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The papillomavirus E2 proteins: Master regulators of viral replication and gene expression

Thesis Submitted for the Degree of Doctor Philosophiae

Candidate:
Antonella Piccini

Supervisor:
Dr. Lawrence Banks

Academic year 1997/1998

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Synopsis

The papillomaviruses constitute a large family of DNA viruses which cause proliferative lesions (papillomas or warts) in higher vertebrates. These lesions generally remain benign, but can progress to malignancy in the case of certain viral types and give rise to for example, cervical carcinomas. There is considerable interest in investigating the mechanism of papillomaviral DNA replication in order to identify means to suppress or eradicate persistent infection. In addition, papillomaviruses provide a useful model to understand eukaryotic DNA replication, since their structure is relatively simple and they replicate their DNA only in the S-phase of the host cell cycle.

Papillomavirus DNA replication is strictly dependent upon two viral proteins, the full-length products of the E1 and E2 early genes. As suggested by protein sequence homology with the SV40 and polyoma T proteins, the E1 proteins have ATPase and helicase activities and the full-length E2 proteins are transcription regulatory proteins. Work with bovine papillomavirus has shown that the E2 protein can bind directly to the E1 protein and enhance the origin *(ori)* binding activity of E1. Little is known about the mechanism of interaction between the human papillomavirus (HPV) E1 and E2 proteins. In this study, I have analysed in detail the role of the human papillomavirus type 16 (HPV-16) E2 protein in viral DNA replication. Initially, I investigated the association between E1 and E2 using a purified glutathione S-transferase-HPV-16 E1 fusion protein from *E.coli* and *in vitro* translated HPV-16E2 proteins, mutated in conserved regions of the N-terminal domain. This assay enabled me to identify two widely separate regions of the HPV-16E2 protein essential for binding to E1.

I next tested the *in vivo* activity of these HPV-16E2 mutants in transcriptional activation and stimulation of viral DNA replication. Several of these proteins revealed that the two functions of E2 can be separated. These studies define three widely spaced regions of the N-terminal domain which are important for DNA replication, two of which retain E1-binding activity. These results point to an additional role of E2 in viral DNA replication other than simply localizing E1 to the origin of replication.

Furthermore, I show that an important element for regulating viral DNA replication might be glucocorticoid hormones.

In the last part of this work, I provide additional evidence for the importance of E2 as an auxiliary factor in viral DNA replication. An extensive set of mutations inactivating the cellular transcription factor binding sites present in the vicinity of the *ori* were tested. None of these mutations had a significant effect on replication, indicating that these sites and factors that bind to them, at least in the presence of E2, are dispensable for replication.

Therefore, the HPV-16E2 protein plays a key role in papillomavirus transcription regulation and DNA replication. These two functions are clearly separable. The E2 protein appears to be the only auxiliary factor involved in stimulation of viral DNA replication and the cellular transcription factors which bind the origin do not affect transient DNA replication. These studies demonstrate that E2 has other activities in replication other than tether E1 to the origin.

Introduction

Viruses may contribute to the development of human tumors by different mechanisms: indirectly by inducing immunosuppression or by modifying the host cell genome without persistance of viral DNA; directly by inducing oncoproteins or by altering the expression of host cell proteins at the site of viral DNA integration. In particular tumor development appears to be a direct consequence of papillomavirus, hepatitis B virus, Epstein-Barr virus, and human T cell leukemia-lymphoma virus infections and represents 15% of the world wide cancer incidence (zur Hausen, 1991). Although these viruses are consistently linked to specific malignances, none of these infections per se is sufficient to induce cancer. These tumors are an example of cooperative effects of specific virus infection and environmental carcinogens.

Papillomaviruses (PV) are a group of small DNA viruses widespread in nature and have been recognized primarily in higher vertebrates. Infection by papillomaviruses in humans produces cutaneous and mucosal squamous epithelial lesions that can eventually become malignant and give rise to, for example, cervical carcinomas.

Carcinoma of the uterine cervix has been found to be associated with concomitant infection by one (or rarely more than one) of several types of human papillomaviruses (HPVs) that infect and cause proliferations in the cervical mucosa. More than 95% of cervical carcinoma specimens contain HPV genomes or their fragments.

HPV infection was recognized as the major cause of cervical cancer by the World Health Organization (WHO) in 1994 and, the search for treatment

of papillomavirus infections provides the main rationale for studying papillomaviral DNA replication.

Because they encode few viral gene products, papillomaviruses rely heavily on the host cell machinery for replication thus providing a simple model for studying regulation of eukaryotic replication.

In addition, the viral DNA is maintained as a freely replicating plasmid with a constant, relatively low, copy number in transformed cells (Ravnan et *al.*, 1992) and therefore replication of papillomaviruses offers some unique aspects for study that are different from replication in lytic viral systems but more similar to cellular DNA replication.

1. Brief historical overview

The viral etiology of human skin warts (papillomas) was established in 1907 (Ciuffo, 1907). 50 years later Richard Shope described the cottontail rabbit papillomavirus (CRPV) and provided one of the first experimental examples of a mammalian cancer virus and of an oncogenic DNA virus (Shope, 1933). Soon after that, Peyton Rous showed that the progression of CRPV-induced papillomas was accelerated and was more frequent if the papillomas were painted with either coal tar or methylcholanthrene, establishing the role of cofactors in papillomavirus-associated carcinogenesis.

Since 1976 it has been recognised that papillomaviruses are pathogens of major importance in a wide variety of animals, including humans, as HPV infections in the anogenital tract are frequently associated with precancerous lesions and invasive squamous cell carcinomas (Syrjänen, 1986).

In the late 1970s the molecular cloning of viral DNAs permitted investigators to circumvent the impediment of the lack of a cell culture system

that allows for expression of the complete viral life cycle, therefore a detailed analysis of the molecular biology of this group of viruses could start.

The study of papillomaviruses was spurred during 1980s by the development of *in vitro* transformation assays which permitted the analysis of the viral functions involved in the induction of cellular transformation (Thomas *et al.*,1964; Geraldes, 1969; Dvoretzky *et al.*, 1980; Lowy *et al.*, 1980; Moar *et al.*,1981).

Sequencing of the DNAs and their availability as labeled probes provided much information on the biochemistry of papillomaviruses, but our knowledge about molecular mechanisms of replication and transformation is still behind that of the related polyomaviruses.

2. Classification

Papillomaviruses belong to the Papovaviridae family, by virtue of their capsid structure and biochemical composition (Mattews, 1982). The icosahedral particles contain a single molecule of double stranded circular DNA, approximately 8,000 bp in size, contained in a capsid of 72 capsomeres. The Papovaviridae family comprises two virus subfamilies: the polyomaviruses and the papillomaviruses.

A characteristic feature of the papillomavirus genome is that all major open reading frames (ORF) are located on the same DNA strand. This is in contrast to polyomaviruses and SV40 whose early and late genes are transcribed in opposite directions (Tooze, 1981).

Each ORF features characteristic patterns which have been conserved between types despite the general heterogeneity within this virus group. The L1 ORF codes for the major structural protein and it is highly conserved,

therefore it has been decided to define a new type based on similarity of less than 90% in the L1 ORF only.

More than 70 humanpathogenic papillomavirus (HPV) genotypes have been isolated thus far (de Villiers, 1989).

3. Papillomaviruses in naturally occurring cancers

Papillomaviruses are associated with naturally occurring cancers in a number of animal species and in humans. However, only some of the many virus types that infect a given species may have oncogenic potential.

Species	Type of proliferation	Predominant viral type
Humans	Skin carcinomas in EV patients	HPV-5, -8
	Cervical cancer	HPV-16, -18
	Condyloma acuminatum	HPV-6, -11
Cattle	Alimentary tract carcinoma	BPV-4
Cottontail rabbit	skin carcinoma	CRPV

In humans, this is best exemplified in skin cancers that develop in lesions of the rare dermatological disorder epidermodysplasia verruciformis (EV) and in genital tract cancers. Whereas the EV patients harbor a large number of

HPV types, squamous cell carcinomas arise mostly in lesions caused by HPV-5 and HPV-8 (Jablonska and Majewski, 1994; Orth, 1987). Similarly, although about 25 HPV types infect the genital tract, only four HPV types (HPV-16, HPV-18, HPV-45, and HPV-31) are detected frequently in cervical cancers (zur Hausen, 1991). HPV types rarely found in invasive cancers are characterized as *low-risk* HPVs, in contrast, *high-risk* HPV types are found frequently in invasive cancers.

In addition, the papillomavirus-related cancers are not uniform with respect to the state of the viral genome in the cancer. The viral genomes are present and remain extrachromosomal in the skin carcinomas of EV patients and in carcinomas arising in the rabbit papilloma. In contrast, HPV-16 and HPV-18 genomes are frequently integrated into the cellular genome in the genital tract cancers (Cullen *et al*, 1991).

4. Genome organisation

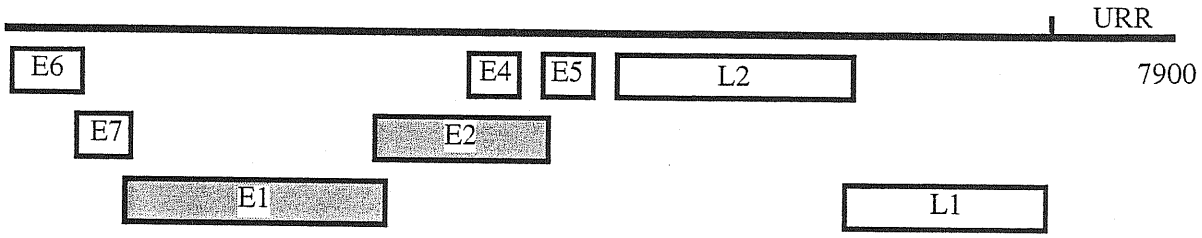
Papillomavirus genomes consist of a double stranded circular DNA genome of about 7900 base pairs. All papillomaviruses have a similar genetic organization (Fig.4). Most major ORFs occupy similar positions relative to each other and there is no substantial difference in the length of homologous ORFs which can be used to align the genome.

One characteristic of the genomic organisation of the papillomaviruses is that all of the ORFs are located on one strand.

The coding strand contains 8-10 designated translational ORFs classified as "early"(E) or "late"(L) by analogy with other DNA viruses where genes are turned on according to a specific time schedule in the course of a productive infection. The so-called early genes are expressed shortly after infection and

prior to the onset of DNA replication. 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 structural proteins of viral particles and are activated during the final stages of the viral life cycle.

Fig.4



genome organisation (circular dsDNA)

With papillomaviruses, there is actually no experimental basis for discriminating between early and late, because replication *in vivo* is highly dependent on the differentiation of keratinocytes, and replication *in vitro* has not been analysed in a productive cell culture system. For convenience, however, we call those ORFs early which are expressed in the nonproductive, basal part of a wart. ORFs, which are specifically expressed in differentiated productive cells of a wart, are regarded as late (Syrjänen *et al.*, 1987).

All papillomavirus genomes have a non-coding region of 500-1000bp which contains several viral promoters, enhancer elements and *cis*-acting sequences important for viral DNA replication. This region has been referred to upstream regulatory region (URR).

4.1 Functions assigned to the papillomavirus open reading frames

The E1 ORF is the largest ORF in all papillomaviruses analysed and it is relatively well conserved among all of the papillomaviruses. BPV-1 initially served as the prototype for studies on the E1 protein. The product of the BPV-1 E1 gene is a nuclear phosphoprotein of about 70 kD that is directly involved in viral DNA replication. It has ATPase and helicase activities (Seo *et al.*, 1993a) as was suggested by protein sequence homology with the SV40 and polyoma large T proteins (Clertant and Seif, 1984; Seif, 1984).

In addition to the protein encoded by the full-length gene, the 5'-end of BPV-1 E1 encodes a protein with an apparent molecular weight of 23kD, but no function has yet been ascribed to this N-terminal E1 protein (Hubert and Lambert, 1993).

The recognition that specific HPVs are closely linked with certain human cancers has focused interest on the specific subgroup of HPVs which are associated with, for example, cervical carcinoma. Although HPV and BPV-1 E1 clearly have analogous function, there are sequence differences, particularly in the N-terminal region, which would suggest that they are not identical. For example, HPV-16 E1 has been found to possess ATPase activity (Storey *et al.*, 1995), but no helicase activity encoded within the HPV-16E1 has so far been reported.

The E2 ORF encodes a transcription factor which interacts directly with a palindromic sequence present in multiple copies in papillomavirus genomes. This interaction results in increased transcription from the viral promoters.

The BPV-1 E2 protein has been well characterised structurally and functionally. The protein is a 48 kD nuclear phosphoprotein (Meneguzzi *et al.*,

1989; McBride *et al.*, 1989a). The BPV-1 E2 ORF also encodes two transcriptional repressors that function through the same enhancer elements but inhibit E2-dependent transactivation. In contrast, HPV-16 E2 has been reported to encode only one transcriptional repressor form of E2 (Bouvard *et al.*, 1994). The full-length E2 protein has also an auxiliary role in DNA replication as will be discussed in more detail in the following sections.

Conservation of the papillomavirus E2 proteins appears low at the primary sequence level, but becomes more marked and apparent at the level of secondary structure and function. Three distinct functional domains have been defined: a C-terminal DNA binding and dimerization domain is separated from the N-terminal transactivation domain by a flexible proline rich " hinge". The N-terminal domain is more extensively conserved than the C-terminus, and the hinge shows little conservation overall, being highly variable in both sequence and length (Giri and Yaniv, 1988).

Sequence and structural homology predictions for the HPV and BPV-1 proteins related to replication and transcription are conserved: matched or mixed combinations of E1 and E2 proteins from HPV-11 or BPV-1 replicated either *ori* in several different eukaryotic cell lines, albeit with varied efficiencies (Chiang *et al.*, 1992b). However, the differences between human and bovine viruses are sufficiently pronounced to warrant an independent definition of the biological and biochemical activity in each native viral system.

The function of the E4 protein is poorly understood. It is expressed at very high levels in wart tissues. E4 is the major transcript produced from the BPV-1 late promoter which also directs expression of the virion structural proteins, strongly suggesting that E4 function is linked to the productive stage

of infection (Baker and Howley, 1987). Expression of the HPV-16 E4 protein in human keratinocytes results in the collapse of the cytokeratin matrix, suggesting a role in releasing virus particles from skin surface cornified epithelial cells (Doorbar *et al.*, 1991).

Despite the fact that there is no efficient *in vitro* tissue culture system for the propagation of the papillomaviruses, cellular transformation by certain of these viruses has permitted researchers to study the viral functions involved in the induction of cellular proliferation by these viruses.

Different papillomaviruses have different cell transformation potentials. Some viruses can fully transform primary cells with no need for additional oncogenes, such as BPV-1 and BPV-2 (Morgan and Meinke, 1980; Jarrett, 1985). Other viruses, such as the *high-risk* genital HPVs, can fully transform primary rodent kidney cells only in cooperation with another oncogene, typically activated *ras* (Matlashewski *et al.*, 1987).

The major oncoprotein of BPV-1 was initially identified as the 44 residue BPV-1 E5 very hydrophobic protein (Campo, 1992), which has been shown to induce cellular DNA synthesis, to activate EGF, PDGF and CSF-1 receptors, and to bind a 16 kD cellular protein of the ductin family of proteolipids. Although there is limited conservation of amino acid sequences between the papillomavirus E5 protein, the HPV E5 gene also encodes a very hydrophobic protein. The product of the HPV E5 gene can promote anchorage independent growth of NIH 3T3 cells in cooperation with ligand stimulated EGFR similarly to BPV-1 E5. However HPV E5 transforming activity in comparison to BPV-1 E5 is much weaker, showing clear differences between the BPV-1 and HPV E5 proteins (Banks and Matlashewski, 1996).

More recently, it has become apparent that BPV-1 E6 and E7 also show strong transforming activity when transcriptional regulation by E1 and E2 is altered (Vande Pol and Howley, 1995).

The E6 and E7 proteins are themselves structurally related. They contain domains of almost identically spaced Cys-X-X-Cys motifs (four in E6 and two in the carboxy-terminal portion of E7). The Cys-X-X-Cys motifs found in a number of nucleic acid binding proteins are characteristic of zinc binding proteins.

The E6 protein of BPV-1 is present at low levels in stably transformed cells, and it is located in the nucleus and in nonnuclear membranes of transformed cells (Androphy *et al.*, 1985). It also binds E6-AP, a ubiquitin ligase necessary for HPV-16 and 18 E6-induced degradation of p53, although BPV-1 E6 appears unable to promote the degradation of p53 (Chen *et al.*, 1997). In addition, using the yeast two-hybrid system it was found that BPV-1 E6 interacts with E6BP (E6 binding protein), a calcium binding protein localised in the endoplasmic reticulum (Chen *et al.*, 1995). Additional functions of BPV-1 E6 is stimulation of transcription when targeted to a promoter, but cellular promoters responsive to BPV-1 E6 have not been identified (Chen *et al.*,1997).

The product of the E7 ORF has been detected in BPV-1 transformed cells and its integrity is necessary for the fully transformed phenotype as assayed by anchorage independence and by tumorigenicity (Neary and DiMaio, 1989).

The HPV genomes are not as efficient as BPV-1 at inducing transformation of established rodent cells, however, alternative assays have been employed, using primary rodent cells, primary human fibroblast and keratinocyte culture (Howley, 1996). In these assays, those *high-risk* HPVs

associated with clinical lesions that are at an increased risk for malignant progression such as HPV-16 and HPV-18 are transformation-positive, whereas the *low-risk* viruses such as HPV-6 and HPV-11 are not (Howley, 1996). These assays have permitted the mapping of the viral genes directly involved in cellular transformation to the HPV E6 and E7 ORFs.

Similar to the transforming gene products of other DNA viruses, both the E6 and E7 proteins interact with host-encoded tumour suppressor proteins and functionally inactivate several eukaryotic cell-cycle checkpoints.

Like SV40 large T and adenovirus E1B, *high-risk* HPV E6 proteins form a stable complex with wild type p53 (Werness *et al.*, 1990). Complex formation between E6 and p53 requires a third protein denoted E6-AP that functions as an E3 ubiquitin ligase and formation of the complex results in ubiquitin-dependent proteolysis of p53 (Sheffner *et al.*, 1993). Wild-type p53 has cell-growth suppressive and tumor suppressive properties. This growth inhibitory activity may be related to the ability of wild-type p53 to act as a positive as well as a negative modulator of transcription. HPV-16 E6 has been shown to abrogate both the transcriptional activation and repression properties of p53 and to disrupt the ability of p53 to mediate cell cycle arrest (Lechner *et al.*, 1992; Mietz, *et al.*, 1992).

Another clear candidate for a p53 function which may be inhibited only following E6 directed degradation is the induction of apoptosis or programmed cell death.

It is worth noting that HPV E6 has also p53-independent transforming activity: during mouse lens development HPV-16 E6 modulates apoptosis through a p53-independent mechanism (Pan and Griep, 1995), whilst another possible mechanism of transforming activity is through the interaction with the calcium-binding protein E6AP (Chen *et al.*, 1997).

E7 proteins encoded by the *high-risk* HPV types associate efficiently with the retinoblastoma gene product (pRB, also designated p105) and with its relatives p107 and p130. Expression of the *high risk* E7 proteins has been shown to interfere with the normal E2F-containing complexes, resulting in increased levels of free, transcriptionally active E2F (Chellappan *et al.*, 1992).

E6 and E7 proteins of the cancer-associated HPV types 16 or 18 are more effective in these interactions than those of HPV types 6 or 11 from benign lesions. It is widely believed that these activities of E6 and E7 are manifested in their ability to cooperate in the immortalization of primary human keratinocytes, the natural target cells of the virus *in vivo*.

The L1 (54kD) and L2 (62kD) proteins constitute the major and minor components, respectively, of the viral capsid, their transcription occurs exclusively in differentiated keratinocytes (Howley, 1996).

L1 is highly conserved among all the papillomavirus types. Specific domains on L1 are probably responsible for viral attachment to susceptible cells, but none has yet been identified.

Papillomavirus capsids have species-specific antigenic determinants located on the virion surface and these determinants are very likely located on L1 (Howley, 1996). Indeed, vaccination of calves with L1 induces production of serum neutralizing antibodies that prevents tumor formation, showing that L1 contains B-cell-specific epitopes (Jarrett *et al.*, 1991).

L2 accounts for only a small proportion of the virion mass and its function is not yet clear. Very recently, it has been reported that L2 has the intrinsic capacity to localize to POD (PML oncogenic domain) macromolecular structures, and the presence of L2 in PODs is associated with the recruitment of the major capsid protein L1 and the nonstructural protein

E2 (Day *et al.*, 1998). Therefore one possibility is that L2-dependent colocalization might serve as a mechanism to promote the assembly of papillomaviruses.

Calf vaccination with L2, whether delivered before or after challenge, promotes early tumor rejection (Jarrett *et al.*, 1991). Rejection follows ulceration of the lesion: this is consistent with L2 being internal to the virion and therefore not readily exposed to the host immune system (Jarrett *et al.*, 1991).

