Functional studies of the Human Papillomavirus E7 protein.

Thesis

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FUNCTIONAL STUDIES OF THE HUMAN PAPILLOMAVIRUS E7 PROTEIN

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A Thesis Submitted for the Degree of Ph.D.
at the Open University

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International Center for Genetic Engineering and Biotechnology
TRIESTE

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Second Supervisor : K. Powell Ph. D.

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A Cesare e alla mia cara famiglia.
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SUMMARY

E7 is the major Human Papillomavirus transforming protein. Mutations in regions of E7 result in a loss or reduction of transforming activity even through wild type levels of pRB binding are retained, indicating the existence of additional cellular targets.

I performed a series of experiments to investigate whether HPV E7, like AdE1a, can bind to TBP. E7 from both benign- and malignancy-associated HPVs binds to TBP, both in vitro and in vivo. Alignment of E7 and E1a sequences shows that the core TBP binding domain on E1a is close to its CKII recognition site, and I found that the phosphorylation can increase the binding of both E7 and E1a to TBP. Mutational analysis confirmed that the highly conserved carboxy-terminal region of TBP is involved in the interaction with E7, and also in the binding with other proteins, such as E2, E6 and p53. In addition, two carboxy terminal mutants of E7 were found to be defective in the binding with TBP.

Two possible biological consequences have been shown for the E7-TBP interaction:
1) E7, like AdE1a, inhibits p53’s transcriptional activity through the formation of a tripartite complex between E7, TBP and p53.
2) E7 mutants which are defective in TBP binding are less transforming, indicating a partial role for TBP in the transformation function of E7.

Considering the importance of E7 phosphorylation in transformation and in regulation of the association with TBP, I dedicated the second part of the study to analysing the E7 phosphorylation state in vivo. I identified a second E7 phosphorylation site which lies at Ser 71, and, more interestingly, I found changes
in the phosphorylation level of E7 protein which could regulate the specificity of the E7-TBP interaction through well-defined phases of the cell cycle.
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INTRODUCTION

1. HISTORICAL ASPECTS

The papillomaviruses (PVs) induce diseases which are characterised by proliferation and transformation of epithelial cells in either skin or mucosa. These cutaneous warts or mucosal papillomas may in rare cases progress to malignancy. The lesions, which are widespread in animal and human populations, are caused by small DNA tumour viruses, named Papillomaviruses from the latin *Papilla*, meaning “nipple” or “pustule”, to denote their pathogenic activity. Specific viral subtypes are associated with both benign and malignant epithelial lesions at different anatomical sites. The infectious nature of warts was first established in 1907 by Ciuffo, who was able to induce common cutaneous warts in “volunteers” by injecting cell-free filtrates of wart extracts (Ciuffo, 1907). Early studies on skin warts induced by cottontail rabbit papillomavirus (CRPV) established the oncogenic potential of this group of viruses (Shope, 1933). This was further confirmed by the presence in the Western Highlands of Scotland of cattle infected by bovine papillomavirus with a high incidence of bladder/alimentary canal cancer (Olson et al., 1969; Jarret et al., 1984). Until recently the bovine papillomavirus system, in particular BPV1, has served as the prototype for studying the molecular biology of the virus. Progress in characterisation of the human papillomavirus was initially quite slow, since it is an exclusively intra-epithelial pathogen, with an infectious cycle totally dependent upon the growth and differentiation of the host keratinocyte. It cannot be propagated in...
normal tissue culture and the concentration of viral particles in warts is highly variable. One of the first successful systems of propagation of HPV was provided by the nude mouse xenograft system, (Kreider et al., 1987; Kreider et al., 1990). Fragments of human neonatal foreskin were incubated with an extract from an HPV11-containing vulvar condyloma, and then grafted under the renal capsule of nude mice. These grafts developed into condylomatous cysts from which virions could be extracted and used to initiate new cycles of cyst formation. However, the system, restricted to a single isolate of HPV11, was not amenable to genetic manipulation and the limited number of virions obtained have been used only as targets to detect antibodies. More recently, several groups have reported virus growth in organotypic (or raft) cultures of epithelial cells (McCance et al., 1988; Lechner et al., 1991; Regnier et al., 1991; Meyers et al., 1992; Dollard et al., 1992). In two or three weeks the epidermal cells at the air-liquid interface stratify and differentiate, and the “artificial epidermis” closely represents its in vivo counterpart (Asselineau and Prunieras, 1984). At the moment, the raft culture is still the most widely used system for studying vegetative viral replication and virus-cell interactions unique to papillomaviruses.

Until the 1970s, the general view was that there was only one HPV and the tissue location, rather than virus type, dictated the morphology and behaviour of the warts at a specific epithelial surface. Largely due to advances in DNA cloning techniques, it was possible to confirm a great plurality of both human and animal papillomaviruses and the viruses were shown to be not only species specific, but also tissue trophic (Gissmann et al., 176; Meisels et al., 1984; Kadish et al., 1986).
2. EPIDEMIOLOGY AND CLASSIFICATION

Papillomavirus-induced tumours are initially benign, but may become malignant (Rous and Beard, 1935) and, using radioactively labelled DNAs of newly characterised types as probes, it has been possible to detect HPVs in a series of tumours of previously uncertain aetiology. The history of HPV lesions is characterised by infection of a germinal cell of the basal layer to establish the virus-infected cells in the epidermis (Ciuffo, 1907), through microtraumas or within “transformation zones”, where columnar epithelium changes to squamous epithelium and where the target cells are steadily exposed (e.g. nasal mucosa, cervix uteri and larynx). In low-grade HPV-induced lesions, the basal layer, which normally consists of one to two layers of mitotically active cells, occupies up to one-third of the epithelium. The remainder of the epithelium undergoes a slightly altered differentiation programme, with virion production occurring in the highly differentiated suprabasal cells. In the epidermal differentiation the proliferating cells are confined to the basal monolayer. The suprabasal daughter cells do not divide, they become committed to differentiate, and begin a succession of changes in keratin gene expression (Franke et al., 1986). The activation or inactivation of specific keratins leads to the set of intermediate filaments characteristics of the spinous and granular layers. The superficial cornified cells are anucleate and no further papillomaviral gene expression is possible. This layer constantly desquamates and it is replaced from beneath (Figure 1).

In the basal layer the viral gene expression is limited to the early region, and the viral DNA is maintained in a latent form characterised by episomal replication at a relatively low copy number. As the cells differentiate and migrate toward the outer
layers of the papilloma, substantially higher copy numbers and assembled virions can be detected (Stoler et al., 1990; Dollard et al., 1992; Meyers et al., 1992). The expression of late genes and viral DNA amplification is thus restricted to the upper, more differentiated, layers of the epithelium. Replication of the viral DNA occurs only in terminally differentiating layers, co-ordinately with cell replication, maintaining a relatively constant copy number per cell (LaPorta and Taichman, 1982). As result, differentiating keratinocytes become increasingly permissive for papillomaviruses, while fibroblasts are non-permissive, but can be transformed in vitro, a system which has been extensively used for genetic analysis.

Figure 1. Viral life cycle coupled with the differentiation of the keratinocytes.
Some warts remain benign with cell division confined to the basal layer, whilst others reach various degrees of dysplasia with continued replication and division, abnormal mitoses, possible shifts in chromosome numbers, chromosomal breakage and aneuploidy through the entire thickness of the epithelium (Fu et al., 1981; Reid et al., 1984). In some cases these lesions can become invasive with penetration of the transformed epithelial cells through the basement membrane, cell metastasis to remote sites and death of the host. Conversion of human papillomas into squamous cell carcinomas mainly occurs in three conditions: the skin disease epidermodysplasia verruciformis (EV) (Jablonska et al., 1972); the cervical dysplasia (zur Hausen, 1977) and, in a lower percentage, the laryngeal papilloma of adults (Kleinsasser et al., 1973).

In infection of the cervix, the low-grade lesions are called condyloma or cervical intraepithelial neoplasia I, CIN I (mild dysplasia) (Koss, 1987), and can progress to higher-grade CIN II (moderate dysplasia) and CIN III lesions (severe dysplasia) (zur Hausen, 1985), to CIS (carcinoma in situ). In CIN III lesions, the ability of the infected cells to differentiate is lost, and basal-like cells occupy the entire thickness of the epithelium. No virus is produced in high-grade CIN III or carcinoma in situ.

Papillomaviruses sometimes establish their genomes in transformed cells as nuclear episomes (30% of cases). In most cases, however, the viral DNA becomes integrated into the cellular genome, with frequent major deletions of viral sequences coding for E1 and E2 proteins (Schwarz et al., 1985; Schneider-Gadicke and Schwarz, 1986; Dürst et al. 1987; Choo et al., 1987). However, HPV infection alone is not sufficient for neoplastic progression, for example the E6 and E7 genes of the high risk HPVs 16 and 18 efficiently immortalise genital keratinocytes in vitro (Kaur et
al., 1989; Barbosa et al., 1989b; Woodworth et al., 1990), but these immortalised
lines are not tumorigenic in the nude mouse assay (Dürst et al., 1987; Kaur et al.,
1988). Progression to the fully transformed malignant phenotype is an infrequent
event requiring prolonged passage (Pecoraro et al., 1989; Hurlin et al., 1991) or
additional insults such as the introduction of activated oncogenes (DiPaolo et al.,
1989) together with the inactivation of tumour suppressor genes: the total
accumulation of changes is the critical determinant in progression (Vogelstein et al.,
1993). Cancer is a multi-step process and a number of endogenous and exogenous
factors may contribute, directly or indirectly, to the development of cervical
intraepithelial neoplasia and malignancy in HPV-infected individuals. The role of
cofactors is underlined by the fact that, for example, CRPV is only weakly
oncogenic by itself, but the progression of induced carcinomas can be accelerated by
the application of chemical carcinogens to the skin of the animals (Rous and
Friedwald, 1944). Moreover, the dietary requirement of bracken fern (an
immunosuppressant) for the formation of fibropapillomas in cattle in association
with BPV1 (Jarret et al., 1978a, 1978b), confirmed the synergism between the virus
and other agents to give rise to the cancer.

Cancer of the cervix is the fifth most common cancer in the world and the second
most frequent cancer in women world-wide (500,000 cases/year of cervical cancer
against 545,000/year of breast cancer), ranking first in developing countries (Parkin
et al., 1988). Epidemiological evidence distinctly indicates that cervical carcinoma
and other high-grade lesions of the genital tract are caused by a sexually transmitted
agent (zur Hausen, 1985). Genital papillomavirus infections are now found in the
population at a very high frequency, estimated to be as high as 30% of the sexually
active men and women in Western countries (King et al., 1980; Boon et al., 1986).

Based on polymerase chain reaction, HPV DNA has been found in around 98% of patients with cervical cancers (60% of these are HPV16 positive), and in 10-20% of normal individuals (only 5% of these are HPV16 positive). 70% of anal cancers contain HPV DNA, although it is possible to find HPV in only 10% of anal specimens from normal individuals (de Villiers et al., 1987; Gergely et al., 1987).

Human papillomaviruses are classified into distinct types on the basis of nucleotide sequence homology rather than serotypically, the distinction being based on less than 50% cross-hybridization of their DNAs in liquid phase under conditions of high stringency. More than seventy types of HPVs have been molecularly cloned from a variety of lesions. All HPVs have a monophyletic origin, but there are two major branches of an hypothetical phylogenetic tree. One contains HPVs associated with cutaneous lesions, the other group of viruses are mainly associated with lesions of mucosal origin. Some of them appear to have greater oncogenic potential, correlating with the in vitro biological activity of the E6 and E7 genes (see below).

For example, HPV6 and 11 are regularly found associated with benign lesions, in genital warts (condylomata), and occasionally with low-grade dysplasias of the cervix. HPV 16, 18, 31, and 33 are more frequently found associated with malignant lesions of the cervix, in cervical dysplasias (intraepithelial neoplasias), and in Bowenoid lesions at external genital sites, and occasionally in extragenital sites (most frequently in the oral or vocal cord mucosa). HPV16 is the most prevalent genotype, being found in more than 50% of these lesions. HPV6 and 11 are therefore considered low-risk types, while HPV16, 18, 31, and 33 are high-risk types (Dürst et al., 1986). The correlation between the phylogenetic grouping and
the *in vitro* and *in vivo* HPV-related manifestations supports the theory that the different pathogenicities are determined, at least in part, by differences in the viral sequences.

### 3. GENOME ORGANISATION

This section briefly reviews the genes of HPV encoding the proteins involved in the structural assembly of the virion and in transcription-replication processes (L1, L2, E4, E1, E2), and the portion of the genome, called the URR (Upstream Regulatory Region), which controls these events. Since the focus of this thesis is in investigating the E7 protein functions, the genes of HPV involved in transformation activity (E5, E6, E7) are covered in greater depth.

Papillomaviruses contain a double stranded circular DNA genome of about 7900 base pairs. The DNA is encapsidated in a virion of icosahedral symmetry composed of 72 protein capsomers, which lacks a lipid-containing envelope (Orth *et al.*, 1977; Chen *et al.*, 1982; Danos *et al.*, 1982; Schwarz *et al.*, 1983; Liddington *et al.*, 1991; Griffith *et al.*, 1992). A comparison of the nucleotide sequences reveals a well-conserved general organisation of the genome of human papillomaviruses. All putative protein coding sequences, called Open Reading Frames (ORFs), are restricted to one strand (Engel *et al.*, 1983; Pfister, 1984), and the individual ORFs are classified as Early (E1 to E7), and Late (L1 and L2), by analogy with other DNA viruses where genes are expressed at different times during the course of a productive infection.
The so-called 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. Early gene products are also involved in transformation of the host cell. The Late genes code for the 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 (Syrjänen et al., 1987). It should be emphasised, however, that alternative splicing generates a complex pattern of protein expression. A scheme of the different transcripts encoded by the HPV genome is shown in Figure 2.

![Figure 2](image-url)

**Figure 2.** Physical map of HPV16 showing genetic organisation. The 7950 base pair circular genome is represented in a linear manner. The positions of the ORFs in three possible translation phases are based on the complete DNA sequence of HPV16 (Seedorf et al., 1985), including more recent revisions. The upper part of the figure shows the mRNA species. The RNA transcripts are represented by arrows in the 5’ to 3’ direction, with gaps signifying introns spliced out of the mRNA.
3.1. **URR (Upstream Regulatory Region)**

The URR, also called the LCR (Long Control Region), of papillomaviruses extends between the L1 and E6 genes and covers approximately 800 bps. This is a non-conserved non-coding region, containing promoters and enhancer elements for the control of transcription, as well as for viral DNA replication. A summary of some of the major factors binding is shown in Figure 3.

![URR map with some of the transcriptional and replication regulatory elements.](image)

In HPV6 and 11, the major promoter is located in E7, close to the boundary of E1; minor promoters are just upstream of the E6 ORF and in the E1 ORF (Chow *et al.*, 1987b). All HPV16 RNA transcripts from malignant tissues, that have been studied, are produced from one promoter at nucleotide 97, located just upstream of the E6 ORF (Smotkin *et al.*, 1989). For HPV18 the transcription of the viral mRNAs initiates at P105. Both of these promoters are known as early promoters. Several reports have demonstrated the existence of another promoter within the E7 gene, P747 for HPV16 (P742 for HPV31b). This promoter is active throughout stratified
epithelium and is induced upon epithelial cell differentiation (Hummel et al., 1992; Grassmann et al., 1996); for this reason it is possible to define P747 as a viral promoter active during the productive stage of the infection. It promotes the expression of E1^E4 and E5 transcripts and also induces a low-level of transcription from the late region which is capable of encoding L1 and L2 proteins. The promoter proximal regions, themselves, do not have any intrinsic transcriptional activity, despite the presence of a number of binding sites for cellular transcription factors, which are involved in a very complex interplay, controlling the viral oncogene expression and playing a key role in development of cervical cancer. Moreover, recent reports have demonstrated that the chromatin structure of the URR region of human papillomavirus type 16 represses the viral oncoprotein expression (Stunkel and Bernard, 1999).

Studies with BPV1 showed that E2 protein, which can influence the rate of transcription from several promoters, binds to the palindromic sequence ACC(N)_6GGT (Androphy et al., 1987a; Moskaluk and Bastia, 1988; Li et al., 1989). This E2-responsive sequence (E2RS) occurs several times in the URR of all PVs, and multimerisation of this element resulted in increased enhancer activity (Haugen et al., 1987; Hawley-Nelson et al., 1988; Hermonat et al., 1988).

Additional elements which have been shown to influence gene expression include API consensus binding sites (Chan et al., 1990) and a cytokeratin octamer sequence (Hoppe-Seyler et al., 1991). This element of the PV enhancer has been demonstrated to confer the cell-type preference of the virus, determining the keratinocyte specific nature of the Papillomaviruses (Butz and Hoppe-Seyler, 1993). Interestingly, HPV16 has also a GRE-glucocorticoid responsive element. (Gloss et
al., 1987), which not only confers responsiveness to glucocorticoids, but also to the progesterone (Pater et al., 1988). PVs with GREs are transcribed more efficiently in cells which are responsive to corticoids, such as those of the cervical mucosa. While the wild-type URR exhibits a dose-dependent, dexamethasone-induced transcriptional activity, mutation of the GRE completely abolishes hormone-dependent stimulation of the HPV18 E6/E7 promoter (Butz and Hoppe-Seyler, 1993). Another important element of HPV16 URR is the Sp1 recognition element, (Hoppe-Seyler and Butz, 1992), activation of which leads to a strong reduction in the transcriptional activity of the HPV18 URR in both, HaCaT and HeLa cells, strongly suggesting that it is important for the transcriptional activation of E6/E7 oncogene expression (Gloss and Bernard, 1990; Butz and Hoppe-Seyler, 1993).

Most interestingly, the HPV URR also contains a YY1 element, which is a transcriptional repressor that inactivates the AP-1 site and blocks the response to TPA (tetradecanoyl-phorbol acetate) (Shi et al., 1991). Mutation of the YY1 binding site leads to an enhanced activity of the HPV18 promoter. Therefore YY1 can be defined as a silencer element working at the level of transcriptional initiation. Thus, HPV expression is subject to a dual control by antagonistic cellular transcription factors: an activation by AP-1 in response to extracellular signals and a strong dominant negative activity by YY1.
3.2. Late Genes

The papillomavirus capsid consists of two structural proteins, encoded by the Late Genes: L1, the major capsid protein of 54-58 kDa (Orth et al., 1977; Gissmann et al., 1977; Müller and Gissmann, 1978), which represents about 80% of the total virion protein (Favre et al., 1975), and L2, of 63-78 kDa (Doorbar and Gallimore, 1987; Jin et al., 1989), the minor capsid component. Translocation of these proteins to the nucleus, where the viral assembly takes place (Orth et al., 1977; Zhou et al., 1993), is mediated by nuclear localisation signal sequences (NLS) (Zhou et al., 1991b; Silver, 1991). HPV16 L1 contains two NLS sequences at the carboxyl terminal end (Zhou et al., 1991b), and alone can assemble into virus-like particles (VLPs), when expressed at sufficiently high levels in eukaryotic cells (Kirnbauer et al., 1992). VLPs present the conformational epitopes required for generating high titre neutralising antibodies, but are devoid of the potentially oncogenic viral genome. L2 also has a NLS located at its carboxyl-terminus and, after complexing with the major capsid protein L1 in the cytoplasm, contributes to its transport into the nucleus. It has been reported that, when the two capsid proteins are expressed separately, L1 shows a diffuse nuclear distribution, while L2 localises to punctate nuclear regions identified as PML (Promonocytic Leukemia Protein) oncogenic domains (PODs) (Day et al., 1998). Coexpression of L1 and L2 induces a relocation of L1 into the PODs, leading to the co-localisation of L1 and L2. Moreover, whereas the localisation of the E1 protein is unaffected by L2 expression, E2 is shifted from a dispersed nuclear locality into the PODs and co-localised with L2, suggesting a possible interaction between E2 and the minor capsid protein. Furthermore, with this mechanism, L2 affects the distribution of the viral DNA and contributes to its
incorporation into the virion through gradual addition of capsid proteins around the
PV chromatin (Day et al., 1998).

The E4 gene is entirely contained within the central portion of the E2 ORF and is
the most abundant HPV protein, constituting up to the 30% of the total infected cell
proteins (Doorbar et al., 1986, 1988; Breitburd et al., 1987). The protein is
expressed quite early, but is classed as late due to its abundance later on, yet it does
not constitute a part of the virus particle (Doorbar et al., 1986; Breitburd et al.,
1987). At least eight E4-derived species have been reported (Doorbar et al., 1986;
Breitburd et al., 1987) and the primary product is a 17 kDa polypeptide, which is
expressed from a spliced mRNA (E1^E4) encoding five N-terminal amino-acids
from the E1 ORF (Doorbar et al., 1988; Chow et al., 1987a). The other species are
derived from this by progressive proteolytic cleavage at the N-terminus, and/or by
dimerisation (Breitburd et al., 1987; Doorbar et al., 1988). E4-specific antibodies
have located E4-specific antigens in cytoplasmic inclusion bodies in cells of the
granular layer of HPV1 induced warts and, as a late protein, its expression is first
detected in cells of the parabasal layer, coinciding with the onset of vegetative viral
DNA replication. The E4 proteins are generally expressed in mucosal lesions at
much lower levels than in cutaneous ones (Brown et al., 1991; Palefsky et al., 1991;
Tomita et al., 1991; Pray and Laimins, 1995). E4 is not required for L1 and L2 to
assemble into virus-like particles in vitro (Zhou et al., 1991a, b), but it may however
be required for virus production in vivo. By altering the normal cytokeratin matrix of
an infected cell, the virus, through E4, may prevent the formation of highly cornified
squames, so facilitating the process of release of virions when the cell is ultimately
shed from the surface of the skin (Zhou et al., 1991a; Roberts et al., 1993).
3.3. Early Genes Involved in Transcription and Replication Processes

E1 is the largest ORF of papillomaviruses, and most of the information concerning its function has been obtained from BPV. The amino acid sequences of the carboxy terminus of E1 are highly homologous (up to 60%) between different PV-types, in contrast the amino terminal region is rather variable. The carboxy-terminus of BPV E1 is essential for BPV replication and the amino-terminus is required for establishment of the genome as a latent nuclear plasmid (Lusky and Botchan, 1985; Hubert and Lambert, 1993). A comparison with the sequence of the large T antigen of SV40 demonstrates significant homologies within more than 200 amino-acid residues, close to the carboxy-terminus of E1 (Clertant and Seif, 1984). It is therefore interesting that E1 is essential in the episomal replication of the viral genome (Lusky and Botchan, 1985), suggesting that conserved structural features of E1 and large T reflect common functions. The HPV E1 protein is a nuclear ATP-binding site phosphoprotein of about 68 kDa. It has been shown to have both ATPase and helicase activity (Seo et al., 1993; Hughes and Romanos, 1993; Bream et al., 1993), and to be the main viral replication protein, able to bind to the origin of replication and to unwind DNA (Blitz and Laimins, 1991; Wilson and Ludes-Mayers, 1991). However, papillomavirus replication also requires the full length E2 protein, which forms a protein complex with E1 at the origin of DNA replication (Ustav and Stenlund, 1991; Ustav et al., 1991; Chiang et al., 1992), and E2 seems to increase the level concentration of E1 at the ori which has an intrinsically low affinity for its recognition site.

The full-length E2 protein is about 50 kDa, and can be functionally divided into three different domains: an N-terminal region which includes the transcriptional
activation domain, a central hinge region, and a C-terminal domain, involved in specific DNA binding to the viral URR, as well as in E2 dimerisation (Giri and Yaniv, 1988). HPV16 E2, like BPV1 E2, is a transcriptional activator (Spalholz et al., 1985). Transactivation by the E2 protein depends upon binding, as a dimer, to a palindromic sequence ACCG(N)_6 CGGT, which is present in multiple copies in PV genomes (Li et al., 1989). Twelve E2 binding sites are clustered within the URR of BPV, forming the E2 responsive-enhancer elements E2RE1 and E2RE2, which activate early promoters co-operatively (Spalholz et al., 1987). It has been demonstrated that, at low levels, the full-length HPV16 E2 protein can transactivate the P97 promoter in cervical keratinocytes with consequent up-regulation of the levels of viral oncoprotein E6 and E7 expression, whereas increased intracellular levels of E2 result in repression of HPV transcription (Bouvard et al., 1994a; Ushikai et al., 1994; Stubenrauch et al., 1996; Steger et al., 1997). The most likely explanation for this difference in observations is the diversity in binding site occupancy at different concentrations of the E2 protein. Thus, at low concentration E2 protein binds to the distal region of the promoter and activates transcription, whereas at high concentration E2 binds to the proximal region of the P97 promoter and represses transcription (Stubenrauch et al., 1996; Steger et al., 1997).

Alternative splicing also results in truncated forms of BPV1 and HPV16 E2 proteins which function as transcriptional repressors (Lambert et al., 1987; Bouvard et al., 1994a). Both full length and spliced forms of E2 have a common domain that mediates their ability to bind DNA specifically, and to form both protein homodimers and heterodimers, and the ratio of the two forms of E2 is critical in determining the levels of transcriptional activation. The recent finding that the
3.4. Early Genes Involved in Transformation Activity

The HPV E5 gene encodes a very hydrophobic protein with a molecular weight of approximately 10 kDa (Halbert and Galloway, 1988; Bubb et al., 1988). The E5 protein has been localised to the Golgi apparatus, the endoplasmic reticulum, and the nuclear membranes of transfected cells (Conrad et al., 1993). Since HPV E5 sequences are frequently deleted during tumour development, E5 was thought not to be involved in the development of HPV associated malignancy. However, numerous studies have now shown that the HPV E5 proteins possess intrinsic transforming activities and it seems likely that E5 may contribute to the initial stages of malignant transformation prior to viral DNA integration.

The first indication that HPV E5 might have transforming potential come from studies where a higher frequency of transformation was noted in transfection experiments with the entire HPV genome, rather than with the E6 and E7 genes alone (Bedell et al., 1989). HPV6 E5 was then shown to be able to transform NIH3T3 cells to anchorage independent growth, and these cells were capable of forming tumours in nude mice (Chen and Mounts, 1990). Although the first observation of a link between E5 and the growth factor receptor was provided by studies on BPV1 E5 (Martin et al., 1989), in subsequent experiments HPV16 E5 was also found to promote anchorage independent growth of NIH3T3 cells in cooperation with ligand stimulated EGFR (Epidermal Growth Factor Receptor) (Pim et al., 1992; Leechanachai et al., 1992). E5 was found to perturb the processing of the activated receptor, increasing its half life and hence the duration of the mitogenic signal (Straight et al., 1995).
In the case of HPV6 E5, most of its activity appears to be via the EGFR, although HPV6 E5 has been reported to complex with several growth factors receptors, including EGFR, c-erb B2 and PDGFR (Conrad et al., 1993, 1994). The transforming activity of HPV E5 in co-operation with PDGFR is much weaker, showing clear differences between BPV and HPV E5 proteins. In a manner analogous to that of BPV1 E5, HPV16 E5 has also been shown to perturb EGFR processing in human keratinocytes (Straight et al., 1995).

The cells containing E5 also express high levels of c-fos and c-jun in response to EGF, suggesting that E5 expression enhances the EGF mediated signal transduction to the nucleus (Leechanachai et al., 1992). The up-regulation of the c-fos mRNA is concomitant with an increased activation of MAP-kinase activity (MAPK), in cells expressing the HPV E5 gene (Bouvard et al., 1994b; Gu and Matlashewski, 1995; Crusius et al., 1997). There is also evidence that E5 may activate the Protein Kinase C (PKC) pathway, giving rise to an increase in early gene expression (Crusius et al., 1997). In this way, E5 may activate a series of processes in the early part of the G1 phase of the cell cycle, priming the cell for the stimulatory effects of E7, which upregulates the cellular DNA replication machinery.

The most likely explanation for E5 functions has come from studies which have shown that HPV E5 can interact with the 16 kDa component of the vacuolar H\(^+\)-ATPase (Goldstein et al., 1991, 1992; Conrad et al., 1993), which is involved in decreasing the pH of vacuoles. The fact that the vacuolar H\(^+\)-ATPase may be a common target for all papillomavirus E5 proteins (BPV1, HPV6, HPV11 and HPV16), was supported by studies which showed that HPV16 E5 expression in human keratinocytes results in a block of 16 kDa function, with a reduction of
BPV1 E2 family of proteins can associate directly with two components of the cellular transcriptional machinery, TFIID and TFIIB, suggests that E2 mediated transactivation occurs, at least in part, via direct contacts with cellular components of the transcriptional complex (Miller Rank and Lambert, 1995).
endosome acidification, and a subsequent prolonged retention of un-degraded EGFR in intracellular vesicles. This increases the amount of EGFR recycling back to the cell surface, thus amplifying the mitogenic response to EGF (Straight et al., 1995). It is also interesting to note that EGFR is overexpressed in more than 80% of cervical lesions that progress to CINIII (Bauknecht et al., 1989), most of which have lost E5 by this stage because of the DNA integration event (Schwarz et al., 1985; Baker et al., 1987). This is suggesting that the EGFR accumulation is able to compensate the lack of E5 at this stage of the viral life cycle.