Expression of L1 and L2 makes it possible to generate abundant virus-like particles (VLPs) that lack viral DNA. The VLPs that are formed in culture appear to be identical in size and structure to authentic HPV virions, or more correctly to empty capsids. Interestingly, capsids assembly can be obtained by overexpression in mammalian cells of only the L1 protein, but it is enhanced by coexpression of L1 and L2 (Rose *et al.*, 1993). VLPs made in culture have already demonstrated their utililty as reagents to generate polyclonal and neutralising antibodies (Kirnbauer *et al.*, 1992).

5. Virus-host interactions

The papillomaviruses have a high degree of species specificity. There are no known examples of natural transmission of human papillomaviruses to other species. In the case of animal papillomaviruses there is only one exception as the bovine papillomaviruses can be transmitted from cattle to horses (Lancaster *et al.*, 1977). Papillomaviruses also display a marked degree of cellular tropism.

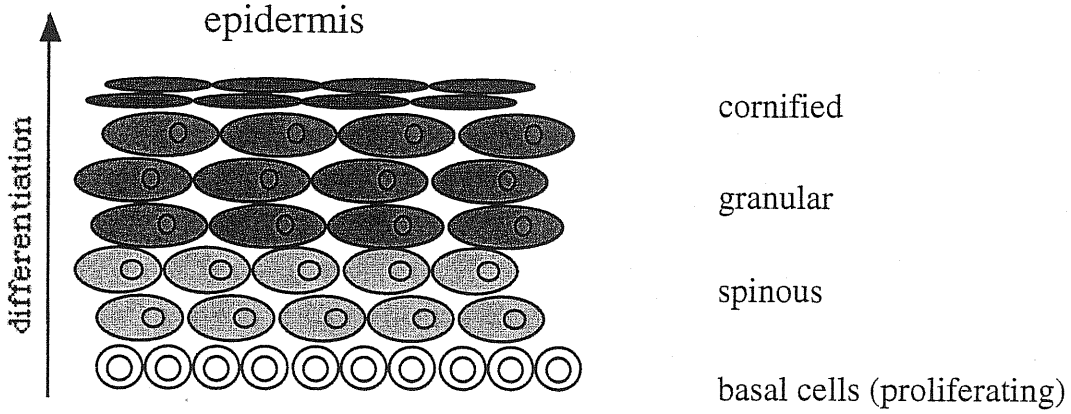
Induction of cellular proliferation is the first response of susceptible cells to viral infection or to the introduction of cloned papillomaviral DNAs. The

most common clinical manifestation of infection with papillomaviruses is the production of warts, which are self-limiting benign tumors that regress after a period of time.

The natural life cycle is complex and not understood in detail. However, in the basal cells of the epithelium, viral DNA is maintained in a latent form characterized by episomal replication at a relatively low copy number. At this stage, expression of viral capsid proteins cannot be detected. 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). This is probably a result of the evolution of a viral life cycle that is tightly coupled to the differentiation program of epithelial keratinocytes in which virion production is limited to differentiating suprabasal cells.

A brief description of differentiation of the epithelium is necessary at this stage (Fig.5).

Fig.5



The epidermis generally matures over a 10-14 day interval by progressive vertical differentiation. The proliferating cells are confined to the

basal monolayer. The suprabasal daughter cells do not divide again, become committed to differentiate, and begin a succession of changes in keratin gene expression (Franke *et al.*, 1986). The genes for sequentially higher molecular-weight species of keratins are activated and, in turn, inactivated to yield the sets of intermediate filaments characteristics of the spinous layer and granular layer. In cutaneous epithelia, the process culminates with dense cross-linking of keratins via disulfide linkages to form the macrofibrils in the tough and relatively impermeable cornified layer. The superficial cells are anucleate and no further papillomaviral gene expression is possible. This layer constantly desquamates and it is replaced from beneath. Internal mucosal epithelium expresses somewhat different sets of keratins as it differentiates and does not develop a cornified surface. It is kept moist by association with interspread secretory glands. Infection with papillomaviruses apparently requires direct physical access to the basal cells exposed in a wound. (Broker and Botchan, 1986).

Specific viral types appear to have a preference for either cutaneous or mucosal types.

In benign and premalignant lesions, papillomavirus genomes persist in the nuclei of the infected cells in the form of unintegrated supercoiled circular DNA. In contrast, approximately 70% of cervical carcinomas contain HPV genomes that have become integrated in the host cell DNA. The integrated HPV fragments invariably preserve the E6 and E7 genes together with the entire URR, and often part of the adjacent late genes. While there do not appear to be preferred integration sites in the cellular DNA, integration usually disrupts the E2 and/or E1 ORFs. In cervical carcinomas containing extrachromosomal viral DNA, where the E1 and E2 ORFS are intact,

mutations are often found in the binding site for cellular transcription factors on the URR.

6. Papillomavirus Replication

A considerable amount of information about the basic cellular enzymatic machinery and the process of initiation of DNA replication has been learned from the study of lytic mammalian viruses (Stillman, 1989; Challberg and Kelly, 1989). But lytic viruses, in most of cases, lack a clearly defined replication control and replicate their DNA exponentially in a single S-phase such that it usually kills its host cell (Tooze, 1980).

Two completely unrelated viruses associated with human cancers, Epstein-Barr virus (EBV) and papillomaviruses, represent an exception to this rule. Both have a latent stage where the viral DNA replicates in the cell as a nuclear plasmid with a regulated copy number and appears to be stably inherited (Mecsas and Sudgen, 1987).

EBV is the only herpesvirus that latently infects B lymphocytes and some epithelial cells in humans and in culture. Its DNA is maintained as a plasmid in the nuclei of infected cells between 1 and 500 copies, when measured in different clones.

In the case of papillomaviruses, they latently infect basal cells of stratified epithelia, while the lytic phase is confined to the differentiated progeny of the basal cells. After latent infection has been established by BPV in cultured cells, the copy number of the plasmids is maintained at approximately 100.

Both BPV and EBV plasmids replicate only during S-phase of the cell cycle distinguishing them from the better characterized mammalian plasmid of mitochondria and from SV40 (Mecsas and Sugden, 1987).

6.1 Assays for papillomavirus replication

At present, the understanding of papillomavirus replication is relatively primitive compared to, for example, the well-studied SV40 system, a significant impediment to its study having been the lack of a cell culture system for expression of the complete viral life cycle.

Recently, however, it has become possible to develop systems to study their replication properties. The basis for all papillomavirus replication assays originates in the early observation that the introduction of BPV into some established rodent cells induces a detectable phenotype, morphological transformation. In the BPV transformed cells, the viral DNA replicates as a plasmid with a modest copy number (Dvoretsky *et al.*, 1980; Lancaster, 1981; Law *et al.*, 1981). Mainly for this reason, bovine papillomavirus has been used as a model system for studying papillomaviral DNA replication *in vivo.*

The replication that takes place in these transformed cells is believed to resemble the latent stage of infection where replication occurs only in the basal layers of the papilloma. Moreover, replication of BPV-1 is regulated by the cell-cycle in that replication of the viral DNA is strictly confined to S phase (Ravnan *et al.*, 1992).

6.1.1 Stable replication assay

BPV-1 can replicate in transformed cells as a multicopy nuclear plasmid, which can persist in the tissue culture cells over long periods of time (Law *et al.*, 1981). The mode of replication of extrachromosomal DNA molecules in eukaryotic cells can be either once-per-cell-cycle in parallel with the chromosome as is observed for the Epstein-Barr virus (Adams, 1987), or by a random-choice mechanism, as is seen for some bacterial plasmids.

If replication is once-per-cell-cycle, postreplicative DNA must be marked to ensure that it does not replicate again until after cell division has occurred. In contrast, replication by random-choice is independent of whether a particular molecule has undergone a round of replication in that cell cycle.

Since the suitability of papillomaviruses as models for chromosomal DNA replication depended upon the assumption of once-per-cell-cycle replication, several experiments were performed to address this point. The final results, however, are consistent with a random-choice replication mechanism and not with a once-per-cell-cycle (Ravnan *et al.*, 1992) supporting the idea that replication and segregation of BPV molecules in cultured cells are not perfectly controlled processes.

An alternative approach to study stably maintained papillomavirus DNA has been to identify, in cervical carcinoma biopsies, cells that contain HPV DNA in an episomal form. These cells are usually derived from low-grade cervical intraepithelial neoplasms (CIN), are morphologically normal and retain the growth requirements of normal cervical keratinocytes (Stanley and Parkinson, 1979), but have an extended life span. An example is a human cervical keratinocyte cell line named W12, containing approximately 100 copies of episomal HPV-16 DNA per cell. W12 cells can form a stratified differentiated epithelium by grafting them onto richly vascularized sites on the flanks of nude mice. Long-term grafts display the histological features of a low-grade cervical dysplasia and, very interestingly, terminally differentiated cells contain amplified levels of HPV-16 DNA, virus capsid antigen, and virus particles (Sterling *et al.*, 1990). The W12 cell seems thus operationally equivalent to a basal epithelial cell latently infected with HPV-16 DNA as it

retains the capacity to complete the virus life cycle and assemble progeny particles.

Obviously, propagation of HPVs *in vivo* by grafting infected tissue under the flank skin of a nude mouse has been considered a very important goal, but it is not a convenient system to study the complete viral life cycle. A lot of attention has been paid to *in vitro* systems that mimic in cell culture the proliferation/differentiation cycles of the the squamous epithelium, like raft cultures.

Organotypic (raft) cultures recreate important features, both morphological and physiological, of epithelial differentiation *in vitro* by raising the cells to an air-liquid interface. This has been done by either the recombination of epidermal cells with dermal elements or through the use of a collagen matrix maintained on a rigid support. Growing keratinocytes in either of these systems allows for a more complete differentiation programme to occur than is observed in monolayer (Meyers *et al.*, 1992).

The induction of the complete vegetative life cycle of HPVs was eventually achieved in raft cultures with a cell line (CIN-612) derived from a cervical intraepithelial neoplasia lesion that maintains episomal copies of HPV-31b DNA (Meyers *et al.*, 1992). It therefore seemed possible that virion synthesis could also be achieved using cloned viral sequences as templates if cell lines could be isolated which stably maintained transfected viral DNA as episomes. Very recently it has, indeed, been possible to propagate HPV31b from transfected cloned DNA, thus confirming the power of the raft culture technique (Frattini *et al.*, 1996).

6.1.2 Transient replication assay

A clear picture of the viral genes involved in papillomavirus DNA replication was first obtained using this system. The transient replication assay is based on the prediction that if a sufficiently large fraction of the cells can be transfected with viral DNA, detection of replication at early times (1-3 days) after transfection should be possible without the need to select for transformed foci. DNA is considered to have replicated if it has acquired resistance to the restriction enzyme *DpnI* which will not cut DNA replicated in mammalian cells because of the lack of a specific methylation (Peden *et al.*, 1980).

Using a series of mutations throughout the BPV early region it was possible to establish unequivocally that two viral proteins, the full-length products from the E1 and E2 open reading frames, are required for viral DNA replication (Ustav and Stenlund, 1991), all other replication proteins are derived from the host cell. Furthermore, provision of these two factors from heterologous expression vectors allowed the identification of viral *cis*-acting elements that are required for replication (Ustav *et al.*, 1991).

6.1.3 Cell-free replication assay

The identification of the two viral proteins required in a transient replication assay (i.e. an *in vivo* system) has also allowed the development of a cell-free replication assay along the lines of the systems previously established for SV40 and polyomavirus (Yang *et al.*, 1991; Seo *et al.*, 1993b; Bonne-Andrea *et al.*, 1995). This *in vitro* replication system consists of a nuclear extract preparation from a permissive cell line supplemented with the viral E1 and E2 proteins overexpressed and purified from baculovirus-infected insect cells, or from *Escherichia coli,* and it is strictly dependent on the presence of a

papillomavirus *ori*. The *in vitro* results showed that E2 is not required to stimulate transcription of host genes encoding replication factors, but that it is a replication factor. In addition, E1 is clearly sufficient for *ori*-specific replication in this system. The resolution to this paradox is that E2 serves an auxiliary role in replication and that this requirement can be bypassed *in vitro*. As will be mentioned in the next sections, E2 functions to increase the selectivity of binding of E1, and the presence of vast quantities of competing cellular DNA sequences could then explain why E2 is required for replication *in vivo* but not *in vitro*.

The results from both the transient replication assay and the cell-free replication assay also demonstrated that the requirements for replication are similar for most, if not all, the different virus types that have been tested. Moreover, unlike the stringent species-specificity demonstrated in cultured cells by other members of the papovavirus family, SV40 and polyomavirus, and the strict host species specificity of papillomaviruses in natural infections, mixed and matched HPV and BPV E1 and E2 proteins can replicate the *ori*s of many types of HPVs as well as BPV and CRPV in transient replication assays performed in human, monkey and rodent cells (Chiang *et al.*, 1991). These results indicate a high degree of similarity among the E1 and E2 proteins and the *ori* sequences of all papillomaviruses, as well as promiscuity in the ability of the viral proteins to engage the mammalian replication machinery, regardless of species.

6.2 The E1 protein is the papillomaviral initiator

The E1 open reading frame is the largest in the papillomavirus genome and it is relatively well conserved among all of the papillomaviruses.

The full-length product of the E1 ORF is a nuclear phosphoprotein with an apparent molecular size of about 70kD (Sun *et al.*, 1990; Storey *et al.*, 1992).

In addition to the 70 kD protein encoded by the full length gene which is absolutely required for viral DNA replication, in BPV-1 transformed cells a protein with an apparent molecular weight of 23kD has been detected (Thorner *et al.*, 1988), encoded by the 5' end of E1. However, no function has yet been ascribed to this N-terminal E1 protein (Hubert and Lambert, 1993).

Initiator proteins, through sequence specific DNA binding, recognize the origin of replication within the genome and mark the region of replication initiation. They allow the initiation of DNA synthesis through localized melting of the DNA helix, and they may also serve as replicator helicases that travel in front of the DNA polymerases, unwinding the duplex DNA (Fanning and Knippers, 1992). Based on a number of these criteria, the E1 protein serves as the initiator of viral DNA replication.

6.2.1 E1 is a DNA binding protein

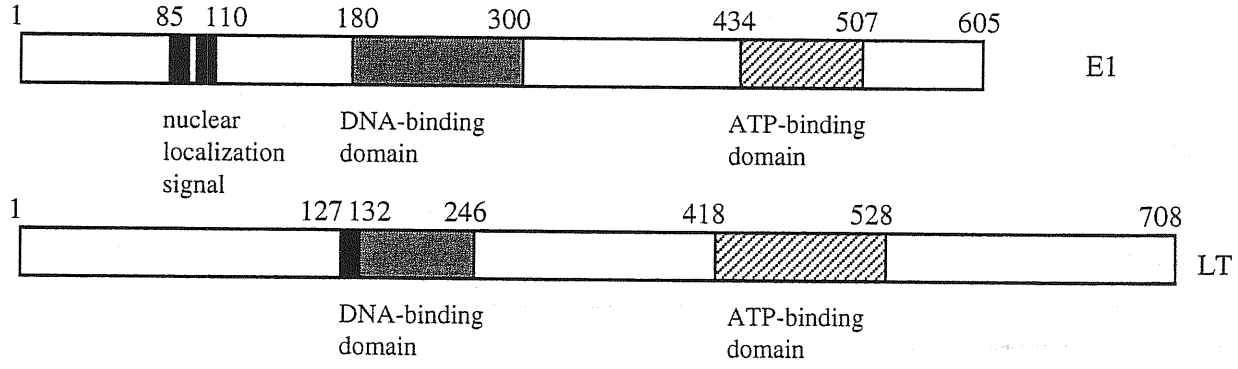
The E1 70kD polypeptide has specific DNA binding activity and serves to recognize the origin (*ori*) of replication. The binding site for the E1 protein has been defined, using DNase footprint experiments in BPV-1, as an imperfect palindromic sequence 18 nucleotides in length with the sequence ATTGTTGTTAACAATAAT (Wilson and Ludes-Meyers, 1991; Ustav *et al.*, 1991;Yang a*t al.*, 1991; Holt *et al.*, 1993) and it has been shown that the DNA-binding activity is stimulated by ATP and $MgCl_2$ (Seo *et al.*, 1993a; Yang et *al.*, 1991; Yang *et al.*, 1993). In addition, mutations in the *ori* that affect E1

binding are defective for replication (Ustav *et al.*, 1991). A related sequence is present in all papillomavirus examined, but binding of other E1s to these putative E1 binding sites has not been characterised in detail. Therefore, the identity of the E1 binding site in HPV *ori* is largely based on homology with the BPV E1 binding site. Obviously, the conservation of the E1 binding site argues that it is likely to play a role in replication of the viral genome.

6.2.2 E1 shows functional homology to the T antigens

Although the E1 protein shows limited sequence homology to the T antigens (Clertant and Seif, 1984; Seif, 1984) the linear arrangement of functional domains on each polypeptide is highly conserved. For example, a domain with ATPase and DNA helicase activities with limited sequence homology is present in the C-terminus of both proteins, while a nuclear localization signal as well as the DNA-binding domain are present in the N-terminal half of the protein (Fanning and Knippers, 1992; Lentz *et al.*, 1993; MacPherson *et al.*, 1994; Thorner *et al.*, 1993).

Fig.6.2.2



Like SV40 large T antigen, E1 is a multifunctional protein that possesses a number of different activities required for the replication of the viral

genome. These include a DNA-dependent ATPase and a helicase that translocates in the 3'-5' direction: it can unwind double-stranded DNA substrates in the presence of a topoisomerase and a single-stranded-DNA-binding protein. What was originally observed as a very limited sequence homology between the E1 proteins and SV40 and polyomavirus T antigen is now well established as a functional homology. However, despite the analogous roles played by both E1 and T-antigen in viral DNA replication, differences exist between the two proteins. Unlike T-antigen, which is the only viral protein necessary for SV40 replication, E1 alone is not sufficient to initiate papillomavirus replication *in vivo*, the viral transcription factor E2 is also required.

6.2.3 The E1 protein binds specifically DNA polymerase α

Another function of initiator proteins, in addition to specific recognition of the replication origin, induction of localized DNA unwinding and helicase delivery, is recruitment of enzymes required for DNA synthesis. This localization appears to be accomplished through a series of protein-protein interactions in a variety of biological systems (Kornberg and Baker, 1992).

The Papovaviruses, unlike larger DNA viruses that encode numerous DNA replication proteins, have evolved a specific strategy. In the case of SV40 and polyoma, the viral large tumor antigen associates with the cellular pol α-primase, and this interaction may aid in localizing pol α to the *ori* (Dornreiter *et al.*, 1990; Dornreiter *et al.*1992). Sequestering pol α-primase at an active *ori* is critical, as this is the only eukaryotic polymerase capable of initiating new DNA chains by virtue of its associated primase activity (Kornberg and Baker, 1992). In the case of papillomaviruses it has been shown that the amino

terminal amino acids of the E1 polypeptide can form a specific protein complex with the p180 subunit of the cellular polymerase α (Park *et al.*, 1994).

Initial *in vitro* studies of SV40 DNA and polyoma DNA replication performed with soluble cell extract showed that the capacity of the extract prepared from cells of different species reflected the ability of those cells to replicate DNA *in vivo,* and subsequent studies suggested that the host cell specificity was determined by the interaction of the T antigen and origin DNA with the host cell DNA polymerase α-primase complex. While SV40 large T binds to primate pol α-primase, it cannot form a productive complex with the murine enzyme. Polyoma large T antigen, on the other hand, recognizes the murine machinery but does not recognize the human equivalent (Murakami *et al.*, 1986). In contrast, the papillomaviruses achieve DNA replication in different mammalian cell lines with an apparent lack of host cell specificity. Replication of papillomavirus origin DNA is possible in normally nonpermissive cells when the viral proteins required for replication are provided from heterologous expression vectors (Chiang *et al.*, 1992b).

These results suggest that the viral protein(s) involved in DNA replication can interact with a different, more highly conserved region of the polymerase-primase complex and that *in vivo* replication specificities are likely due to restrictions at the level of gene expression.

6.2.4 E1 binds the *ori* in at least two different forms

The initiator E1 is capable of binding to the *ori* in at least two different forms. By virtue of a protein-protein interaction between the E1 and E2 proteins, monomeric E1 can bind cooperatively with an E2 dimer to form an

E1/E2/*ori* complex when binding sites for both proteins are present (Mohr *et al.*, 1990; Blitz and Laiminis, 1991; Lusky and Fontane, 1991; Yang *et al.*, 1991; Seo *et al.*, 1993b; Sedman and Stenlund, 1995). Binding in this form is highly sequence specific.

At higher concentrations E1 alone can bind to the *ori* in a trimeric form that encircles the DNA (Sedman and Stenlund, 1996); this complex forms with relatively high affinity but shows low sequence specificity. The E1 ring is generated by the assembly of individual monomers onto DNA, as the E1 protein is not able to form trimers spontaneously in the absence of DNA (Sedman and Stenlund, 1995). An hexameric E1 complex which forms in the presence of single stranded oligonucleotides has also been characterized (Sanders personal communication). This complex co-fractionates with DNA helicase and ATPase activity.

Based on these observations and on *in vitro* replication experiments performed in the presence of neutralizing antiserum (Liu *et al.*, 1995), an assembly pathway has been proposed where the E1/E2/*ori* complex, which has high sequence specificity, serves to specifically recognize the *ori* and lacks other replication-related activities. The E1 protein is required for both initiation and elongation of viral DNA replication and most likely remains at the replication forks, unwinding the parental DNA strands in an ATP-dependent manner. This complex serves as a seed for the larger E1-*ori* complex which has a low degree of sequence specificity, but can distort the *ori* in ATP-dependent manner as elongation proceeds. A similar conclusion has been reached for the SV40 T antigen (DePamphilis and Bradley, 1986) and is consistent with the relatively strong nonspecific DNA affinity of the E1 protein. The addition of E2 antiserum after initiation does not inhibit replication, suggesting that the E2 protein is needed only for initiation and it is

not maintained stably in a multimeric replication-competent E1 complex at the origin.

6.3 The E2 protein

The full-length product of the E2 open reading frame is the only other viral protein in addition to E1 required for viral DNA replication *in vivo* (Ustav and Stenlund, 1991). The E2 protein plays a central role in regulation of viral transcription when bound to a palindromic DNA sequence present in several copies in the regulatory region of all papillomaviruses. Bovine papillomavirus type 1 has been extensively studied as a model for papillomavirus transcription. The product of the BPV translational ORF E2 is a family of 3 genetically related proteins which strictly regulate papillomavirus gene expression.

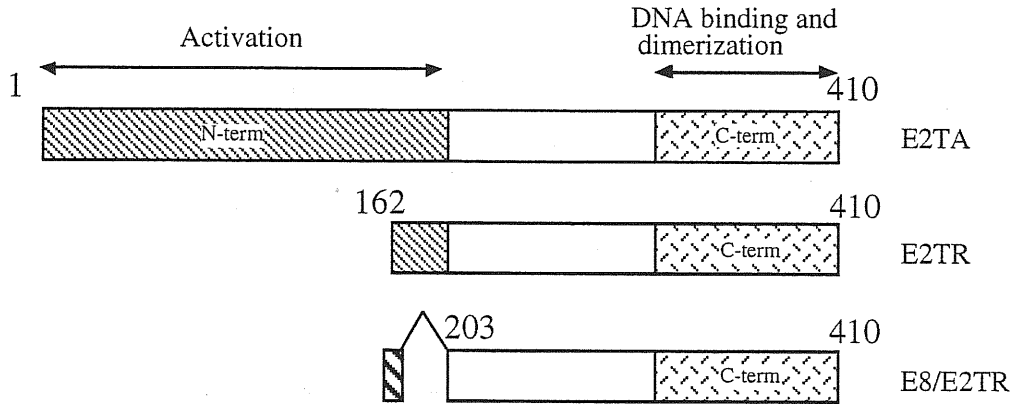


Fig.6.3: E2 proteins and their functional domains. Numbers indicate the amino acid residues in the BPV-E2 ORF. Shown are the regions of the E2 proteins with identified biochemical activities.

The E2 ORF full-length product is a 48kD protein that has been reported to be an activator of viral gene expression. Transactivation depends upon its binding as a dimer to the palindromic sequence $ACC(N)_6GGT$. E2 can also

activate transcription from heterologous promoters, such as those of the herpes simplex virus (HSV) thymidine kinase (tk) gene or the early region of SV40, in the presence of binding sites either upstream or downstream of the promoter sequence (Hawley-Nelson *et al.*, 1988; Thierry *et al.*, 1990).

In addition to the full-length E2 protein (E2TA), two N-terminally truncated E2 ORF gene products have been identified in BPV-1: E2TR and E8/E2 (Fig.6.3). They are generated by alternative splicing and both share the C-terminal region of E2 responsible for DNA binding and dimerization. E8/E2 also contains 23% of the BPV-1 E8 ORF.

These truncated proteins can bind DNA and have been described as repressors because they can inhibit the transactivation function of the full-length E2 (Lambert *et al.*, 1987, 1989).

Surprisingly, E2 appears to have activities unrelated to its role in transcription. It has been reported that overexpression of BPV E2 into HeLa cells can dramatically inhibit cell proliferation and that cells accumulate in the G_1 phase of the cell cycle (Hwang *et al.*, 1996), suggesting that it is not due to a toxic effect. In the HeLa cervical carcinoma cell line, several copies of the HPV-18 DNA are integrated into the genome and the P_{105} promoter constitutively transcribes the E6 and E7 oncogenes, although these cells do not express the endogenous E2 protein owing to an interruption in the E1-E2 ORFs (Schwarz *et al.*, 1985). The mechanism through which the BPVE2 protein inhibits cell growth is not clear but is due, in part, to an increased level of p53, which induces expression of the p21/WAF1 cdk inhibitor. It has also been reported that both BPV and HPV18E2 proteins can trigger cell death when transiently expressed in HeLa cells (Desaintes *et al.*, 1997). The pathway leading to E2-mediated cell death shares many characteristic features of apoptosis but seems to diverge, at least partly, from the one involved in E2-

induced G_1 growth arrest, since it does not require the p53 transcriptional activity.

In addition, very recently, Frattini *et al.* (1997) showed that the E2 transcription factor is able to override the normal regulatory mechanism that ensures faithful completion of S-phase when overexpressed in the CIN 621-9E human keratinocyte cell line which stably maintains HPV episomes (Frattini *et al.*, 1997). In this study, the product of full-length HPV-31E2 gene was found to induce an S-phase arrest that allowed re-replication of cellular DNA. Following infection by the E2-expressing vector, the CIN612-9E cell line exhibited a 5 to 9 fold increase in viral copy number, therefore E2 seems to target cell cycle progression to facilitate the replicative cycle of the virus.

It will obviously be important to determine whether all these reported activities of E2, apparently unrelated to its role as a transcription factor, represent true *in vivo* functions of the protein or indirect consequences of E2 overexpression.

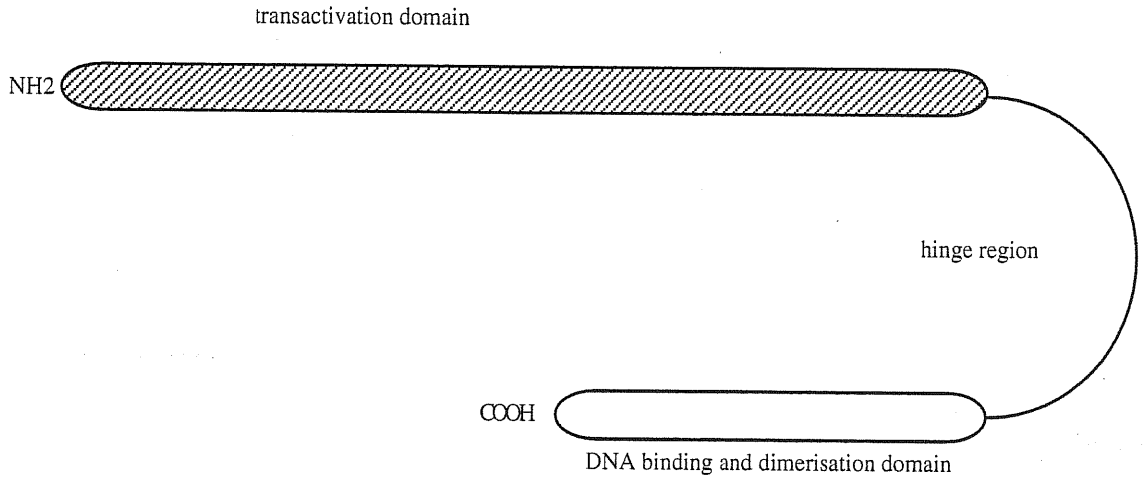
6.3.1 The E2 proteins show conservation in two functional domains

The E2 proteins are relatively well conserved among papillomaviruses, as can be seen by multiple sequence alignment of the deduced amino acid sequences from different papillomavirus types. In particular, the E2 proteins are conserved in two functional domains overlapping the three separate domains based on structure prediction. The N-terminal domain, consisting of approximately 200 amino acids, is required for transactivation. This domain is followed by a hinge region that varies in length and it is not conserved between different papillomaviruses, and a C-terminal DNA binding and dimerization domain of about 85 amino acids (Giri and Yaniv, 1988). The role

of the central region is still unclear, but its high proline content suggest that it might act as a flexible link between the two conserved regions. (Fig. 6.3.1).

A very good support for the idea that E2 is a modular protein resulted recently from the *in vitro* production of functional chimeric E2 proteins by generating a swap point between the transactivation domain, the hinge region or the DNA binding domain of E2 from BPV-1 and from HPV-11 (Berg and Stenlund, 1997).

Fig. 6.3.1



The shorter E2 proteins contain the DNA binding and dimerization domains of the C-terminus, but lack the transactivation domain. E2TR and E8/E2 can inhibit the transcriptional transactivating function of the full-length polypeptide by competing for its cognate DNA binding sites and by forming inactive heterodimers with the full-length transactivation protein (McBride *et al.*, 1991; Bouvard *et al.*, 1994).

Only the full-length E2 protein enhances replication and transcription, the other two proteins function as repressors of transcription and possibly also of DNA replication. However the regions of E2 required for enhancement of

replication and transcription are not identical (Piccini *et al.*, 1997; Sakai *et al.*, 1996).

The crystal structure of the BPV-1 E2 DNA binding domain (containing the C-terminal 84 amino acids of the protein), bound to its DNA target, has been published (Hegde *et al.*, 1992). It reveals novel types of interactions between the two E2 monomers which constitute, upon dimerization, an eight-stranded antiparallel, β-barrel structure. This type of structure is unique amongst eukaryotic transcription factors.

Little structural work has been performed on the amino terminal domain, which contains the transcriptional activation activity.

6.3.2 E2 binds directly components of cellular transcriptional machinery

Transactivators are believed to function, at least in part, through contacting components of the cellular transcriptional machinery and affecting the formation and/or stability of the preinitiation complex (Choy and Green, 1993). In many cases, virally encoded transcriptional activators have been found to contact directly components of the host cell's transcriptional machinery; these interactions may be important, though not necessarily sufficient, for mediating viral transactivation.

Induction of gene expression by the papillomavirus E2 protein requires its 200 aminoacid amino terminal transactivation domain. E2 can localize the transcription complex to the target promoter through its carboxy-terminal sequence specific DNA binding domain, however the cellular factors that lead to the formation of an activated RNA polymerase complex have yet to be identified and characterized.

TBP and TFIIB have separately been described to be rate-limiting for preinitiation complex formation (Klein and Struhl, 1994; Lin *et al.*, 1991) and it has been shown that indeed E2 is able to bind directly to the TBP protein of the TFIID complex and to TFIIB. These interactions have been mapped to the C-terminal portion of E2, a region also present in the other E2 protein family members, E2TR and E8/E2TR (Rank and Lambert, 1995). However, the biological relevance of the binding to TFIID and TFIIB *in vitro* has not been established *in vivo*, while the N-terminal half of the E2 protein is clearly important for the transactivation function: the N-terminal domain can stimulate transcription in eukaryotic cells when cloned onto a heterologous DNA binding domain (Haugen *et al.*, 1988).

More recently, direct physical and functional interaction has been reported between the E2 N-terminal domain and TFIIB (Yao *et al.*, 1998) and it has been shown to be necessary for E2 transactivation function *in vivo*.

In addition, using the yeast two-hybrid screen, a 37kD nuclear protein has been identified (AMF-1) that interacts with E2 transactivation domain and a strong correlation appears to exist between the ability of the E2 protein to stimulate transcription and the ability to interact with AMF-1 (Breiding *et al.*, 1997).

Surprisingly, only in the context of a subclass of HPVs such as HPV-16, HPV-18 and HPV-11, E2 is able to repress transcription of the viral E6 and E7 genes (Cripe *et al.*, 1987; Dong *et al.*, 1994; Thierry and Yaniv, 1987). Four E2 binding sites (BSs) are found in the URRs of these HPVs, with conserved relative positions. Two of them, E2 BS1 and BS2, form a tandem repeat located three or four nucleotides upstream of the TATA box of the E6/E7 promoter and a conserved Sp1 binding site is situated in close proximity to E2 BS2 (Gloss and Bernard, 1990). The Sp1 binding site may overlap

E2BS2, supporting the hypothesis that E2 might interfere with Sp1 transactivation ability through binding to this site (Dong *et al.*, 1994; Tan *et al.*, 1991). Alternatively, it was suggested that E2-mediated transcriptional repression is due to steric hindrance between the TATA box binding protein and E2 at E2BS1. Different conclusions were reached by groups studying E2 transcriptional activity, therefore the mechanism of down-modulation by full-length E2 is still controversial.

6.4 The upstream regulatory region (URR)

The URR, where the origin of replication is located, is positioned between the end of the late ORF and the beginning of the E6 early gene, and is about 1 kb in length. The HPV-18 URR has been subdivided by *RsaI* digestion into three functional units (Gius *et al.*, 1988): (i) a 5'-terminal 389-bp region of unknown function, encompassing almost half of the URR but contributing only marginally to the transcriptional stimulation of the P_{105} promoter that regulates transcription of the E6 and E7 genes (Hoppe-Seyler and Butz, 1993; Thierry *et al.*, 1987), (ii) a 230-bp constitutive enhancer region that plays a key role in the efficient activation of HPV18 E6/E7 transcription (Gius *et al.*, 1988), and (iii) a 206-bp promoter-proximal region containing the E6/E7 promoter at its 3' terminus (Butz and Hoppe-Seyler, 1993).

The HPV-18 enhancer was detected in a study aimed to isolate enhancer elements of human origin randomly from the HeLa genome by functional selection (Swift *et al.*, 1987): the result is not surprising since it is known that HeLa cells contain 20-50 copies of integrated HPV18 DNA (Schwarz *et al.*, 1985).

The function of the enhancer, referred to as the constitutive enhancer, is dependent solely on cellular factors. It directs expression to high levels in squamous epithelial cells, but is only weakly active in other cell types: the keratinocyte transcriptional factor KRF-1 being a major determinant of this cell-type specificity, and necessary for HPV-18 enhancer activity (Mack and Laimins, 1991).

A variety of cellular transcription factors, including NF1, AP1, KRF-1, Oct-1, Sp1 and the glucocorticoid receptor, have been shown to bind to the HPV-18 URR and to participate in the transcriptional regulation of the E6/E7 promoter located at the 3' terminus of the URR (Butz and Hoppe-Seyler, 1993).

As the *cis* elements that control the initiation of viral DNA replication are overlapping with *cis* elements that control the viral early transcription, the possibility exists that these different elements involved in HPV transcription are also involved in the control of DNA replication.

6.5 The papillomavirus origin of replication

The initiation of DNA synthesis is a precisely orchestrated event performed at defined genetic loci. Origins of replication are *cis*-acting elements that direct the assembly of specialized nucleoprotein complexes that function in duplicating the genome (Kornberg and Baker, 1992).

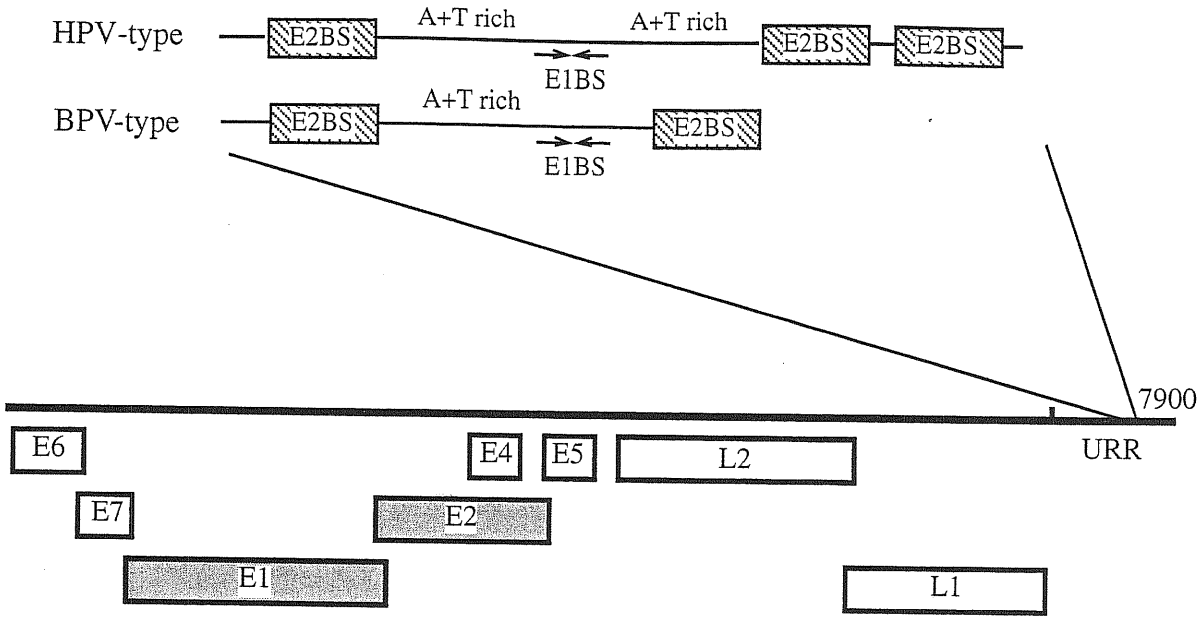
Numerous studies have been directed toward understanding the role of DNA sequences in the replication of BPV-1 and HPVs. These studies have shown that the origin of replication is located within the upstream regulatory region (URR), which contains regulatory signals for viral transcription, and the *cis*-acting sequences have been defined for a number of papillomaviruses

using transient replication assays where the respective E1 and E2 proteins have been expressed from heterologous expression vectors (Ustav *et al.*, 1991; Chiang *et al.*, 1992b; Del Vecchio *et al.*, 1992; Remm *et al.*, 1992; Lu *et al.*, 1993; Sverdrup and Kahn, 1994). The position of the viral origin of replication appears to be completely conserved between a number of different virus types.

A general feature of hitherto characterized eukaryotic replicons is a link between replication and transcription, such that transcription factor binding sites form a part of the *cis*-acting elements required for initiation of replication *in vivo* (DePamphilis, 1993). In the case of papillomaviruses, the required transcription factor, E2, is encoded by the virus and other transcription factors that have been tested are inactive.

The small noncoding region containing all the sequences required in *cis* for DNA replication *in vivo* presents three recognizable elements, an A+T rich region, a binding site for the E1 polypeptide and a binding site for the E2 polypeptide (Ustav *et al.*, 1993, Remm *et al.*, 1992). The papillomavirus *ori* sequences can be classified according to two schematic variants. Some animal papillomaviruses, including BPV-1, are organized as illustrated in Fig. 6.5 where two E2 binding sites flank an A+T rich sequence and the palindromic sequence that constitutes the E1 binding site. In the natural context, most human papillomaviruses have three high-affinity E2BSs located in the origin fragment. Deletion or mutation of any one of these sites results in a small reduction of replication (Chiang *et al.*, 1992a; Remm *et al.*, 1992; Lu *et al.*, 1993; Sverdrup and Kahn, 1994).

Fig. 6.5



On the other hand, use of a larger fragment containing additional sequences from the URR region commonly results in higher replication activity than for a smaller fragment, but whether these effects are due to specific elements in the URR is unknown.

The definition of a minimal *ori* fragment has been complicated by apparent redundancies concerning the E2BS. For example in the BPV *ori*, a fragment comprising the A+T rich sequence, the E1BS, and the downstream E2BS (right) was originally defined as the minimal *ori* (Ustav *et al.*, 1991), but another fragment containing the upstream E2BS (left), the A+T sequence, and the E1BS is also active for replication albeit at lower levels.

Definition of a minimal HPV *ori* is even more complex in this regard; deletion analysis of the HPV-11 and HPV-18 *oris* demonstrated that even non overlapping fragments have replication activity, indicating a degree of redundancy (Chiang *et al.*, 1992a; Lu *et al.*, 1993; Sverdrup and Kahn, 1994).

These results indicate that the requirement for an E2BS is absolute, but a great deal of flexibility exists in terms of both the position and the affinity of the E2BS. A relationship appears to exist however between the affinity of the E2BS and the ability to function at a distance from the binding site for E1 (Ustav *et al.*, 1993).

Thus, a search for a minimal sequence that can function as an origin may be less informative than a more quantitative approach where all elements that affect the origin activity are identified.

6.6 E1 interacts directly with E2

It is well established, by coimmunoprecipitation assay and two hybrid analysis, as well as GST-pull down assay, that the E1 replication protein can form a complex with the E2 transcription factor (Mohr *et al.*, 1990; Lusky and Fontaine, 1991; Bream *et al.*, 1993; Storey *et al.*, 1995).

Formation of the complex between BPV-1 E1 and E2 can be modulated by phosphorylation of the E2 protein, as the binding is stimulated 2- to 3- fold when the E2 protein is treated with alkaline phosphatase prior to incubation with E1 (Lusky and Fontaine, 1991). It is not known at present whether phosphorylation of E1 influences the complex formation.

Studies have been performed to determine what regions in E2 are required for interactions with the E1 protein, using a variety of methods, and they all indicate that mutations in the N-terminal activation domain of E2 are deleterious for this interaction (Benson and Howley, 1995; Hibma *et al.*, 1995; Piccini *et al.*, 1995; Storey *et al.*, 1995).

The E1-E2 interaction results in the formation of a specific complex on the *ori* (Sedman and Stenlund, 1995). The ability to form this particular

complex shows a very strong correlation with replication *in vivo* and is dependent on specific interactions between the two proteins and their recognition sequences in the DNA, as well as interaction between the two proteins.