The HPV E6 proteins vary from 137 to 158 residues in length, and the most striking structural feature is the repetitive motif Cys-X-X-Cys. Four repeats can be found with exact intervals of 29, 35/36, and 29 amino acids, suggesting a well conserved tertiary structure, shown in Figure 4.

Figure 4. Schematic representation of the HPV18 E6 protein with the principal functional domains. Boxes represent regions of E6 which, if mutated, result in loss of the indicated functions.
This Cys-X-X-Cys motif, present in several proteins (T/t SV40 antigens, t Polyoma antigens, early Adenovirus proteins), is a characteristic of Zinc finger domains, and indeed HPV18 E6 and BPV1 E6 proteins have been shown to bind zinc in vitro (Barbosa et al., 1989a; Grossman and Laimins, 1989). The first functional analysis of E6 was achieved by genetic studies on BPV1, which has been shown to be able to mediate morphological transformation of fibroblasts (Schiller et al., 1984; Androphy et al., 1987b). The very low levels of the protein makes the determination of the intracellular location extremely difficult (Androphy et al., 1985; Banks et al., 1987).

One report locates HPV18 E6 in the nuclear matrix and in the non-nuclear membrane if the protein is expressed in baculovirus (Grossman et al., 1989). Subsequent assays found BPV and HPV E6 proteins to be localised in nuclear and membranous compartments (Androphy et al., 1985; Kanda et al., 1991; Chen et al., 1995; Sherman and Schlegel, 1996).

The E6 and E7 proteins of high-risk HPVs (16 and 18) are retained and expressed in cervical carcinoma cells, suggesting that they participate in the initiation and/or maintenance of the tumour (Schwarz et al., 1985; Schneider-Gadicke and Schwarz, 1986). In support of its apparent involvement in the development of cervical cancer, the E6 gene of high-risk HPVs has been shown to co-operate with ras in the immortalisation of primary BMK (Baby Mouse Kidney cells) (Storey and Banks, 1993; Pim et al., 1994) and to be required, with E7, for immortalisation of primary normal human cervical or foreskin keratinocytes (Smotkin and Wettstein, 1986; Banks and Crawford, 1988; Münger et al., 1989a; Hawley-Nelson et al., 1989; Hudson et al., 1990). HPV6 and HPV11, as low risk types, also infect the genital tract, but are rarely associated with cervical carcinomas, and their E6 and E7
proteins do not co-operate to immortalise human keratinocytes (Barbosa et al., 1991). Both E6 and E7 have each been shown to override a number of eukaryotic cell-cycle checkpoint controls and it is widely believed that it is through these pathways that E6 and E7 co-operate in the immortalisation of primary human cells. One of the cellular targets of E6, as well as of SV40 TAg (Lane and Crawford, 1979; Linzer and Levine, 1979) and of E1b 55kDa protein (Sarnow et al., 1982), is the tumour suppressor protein, p53 (Werness et al., 1990). The demonstration that in contrast to many human cancers, p53 mutations are rare in cervical cancers (Crook et al., 1992; Helland et al., 1993) and that the protein could not be detected in HPV-transformed cell lines, such as the cervical tumour line Hela (Matlashewski et al., 1986; Scheffner et al., 1991), suggested that the effects of HPV E6 on p53 are analogous to an inactivating mutation. Following the observation that high-risk HPV E6 proteins form a stable complex with p53 (Werness et al., 1990), it was shown that, complex formation between E6 and p53 resulted in ubiquitin-dependent proteolysis of the latter (Scheffner et al., 1990). This is characterised by the fact that E6-expressing cells lose the G1 checkpoint activity very early (Dulic et al., 1994) and are resistant to p53 induced growth arrest and apoptosis as a result of DNA damage (Kessis et al., 1993; Foster et al., 1994; Pan and Griep, 1995; Thomas et al., 1996; Cai et al., 1997). The ubiquitin-dependent proteolysis consists of two ATP-dependent processes (Ciechanover et al., 1990): multiple ubiquitin molecules, activated by an E1 enzyme, are transferred and covalently linked to the substrate protein by ubiquitin conjugating E2 enzymes. Then the highly ubiquinated protein is recognised and degraded by a protease complex. In the E6-p53 system, it was shown that a 100 kDa cellular protein, called E6-AP (for E6-Associated Protein) (Scheffner
et al., 1990), is essential to the process and the E6/E6AP complex constitutes an E3 ligase, which confers the substrate specificity on the reaction. No study has yet shown any association between p53 and E6-AP in the absence of HPV E6, and anti-sense inhibition of E6-AP results in increased p53 levels only in those cells which express E6 (Beer-Romero et al., 1997). Several studies have indicated that the association with p53 and its subsequent degradation are independent activities of E6 (Crook et al., 1991; Thomas et al., 1995), and similarly the domain of p53 required for the association with E6 (376-384 amino acids) is separable from the region of the protein required for degradation (66-326 amino acids) (Mansur et al., 1995).

The complexity of the E6-p53 interaction was further highlighted by the results of mutational analysis of the E6 protein. At least four regions of E6 appear to be involved in the interaction, with domains required for binding and degradation being quite separate (Crook et al., 1991; Mietz et al., 1992; Pim et al., 1994). In addition, many of the data generated by in vitro assays has been shown to be not representative of the in vivo situation (Foster et al., 1994; Crook et al., 1996; Gardiol and Banks, 1998). Recent studies have show that the N-terminal 43 amino acids of E6 are required for binding to E6-AP and that this region is required for degradation activity, while the C-terminal half of E6 is responsible for binding p53 (Pim et al., 1994; Li and Coffino, 1996). Interestingly, the ability of E6 to induce p53 degradation would also appear not to be essential for E6’s transforming activity indicating the existence of additional functions of E6 which are important for transformation (Pim et al., 1994).

The ability of E6 to target p53 for degradation would also appear to be influenced by p53. Many mutants of p53 are not susceptible to E6 induced degradation and some
studies have demonstrated that certain p53 polymorphisms (Matlashewski et al., 1987a) may also affect its susceptibility to E6 induced degradation. In particular, the codon 72 polymorphism, encoding either Pro or Arg, widely affects the susceptibility to degradation, with the Arg form being significantly more susceptible to degradation than the Pro form (Storey et al., 1998). It remains to be determined whether the array of post-translational modifications, which have been reported for p53, could also affect its susceptibility to E6 induced degradation. As well as targeting p53 for degradation, E6 can also inhibit p53 binding to its cognate DNA recognition sequence (Lechner and Laimins, 1994; Thomas et al., 1995; Molinari and Milner, 1995). The strength of the binding of p53 to the DNA is dependent upon the precise target sequence, but E6 is able to disrupt each complex (Thomas et al., 1996), inhibiting in this way all the transcriptional activities of the tumour suppressor protein. The low-risk E6 proteins show only a weak p53 binding function and no clear degradative activity, and so far no obvious inhibitory effect on p53 function has been described (Werness et al., 1990; Scheffner et al., 1990). The expression of high-risk E6 in normal diploid human fibroblasts results in genomic instability, presumably as a consequence of inhibition of p53 function (White et al., 1994). This activity of E6, may be critical in allowing the accumulation of genetic changes which contribute to malignant progression.

Although a major function of E6 is abolition of p53 function, a very large number of additional targets have been identified. Recent studies have identified Bak (bcl-2 family) and c-Myc as being targets for E6 mediated degradation, confirming that the E6 protein of the oncogenic HPV types is able to prevent apoptosis by both p53-dependent and p53-independent pathways. The E6/E6-AP complex has also been
implicated in these interactions (Gross-Mesilaty et al., 1998; Thomas and Banks, 1998), the consequence of which is a reduction in the number of cells entering apoptosis and an increase in the length of cell survival. Recent studies have also shown an interaction between E6 proteins and hDlg/SAP97 (DLG) (Lee et al., 1997; Kiyono et al., 1997). This is a PDZ-domain containing protein, which recruits plasma membrane and cytoskeletal proteins to regions of cell-cell contact, mediating association between the cytoskeleton and signalling molecules. The binding to DLG is mediated by sequences in the carboxy terminus of the high risk E6 proteins and results in rapid degradation of the DLG through the proteosome (Gardiol D., in press). Whether E6-AP is involved in this activity remains to be determined. Recent studies identified another target of E6 in a novel putative GAP protein, E6TP1 (E6-targeted protein 1), which is involved also in signalling pathway. High-risk but no low-risk HPVs are able to degrade this novel E6-binding protein (Gao et al., 1999).

Among the several proteins which interact with E6 is E6BP (E6 Binding Protein), which only binds to E6 proteins with transforming potential (Chen et al., 1995). This calcium binding protein is localised to the endoplasmic reticulum and, although the consequences of the E6/E6BP interaction are not yet known, there is clear potential for a role in the regulation of normal cellular differentiation, proliferation and transformation (Solomon et al., 1998). A yeast two-hybrid screen also identified MCM7, which is involved in the control of initiation of DNA replication (Thömmes et al., 1997), as a target of the E6 proteins derived from both benign and oncogenic HPV types (Kukimoto et al., 1998; Kühne et al., 1998). However the precise biological consequences of this interaction remains to be determined. Many of these
interactions with E6 would appear to be mediated by the presence of a conserved E6-binding motif (ELLG) with a vital alpha-helical structure in several of the target proteins of E6 (Elston et al., 1998).

In contrast to the simple mono-cistronic mRNAs which encode E6 from the non-oncogenic HPVs (Chow et al., 1987b; Smotkin et al., 1989), the splicing pattern for the early viral transcripts of the oncogenic types, such as HPV 16 and HPV 18, is complex. These HPVs produce alternatively spliced mRNAs (I to IV) from which a large portion of E6 has been removed, with the mRNA splicing back into a different reading frame before reaching an adjacent termination codon, as shown in Figure 5.

![Figure 5](image)

Figure 5. Schematic representation of the E6*I protein in comparison with the full length E6.

These proteins are termed E6* and form the majority of early viral transcripts both in cervical tumours and in tumour-derived cell lines (Schneider-Gadicke and Schwarz, 1986; Smotkin and Wettstein, 1986). HPV18 E6*I protein interacts both with the full-length HPV16 and 18 E6 proteins and also with E6AP, and subsequently blocks the association of full-length E6 with p53, inhibiting p53 degradation in vitro and in vivo. The consequence is an increased transcriptional activity on p53-responsive promoters and inhibition of cell growth in cells
transfected with E6* (Pim et al., 1997). This may represent the first potential biological function for this polypeptide by which HPV is able to regulate the activity of the full-length E6 protein with respect to p53 during viral infection.
3.5. E7 PROTEIN

The E7 protein was first identified in 1986 (Smotkin and Wettstein, 1986) in a cancer-derived cell line. Subsequently, it has been shown to be retained and expressed in cervical tumours and in cervical tumour derived cell lines (Schwarz et al., 1983; Smotkin and Wettstein, 1986) in contrast with E1 and E2 proteins (Schwarz et al., 1983; Schneider-Gadicke and Schwarz, 1986).

The E7 ORF encodes a small acidic protein of 98 amino acids (Seedorf et al., 1985), and some of its key features are shown in Figure 6.

Figure 6. The HPV16 E7 amino acid sequence compared with the homologous regions of E1a and SV40 large T Antigen. The boxes represent regions of E7 which, if mutated, result in loss of the principal functions of the protein.
From the primary amino acid sequence the protein can be tentatively divided into three regions. Region 1 (the N-terminal 20 amino acids) and Region 2 (amino acids 21 to 40) show high homology to parts of Adenovirus E1a (Moran et al., 1986; Whyte et al., 1988).

Amino acids 6 to 20 and amino acids 21 to 40 of HPV 16 E7 resemble residues 41 to 56 (in conserved domain 1, CR1) and residues 121 to 139 (in conserved domain 2, CR2), respectively, of Adenovirus 5 E1a, both of which constitute the essential region for the E1a transforming function (Moran et al., 1986; Moran and Mathews, 1987; Lillie et al., 1987; Whyte et al., 1989). The homology with conserved domain 2 is also found in SV40 large T (LT). Region 3, which comprises the C-terminal half of the protein (amino acids 41 to 98), contains a zinc-finger structure (Evans and Hollenberg, 1988; Barbosa et al., 1989a) and two metal binding, Cys-X-X-Cys motifs, which are separated by a stretch of 29 amino acids.

Although the E7 C-terminal region has very little homology with the E1a C-terminal region, both of the proteins contain the Cys-X-X-Cys motifs. Similar structural features are also present in the E7 proteins from HPV 18 (Boshart et al., 1984; Cole and Danos, 1987) and HPV 6 (Schwarz et al., 1983).

The metabolic stability of the protein was determined by pulse-chase experiments (Smotkin and Wettstein, 1987), and the half-life was calculated at approximately 60 minutes. The lack of changes in mobility during the chase suggested that there are no major post-translational modifications, such as glycosylation or proteolytic cleavage. Spectroscopic analyses and biochemical studies have also indicated that the carboxyl-terminal zinc-binding domain may be involved in mediating dimer/multimer formation (Roth et al., 1992; McIntyre et al., 1993). Using the two-
hybrid system, it was demonstrated \textit{in vivo} that the C-terminus of E7 is responsible for the formation of oligomeric complexes, although the N-terminus also contributes to this function (Clemens \textit{et al}., 1995; Zwerschke \textit{et al}., 1996). An E7 mutant (16E7 G77) that maintained the ability to form oligomeric complexes also retained transforming and transcriptional activities in mammalian cells (Phelps \textit{et al}., 1992).

In contrast, the dimerisation-negative mutants (SS58/59, R67) were impaired in their biological activities, suggesting that the oligomerisation could be required as an indicator for the structural and functional integrity of the protein (Clemens \textit{et al}., 1995). As with the high-risk HPV E7 proteins, the low-risk HPV E7 proteins, are also able to form oligomeric complexes and, even here, the interaction is dependent on carboxyl-terminal sequences, since the HPV6 E7 amino-terminal region cannot dimerise with the full-length HPV16 E7 protein (Zwerschke \textit{et al}., 1996).

There are several conflicting reports concerning E7 localisation. Using a variety of techniques E7 has been found both in the cytoplasm (Smotkin and Wettstein, 1987; Sato \textit{et al}., 1989b) and in the nucleus (Tommasino \textit{et al}., 1990; Greenfield \textit{et al}., 1991; Tommasino \textit{et al}., 1992; Fujikawa \textit{et al}., 1994; Smith-McCune \textit{et al}., 1999). Moreover, using both immunofluorescence and electron microscopy, E7 has also been found to be associated with the nucleolus in CaSki cells (Zatsepina \textit{et al}., 1997). This localisation appeared to be cell cycle dependent, since it was considerably reduced during the G2 phase, although the total level of E7 protein remained constant during all phases of the cell cycle. The nucleolar localisation was also observed in fission yeast \textit{Schizosaccharomyces pombe}, suggesting that a targeting mechanism of the oncoprotein into the nucleolus could be common to both mammalian and yeast systems (Zatsepina \textit{et al}., 1997).
E7 is phosphorylated by casein kinase II (CKII) at the consensus sequence between residues 30 and 37 in Region 2 of the protein (Smotkin and Wettstein, 1987; Firzlaff et al., 1989; Barbosa et al., 1990). It shares this property with large T antigen of SV40 (Tegtmeyer et al., 1977), large and middle T antigens of polyomavirus (Schaffhausen and Benjamin, 1979), and E1a of Adenovirus (Vousden and Jat, 1989). Barbosa et al. (1990) established that HPV16 E7 was a substrate of CKII, the enzymatic activity of which, can be distinguished from that of the other kinases by its ability to use GTP and ATP with similar efficiency, and to be inhibited at low concentrations of heparin. The Serine residues at positions 31 and 32 are the two amino-acids which are phosphorylated, and mutation of either Serine alone results in a >2-fold reduction in the efficiency of phosphorylation, suggesting a synergism between the two adjacent residues in the susceptibility to the phosphorylation. The examination of different genital HPV types found a correlation between the oncogenic potential of the virus and the rate of phosphorylation. HPV18 E7, believed to have the most oncogenic potential, is phosphorylated more rapidly than HPV16 E7. In comparison, HPV6 E7, mostly associated with benign lesions, showed the slowest rate of phosphate incorporation. Probably sequence divergence within their respective CKII recognition sequences accounts for the differences in phosphorylation.
3.5.1. E7 Transformation Activity.

All the epidemiological evidence which implicates HPV in cervical cancer also indicates that other factors must be involved; for example, exposure to chemical carcinogens, cigarette smoking or X-rays. In this respect, it is similar to other types of cancer in which carcinogenesis appears to occur through a series of steps. In support of this concept, a number of reports have shown that multiple oncogenes are required for the morphological transformation of normal primary cells in vitro (Land et al., 1983; Ruley, 1983; Van der Eb and Bernards, 1984). For example, transformation of primary cells by Adenovirus requires the co-operation of the E1a and E1b genes (Van der Eb and Bernards, 1984). In in vitro assay systems, the E1a gene alone is capable of the establishment of primary cells and co-operating with other oncogenes such as the E1b gene or activated ras gene to effect complete morphological transformation of these cells (Ruley, 1983). Using this type of co-operation assay, different oncogenes have been operationally classified into two groups (Land et al., 1983; Ruley, 1983; Rassoulzadegan et al., 1983). One group, referred to as the establishment genes, consists of those genes that can establish primary cells in vitro and can co-operate with the ras gene; this group includes E1a, C-myc, N-myc, mutant p53, polyoma large T and SV40 large T. The second group, referred to as the transforming genes, which require the co-operation of an establishment gene in order to transform primary cells, includes the three ras oncogenes (H, K, N) and the polyoma middle T gene.

The first indication that HPV harboured a transforming capability in vitro was demonstrated in rodent cells. These studies can be divided into two groups, based on whether the cells were derived from established lines or primary cultures. In the case
of established rodent cells, NIH 3T3 cells were normally used. The measure of transformation usually takes one of two forms: either the ability of the transfected cells to lose their contact inhibition and form dense foci, which overgrow the cell monolayer, or alternatively, the ability of the transfected cell to grow in semisolid medium and form tumours in animals. Studies using the entire viral early region of HPV16, expressed in retroviral constructs, transfected into mouse cells, demonstrated that the HPV16 genome contained a region encoding transforming activity. This caused the formation of rapidly proliferating foci capable of forming tumours in nude mice (Tsunokawa et al., 1986; Yasumoto et al., 1986; Matlashewski et al., 1987b). Transformation of primary cells, such as Baby Rat Kidney (BRK), Rat Embryo Fibroblasts (REF) and Baby Mouse Kidney (BMK) by HPV, represent more relevant assays with respect to the transforming activity in vivo. Of these, BMK and BRK cells are preferable in that the target cells are primarily of epithelial origin. Matlashewski et al. (1987b), found that the early genes of HPV16 under the control of the Moloney Murine leukaemia virus long terminal repeat (Mo MuLV-LTR) could bring about immortalisation of BRK cells in the presence of the co-operating activated oncogene, Ha-ras. The HPV types most commonly found in carcinomas (types 16, 18, 31 and 33) are able to co-operate with ras to transform primary cells, but those types most commonly found in benign lesions (types 6 and 11) are not (Pirisi et al., 1987; McCance et al., 1988; Storey et al., 1988; Woodworth et al., 1989). Dissection of the HPV16 genes found that the major activity in these assays was encoded by the E7 gene (Phelps et al., 1988; Storey et al., 1988), and indicated functional similarity between E7 and Adenovirus E1a. The sequence and functional homology between HPV16 E7, SV40 large T and
Ad E1a, was further confirmed by experiments demonstrating complementation between these different oncogenes in immortalisation assays. Cells transformed with a mutant SV40, which confers a temperature sensitivity for cell growth (Vousden and Jat, 1989), could be rescued at the non-permissive temperature, not only by the introduction of wild-type SV40 or Ad E1a, but also by the co-transfection with HPV16 E6 and E7 (although the major activity appears to be localised to E7). Using an inducible E7 expression system, E7 was also found to be continually required for maintenance of the transformed phenotype (von Knebel-Doeberitz et al., 1988; Crook et al., 1989) in cells transformed with HPV16 E7 and EJ-ras. This is consistent with continual presence of E7 in cervical tumour derived cell lines. (Schwarz et al., 1985; Smotkin and Wettstein, 1986) many years after the initial transforming event, suggesting that E7 is required to confer a growth advantage upon the transformed cells. All these studies appear to demonstrate that blocking E7 function is likely to have very good therapeutic potential, and this is a driving force for dissecting the mechanisms of action of the E7 protein.

In BRK cells the principal immortalising activity is encoded by the E7 gene, although, unlike Ad E1a, E7 does not encode a function allowing establishment of these cells in culture in the absence of a co-operating oncogene. In BMK cells, both E6 and E7 genes will co-operate with activated ras to immortalise these cells (Storey and Banks, 1993). However, the most relevant system with respect to activity of E7 in human tumours is the immortalisation of primary human keratinocytes. In these cells E7 alone can induce hyperproliferation, but these cells eventually undergo senescence. E6 alone exhibits no transforming activity. Co-transfection of E6 and E7 generated keratinocyte lines with indefinite growth
potential, demonstrating that E6 and E7 co-operate to immortalise human keratinocytes in vitro, and that the maintenance and expression of E6 and E7 in human cervical carcinomas could have pathological significance (Hawley-Nelson et al., 1989; Hudson et al., 1990; Barbosa et al., 1991; Halbert et al., 1991).

3.5.2. E7 in the Cell Cycle.

To study the role of E7 both in cell transformation and viral replication, a number of studies have been done to investigate the points of action of E7 in the cell cycle. Using an inducible expression system, it was demonstrated that E7 can induce DNA synthesis in serum starved cells, and that E7 functions at the transition from G0/G1 to S phase of the cell cycle (Banks et al., 1990a). This adds further support to the hypothesis of a common pathway of transformation shared by the DNA tumour viruses HPV, SV40 and Adenovirus. Both Adenovirus and HPV infect terminally differentiating epithelial cells that are arrested in G0. To undergo DNA replication, the virus makes use of the host enzymes for DNA synthesis. Therefore the common feature of inducing cellular DNA synthesis, with concomitant cellular proliferation, fits well with the events required for viral replication. This is, moreover, supported by a mutational analysis which confirmed that the region responsible for induction of DNA synthesis localised to the N-terminal part of the protein which shares homology with the CR1 and CR2 regions of E1a (Rawls et al., 1990). Several studies have also shown that E7 expression in keratinocytes results in a substantial increase in DNA synthesis without inducing a marked alteration in the normal pattern of keratinocyte differentiation (Blanton et al., 1992; Cheng et al., 1995).
Recently, E7 was demonstrated to be able to disrupt the response of epithelial cells to three different negative growth arrest signals: quiescence imposed upon suprabasal epithelial cells, G1 arrest induced by DNA damage, and inhibition of DNA synthesis caused by treatment with transforming growth factor β (Pietenpol et al., 1990; Hickman et al., 1994; Demers et al., 1994; Cheng et al., 1995; Morosov et al., 1997). Mutant proteins, that failed to abrogate growth arrest signals, were transformation deficient, and E7 protein from the low-risk virus types was not able to bypass any of the growth arrest signals (Demers et al., 1996). The DNA synthesis induction is due, first of all, to the effect of E7 on the function of cyclin/cdk2 inhibitors (p27 and p21). E7 expression indeed antagonises the ability of p27 to inhibit cyclin E associated kinase activity and, can overcome p27 inhibition of cyclin A gene expression (Zerfass-Thome et al., 1996). These functions correlate with the ability of the carboxy terminal half of E7 to interact with p27 (Zerfass et al., 1996). Moreover, p21-mediated growth arrest in vivo, which is inducted by DNA damage (p53-dependent pathway) (El-Deiry et al., 1993) or by TGF-β (p53-independent pathway) (Missero et al., 1995; Butz et al., 1998), can be blocked by E7 expression, via the interaction between the two proteins. This function occurs through the prevention of p21 inhibition of cyclin/cdk2 activity and the block of p21 inhibition of PCNA (Proliferating Cell Nuclear Antigen)-dependent DNA replication (Funk et al., 1997; Jones et al., 1997a). Interestingly, HPV 16 E7 induction of DNA synthesis in quiescent cells can be stimulated by certain growth factors (Morris et al., 1993), and this is the most likely explanation for the reported co-operativity between E5 and E7 (Bouvard et al., 1994b; Valle and Banks, 1995).
3.5.3. E7 Transcription Activity.

In the course of experiments initially designed to characterise the HPV16-encoded transcriptional regulatory function, a novel papillomavirus transactivating function was detected that could activate the Adenovirus E2 early promoter. This function was genetically mapped to the E7 ORF and was found to be distinct from the HPV16 E2 transactivation function, previously described (Phelps and Howley, 1987). The target in the AdE2 promoter for this novel function was similar to that required for Ad E1a activation, suggesting that these two different viral factors might act as transcriptional activators through a similar mechanism. Indeed, a common target of E1a and E7 is the RB protein. Deletion or mutation of the retinoblastoma gene, RB1, are common features of many tumours and tumour cell lines, and the loss of the RB1 gene, or the inability to synthesise the gene product p105-RB, is correlated with increased cell proliferation and oncogenesis. The RB protein binds to several critical cellular proteins, including the transcription factor E2F, myc, and D-cyclins (Bandara et al., 1991; Chellappan et al., 1991).

The E2F proteins comprise a family of proteins involved in controlling transcription of several genes that are required in DNA replication, and exists as a series of heterodimers formed by E2F and DP protein (Lam and La Thangue, 1994b). Some E2F proteins bind RB (E2F1, E2F2, and E2F3), whilst others bind only RB-related proteins, such as p107 or p130 (E2F4 and E2F5) (Cao et al., 1992; Devoto et al., 1992; Shirodkar et al., 1992; La Thangue et al., 1996; Ferreira et al., 1998). RB is phosphorylated in a cell-cycle dependent manner and, as cells approach the G1/S transition, RB is phosphorylated by a protein kinase complex consisting of a Cyclin-Dependent-Kinase (CDK) and a cyclin, although it is hypophosphorylated in G0/G1.

Aspects of the RB pathway are shown in Figure 7 (Tommasino and Crawford, 1995).

![Figure 7](image_url)

Figure 7. Schematic representation of some aspects of the pRB pathway. It shows the pRB interaction with the E2F family of transcription factors, and the function of E7, which sequesters pRB allowing release of free E2F and activation of transcription. The p53 pathway and the mechanism by which the papillomavirus, through E6 mediated p53 degradation, overcomes the tumour suppressor function are also indicated.

In cells transformed or infected with Adenovirus or SV40, the transforming proteins of these viruses form complexes with p105-RB (White et al., 1988; DeCaprio et al., 1988). It has been speculated that this interaction inactivates the RB protein, thus
mimicking the loss of the RB1 gene as seen in the genetic predisposition to retinoblastoma. *In vitro* co-immunoprecipitation mixing assays have been used by Dyson *et al.* (1989) to demonstrate that HPV16 E7 also interacts with p105-RB, underlining that all the three viruses (HPV, SV40 and Adenovirus) may use similar mechanisms in transformation, with sequestration of pRB and consequent release of free E2F, which activates transcription. Therefore, RB binding is a possible step in HPV associated carcinogenesis.

The E7 proteins of high-risk HPV types, (16 and 18), form complexes with p105-RB with equal affinities. The E7 proteins, encoded by the HPV types 6b and 11, which are associated with clinical lesions with a lower risk of progression, bind to pRB with lower affinity (Münger *et al.*, 1989b; Gage *et al.*, 1990). This characteristic may help to explain the difference in the oncogenic potential of the oncogenic and non-oncogenic types of genital papillomaviruses (Münger *et al.*, 1989b). The domain of pRB interaction has been mapped on E7 to a small stretch of amino-acids (LXCXE) surrounding the cysteine residue at sequence position 24 (C24) and the glutamic acid at sequence position 26 (E26) (Münger *et al.*, 1989b; Gage *et al.*, 1990; Dyson *et al.*, 1992).