One of the striking consequences of these interactions is a substantial increase in the sequence specificity of the complex compared with that of the individual components. In the presence of E2, E1 binds to the origin in a form that in the absence of E2 lacks, or has very low, DNA binding activity, demonstrating that the interaction with E2 alters the DNA binding activity of the E1 protein (Sedman and Stenlund, 1996). The two proteins that individually bind to their respective binding sites with a low degree of selectivity, bind in combination with several hundred-fold greater sequence specificity.

6.7 E2 is an auxiliary factor in DNA replication

In recent years it has become clear that several transcription factors are multifunctional and also directly influence initiation of DNA replication. Many viral origins in fact consist of a core origin and auxiliary regions that contribute to the initiation of replication and are required for optimal viral growth (DePamphilis, 1993). These auxiliary regions contain transcription factor binding sites. As a general rule it seems that transcription factors enhance replication by facilitating rate-limiting steps in the initiation process. These steps include recruiting of initiation proteins, changing the activity of initiation proteins, induction of structural changes in origin DNA and global changes in the chromatin structure surrounding the origin. Of course the

various mechanisms are not mutually exclusive and may operate together to optimize the initiation process.

A peculiarity of papillomavirus DNA replication is the absolute requirement *in vivo* for the viral transcription factor E2. This requirement is highly specific, and various other transcription factors that have been tested are inactive (Ustav *et al.*, 1991; Li and Botchan, 1993). For example, a hybrid activator, VP16-E2, which contains the activation domain from the herpes simplex virus protein VP16 fused to the DNA-binding domain of E2, fails to support replication (Ustav *et al.*, 1991). In contrast, SV40 and polyomavirus are not limited to any one particular factor, although both viruses show some specificity in that not all transcription factors are capable of providing the auxiliary function (DePamphilis, 1993).

The apparent requirement for the E2 protein for replication *in vivo* presents a paradox of sorts: in a cell-free replication system that has recently been developed, E1 is clearly sufficient for *ori*-specific replication (Yang *et al.*, 1991). Since E2 is not required in this system, the E1-E2-*ori* complex is not required, hence replication can be initiated *in vitro* without the formation of the E1/E2/*ori* complex.

The E2 protein falls in the category of replication auxiliary factors simply because this factor is not required for replication under all conditions: ori specific replication *in vitro* can somehow bypass the requirement for E2. Since E1 appears to have a modest sequence specificity, E2 functions to increase the selectivity of binding of E1 between "specific" and "nonspecific" DNA. In fact, by challenging the sequence specificity of E1 by introducing nonspecific competitor DNA, a standard *in vitro* replication system that shows little dependence on E2, can be made highly dependent on E2 and an

appropriately positioned E2 binding site, closely reproducing the *in vivo* requirements (Sedman and Stenlund, 1995).

The major function of the auxiliary factor E2 is therefore to participate in the assembly of an active replication complex by increasing the binding specificity of the initiator. Other functions that have been proposed for the E2 protein *in vivo*, such as interaction with replication protein A (RPA) (Li and Botchan, 1993), a single-stranded DNA binding protein. Derepression of chromatin templates, might also be an important E2 function, but certainly shows little specificity since many different transcription factors can provide these functions, but cannot replace E2 *in vivo* (Li and Botchan, 1994).

Materials and Methods

1. Plasmid constructions

1.1 Expression vectors

The full-length HPV-16 E1 gene was amplified by PCR using Vent polymerase (Biolabs) from the plasmid pW12 (Storey *et al.*, 1992) which contains HPV16 DNA from W12 cells. Unlike other HPV-16-containing cell lines derived from carcinomas which contain HPV-16 genomes integrated into the host chromosome (e.g. SiHa, CaSki), W12 cells contain HPV-16 genome as a multicopy stably replicating episome (Stanley *et al.*, 1989) and are able to produce HPV-16 virions when grafted onto nude mice (Sterling *et al.*, 1990). As E1 encodes functions necessary for episomal replication (Lusky and Botchan, 1986) we assumed (and later confirmed experimentally) that the W12 E1 ORF could code for a fully functional product.

The HPV-16 E1 gene was cloned between the *BamHI* and *EcoRI* sites of the plasmid pGEX2T (Smith and Johnson, 1988) to generate the plasmid pGEX2T16E1 which produces a fusion protein between glutathione S-transferase (GST) and E1. The HPV-16 E1 gene was also cloned between the *BamHI* and *EcoRI* sites of the plasmid pJ4Ω (Lees *et al.*, 1990) to give pJ4ΩE1.

Full-length E2 was cloned into pJ4Ω to give pJ4ΩE2 (Lees *et al.*, 1990) and consists of nucleotides 2755-3853 from the HPV-16 genome. Mutants of E2 (kindly provided by A. Storey, ICRF, London) were generated by cloning the full-length gene into M13mp19 and mutagenesis reactions were performed

using an *in vitro* system (Amersham). For single point mutations 21 base oligonucleotides were used. Where larger deletions were required, 28-mers were used so that a 14 base anchor was present on each side of the sequence which was to be deleted (Storey *et al.*, 1995; Piccini *et al.*, 1995).

Full-length E2 or a mutated form of the E2 protein were cloned between the *BamHI* and *EcoRI* sites of the plasmid pGEX2T to give pGEX2T16E2.

Protein expression vector pCGE1B was constructed by inserting HPV-18 sequences from 119-4743 (*BamHI/DpnI*) to the eukaryotic expression vector pCG-ATG$^-$(Tanaka and Herr, 1990). Deletions of E2 and E5 ORFs were made by deleting the coding sequences 3' from *DraIII* (nt. 2999) and *NdeI* (nt. 3918) respectively to give rise to the pCGE1BΔE2 and pCGE1BΔE5 plasmids. Numbering of nucleotides is according to Cole and Danos (1987). These expression vectors have been kindly provided by Dr. Mike Romanos (Glaxo Wellcome, Stevenage, UK).

For expression of proteins *in vitro* each of the HPV genes were individually subcloned into the plasmid pSP64. The full-length E2 sequence was derived from the plasmid pJ4Ω16E2, and a truncated form of E2 encoding only the activation domain was derived from the plasmid pJ4ΩACE2 (Storey *et al.*, 1992). The putative spliced repressor form of E2 encoding DNA binding domain (nucleotides 1264-1301^3357-4222) was from the pTZ18uE2C,E5 plasmid described by Doorbar *et al.* (1990). Both the N-terminal ACE2 and carboxyl spliced repressor from E2 were cloned into pGEX2T to generate GST fusion proteins.

1.2 CAT reporter plasmid

The CAT reporter plasmids used in this study consists of the HPV-16 URR from nucleotides 7403 to 114 cloned into the *HindIII/BamHI* sites of pBLCAT2 where the CAT gene is under the control of the TK promoter and is termed 16URR:TK:CAT (Bouvard *et al.*, 1994).

1.3 *Ori* plasmids

The HPV-16 origin containing replicon consists of the HPV-16 URR from nucleotides 7403 to 114 cloned into the *HindIII/BamHI* sites of pBLCAT3 (Lukow and Schutz, 1987) and it is termed 16URR::CAT.

The HPV-18 origin-containing plasmids consists of the HPV-18 enhancer and promoter proximal region from nucleotides 7391 to 88 (numbering according to Cole and Danos, 1987) cloned into the *BamHI/HindIII* sites of vector pBL (Hoppe-Seyler *et al.*, 1991) and it is termed p18URRL. Site-specific mutagenesis of p18URRL was performed by polymerase chain reaction. DNA sequences of oligonucleotides used for *in vitro* mutagenesis are listed below (only the sense strand of each oligonucleotides is shown). The name of each oligonucleotide corresponds to the name of the mutated HPV-18 origin replicon. Mutated cellular factor binding motifs are underlined.

18NF1M 5'-TTAAGCTAATTGCATACTTGGtaTGTACAACTAC
TTTCATGTCCAACATTCTGT
18OctM 5'-TTAAGCTAAgTaaATACTTGGtaTGTACAACTACTTT
CATGTCCAACATTCTGT
18KRFM 5'-TGCTTaacgAACTATATCCACTaaaTATGT
18AP1PM 5'-GAACTATAATATaAgcttGCTGTGC

18APIEM 5'-TGGCGATACAAGGCGCACCTGGTATctagacTTTTCCTGTCC

18Sp1M 5'-GTAGTATATAAAAAAactAGTGACCGA

18GREM 5'-AGGTTGGGCAGCggATACTATACTTTTC

The HPV-18 origin containing plasmids have been kindly provided by Felix Hoppe-Seyler (Deutsches Krebsforschungszentrum, Heidelberg).

2. Production and purification of GST fusion proteins

Escherichia coli KC1101 protease deficient (lon^-) strain were transformed with pGEX2T base expression plasmids. Cultures were grown overnight at 37°C in 100 ml of LB medium supplemented with 50 μg/ml ampicillin. The next day the culture was added to 700 ml of fresh medium, grown until the optical density at 600 nm reached 0.6, and proteins were then induced by the addition of 1mM isothiogalactopyranoside (IPTG) for 5 hours. Bacteria were harvested by centrifugation in a Sorvall GSA rotor at 4000 r.p.m. for 10 mins. Bacterial pellets were resuspended in 10 ml of PBS and $MgCl_2$ added to a final concentration of 15 μM plus Triton X-100 to 1%. Bacteria were then lysed by sonication and insoluble material was removed by centrifugation at 10000 r.p.m. The supernatant was incubated with glutathione agarose beads (Sigma) at 4°C for 2 hours. The beads were washed 5 times with 10 bead volumes of PBS containing 1% Triton X-100.

Escherichia coli DH5α strain were transfomed with pGEX2T containing the full-length wild-type E2, mutated forms of the full-length E2, the N-terminal activation domain or the C-terminal DNA binding domain. The GST fusion proteins were purified as described above.

3 *In vitro* phosphorylation of GST fusion proteins:

Casein kinase II reactions were performed in 20 mM HEPES pH 7.5, 20 mM $MgCl_2$, 0.3 aprotinin, 1 µM pepstatin, 10 µM ATP, 0.3 µCiγATP in a final volume of 10 µl. One unit of enzyme (Promega) was added to each reaction. After incubation at 37°C for 15 minutes, phosphorylation was monitored by SDS/PAGE and autoradiography.

4. Transcription and translation of proteins *in vitro*

Proteins were expressed by using the TNT coupled reticulocyte lysate transcription-translation system as specified by the manufacturer (Promega). The pSP64 contructs were used as templates for the transcription reactions and the proteins were radiolabeled by inclusion of [^{35}S] cysteine in the reaction mixture.

5. Pull-down experiments

5.1 GST pull-down experiments

Radiolabeled, *in vitro* translated proteins were quantitated with a Packard Instant Imager and incubated with equal amounts of GST fusion proteins immobilised on beads. Beads were subsequently washed several times in PBS to remove the detergents used in purification. After incubation at 4°C or at room temperature for 1 h, the beads were washed four times with PBS containing 0.1% NP40.

Proteins specifically bound to the beads were removed by boiling in an equal volume of 2x SDS sample buffer and subjected to SDS-PAGE.

5.2 Biotin pull-down experiments

In vitro translated radiolabeled E1 and E2 proteins were directly mixed. After allowing the proteins to associate by incubation at 4°C for 1 h, 0.5 µg of biotinylated oligonucleotide containing two E2 binding sites was added and the incubation was allowed to continue for a further 20 min. As a control, an identical but unbiotinylated oligonucleotide was added in place of the biotinylated oligonucleotide. Proteins bound to the biotinylated oligonucleotide were recovered by the addition of 20 µg of avidin-agarose beads (Sigma). The beads were washed and proteins were eluted as described above.

The sequence of the E2 oligonucleotide used was:

5' GCTTCAACCGAAATCGGTTGAACCGAAACCGGTTGCATG

together with its complement which were hybridized together prior to incubation with E2. The E2 binding sites are underlined. The oligonucleotide was biotinylated at the 5' end where required.

6. ATPase assays

The ATPase activity of the purified E1 fusion protein was detected by release of P_i from [γ-^{32}P]ATP. Reactions were performed in a total volume of 20 µl in 50 mM Tris-HCl pH 8, 100 mM NaCl, 10 mM $MgCl_2$, 1mM dithiothreitol, 0.1 µCi [γ-^{32}P]ATP. About 1 µg per 10 µl of either the GST-16E1 or the GST-cyclin D proteins were used per assay. After incubation at 37°C for 1 h, 1 µl was spotted onto a polyethyleneimine thin-layer plate and chromatographed in 0.5 M formic acid-0.5 M LiCl. The plate was then dried and exposed to Kodak XAR film for 20 min to 1h. Radioactive spots were then excised and quantified by liquid scintillation counting.

7. Gel retardation assays

Oligonucleotides corresponding to two E2 recognition sites,

5'-GCTTCAACCGAAATCGGTTGAACCGAAACCGGTTGCATG

were labelled in the presence of [γ-^{32}P]ATP and polynucleotide kinase. Gel shifts were carried out by mixing the probe (40000 cpm, 100ng) with the specific proteins expressed *in vitro* using the TNT coupled transcription-translation system (Promega) in a buffer containing 20 mM potassium phosphate pH 7.4, 0.1 M NaCl, 1 mM EDTA, 0.1% NP-40, 3 mM DTT, 0.7 mg of bovine serum albumin/ml, 10 mM $MgCl_2$, 10% glycerol and in the presence of 20 ng of non-specific competitor DNA (pUC19). After incubation at room temperature for 20 min, loading dye was added and the samples were resolved on non-denaturing 6% acrylamide (acrylamide: bis, 40: 1) gels in 0.5X Tris-borate-EDTA. The gels were then dried and processed by autoradiography.

For supershift analysis 1 µl of the polyclonal anti-E2 antibody raised against the C-terminal domain of the protein (kindly provided by Lutz Gissmann) was added to the E2-oligonucleotide complex after the former 20 min binding reaction and the mixture incubated for a further 30 min at 4°C. As a control 1 µl of a polyclonal antibody raised against GST-HPV-16E1 was used.

8. Cell culture and transfections

HT1080 is a cell line derived from a human fibrosarcoma (Laug *et al.*, 1983). The C-33 I cell line is derived from a human cervical carcinoma. The 293 cell line is derived from primary human embryonal kidney cells transformed by sheared human adenovirus type 5 (Ad 5) DNA that contains

and express the transforming genes of Ad 5. Cells were cultured in DMEM supplemented with 10% foetal calf serum. Transfection with plasmid DNA was done by standard calcium phosphate precipitation.

Determination of transfection efficiency was carried out by *in situ* staining of transfected cells 24 h after transfection with the β-galactosidase expressing plasmid pCH110 (Pharmacia). Plates were washed thoroughly with PBS, the cells were fixed with 0.2% glutaraldehyde , 2% formaldehyde for 10 min, washed again with several changes of PBS and stained with a staining solution containing 0.4 mg/ml X-gal, 2 mg/ml potassium ferrocyanide, 2 mg/ml potassium ferricyanide and 0.5 mM $MgCl_2$ in PBS. The plates were examined by light microscopy after overnight incubation at 37°C.

Dexamethasone (DEX) was used at a concentration of 10^{-6} M.

9. CAT assays

Cells were transfected with 5μg of CAT reporter plasmid and 5 μg of E2 expression plasmid unless otherwise indicated. Where titrations were performed, DNA input was standardized by addition of increasing amounts of vector only DNA. Cells were harvested after 48 h in 400 μl of 40 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA and subjected to three freeze-thaw cycles, followed by incubation at 65°C for 10 min. Samples were clarified by centrifugation at 14000 r.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 [^{14}C] chloramphenicol (50 mCi/mmol; Amersham) in a final volume of 50 μl at 37°C for 30-60 min. Following extraction with ethyl acetate, samples

were analysed by thin layer chromatography and visualized by autoradiography. Samples were quantified with a Packard Instant Imager.

10. Transient replication assays

Transient replication assays were performed with $8x10^5$ 293 cells in 90-mm-diameter plates. Transfection efficiencies typically ranged from 30 to 40% for 293 cells and 10 to 20% for C33-I cells.

For the E2 assays 5µg of either the mutated or the wild-type pJ4ΩE2 expression plasmid DNA plus 5µg of E1 expression plasmid pCGE1BΔE2 and 1µg of the replicon DNA 16URR::CAT were transfected into 293 cells.

For the study of transient replication in the presence of DEX, 5µg of pCGE1BΔE5 vector, expressing both E1 and E2 was transfected together with 1µg of the replicon DNA 16URR::CAT. For the analysis of mutated origins in transient replication, 5µg of pCGE1BΔE5 vector were transfected together with the indicated amount of origin-containing plasmids.

Three days post-transfection, low molecular mass DNA was isolated by the Hirt extraction procedure (Hirt, 1967). Samples were digested overnight with a restriction enzyme that linearizes the ori plasmids and treated with an excess of *DpnI* to remove the unreplicated input dam^+ methylated DNA. Total digestion products were separated on a 0.8% agarose gel in Tris-acetate-EDTA buffer. The DNA products were transferred to a Hybond-N^+ membrane and subsequently hybridized to ^{32}P-labelled replicon probe generated by random priming. Blots were hybridized overnight at 42°C in a solution containing 6x SSC, 5x Denhardt's solution, 0.5% SDS, 50% formamide and 500 µg/ml herring sperm DNA. Blots were washed three times for 30 min at 42°C in 2x SSC and 0.1% SDS and then twice for 40 min at 55°C in a solution containing

0.2x SSC and 0.1% SDS. Blots were usually exposed for 10 min and quantitated with a Packard Instant Imager.

11. Western blot analysis

The relative stability of the different E2 mutant proteins was determined by Western blot analysis following transfection of 293 cells. Cells were harvested after 24 h and extracted in a solution containing 50 mM Hepes pH 7.0, 250 mM NaCl, 0.1% NP40, 32 µg/ml phenylmethylsulfonylfluoride (PMSF) and 1% aprotinin. Protein concentrations were determined using the Bio-Rad protein assay and equal amounts were run on 12% polyacrylamide gels and transferred to a nitrocellulose membrane. HPV-16 E2 protein was detected using a pool of the anti-E2 monoclonal antibodies TVG261 and TVG271 (Hibma *et al.*, 1995), followed by incubation with rabbit anti-mouse biotin conjugate (DAKO) and avidin peroxidase conjugate (DAKO). Blots were developed using the Amersham ECL system according to the manufacturer's instructions. The anti-E2 monoclonal antibodies TGV261 and TGV271 are a kind gift of Merilyn Hibma (University of Otago, Dunedin, New Zealand).

Results

Papillomavirus DNA replication is strictly dependent upon two viral gene products, E1 and E2. Work with bovine papillomavirus has shown that the E2 protein can bind directly to the E1 protein and enhance the binding of E1 to the ori. However, little is known about the mechanism of interaction between human papillomavirus type 16 (HPV-16) E1 and E2 proteins.

In the first part of my study, the association between HPV-16E1 and E2 was examined, with the aim to identify the region of E2 important for binding E1. This was determined using a purified glutathione S-transferase-HPV-16 E1 fusion protein from *Escherichia coli* and HPV-16E2 proteins expressed by *in vitro* transcription-translation. Subsequently, the E2 mutants were analysed for their ability to transactivate an E2 responsive promoter and to support E1-dependent DNA replication *in vivo*. In the final section, the contribution of cellular transcription factors in DNA replication was assessed.

1. Purification of biochemically active GST-HPV-16E1 fusion protein

The GST-HPV-16E1 fusion protein was purified to near homogeneity from bacteria as is shown in Fig.1(A). The GST-HPV-16E1 protein migrated on 10% polyacrylamide gels with an apparent molecular mass of 98kD, in good agreement with the predicted size of the fusion protein. Under the conditions used, a small amount of protein breakdown was usually observed as faster migrating bands. Western blot analysis with an anti GST antibody confirmed that the 96kD protein was GST derived (Fig.1(B)).

Since the GST-HPV-16E1 fusion protein was intended for use in protein binding studies, I wanted to determine if a proportion of the purified protein was in a native and active conformation. An ATPase assay was employed as a measure of E1 activity and, as can be seen in Fig.1(C), GST-HPV-16E1 clearly possessed ATPase activity. In contrast, no ATPase activity was found to be associated with similar extracts prepared from bacteria over-expressing a GST-human cyclin D protein. These results indicated that, at least a proportion of the purified E1 fusion protein was correctly folded.

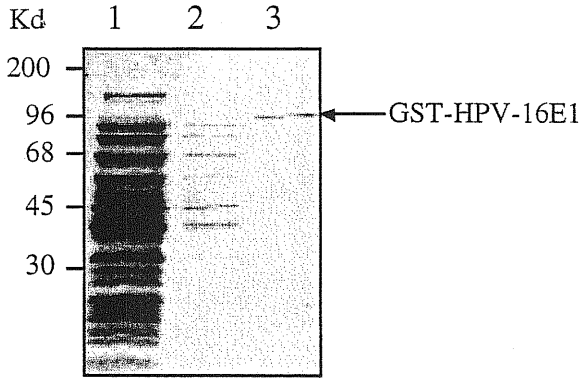


Fig.1(A): purification of GST-HPV-16E1 fusion protein. Samples of supernatant from the first (lane 1) and third (lane 2) washings of the glutathione-agarose resin in PBS-1% Triton X-100. Bound GST-16E1 (about 2 μg) was eluted from 25 μl resin in SDS sample buffer (lane 3).