HPV16 E7, like SV40 LT, has been demonstrated to bind preferentially to the underphosphorylated form of RB (Imai *et al.*, 1991), which is considered to be the active form, restricting cell proliferation, whereas E1a has been shown to bind both forms of pRB (Ludlow *et al.*, 1989; Whyte *et al.*, 1989). It is interesting to note that the underphosphorylated form of pRB is the predominant form found in differentiating keratinocytes (Nead *et al.*, 1998). Taking into account the cell-cycle
dependent phosphorylation of RB, the effect of the E7/RB interaction is to release free E2F, which is then able to activate the transcription of genes required in cell cycle control, DNA synthesis and cellular proliferation (Phelps et al., 1991; Bandara et al., 1991; Hiebert et al., 1991; Mudrji, et al., 1991; Shirodkar et al., 1992). These include DNA polymerase α (Polα), thymidine kinase (TK), dihydrofolate reductase (DHFR), c-myc and c-myb. PCNA is also upregulated in an E7-dependent manner (Cheng et al., 1995), but it is not clear at present whether this is as a direct result of transcriptional activation by E2F or by another protein (Huang and Prystowsky, 1996). More interestingly, the stimulation of E2F responsive promoters by E7 is not solely due to the release of free E2F. In G1 phase, pRB actively represses genes required during G2 by recruiting a histone deacetylase (HDAC1) (Brehm et al., 1998; Magnaghni-Jaulin et al., 1998). Thus, G2 phase-specific, E2F1-responsive promoters are suppressed during G1 by the E2F/pRB/HDAC1 complex at the promoter. It is thought that HDAC1 might convert surrounding chromatin from a transcriptionally active (hyperacetylated) to a transcriptionally repressed (hypoacetylated) state, and E7 has been shown to be capable of disrupting this complex (Brehm et al., 1998, 1999).

It is thought that complex formation of viral oncoproteins with pRB and p53 (Lane and Crawford, 1979; Sarnow et al., 1982; Werness et al., 1990) prevents these proteins from performing their normal cellular function, and therefore accounts at least in part for the transforming properties of the various viral oncoproteins. Moreover E7 stimulates DNA replication in growth-arrested rodent cells in the absence of any additional stimuli (Sato et al., 1989a; Banks et al., 1990a), and this activity correlates with the ability of E7 to bind pRB (Banks et al., 1990b) and
release E2F (Pagano et al., 1992; Morris et al., 1993). Although interaction with pRB may appear to be sufficient to release functional E2F, recent studies have indicated that E7 can directly target pRB for degradation. Infection of human keratinocytes with recombinant retroviruses expressing HPV16 E7 produce a reduction in pRB levels (Demers et al., 1994), and in E7 immortalised human mammary epithelial cells, pRB protein level are reduced, even though RB mRNA levels remains high (Wazer et al., 1995). More recently, it has been shown that E7 can target pRB for degradation, mediated via the ubiquitin-dependent proteolysis system (Boyer et al., 1996). This was further supported by the demonstration that E7 interacts with the S4 subunit of the 26S proteasome (Berezutskaya and Bagchi, 1997). As a consequence of this interaction there is an increase in the ATPase activity of the S4 enzyme, which is involved in the assembly of the 26S proteasome, and this suggests a mechanism by which E7 may stimulate the degradation of pRB.
3.5.4. Mutational Analysis of E7.

A point mutational analysis of HPV16 E7 (Edmonds and Vousden, 1989) revealed important features regarding the transactivation and transforming functions of the protein (Figure 8).

Figure 8. Schematic representation of the HPV16 E7 protein, showing the domains responsible for pRB binding and CKII phosphorylation.

pRB expressing constructs, carrying mutations in the region of E1a/LT homology (pRB binding mutants, p24Gly and p26Gly; the phosphorylation defective mutant, p31Arg), are severely reduced in transactivation activity; although, p24Gly and p26Gly are negative for transformation, and p31Arg, retains transforming activity. Mutants with lesions in the acidic stretch of amino-acids characteristic of the CKII site (p35/36Asp/His) retain a low level of transactivating activity, and are transformation competent. The replacement of the two phosphorylated serines by uncharged alanine residues drastically reduces the ability of E7 to co-transform primary cells with ras, whereas negatively charged aspartic acid at the same positions produces only a slight effect. This difference is not reflected in the
p105/RB binding, suggesting that negative charges at position 31/32 provided either by phosphorylation or by negatively charged amino-acids are necessary for efficient transformation, without significantly affecting pRB binding (Edmonds and Vousden, 1989; Barbosa et al., 1990; Firzlaff et al., 1991). Neither phosphorylation of E7 nor its ability to transactivate are required for transformation (Edmonds and Vousden, 1989; Watanabe et al., 1990), and the domains required for the transactivation function are not fully separable from those required for transformation. All these data indicate that efficient transactivation is not required for transformation by HPV16 E7 (Storey et al., 1990).

Although focal transformation of rat 3Y1 cells by E7 is eliminated by changing His2 to Asp or Cys24 to Gly (pRB binding) and is greatly impaired by changing Cys61 or Cys94 to Gly, the transforming function survives mutations at Leu13 and Cys68 and deletion of Asp-Ser-Ser (amino acids 30 to 32) (Watanabe et al., 1990). Mutations which alter cysteine residues in the Cys-X-X-Cys motif decrease transformation markedly, although they do not abolish it entirely, and display a decreased ability to transactivate the AdE2 promoter (Edmonds and Vousden, 1989; Watanabe et al., 1990; Demers et al., 1996). Mutations in the second region (Edmonds and Vousden, 1989), which affect only one of the two Cys-X-X-Cys motifs (p58Gly, p91Gly, and p88End), severely reduce but do not abolish transformation. Double mutants, in which both Cys-X-X-Cys motifs are disrupted (p58+88Gly+End and p58+91Gly+Gly), also retain a very low level of transforming activity, thus the presence of the two repeats enhances the transforming potential, but is not essential for it.
Studies with chimaeric E7 genes, constructed by the exchange of the amino and carboxyl coding halves of the HPV6 and HPV16 E7 genes, suggested that the amino-terminal half of E7 determines the affinity for binding to pRB, the transformation properties and the ability to abrogate transforming growth factor β (TGF-β)-mediated repression of the c-myc promoter. These experiments also underlined that the ability to transactivate the AdE2 promoter is a common property of E7 proteins of both the low-risk and the high-risk HPV types (Münger et al., 1991). In agreement with earlier work (Clemens et al., 1995), the transactivation domain of E7 has been mapped primarily to the amino-terminal 37 amino-acids of the protein, while the carboxy-terminus (amino acids 38-98) was found to have a much lower activation potential, although recent studies have revealed the presence of a possible second pathway for transcriptional activation by HPV16 E7, located in the C-terminus part of the protein, independent of its interaction with pRB-E2F complexes (Zwerschke et al., 1996). While the transcriptional activation function has been clearly demonstrated in the heterologous system of the Adenovirus, its role in the transcriptional regulation of the HPV genes themselves remains to be determined. In fact, E2F binding sites have not been found in the HPV16 and HPV18 genomes, nor has E7 been demonstrated to regulate the P105 or P97 promoters (Phelps et al., 1991).

Fragments of E7 protein derived from its conserved region 2 domain exhibit reduced affinity for pRB compared with the full-length protein and do not dissociate the pRB/E2F complex, suggesting the presence of additional contact sites between E7 and pRB. A number of studies (Jones et al., 1990; Patrick et al., 1994) have
identified a region of E7, distinct from the CR2 domain, which is sufficient to bind pRB, and which encompasses the zinc-binding conserved region 3 domain. The amino-acids on pRB responsible for binding E7 are localised on residues 649-772 in the B pocket of the protein (Kaelin et al., 1990; Hu et al., 1990), and mutations in these regions of pRB are found in many tumours. These results are consistent with a model in which optimal binding of E7 to pRB requires at least two distinct contact sites: the identified high affinity interaction CR2 and pRB pocket region, and a second interaction between CR3 of E7 and the COOH-terminal region of pRB. The latter interaction is sufficient for E7’s inhibition of E2F binding to pRB.

The CRPV (Cottontail Rabbit Papillomavirus) E7 protein also interacts with pRB in a manner similar to that of HPV E7 (Haskell et al., 1993), and CRPV E7 mutants defective for this interaction are nonetheless able to induce wart formation in the infected rabbits (Defeo-Jones et al., 1993). Moreover, the interaction of HPV E7 and pRB has been found to be insufficient for efficient transformation of NIH3T3 (Banks et al., 1990b). Thus, mutations in the extreme amino terminus of the protein (CR1) lead to a loss or reduction of transforming activity even while retaining wild type levels of interaction with pRB (Banks et al., 1990b; Barbosa et al., 1990; Watanabe et al., 1990). Again, mutants of E7 defective in pRB binding are still able to immortalise keratinocytes (Jewers et al., 1992). These observations demonstrate that, although the pRB interaction is vital for a number of E7’s activities, interactions with other cellular proteins play a part in E7 functions.

Two other RB related proteins, p107 and p130, negatively regulate the transcriptional activity of members of the E2F family (Dyson et al., 1992; Schwarz et al., 1993; Mayol et al., 1993), probably by recruiting histone de-acetylases
Ferreira et al., 1998), and overexpression of either p107 or p130 can cause a cell cycle block. p130 exerts its transcriptional regulatory function during the G0/G1 transition (Cobrinik et al., 1993), while p107 is active in the G1/S transition and in the G2 phase. p107 binds to cyclin E or cyclin A/CDK2 in late G1 and in S phase, but it is not yet clear what role the two cyclins have in this complex (Motokura and Arnold, 1993). HPV16 E7 binds both p130 and p107 proteins (Davies et al., 1993) and the E7s from the low-risk HPVs bind p107 with a lower affinity than those from the high risk E7s, as found for RB. In the S phase of the cell cycle, E7 binds the cellular complex p107/cyclinA/CDK2; this S phase cellular complex is normally associated with E2F, as shown in Figure 9 (Tommasino and Crawford, 1995).

Figure 9. Schematic representation of the p107 pathway together with the ones of the cyclins A and E.
Under these conditions, E7 and E1a seem to behave differently. When E1a binds p107 it releases free active E2F, whereas E7, although it associates with p107, does not cause dissociation of the p107/E2F complex (Pagano et al., 1992; Arroyo et al., 1993). Recent reports suggest that E1a is able to disrupt both p107/E2F complexes of G1 and S phases. In contrast E7, although it binds both p107/E2F complexes efficiently, causes dissociation of the G1 phase complex only. This suggests that E1a and E7 may function in cellular transformation in similar, but not identical ways (Zerfass et al., 1995). Moreover, the indications are that E7/pRB binding is more important for transformation than the interaction between E7 and p107. This is supported by the fact that there is no evidence that p107 is a tumour suppressor protein.

HPV 16 E7 has also been reported not only to be able to repress the inhibitory function of cyclin/cdk (Zehbe et al., 1999), but also to be involved in controlling other cell cycle checkpoints, after DNA replication. In transformed BRK cells, E7 expression leads to an increase in the levels of cyclin E and cyclin A, but not cyclin D1, activating sequentially both the cyclin E and the cyclin A promoters (Zerfass et al., 1995; Schulze et al., 1998). Increased levels of cyclin E expression require sequences within the CD2 domain of E7, while cyclin A up-regulation require sequences in both CD1 and CD2 domains of E7. HPV E7 is able to bind cyclin A in a cell-cycle dependent manner during the S phase (Tommasino et al., 1993), and may interact with the associated kinase complex through p107, although some in vitro data with mutants of cyclin A support the idea that the interaction could also be direct (Tommasino et al., 1993; Ciccolini et al., 1994). Although wild type cyclin A binds E7, various deletion mutants that lack the ability to bind and to activate
p34/cdc2 and p33/CDK2 kinases, also fail to bind E7. This suggests that a similar conformation of cyclin may be required both for binding E7 and for associating with p34/cdc2-related kinase(s) (Tommasino et al., 1993). Mutational analysis of E7 has show that an amino-acid substitution (Glu 26 to Gly) which had no effect on binding to p107, completely abolishes the ability of E7 to bind the cyclin/CDK complex (Davies et al., 1993). CyclinA/CDK2 binds directly to the N-terminus of E2F in S phase, with a consequent loss of E2F-DNA binding activity and a shut-down of transcription. E7 may have a role in this pathway, which controls the fidelity and the timing of DNA replication. Again as with the p107 complex unlike Adenovirus E1a, which dissociates the cyclin A complex to release free E2F, E7 does not dissociate the complex but becomes part of it (Arroyo et al., 1993). In this way, E7 may prevent the inactivation of the E2F in S phase, allowing the cell to escape the normal checkpoints, with consequent loss of the DNA replication fidelity. In support of this E7 is implicated in induction of chromosomal abnormalities (White et al., 1994; Thomas and Laimins, 1998). The constitutive activation, in E7 expressing cells, of b-myb (Lam et al., 1994a), which is regulated by p107/cyclin A/E2F complexes, may be also explained by the overriding of the cell cycle control of this complex by E7.

A recent report has demonstrated binding between HPV18 and 16 E7 and cyclin E, which controls the G1/S transition (McIntyre et al., 1996; Martin et al., 1998); this binding is mediated by p107. Both E7/cyclin E and E7/cyclin A complexes exhibit kinase activity through associated CDK2 proteins, which can contribute to phosphorylation of p107. Another explanation of the E7/cyclin interaction may be that E7 can target the catalytic subunit to other specific substrates; this might lead to
phosphorylation of viral proteins such as E1, thus stimulating virus DNA replication. This would be analogous to the regulation of SV40 DNA replication by p34/cdc2 through phosphorylation of the viral LT antigen (McVey et al., 1989).

Considering that HPVE7 is not able to bind to the DNA, it is obvious to think that the protein could exert its numerous functions through several pathways involving protein-protein interactions. The objective of my research was to find a potential new role of E7 in the transcriptional machinery cascade, constituted from a incredibly number of transcription factors, which have been already shown to be involved in several interactions with some of other viral oncogenes. Between the numerous already studied binding that E7 has with a great number of cellular proteins, the one with TBP, analysed in this thesis, could give some efforts in the studies which regard the transcriptional and transformational pathways of E7. The role that E7 could indeed play bringing TBP on its DNA binding motif or displacing TBP from its consensus, or sequestering TBP from other complexes, could be of extremely importance in the ability of the protein to activate or repress downstream genes, and to switch off or on other pathways involved in the development of the viral life process in the host cell.
MATERIALS AND METHODS

1. RECOMBINANT DNAs.

1.1. GST Expression Constructs.

The HPV16 E7 DNA was produced by PCR using the following oligonucleotides: ACGTAGGGATCCCATGTAATC at the 5’ end with the Bam H1 restriction site, and CTCTTCCGAAATTCTGACCTAAG at the 3’ end with the EcoRI restriction site. The 340 bps fragment obtained was further cloned in-frame in the pGEX-2T plasmid (Smith and Johnson, 1988) (Figure 10) at the corresponding restriction sites, to generate the construct pGEX2T16E7 which produces a fusion protein between glutathione S-transferase (GST) and E7.
Figure 10A, B. PGEX fusion protein expression vectors which express a cloned gene as a fusion protein to GST. The lac repressor (product of the lacI gene) binds to the P_{lac} promoter, repressing expression of GST.

The HPV11 E7 DNA was produced by PCR using the following oligonucleotides: GTGGAAGGATCCTTGCTTACACTG at the 5’ end with the Bam H1 restriction site, and TACCGAATTCGTCCGCCATCCTTG at the 3’ end with the EcoR1 restriction site. The 303 bps obtained fragment was cloned in-frame in the pGEX-2T plasmid at the corresponding restriction sites.

The HPV16 E7 5’ half DNA, constituted by the first 150 bps, was obtained by in vitro directed mutagenesis using PCR with the following oligonucleotides: ACGTAGGGATCCCATCCTGGTAATC at the 5’ end with the BamH1 restriction site, and CTGGAATTCCAGCTGGACCATCTAT at the 3’ end with the EcoR1 restriction site. The mutated DNA fragment was cloned in-frame in the pGEX-2T plasmid at the correspondent restriction sites.

The HPV16 E7 3’ half DNA, constituted by the last 192 bps, was obtained by in vitro directed mutagenesis using PCR with the following oligonucleotides: CCAGGATCCCAAGCAGAACCAGAC at the 5’end with the BamH1 restriction site, and CTCTTCCGAATTCGTACCTGCAGG at the 3’ end with the EcoR1
The restriction site. The mutated DNA fragment was cloned in-frame in the pGEX-2T plasmid at the correspondent restriction sites.

The **HPV16 E7 p31/32** mutation DNA, characterised by the substitution of the two Serines with Arginine at position 31 and Proline at position 32 (described before by Barbosa *et al.*, 1990), was produced by PCR using the following oligonucleotides: ACGTAGGGATCCACGTGAAT TC at the 5’ end with the BamH1 restriction site, and CTCTTCCGAATTCCGTACCTGCAGG at the 3’ end with the EcoRI restriction site. The 340 bps fragment obtained was cloned in-frame in the pGEX-2T plasmid at the corresponding restriction sites.

The **HPV16 E7 Aspartic Acid** mutation, characterised by the substitution of the two Serines at positions 31 and 32 with Aspartic Acids (described before by Firzlaff *et al.*, 1991), was obtained by *in vitro* directed mutagenesis using PCR with the following internal oligonucleotides: TTCATCCTCCTCCTCGTCGTCGTCATT at the 5’ end and GAGCAATATAATGACGACGACGACGAGGAG at the 3’ end. The external oligos were the same as the wild type 16E7: ACGTAGGGATCCACGTGAATTC at the 5’ end with the BamH1 restriction site and CTCTTCCGAATTCCGTACCTGCAGG at the 3’ end with the EcoRI restriction site. The mutated DNA fragment of 340 bps was cloned in-frame in the pGEX-2T plasmid at the corresponding restriction sites.

The **HPV16 E7 Δ1** (deletion aa52-56), Δ2 (deletion aa 65-67), Δ3 (deletion aa 75-77), and Δ4 (deletion aa 79-83) DNAs, were obtained using PCR with the following oligonucleotides: ACGTAGGGATCCACGTGAATTC at the 5’ end with the BamH1 restriction site, and CTCTTCCGAATTCCGTACCTGCAGG at the 3’ end with the EcoRI restriction site. The template DNAs pJ4Ω E7 Δ1, Δ2, Δ3 and Δ4 were available in the laboratory. The construct have been done by Dr. David Pim.
The mutated DNA fragments of respectively 325 bp, 331 bp, 331 bp, and 325 bp were cloned in-frame in the pGEX-2T plasmid at the corresponding restriction sites.

The **HPV16 E7 Ser 71** DNA, characterised by the substitution of the Serine at the position 71 with a Glycine (described by Storey *et al.*, 1990), was produced by PCR using the following oligonucleotides: ACGTAGGGATCCCCAGCTGTAATC at the 5’ end with the BamHI restriction site, and CTCTTCCGAATTCTACCTGAGG at the 3’ end with the EcoRI restriction site. The template of mutated DNA was available in the laboratory. The 340 bps fragment obtained was cloned in-frame in the pGEX-2T plasmid at the corresponding restriction sites.

The **HPV16 E7 3’ end Ser 71**, characterised by the Ser71 mutation inserted only in the 3’ half of the E7 protein, was produced by PCR using the following oligonucleotides: CCAGGATCCCAAGCAGAACCGGAC at the 5’ end with the BamHI restriction site, and CTCTTCCGAATTCTACCTGAGG at the 3’ end with the EcoRI restriction site. The 192 bps fragment obtained was cloned in-frame in the pGEX-2T plasmid at the corresponding restriction sites.

The **Adeno 5 E1a** DNA was produced by PCR using the following oligonucleotides: ATTCGATCGGATCCAAATGAGACATATT at the 5’ end with the BamHI restriction site, and TACGTACCAGAATTCCGTACTACTAT at the 3’ end with the EcoRI restriction site. The 795 bps fragment obtained was cloned in-frame in the pGEX-2T plasmid at the corresponding restriction sites.

The **TATA Box Binding Protein** DNA was produced by PCR using the following oligonucleotides: CGCAAGGGATCCTGGTTTGCCAAG at the 5’ end with the BamHI restriction site, and GGTACATGAATTCCATTACGTCGT at the 3’ end with the EcoRI restriction site. The template DNA was obtained by reverse transcription of HeLa mRNA using the same oligonucleotides followed by PCR.
amplification and subsequent cloning into an expression vector. The 1057 bps fragment obtained was cloned in-frame in the pGEX-2T plasmid at the corresponding restriction sites.

The **GST16 E1** construct was available in the laboratory (Storey *et al.*, 1995).

The **TBP mutant 1** DNA, characterised by the deletion of aa 1-201, was obtained by *in vitro* directed mutagenesis using PCR with the following oligonucleotides: GCTGCAGGGATCCATGAGGATAAGA at the 5’ end and GGTACATGAAATTCATTACGTCGT at the 3’ end. The mutated DNA fragment of 426 bps was cloned in-frame in the pGEX-2T plasmid at the BamH1 and EcoR1 restriction sites.

The **TBP mutant 2** DNA, characterised by the deletion of aa 100-339, was obtained by *in vitro* directed mutagenesis using PCR with the following oligonucleotides: CGCAAGGGATCCTGGTTTGCCAAG at the 5’ end, and AGGCGTGTGAATTCATTACGTCGT at the 3’ end. The mutated DNA fragment of 343 bps was cloned in-frame in the pGEX-2T plasmid at the BamH1 and EcoR1 restriction sites.

The **TBP mutant 3** DNA, characterised by the deletion of aa 272-339, was obtained by *in vitro* directed mutagenesis using PCR with the following oligonucleotides: CGCAAGGGATCCTGGTTTGCCAAG at the 5’ end, and TGGCTCAGAATTCATTACGTCGT at the 3’ end. The mutated DNA fragment of 876 bps was cloned in-frame in the pGEX-2T plasmid at the BamH1 and EcoR1 restriction sites.

The **TBP mutant 4** DNA, characterised by the deletion of aa 298-339, was obtained by *in vitro* directed mutagenesis using PCR with the following oligonucleotides: CGCAAGGGATCCTGGTTTGCCAAG at the 5’ end, and
AATTCTGGAATTCTCATTCTGTAG at the 3' end. Further the mutated DNA fragment of 922 bps was cloned in-frame in the pGEX-2T plasmid at the BamH1 and EcoRI restriction sites.

The **human p53** DNA was produced by PCR using the following nucleotides oligonucleotides: CTGGTTCGCGTGGATCCCGGGTCACTGCCATG at the 5' end with the Bam HI restriction site, and AAGTGGAATTCTCAGGTGAC at the 3' end with the EcoR1 restriction site. The 1237 bps fragment obtained was cloned in-frame in the pGEX-2T plasmid at the corresponding restriction sites.

### 1.2. *In vitro* Expression Vectors.

To obtain the **SP64 16E7** construct for expression of protein *in vitro* the HPV16 E7 above cloned in pGEX-2T plasmid was subcloned into SP64 expression vector (Melton *et al.*, 1984) (Figure 11), at the BamH1 and EcoR1 restriction sites.

![Figure 11. pSP64 vector, which is carrying the promoter for the bacteriophage-encoded DNA-dependent SP6 RNA polymerase transcription unit.](image)
To obtain the **SP64 TBP** the full length TBP clone was obtained by reverse transcription of HeLa mRNA as described before, followed by amplification obtaining the fragment of 1057 bps which was subsequent cloning into plasmid SP64, at the BamH1 and EcoR1 restriction sites.

The **carboxy terminal TBP** expression plasmid was kindly provided by T. Kouzarides and encodes amino acids 165-339 of TBP in pING (Metz et al., 1994).

The **SP64 16 E2, SP64 18 E6** and the **SP64 p53** (Bouvard et al., 1994a; Pim et al., 1994) were available in the laboratory.

### 1.3. *In vivo* Eukaryotic Expression Vectors.

For eukaryotic expression **pJ4Ω** (Matlashewsky et al., 1987b) and the **pJ4Ω16 E7, pJ4Ω11 E7, pJ4Ω26 E7 and pJ4Ω18 E7** (Storey et al., 1988) were available in the laboratory, as was the **Adenovirus E2 CAT** reporter plasmid (Murthy et al., 1985; Storey et al., 1990), the **EJ-ras** expressing plasmid (Matlashewsky et al., 1987b), the plasmid expressing the G418 (Geneticin) selectable marker, **pSV2neo**, the p53 expression vector **RSVp53**. The **pG13 CAT** p53 responsive CAT reporter plasmid was kindly provided by Bert Vogelstein (Kern et al., 1992; Steegenga et al., 1996).

The HPV16 E7 mutants, called **566** (aa 2 His-Pro), **631** (aa 24 Cys-Pro), **638** (aa 26 Glu-Gly), **p32** (aa 32 Ser-Trp), **p31/32** (aa 31 Ser-Arg and aa 32 Ser-Pro), **p63** (aa Ser-Ala), **p71** (aa 71 Ser-Gly), **p95** (aa 95 Ser-Ala), were all described previously (Edmonds and Vousden, 1989; Banks et al., 1990b; Barbosa et al., 1990) and were available in the laboratory cloned in **pJ4Ω**. To obtain the **pJ4ΩΔ1, pJ4ΩΔ2, pJ4ΩΔ3, pJ4ΩΔ4**, all the previous E7 mutants cloned in the pGEX-2T plasmid were subcloned, into pJ4Ω plasmid at the BamH1 and EcoR1 restriction sites.
1.4. **Large Scale DNA Amplification and Purification.**

All the plasmids used in the project were amplified in Luria Broth supplemented with 50μg/ml of Ampicillin. The bacteria were lysed by alkaline solutions (Birnboim and Doly, 1979), and the closed circular DNA was purified by centrifugation to equilibrium in cesium chloride-ethidium bromide gradients (Maniatis *et al.*, 1982).

1.5. **Small Scale DNA Amplification and Purification.**

The small scale DNA amplification and purification was done also in presence of alkaline lysis, followed by a phenol/chloroform extraction and ethanol precipitation supplemented with 0.3M Na Acetate (Maniatis *et al.*, 1982).

1.6. **Polymerase Chain Reaction.**

The GST constructs and the *in vivo* expression constructs were obtained by polymerase chain reaction using a mix with 500ng of the template DNA, the specific primers at the concentration of 1μM each, a concentration of 400 μM of each dNTP, 1 unit of enzyme (Taq polymerase, by Perkin Elmer) and the PCR buffer (final concentration of 50mM KCl, 10mM Tris pH 8.3, 1.5mM MgCl₂, 0.01% Gelatin). The reactions were completed with a total of 30 cycles of the Perkin Elmer thermal-cycler instrument. To maximise the specificity, the annealing reaction was carried out for 1min. at the highest temperature permitted by the primers. All the final products were controlled in agarose gel electrophoresis.

The constructs for which the template DNAs were not available has been obtained by M-MuLV (Moloney Murine Leukemia Virus) **Reverse Transcriptase** reaction, using HeLa cells RNA as template with the same PCR conditions of the other
reagents in the reaction, carried out in the specific buffer (50 mM Tris/HCl pH 8.3, 8mM MgCl₂, 10 mM dithiothreitol). The mix was heated at 56 °C for 5 mins. The enzyme was added and the reaction was completed at 42 °C for 1 h. The product of the transcription was used as template for the final PCR reaction. All mutants were verified by DNA sequencing, following the Sequenase kit version 2.0 protocol using the T7 DNA polymerase (United States Biochemical).

2. CELL CULTURE TECHNIQUES.

All cells used during the project were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) of heat inactivated (56 °C for 30 mins.) Foetal Calf Serum, 1% (v/v) Glutamine, and 200 units/ml Penicillin plus 100 µg/ml Streptomycin. Cells were grown at 37 °C in 10% CO₂.


The cell lines used during the project were the following:

**Cos-1** → fibroblast-like cell line established from simian cells CV-1, which were transformed by an origin-defective mutant of SV40 which codes for wild-type T antigen (Gluzman, 1981).

**HT1080** → cell line derived from human fibrosarcoma (Rasheed et al., 1974). These cells do not contain HPV genome DNA.

**Saos-2** → cell line derived from primary human osteogenic fibrosarcoma (Fogh and Trempe, 1975). These cells are pRb null and p53 null.

**NIH 3T3** → Embryo, contact-inhibited, NIH Swiss mouse cells.
CaSki ➞ cell line derived from human cervical epidermoid tumour (Smotkin and Wettstein, 1986). These cells have contain an integrated HPV16 genome, about 600 copies per cell. They are a source of high level E7 expression.

BRK ➞ Baby Rat Kidney primary cells derived from the kidneys of 9 day-old Wistar rats.

14/2 ➞ BRK cell line transformed with a construct containing dexamethasone inducible HPV16 E7 (Crook et al., 1989),

HaCat ➞ Immortalised epidermal keratinocytes.

2.2. Extraction of BRK Primary Cells.

The BRK cells were obtained from the kidneys of 9 day-old Wistar rats. The tissues were first washed several times in DMEM without serum, then homogenised and finally trypsinised using 0.25% Trypsin solution in PBS. After at least three steps of trypsinisation, 15mins. each at 37 °C, the reaction was blocked with 50% Foetal Calf Serum. The cells were then centrifuged for 10mins. at 1200 r.p.m. and the pellet was resuspended in DMEM supplemented with 10% FCS, 1% Glutamine, 200 Units/ml Penicillin and 100 µg/ml Streptomycin. From one rat we normally obtained enough cells to plate four tissue culture dishes of 100mm in diameter. After two days in culture the BRK cells are ready to be transfected.

2.3. Transfection of BRK Primary Cells for Transformation Assays.

The transfection was carried out using the standard calcium phosphate precipitation procedure (Matlashewski et al., 1987b). The transformation assays were done by co-transfecting the cells plated on 100mm dishes with 5 µg pJ4Ω16 E7 or the appropriate mutant, together with 5µg EJ-ras expressing plasmid and 3 µg pSV2neo
expressing the G418 (Geneticin) selectable marker (Southern, 1982). After glycerol treatment the cells were cultured in DMEM containing 10% Foetal Calf Serum and 200 μg/ml of G418 selection and the colonies allowed to develop. After about two weeks the dishes were fixed with 10% Formaldehyde (Carlo Erba) and stained with 10% Giemsa-Blue (Merck), and colonies were counted. In parallel other BRK cells were transformed with the same plasmids to obtain stable lines expressing the E7 wild type and the mutants. To assess the capacity of the cells to express the proteins of interest we tested each line, derived from a pool of colonies, by Western Blot and immunoprecipitation analyses using a polyclonal anti-GST16E7 antibody.

3. CAT ASSAY.

3.1. p53 CAT Assay.
Saos-2 cells plated at the density of 1 X 10^5 in 60mm tissue culture dishes the day before were transfected with 1μg of the p53-responsive CAT reporter plasmid, pG13CAT, plus 1μg of p53 expression plasmid, RSVp53. The co-transfection was done with increasing amounts of pJ4Ω16 E7 (1, 5, 10 μg) or 10 μg of different E7 proteins. Cells were harvested after 48 hours in 200 μl of 40mM Tris/HCl pH 7.5, 150 mM NaCl, 1mM EDTA and subjected to three freeze-thaw cycles, followed by incubation at 65 °C for 10mins.. Samples were clarified by centrifugation at 14000r.p.m. in a microfuge and protein concentration was estimated by the Bio-Rad protein assay. CAT assays were routinely performed with 1-5 μg of protein incubated with 2.5 μl acetyl-CoA (33.3 mg/ml) and 1.5 μl [14C] chloramphenicol (50 mCi/mmol; Amersham) in a final volume of 50 μl at 37 °C for 30-60mins..
Following extraction with ethyl acetate, samples were analysed by thin layer chromatography and visualised by autoradiography. Samples were quantified with a Packard Instant Imager. Each CAT assay was repeated at least three times to obtain the mean percentage CAT conversion. The p53 CAT assays (Figures 27A,B and 28A,B, pp.83-86 of the Results section) have been done by my Director of Studies Dr. Lawrence Banks.

3.2. Adenovirus E2 CAT Assay.

NIH 3T3 cells plated at the density of $1 \times 10^5$ in 60mm tissue dishes the day before were transfected with $3 \mu g$ of the Adenovirus E2 CAT reporter plasmid together with $5 \mu g$ of pJ4Ω control, $5 \mu g$ of pJ4Ω16 E7 and $5 \mu g$ of all the other E7 mutants to be tested expressed in pJ4Ω vector. After 48 hours the cells were harvested and the CAT activity was measured as described above.

4. BROMO-DEOXYURIDINE INCORPORATION.

To determine the different cell cycle phases of the 14/2 cells used for the *in vitro* and *in vivo* phosphorylation experiments, a BUDR incorporation assay was performed on the cells. These were plated on cover slips in a concentration of $3 \times 10^5$ cells/30mm dish. The day after the cells were subjected to serum starvation (medium supplemented with 0.2% Foetal Calf Serum) for a period of 48 hours. Medium was then replaced with 10% FCS for a period of 8 hours. Time points were taken with 1 hour incubation with 20mM of Bromo-deoxyuridine (Sigma). The time points were at 0h, 2h, 4h, and 8 h post addition of serum. Simultaneously a cover slips with cells not subjected to any serum starvation was also used to assess the
level of BUDR incorporation. An immunofluorescence assay was then performed. The cells were fixed with 3.7% Para-formaldehyde (Carlo Erba) for 20mins. at room temperature, and subjected to a denaturation step with 0.5 NaOH (Sodium Hydroxide) in PBS for 10seconds. A neutralisation passage was performed with 0.1 M Glycine/PBS for 5mins. followed by permeabilisation with 0.1% TRITON X-100 in PBS for 2mins. Cells were incubated with anti-BUDR antibody (Sigma, IGγ2 epitope) diluted 1:5 in PBS supplemented with 3% BSA (Bovine Serum Albumine) for 1hour at 37 °C in a humid chamber. After extensive washing with PBS, RITC (Rhodamine)-conjugated anti-IGγ2 antibody was added, diluted 1:50 in PBS/3% BSA for 30mins. Finally, after washing with PBS, cells were stained with Hoechst solution for 10mins., cover slides were mounted with (KPL, Mounting Medium) and analysed with a LEITZ UV-microscope. A number of 20 fields was counted and the means of the percentage cells BUDR positive versus the number of total cells were calculated for each time point.

5. PROTEIN EXPERIMENTS.

5.1 Production and Purification of GST Fusion Protein.

For the production of the GST fusion proteins *Escherichia coli* DH5-α rendered competent by the MgCl₂ and CaCl₂ method, (Maniatis *et al.*, 1982) were transformed with pGEX-2T expression plasmids. Overnight cultures at 37 °C in 100ml of Luria Broth supplemented with 50μg/ml ampicillin, were diluted 1:10 and grown until the optical density at 600 nm reached 0.6 (about 1h). Proteins were then induced by the addition of 1mM IPTG (iso-propyl-β-D-thiogalactopyranoside) for 3 hours. Bacteria were then harvested by centrifugation in a Sorvall GSA rotor at
4000 r.p.m. for 10 mins. The pellet was resuspended in 10 ml of lysis buffer (20 mM HEPES pH 7.2, 100 mM KCl, 5 mM MgCl₂, 0.5% TRITON X-100, 0.15% NP40, 100 U/ml DNAse I, 1 mM PMSF (phenylmethylsulfonylfluoride), 1% Aprotinin, 5 μg/ml Leupeptin). Cells were then lysed by sonication and clarified from the insoluble material by centrifugation in a Sorvall SS 34 rotor at 10000 r.p.m. for 15 mins. The supernatant was then incubated with glutathione agarose beads (Sigma) at 4 °C for 1 h. The beads were then washed at least three times with 10 bead volumes of lysis buffer. The purity of the proteins was monitored by SDS-PAGE gel and staining with either Coomassie blue or the Bio-Rad silver stain system.

5.2 Production of Polyclonal Antibodies against HPV16 E7.

Polyclonal antibodies against HPV16 E7 were obtained using GST.16E7 fusion protein purified from *Escherichia coli*, eluted from the GST beads with 15 mM reduced glutathione in 50 mM Tris pH. For the first immunisation 200 μg of purified protein was injected into two New Zeland rabbits in incomplete Freund’s adjuvant. In the following injections (about 6) 100 μg of protein were used every 21 days. The method of covalent coupling proteins to glutathione beads by dimethylsulphimidate was applied to affinity purify the antibodies, and their subsequent elution was performed on the same column with glycine/HCl 0.2 M solution. The specificity of the antisera was tested with several immunoprecipitations performed on HPV16 E7 translated *in vitro*, and on labelled CaSki cell extract (Smotkin and Wettstein, 1986) in comparison with the pre-immune antibody. In parallel, Western Blot analysis was done on the purified antigen used for the immunisation, at different concentrations and on total cell
extract of CaSki cells. Purified antibody was aliquoted and stored at –80 °C or at 4°C with 0.2% Sodium Azide (NaCN₃).

5.3. Transcription and Translation of Proteins \textit{in vitro.}

Proteins were expressed using the TNT coupled reticulocyte lysate transcription-translation system as specified by the manufacturer (Promega). The pSP64 constructs were used as templates for the transcription translation in a total amount of 500 ng DNA, plus addition of 0.5 µl of TNT buffer, 0.5 µl of amino acids minus cysteine 1mM (final concentration 20 µM), 0.5 µl of RNase inhibitors (40 Units/µl), 12.5 µl of Reticulocyte lysate, 2 µl of $^{35}$S Cysteine 10mCi/ml (final concentration 0.3 mCi/ml) and 0.5 µl of SP64 RNA Polymerase in a final reaction volume of 25 µl. The transcription translations were carried out for 2 hours at 30°C and the final products were assayed by SDS-PAGE and autoradiography. Samples were quantified with a Packard Instant Phosphor Imager.

6. BINDING ASSAYS.

6.1 \textit{In vitro} Binding : GST Pull-Down Experiment.

GST fusion protein were induced and bound to glutathione agarose beads as described above. Glutathione agarose beads together with bound proteins were then washed several times in PBS to remove the detergent used in purification. The beads (approximately 200 ng) were then incubated for 1 hour with \textit{in vitro} translated radiolabelled protein. Complexes were then washed three times in PBS containing 0.5% NP40. Specifically bound proteins were removed by boiling in an equal volume of 2XSDS sample buffer (10mM Tris pH 6.8, 200 mM dithiothreitol,
4% SDS, 0.2% Bromophenol Blue, 20% glycerol) and monitored by SDS-PAGE
and autoradiography.

Where comparative binding assays were done with several GST-fusion proteins,
these were previously balanced by titration of known BSA protein concentrations
run in parallel on a Coomassie-blue or Silver stained SDS-PAGE gel. Where the
comparison was done between different in vitro transcription translations, these
were previously quantified with a Packard Instant Phosphor Imager instrument.
Where the strength of protein-protein interaction was tested, the binding assay was
repeated exactly with the above experimental conditions, with the only change in
the concentrations of the salt ranging from 0.17 to 1M, 2M, and 4M NaCl in the
final washing.

6.2 In vitro Direct Binding.

For the direct binding assays, GST fusion proteins were purified as described above,
balanced with each other and incubated with 40 ng of purified TBP (Promega) for 1
hour at 4°C. Following extensive washing with PBS containing 0.5% NP40, bound
protein was detected by Western Blot analysis using an anti-TBP monoclonal
antibody (Oncogene Science, Inc.) with ECL detection (Amersham).

6.3 In vivo Binding: E7/TBP Co-Immunoprecipitation.

CaSki and HT1080 cells were labelled with 1mCi [35 S] methionine/cysteine trans
label (ICN) per each 100 mm dish for at least 4 hours at 37°C. Cell extracts were
made as described previously (Banks and Crawford, 1988), washing the dishes with
PBS three times, then adding 200 µl of a lysis solution (250 mM NaCl, 0.15% NP40, 50 mM HEPES pH 7, 1% Aprotinin, 100 µM TLCK, 200 µM TPCK). After
30 mins. on ice the cells were scraped off the plates and debris removed by centrifugation at 5000 r.p.m. for 2 mins. Supernatant was removed and proteins were precipitated using either rabbit pre-immune, rabbit anti-GST.E7, or rabbit anti-TBP antibodies kindly provided by A. Berk. Protein A sepharose (Pharmacia) was added and incubated at 4 °C for 1 hour with rocking. After extensive washing in lysis buffer the proteins were run on 15% SDS-PAGE gel and analysed by autoradiography. Alternatively, proteins were immunoprecipitated from unlabelled cell extracts using either rabbit preimmune or anti-GST.16E7 antibodies. Proteins were then separated by 10% SDS-PAGE gel and electrophoretically transferred to nitrocellulose. Co-precipitated TBP was further detected using anti-TBP monoclonal antibody (Oncogene Science, Inc.) coupled with ECL detection system (Amersham).

7. **PHOSPHORYLATION EXPERIMENTS.**

7.1 **In vitro** Phosphorylation of HPV E7 Proteins.

To obtain the *in vitro* Casein Kinase II phosphorylation of the HPV E7 proteins, GST fusion proteins were placed in 20 mM HEPES pH 7.5, 20 mM MgCl₂, 0.3 μM Aprotinin, 1 μM Pepstatin, 50 mM NaF, 4 mM Na₃VO₄, 100 mM K₂HPO₄, 10 μM ATP, 56 nM [γ³²P] ATP (Amersham) in a final volume of 30 μl. Routinely one unit of enzyme (Promega) was added to each reaction. After incubation at 30 °C for 20 mins. the reaction was blocked in ice. Phosphorylation of E7 proteins was monitored by SDS-PAGE gel and autoradiography. For binding assays unlabelled ATP replaced the isotope and phosphorylation was monitored by performing a labelled reaction in parallel.
When the *in vitro* phosphorylation of HPV E7 proteins was performed through the cell cycle, an extract of 14/2 cells was used as source of kinases to incubate with the different GST fusion proteins. These cells were subjected to serum starvation (0.2% Foetal Calf Serum) for 48 hours and then induced to the cell cycle entry with addition of 10% Foetal Calf Serum for a total of 8 hours. The cell extract samples were harvested in the lysis buffer (250 mM NaCl, 0.15% NP40, 50 mM HEPES pH 7, 1% Aprotinin, 100 μM TLCK, 200 μM TPCK, 50 mM NaF, 4 mM Na₃VO₄, 100 mM K₂HPO₄) every two hours. The GST fusion proteins were incubated with equal amount (10 μg) of each time-course cell extract, estimated by the Bio-Rad protein assay, for 20 mins. at 30 °C in a total amount of 30 μl with addition of 10% of phosphorylation buffer (final concentrations: 20 mM HEPES pH 7.5, 20 mM MgCl₂, 0.3 μM Aprotinin, 1 μM Pepstatin), plus 56 nM[^32P] γ ATP (Amersham) and 10 μM ATP. After extensive washing the phosphorylated proteins were monitored on SDS-PAGE gel and autoradiography. The phosphorylation levels were quantitated with a Packard Instant Phosphor Imager instrument.

### 7.2 *In vivo* Phosphorylation of HPV E7 Proteins.

BRK lines expressing HPV16 E7, HPV16 p31/32, and HPV16. Asp were cultured for 2 hours at 37°C in phosphate-free DMEM medium (Flow) supplemented with 10% dialysed Foetal Calf Serum. After this time, the cells were incubated for 3 hours with 1 mCi per dish of[^32P] orthophosphate (Amersham). Cells were then scraped from the dishes (Banks and Crawford, 1988) in lysis buffer (250 mM NaCl, 0.15% NP40, 50 mM HEPES pH 7, 1% Aprotinin, 100 μM TLCK, 200 μM TPCK, 50 mM NaF, 4 mM Na₃VO₄, 100 mM K₂HPO₄) and placed on ice for 30 mins.
The extracts were cleared by centrifugation at 14000 r.p.m. for 5mins., then the protein concentrations were measured by a Bio-Rad protein assay system. Equal amounts of extracts were further incubated with anti-16E7 polyclonal antibody for 3 hours at 4 °C with rocking. 100 µl of Protein A-sepharose (Pharmacia) were added to each immunoprecipitation for other 40mins. at 4 °C with rocking, and then the beads were washed several times with the 10 X volume of lysis buffer. Finally, the bound proteins were separated on 15% SDS-PAGE gel and analysed by autoradiography.

8. WESTERN BLOT ANALYSIS.

The proteins of interest were separated on the required percentage SDS-PAGE gel, then electrophoretically transferred to nitrocellulose membrane following the Maniatis protocol (Maniatis *et al.*, 1982). Membranes were then probed with the specific antibody at the optimum dilution in PBS supplemented with 2% milk powder (1:200 anti-16 E7 polyclonal antibody, 1:100 anti-TBP monoclonal antibody by Oncogene Science, 1:100 anti-p21 monoclonal antibody by Oncogene Science) for at least 2 hours. The next incubation was done with anti-mouse or anti-rabbit biotinylated antibodies diluted 1:1000 in PBS with addition of 10% milk powder for another hour. The final incubation was done with Avidin-peroxidase conjugated diluted 1:1000 in 10% milk powder/PBS solution. The reaction was then developed using the ECL system (Amersham), according to the manufacturer’s instructions.
9. **NUCLEIC ACID HYBRIDISATION.**

9.1 **Southern Blot.**

Genomic DNA was isolated by sodium dodecyl sulfate (SDS) lysis from BRK cells grown in 100 mm tissue culture dishes. High molecular weight DNA was separated by 24 hours digestion with proteinase K. Finally the DNA was recovered from the aqueous phase after phenol/chloroform extraction by spooling through ethanol. DNA samples (20 µg) were digested with Bam H1/EcoR1 enzymes, separated by electrophoresis through 1% agarose gel and transferred to Hybond-N nylon filters (Amersham) by capillary blotting in 50 mM NaOH, following the Maniatis protocol (Southern, 1975; Maniatis et al., 1982). The agarose gel was stained with Ethidium Bromide to confirm that approximately equal amounts of nucleic acid were loaded in each lane. The filter was prehybridised in 50% deionised formamide, 5 X Denhardt’s solution, 5 X SSC (150 mM NaCl, 15 mM Trisodium Citrate, pH 7.0) and 500 µg/ml denatured salmon sperm DNA for 4 hours at 42 °C. Hybridisation was performed at 42 °C for at least 36 hours in the above solution plus the probe. The probe consisted of HPV16 E7 digested with BamH1 / EcoR1 enzymes and labelled by random priming using [$^{32}$P] dCTP and the NEBlot ™ Kit (Biolabs). After hybridisation the filter was washed for 30mins. at 42 °C in 2 X SSC plus 0.1% SDS, then for 30mins. at 55 °C in 0.2 X SSC plus 0.1% SDS and then for another 20mins. at 55 °C in 0.1 X SSC plus 1% SDS. Finally the membrane was exposed to Kodak X-ray film with screen.

9.2 **Northern Blot.**

Total cellular RNA was prepared from 100 mm tissue dishes of BRK lines by a phenol/guanidinium isothiocyanate extraction procedure using RNAzol
(Biogenesis, Bournemouth, UK). 10 µg of total RNAs were fractionated on 1.2 % formaldehyde (Carlo Erba) agarose gel, where 28S and 18S ribosomal RNAs were used as size markers. The RNA was transferred to a nylon membrane by capillary blotting in 50 mM NaOH following the Maniatis protocol (Maniatis et al., 1982). The membrane was probed with the HPV16 E7 fragment which was labelled by random priming using $[^{32}P]$ dCTP and the NEBlot™ Kit (Biolabs). The membrane was then washed following the same procedure as the Southern Blot, described above, and finally exposed to autoradiography. To ensure the equal amounts of RNA had been loaded, the membrane was stained for RNA using methylene blue.
RESULTS

1. Production of Polyclonal Antibodies against HPV16 E7.

One of the principal obstacles in carrying out the project was the lack of highly specific polyclonal antibodies against the HPV16 E7 protein. To address this, the production of such an antibody was obtained using a purified GST.16E7 fusion protein. In Figure 12A there is an example of the induction of GST.16E7 fusion protein with IPTG, run on 15% SDS-PAGE gel and stained with Coomassie blue. Positive clones were identified by the presence of a protein band of the correct molecular weight in the induced, but not in the uninduced, sample. Tracks 7 and 9 clearly show a 44 kDa band that is absent in the uninduced sample (track 1). The molecular weight of the fusion protein, of about 44 kDa, is due to the sum of the molecular weight of GST alone, which is of 27.5 kDa, and the molecular weight of E7 of about 17 kDa.

The GST.16E7 protein fusion was then purified for inoculation into rabbits. The purity of the protein was monitored on a 10% SDS-PAGE gel stained with either Coomassie blue or the Bio-Rad silver stain system. The profile of the protein fractions obtained is shown in Figure 12B. Different concentrations of BSA were also run on the same gel to estimate the amount of the protein in each fraction.

For the first immunisation a concentration of 200 µg of purified protein was injected into two New Zealand rabbits. In the following injections 100 µg of protein were used every 21 days. The antibodies were purified using the method of covalent coupling proteins to glutathione beads by dimethylsuberimidate and their
**Figure 12A.** Induction with 1mM IPTG of *E.coli* transformed with recombinant plasmid pGEX-16E7. The positive clones are visible in the 7 and 9 tracks. The SDS-PAGE gel is stained with Coomassie blue.

**Figure 12B.** 10% SDS-PAGE gel of the eluted fractions with 15mM reduced glutathione from the column of GST.16 E7 fusion protein. On the left, there are scalar dilutions of BSA used for calculation of the concentrations of the eluted 16 E7 in the different fractions. For example, in the 7 track there is a concentration of about 200 ng of the GST E7 protein.
subsequent elution was performed on the same GST16 E7 column with glycine/HCl 0.2 M solution. To test the specificity of the antisera, several immunoprecipitations were performed on HPV16 E7 translated in vitro, and Western Blot analyses were also done on the purified antigen, used for the immunisations, at different concentrations. The results of these experiments are shown in Figure 13A and Figure 13B. It is clear that almost all the fractions of the purified antibody are able to immunoprecipitate HPV16 E7 protein, and can detect as little as 20pg of the GST fusion protein in Western Blot analysis. Since a percentage of the polyclonal antibody might be expected to react against the GST protein alone, a Western Blot on a total cell extract of CaSki was also performed and the result is shown in Figure 13C. This reveals the specificity of the purified fraction used at different dilutions 1:200, 1:1000; 1:1000 in PBS/2% milk powder, followed by anti-rabbit antibody at 1:1000 plus peroxidase-conjugated Avidin at 1:1000 in PBS/10% milk. The blot was developed by the ECL reaction (Amersham). Moreover, to determine whether the polyclonal antibody could detect E7 in HPV transformed cells, an immunoprecipitation of labelled CaSki cells was done with both the anti-E7 and the preimmune antibodies. The result is shown in Figure 13D, where it is possible to see the presence of the specific band at 17 kDa of E7 only in the track immunoprecipitated with the anti-E7 antibody. No band at the same molecular weight is visible in the immunoprecipitation with preimmune antibody.

The polyclonal antibody did not cross-react with the E7 proteins of other HPVs nor did it react against other proteins of HPV16, demonstrating high specificity for HPV16 E7.
Figure 13A. 15% SDS-PAGE gel of the immunoprecipitations of HPV16 E7 translated in vitro, using the TNT Promega system, with the different fractions of affinity-purified anti-GST.16 E7 antibodies. Almost all the fractions of the purified antibody are able to immunoprecipitate in vitro translated HPV16 E7. On the right side of the gel the immunoprecipitation done with the total serum before purification is shown.

Figure 13B. Western blot on different dilutions of the GST.16 E7 antigen that was used for the immunisation. The blot was probed with one of the most reactive fractions of the purified antibody (n°.1 of Fig.13A) at a dilution of 1:2000 in PBS added with 2% milk. The amplification of the reaction was done with a dilution 1:100 of the secondary anti-rabbit antibody, and with a dilution of 1:1000 of the peroxidase-conjugated Avidin; both of them were in PBS added with 10% milk powder. As negative control we used COS cell extract.

Figure 13C. Western Blot on total CaSki cell extract with different dilutions of the purified polyclonal antibody. The band corresponding to E7 is arrowed.

Figure 13D. Immunoprecipitation of labelled cell extract of CaSKi cells with anti-E7 and pre-immune antibodies. The band corresponding to E7 is arrowed.
2. Production of GST Fusions Proteins and Purification.
A number of studies have shown the ability of Ela to bind TBP (Horikoshi et al., 1991; Lee et al., 1991). Considering the high degree of conservation of functions between Ela and E7, we were interested in analysing whether E7 proteins could also bind to TBP. To investigate this the HPV16 E7, HPV11 E7, and Ad5 Ela proteins, were expressed in bacteria as GST-fusion proteins; Ela being included as a positive control. The protein profiles of the fusion proteins are shown in Figure 14A, which shows purified GST alone of 27.5 kDa, GST.16 E7 protein at 44 kDa, GST.11 E7 at about 41 kDa and GST.E1a at 67 kDa. The profile demonstrates purification to near homogeneity. The lower molecular weight bands in each preparation correspond to breakdown products which was confirmed by Western Blot analysis, using anti-GST.E7 antibody, where the antibody reacted strongly with the lower molecular weight bands (Figure 14B).

3. HPV E7 Complexes with the TATA Box Binding Protein.
To assess the ability of TBP to bind to these proteins, full length TBP was then translated in vitro and incubated with the fusion proteins bound to glutathione agarose beads for 1h at 4°C in the presence of DNase I to avoid artifactual binding mediated by DNA. Complexed proteins were then washed three times in PBS containing 0.5% NP40 and bound proteins were monitored by PAGE and autoradiography. A schematic representation of the pull down assay is shown in Figure 15A and the results of the E7/TBP binding are demonstrated in Figure 15B. The gel of the binding assay was also stained with Coomassie blue to confirm equal loading of GST-fusion proteins (Figure 15C). It is clear that both GST.E7 fusion
Figures 14A and 14B. The left hand panel shows the protein profile of the fusion proteins run on 10% SDS-PAGE gel and stained with Coomassie blue. Arrows indicate positions of the full length proteins. 1. GST; 2. GST.16 E7; 3. GST.11 E7; 4. GST.E1a. The right hand panel shows the Western Blot on GST.16 E7 purified protein, to demonstrate that the lower molecular weight bands are breakdown products of the full length fusion protein. The blot was probed with anti-GST.16 E7 antibody.
proteins and the Ad5 E1a fusion protein bind to the \textit{in vitro} translated TBP in comparison with the GST control. This result shows that E7 proteins from both benign and malignancy-associated HPVs share with the Ad5 E1a protein the ability to bind TBP, indicating a further conservation of function among these proteins. By performing Phosphor Imager analysis of the E7-TBP binding assays it was possible to quantify the percentage of input protein binding in all the experiments, which is shown in Figure 15D. For HPV16 E7 it was routinely about 20\% of the input, whereas HPV11 E7 bound somewhat less efficiency and was approximately 10\% of the input.

To further investigate the specificity of the binding, the pull-down assay was performed using the full length TBP expressed as a GST fusion protein together with the \textit{in vitro} translated HPV16 E7, and the results are show in Figure 16. In this assay HPV16 E1 protein was also introduced as a further negative control, together with GST alone. It is clear from the result that the interaction between HPV16 E7 and TBP also occurs under these conditions. HPV16 E1 shows no binding to GST.TBP, confirming the specificity of the assay.

4. Binding of HPV16 E7 to TBP is Direct.

The above results show that HPV16 E7 complexes with TBP as efficiently as Ad E1a. However, since the reactions were performed using \textit{in vitro} translated proteins, the possibility exists that the protein-protein interactions obtained were via an unknown intermediate protein. To investigate this possibility, 40 ng of purified TBP (Promega), was incubated with purified GST.16 E7, GST.16 E1 and GST alone, each bound to glutathione agarose columns. Following extensive washing, the
Figures 15A, 15B, 15C and 15D. Panel A shows a schematic model of the pull-down assay. The radiolabelled TBP protein is mixed with the GST fusion protein immobilised on the beads; after several washes the retained protein is assessed by SDS-PAGE and autoradiography.

Panel B shows the experiment done with GST, GST.16 E7, GST.11 E7, and GST.E1a as positive control. All the proteins tested are balanced as show in panel C where the same gel was stained with Coomassie Blue. Panel D shows the quantitation of the percentage input TBP binding to the different GST fusion proteins from four different experiments.
Figure 16. Binding between GST.TBP and *in vitro* translated E7. On the left side are the inputs of the translations of both E7 and E1, the latter one being used as negative control. In the following tracks there are: 1. GST + E7; 2. GST + E1; 3. GST.TBP + E7; 4. GST.TBP + E1.
amount of TBP retained was determined by electrophoresis and Western Blotting using an anti-TBP monoclonal antibody (Oncogene Science). The results obtained are shown in Figure 17. As can be seen, quantitative recovery of TBP was obtained on the GST.16 E7 column, with little or no binding to the GST.16 E1 fusion protein or to the GST alone. These results demonstrate that the interaction between E7 and TBP is direct and not mediated by any intermediate proteins. The weaker binding with E1, seen only in this assay, was probably due to the higher sensitivity of the Western Blot compared with the in vitro experiment done with the labeled protein.