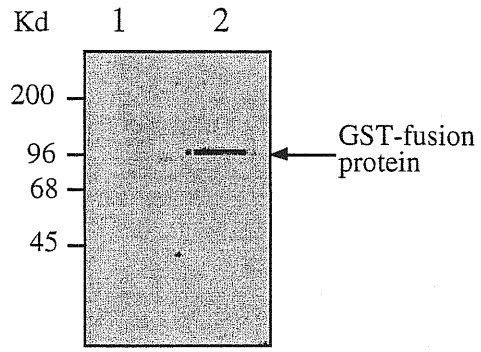


Fig.1(B): western blot performed using anti-GST polyclonal antibodies. Total protein samples from bacterial cultures induced with 1mM IPTG (lane 2) for 5 h or uninduced (lane 1).

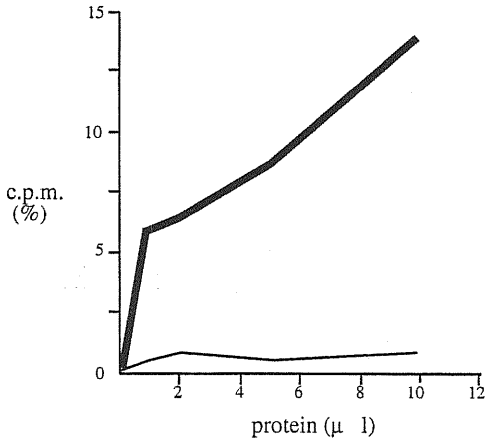


Fig.1(C): the ATPase activity associated with GST-HPV-16E1 protein (**bold line**) was monitored by the release of $^{32}P_i$ from [γ-^{32}P]ATP. No ATPase activity was detected in samples of GST-human cyclin D (plain line) that had been prepared in an identical manner. The maximal amount of protein used per assay was 1 μg contained in a volume of 10 μl. Proteins being diluted in buffer just prior to use as required.

2. GST-HPV-16E1 is a substrate for casein kinase II phosphorylation

Casein kinase II (CKII) is present in the nucleus and in the cytoplasm of all eukaryotic cells that have been studied (Allende and Allende, 1995). CKII is able to phosphorylate serines and threonines immersed in acidic sequences within proteins and peptides. The minimum requirements for phosphorylation is depicted by the sequence S*/T*XX D/E, in which the asterisk denotes the phosphorylated serine or threonine and X any non-basic amino acid. Since the HPV-16E1 protein sequence contains nine potential CKII phosphorylation sites, it is thus a strong candidate substrate for this enzyme.

To test this hypothesis, an *in vitro* phosphorylation assay was performed on the purified GST-HPV-16E1 and E2 fusion proteins using purified CKII enzyme. As is shown in Fig.2(B), the GST-HPV-16E1 is phosphorylated while no phosphorylation is detected on the GST-HPV-16E2 protein. This result therefore demonstrates that HPV-16E1 is an excellent candidate for CKII

phosphorylation and further indicates structural integrity of the purified GST-HPV-16E1 fusion protein.

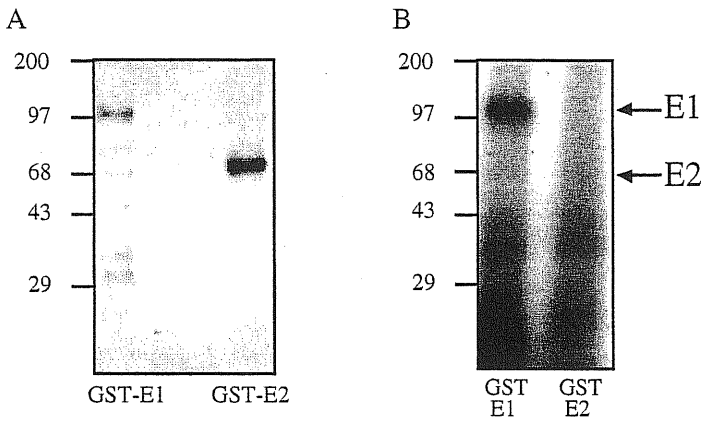


Fig.2(A): coomassie blue stained 12% PAGE of the purified GST-HPV-E1 and GST-HPV-E2 fusion proteins. (B) CKII phosphorylation of the fusion proteins bound to the glutathione agarose beads. Arrows indicate the position of the different fusion proteins.

It should also be noted that the HPV-16E2 primary sequence has five potential CK2 phosphorylation sites, but nonetheless, HPV-16E2 is not a good substrate for CK2 phosphorylation. CK2 dependent phosphorylation of HPV-16E2 has never been detected, either during the course of this study, nor by other groups. An obvious explanation for this could be that the presence of a kinase consensus site in the primary sequence of a protein does not necessarily imply that the given kinase is able to phosphorylate that protein. In addition, it has previously been shown that for BPV-1 E2, major phosphorylation sites are two serine residues at amino acid position 298 and 301, within the hinge domain of the protein (McBride *et al.*, 1989a). These target residues are within an acidic region and are potential CK2 recognition sites. However, this region of BPV-1 E2 is not conserved: the homologous region of HPV-16E2 is not highly acidic and bears little resemblance to the potential CK2 target sequence in BPV-1.

3. HPV-16E2 binds to HPV-16E1

Having expressed and purified the HPV-16E1 protein, I proceeded to investigate its ability to associate with the HPV-16E2 protein. The HPV-16E2 protein was transcribed and translated *in vitro* in the presence of [^{35}S]cysteine and mixed directly either with beads containing the GST-HPV-16E1 fusion protein or with beads containing GST alone. The mixtures were incubated at either room temperature or at 4°C for 1 h to assess the effect of temperature on the binding reaction, since it had been previously reported for the BPV-1 E1/E2 interaction that it was cold sensitive (Benson and Howley, 1995). The beads were washed and bound proteins were eluted and analysed by SDS-PAGE. The efficiency of binding was assessed by comparing the level of recovered E2 to a sample of the input translation mix. The results obtained are shown in Fig.3 and the HPV-16E2 protein was found to bind strongly to the GST-HPV-16E1 fusion protein at 4°C, where the majority of the input E2 protein was recovered. Since a reduced level of binding, was observed if the incubation was carried out at room temperature, all further binding experiments were performed at 4°C.

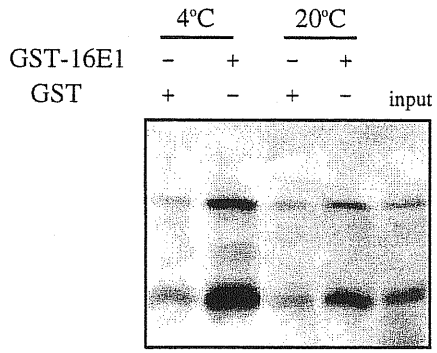


Fig.3: binding of *in vitro* translated HPV-16E2 to purified GST-HPV-16E1. Radiolabelled full-length E2 protein (4 µl) synthesized *in vitro* was mixed with either GST or GST-HPV-16E1 bound to glutathione-agarose resin. Following incubation at either 4°C or 20°C for 1 h the resin was washed and bound proteins were eluted. A 1 µl sample of the translation mix (input) was also electrophoresed to assess the recovery of E2.

A faster migrating band, with an apparent mobility of a 27 kD protein, was observed in all transcription-translation reactions of E2 (Fig.3). This

shorter product reacted specifically with monoclonal antibodies against the region of amino acid 18-41 of E2, but not with polyclonal antibodies directed against the C-terminus (data not shown). Therefore, this product is E2 related and this assumption is further supported by its ability to interact strongly with the E1 protein.

4. E1 and E2 form complexes which bind DNA

All papillomavirus origins of replication include one or more binding sites for the virus-encoded transcription factor E2. The E2 protein is known to recognize with high affinity the consensus sequence $ACCG(N)_6CGGT$ (Androphy *et al.*, 1987). A possible explanation for the strict requirement for E2 for DNA replication *in vivo* has been the generation of a specific E1/E2/*ori* complex. Having shown that the HPV-16E1 and E2 proteins associate *in vitro*, I wanted to address whether an affinity column containing the E2 binding site is able to retain both the E2 and the E1 proteins. To perform this binding experiment, *in vitro* translated radiolabelled E1 and E2 proteins were mixed together in PBS. After incubation at 4°C and addition of either biotinylated or unbiotinylated E2 binding site oligonucleotide, the E2 protein bound to the biotinylated oligonucleotide was recovered by the addition of avidin-agarose resin which was gently mixed with the proteins for 20 min. The resin was extensively washed and bound proteins were anlaysed by SDS-PAGE. Fig.4 shows that the full-length E2 protein was only recovered if a biotinylated oligonucleotide was used (lanes 2 and 3). In addition, the E1 protein was only detected if both E2 and the biotinylated oligonucleotide were present in the reaction (lane 2). The faster migrating species, always generated in the E2 transcription-translation reactions, was unable to specifically bind to

the oligonucleotide, consistent with it containing only N-terminal derived sequences. These results demonstrated that E1 and E2 can form protein-protein complexes that do not interfere with the ability of E2 to bind to its cognate recognition sequence.

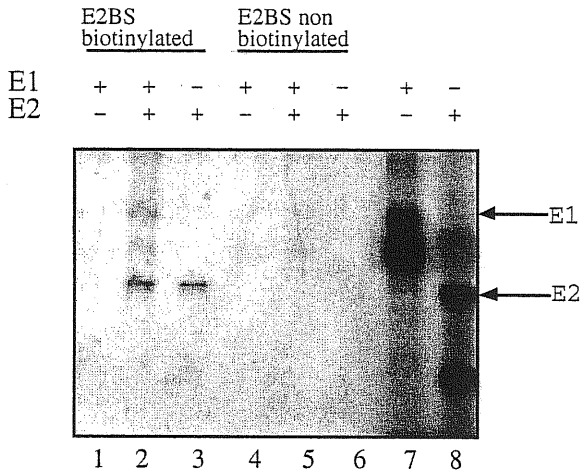


Fig.4: Radiolabelled E1 (4 µl) and E2 (2 µl) proteins were mixed at 4°C for 1 h. A 0.5 µg sample of either a non-biotinylayed or biotinylated oligonucleotide containing two E2 binding sites was then added and incubated for a further 1.5 h. Proteins bound to the biotinylated oligonucleotide were recovered by the addition of 10 µl of avidin-agarose beads. Sample were washed three times, eluted and resolved on an SDS-10% polyacrylamide gel. The positions of the E1 and E2 proteins are indicated. Unprogrammed lysate was added to incubations where E2 was omitted. Samples (1 µl) of the E1 and E2 translation mixtures are shown in lanes 7 and 8, respectively. All E1 and E2 proteins produced by the transcription-translation reaction were stable for the course of the incubation.

5. Identification of a region of the HPV-16E2 protein important for E1 binding

Having established assays for monitoring the HPV-16 E1/E2 interaction *in vitro*, I wanted to identify the region of E2 responsible for this association. First of all, I aimed to define whether regions in the amino terminal or carboxy terminal half of E2 could mediate the interaction with E1.

To address this question, the ability of GST fusion proteins containing either the full-length E2, the N- terminal activation domain or the C-terminal DNA binding domain were tested for binding to the radiolabelled *in vitro*

translated E1 protein. The regions of E2 encoded by the truncated proteins and their relative mobility and purity is shown in Fig.5(A) and (B) respectively.

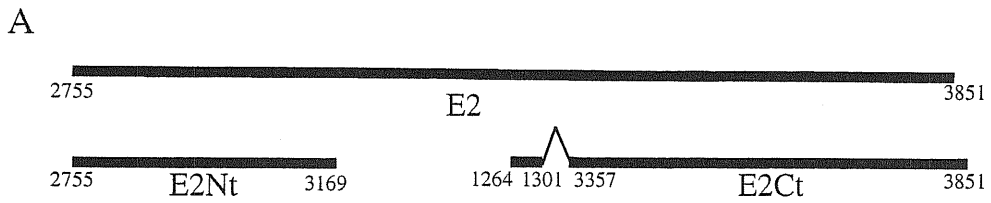


Fig.5(A): E2 fusion proteins used in this study. Numbers indicate nucleotides of the HPV-16 genomic sequence.

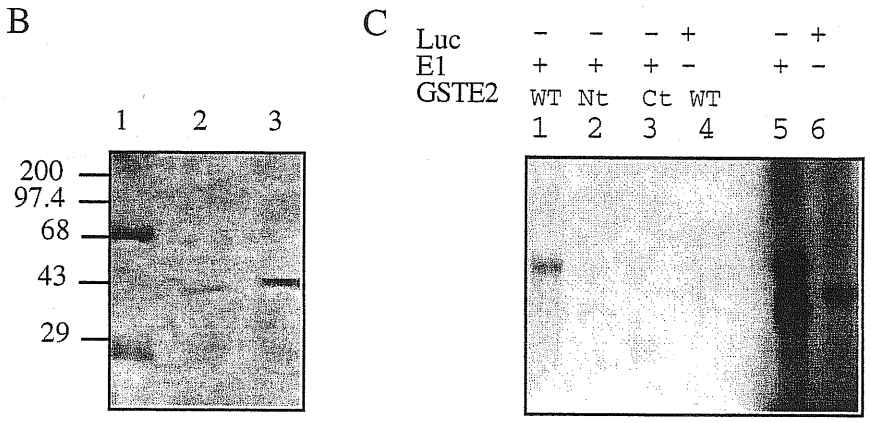


Fig.5(B): purification of GST-HPV-16E2 proteins resolved on a 12% polyacrylamide gel and stained with Coomassie blue. Lane 1, GST fused to full-length E2; lane 2, GST fused to the N-terminal domain of E2 (GST-HPV-16E2Nt) derived from pJ4Ω ACE2; lane 3, GST fused to the E2 C-terminal DNA binding domain (GST-HPV-16E2Ct) derived from pTZ18uE2C,E5. Fig.5(C): binding of *in vitro* translated HPV-16 E1 protein to GST-HPV-16E2 fusion proteins: *in vitro* translated E1 (2 µl) was incubated with equal amounts of either the full-length GST-HPV-16E2 or truncated GST-HPV-16E2 proteins. Binding of E1 was only observed with the full-length E2 protein. The input levels of E1 and luciferase are shown in lanes 5 and 6 respectively (2 µl of the respective translation mix).

No binding was observed between E1 and the short C-terminal repressor form of E2 (GST-HPV-16E2Ct in Fig.5(C)), which encodes only the DNA binding domain, but which lacks the activation domain. In addition, no binding of E1 to the GST-HPV-16NtE2 fusion protein, encoding the first 138 amino acids of the activation domain, was observed. This finding is not necessary in contrast with the previuos observation (Fig.3) that a shorter product of the

HPV-16 E2 *in vitro* transcription-translation reaction, corresponding to the N-terminal portion of the E2 protein is specifically retained on the GST-HPV-16E1 column. This shorter N-terminal portion of the E2 ORF migrates with an apparent molecular weight of 27 kD, suggesting the presence of a species about 243 amino acids long. The N-terminal portion of the E2 protein produced in the *in vitro* transcription-translation reaction is longer than the GST-HPV-16NtE2 fusion protein encoding the first 138 amino acids of the activation domain and may therefore include a region important for the E1-E2 interaction.

To confirm the absence of aspecific co-sedimentation, the internal luciferase (Luc) standard was added to the GST-HPV-16E2 protein.

Taken together, the results of this crude analysis indicate that the region of E2 responsible for the interaction with E1 lies between residues 138 and 201, as defined by the NtE2 and CtE2 truncations.

6. Heteromeric complexes of the full-length E2 and C-terminal E2 can bind E1

Although no binding was observed between the GST-HPV-16E1 and the CtE2, I was interested to determine if heteromeric complexes between the full-length E2 and the CtE2 were still capable of binding to E1. This seemed to be of particular interest since a truncated form of HPV-11 E2 had been shown to inhibit viral DNA replication (Liu *et al.*, 1995) and a possible mechanism might be by inhibiting the association of E1 and E2. This would be most likely if E2 bound to E1 only as a multimer. In order to assay for the different forms of E2 protein, a series of gel retardation assays were performed on an oligonucleotide derived from the HPV-16 URR, containing the two E2

recognition sequences placed immediately upstream of the P_{97} promoter.

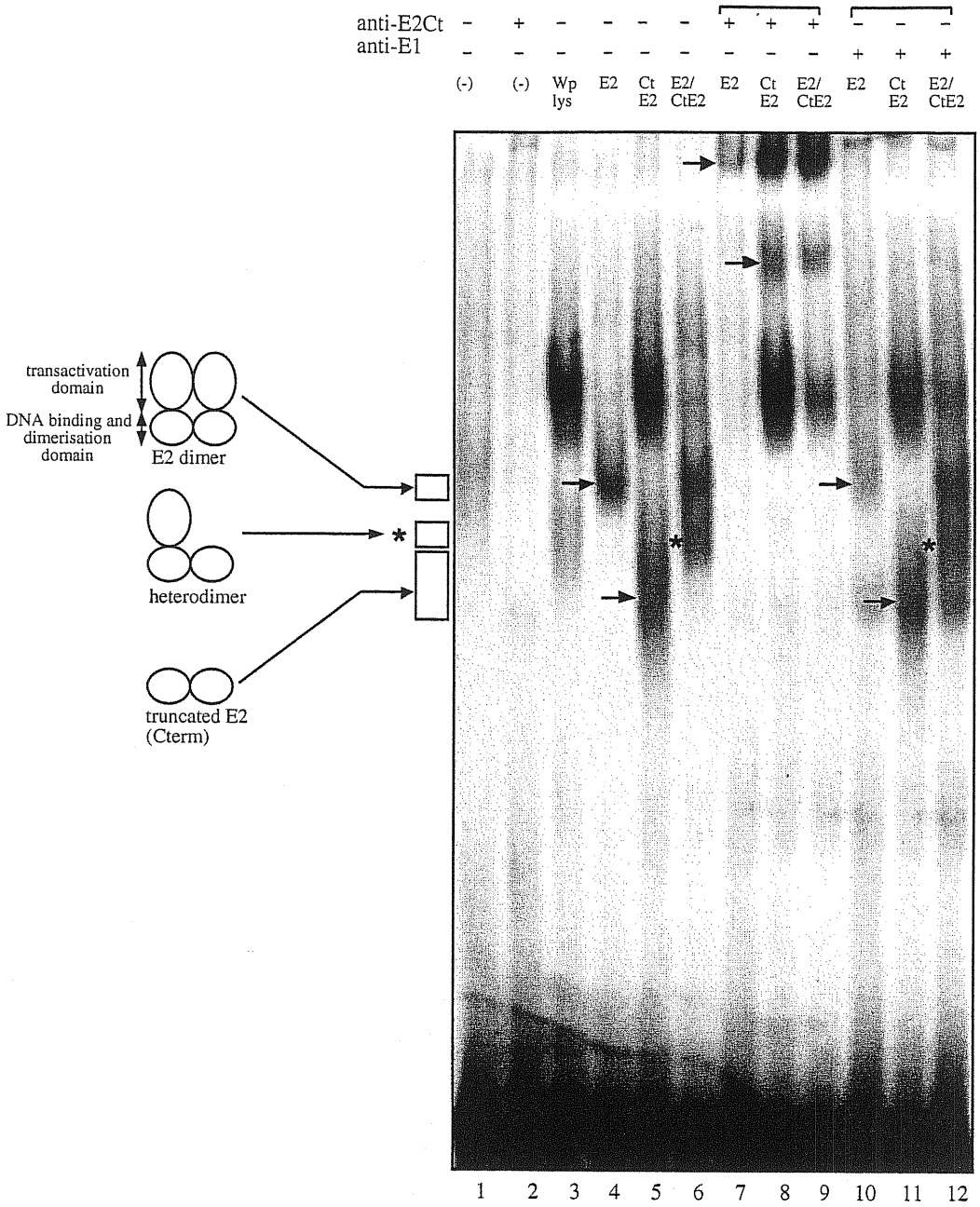


Fig.6(A): gel retardation and supershift analysis of a labelled E2 recognition site oligonucleotide (E2 BS) with *in vitro* translated E2 proteins. HPV-16 full length E2 and C-terminal E2 were translated individually (lanes 4 and 5, 7 and 8, 10 and 11 respectively) or together (lanes 6, 9 and 12) and incubated in the presence of anti-C-terminalE2 serum or anti-E1 serum as indicated. Supershift can be seen in lanes 7, 8 and 9. Lane 3 represents aspecific retardation of labelled E2 BS detected when using water primed reticulocytes lysate (Wp lys) in the absence of the E2 protein. Full-length E2 and C-terminal E2 homomers and heteromers(*) retarded bands are indicated on the left of the gel. and on the left of each lane.

The full-length E2 and the CtE2 were *in vitro* transcribed and translated before mixing with the labelled oligonucleotide. The results are shown in Fig.6(A) and demonstrate that both forms of E2 specifically retarded the labelled oligonucleotide. The full-length E2 protein-oligonucleotide complex migrated as a single discrete band. In contrast, the CtE2-oligonucleotide complex migrated and was more heterogeneous. These results suggested that only one configuration of full-length E2 was binding to the oligonucleotide, possibly as a tetramer, whereas the short form bound in a number of different configurations. To investigate the ability of the different forms of E2 to produce heteromeric complexes, the two forms of E2 were *in vitro* translated in the same reaction mixture and then used in the gel retardation assay. As can be seen from Fig.6(A), upon co-translating the two E2 proteins there was a marked shift in some of the retarded oligonucleotide, resulting in a complex which migrated with relative mobility between that of the full-length and the short E2 complexes. The identity of these complexes was confirmed by the supershift analysis using anti-E2 antibodies (Fig.6(A)). These observations are therefore consistent with the full-length and CtE2 proteins forming heterodimers.