5. HPV16 E7 Binds TBP in vivo.

Having shown that HPV16 E7 will complex with TBP in vitro it was necessary to verify that this interaction takes place in vivo. In the following experiments the CaSki cervical tumour-derived cell line was used as a source of high level E7 expression (Smotkin and Wettstein, 1986). To attempt to co-immunoprecipitate HPV16 E7 and TBP from extracts of these cells, in total 3 x 90 mm dishes of CaSki cells at 80% confluency were used per antibody reaction. Each dish was labelled with 1mCi of $^{35}$S methionine/ cysteine (ICN translabel) for 4 hours. Cell extracts were made as described previously (Banks and Crawford, 1988) and immunoprecipitation performed using anti-16 E7, anti-TBP (Oncogene Science) and pre-immune antibodies, followed by SDS-PAGE gel and autoradiography. The results obtained are shown in Figure 18A. Clearly the major protein species precipitated by the anti-E7 antibody migrates at approximately 17 kDa, and corresponds to HPV16 E7. A number of higher molecular weight proteins are also co-precipitated, including a protein of 105 kDa (visible only at a lower exposure),
B.

Figure 17. GST.16 E7 and GST.16 E1 fusion proteins and GST alone were purified on glutathione agarose columns. These were then incubated with 40ng of purified TBP. After extensive washing, bound TBP was assessed by Western Blot analysis with an anti-TBP monoclonal antibody at a 1:500 dilution, followed by an anti-mouse at 1:1000 and avidin-peroxidase 1:1000, both in PBS/10% milk powder. The left hand lane represents half of the TBP input. 1. GST.16 E1 + purified TBP; 2. GST.16 E7 + purified TBP; 3. GST + purified TBP.
which might correspond to pRB. Most interestingly, significant amounts of a protein migrating about at 43 kDa are also co-precipitated by the anti-E7 antibody. This protein co-migrates with the major protein species precipitated by the anti-TBP antibody and probably corresponds to TBP. The anti-TBP antibody also weakly precipitates a protein co-migrating with the E7 protein. Neither E7, TBP nor pRB is precipitated by the pre-immune antibody.

In order to verify that the protein migrating at about 43 kDa precipitated with the anti-E7 antibody corresponded to TBP, the experiment was repeated using unlabelled cell extract of CaSki and HT1080 cells which lacks HPV DNA sequences. Following immunoprecipitation with anti-E7 or pre-immune antibodies, bound proteins were resolved by electrophoresis and transferred to nitrocellulose. Bound TBP was then determined by Western Blot analysis with anti-TBP antibody. The results shown in Figure 18B demonstrate that only in the CaSki cell extract immunoprecipitated with anti-E7 antibody was a significant amount of TBP detectable. These results confirm that the protein migrating at about 43 kDa co-precipitating with HPV16 E7 is TBP. Obviously these observations indicate that the percentage of E7 bound to TBP at any one time is very low, but nonetheless show that the binding also occurs in vivo.


Since the E7 protein of both benign and malignancy associated HPVs is able to bind TBP, I decided to analyse the potential difference in the strength of the interaction between HPV16 E7/TBP and HPV11 E7/TBP. To study this, the binding reactions were done as before, but following incubation the bound proteins were exposed to
Figures 18A and 18B. Co-immunoprecipitation of HPV 16 E7 and TBP.

A. CasKi cells were labelled with $^{35}$S methionine/ cysteine for 4 h, extracts were prepared and proteins precipitated with pre-immune (P.I.), anti-TBP and anti-E7 antibodies. Precipitates were analysed by SDS-PAGE gel and autoradiography. Protein species precipitated with the antibodies corresponding to E7 and TBP are indicated.

B. CasKi and HT1080 cell extracts were precipitated with pre-immune (P.I.) or anti-E7 (α-E7) as indicated. Co-precipitated TBP was then detected by Western Blot analysis with an anti-TBP monoclonal antibody at a dilution of 1:500 in PBS added with 5% milk. The amplification was done with antimouse biotinylated antibody and peroxidase-conjugated Avidin, both of them diluted 1:1000 in PBS/10% milk.
increasing concentrations, ranging from 0.05% to 0.5%, of Triton X-100 in the washing step. The results are shown in Figure 19A. As can be seen there are clear differences in the relative affinities of 16 E7 and 11 E7 for TBP. The interaction between 16 E7 and TBP appears to be stronger than the one between 11 E7 and TBP; in fact, it is possible to increase the concentration of Triton X-100 to 0.5% without losing the 16 E7/TBP interaction, whereas the 11 E7/TBP interaction is abolished by addition of 0.05% Triton X-100. The same experiment was repeated but, this time, altering the concentration of NaCl in the washes. The results are shown in Figure 19B. Again it is possible to see that the 16 E7/TBP interaction is stronger, since significant binding is obtained following 4M NaCl wash, whereas the 11 E7/TBP binding is abolished by washing with 1M NaCl. The conclusion from these set of assays is that the interaction between 16 E7 and TBP is significantly stronger than the one between 11 E7 and TBP.

7. E7 Binding to TBP is Enhanced by CKII Phosphorylation.

Since both E7 and Ad E1a bind to TBP it was interesting to determine whether the region of Ad E1a known to be responsible for the binding to TBP (Lee et al., 1991) was conserved in the HPV 16 E7 protein. An alignment of the relevant sequences is shown in Figure 20A. As can be seen, the core TBP binding domain on E1a is close to its Casein Kinase II (CKII) recognition site and, although there is not a high degree of conservation between the core TBP binding domain of E1a within the corresponding E7 sequence, it did not seem unreasonable that phosphorylation may affect binding of these viral proteins to TBP.
Figure 19.
Panel A. Binding of 16 E7 and 11 E7 to TBP following washing with different concentrations of Triton-X 100.

Panel B. Binding of 16 E7 and 11 E7 to TBP following washing with different concentrations of NaCl.
In order to investigate the above hypothesis, the purified GST.16 E7 fusion protein was phosphorylated \textit{in vitro} with purified Casein Kinase II enzyme (Promega). To assess the optimal conditions of phosphorylation, a time course experiment was first performed. The incubation was carried out with approximately 80ng of the GST fusion protein, and protease inhibitors (0.3 \mu M Aprotinin, 1 \mu M Pepstatin, 50 mM NaF, 4 mM Na$_3$VO$_4$, 100 mM K$_2$HPO$_4$), BSA (1\mu g/\mu l), $[^{32}\text{P}]$ radiolabelled $\gamma$ ATP (56 nM) (Amersham), and the purified CKII enzyme (1 Unit/reaction) were also added to the reaction mix. The specificity of the reaction was improved by the introduction of an excess of cold ATP (10 \mu M). The time course was done with incubations at 2, 5, 10, 15, and 30 mins. and the results are shown in Figure 20B. From this experiment it is possible to deduce that the CKII can phosphorylate 16 E7 \textit{in vitro}, in agreement with previously published observations (Barbosa \textit{et al.}, 1990). In the time course the maximum phosphorylation activity was obtained between 10 and 15 mins. Moreover, considering the concentration of free cold and radiolabelled ATP in the reaction, it was possible to calculate the percentage of E7 that was phosphorylated at each time course point (see Material and Methods). The kinetic analysis, shown in Figure 20C and 20D, indicates that between 35\% and 40\% of the available GST.16 E7 is phosphorylated within 15 mins. of the incubation, confirming that E7 is an extremely good target for CKII phosphorylation.

Having established the optimal conditions for the phosphorylation reaction it was possible to proceed in the investigation of the potential effects of the phosphorylation upon the E7-TBP interaction. To do this the binding assay was repeated pre- and post-phosphorylation of the GST.16 E7 fusion protein. As well as HPV16 E7, HPV11 E7 and a mutant of E7, p31/32, which cannot be
Figures 20A, 20B, 20C and 20D. *In vitro* GST 16 E7 phosphorylation. Panel 20A shows the sequence alignment of AdE1a and HPV16 E7, and the residues of the E1a-TBP binding domain. Note also the homology between the two CKII recognition motifs. Panels 20B, 20C and 20D report the time course experiment, which shows the maximum E7 phosphorylation between 10 and 15 mins. The profile of the phosphorylation was obtained with the cpm/min (Y axis) against each time course point (X axis).
phosphorylated by CKII (Barbosa et al., 1990), were included in the experiment. All the proteins were expressed as GST fusion proteins and purified as described before. The results of the phosphorylation reaction is shown in Figure 21A. In agreement with previously published data (Barbosa et al., 1990) there is a strong phosphorylation of the HPV16 E7 and HPV11 E7 fusion proteins and no phosphorylation of the GST control, nor of the p31/32 mutant of E7. The phosphorylated proteins were then incubated with in vitro translated carboxy terminal (aa 165-339) TBP and, following extensive washing, the bound TBP was assayed by SDS-PAGE gel and autoradiography. The results obtained are shown in Figure 21B. In the absence of phosphorylation the TBP is retained in similar amounts on both GST.p31/32 protein and the wild type GST.16 E7 protein and, to a lesser extent, on the GST.11 E7 protein. However, after phosphorylation it is clear that, although the amount of TBP retained by the GST. p31/32 protein remains the same, there is a marked increase in the amount of TBP binding to the wild type HPV16 E7 and HPV11 GST E7 protein. These results show that the CKII phosphorylation of HPV E7 increases its ability to complex with TBP and, further, that the binding site on TBP is within the conserved carboxy terminal region of the protein contained within residues 165-339. Figure 21C shows the same gel of the binding assay rehydrated and stained with the Silver stain system (Biorad), to verify that the amount of the proteins used was balanced.

8. Calculation of the Ka of the E7/TBP Interaction.

Having shown that the phosphorylation of E7 increases the binding to TBP, it was interesting to determine the association constant (Ka) of the E7/TBP interaction. To
Figures 21. Phosphorylation of E7 by CKII increases TBP binding. Panel A shows CKII phosphorylation of the fusion proteins bound to the glutathione agarose beads. Arrow indicates the position of the labelled E7 fusion protein. Panel B shows the amount of TBP bound to the different fusion protein columns before and after CKII phosphorylation. In this case the carboxy terminal portion of TBP, comprising residues 165-339, was used. Bound TBP is indicated. Panel C is the same gel of the binding assay rehydrated and stained with the Silver stain system (Biorad).
do this a titration experiment was done using several dilutions of the GST. 16 E7 fusion protein pre and post phosphorylation. The results of the binding are shown in Figure 22A. It is clear that the interaction between the phosphorylated 16 E7 and TBP is stronger than the one between the non-phosphorylated E7 and TBP over all the range of the different concentrations used. Quantification of the binding assays using a Phosphor Imager analysis of each pull-down experiment before and after phosphorylation allowed the calculation of the association constant of the E7/TBP interaction. This corresponds to the minimum molarity of the E7 protein which is necessary to obtain a binding with TBP in a pull-down experiment. In this case the value of the E7/TBP Ka is 31.2 nmol. From the graph in Figure 22B, the Ka is calculated as the reciprocal of of the Km (-1/Km). The Km is the reciprocal of half of the value obtained from the point where line A crosses the X axis. The line A is obtained from the interpolation between the reciprocal of the cpm/min and the reciprocal of the concentrations of E7 used in the pull-down experiment, and the point where the line A crosses the X axis defines the concentration for the calculation of the Ka. The line A, obtained from the values of the phosphorylated E7 is crossing the X axis, instead no immediate crossing is evident between the line B (obtained from the values of the non- phosphorylated E7) and the X axis. This confirms that it is easier to define a Ka for the E7/TBP association in presence of phosphorylated E7, which condition is probably also the more favourable for the binding of the two proteins in vivo. In contrast, the Ka calculated for line B is of 384 nM, value which is still quite reasonable for a protein-protein interaction but is nevertheless about 12 fold higher than the Ka obtained with the phosphorylated E7.
Figures 22. Calculation of the Ka of the E7/TBP binding. Panel A shows the binding between titrated concentrations of E7 and \textit{in vitro} translated TBP. Increasing concentrations of E7 correspond to increasing amounts of TBP pulled-down in the assay. Panel B shows the calculation with the mathematic formula of the association constant of the E7/TBP interaction. In the graph line A is made with the values of the binding with phosphorylated E7 and line B is due to the interpolation of the values obtained from the binding with unphosphorylated E7. In the calculation the critical value is obtained from the line A crosses the X axis.
9. CKII Phosphorylation of Ad E1a Increases Binding to TBP.

From the sequence alignment in Figure 20A it is clear that the core TBP binding domain on Ad E1a is close to its potential CKII phosphorylation site. Hence it was reasonable to analyse whether CKII phosphorylation of Ad E1a could also affect its ability to bind TBP. In addition, previous studies had indicated that mutation of the CKII site of E7 to two acidic residues gave rise to levels of transforming activity similar to that of wild type (Firzlaff et al., 1991), presumably by replacing the two negative charges provided by CKII phosphorylation. Therefore, it was also interesting to see what effect the conversion of the two Serine residues to two Aspartic Acid residues would have on the ability of HPV16 E7 to bind TBP (Figure 23A). The Aspartic mutant, wild type HPV16 E7, the mutant p31/32 and also Ad E1a were produced and purified as GST fusion proteins and the protein profile is shown in Figure 23B. The proteins were then incubated with purified CKII, as described above, and their ability to bind to the carboxy terminal region of TBP was assessed. The results, in Figure 23C, show that in the absence of the phosphorylation, significant binding of E7 and E1a to TBP was seen, although decreased p31/32 interaction was seen in this assay. After phosphorylation of the proteins the amount of TBP binding E7 and E1a increased dramatically; at the same time there was no difference in the amount of TBP binding to the p31/32 mutant of E7. The conclusion was that the phosphorylation of E1a also increases its ability to bind to TBP. Of additional interest are the results obtained with the double Aspartic Acid mutant which cannot be phosphorylated by CKII but whose ability to bind TBP is very similar to that of phosphorylated wild type E7. These results demonstrate that the incorporation of two negative charges into the region of the
Figures 23A, 23B and 23C. CKII phosphorylation of Ad E1a increases its binding to TBP. Panel 23A shows the sequence alignment between HPV16 E7, Ad E1a and SV40 large T antigen, with the double mutation at the phosphorylation sites used in this experiment. Panel 23B indicates a Coomassie Blue stained gel of the GST fusion proteins. The proteins were purified on beads and their concentrations were equalised and subjected to CKII phosphorylation. Binding of *in vitro* translated carboxy terminal TBP was then assessed by SDS-PAGE gel and autoradiography, as is shown in panel 23C. The last track shows the binding between TBP and the GST.E7 mutated to double Aspartic Acid residues at the CKII recognition sites.
CKII site is the critical event in regulation of the binding with TBP, and not the presence of the phosphate moieties per se.

10. HPV16 E7 Binds to the Conserved Carboxy Terminal Region of TBP.

Previous studies have shown that a number of proteins like SP1, p53 and SV40 large T antigen can interact with the same carboxy terminal region of TBP (Emily et al., 1994; Martin et al., 1993). Since this domain of TBP seemed to be important for different protein-protein interactions it was therefore necessary at this point to investigate which region of TBP was bound by E7.

In order to do this, a series of TBP deletion mutants was first constructed and expressed as GST fusion proteins. The DNA fragments corresponding to mutant 1 (deletion aa 1-201), mutant 2 (deletion aa 100-339), mutant 3 (deletion aa 272-339), and mutant 4 (deletion aa 298-339) were obtained by PCR amplification using full length TBP as the template, followed by cloning into the BamH1/EcoR1 sites of pGEX- 2T. These mutants are shown schematically in Figure 24A. The fusion proteins were expressed and purified as described previously and the protein profiles are shown in Figure 24B.

In order to determine which region of TBP is involved in the interaction with E7, an in vitro binding assay was then performed using equal amounts of the different fusion protein deletion mutants of TBP immobilised on glutathione agarose columns, and in vitro translated E7. The result is shown in Figure 24C and it is evident that E7 is not able to bind to the mutant 2 which lacks the carboxy terminal part of TBP. In contrast, E7 binds M1, M3 and M4 almost as strongly as the full
Figures 24A, 24B and 24C. Panel 24A shows the different stretches of TBP which were cloned in pGEX-2T to produce the full length GST.TBP and the mutant GST.TBP fusion proteins. Panel 24B shows the protein profiles of the GST.TBP fusion proteins, expressed in bacteria, then purified on glutathione agarose beads and analysed by SDS-PAGE stained with Coomassie Blue. Panel 24C shows the binding experiment between the GST fusion TBP mutants and the \textit{in vitro} translated E7. This demonstrates that the region of TBP involved in the interaction with E7 lies within residues 201-272.
length TBP. These results enable us to define a stretch of amino acids between residues 201-272 of TBP which are essential for complex formation with E7.

11. Comparison of the Ability of Different Human Papillomavirus Proteins to Bind TBP.

Many viral and cellular proteins have been shown to bind TBP. Of particular relevance for HPV are the studies which have shown binding between TBP and the viral BPV1 E2 protein (Steger et al., 1995; Miller-Rank and Lambert, 1995) and between TBP and the cellular tumour suppressor p53 (Seto et al., 1992; Liu et al., 1993). In addition, during the course of these studies, I also noted that HPV18 E6 could bind TBP in vitro. Therefore, it was interesting to determine if the sites of interaction of this diverse group of proteins with TBP were the same as those already found for E7. The binding reactions were done as before using the purified deletion mutants of TBP on glutathione agarose beads all balanced and the different HPV proteins translated in vitro. The results of the experiment are shown in Figure 25 and reveal a number of interesting points. First, none of the proteins tested show any significant binding to the M2 fusion protein; this demonstrates that E2, E6 and the p53 all bound to the some conserved carboxy terminal region of the TBP molecule, as does E7. Binding of p53 is much weaker on the M1, M3 and M4 fusion proteins compared with the full length TBP (as visible also from the percentage bindings of each interaction, as determined by a Phosphor Imager analysis of the gel), but it is nonetheless detectable and indicates that residues 201-272 of the TBP molecule are required for p53 binding, although residues 100-201 would also seem to be important. Interestingly, a very similar pattern of binding is
Figure 25. Comparison of the binding sites of p53, E2, E6 and E7 on the TBP molecule. GST.TBP fusion proteins (FL being full length TBP) were induced and purified to the levels shown in Figure 24B, and then incubated with \textit{in vitro} translated radiolabelled E2, E6, E7 and p53 as indicated. After extensive washing the bound proteins were determined by SDS-PAGE gel and autoradiography. By Phosphor Imager analysis the cpm were measured for each interaction and the values are reported at the bottom of each experiment.
obtained with the E6 protein and this suggests that E6 and p53 can bind TBP in a very similar way. A pattern identical to that found with E7 was obtained with the E2 protein, indicating a strong similarity between E7 and E2 in their binding with TBP. The data demonstrate that all the proteins tested bind within the same carboxy terminal region of the TBP molecule, but that there are differences in the precise residues involved.

12. Strength of the Different Interactions with TBP.

Having shown that differences exist in the precise nature of the interaction between the viral proteins and TBP, it was interesting to investigate further the relative strengths of these interactions. Obviously, in the context of a viral infection, differences could have important implications in terms of competition for the available TBP. To study this, the binding reactions were done as described before but, following incubation, the bound proteins were exposed to increasing concentrations of NaCl ranging from 0.17 M to 4 M in the washing step. The results obtained are shown in Figure 26 and demonstrate interesting differences in the relative affinities of the viral proteins for TBP. The weakest interaction is that between E2 and TBP, since 1 M NaCl almost abolishes this interaction. Considering that E2 is a major regulator of viral gene expression and its diverse effects have been postulated to occur through interaction with the basal transcriptional machinery, including TBP (Ushikai et al., 1994; Miller-Rank and Lambert, 1995; Steger et al., 1995), this is a surprising result, even if there are reports suggesting that the ability of TBP to interact with an activation domain in vitro is not directly relevant to its ability to support activated transcription in vivo (Tansey et al., 1995;
Figure 26. Comparison of the relative strengths of p53, E2, E6 and E7 binding to TBP. Full length GST.TBP fusion protein was prepared as described in the text and incubated with \textit{in vitro} translated radiolabelled p53, E2, E6 and E7 as indicated. Bound proteins were then washed in increasing concentrations of NaCl, as shown, and remaining protein determined by SDS-PAGE and autoradiography.
Thut et al., 1995). In the same experiment, HPV18 E6 and p53 show very similar strengths of interaction with TBP, with weak binding seen following 1 M NaCl treatment, but complete disruption by 2 M NaCl. The strongest interaction is that between E7 and TBP where there is still strong binding after 2 M NaCl washing. Allowing for the fact that the ranking of the different proteins may be specific to this assay, due to the anomalous salt concentrations used, these results nonetheless indicate a hierarchy between the HPV proteins for binding TBP, with E7 being strongest, followed by E6 and finally the weakest interaction being observed with E2.


Numerous cellular targets of HPV E7 have now been identified, including pRB, p107, cyclin A, TBP and members of the AP-1 transcription factor family (Whyte et al., 1988; DeCaprio et al., 1988; Dyson et al., 1989; Münger et al., 1989b; Thierry et al. 1992; Ciccolini et al., 1994; Morosov et al., 1994; Nead et al., 1998; Smith-McCune et al., 1999). As with Adenovirus E1a, many of these interactions are important for the ability of E7 to transform cells. Recent studies with Ad E1a have demonstrated that E1a can inhibit the transcriptional activity of the cellular tumour suppressor protein, p53 (Steegenga et al., 1996). This report suggested that this may be brought about through an increase in the molecular weight of p53 protein complexes within the cell, in response to the presence of E1a. Since TBP binding by p53 was reported to play a role in p53 transcriptional activity (Seto et al., 1992; Martin et al., 1993) and considering that E1a and E7 are both able to bind TBP, it
was extremely intriguing to investigate whether E7 had any effects on p53 transcriptional activity. To investigate this, a series of experiments were first performed to see whether HPV16 E7 could modulate p53 transcriptional activity in p53-null Saos-2 cells (Osteosarcoma cells). 1 x 10^5 cells were transfected with 1 µg of the p53 responsive CAT reporter plasmid, pG13CAT, plus 1 µg of p53 expression plasmid. Increasing amounts of the HPV16 E7 expression plasmid, pJ4Ω.16E7, were included and the cells were harvested after 48 hours. CAT assays were performed as described previously (Pim et al., 1994) and the results obtained are shown in Figure 27A. All the transfections were controlled by co-transfection with a β-galattosidase expressing plasmid, which controls possible squelching and transfection frequencies. It is clear that increasing amounts of 16 E7 produce a marked decrease in p53 transcriptional activity in a manner similar to that previously reported for E1a (Steegenga et al., 1996).

To determine whether this function of E7 was restricted to the tumour associated E7 proteins or was conserved throughout the genital HPV types, the above assay was repeated including HPV18 E7, HPV6 E7 and HPV11 E7. The results, in Figure 27B, demonstrate that all four of the proteins possess the ability to inhibit p53 transcriptional activity. In conclusion this function of E7 is conserved between both benign and tumour-associated HPV types, and does not appear to be directly associated with the ability of E7 to transform cells.
Figures 27A and 27B. Abolition of p53 mediated transcriptional activation by HPV E7 proteins. Saos-2 cells were transfected with 1μg of the p53 responsive CAT plasmid, pG13CAT, 1μg of the RSVp53 plasmid together with pJ4Ω or pJ4Ω.E7 plasmids as indicated. Cells were harvested and CAT activity measured after 48hours. Panel A. Titration of pJ4Ω.16 E7 as indicated containing 10, 5, and 1μg of the transfected E7 expression plasmid. Panel B. Comparison of the different E7 proteins derived from benign and tumour associated HPVs. In each case 10μg of the E7 expression plasmid was transfected. The histograms represent the means of three CAT assays.

Having shown that HPV16 E7 proteins inhibit p53 transcriptional activity it was interesting to identify the region of the E7 protein which was responsible for this function. A series of well characterised HPV16 E7 mutants (Edmonds and Vousden, 1989; Banks *et al*., 1990b; Barbosa *et al*., 1990) were assessed for their ability to inhibit p53 transcriptional activity. The results shown in Figure 28A suggest that mutants of E7 which are defective in transformation, such as 566 and 631, still retain the ability to inhibit p53 transcriptional activity. It is important to underline that 631 mutant is defective in pRB binding, and this demonstrates that inhibition of p53 transcriptional activity is independent of the capacity of E7 to bind pRB. Moreover, only the mutants 638 and p31/32 have a reduced ability to inhibit p53 transcriptional activity. Mutant 638 seems to encode an unstable protein (Edmonds and Vousden, 1989) and mutant p31/32 encodes a stable E7 protein which is defective for CKII recognition. This data supports the conclusion that phosphorylation of E7 by CKII may play a role in its inhibition of p53 transcriptional activity. Previous studies raised the possibility that E7 could be phosphorylated at other residues within the protein (Storey *et al*., 1990), and it was clearly important to determine whether potential phosphorylation at these sites might be important for the ability to suppress p53 transcriptional activity. The results are shown in Figure 28B, and it is clear from the analysis that only phosphorylation of the CKII sites 31 and 32 appears to be important for this function of E7. Table 1 shows a summary of the above assays from a series of at least three different independent transfections.
Figures 28A and 28B. Localisation of the region of HPV16 E7 responsible for inhibition of p53-mediated transcriptional activation. Saos-2 cells were transfected and processed as described in the legend of Figures 27A and 27B. In panel 28A, mutants of E7 spanning the conserved amino terminal region of the protein are tested. In panel 28B, potential phosphorylation-defective E7 mutants which are spread throughout the protein are assessed. The histograms represent the means of three CAT assays.
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<th>Expression plasmid</th>
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<tr>
<td>pJ4 Ω</td>
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<tr>
<td>pJ4 Ω.. 16E7</td>
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<tr>
<td>pJ4 Ω.. 18E7</td>
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<td>3.1</td>
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<tr>
<td>pJ4 Ω.. 638</td>
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<tr>
<td>pJ4 Ω.. p32</td>
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<tr>
<td>pJ4 Ω.. p31/32</td>
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<td>pJ4 Ω.. p95</td>
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Table 1. Summary of the fold reductions of a series of at least three different independent transfections. The mutants of E7 are as follows: 566 represents amino acid residue (aa) 2 His-Pro. 638 represents aa 26 Glu-Gly. p32 represents aa 32 Ser-TrP. p31/32 represents aa 31 Ser-Arg and aa 32 Ser-Pro. p63 represents aa 63 Ser-Ala. p71 represents aa 71 Ser-Gly. p95 represents aa 95 Ser-Ala.

a Numbers show fold reduction in p53 transcriptional activation obtained by cotransfecting 1 μg of pG13CAT and 1 μg of p53 expression plasmid. These represent the mean values from between 3 and 6 individual experiments.
15. Inhibition of p21 Protein Induction by HPV16 E7.

The question at this point was: can E7 affect the induction by p53 of a naturally occurring target gene? One such gene encodes the p21/WAF-1 protein (Harper et al., 1993; El-Deiry et al., 1993). The p21 protein is a universal inhibitor of cyclin-dependent protein kinases (inhibitor of CDK2, CDK4; Xiong et al., 1993), and cyclin-dependent protein kinases are known to be engaged in phosphorylating the retinoblastoma protein RB and in positively regulating the transcriptional activity of E2F by releasing it from its association with pRB. In this way p53 is thought to trigger G1 arrest via p21 and pRB-dependent inhibition of E2F activity (Harper et al., 1993; Dulic et al., 1994). The effects of E7 expression upon p21 induction were investigated in Saos-2 cells following transfection with p53 expression plasmid. Cells were transfected and extracted after 48 hrs with lysis buffer (Thomas et al., 1996), and the p21 and p53 levels were determined by Western Blot analysis. The results obtained are shown in Figure 29. Panel A it is shown the level of p53 protein obtained following transfection of Saos-2 cells with 1μg of RSVp53 plus 10μg of pJ4Ω.16E7. As can be seen, a slight increase in the level of p53 protein is obtained in cells containing E7, in agreement with previous studies (Demers et al., 1994). In contrast, when the same blot was re-probed for p21 (Panel B), it is quite clear that there is a decrease in the level of p21 protein in the E7 containing cells, compared with the cells transfected with p53 alone. In such transient transfection assays, E7 can thus significantly reduce the level of expression of a naturally occurring p53-induced protein.
Figure 29A and 29B. Inhibition of p21 protein induction by pJ4Ω.16E7. Saos-2 cells were transfected with either 1μg of RSVp53 (p53) plasmid plus 10μg of pJ4Ω.16E7 (E7) plasmid, as indicated, or vector alone sequences (-). After 48 hours the cells were extracted and the p53 protein (Panel A) levels measured using a pool of anti-p53 monoclonal antibodies pAb1801, 1802, and 1803 (Banks et al., 1986). p21 protein levels (Panel B) were measured using an anti-p21 antibody (Oncogene Science Inc.). Western blots were developed using the Amersham ECL detection system according to the manufacturer’s instructions.
16. **Complex Formation between E1a and E7 with p53 and TBP.**

Since both E1a and p53 associate with TBP, a possible explanation for the higher molecular weight forms of p53 seen in E1a expressing cells (Steegenga et al., 1996) could be co-association of E1a -TBP and p53, which may indicate a mechanism for inhibition of p53 transcriptional activity. Since E7 can also interact with TBP and the CKII phosphorylation plays an important role in this interaction it was obvious to investigate whether the inhibition of p53 transcriptional activity by both E1a and E7 was due to a tripartite complex with TBP, and whether the phosphorylation of E7 could also play a key point in this pattern. To test this hypothesis a series of *in vitro* assays were performed to determine whether E1a, E7 and p53 could associate in the presence of TBP. Ad E1a, HPV16E7 and p53 were expressed as GST fusion proteins and purified on glutathione agarose beads, and the binding assays were performed with the appropriate *in vitro* translated proteins. The results obtained from a series of binding experiments are shown in Figure 30 and Figure 31A. A very small amount of p53 binds to GST.E1a or GST.16 E7 protein when compared with the GST control. However, upon addition of *in vitro* translated TBP, there is a dramatic increase in the amount of p53 retained on both GST.E1a and GST.16 E7. The converse experiment using GST.p53 is shown in Figure 31B where, in the absence of additional TBP, no E7 is bound to the GST.p53 column; upon addition of TBP, a stimulation of E7 binding is seen. These results demonstrate that E7 and p53 will normally not associate directly. Only in the presence of TBP can a tripartite complex form, thus indicating that both E7 and p53 can bind TBP simultaneously.
Figure 30. Complex formation between E1a, HPV16 E7, p53, and TBP. *In vitro* translated p53 (p53*) was added to GST.16 E7 or GST. E1a resin and the binding was monitored. In the lanes indicated, radiolabelled TBP (TBP*) was also included. The location of p53 and TBP proteins are shown.
Figure 31A and 31B. Complex formation between p53, TBP and E7. In panel A, *in vitro* translated labelled p53 (p53 *) was added to GST resin or GST.16 E7 resin and binding monitored. In the last lane radiolabelled TBP (TBP *) was also included. The location of the p53 and TBP proteins are shown. Panel B shows the binding of labelled *in vitro* translated HPV16 E7 (E7 *) to GST.p53 resin. In the first lane cold TBP was also added to the reaction. The presence of the E7 protein is indicated.
17. CKII Phosphorylation can Affect the Tripartite Binding between E7, p53 and TBP.

Since CKII phosphorylation increases the interaction between E7 and TBP and, it was extremely important to determine whether phosphorylation of E7 by CKII had any effect on the E7-TBP-p53 complex formation. The p31/32 mutation of E7 which has a weak effect on p53 transcriptional activity, cannot be phosphorylated, and has reduced TBP binding activity was included as a control. The above binding assay was repeated with the GST fusion proteins before and after CKII phosphorylation. The results are shown in Figures 32A and 32B. In agreement with previous studies, it is clear that CKII phosphorylation of the wild type E7 increases the level of TBP binding. In addition this stimulation of TBP binding is accompanied by a dramatic increase in the amount of p53 protein retained, further supporting the notion that the binding of p53 to E7 is via TBP. In contrast, performing the same analysis with the GST.p31/32 mutant, shows that, although p53 is bound weakly to E7 in the presence of TBP and in the absence of phosphorylation, the level of binding of either TBP or p53 does not change following the CKII phosphorylation reaction. It is noteworthy that the p31/32 mutant of E7 retains the ability to bind TBP, and hence p53, in a wild type manner in the absence of phosphorylation. This is not surprising considering the result of the in vivo assays where this mutant also weakly inhibits p53 transcriptional activity. However, phosphorylation of E7 greatly increases its ability to bind TBP and this correlates very closely with its increased ability to inhibit p53 transcriptional activity in vivo. Quantitation of the bound proteins with a Phosphor Imager indicates that the percentage of TBP retained rises from 6% to 19% following phosphorylation of wild type E7, and the amount of bound p53 goes from
Figures 32A and 32B. Panel A shows the binding of *in vitro* translated labelled p53 (p53\(^*\)) and *in vitro* translated TBP (TBP\(^*\)) to GST.16E7 resin and mutant GST.p31/32 resin, before and after CKII phosphorylation (P). Binding reactions were performed in PBS, and the p53 and TBP inputs are shown. In panel B the same gel was stained with Coomassie blue to confirm that levels of GST fusion proteins were equivalent.
6% to 19% at the same time. The level of proteins retained on the p31/32 resin does not change following phosphorylation and remains at 4.8% for TBP and 5% for p53.

18. Localisation of the Region of E7 that Binds TBP.

At this point of the research it was obvious to determine which region of E7 was involved in the interaction with TBP. Attention was focused on the carboxy terminus of E7 where there are regions well conserved between the different HPVs, keeping in mind that the non oncogenic forms of HPV are also able to interact with TBP. To this aim four different deletion mutants were constructed in the carboxy terminal part of the E7 protein; three of them have deletions located in the Zinc finger domain, the other has a deletion just before this region of the protein. The reason behind the construction of these mutants was that the available amino-terminal mutants of E7 all retained the ability to bind to TBP (personal observations) and, moreover, mutations in the 3' end of E7 had not yet been analysed in depth. In Figure 33A we can see the panel of the proteins: mutant 1 is a deletion from aa 52 to aa 56; mutant 2 from aa 65 to aa 67; mutant 3 from aa 75 to aa 77 and mutant 4 from aa 79 to aa 83. The deletion mutants were cloned into vectors suitable either for in vitro transcription translation (SP64) or also for GST fusion protein production.

The in vitro binding assay was first done with equal amounts of each in vitro translated mutant mixed with a constant amount of GST.TBP (carboxy terminal region), and the result after PAGE analysis is shown in Figure 33B. It is clear from the results of this experiment that mutant 2 and mutant 3 bind to the TBP as
Figures 33A and 33B. Mutational analysis of the carboxy terminal half of the E7 protein regarding the TBP binding domain. Panel 33A shows the location on the protein of the four deletions introduced into the HPV16 E7. Also shown for comparison is the location of the pRb binding pocket and the CKII recognition site. In panel 33B, in vitro translated radiolabelled wild type E7 and the four deletion mutants were incubated with purified GST.TBP fusion protein. After extensive washing the bound proteins were analysed by SDS-PAGE gel and autoradiography. On the left side of the gel are the protein inputs. On the right side are the amounts of proteins retained on the column.
strongly as the wild type E7; in marked contrast, the mutant 1 and the mutant 4 retain only minimal binding to TBP. This result allows us to say that there is probably more than one domain on E7 involved in the interaction with TBP. The reverse binding assay was also done, using the single deletions as GST fusion proteins, as shown in Figure 34A, plus in vitro translated TBP. Again, the above results were confirmed, since (Figure 34B), mutants 2 and 3 bind TBP as strongly as wild type E7 and mutants 1 and mutants 4 are defective.

To verify whether the mutants bound directly to TBP in similar manner as wild type E7, the direct binding experiment was again performed, using the purified TBP (Promega) protein and the different mutants of HPV16 E7 as GST fusion proteins. In this way other potential factors present in the translation, which could mediate the association were avoided. 40 ng of the purified TBP were mixed with balanced amounts of GST fusion mutants 1, 2, 3, and 4. After extensive washing, the bound protein was assessed on a SDS-PAGE which was then subject to a Western Blot analysis. The membrane was probed with commercial monoclonal anti-TBP antibody (Oncogene Science), and the results obtained are shown in Figure 34C. As can be seen the binding between mutants 2 and 3 and TBP is also direct, similarly to what was observed between wild type E7 and the TBP.

Considering the strong influence of CKII phosphorylation on the E7-TBP interaction, it was extremely interesting to analyse whether the CKII phosphorylation could have any effects also on the binding between TBP and these E7 mutants. Therefore the binding experiment was done before and after the phosphorylation of the E7 mutants, and the result is reported in Figures 35A and 35B. Again, the binding between mutants 2 and 3 and TBP increases after the phosphorylation, as seen with the interaction with wild type E7. The experiment
Figures 34A, 34B and 34C. Binding between mutants of E7 and TBP. Panel 34A shows the profile of the mutant E7 GST fusion proteins purified to near homogeneity. The molecular weight of each is about 45 Kd because the deletions are only of 3 or 5 amino acids. Panel 34B shows the binding between equal amounts of GST fusion proteins and in vitro translated carboxy terminal region of TBP. In panel 34C the Western Blot demonstrates that in the case of E7 deletions the binding is also direct and the conditions of the E7/TBP association remain the same.
Figures 35A and 35B. Phosphorylation of E7 mutants defective for TBP binding still increases binding. Equal amounts of the different E7 deletion mutants were bound to glutathione agarose beads and binding assays to radiolabelled TBP were performed. Upper panel shows binding before phosphorylation, and lower panel shows binding after phosphorylation.
also suggest that the association between TBP and mutants 1 and 4, which normally bind TBP to a much lesser extent than wild type E7 can also be increased after the phosphorylation reaction. These results demonstrate that mutants of E7 which are greatly reduced in their ability to complex with TBP can nevertheless be stimulated to bind following CKII phosphorylation.

19. **HPV16 E7 Binding to TBP Contributes to its Transforming Activity.**

Having characterised the region of TBP bound by E7 and the domains of E7 involved in the interaction with TBP, it was of additional interest to determine whether E7 binding to TBP contributes to its transforming activity. Indeed, previous studies using an E7 mutated in its CKII recognition site, which has reduced binding to TBP, would predict that the TBP association may play a role, since the non-phosphorylatable mutant exhibits reduced levels of transforming activity (Barbosa et al., 1990). To investigate this, the ability of the E7 carboxy terminal mutants to cooperate with an activated ras oncogene in the transformation of primary Baby Rat Kidney (BRK) cells was tested. These cells were prepared from 9 day-old Wistar rats and transfected with the different mutant E7 genes cloned in plasmid pJ4Ω (10μg), together with EJ-ras (3μg) and a selectable marker (1μg) for geneticin (G418). After two weeks of selection with 200 μg/ml G418 the cells were fixed, stained and the number of the colonies for each transfection were counted. In Table 2 there is a summary of results of seven different experiments. We obtained a considerable number of colonies with the wild type E7 and a low number of colonies with the p31/32 mutant, in agreement with previous studies (Edmonds and Vousden, 1989; Storey et al., 1990; Firzlaff et al., 1991). Doing a comparison
Table 2. Transforming activity of the E7 deletion mutants compared with wild type E7. Primary BRK cells from 9-day-old Wistar rats were transfected with the indicated plasmid together with EJ-ras and pSV2neo. After 2 weeks of selection in 200 μg/ml G418 the cells were fixed and stained and the colonies counted. Numbers show the colonies obtained from seven separate transformation assays. ND: not determined.
between the four mutants, we can see some differences. It is clear that mutants Δ 65-67 (Δ2) and Δ 75-77 (Δ3) have essentially wild type levels of transforming activity. In contrast mutant Δ 52-56 (Δ1) and Δ 79-83 (Δ4) have a significantly reduced (P < 0.001 by χ²) ability to cooperate with EJ-ras in the transformation of primary BRK cells. These results indicate that the regions of the E7 protein spanning residues 52-56 and 79-83 have a role in its transforming activity and suggest that, although binding TBP is not essential for the transforming activity of E7, it nevertheless contributes to some extent. This is further borne out by the CKII mutant of E7 which has reduced transforming activity and reduced ability to complex with TBP.


At this point it was necessary to determine whether the different E7 mutants were expressed at levels similar to the wild type E7 protein. Total cell extracts of the transformed lines were assessed by Western Blot analysis, using anti E7 polyclonal antibody and the ECL protocol (Amersham). The results are shown in Figure 36A. It is clear that in comparison with the negative control, all of the extracts contain the E7 protein. However, the levels of the E7 protein that was found in the lines transformed with Δ2 and Δ3 is striking. In these lines the level of E7 is 8 to 10 fold higher than in the wild type E7-containing cells and the Δ1 and Δ4 lines. To further investigate the reason underlying the very high levels of expression of the mutant proteins, Southern Blot and Northern Blot analyses were performed on the different cell lines.
Figures 36A, 36B and 36C. Expression of the E7 deletion mutant proteins. Panel 36A shows the Western Blot analysis probed with the anti-E7 polyclonal antibodies diluted 1:200 in PBS/2% milk powder, followed by anti-rabbit and avidin-peroxidase conjugate, both at 1:1000 in PBS/10% milk powder. It is clear that each of the mutants expresses E7 protein, but that Δ2 and Δ3 express higher levels of protein. The number under each track is that of the clone analysed. Panel 36B shows a Southern Blot analysis of the same clones. The DNA copy number of each clone can be seen. Panel 36C shows the Phosphor Imager analysis of panel 36B.
The results of the Southern Blot analysis are reported in Figures 36B and 36C. It is clear, also from the data obtained with the Phosphor Imager analysis, that there is a very high DNA copy number of Δ2 line compared with the wild type E7 line. This might explain the high levels of Δ2 protein seen in the Western Blot analysis. However, the Δ1 DNA copy number is about double that of wild type E7, yet the E7 protein levels in Δ1 cells is equal that one of the wild type cells. The same argument is true for the Δ3 line: the DNA copy number is slightly higher than that of wild type E7, but the level of the protein is at least 8 to 10 fold higher. It is therefore possible to conclude the DNA copy number does not reflect the final levels of production of the protein in the transformed clones that were analysed. A Northern Blot experiment was also performed to assess the amount of the mRNA present in the different lines, and the results obtained are shown in Figures 37. In panels 37A and 37C we can see that the amount of mRNA of clone Δ3(6) appears to be twice that of clone E7(7). However, upon Coomassie Blue staining of the Northern Blot it became clear is that the amount of total RNA loaded for the Δ3(6) clone is also twice as much as that loaded for the E7(7) clone. Thus the amount of mRNA encoding E7 present in the different lines is very similar and does not account for the difference in the amount of E7 protein expressed in the cells, and suggests that may be an intrinsic difference in protein stability. To address this aspect of the overproduction of the E7 mutants a test of the protein half life by a pulse-chase experiment was performed. The results are shown in Figures 38A and 38B. It is evident, from the autoradiograph and also from the data obtained by the Phosphor Imager analysis, that the half life of E7 wild type protein is about 1 hour, in agreement with previous studies (Smotkin and Wettstein, 1987). In contrast, the half
Figures 37A, 37B, and 37C. Northern Blot analysis of the deletion clones of E7. Panel 37A shows the Northern Blot to demonstrate the amount of mRNA present in the different E7 mutants. The mRNA of Δ3 mutant appears to be about 2 fold higher than that of wild type E7. Only after staining the same blot, shown in Figure 37B, it is clear that the amount of total Δ3 RNA loaded was double that of the wild type E7. The data obtained with the Phosphor Imager analysis are shown in Figure 37C.
Figures 38A and 38B. Half life experiment on wild type E7, Δ2 and Δ3 clones. Panel 38A shows a pulse-chase assay done on the three different cell lines. It is also clear from the data obtained by Phosphor Imager analysis, shown in panel 38B, that the half life of wild type E7 protein is of about 1 hour. In comparison the half life of the Δ2 and Δ3 clones is at least of about 2 hours, indicating that the E7 mutants are more stable. I have to stress the fact that the data in panel B are calculated from the mean of the results of three different experiments and are not specially referring to the experiment shown in panel A.
life of the Δ2 mutant is about 2 hours. The same experiment was repeated for the Δ3 deletion mutant with similar results (Figures 38A and 38B). Taken together, these results indicate that the Δ2 and the Δ3 mutants are intrinsically more stable than the wild type E7 protein.

21. Transcriptional Activity of E7 Mutants.

Another possible explanation for the reduced transforming activity of the Δ1 and Δ4 mutants could be reduced binding to pRB and consequent reduced induction of E2F transcriptional activity. To investigate this possibility, a series of transcriptional activation assays were performed on the Adenovirus E2 promoter with the different E7 mutants. Cells were transfected with the Ad E2 CAT reporter plasmid together with wild type and mutant E7 expressed in pJ4Ω. The results obtained are shown in Figure 39A. All the four mutants possess a similar ability to activate the adenovirus E2 promoter as the wild type E7 protein and, furthermore, the overexpression of the Δ2 and Δ3 mutants, does not affect their transcription activity. It was of additional interest to analyse, at this point, the capacity of the mutants to degrade pRB. With this aim, a Western Blot analysis on total cell extracts was performed using monoclonal anti-pRB antibody (Santa Cruz). The result, shown in Figure 39B, demonstrates that all the clones are able to target pRB to the same level. As positive control we used a BRK cell line transformed only with activated ras oncogene. The pRB protein is not detectable in any of the lines which express either the wild type E7 or the mutants, suggesting that all the E7 proteins tested are able to bind pRB and to target it for degradation, in agreement with previous reports (Demers et al., 1994; Wazer et al., 1995). CaSki cells were used to confirm the level of pRb protein
Figures 39A and 39B. Comparison of the abilities of the different E7 mutants to activate transcription. Panel 39A shows the CAT assay on NIH3T3 cells transfected with 3µg of AdE2 promoter together with 5µg of pJΩ4 control, wt E7 or indicated mutant. It is clear from the mean percentage CAT conversion, calculated from at least three experiments, that all the deletion DNAs are able to activate transcription like wild type E7. Standard deviations are shown. Moreover, all the E7 mutants are also able to degrade pRB like wild type E7, as demonstrated in panel 39B with the Western Blot analysis, using monoclonal anti-pRb (Santa Cruz) antibody on the total cell extracts.
present in a E7 positive line and Saos-2 cells were introduced as negative control. Taken together these results demonstrate that the reduced levels of transforming activity obtained with Δ1 and Δ4 mutants is not due to low level of expression, nor to a defect in the pRB association. It is most likely a reflection of the reduced ability to interact with TBP.

22. Phosphorylation State of the E7 Protein in vivo.

In this project several new observations have been shown, so far, regarding E7 functions.

❖ a new direct strong interaction between E7 and the important transcription factor TBP;
❖ the E7 phosphorylation by CKII increases the interaction between the two proteins;
❖ the E7 binding domain on TBP is confined to residues 202-271;
❖ the binding domains on E7 are limited to the carboxy terminal region of the protein (52-56 aa and 79-83 aa);
❖ the interaction between E7 and TBP can influence the inhibition by E7 of p53 transcriptional transactivation activity, through the formation of a tripartite complex;
❖ the E7-TBP interaction can affect the transforming activity of E7.

Considering the importance of the phosphorylation event in the E7-TBP interaction, it was extremely intriguing at this point to analyse in more detail the phosphorylation state of the E7 protein in vivo. Furthermore, since a large number of cellular targets for E7 have now been identified, it was reasonable to think that
differential phosphorylation of E7 may be a means of providing specificity for a number of these interactions during different phases of the cell cycle and/or differentiation. Moreover, although there are reports (Barbosa et al., 1990) of E7 phosphorylation by the CKII enzyme, no observations have been made on the potential changes in the level of E7 phosphorylation during the cell cycle nor on other possible phosphorylation sites of the protein.

It was firstly of interest to determine whether phosphorylation of E7 was only at the CKII site, or whether there were other regions of the protein which could be phosphorylated. To do this BRK cell lines were produced expressing wild type E7, the mutant of E7 in the CKII recognition site, p31/32, and the double Aspartic Acid mutant at the CKII recognition site. These cells were all labelled with $^{32}$P orthophosphate. A equal amount of total cell extract from each line was then immunoprecipitated with monoclonal anti-E7 antibody. The Protein A Sepharose bound complexes were assessed on a 15% SDS-PAGE gel and autoradiography. Ras alone transformed cell line was included in the assay as negative control. The results are shown in Figures 40A and 40 B. The autoradiograph shows that in the wild type line the E7 protein is phosphorylated. More surprising is the presence of a radiolabelled band of the same molecular weight in the p31/32 and Aspartic Acid cell lines. This result demonstrates that mutants which are defective for CKII phosphorylation are nevertheless phosphorylated in vivo. This shows conclusively that E7 can be phosphorylated at residues outside the CKII recognition sequence.
Figures 40A and 40B. Phosphorylation of HPV-16 E7 protein in vivo. BRK cells were transfected with wild type HPV-16 E7 plus EJ-ras and two mutants of E7 in the CKII recognition site; p31/32 and double Aspartic Acid. The cells were labelled with ImCi of $^{32}$P orthophosphate and E7 immunoprecipitated using anti-E7 polyclonal antibody. As can be seen from two separate experiments (Panels A and B) both of the E7 mutants in the CKII recognition site show incorporation of $^{32}$P indicating phosphorylation of E7 at residues different from the CKII recognition site.
23. The E7 Phosphorylation State Changes during the Cell Cycle.

Having demonstrated that HPV16 E7 is phosphorylated in BRK cells by at least two different kinases, it was then interesting to analyse the possible changes in the phosphorylation events during the different phases of the cell cycle.

In order to do this the 14/2 cell line was used, available in the laboratory. These are BRK cells transformed with a construct which has the mouse mammary tumour virus long terminal repeat (MMTV-LTR) in front of the E7 gene. This contains a glucocorticoid response element (GRE) which allows E7 expression to be induced by the presence of glucocorticoid hormone (dexamethasone $10^{-6}$ M) (Crook et al., 1989). The 14/2 cells expresses EJ-ras constitutively, and the HPV16 E7 inducibly. In the absence of dexamethasone the cells cease to express E7 and cease to proliferate and after 14 days the majority of them are not viable. Previous studies have shown the capacity of E7 to stimulate DNA synthesis (Banks et al., 1990a), using this E7 inducible system. Using either serum starvation or dexamethasone withdrawal, these cells enter S phase in 6-8 hours after addition of Foetal Calf Serum (FCS), or dexamethasone. To perform the E7 in vivo phosphorylation assay through the different phases of the cell cycle, the 14/2 cells were starved for 48 hours in low serum concentration medium (0.2% FCS) with normal hormone growing conditions ($10^{-6}$M dexamethasone), and then were incubated with 10% FCS for a total period of 8 hours. At the same time $^{32}$P orthophosphate was added to the medium. Samples were taken every 2 hours and equal amounts of cell extracts for each time point were immunoprecipitated with monoclonal anti-E7 antibody. Simultaneously two other assays were performed on a parallel unlabelled set of cells: A) BUDR (Bromo-deoxy uridine) incorporation was assessed to monitor DNA synthesis to confirm the G0 block of the cells after the 48 hours and monitor
the S phase entry; B) Western Blot analysis on the same cell extracts to verify equal E7 protein expression at each time point. The results are shown in Figures 41A, 41B and 41C. Panel A shows the profile of BUDR incorporation, made with the percentages of cells positive to the stain at each time point. In agreement with previous observations (Banks et al., 1990a), it is clear that the 14/2 cells enter S phase 6 hours post addition of serum, with 64% of cells positive for the BUDR staining, compared with the G0/G1 block of only 12% of BUDR-positive cells. Panel B shows the results of two different in vivo phosphorylation experiments. As can be seen there is a clear change in the phosphorylation level of the E7 protein during the different phases of the cell cycle. Indeed, there is a high level of phosphorylation at the 0h time point (G0/G1), with a decrease of the level of phosphorylation after 2 hours, followed by another increase after 6 hours (S phase). In panel C is shown the result of the Western Blot analysis on the same cell extracts demonstrating the absence of variability in the E7 protein expression throughout this part of the cell cycle, suggesting that the differences in E7 phosphorylation level is not a reflection of changes in the levels of E7 protein expression. These data allow us to say that there is a change in the E7 phosphorylation levels through the different phases of the cell cycle.


Having demonstrated differences in the E7 phosphorylation in vivo, it was necessary to establish an in vitro assay for further dissecting these activities. To do this, GST.16E7 protein was incubated for 10 mins. at 30°C with extracts of 14/2 cells (made as described in Material and Methods), as a source of kinases, plus
Figures 41A, 41B and 41C. Panel A shows the cell cycle profile of 14/2 cells done after BUDR staining on cells starved for 48 hours in a low concentration of serum (0.2%FCS) and then induced with 10% FCS for a total time course of 7 hours. It is clear that the 14/2 cells show maximum S phase entry after 6 hours.

Panel B shows the in vivo E7 phosphorylation state in 14/2 cells during a time course assay. It is clear from the data obtained by Phosphor Imager analysis that there is a variation in the level of E7 phosphorylation during the different phases of cell cycle. In panel C is the Western Blot analysis of the same time course which demonstrates an equal amount of E7 protein expression during the time course.
labelled $^{32}$P γ ATP (Amersham) (100μM) and protease inhibitors (0.3 μM Aprotinin, 1 μM Pepstatin, 100 μM TLCK, 200 μM TPCK). After extensive washing the proteins were run on SDS-PAGE gel. The result of the autoradiography is shown in Figure 42A. The experiment was done on an equal amount of GST.16 E7 with decreasing amounts of cell extract of growing 14/2 cells, to determine the minimum quantity of extract necessary to obtain phosphorylation. The E7 phosphorylation occurs in vitro even with 200ng of 14/2 cell extract.

Having established optimal conditions for phosphorylation in vitro, the phosphorylation of the GST.16E7 wild type fusion protein was performed using extracts of 14/2 cell harvested at different points in the cell cycle. The cells were starved, as before, for 48 hours in low serum, and then were induced with 10% FCS for 8 hours. Cell extracts were made for each time point. Equal amounts of GST.E7 protein were mixed for 10 mins. at 30°C with the same amount (2μg) of each cell extract, with addition of labelled ATP and protease inhibitors. After extensive washing, the labelled GST.E7 proteins were assessed on SDS-PAGE gel and autoradiography. The results are shown in Figure 42B, and demonstrate that there are clear changes in the in vitro phosphorylation of GST.16 E7 by 14/2 cell extracts from the different points of the cell cycle. Indeed, GST.E7 is highly phosphorylated at time point 0 (G0) then the level of phosphorylation decreases, followed later by another increase of the E7 phosphorylation state at time points 6 and 8 hours (S phase). Interestingly the pattern of phosphorylation seen in the in vitro assay follows very closely the pattern seen in the in vivo assay. A slight difference is visible in the levels of phosphorylation at the 6-8 hrs time points in this particular assay (Figure 42), but the following experiments (Figures 43-46) all show a decrease in E7 phosphorylation at the later time points very similar to that seen in
Figures 42A and 42B. *In vitro* phosphorylation of GST.16E7 fusion protein using 14/2 cell extracts.

The top panel shows the titration of the 14/2 cell extract to determine the optimal conditions for GST.E7 *in vitro* phosphorylation. The bottom panel shows the GST16.E7 phosphorylation following incubation with extracts of 14/2 cells at different times during the cell cycle. As can be seen there are clear changes in the levels of E7 phosphorylation during the time course. The data obtained by Phosphor Imager analysis are also shown.
the in vivo assay. Taken together, this data suggests that E7 is differentially phosphorylated in the progress from G0/S phases, with reduced phosphorylation at later times during the cell cycle. The assay was repeated under the same conditions with HaCat cell extract (Figure 43) and the results confirm that the previous data was not only specific for the BRK 14/2 cells.