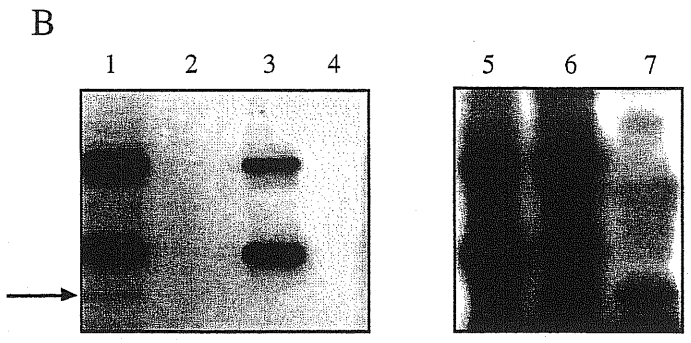


Fig.6(B): binding of CtE2/E2 complexes to GST-16E1. Proteins were either translated separately or together and mixed with GST-16E1 or GST resin. GST-16E1 resin was used in lanes 1-3. The input E2 proteins were: lane 1, 1 µl E2 plus CtE2 co-translated mixture; lane 2, 1 µl CtE2 alone; lane 3, 1 µl E2 alone. Lane 4 GST resin plus 1 µl E2 protein. lanes 5-7, 1 µl of each translation reaction as follows: lane 5, E2 alone; lane 6, E2plus CtE2; lane 7, C-tE2 alone.

The different forms of the E2 protein were then tested for their ability to bind both GST and GST-HPV-16E1. This was done by translating full-length E2 and E2Ct alone or in combination and then incubated with GST-HPV-16E1. It should be noted that the CtE2 protein contains only five cysteines as compared to the 11 cysteines present in the full-length E2 protein, and this probably accounts for the fact that the CtE2 portion always appears as a weaker band in the co-translating mixtures. Nonetheless, the results of the binding assays, shown in Fig. 6(B), demonstrate that a proportion of the CtE2 was retained on the GST-16E1 resin, when it has been co-translated with the full-length E2 protein. A densitometric scan of the autoradiogram revealed that the absorbance ratio of E2: CtE2 in the input is 1.8 (Fig.6(B) lane 6) and 4.1 in the proteins bound to GST-HPV-16E1 (Fig.6(B) lane 1), suggesting that just under half of the Ct E2 produced in the *in vitro* translation mix is bound to full-length E2. As before, no binding of the E2Ct alone is observed in the absence of the full-length E2 protein. This observation suggests that the heteromeric HPV-16E2 protein complexes can interact with the HPV-16E1 protein.

7. A stretch of highly conserved amino acids of HPV-16E2 are involved in binding to E1

The binding studies reported above indicated that a region spanning amino acid 138-201 of E2 was necessary for binding E1. To identify the precise region of E2 required for this interaction, I made use of a series of mutated E2 proteins which had been previously generated throughout the transactivation domain of HPV-16 E2. These are shown schematically in Fig.7(A).

All the E2 mutants were translated to the same efficiency as the wild-type (WT) protein. Equal amounts of either labelled wild-type or mutant E2 protein were mixed with GST-16E1 and assayed for binding. The results obtained are shown in Fig.7(B). Of the four E2 mutants tested in these assays only one mutant, which had a deletion of amino acids 156-159, completely failed to bind to the GST-HPV-16E1. All the other mutants tested bound strongly to GST-HPV-16E1. As before, no non-specific binding of either wild-type or mutant E2 proteins to the GST resin was observed. The deletion present in the E2 M9 mutant (Δ156-159) occurs in a small but highly conserved hydrophobic region of the E2 activation domain (Fig.7(C)).

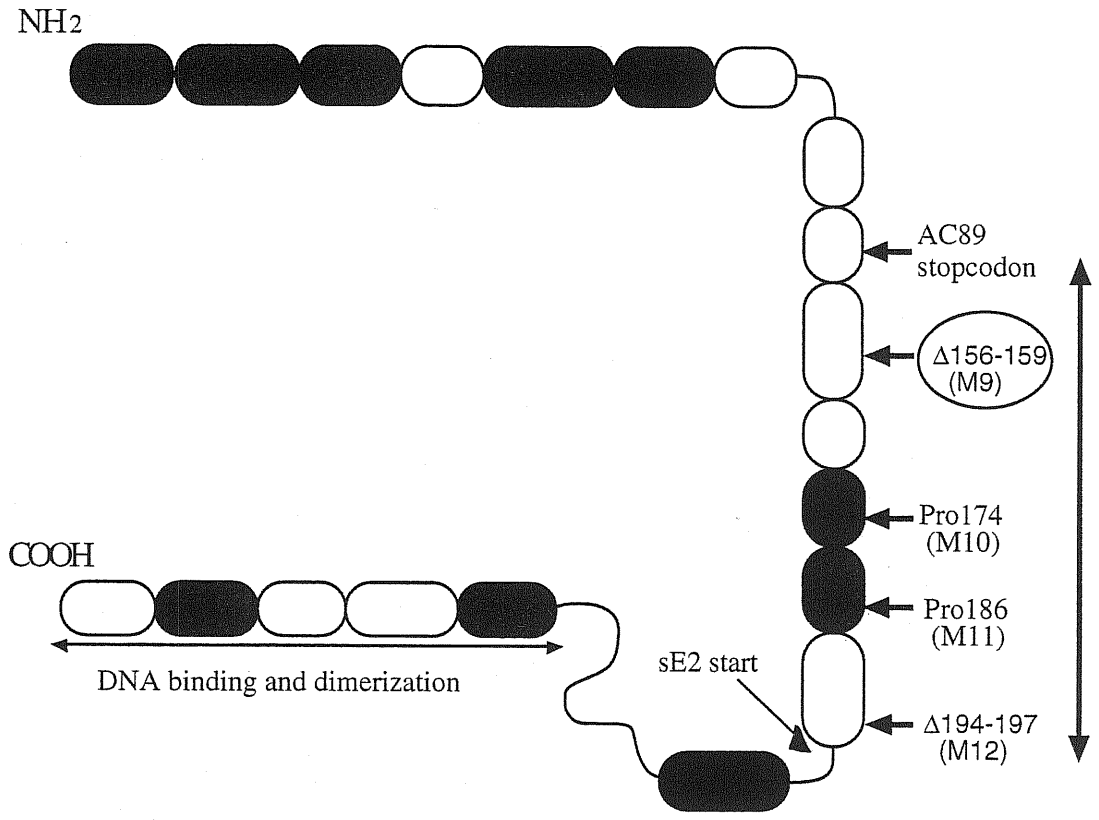


Fig.7(A): schematic representation showing location of E2 mutants with respect to the E2 functional domains. Numbers identify the amino acid residue(s) mutated together with the new residue at that position, Δ indicates amino acids deletion. AC89 stop codon defines NtE2. sE2 start defines CtE2. Secondary structure of the HPV-16 E2 is based on computer prediction. α-helices are schematized by ●, β-sheets are schematized by ○ .

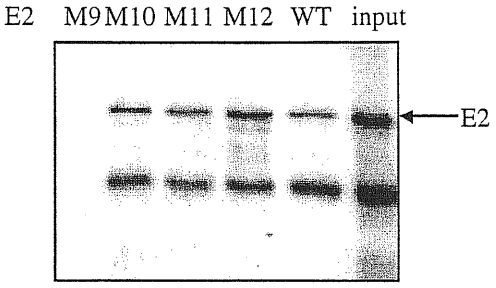


Fig.7(B): binding of HPV-16E2 mutants to GST-HPV-16E1 resin. All the E2 mutants were translated to the same efficiency and were stable as the wild-type protein in the translation mixture. Each radiolabelled E2 protein (2 µl) was mixed with glutathione resin which had bound to it GST-HPV-16E1. E2 input (1 µl) is shown. The position of the full-length E2 protein is indicated.

							E2Δ156-159 (M9)								
HPV-16	E2	Q	**V**	**D**	Y	Y	**G**	**L**	**Y**	**Y**	V	-	H	E	**G**	I
HPV-18	E2	C	**V**	S	H	R	**G**	**L**	**Y**	**Y**	V	-	K	E	**G**	Y
HPV-6	E2	M	**V**	**D**	A	K	**G**	**I**	**Y**	**Y**	-	**T**	C	-	**G**	Q
HPV-11	E2	S	**V**	**D**	A	K	**G**	**I**	**Y**	**Y**	-	**T**	C	-	**G**	Q
BPV-1	E2	G	A	**D**	G	T	**G**	**L**	**Y**	**Y**	C	**T**	M	A	**G**	A
HPV-5	E2	G	**V**	N	Q	T	**G**	**I**	**Y**	**Y**	M	Q	G	S	F	K
HPV-8	E2	G	**V**	N	Q	T	**G**	**I**	**Y**	**Y**	M	Q	G	S	F	R
HPV-10	E2	K	**V**	S	Y	E	**G**	**L**	**Y**	**Y**	-	**T**	H	E	N	M

Fig.7(C): alignment of papillomavirus E2 sequences showing conserved residues in bold letters. The deleted region of HPV-16 E2 implicated in binding to E1 is highlighted.

8. Multiple regions of the E2 protein are involved in binding to E1

At the time these studies were being performed, a separate study, using anti-E2 monoclonal antibodies, indicated that a region of E2 important for the interaction with E1 was present within the extreme amino-terminal portion of the protein (Hibma *et al.*, 1995). This seemed to indicate that more than one region of the E2 molecule might contribute to E1 binding.

To further characterize the mechanism of interaction between these proteins additional mutants of E2 lying in the extreme amino terminal region of the transactivation domain, were assessed for their ability to bind to HPV-16E1. These mutants were assigned the numbers 1-15 and are depicted schematically in Fig.8(A).

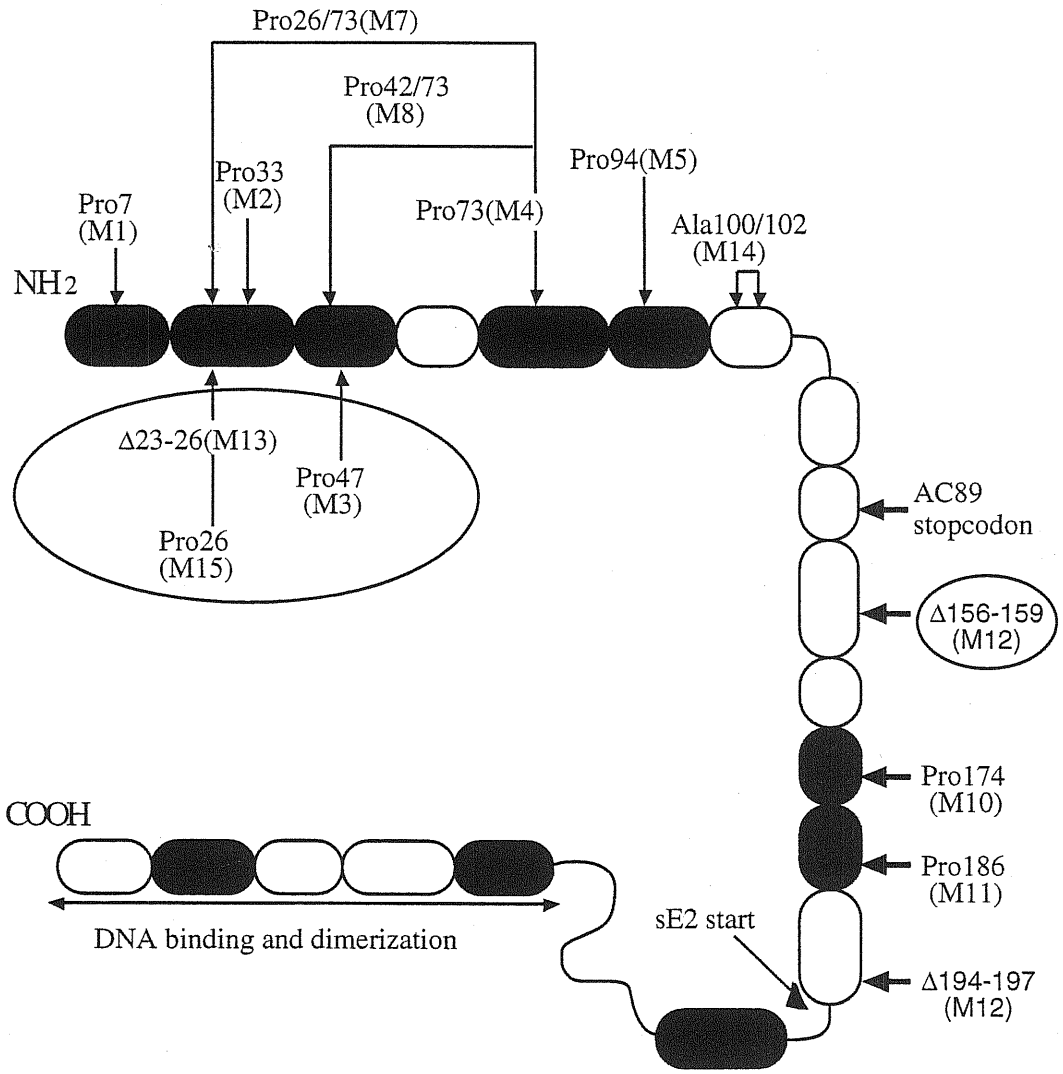


Fig. 8(A): Schematic representation illustrating all the E2 mutants used throughout this study. Symbols are as described in Fig. 7(A).

As I had previously observed that M9(Δ156-159) is completely defective for binding E1, this mutant protein was used as a negative control for a new series of GST pull-down assays. The results obtained are shown in Fig.8(B) and it is clear that mutants within the extreme N-terminal region were also defective in binding E1. Thus M13(Δ23-26) is completely negative whilst M3(Arg-Pro47) is clearly reduced and M4(Ile-Pro73) is reduced. These experiments indicate that residues spanning 23-73 contribute to the E2 association with E1.

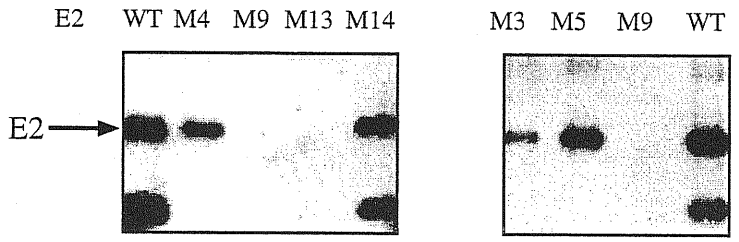
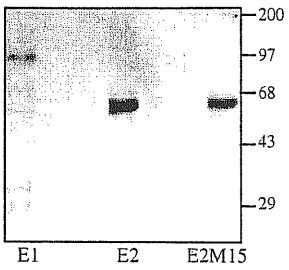


Fig 8(B): association between GST-E1 and HPV-16E2 mutant proteins. The GST-E1 fusion protein was tested for binding to six different *in vitro* translated E2 mutants and to the wild-type (WT) as indicated. Protein inputs were equalized throughout prior to the binding assay.

In order to monitor further the specificity of these associations, the same assay was also performed by mixing GST-HPV-16E2 beads with radiolabelled E1. Since the mutational analysis defined residues 23-26 as being essential for E1 binding and leucine 26 is conserved between all genital HPVs, I was interested in determining if this amino acid was important for the E1-E2 interaction. Therefore an additional point mutation was made within E2 replacing the leucine at position 26 with proline (M15). This time wild-type E2 and E2M15 were expressed as GST fusion proteins. The protein profiles are shown in Fig.8(C). The results for the E1 binding assay are shown in Fig.8(D) and it is clear that while wild type E2 binds E1 strongly, little or no binding is seen with the GST-M15 mutant E2. These data are in agreement

with the results of Hibma *et al.* (1995), and demonstrate that an additional region of the E2 protein is involved in the interaction with E1.

C



D

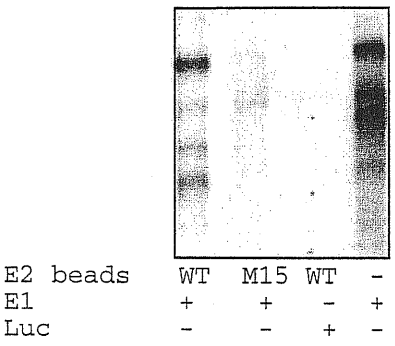


Fig.8(C): coomassie blue stained 12% PAGE of the purified GST-E2, GST-M15 and GST-E1 fusion proteins. (D) Binding of in vitro translated E1 to GST-E2 and GST-M15 fusion proteins. Equal quantities of the GST-E2 and the GST-M15 proteins were tested for binding to 2 µl of the in vitro translated E1 protein. The last lane shows 1 µl of the input E1 in vitro translated protein. Luciferase (Luc) was also included as a negative control on the GST-E2 beads.

9. Modulation of HPV-16E2 transcriptional activity by HPV-16E1

One of the major functions of E2 is the regulation of viral gene expression (Cripe *et al.*, 1987). Recent studies have shown that BPV-E1 can modulate the transcriptional activity of the BPV-1 E2 protein (Le Moal *et al.*, 1994). To determine whether the HPV-16E1 protein possessed a similar activity with respect to the HPV-16E2 protein, a series of CAT assays were performed. The full-length E2 expression plasmid, pJ4Ω16E2 (Lees *et al.*, 1990) together with increasing amounts of the E1 expression plasmid pJ4Ω16E1 (containing nucleotides 864-2809 of HPV-16 cloned into pJ4Ω), were cotransfected with the HPV-16 URR::TKCAT reporter plasmid (Bouvard *et al.*, 1994) into HT1080 cells. The results obtained are shown in Fig.9. Cotransfection of E1 results in a significant increase in HPV-16E2 transcriptional transactivation. This indicate a similar pattern of interaction

between the HPV-16E1 and E2 proteins exists to that reported for the BPV-1 E1 and E2 proteins (Le Moal *et al.*, 1994).

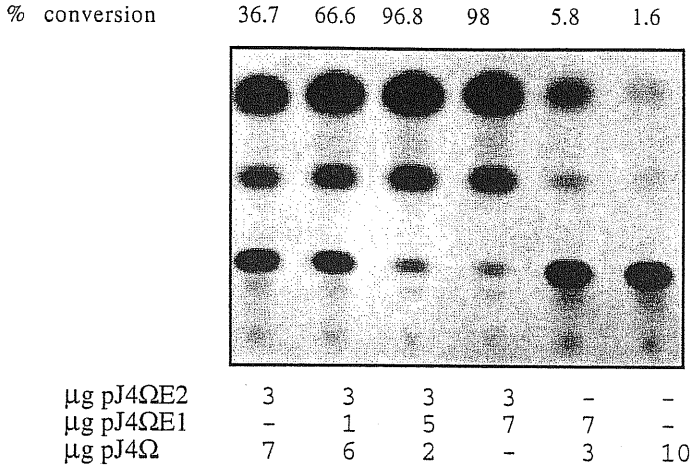


Fig.9: modulation of HPV16-E2 transcriptional activity by HPV-16E1. HT1080 cells were transfected with the reporter plasmid 16URR::TK CAT together with the indicated amounts of control pJ4Ω or E2 expression plasmid pJ4ΩE2, plus increasing amounts of the E1 expression plasmid pJ4ΩE1. After 48h cells were harvested and CAT activity was assayed. Numbers show percentage CAT conversion.

Surprisingly, also HPV-16 E1 routinely showed intrinsic transactivation capacities (Fig.9), although it was much less efficient than E2. This may contribute to the synergistic transactivation by E1 and E2.

Recently, a similar weak transcriptional activation function has been shown for the HPV-18 E1 protein (Demeret et al., 1998). Clearly, the intrinsic E1-dependent transcriptional activity is not fully elucidated and mutational analysis will be required to characterise this function of E1.

10. Transcriptional activation by the HPV-16E2 mutant proteins

Having identified regions of E2 important for binding E1 *in vitro* I next wanted to assess the activities of these proteins *in vivo*. In particular, I was interested in determining whether these E2 mutants would enable me to

identify regions of E2 important for transcriptional activation and for supporting viral DNA replication.

I first assayed the ability of the E2 mutants to activate transcription of the E2 responsive reporter plasmid, HPV-16URR::TK CAT (Bouvard *et al.*, 1994). Human 293 cells were transfected with the different E2 mutants together with the HPV-16URR::TK CAT reporter. After 48 h cells were harvested and CAT activity was measured. Results obtained from a series of assays are shown in Table 10.

Mutant	Activation(-fold)*
WT	2.6 +/- 0.5
M1	1.7 +/- 0.28
M2	1.2 +/- 0.21
M3	1.3 +/- 0.4
M4	1.0 +/- 0.33
M5	2.1 +/- 0.26
M7	1.1 +/- 0.26
M8	1.0 +/- 0
M9	1.1 +/- 0.16
M10	1.3 +/- 0.36
M11	1.9 +/- 0.54
M12	1.1 +/- 0.39
M13	1.2 +/- 0.23
M14	1.3 +/- 0.31
M15	1.4 +/- 0.53

Table 10.
*Numbers represent the mean level of transactivation from at least three independent experiments. Standard deviation is indicated on the right of each activation fold value.