25. Behaviour of the E7 p31/32 Mutant in the in vitro Phosphorylation Assay. Having shown that mutants of E7 in the CKII recognition site are nevertheless highly phosphorylated in vivo, an in vitro assay was performed to determine which kinase exhibited cell cycle dependent activity. There are previous reports suggesting the regulation of CKII kinase activity by the serum conditions in which the cells are grown, with an induction of the CKII activation in G0-G1 phase of the cell cycle, then a decrease of activity followed a further activation in G1-S phase transition. (Carroll and Marshak, 1989). To verify whether the cell cycle dependence of E7 phosphorylation was due to the cyclic activation of the CKII enzyme during the cell cycle or to another kinase, phosphorylation of the p31/32 mutant of E7 was assessed. The experiment was done as described above, with incubation of equal amounts of GST. p31/32 fusion protein and balanced aliquots (2μg) of the 14/2 time course cell cycle extracts. GST. 16 E7 was included in the assay to confirm the previous E7 phosphorylation pattern and to permit a direct comparison with the mutant. The result is reported in Figure 44A and 44B, and reveals a number of interesting observations. First of all, the phosphorylation level of wild type E7 still changes through the different phases of the cell cycle, with a strong phosphorylation at time point 0h (G0/G1), followed by a decrease and again by an increase at time
Figure 43. Differential *in vitro* phosphorylation of GST.16E7 fusion protein, by HaCat cell extracts. Equal amounts of the fusion protein were incubated with equal aliquots of HaCat cells extracts harvested at different times following serum addition. The results show that the phosphorylation state of E7 protein also alters during the cell cycle in human epithelial cells.
Figures 44A and 44B. *In vitro* phosphorylation of the p31/32 mutant in comparison with wild type E7. The top panel shows the wt GST.E7 phosphorylation during the phases of the cell cycle. The bottom panel shows the GST. p31/32 phosphorylation during the same time course. It is clear that there is a difference in the pattern of phosphorylation between the two proteins, indicating phosphorylation of E7 by two different enzymes during the cell cycle.
points 6h (S phase). More surprising is the p31/32 result, which shows a completely different pattern of phosphorylation. Indeed, the E7 mutant is not phosphorylated at time point 0 (G0/G1) and its phosphorylation appears to happen only later in the cell cycle, after 6 hours of serum induction. Thus, it is possible to say that there are two different phosphorylation sites on E7: one responsible for the E7 phosphorylation in the first stages of the cell cycle, and another one responsible for the E7 phosphorylation during the later phases of the cell cycle. Thus, the CKII enzyme would appear to phosphorylate E7 in the G0/G1 phase of the cell cycle, and another, unknown kinase, phosphorylates E7 later on when the cells undergo S phase entry. However, it seems likely that this later phosphorylation level consists of both CKII and the other unknown kinase, since the levels of phosphorylation on the wild type E7 is considerably higher than that on the p31/32 mutant at the S phase transition.

26. Identification of a Novel E7 Phosphorylation Site.

Since the behaviour of the p31/32 mutant suggested the presence of another potential phosphorylation site of the E7 protein, it was necessary at this point to perform a mutational analysis of E7 to determine which is/are the new residue/s capable of being phosphorylated. A previous report (Storey et al., 1990) had identified the presence of potential phosphorylation sites within the 3’ half of the E7 protein. For this reason, the 3’E7 end was cloned into the pGEX-2T plasmid, and the fusion protein was produced and purified as described above. The in vitro phosphorylation experiment was then performed with the GST. 3’E7 end and the labelled proteins were run on SDS-PAGE gel. The result of the autoradiography is
reported in Figure 45. It is clear from the assay that the phosphorylation pattern of the carboxy-terminus region of E7 (Panel B) is different from that of the full length wild type protein (Panel A). Furthermore, the phosphorylation evidently occurs only later on (4-6 hrs) after serum induction, suggesting a correlation with the results obtained with the p31/32 mutant. The conclusion is that the second potential phosphorylation site of E7 is located in the carboxy-terminus half of the protein.


Having found that the second potential phosphorylation residue of E7 is located in the carboxy-terminus part of the protein, a more detailed mutational analysis of this region was performed. The potential Serines which could be phosphorylated in the 3'E7 end of the protein, are at position 71 and 95. Previous authors have speculated that Ser71 could constitute a potential phosphorylation site (Storey et al., 1990). Therefore to address this possibility, a full length E7 with a Ser71 mutation (Glycine instead the Serine) was cloned into the pGEX-2T plasmid and the protein was produced and purified as described before. Furthermore, the same mutation was introduced into the 3'E7 end of the protein and was cloned in pGEX-2T. The assay was performed as before and the results of the autoradiographies are shown in the left hand panels of Figures 46A, 46B and 46C. The right hand panels show the same gel rehydrated and stained with Coomassie Blue to confirm that the variations seen in the phosphorylation pattern were not due to differences in the amounts of the GST fusion proteins used. It is clear from the autoradiography, that the Ser71 mutant in the full length protein is phosphorylated only at time point 0h (G0/G1).
**Figures 45A and 45B.** *In vitro* phosphorylation of the GST. 3′E7 end fusion protein. The cell cycle experiment was repeated as described in the text. The top panel shows wt E7 phosphorylation and the bottom panel shows phosphorylation of the carboxy-terminal half of E7. These results demonstrate that the second phosphorylation site on E7 lies within its carboxy-terminal half.
Figures 46A, 46B and 46C. In vitro phosphorylation of GST. Ser71 and GST.3’end Ser71. It is clear from the autoradiography and from the data obtained with the Phosphor Imager analysis (at the bottom of each assay), that the Ser71 mutant is phosphorylated only at time point 0 (G0/G1). In contrast, 3’end Ser71 is not phosphorylated at any point during the time course. These results demonstrate that phosphorylation of E7 at G0/G1 is by CKII and that later phosphorylation of E7 is at Ser71 by an unknown kinase which shows cell cycle regulation.
No other phosphorylation is present at any later time point. Moreover, in the *in vitro* phosphorylation of the Ser71 mutant in the 3′E7 end part of the protein there is no evidence of any phosphorylation events, suggesting that the second phosphorylation site of E7 is located at residue 71 in the carboxy-terminal half of the protein.
DISCUSSION

E7, the major transforming protein of HPV16, is a multifunctional protein. It has been shown previously to complex with a number of the cellular proteins intimately involved in the control of the cell growth. These include pRB, p107 and the Cyclin A/CDK2 complex (Dyson et al., 1989; Davies et al., 1993; Tommasino et al., 1993). Mutations in a number of regions of E7 result in a loss or reduction of transforming activity even though wild type levels of pRB binding are retained (Banks et al., 1988; Barbosa et al., 1990; Storey et al., 1990). Although the principal transcriptional activity of E7 is via pRB, there are several reports of E7 activating transcription via pRB-independent mechanisms (Edmonds and Vousden, 1989; Phelps et al., 1991; Zwerschke et al., 1996), indicating that E7 has the potential to bind to components of the cellular transcriptional machinery.

E7 has been shown to interact also in vitro with the members of AP-1 family of transcription factors, such as c-jun, junB, junD and c-fos (Thierry et al., 1992; Morosov et al., 1994; Satoru et al., 1995; Antinore et al., 1996). Mutations in the zinc-binding CD3 domain, but not in the pRB-binding pocket, of E7 abolish the interaction with c-jun, which as a consequence results in the down-regulation of c-jun responsive promoters (Antinore et al., 1996) and mediates the E7 transformation activity. The use of a dominant negative c-jun abolishes the cooperation between E7 and EJ-ras in primary rodent cells and suppress the anchorage-independent growth of HPV transformed keratinocytes (Li et al. 1998). Keeping all these observation in mind, it was of additional interest to identify other
cellular proteins related to the transcriptional machinery with which E7 may interact. We considered potential candidates including the basal elements of the cellular transcriptional machinery, with particular consideration of previous work carried out on the Adenovirus E1a, in particular TBP (Horikoshi et al., 1991; Lee et al., 1991).

I have shown that a new target of E7 is the TBP. The interaction is regulated by CKII phosphorylation which in turn is cell cycle regulated. These results are the first demonstration of a role for the CKII phosphorylation of E7. This mechanism provides specificity for the recruitment of one of the target proteins of E7 during different phases of the cell cycle, and also potentially during the replicative cycle of the viral infection.

Since a large portion of the project was dependent up the availability of an effective anti-HPV16 E7 antibody, I should start the discussion of my results with the production of anti-HPV16 E7 polyclonal antibodies, which was obtained by regular immunisation with GST.16 E7 fusion protein into rabbits. Keeping in mind that anti-E7 antibodies can be detected in the serum of 20-30% of patients with HPV16-associated cervical lesions (Jochmus-Kudielka et al., 1989; Tindle et al., 1990; Suchánková et al., 1991), the E7 protein is easily recognised as foreign by the immune system.

The immunisation was successful since the antibodies obtained were reactive even with very low concentrations (20 pg) of GST.16 E7 protein on a Western Blot. The antibody also reacted in the same assay with the E7 protein present in the total crude cell extract of CaSki cells which contain multiple copies of integrated HPV16, recognising a protein of at 17 kDa. This corresponds to the observed molecular weight of the E7 protein as discussed by others (Smotkin and Wettstein, 1987). The polyclonal anti-E7 antibody has been shown to be reactive with high affinity and specificity also in immunoprecipitation analysis, because of its ability to precipitate in vitro translated E7 protein, to recognise the eukaryotic E7 protein from radiolabelled CaSki cells, and to no cross-react with other HPV E7s or other HPV proteins.

2. GST Fusion Protein Production and Purification.

To analyse the presence of possible interactions between HPV16 E7 and factors of the transcriptional machinery, the E7 proteins and the other proteins which needed
to be compared with E7 (E7 mutants and E1a) were expressed as fusions with glutathione S-transferase (GST).

The production of this kind of fusion proteins was relatively easy and resulted in a very useful system for in vitro pull-down experiments, considering that the produced proteins maintained an active conformation similar to the native one. This emerged clearly from the results obtained by the immunological use (as above described) and by the experiments done on the phosphorylation of E7. The protein had to be cloned in frame with the GST expression vector, which has a lac repressor (product of the lacI gene) which binds to the P_{ac} promoter, repressing the expression of GST. All the fusion proteins presented a total molecular weight due to the sum of the GST molecular weight of 27,500 Daltons and the molecular weight of the single protein of interest, about 17,000 Daltons for E7. Such fusion proteins were all soluble thanks to the absence of strongly hydrophobic regions in the E7, and all of them were easily purified from lysed cells under nondenaturing conditions by absorption onto glutathione agarose beads, with the following possibility of elution in the presence of free glutathione at neutral pH.

After the production of the GST fusion proteins the purification, tested on SDS-PAGE gel stained either with Coomassie Blue or with the Silver stain system (Bio-Rad), was shown to be near homogeneity, since for most of the proteins only the single band of the fusion protein was obtained. In some of the cases, other lower molecular weight bands were present, which were demonstrated by Western Blot analysis to be breakdown products of the full length GST fusion protein.
3. *In vitro* Interaction between HPV E7 and TBP.

In the first part of the project the TATA Box Binding Protein (TBP) was found to be an additional cellular target for HPV-16 E7. This was first demonstrated using GST.16E7 fusion protein plus *in vitro* translated TBP. To analyse the interaction in more detail the experiment was done also with comparison between HPV16 E7, HPV11 E7 and Ad E1a. With all the proteins significant binding to the TBP protein was observed. The amount of TBP retained on the GST.16E7 beads was routinely about 20% of the total input, confirming the presence of a strong interaction between the two proteins. The *in vitro* assay revealed a sort of hierarchy in the strength of the interactions, demonstrating a stronger binding between E1a and TBP (21% of the input), followed by the binding between 16 E7 and TBP. Finally, the weakest interaction is the one between 11 E7 and TBP (10% of the input).

Furthermore, the data obtained confirmed the presence of a strong binding, with 21% of retained protein, between Ad E1a and TBP as had previously been demonstrated (Horikoshy *et al.*, 1991; Lee *et al.*, 1991; Mazzarelli *et al.*, 1995; Enzenauer *et al.*, 1998).

Two further observations can be made from the above results. First, that binding to TBP is a conserved function of the HPV E7 proteins and is not confined to the oncogenic-associated HPVs. A different situation has been found for other proteins bound by E7, such as pRB, p107 and Cyclin A where the association is weaker for the “low risk” HPV6 and 11 E7 proteins as compared to “high risk” HPV16, 18 and 33 E7 protein (Münger *et al.*, 1989b; Ciccolini *et al.*, 1994). Recent reports have suggested that E7 may stimulate transcription from the c-*fós* promoter (Morosov *et al.*, 1994), through its interaction with some transcription factors, like TBP or dTAFII 110. Moreover, another study underlined recently the presence of an
interaction between E7 of HPV 8, an oncogenic HPV type specifically associated with skin cancer of epidermodysplasia verruciformis, and TBP, together with other transcription associated factors, such as TAFI120, TAFI128, TAFI155, TAFI1DeltaN135 (Enzenauer et al., 1998). Our results demonstrating the conservation of the E7-TBP binding between malignant and benign HPVs are however in contradiction with the fact that only E7 from the high-risk HPVs, but not low-risk HPVs, stimulates the c-fos promoter possibly contributing to the oncogenic potential of the Papillomavirus indicating either that this activity is not solely due to the ability of E7 to bind TBP and that this reflects the differences in the respective affinities of 16 E7 and 11 E7 for TBP. Second, the E7 function is also conserved with the Ad E1a protein, confirming once more the conservation of functions between these two oncogenic proteins. Moreover, the E1a-TBP interaction is known to be necessary, although not sufficient, for E1a transactivation. Indeed, E1a has two apparent modes of stimulating transcription, one involving the pRB/E2F mechanism and the other involving transcriptional stimulation through interaction with transcription complexes bound at the promoter (Lee et al., 1991).

Finally, the fact that the binding was very strong using the TBP expressed as a GST fusion protein and the in vitro translated E7, suggested that the above interaction was specific and not due to a particular conformation assumed by the fusion protein. The binding experiments performed with purified commercial TBP and not an in vitro TBP transcription translation product, allowed the analysis to be done in the absence of any additional factors which could mediate the interaction, permitting the conclusion that the E7-TBP binding was also direct. Moreover, the GST fusion proteins used were produced with the addition of DNAse I in their purification,
which allowed us to eliminate also the DNA as a possible mediator of the binding. Considering that E7 protein is an acidic protein and apparently does not have DNA binding ability (Imai et al., 1991), it is likely that it is exerting its biological functions through direct protein-protein interactions.

*In vitro* analysis of the E7-TBP interaction was done to define the strength of the binding between the two proteins. By incubating the E7-TBP complex with several different types of washes, where the detergent or the salt concentrations varied, it was possible to see that the binding was resistant to very stringent conditions. Indeed, HPV16 E7 was able to bind to TBP even at 1% TRITON-X 100 or 4M NaCl concentrations, whereas the HPV11 E7/TBP binding was abolished by washing with 0.05% TRITON-X 100 or 1M NaCl. This underlines that the HPV16 E7/TBP interaction is significantly stronger and more stable than that between HPV11 E7 and TBP.

4. *In vivo* Interaction between E7 and TBP.

Having demonstrated the presence of an *in vitro* interaction between E7 and TBP, it was necessary to confirm the existence of the binding also *in vivo*. The results obtained from the co-immunoprecipitation of radiolabelled CaSki cells showed that the major protein species precipitated by the anti-E7 antibody migrates at 17 kDa approximately, and corresponds to HPV16 E7. Interestingly, significant amounts of a protein migrating at 43 kDa are also co-precipitated by the anti-E7 antibody, and this protein co-migrates with the major protein species precipitated by the anti-TBP antibody. On the other hand the anti-TBP antibody weakly precipitates a protein co-migrating with the E7 protein. Unfortunately, the amount of protein co-precipitated
with either the TBP or the anti-E7 antibodies is very low, indicating that in vivo the percentage of both proteins involved in the complex is small or that the interaction does not survive the immunoprecipitation procedures. Other authors have found the same problems in the in vivo E7/pRB binding (Dyson et al., 1989). This, however, is not particularly surprising since studies with c-Fos and c-Rel, which are also known to bind TBP, indicate that the percentage of total protein complexed to TBP at any one time is approximately 1% (Metz et al., 1994; Kerr et al., 1993). These authors speculate that this may be due to TBP being bound to a large number of other proteins thus reducing the amount available for binding c-Fos at any one time. This is even more applicable to E7 which itself binds to a number of other cellular proteins. The in vivo experiments are further technically difficult firstly because of the relatively low cellular level of the E7 protein, like those reported for most transcription factors (Imai et al., 1991), which would limit complex formation. Secondly the E7 interaction with other factors could also depend on cell cycle regulation or modification of any of the proteins involved. In addition, based on the phosphorylation data (as discussed below), it should also be borne in mind that the level of interaction may vary depending upon the accessibility of E7 to CKII, or on the overall levels of CKII activity, which is itself cell-cycle regulated (Carroll and Marshak, 1989; Lorenz et al., 1994). The percentage of phosphorylated E7 in vivo is not known and since the interaction is in part dependent on this modification (see next), it is reasonable that in the in vivo experiment the amount of E7 retained is low.

The specificity of the interaction was also confirmed by using a pre-immune antibody which failed to immunoprecipitate either E7 or TBP proteins. Moreover, a number of higher molecular weight proteins, including a protein of 105 kDa (which
is visible only at lower exposure of the gel and could be pRB), were also co-precipitated by the anti-E7 antibody and not by the preimmune antibody, demonstrating the specificity of the assays. Moreover, the immunoprecipitation followed by the Western blot assays done on unlabelled CaSki and HT1080 cells confirmed that the band at approximately 43 kDa co-precipitating with HPV16 E7 was TBP. In conclusion, the above observations indicate that, even if the percentage of E7 bound to TBP at any one time is very low, the interaction in vitro is supported by the presence of the binding in vivo. Moreover, the results described above are in agreement with studies using the yeast two-hybrid system which has also demonstrated functional interaction between the HPV E7 proteins and TBP (Phillips and K. Vousden, personal communication).

5. E7 and E1a Binding to TBP are Enhanced by CKII Phosphorylation.

Based on the proximity of the Ad E1a-TBP binding domain to its site of Casein Kinase II phosphorylation, the obvious question was what effect would CKII phosphorylation have upon the ability of E7 and E1a to bind TBP.

The first analysis investigated the ability of the GST16.E7 fusion protein to be phosphorylated in vitro by the CKII enzyme. The results were in agreement with previously published observations (Barbosa et al., 1990), demonstrating the capacity of the GST16.E7 protein to reach maximum phosphorylation in vitro (35% to 40% of the total protein) by CKII after 10-15 mins. of incubation. This confirmed that the E7 protein is an extremely good target for CKII phosphorylation even when is in the form of a GST fusion protein; the specificity of the assay was confirmed by the use of the p31/32 mutant of E7 which could not be phosphorylated by CKII.
Having established the optimal condition for the phosphorylation reaction the following experiments showed that CKII phosphorylation of E7 significantly increases the affinity of E7 for TBP. Interestingly, this effect was also obtained with the Ad E1a protein and revealed a striking conservation of function between these two viral proteins. The fact that the binding with the p31/32 mutant is not affected at all by the CKII phosphorylation, and the binding between HPV11 E7 and TBP is affected to a lesser extent, is probably a consequence of the different levels of phosphorylation presented by the proteins tested: null, that of the p31/32 mutant, and low, that of HPV11E7, when compared with HPV16 E7. Our results demonstrate the importance of the phosphorylation state of E7 in the TBP interaction which, in contrast, is not an essential modification for the E7/pRB binding (Barbosa et al., 1990), underlining the difference between the two protein association events. There are, nevertheless, reports showing that pRB protein preferentially binds the phosphorylated form of HPV6b E7 (Gage et al., 1990), and this could provide an explanation for why HPV6b is associated with cancer in rare cases, where changes in expression of cellular kinases may lead to a higher level of E7 phosphorylation and stronger binding of the RB protein. In our situation the cellular kinases could give an increase in the percentage of phosphorylated E7, with a subsequent increase of E7 interacting with TBP. This could finally cause an activation of the cascade of events related to the transcription or transformation activities of E7.

One unexpected feature of many transcription factor domains, especially eukaryotic ones, is that they sometimes show only a modest degree of specificity and affinity in their interactions with ligands. For example, λ repressor binds to specific operator sequences with affinities as high as $10^{-13}$ M and binds to specific DNA
sequences 500,000-fold better than non-specific DNA (Sauer et al., 1990). In contrast, the binding to DNA by some steroid receptors occurs with nanomolar affinity and with a specificity of less than 100-fold (Schauer et al., 1989). The increase of the E7-TBP interaction due to the phosphorylation event allowed the calculation of the association constant (Ka) between the two proteins, which was determined to be 31.2 nM. This means that the minimum E7 concentration necessary to get an interaction with TBP in vitro is of 31.2 nM, confirming the high strength of the binding between the two proteins, and that the phosphorylated state is also the most favourable condition for the binding of the two proteins in vivo. On the other hand, it is possible for interaction to be very specific even if the affinity is low, and it is probably specificity, rather than affinity, that is the key ingredient for assembling functional transcription complexes. Moreover, at present it is not clear whether CKII phosphorylation promotes binding of an additional protein to E7 which then enhances TBP binding, keeping in mind that co-operative and multiple interactions ensure that the overall specificity of the transcription complex is high, even if some individual interactions are of low specificity. Nonetheless, these results were particularly interesting since it was the first demonstration of an actual role for the CKII phosphorylation of E7 and indicates a precise mechanism for regulating both E7 and E1a functions in vivo. Previous experiments with mutants of E7 defective for CKII phosphorylation have indicated a reduction both in transforming activity (Barbosa et al., 1990) and in the ability to activate transcription from the Adenovirus E2 promoter (Edmonds and Vousden, 1989; Phelps et al., 1991), confirming that the phosphorylation event contributes to the biological activities of the HPV E7 protein. The studies described here provide a clear molecular explanation for these observations.
Of further interest were the studies with the double Aspartic Acid mutant of HPV16 E7, which mimics a fully phosphorylated state of the protein and has already been analysed in previous studies (Firzlaff et al., 1991). The data presented here indicate that the incorporation of a negative charge into this region of the E7 protein is sufficient to increase binding to TBP. Thus, the double Aspartic Acid mutant, is able to bind TBP with a similar affinity to that seen with the phosphorylated wild type protein. These results indicated that the phosphate moieties incorporated at the CKII site are not themselves important in binding TBP but rather, that the net negative charge at this point on E7 is the critical factor. Interestingly, previous studies using this same double Aspartic Acid mutant of E7, have shown it to possess wild type levels of transforming and transactivation activities (Firzlaff et al., 1991), and it should be also emphasised that unphosphorylated E7 is still capable of binding TBP. Moreover, since the CKII site is adjacent to the pRB binding site, it had been suggested that CKII might regulate association with pRB (Firzlaff et al., 1989). Subsequent studies have demonstrated no difference between wild type, Alanine and Aspartic Acid mutants in their in vivo binding to pRB (Barbosa et al., 1990), suggesting that the binding to pRB protein is probably not sufficient for the transformation activity of E7, keeping in mind that there are also other mutations in the N and C termini of E7 which can affect the transformation activity of the protein (Edmonds and Vousden, 1989).

6. HPV16 E7 Binds to the Conserved Carboxy Terminal Region of TBP.

The in vitro analysis of the E7-TBP interaction was done in part using a truncated version of the TBP molecule, comprising the conserved carboxy terminal half of the
protein (amino acids 165-339). In order to study this interaction in more detail, a TBP mutational analysis was done to define which domains of the TBP protein were involved in the E7 binding. The results identified the region of TBP essential for complex formation with E7 in the stretch of amino acids between residues 201 and 272, confirming that the E7 protein is one of a number of proteins (including E1a, p53, and SV40 T antigen) which can interact with the same carboxy terminal region of TBP (Lee et al., 1991; Emily et al., 1994; Martin et al., 1993). This domain contains a repeat of basic residues highly conserved throughout evolution and has been postulated to be a site of interaction between TBP and acidic activation domains (Horikoshi et al., 1989). Moreover, in the case of T antigen and p53 it has been suggested that these two proteins may compete with each other determining a situation of antagonism for the binding to TBP.

The transcription initiation on protein-encoding genes is mediated by RNA Polymerase II, and a complex array of general initiation factors (TFIIA, B, D, E, F, H) (Zawel and Reinberg, 1992; Hernandez, 1993; Parvin and Sharp, 1993), which are highly conserved from yeast to man. Polymerase II promoters are composed of a core and regulatory regions (enhancers and silencers). The core comprises a TATA box and a transcription start site. The regulatory elements are highly varied and gene-specific and contain one or more sequences for interaction with DNA-binding regulatory proteins. The general initiation factors constitute the basal apparatus, able to recognise the core promoter and to initiate transcription through a cascade of events (Figure 47).
First TBP binds to the TATA box, forming a transcription pre-initiation complex (PIC). Second, TFIIB bridges to the Polymerase II and, with TFIIA, stabilises the TBP binding to the DNA. Further, the interaction with TFIIF is a determinant in promoter targeting of RNA PolII. The next step in the pathway is the binding with TFIIE, which has ATPase and kinase activities and which plays a role in promoter melting. Binding of TFIIH completes the assembly of the PIC (Figure 48) and is responsible for DNA melting, phosphorylation of the CTD (carboxy-terminal domain) of RNA PolIII, and elongation/promoter clearance steps.
Figure 48. Cascade events of the transcriptional machinery (B) (Roeder, 1996).
TBP forms, together with certain TBP-associated factors (TAFs), the TFIID general initiation factor. The assembly of TFIID is one of the major regulatory points in controlling gene expression from class II promoters (Zawel and Reinberg, 1992). It is known that, although TBP can elicit basal activity on core promoters, transcriptional activity at comparable molar inputs is usually greater with the whole TFIID complex than with TBP alone (Sun et al., 1994; Kaufmann and Smale, 1994; Martinez et al., 1994), because some of the TAFs can function as co-activators mediating the activation signal from enhancer-bound regulators. At present, it is known that the human and Drosophila TAFs are highly conserved and several homologues to these have been found in yeast (Reese et al., 1994; Verrijzer et al., 1994; Poon et al., 1995).

A large number of cellular and viral transcriptional regulators have now been shown to bind to the basic subunit of the TFIID complex, TBP (Zawel and Reinberg, 1992; Stringer et al., 1990; Lieberman and Berk, 1991; Metz et al., 1994) and this interaction can result in either transcriptional activation or transcriptional repression depending upon the nature of the binding.

Recent reports (Tansey et al., 1994) have demonstrated that mutations in the conserved regions of TBP can attenuate the in vivo activity of the transcription factor, and can differentially affects its response to different activation domains. Although the activity of TBP mutants in vivo did not correlate with DNA binding or basal transcription in vitro, it did correlate with binding in vitro to the largest subunit of TFIID, hTAF 250, which recruits TBP into TFIID (Weinzierl et al., 1993; Chen et al., 1994; Ruppert et al., 1995), suggesting that TBP utilises multiple interactions across its surface to respond to RNA polymerase II transcriptional activators in vivo. Further, some of these interactions appear to involve recruitment
of TBP into TFIID, whereas others are involved in response to specific types of cellular activators, like E7. What kind of effects has E7 in the transcriptional machinery? It is activating some promoters and in which phases of the cell cycle? The biological consequences of the E7/TBP binding that we found are not answering to all these questions, even if the post-transcriptional modifications of E7 seem to play an important role regarding the correlation between E7 functions and cell cycle.

7. Different Human Papillomavirus Proteins are Able to Bind TBP.
Many viral and cellular proteins have been show to bind TBP and of particular relevance for papillomaviruses are the studies which have shown binding between TBP and the viral E2 protein (Steger et al., 1995; Miller-Rank and Lambert, 1995; Enzenauer et al., 1998) and between TBP and the cellular tumour suppressor p53 (Seto et al., 1992; Liu et al., 1993). In addition, during the course of my studies, HPV18 E6 was also demonstrated to bind to TBP in vitro. Recently this interaction was also demonstrated in vitro for the HPV8 E6, which is specifically associated with skin cancers of epidermodysplasia verruciformis patients (Enzenauer et al., 1998). It was therefore interesting first to identify the region of TBP bound by E7, second to compare this with other proteins known to associate with TBP, and third to determine the relevance of the E7-TBP interaction to the transforming activity of E7. In this study it was shown that HPV16 E7, HPV16 E2, HPV18 E6 and the cellular tumour suppressor p53 all bind to the same conserved carboxy-terminal region of the TBP molecule. p53 was included in the experiments because, although similar studies were performed previously (Martin et al., 1993), it was nevertheless
interesting to compare directly the ability of p53 to bind different regions of TBP with that of the viral proteins. The results provided a number of interesting finding. Firstly, E6 and p53 presented a very similar pattern of binding. Indeed, residues 201-272 of the TBP molecule are required for p53 and for E6 binding, although residues 100-201 would also seem to be important. This suggested that p53 and E6 may bind TBP in a very similar manner. For p53, the binding with TBP has been postulated to be a way to reduce the efficiency of transcriptional initiation. P53 might contain a domain that actively interferes with some aspect of initiation when it is bound to TBP, or it could block another, positively acting factor from binding to TBP (Seto et al., 1993). Until now, no biological consequences has been found for the E6/TBP interaction.

On the other hand, the data obtained with E7 indicated that residues 100-201 on TBP had no role in the E7 interaction and, further, that the region of TBP responsible for E7 binding lies entirely within residues 201-272. A similar situation holds true for HPV16 E2, indicating a strong similarity between E2 and E7 in their binding to TBP. Therefore all the proteins analysed bind TBP within the same carboxy-terminal region of the TBP molecule, but there are clear differences in the precise residues involved. Moreover, the differences seen in binding assays done under different salt concentrations, confirmed the relative stability of the interactions and that, within the context of a viral infection, differences could have important implications in terms of competition for the available TBP. In addition, the stability of the binding to TBP does not necessarily reflect the known activities of these proteins in vivo. The HPV16 E7, HPV16 E2, HPV18 E6 and p53 proteins all play an important role in the transcriptional machinery (Phelps and Howley, 1987; Phelps et al., 1988; Sedman et al., 1991; Kern et al., 1991) and for p53 and
BPV1 E2 this has been show to be related, in part, to their ability to bind TBP (Seto et al., 1992; Liu et al., 1993; Steger et al., 1995). The fact that the most easily disrupted binding was that between E2 and TBP was a quite surprising result, considering that E2 is a major regulator of viral gene expression and its diverse effects have been postulated to occur through interaction with the basic transcriptional machinery, including TBP (Ushikai et al., 1994, Miller-Rank and Lambert, 1995; Steger et al., 1995). Keeping in mind the important roles of TFIID complex in transcriptional initiation by RNA Polymerase II (Greenblatt, 1991; Sadowski et al., 1993) and that the C-terminal 180 amino acids of TBP can replace a TFIID fraction for basal transcription in vitro, thanks to the two DNA-binding repeats in the same region (Horikoshi et al., 1990; Peterson et al., 1990), there are nevertheless reports suggesting that the ability of TBP to interact with an activation domain in vitro is not directly relevant to its ability to support activated transcription in vivo (Tansey et al., 1995; Thut et al., 1995).

In conclusion, the data allow to us to define a hierarchy between the HPV proteins for binding TBP, with E7 being the strongest, followed by E6 and finally the weakest interaction being observed with E2.

8. Abolition of p53 Mediated Transcriptional Activation and Inhibition of p21 Protein Induction by HPV E7 Proteins.

In the case of E7, however, it was possible to demonstrate that binding with TBP may play a role in some of its functions. HPV16 E7 has been shown to be capable of immortalising human cells at low frequency in the absence of other co-operating oncogenes (Hudson et al., 1990; Halbert et al., 1991). This can be explained by the
fact that E7 can overcome p53-induced growth arrest which is mediated by p21, through the premature release of E2F from pRB, and thus E7 shortcuts the inhibitory function of the p21 protein (Harper et al., 1993; Demers et al., 1994; Slebos et al., 1994). A similar strategy is also used by Adenovirus E1a, therefore both viral proteins have evolved a mechanism for overcoming a p53 regulatory pathway indirectly. Recent studies with SV40 large T and Adenovirus E1a demonstrated an additional activity of this protein with respect to p53 function, the ability to inhibit the transcriptional activity of p53 both in transient transfections and in stably transfected cell lines (Fields et al., 1990; Farmer et al., 1992; Steegenga et al., 1996). Indeed, one of the p53 functions is the capacity to strongly activate transcription thanks to the presence of a sequence-specific DNA-binding domain (Kern et al., 1991), with a powerful activation domain (Raycroft et al., 1990). E1a-induced repression of the transcriptional activation potential of p53 is most likely not caused by the disruption of the TBP binding to the transcription activation domain of p53. Thut et al., (1995) have shown that other human co-activators TAFII32 and TAFII70, two subunits of the TFIID complex, can also bind to the transcription activation domain of p53 in vitro, and these factors are important for stimulation of the basal transcription initiation complex. On the other hand, the study of Steegenga et al., (1996) suggested that there is an increase in the molecular weight of p53 protein complexes within the cell, in response to the presence of E1a. Apparently, expression of AdE1a drives p53 into high-order complexes. This increased complex formation of p53 is most probably an indirect effect, since no direct interaction between p53 and AdE1a has been detected (Egan et al., 1988; Whyte et al., 1989; Lowe et al., 1993). Thus the appearance of high-order complexes might be due to increased homo-oligomerisation of the p53 protein.
via its oligomerisation domain (Sturzbecher et al., 1992) but also might involve binding to other proteins. Recent reports have demonstrated that an E1a mutant, incapable of physically interacting with pRB, retained the capacity to inhibit transactivation by p53, whereas E1a mutant in the p300/CBP-interacting domain failed to inhibit p53 (Somasundaram and El-Deiry, 1997), underlining the possibility of a mediator in the E1a function. These previous observations constituted the basic elements for performing a similar analysis for the HPV16 E7 protein. Indeed, the results indicated that E7 was also able to inhibit p53 transcriptional activity, with this function conserved between both benign and tumour-associated HPV types, underlining that this activity of E7 is most probably related more to viral replication than to transformation. Moreover this hypothesis was confirmed by the mutational analysis done with the mutants 566 (Banks et al., 1990b) and 631 (Edmonds and Vousden, 1989) which, even though defective in transformation, still retained the ability to inhibit p53 transcriptional activity. It was of particular note that the inhibition of p53 transcriptional activity was also independent of E7's ability to bind pRB, as in the case for E1a, as observed when we tested the 631 mutant, defective in pRB binding. Only two of the mutants tested, 638 (Edmonds and Vousden, 1989) and p31/32 (Barbosa et al., 1990), appeared to have a reduced ability to inhibit p53 transcriptional activity. This could be explained by the fact that 638 lies within the pRB binding pocket, but it has been shown previously to encode an unstable protein (Edmonds andVousden, 1989). Mutant p31/32, on the other hand, encodes a stable E7 protein which is defective for CKII recognition (Barbosa et al., 1990), and this support the conclusion that CKII phosphorylation of E7 may play a role in its inhibition of p53 transcriptional activation. The analysis of four additional mutants to see if any other potential sites
of E7 phosphorylation could have any effect on p53 transcriptional activity, suggested that only the CKII phosphorylation was involved in this function of E7 (Storey et al., 1990).

Although the above studies show that E7 will inhibit p53 transcriptional activity on a CAT reporter construct, a major point was whether or not E7 can affect p53 induction of a naturally occurring target gene. One such gene encodes the p21/WAF-1 protein (Harper et al., 1993; El-Deiry et al., 1993). In stable transfectants expressing AdE1a no induction of WAF 1 has been found, and hardly any induction of MDM2 after X-ray irradiation. The inhibition of p53-dependent p21 induction by E1a not only relieves a block at the G1/S boundary through cyclin-CDK-inhibitory effects by p21, but also relieves a block during DNA replication through interaction between p21 and PCNA and its effect on processive DNA synthesis (Waga et al., 1994). Therefore it was decided to investigate the effects of E7 expression upon p21 induction in Saos-2 cells following transfection with p53 expression plasmid. The results, which demonstrated an increase of the p53 level in cells containing E7, agree with previous studies (Demers et al., 1994) and a correspondent decrease in the p21 protein level in the same cells, compared with the cells containing p53 alone, demonstrate that, in a transient transfection, E7, like E1a, can significantly reduce the level of expression of a naturally occurring p53-induced protein. Our results are nevertheless in contrast with some recent reports, which show in E7 expressing cells an enhanced expression of p21 correlated with a reduction in cyclin E- and A-associated kinase activities (Jones et al., 1997; Hickman et al., 1997; Jian et al., 1998, 1999), and in agreement with other reports which underline the ability of high and low risk HPV E7 proteins to neutralise the inhibitory effect of p21 in vivo (Schmidt-Grimminger et al., 1998;
Zehbe et al., 1999), and the absence of detectable p21 in organotypic culture immortalised by E7 (Steenbergen et al., 1998). In our case, it is possible that the inhibition of p21 expression by E7, while permitting DNA synthesis and cell cycle progression, allows E7 to suppress important cell cycle checkpoints, independent of its ability to interact with and inhibit the function of pRB. Thus, like SV40 large T antigen and E1a, E7 also targets a function of both pRB and p53. Most human cancers show perturbation of growth regulation mediated by the tumour suppressor proteins pRB and p53, indicating that the loss of both pathways is necessary for tumour development. Loss of pRB function leads to abnormal proliferation related to deregulation of the E2F transcription factors. However, a recent report (Bates et al., 1998) has found a link between the pRB and the p53 tumour suppressor in the p14ARF protein. Thus E2F-1 directly activates the expression of the human tumour suppressor protein p14ARF, which binds to the MDM2-p53 complex and prevents p53 degradation, E7 causing the increase of E2F has the contradictory effect of the increase of p53 stability with the activation of an apoptotic cell death pathway (Jones et al., 1997b; Iglesias et al., 1998). In this way E2F-1, a protein inherently activated by cell-cycle progression, is a part of a fail-safe mechanism to protect against aberrant cell growth.

9. Tripartite Complex Formation between E7, p53 and TBP.

Considering that E1a and p53 both associate with TBP, a possible explanation of the higher molecular weight forms of p53 seen in E1a expressing cells (Steegenga et al., 1996) could be co-association of E1a-TBP and p53. The series of in vitro assays done to test this hypothesis, which could indicate a mechanism for inhibition of p53
transcriptional activity, demonstrated that E7 (or E1a) and p53 will normally not associate directly. However, we found that, in the presence of TBP, a tripartite complex can form, thus indicating that both E7 (or E1a) and p53 can bind TBP simultaneously. There are other reports indicating a functional relationship between TBP association and transcriptional repression, underlining the capacity of TBP to act as a bridge for various transcription factors to contact or associate with other proteins, in turn allowing the contribution of their additional functional domains for activation or repression (Kuddus et al., 1995; Zhang et al., 1996). Our results suggest that the formation of the tripartite complex on a promoter is part of a mechanism of repression, and the participation of E7 in the transcription complex may limit the access of co-activators to the complex, which would be necessary for the activator function. The E7 protein could sequester TBP on the promoter in an unproductive complex by inhibiting the further formation of the basal transcription complex (Meisterernst et al., 1991a,b).

Having shown that CKII phosphorylation of E7 stimulates its ability to complex with the core component of TFIID, the TATA Box Binding Protein (TBP), a subsequent experiment to investigate the effect of CKII phosphorylation on the tripartite complex confirmed the previous observations. The conclusion, accompanied by the fact that this stimulation of TBP binding is followed by a dramatic increase in the amount of p53 protein retained, further supports the notion that the binding of p53 to E7 (or E1a) is not direct but occurs via TBP. Moreover, although p53 protein binds to TBP in the absence of phosphorylation, the level of neither protein changes following the CKII phosphorylation reaction; the p31/32 mutant of E7 also retains the ability to bind TBP, and hence p53, in a manner like wild-type E7 in the absence of phosphorylation. This is not surprising considering
the results of the *in vivo* assays where this mutant also weakly inhibits p53 transcriptional activity. However, phosphorylation of the wild-type E7 protein greatly increases its ability to bind TBP and this correlates very closely with its increased ability to inhibit p53 transcriptional activity *in vivo*. All these results together suggest an additional route by which HPV can perturb normal p53 function.

10. **E7 Mutational Analysis to Define the Region(s) Responsible for the TBP Binding.**

The mutational analysis done on E7 to localise the domains involved in the interaction with TBP allowed us to demonstrate that the binding with this transcription factor may play a role in E7’s transforming activity. Attention was focused on the carboxy terminus of E7 where there are regions well conserved between the different HPVs, keeping in mind that the non oncogenic forms of HPV are also able to interact with TBP. The analysis done with the E7 mutants translated *in vitro* and also immobilised on the beads as GST fusion proteins, identified two regions of the carboxy-terminal half of the protein, spanning amino acid residues 52-56 and 79-83 which, when deleted, resulted in a loss of binding to TBP. This result allows us to say that there is more than one domain on E7 involved in the interaction with TBP. Two other mutants within the same region of E7, spanning amino acid residues 65-67 and 75-77, retained wild-type levels of binding to TBP, indicating that disruption of binding was not due to a general perturbation of the structure of the carboxy-terminal half of E7 as a result of the amino acid deletions. Also in this case the bindings between the carboxy terminal E7 mutants and TBP
were all direct, confirming that the conditions of the interaction seemed to remain constant. Moreover, the binding between all the mutants and TBP was increased after the phosphorylation of the E7 proteins and, considering that all the mutants of E7 can be phosphorylated to the same level, the results confirmed that CKII domain contributes in binding to TBP.

11. E7-TBP Interaction Contributes to E7 Transforming Activity.

The analysis of the E7 mutants for their ability to transform primary Baby Rat Kidney cells demonstrated that the deletions 52-56 and 79-83, which were defective in TBP binding, were also reduced in their ability to transform cells in co-operation with EJ-ras. Mutants of E7, resulting from the deletions of amino acids 65-67 or 75-77, which retained wild-type levels of TBP binding, also retained wild-type levels of transforming activity. The importance of TBP binding for the transforming activity of E7 was also supported by the previous results. Indeed, we have shown that CKII phosphorylation of E7 greatly enhances its ability to bind TBP and, as yet, no other function has been proposed for the CKII phosphorylation of E7. It has also been shown that mutation of the CKII site on E7 results in reduced transforming activity (Barbosa et al., 1990), and this is consistent with reduced binding to TBP.

We have shown that the reduction in the transforming activity of the mutants 52-56 and 79-83 was not due to a reduction in induction of E2F, since all four mutants retained wild-type levels of transcriptional activity on the Adenovirus E2 promoter. These results also confirm that the mutants 52-56 and 79-83 are stable in vivo, a
point which we have also verified by Western Blot analysis of the transformed BRK cell lines.
Although mutants 52-56 and 79-83 are reduced in their ability to transform cells, colonies are nonetheless obtained. This is not too surprising since it has been shown that only regions around the pRB binding pocket and within the extreme amino terminus of E7 are absolutely essential for the transforming activity of E7 (Edmonds and Vousden, 1989; Banks et al., 1990b). Therefore, binding TBP is only contributory to the transforming activity of E7 and the results raise the question of how the E7-TBP interaction might be involved in one of the E7 functions, such as the capacity of the protein to transform cells. The obvious conclusion would be that E7, through its interaction with TBP, may activate expression of some, as yet unknown, cellular genes which aid the transformation process. Together with the previous data on the capacity of E7 to inhibit p53 transcriptional activity, through the formation of a tripartite interaction, there is the basis of an alternative mechanism for this activity of E7, since E7 binds TBP with an apparently higher affinity than the binding between TBP and p53, even if there is not competition between the different interactions. This supports the ability of E7 to perturb the p53-TBP-transcription factor complex, resulting in an inhibition of p53 transcriptional activity.

The transforming activity of E7 is sensitive to mutations in both the N-terminal (Phelps et al., 1988) and C-terminal (Jewers et al., 1992) domains, and although the N-terminus mediates binding to the pRB proteins contributes to deregulation of the cell cycle, the function of the C-terminal domains remains to be disclosed. Our results together with the recent reports (Zwerschke et al., 1999) on the capacity of 16 E7 to modulate the M2 pyruvate kinases activity, could explain the important role
of the C-terminal half of the protein in tumour progression event. Indeed, wild type E7, and not carboxy terminal mutants, expression in BRK cells leads to a significant increase in the total glycolitic rate and an increased conversion of glucose into lactate, which implies that expression of E7 insures the channeling of glucose carbons to synthetic processes and at the same time reduces the cell’s requirement for oxygen, two important properties of tumour cells (Kim et al., 1997; Brand et al., 1997).

12. Expression of E7 Mutant Proteins and their Transcription Activity.

Having found different levels of transforming activity with the E7 mutants, it was necessary to demonstrate the level of expression of those proteins in vivo. From the Western Blot analysis it was clear that all the mutants were able to produce the E7 protein, but more interesting was the amount of proteins produced. Firstly mutants 1 (Δ1) and 4 (Δ4), which although reduced in transforming activity produced levels of protein similar to wild type E7. This demonstrates that the reduced transformation activity was not due to low levels of mutant protein expression. More interestingly, however were mutants 2 (Δ2) and 3 (Δ3), which express the E7 protein at a level 8 to 10 fold higher than the wild type E7-containing line. This aspect was analysed more in detail, by Southern and Northern Blot assays which demonstrated that at least for mutant Δ3, the levels of protein expression were not due to high DNA copy number or levels of mRNA expression. Subsequently, half life experiments were performed on these lines and showed that while the half life of wild type E7 was about 1 hour the half life of mutants Δ2 and Δ3 was of about 2 hours, underlining
the increased stability of these mutant proteins when compared with the wild type E7.

At the moment we don’t know the reason of this increased stability. However, there are reports underlining the capacity of E7 to bind the subunit 4 (S4) ATPase of the 26S proteasome (Berezutskaya and Bagchi, 1997), and subsequently increase the ATPase activity of S4 subunit. It is known that the interaction is through the carboxy-terminus region of E7 and that it is independent of E7 sequences involved in binding to pRB. The authors propose the involvement of this protein-protein interaction in the capacity of E7 to target and degrade pRB. Considering the increased half life of our E7 carboxy-termini mutants my hypothesis is that there could be involvement of the S4 (or of other proteasome components) regulating the levels of E7 expression. Subsequently, it will be of extreme interest to test our E7 deletion mutants for their capacity to interact with components of the proteasome pathway, and to analyse whether the turn-over of the E7 protein is due to any of these interactions.

13. Phosphorylation State of E7 Protein in vivo, and its Changes during the Cell Cycle.

Keeping in mind the relevance of the phosphorylation event in the E7-TBP interaction, it was extremely interesting to analyse in more detail the phosphorylation state of the E7 protein in vivo. Moreover, the phosphorylation event seems to be one of the post-translational modifications which could be undoubtedly important in regulating transcription factor function in several ways. First of all, contributing to the translocation of the transcription factors into the
nucleus (Rihs et al. 1991; Jans et al. 1991), second to their DNA binding activity (Lin et al., 1992; Rolley and Milner, 1994) and third to the interaction of transcription factor transactivation domains with the transcriptional machinery (Firzlaff et al., 1991; Fagan et al., 1994; Muller, 1995; Whalen et al., 1997). These possibilities are not mutually exclusive and, in principle, phosphorylation at multiple sites by different protein kinases could result in several distinct levels of regulation (Teodoro and Branton, 1997). In addition, the progression through the cell cycle is dependent on phosphorylation of key regulatory proteins by cyclin-dependent kinases (CDKs) which in turn are regulated in a complex fashion by association with cyclins, phosphorylation and dephosphorylation, and binding of CDK inhibitors (CKIs).

There has been only a few reports about the E7 phosphorylation state (Smotkin and Wettstein, 1987; Barbosa et al., 1990; Storey et al., 1990), and these underlined the presence of an E7 phosphorylation site at Serine residues in position 31/32 which has reduced transforming activity, and the lack of any defect in transformation of other mutants of E7 at other potential sites of phosphorylation. No observations had been made on the potential changes in the level of E7 phosphorylation during the cell cycle. Since a large number of cellular targets for E7 have now been identified, it was intriguing to consider that differential phosphorylation of E7 may be a means of providing specificity for a number of these interactions during different phases of the cell cycle and/or differentiation.

The preliminary results on E7 phosphorylation obtained with stable transformed BRK cell line demonstrated that mutants which were defective for CKII phosphorylation, were nevertheless phosphorylated in vivo, suggesting the presence in the E7 protein of other phosphorylated residues outside the CKII recognition
sequence, as was suggested by previous studies (Storey et al., 1990). The results was confirmed also by the presence of a phosphorylated band in the BRK Aspartic Acid line. The fact that in the cell line produced with the Aspartic mutant E7 appears to be more highly phosphorylated than in the cell line produced with the p31/32 construct is justified by the higher amount of total cell extract precipitated.

The conclusion that HPV16 E7 protein is phosphorylated in vivo by at least two different kinases brought us to the following analysis of possible changes in the phosphorylation events during the different phases of the cell cycle. Using the hormone-inducible 14/2 cell extracts (Crook et al., 1989) we showed a surprising change in the phosphorylation level of the E7 protein during the different phases of the cell cycle. This was characterised by high phosphorylation at the G0/G1 phase, followed by a decrease in the phosphorylation and by another increase at the start of the S phase of the cell cycle. The result was demonstrated to be specific by the absence of variability in the E7 protein expression throughout the cell cycle, suggesting that the differences in the E7 phosphorylation level was independent from the protein production.


For further dissecting these functions it was necessary to establish an in vitro assay. Again, the GST fusion protein system has proved to be very useful. Using E7 protein as wild type or mutant constructs with the 14/2 cell extract it was possible to determine first of all that the E7 phosphorylation occurred also in vitro even with very low amounts of extract. Using extracts of 14/2 cells that had been synchronised by serum starvation and then harvested at different points through the cell cycle, we
were also able to show cell-cycle specific differences in E7 phosphorylation \textit{in vitro}. The reproducibility of the assay with human HaCat cell extracts, demonstrated that the previous data was not only specific for rat cell lines, and confirmed the specificity of the observations. I would like to stress that the aim of the experiments was to analyse the fluctuation of the phosphorylation state of E7 only going from G0/G1 to S phase of the cell cycle. The persistence or no of the phosphorylation after this time of the cell cycle could depend on the single experiment and could require a more detailed analysis extending the time course to 10-16 hrs after serum addition.


CKII is a highly conserved ubiquitous serine/threonine kinase composed of two catalytic subunits ($\alpha$ and/or $\alpha'$) and two presumably regulatory subunits ($\beta$). CKII has numerous cellular functions including a possible role in mitogenic signalling. There are reports suggesting a serum-responsive regulation of the CKII activity in tissue culture, characterised by an induction of the CKII activity in G0-G1 phase, a decrease and then a subsequent activation in G1-S phase (Carroll and Marshak, 1989; Lorenz \textit{et al.}, 1994). Other reports show that the presence of other growth factors like EGF (Sommercorn \textit{et al.}, 1987; Ackermann and Osheroff, 1990; Pepperkok \textit{et al.}, 1991) have a similar effect, with CKII activity rising sharply between 20 and 30 mins., peaking at approximately 50 mins., and returning to basal levels by approximately 120 mins., or IGF (Sommercorn \textit{et al.}, 1987) with a maximal activity after 10 mins. which persisted through 90 mins.. Therefore, to
verify whether the different behaviour of E7 phosphorylation was due to an alternative activation of the CKII enzyme during the cell cycle, we used the \textit{in vitro} phosphorylation assay to test the p31/32 mutant which is defective for the CKII phosphorylation, nevertheless bearing in mind that this mutant was still a target for other unknown kinases, as we saw by the \textit{in vivo} phosphorylation experiment in BRK cells. The results revealed a surprising different pattern of phosphorylation of the CKII mutant in comparison with the wild type E7, demonstrating the lack of phosphorylation at time G0/G1 and a subsequent increase in phosphorylation only after 6 hours of serum induction. This confirms the presence of at least two different phosphorylation sites on the E7 protein. One site is responsible for the E7 phosphorylation in the first stages of the cell cycle, and the other one is responsible for the E7 phosphorylation during the later phases of the cell cycle. In addition, the responsible enzymes seem also to be different, since the CKII appears to phosphorylate E7 in the G0/G1 phase, and other unknown kinase(s) phosphorylates E7 later on when the cells enter S phase. Moreover, considering the amount of phosphorylation obtained after 6 hours of serum induction, it seems that the second phosphorylation event probably reflects phosphorylation at the CKII site and elsewhere. These results brought some important conclusions regarding the E7 phosphorylation state \textit{in vivo}. The phosphorylation of the protein, first of all, changes during the course of the different phases of the cell cycle. Second, the E7 protein has two phosphorylation sites, which are susceptible to phosphorylation at different times. Third, the kinases involved are also different. All these observations confirm the presence of a potential regulation of the E7 protein during the cell cycle by post-translational modifications of the protein itself, which could allow E7 to be involved in different pathways.

The mutational analysis of the E7 protein was, at this point, necessary to identify which was/ were the new residue/s capable of being phosphorylated. There was only one study (Storey et al., 1990) suggesting the presence of a possible second phosphorylatable site on E7 in the carboxy-terminal half of the protein. For this reason the E7 protein was divided in two halves and the 3' end was fused as GST protein and tested in the \textit{in vitro} phosphorylation assay. From the result it is clear that the phosphorylation pattern of the carboxy-terminus region of E7 was different from that of the full length wild type protein and reproduces that obtained with the p31/32 mutant. This allowed us to conclude that the second phosphorylation site of E7 is located in the carboxy-terminal half of the protein. A more detailed mutational analysis of this region revealed the presence of two Serines which could be phosphorylated by a kinase, the first at position 71 and the second at position 95. Since previous authors have speculated on the possibility of Ser 71 being a potential E7 phosphorylation site, this mutant was cloned as a GST fusion protein and analysed in the \textit{in vitro} phosphorylation assay. Strikingly, the Ser 71 mutant presented a pattern of phosphorylation characterised by an high level of phosphorylated protein only at time G0/G1. No other relevant phosphorylation event was present at the following time points. The same phosphorylation experiment was done on a GST construct containing Ser 71 mutation only in the carboxy-termini part of the E7 protein. In this case, no phosphorylation of the mutants was obtained. This allows us to conclude that the Ser 71 residue in the carboxy-terminal half of E7 is the location of the second phosphorylation site of the protein. Unfortunately, we are not able to define which is the phosphorylation enzyme responsible for this event, since the Ser 71 is not part of a known consensus
domain of the most important kinases (Kemp and Pearson, 1990; Wang and Roach, 1995), as it is shown in Table 3. Our sequence is CVQSTHVDI. Several protein kinases have requirements for positively-charged residues in the vicinity of the phosphorylation site, and the presence of basic residues or acidic residues are in some cases essential for defining the specificity of the enzyme.

<table>
<thead>
<tr>
<th>Protein kinase</th>
<th>Sequence</th>
<th>Motifs*</th>
</tr>
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<tbody>
<tr>
<td>CAMP-dependent protein kinase</td>
<td>LRRASLG</td>
<td>RRXS</td>
</tr>
<tr>
<td>Protein kinase C</td>
<td>QKRPSQRSKYL</td>
<td>RXXSXRR</td>
</tr>
<tr>
<td>Ribosomal protein S6 kinase</td>
<td>RRLSSLRA</td>
<td></td>
</tr>
<tr>
<td>Casein kinase I</td>
<td>DDDDVASLPGLRRR PLSRTLS(P)VASLPGL</td>
<td>S(P)XXS</td>
</tr>
<tr>
<td>Casein kinase II</td>
<td>RRREEETEEE RRRDDDSDDD</td>
<td>SXXE</td>
</tr>
<tr>
<td>Glycogen synthase kinase-3</td>
<td>PRPASVPPSPSLSRHSPPHQ(S)EDEEEEP</td>
<td>SXXXS(P)</td>
</tr>
<tr>
<td>CDC2 kinase</td>
<td>KSPAKTPVK</td>
<td>SPXR</td>
</tr>
</tbody>
</table>

* In most cases, R can be substituted by K; S can be substituted by T; and E can be substituted by D

Table 3. Peptide substrates and recognition motifs of some protein kinases.

The residues closed to the second phosphorylation site of E7 are not increasing in particular way the acidity or basicity of the domain, and the fact that our sequence is not present in the number of the usual recognition motifs should not to be discouraging, since not all the peptide substrates have been discovered so far.
The obvious conclusion from these studies is that the CKII phosphorylation regulates the interaction between E7 and TBP. Most interestingly, the different phosphorylation events occurring on E7 could provide the protein with the specificity for the TBP interaction at well-determined stages of the cell cycle. Keeping in mind that E7 is a low abundance protein, this specificity could be extremely important since it will enable different phosphorylation forms of the protein to perform different roles during the cell cycle and/or differentiation. These studies go some way to answering the question of how such small multifunctional proteins can interact with so many target proteins at any given time of the cell cycle or virus life cycle.

Future experiments could be performed to analyse more in details whether there are and which they are the RNAs in the host cell which could be activated or no by E7 through its interaction with TBP and, furthermore, which kind of promoters and down-stream genes could be turned on or off by this new pathway of E7.

Even more, all the obtained results could have a great impact in the construction of a new model of therapy against HPV pointing out the potential blocking of completely new pathways of E7 both in transcription and transformation activities.
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inhibits unscheduled DNA synthesis reactivated in differentiated keratinocytes. 


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From the data of the project the following three papers have been published:

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