These results indicate that wild-type protein induced a mean activation of the enhancer of approximately 2.6 fold and this is in agreement with previously published results (Bouvard *et al.*, 1994). The mutant E2 proteins display a broad range of activities, from close to wild-type (M5 and M11), intermediate (M1) and defective (M4, M7, M8, M9 and M12). This analysis defines regions of the E2 protein around amino acids 73, 156-159 and 194-197 as being essential for transcriptional activity in human 293 cells.

A large proportion of the E2 mutants, however, are defective. As one possible explanation could be the instability of the mutant E2 proteins

themselves, I performed a series of transient transfections into 293 cells. After 24 h cells were extracted and levels of expression of the mutant E2 proteins were determined by Western blot analysis. The results shown in Fig.10 demonstrate that, in contrast, the mutant E2 proteins are relatively stable with respect to the wild-type protein; only mutant 10 appears to be somewhat weaker. Of the remaining mutants analysed, only M7 and M8 appeared to be completely unstable (data not shown).

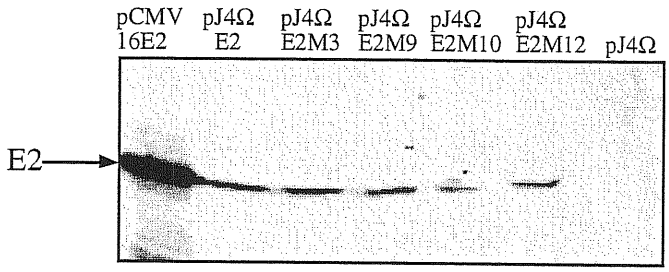


Fig.10: detection of wild-type and mutant HPV-16E2 proteins in 293 cells. Cells were transfected with pJ4Ω containing wild-type and mutant E2 sequences as indicated. After 24 h cells were extracted and E2 protein was detected using the anti-E2 monoclonal antibodies TVG 261 and TVG 271. Positive control cells were transfected with pCMV16E2 and negative control cells were transfected with pJ4Ω plasmid only. The position of the E2 protein is indicated by the arrow.

11. Transient DNA replication assay

I was next interested in assessing E2 mutants ability to support transient viral DNA replication. In order to detect low level replication activities of the mutant E2 proteins, a highly efficient transient replication assay was established.

When the transient replication assay was used for the first time to investigate BPV replication, it was estimated that, with a detection level of 1-10 pg of DNA, one would require 10^3-10^4 cells replicating the origin containing plasmid to detect replication. It was also predicted that replicated viral DNA would be detected transiently only if the transfection efficiency was sufficiently high and therefore cells were transfected using the high efficiency

electroporation procedure (Ustav and Stenlund, 1991). Furthermore, in a recent study (Sakai *et al.*, 1996), the authors transfect cell by electroporation and subsequently PCR amplified the purified replication products in order to increase the detection efficiency.

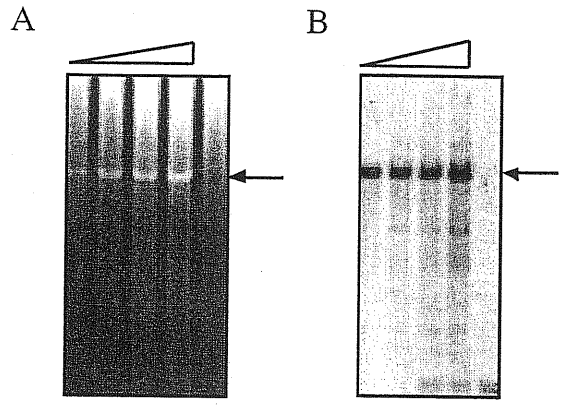


Fig.11: a plasmid containing the HPV-16 URR replicated in 293 cells when cotransfected with increasing amounts (1-7 μg) of protein expression vector pCGE1ΔE5. The last lane of each panel represents the negative control, cells being transfected only with the HPV-16 URR plasmid. The arrow indicates *DpnI* -resistant replication products. (A) Ethidium Bromide-stained agarose gel before blotting on nylon membrane. (B) replication products visualized after hybridization of the membrane with a high specific activity probe.

In the present work, the 293 eukaryotic cell line was transfected by standard calcium phosphate precipitation. Transfection efficiency, as determined by an in situ staining reaction using a β-galactosidase expressing plasmid, typically ranged from 40 to 50%. Three days posttransfection, low-molecular-weight (LMW) DNA was isolated by the Hirt extraction procedure (Hirt, 1967). The samples were first digested with BamHI that cuts the *ori*-containing plasmid at a unique restriction site in order to convert the products of replication into linear molecules of unit length. Then samples were digested with *DpnI* in order to eliminate all molecules that had not undergone at least one round of replication. Full-length linear plasmid DNA was identified by its migration during gel electrophoresis, followed by Southern blotting and hybridization with an *ori*-containing probe.

As can be seen from Fig.11(A) the replication products were detectable already at the stage of DNA fractionation on agarose gel. The DNA products were transferred to a positively charged nylon membrane and probed with a

^{32}P labelled *ori*-containing probe (Fig.11(B)). Replicated viral DNA was routinely detected after 5-10 minutes of membrane exposure. It is clear that this assay is extremely sensitive and has the advantage of not having to rely upon PCR amplification.

12. Replication of an *ori* plasmid is dependent on the relative levels of E1 and E2 proteins

Overexpression of the E2 protein from the pCMV plasmid dramatically affected E2 ability to transactivate gene expression (Bouvard *et al.*, 1994). To determine whether also viral DNA replication was supported more efficiently by a specific level of the E2 protein, I tested three different E2 expression vectors in the transient replication assay.

Typical results are shown in Fig.12 where the arrow indicates *DpnI* undigested replicated DNA; non-replicated input DNA is detected at the bottom of the gel. This analysis demonstrates that, when the E1 protein is expressed from the pCGΔE2 plasmid driven by the CMV promoter and E2 is expressed from the pJ4Ω plasmid driven by the Moloney murine leukaemia virus (MoMLV) LTR, amplification of the replicon takes place. When the E2 protein is expressed from the pCMV plasmid or from the pCDNA3 plasmid, both driven by the CMV promoter, no replicated DNA is detected.

The Western blot shown in Fig.10, indicates that the amount of E2 protein expressed from the latter plasmids is at least one order of magnitude higher than the amount produced by the pJ4Ω-based contructs. These results clearly evidence that overexpression of E2 inhibits plasmid replication and are in agreement with the findings of Svedrup and Khan (1994).

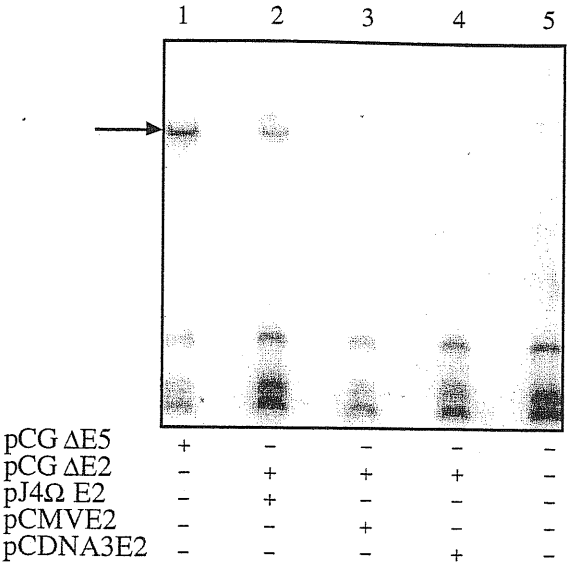


Fig.12: replication of an ori-containing plasmid in the presence of pCGΔE2 and various E2-expressing plasmids (lanes 2- 4) performed in C-33 I cell line. The expression vector pJ4ΩE2 contains the MoMLV promoter; the expression vectors pCMV and pCDNA3 contain the human cytomegalovirus immediate early promoter. Lanes 1 and 5 are positive (pCGΔE5 plasmid expressing both E1 and E2) and negative control (*ori* plasmid only) respectively.

13. Identification of the regions of the E2 protein necessary for transient viral DNA replication

In order to assess the activity of the different E2 mutants in the *in vivo* DNA replication assay, I cotransfected 293 cells with E1 and E2 expression plasmids, along with the HPV-16 origin-containing plasmid 16URR::CAT. The results from a typical assay are shown in Fig.13(A). The averaged results from multiple assays are shown in Fig.13(B).

The mutant E2 proteins display a variety of phenotypes with respect to their ability to stimulate viral DNA replication. It is clear from this analysis that the integrity of several regions of the E2 protein, as exemplified by the mutants M2(Trp-Pro33), M3(Arg-Pro47), M4(Ile-Pro73), M9(Δ156-159), M11(Val-Pro186), and M13(Δ23-26) are absolutely essential for efficient viral DNA replication. The results with M4(Ile-Pro73) are in agreement with previous studies (Sakai *et al.*, 1996) and results with the deletion mutants

encompassing aa 23-26 and 156-159 are not surprising since they are also defective in their ability to bind E1 (Piccini *et al.*, 1995). However, M11 (Val-Pro186) is particularly interesting since it retains wild-type levels of binding to E1 and close to wild-type levels of transcriptional activity. These results suggest that HPV-16E2 protein might have a role in viral DNA replication other than simply targeting E1 to the origin of replication. The M12 deletion mutant (Δ194-197) replicates the HPV-16 *ori* more efficiently as the wild-type E2 protein, yet it is defective in its ability to activate transcription. Interestingly, M12 exhibited a very high replication activity in the particular assay shown in Fig. 13(A), however multiple repeats of the replication experiment consistently indicated an activity close to wild type or slightly higher, as is shown if Fig.13(B). At present, the significance of this observation is still under investigation. A summary of the activities of the mutant E2 proteins with respect to their ability to bind to the HPV-16 E1 protein, stimulate HPV-16 enhancer activity and stimulate transient viral DNA replication is shown in Fig.13(C).

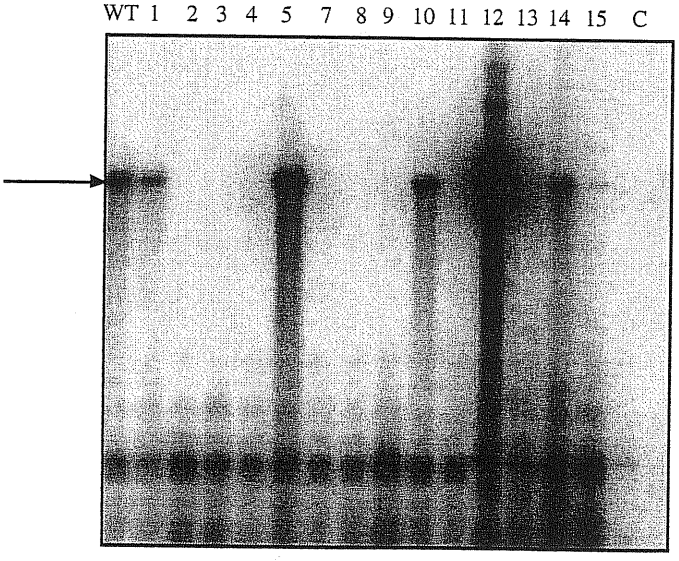


Fig.13(A): identification of regions of the E2 protein necessary for transient viral DNA replication. 293 cells were transfected with an HPV-16 *ori*-containing plasmid together with E1 and the indicated E2 expression plasmids (number indicates the correspondent mutant). After 72 h cells were extracted, DNA was digested with *DpnI* and replicated DNA was detected following Southern blot hybridization. The arrow indicates the replication products.

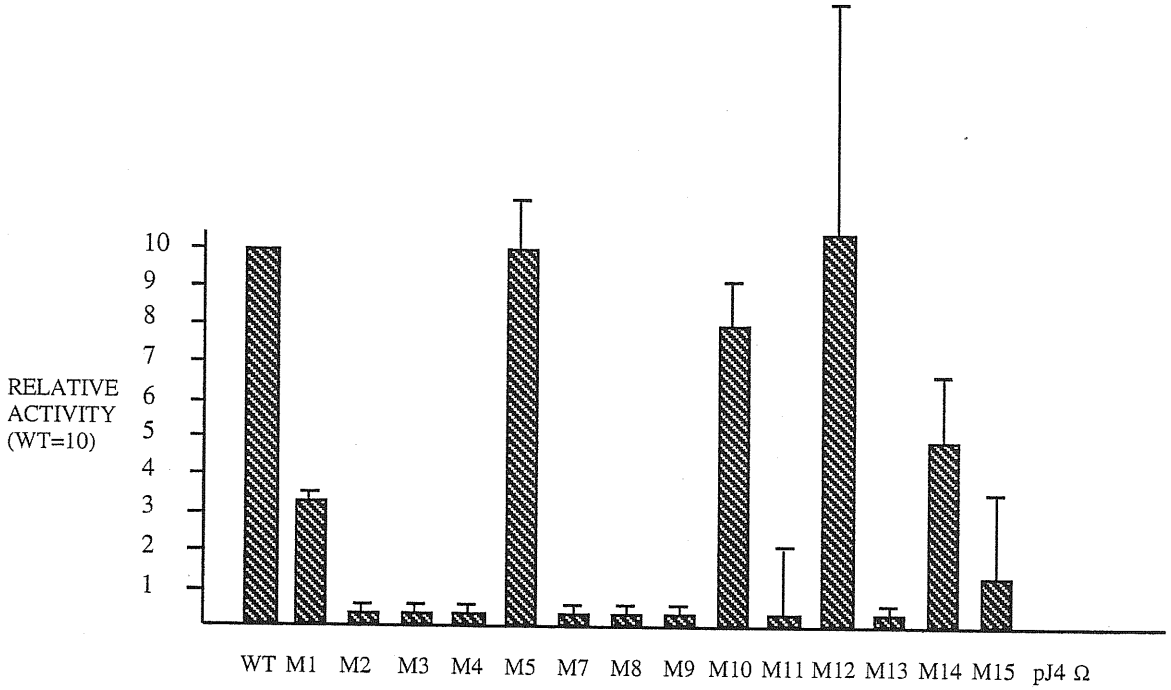


Fig.13(B): relative activity of the mutant E2 proteins with respect to wild-type E2 in supporting transient viral DNA replication. Replication assays were performed as described and the replicated products quantified by PhosphorImager analysis. The numbers represent relative activity of the mutant E2 proteins measured from at least three independent replication assays.

Mutant	E1 binding	DNA replication	Enhancer activation
WT	+++	+++	+++
M1	++	++	++
M2	ND	-	+/-
M3	+	-	+/-
M4	++	-	-
M5	+++	+++	+++
M7	-	-	-
M8	-	-	-
M9	-	-	-
M10	+++	+++	+/-
M11	+++	-	++
M12	+++	++++	-
M13	-	-	+/-
M14	+++	++	+/-
M15	-	+	+/-

Fig.13(C): summary of the relative ability of the different E2 mutants to bind to the HPV-16 E1 protein, stimulate HPV-16 enhancer activity and stimulate *ori*-dependent transient DNA replication.

14. The role of cellular transcription factors in HPV DNA replication

A general feature of hitherto characterized eukaryotic replicons is a link between replication and transcription such that transcription factors binding sites form a part of the *cis*-acting elements (*ori*) required for initiation of replication *in vivo* (DePhamphilis, 1993). A variety of cellular transcription factors, including NF-1, AP1, KRF-1, Oct-1, Sp1, and the glucocorticoid receptor, have been shown to bind to the HPV-18 and HPV-16 URRs and to participate in the regulation of the E6/E7 promoter located at the 3' terminus of the URR in addition to the viral E2 protein (Gloss *et al.*, 1989; Butz and Hoppe-Seyler, 1993). Therefore, the overlapping of the viral origin of replication with promoter elements of early transcription suggested a possible cooperativity or interference between these different elements and HPV DNA replication. A similar organization of core origin with auxiliary elments has been described in other papovaviruses, like SV40 or polyomavirus.

It is known that the activity of a given regulatory element can strongly depend on the overall composition of control region, i.e., on the nature of potentially cooperating *cis* motifs. Furthermore, the proper spacing between different *cis*-regulatory elements can be highly important for their functional cooperation (Butz and Hoppe-Seyler, 1993).

In view of these findings, the functional significance of individual *cis*-regulatory elements defined by the specific transcriptional control elements within the HPV URR was investigated. I made use of a series of HPV-18URR constructs which have been previously described (Butz and Hoppe-Seyler, 1993). Each single element in the HPV-18URR schematically shown in Fig.14(A) was inactivated by site-directed mutagenesis.

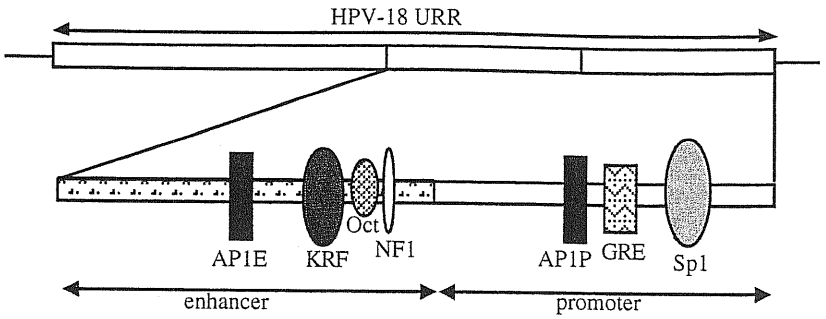


Fig.14(A): symbolic representation of the factors binding to the HPV-18URR enhancer (stippled area) and promoter proximal region (open area).

The mutated origins were tested for their ability to replicate in 293 cells in a transient replication assay. To obtain quantitatively comparable results I cotransfected a reference plasmid harbouring an intact HPV16 URR (reference origin), along with the different mutated HPV-18URR (test origin). The reference plasmid contains an origin and is of a different size, therefore enabling easy discrimination between the test and the reference *oris*. In this assay, the mutants and reference plasmid compete for cellular and viral replication proteins. The replication efficiency of each mutant (test) plasmid can therefore be calculated as a ratio of the amount of replication of the mutant template to that of the reference plasmid.

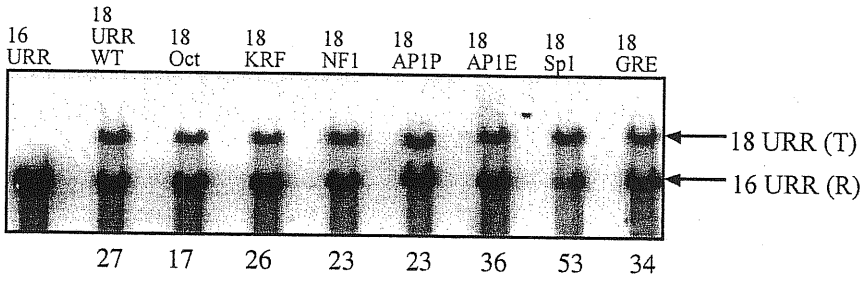


Fig.14(B): replication activity of a replicon containig a HPV-18 URR fragment mutated in the indicated cellular factor binding site was measured in a quantitative replication assay. Test plasmids (T) were cotransfected with reference plasmid (R). Numbers below each lane indicate relative replication efficiency (reference =100).

The results obtained are shown in Fig.14(B). Stimulation of DNA replication by the auxiliary elements of SV40 and polyomavirus is up to 100- and 1000- fold respectively (Guo *et al.*, 1991; Muller *et al.*, 1988), in contrast,

none of the mutants tested significantly affected viral DNA replication. Therefore, relative to the cellular factors considered, HPV18 replication is not dependent on auxiliary elements. The maximum modulation is a 2 fold stimulation in the case of the Sp1 mutated origin, thus confirming the crucial role of the E2 transcription factor in viral replication.

In a previous study performed on the HPV-18 URR mutated in the Sp1 binding site, a weak negative effect of Sp1 mutation was obtained on a viral DNA replication assay (Demeret *et al.*, 1995). In contrast, I routinely observed modest stimulation of viral DNA replication following mutation of the Sp1 binding site. This result was not affected by the relative amount of reference origin transfected (Fig.14(C)) as it was reported by Demeret *et al.* (1995).

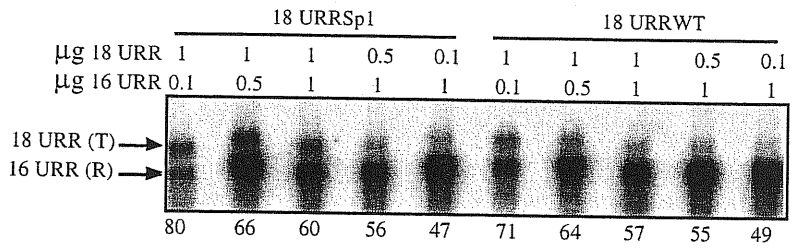


Fig 14(C): replication activity of a plasmid containing a HPV-18 URR fragment mutated in the Sp1 binding site does not susbstantially change at various test/reference concentrations compared to the wild type HPV-18 URR .

However, since the mutations introduced in the Sp1 binding site in the two studies are different, it is difficult compare the two reports.

Based on these data it seems most likely that E2 bound to the core origin, in addition to its interaction with E1, is also able to supply a function similar to that brought about by transcritpion factors bound to auxiliary elements in the SV40 and polyomavirus origin of DNA replication.

15. Effects of glucocorticoid hormone upon viral DNA replication

The HPV-16 URR contains a consensus glucocorticoid responsive element (GRE) located about 300 bases upstream of the P_{97} promoter and transcription of the viral early region can be upregulated by glucocorticoids (Gloss *et al.*, 1987). It was therefore interesting to determine whether this regulator of viral gene expression may also regulate viral DNA replication in the presence of glucocorticoid hormone. To do this, cells were transfected with a wild type *ori*-containing plasmid or an *ori* plasmid containing a deletion in the GRE. Dexamethasone was added to the culture and 72 h later cells were harvested and the level of HPV DNA replication was measured.

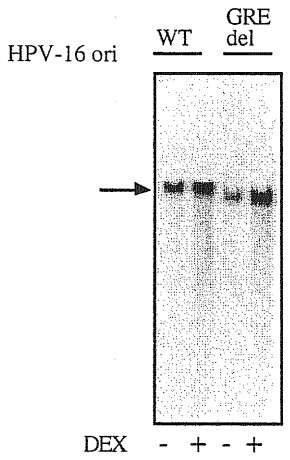


Fig.15. An HPV-16URR containing plasmid as well as an HPV-16 URR plasmid containing a deletion of the GRE element (GRE del) were tested for their ability to support transient DNA replication. The assay was performed in the presence (+) or in the absence (-) of the glucocorticoid dexamethasone.

It is clear that addition of dexamethasone results in a stimulation of viral DNA replication compared with cells lacking hormone. However, a similar level of stimulation is also observed in the GRE deleted *ori* plasmid, indicating that this stimulation is not a consequence of the glucocorticoid receptor (GR) binding to the HPV DNA. In addition, to determine whether the glucocorticoid element located in the URR was responsible for this effect, an HPV-16 *ori*

replicon was generated, with a deletion in the GRE element. The results obtained are shown in Fig.15. It is clear that a deletion of the GRE element in the HPV-16 URR does not abolish dexamethasone mediated upregulation of transient replication. Rather this result likely indicates that the glucocorticoid is having an effect upon the cellular DNA replication machinery. Nonetheless, this result has important implications for the effects of glucocorticoids upon viral replication *in vivo*.

Discussion

From *in vivo* replication studies, two viral proteins have been found to be absolutely essential for DNA replication, the early proteins E1 and E2 (Ustav and Stenlund, 1991). They are sequence-specific DNA binding proteins, and binding sites for E1 and E2 are found in the *ori* sequences conserved among different papillomavirus genomes. In general, the properties of the E1 and E2 proteins have been studied most extensively for BPV-1.

The results presented in this study provide a great deal of information both about the HPV-16E2 protein and about the interaction between HPV-16E1 and E2. The human papillomavirus type 16 was chosen because it is a *high risk* type that is frequently associated with progression to malignancy in the human cervix (zur Hausen, 1991). In addition, when this analysis started, little was known about the HPV-16E1-E2 interaction, and that was mainly based on analogies with the interaction between BPV-1 E1 and E2.

Early studies on the mechanism of action of the HPV-16 and HPV-18 E2 transcriptional regulators suggested that they were substantially different from the related BPV-1 E2 protein. The full-length BPV-1 E2 protein was known to up-regulate viral gene expression, in contrast, the full-length HPV-18 and HPV-16 E2 protein could repress transcription in the same system. This led to the hypothesis that the function of the E2 protein was not conserved among different papillomavirus genotypes. Not surprisingly, this difference in transcriptional regulation by the HPV-16 and HPV-18 E2 produced excitement in the field. The E2 ORF is often deleted in late stage tumors and therefore loss of E2 could result in the deregulation of E6 and E7 gene expression contributing to viral induced tumorigenesis. However, in 1994, it was

separately reported by two groups (Bouvard *et al.*, 1994; Ushikai *et al.*, 1994) that HPV-16 and HPV-18 E2 can act as potent transcriptional activators of the viral promoter in normal keratinocytes when not overexpressed. These studies suggest that they are indeed more similar to the BPV-1 E2 protein. The consensus now seeming to be that overexpression of HPVE2 results in transcriptional repression, whereas lower levels give rise to transcription activation. It remains to be determined which of these activities of E2 are relevant for the viral lifecycle and/or viral tumorigenesis.

One extra relevant issue appeared to me from these new studies: that structural differences between the BPV-1 E2 and the human E2 proteins exist as in a transcriptional assay where the HPV-16URR is placed in promoter configuration, HPV-16 E2 activated gene expression, but BPV-1 E2 strongly repressed gene expression from the human URR (Bouvard *et al.*, 1994).

It has also been shown that the BPV E1 and HPV-11 E2 combination does not function for replication of a BPV minimal *ori*, in spite of a high degree of conservation between the BPV and HPV E2 proteins (Sedman and Stenlund, 1995). The HPV-11E2 protein could interact with BPV-1 E1 in the transient replication assay only if the distance between the E1 an E2 bindig sites on the *ori* was increased. The authors' most likely explanation for the inability of HPV-11 E2 to interact with E1 on the BPV-1 ori was because of sterical constraints. These reports again suggest that the bovine and the human E2s are not always structurally compatible. Furthermore, the position of the E2 binding sites relative to the E1 binding site in the HPV *ori*, is different compared to the BPV *ori*. This provides additional support for the idea that differences between the modes of interaction of the BPV and HPV E1 and E2 proteins on the respective *ori* exist. Very recently, a direct comparison of the binding of the E2 proteins from BPV-1 and HPV-16 to a series of binding

sites, as a function of the sequence of their central spacer was performed (Hines *et al.*, 1998). The BPV-1 E2 was moderately sensitive to the nature of the central spacer, in contrast, the HPV-16 E2 binds to sites containing A:T rich central spacers with significantly increased affinity. The mechanism of DNA-sequence specific binding of the homologous HPV-16 E2 and BPV-1 E2 proteins is therefore different.

Based on these criteria, it seemed important to characterize and map the interaction between the HPV-16 E1 and E2 proteins as speculations based on analogy between the BPV-1 E2 with the HPV E2 would necessarily suffer from limitations.

1. Purification of active GST-HPV-16 E1 fusion protein

The HPV-16E1 protein derived from a low grade cervical lesion (Stanley *et al.*, 1989), was expressed and purified from *E.coli* as a GST-fusion protein. The purified GST-HPV-16E1 retained ATPase activity and was phosphorylated by CKII. These results demonstrated that, at least a proportion of the purified GST-16E1 fusion protein was properly folded and biologically active. One of my objectives, in initiating this study, was to measure the helicase activity of the purified GST-HPV-16E1 protein, as the availability of such an assay would be invaluable in the search for antiviral compounds. This expectation was based on results from other laboratories, indicating that the E1 proteins from BPV-1 and HPV-6b contained helicase activity. To convincingly show that GST-HPV-16E1 is a helicase, extensive purification was required, since even minute contamination by *E.coli* proteins would preclude definitive assignement of the activity to the over-expressed protein. In the present analysis, no helicase activity encoded within purified GST-HPV-16E1 was

found. A resonable explanation for this lack of activity could be due to a steric effect of the GST fusion, as in most cases (i.e. SV40 Tag) formation of a specific multimeric form of the protein is required for the helicase activity. Unfortunately, I could not produce sufficient mature E1 from GST-E1 using thrombin cleavage to test this hypothesis. However, an alternative explanation is simply the very low intrinsic helicase activity of the HPV-16 E1 protein. It was recently observed that the HPV-11 E1 helicase activity is very poor compared to other known helicases and hardly detectable (Phelps, unpublished observations). The sum of these observations helps to clarify why monitoring of HPV-16E1 helicase activity is difficult and has not been yet reported.

Taken together these results suggest major differences in the levels of helicase activities exhibited by the HPV and BPV E1 proteins. This further supports the notion that BPV-1 E1 is not a good model for HPV-E1.

2. Binding of HPV-16E2 to GST-HPV-16E1 protein

Having purified functionally active HPV16-E1 protein, its ability to associate with E2 *in vitro* was demonstrated using a GST pull-down assay. These studies showed that complex formation between GST-HPV16-E1 bound on glutathione beads and *in vitro* translated E2 could readily be obtained. The experimental conditions allowing for optimal recovery of E2 were therefore established.

To determine whether the *in vitro* translated E2 protein was functionally active following association with E1, a modified pull-down assay was developed. The *in vitro* translated HPV-16E2 protein was found to be able to interact with E1 while binding to an oligonucleotide containing the E2

recognition sequence. These results are therefore consistent with the presence of a properly assembled E1/E2 complex.

At this stage, the GST pull-down assay seemed a suitable approach to start dissecting the E1-E2 interaction.

3. Binding of E2 heteromeric complexes to GST-HPV-16E1

A differentially spliced form of E2 was isolated from the W12 cell line (Doorbar *et al.*, 1990). This protein derives few amino acids from the E1 region and the remainder consists of the C-terminal half of the E2 protein encoding the DNA binding and dimerization domains. This is similar to the E8^E2 repressor protein identified in BPV (Lambert *et al.*, 1987) and the E2C in HPV-11 (Chiang *et al.*, 1992a) and it is able to progressively abolish full-length E2-mediated transactivation (Bouvard *et al.*, 1994). The respective levels of the two forms of E2 have been reported to be critical in controlling viral gene expression of HPV-11, HPV-16 and BPV-1, as all E2 proteins devoid of the trans-activating domain function as binding-site-specific transcription repressors (Bouvard *et al.*, 1994; Chiang *et al.*, 1991; Choe *et al.*, 1989; Cripe *et al.*, 1987). In addition, modulation of HPV-11 transient DNA replication occurs both in *vivo* and *in vitro* when HPV-11 E2 repressor is added in the respective assay (Chiang *et al.*, 1992a; Liu *et al.*, 1995) and the role of the full-length E2 in transient replication cannot be fulfilled by the E2 protein devoid of the transactivation domain, rather it inhibited replication.

One hypothesis was that the two forms of E2 were modulating each other through dimerization. Upon *in vitro* transcription and translation of full-length HPV-16E2, an E2 form was generated that specifically retarded a labelled oligonucleotide containing the two E2 recognition sequences placed

immediately upstream of the P_{97} promoter on the HPV-16 URR. The full-length E2 protein-oligonucleotide complex migrated as a single discrete band, suggesting that only one configuration of full-length E2 was binding to the oligonucleotide, possibly as two dimers. In contrast, the C-terminal E2 oligonucleotide complex migrated faster and was composed of at least three different complexes. Upon co-translating both full-length E2 and the C-terminal form, heteromeric complexes of the different forms of E2 were produced, based on the appearance of a new complex which migrated with relative mobility between that of the full-length and the short E2 complexes.

A possible mechanism of inhibition of DNA replication by E2Ct may be by inhibiting the association of E1 and E2. I therefore investigated whether the heteromeric complexes of full-length E2 and E2Ct could bind E1 in a GST pull-down assay. Not surprisingly, E2Ct alone failed to bind to E1. In contrast, a small amount of E2Ct was able to associate with the GST-HPV-16E1 protein when in the presence of the full-length E2. This was most probably due to the formation of heteromeric complexes between the full-length and E2Ct proteins (Bouvard *et al.*, 1994) through the C-terminal dimerization domain, and demonstrates that full-lenght E2 and E2Ct heteromers can still associate with E1 *in vitro*.

In vitro translated BPV-1 E2 proteins form stable dimers (McBride, 1989b), therefore, the obvious implication of this result would be that only one molecule of E2, within the dimer, is sufficient to recognize E1. These data are in agreement with the recent determination of the stoichiometry of E1/E2 binding (Chen and Stenlund, 1998). BPV-1 E2 was able to predominantly bind two E1 monomers at the BPV-1 *ori*. A potential caveat associated with the GST pull-down experiment would be if *in vitro* translated E2 could exist also in stable multimeric forms in solution.

4. E1 modulates E2-dependent transcriptional activity

Having established that an interaction between the E1 and E2 proteins takes place *in vitro* and knowing that one of the consequences of the BPV-1 E1/E2 interaction is an increase in the specificity of E1 binding to the *ori* (Sedman and Stenlund, 1995), a correspondent alteration of the properties of E2 in the E1-E2 complex was hypothesized. In addition to its role in replication, E2 is a transcriptional regulatory protein, therefore the effects of the E1-E2 interaction upon E2 function can be examined by assessing E2-mediated activation of transcription in the presence or absence of the E1 protein.

It was previously shown that BPV-1 E1 could potentiate E2 mediated transactivation of a reporter plasmid containing an E1 binding site and a weak E2 binding site (Le Moal *et al.*, 1994). In the present work the HPV-16E1 and E2 proteins, when coexpressed, synergistically increased E2 mediated transactivation, whilst E1 alone could also activate the same reporter plasmid, but to a much lesser extent. These data are consistent with a mechanism involving cooperative binding of HPV-16E1 and HPV-16E2 to adjacent binding sites on the HPV-16 URR. These studies are further supported by the recent observations that HPV-18 E1 exhibits a low intrinsic transactivation activity and that synergistic activation of transcription between the HPV-18E1 and E2 proteins is detected (Demeret *et al.*, 1998).

5. Identification of the regions of E2 involved in binding to E1

In order to identify regions of E2 involved in binding to E1, mutations within the E2 protein were previously introduced into domains highly conserved amongst different papillomavirus types. The reasoning being that

the E1-E2 interaction was likely to be highly conserved amongst different HPV types. These mutations were then assessed for binding to the GST-16E1 fusion protein.

Two widely spaced regions within the N-terminal transactivation domain of E2 were found to be important for binding to E1. A stretch of aminoacids between residues 156 to 159 was essential for complex formation between E1 and E2. In addition, both a point mutation at amino acid 26, where a leucine is changed to a proline, and a deletion of amino acids 23 to 26 completely abolished the E1-E2 protein complex. The binding between amino acids 23 to 26 seemed more extensive since reduced E1 binding was also obtained with the point mutations of arginine to proline at position 47 and isoleucine to proline at position 73.

These results are in agreement with Hibma *et al.* (1995), which investigated the HPV-16 E1-E2 interaction using monoclonal antibodies against E2 and with two other recent studies (Sakai *et al.*, 1996; Yasugi *et al.*, 1997). In the case of Hibma *et al.*, monoclonal antibodies binding to residues 18-41 were found to block association of E1 with E2, indicating the involvement of this region of E2 in binding to E1. All reports indicate that mutations within the N-terminal activation domain of HPV-16E2 are deleterious for the E1-E2 association, but only in the present study and in the one of Sakai *et al.*, the aminoacids responsible for the binding were identified. A direct comparison with the present analysis is difficult, due to the substantially different nature of the mutations introduced and because mutations are not overlapping. However the important regions of E2 identified to be important for E1 binding are very similar. A substitution with an alanine at the level of amino acid 39 abolished the E1-E2 binding and thus confirmed that the region between amino acid 23 to 73 was required for efficient binding. Two additional point mutations at

amino acid 92 and 122, were found to affect E1 complex formation by Sakai *et al.* In contrast, the results reported in this study argue against the importance of these two regions. A substitution with a proline at amino acid 93 and a double substitution with an alanine at amino acids 100 and 102 did not decrease E2 efficiency to bind to E1.

6. The transactivation function of the E2 mutant proteins

When this study began, there was much speculation regarding the role that E2 plays in papillomavirus DNA replication. One proposal was that the full-length E2 protein could bind viral DNA and recruit replication factors to the origin. Alternatively, E2 could indirectly affect replication by enhancing transcription of either viral or cellular genes necessary for replication. However, when the BPV-1 E1 and E2 proteins were expressed from heterologous promoters on separate expression vectors, E2 was still absolutely required for DNA replication (Ustav and Stenlund, 1991), in addition, BPV-1 DNA replication occurred in cell-free extracts indicating that E2 mediated transactivation of cellular proteins is not required (Yang *et al.*,1991).

To determine whether the transcriptional activation function of the full-length HPV-16 E2 protein could be separated from its replication function, the E2 mutants were firstly assayed for their ability to transactivate the HPV-16 enhancer *in vivo*.

As it was possible that the mutations introduced influenced the secondary structure of the E2 protein altering its stability, the expression level of each of the mutants was assessed. Only two mutants, M7 (Pro26/Pro73) and M8 (Pro42/Pro73) failed to make a protein detectable by immunoblotting, in accordance with their inability to bind to the E1 protein, to transactivate gene

expression and to stimulate DNA replication. The other mutated E2 proteins were of the expected molecular weight and their stability was similar to that seen with wild-type E2, ruling out lack of activity due to lack of expression. As a positive control for the above mentioned Western blot analysis, the pCMV plasmid was included. This expression vector is based on the human cytomegalovirus immediate early promoter and gave strikingly higher levels of E2 expression than the murine LTR promoter in plasmid pJ4Ω. It has previously been reported that high HPV-16E2 expression repressed the HPV-16 promoter in a sequence independent fashion (Bouvard *et al.*, 1994), reminiscent of squelching (Ptashne, 1988), and these expression studies further support that hypothesis.

The results obtained from the transcriptional assays are in broad agreement with previously published observations in that several regions of the N-terminal domain of E2 appear to be essential for transcriptional activity (Sakai *et al.*, 1996). Only mutants M5(Pro94) and M11(Pro186) exhibited levels of transcriptional activity approaching that of the wild-type protein. Mutants M4(Pro73), M9(del156-159) and M12(del194-197) were defective in transcription activation and only reduced activity was seen with M1(Pro7), M2(Pro33), M3(Pro47), M13(del23-26), M14(Ala100/102) and M15(Pro26). These results demonstrate that, in contrast to the interaction with E1, transcriptional transactivation by E2 is very susceptible to mutational inactivation. This supports the notion that multiple regions of the E2 protein may be necessary for transcriptional activation (Breiding *et al.*, 1997)). This is not surprising since many transcription factors have been reported to interact with multiple components of the transcriptional machinery and a prediction from these studies is that the same is true for E2.

At this stage it was difficult to compare this study with earlier analysis based on large deletions of BPV-1 E2, which are more likely to strongly alter protein conformation (Winokur and McBride, 1992).

7. Identification of regions of E2 important for supporting transient DNA replication

The degree to which the mutant E2 proteins could support transient viral DNA replication was next investigated. The present results are consistent with the work of Sakai *et al.*, demonstrating that E2 protein ability to activate transcription is separable from stimulation of viral DNA replication, although additional regions affecting viral DNA replication were found.

The importance of the N-terminal domain of E2 in both DNA replication and transcriptional activation is highlighted by mutants M2(Trp-Pro33), M3(Arg-Pro47), M4(Ile-Pro73) and M13(Δ23-26) which are completely negative for DNA replication and are weak or negative transcriptional activators. Similar mutations to these have been reported previously (Sakai *et al.*, 1996), but some differences exist. The mutations described here are likely to be more disruptive due to insertion of Pro as opposed to Ala in the study by Sakai *et al.* In addition, the assay described in Sakai *et al.*, required PCR amplification to detect the replicated products. In this study, the signal was very efficiently detected after low molecular weight DNA extraction without enrichment, and therefore the data are not altered by PCR cycles.

As can be seen from the comparison in Table 7, mutant M2(Trp-Pro33) is in agreement, mutant M3(Arg-Pro47) is largely defective in this study but the equivalent Arg47Ala mutant exhibits wild-type levels of transcriptional activity and only reduced levels of DNA replication activity. Finally, mutant

M4(Ile-Pro73) is again largely defective in this study but the equivalent Ile73Ala mutant has wild-type DNA replication activity and is defective in the transcriptional assay. The conclusion which can be drawn is that this extreme N-terminal region of the transactivation domain of E2 is critical to both DNA replication and transcriptional activation and, depending upon the severity of the mutation introduced, permits only a partial separation of the transcriptional and DNA replication activities of the E2 protein. It was shown that mutant E2 proteins whithin the region encompassing amino acid residues 26-73 have reduced E1 binding activity (Piccini *et al.*, 1995) and therefore the reduced ability to support DNA replication is not surprising.

P	S	P	S	P	S	P	S
Mutant		E1 binding		DNA replication		Transactivation	
WT	WT	+++	+++	+++	+++	+++	+++
	E2A		+++		+		+
M1(R7P)		+++		++		++	
	D13A		++		++		+++
M15(L26P) | M13(del23-26)		-		+ | -		+/- | +/-	
M2(W33P)	W33A	ND	+	-	+	+/-	+
	R37A		+++		+		-
	E39A		-		-		+++
M3(R47P)	R47A	+	+++	-	++	+/-	+++
	K68A		+++		++		++
M4(I73P)	I73A	++	+++	-	+++	-	-
	L79A		++		+++		+++
	E90A		+++		+++		+++
	W92A		+		+		-
M5(L94P)	T93A	+++	+++	+++	++	+++	+++
M14(E100A/Y102A)	E100A	+++	+++	++	++	+/-	+++
	Y158A		+++		++		+++
	Y167A		++		+++		+++
	D174A		+++		+++		+++
	K177A		++		+++		+++
	Y178A		+		+		
M9(del156-159)		-		-		-	
M10(D174P)		+++		+++		+/-	
M11(V186P)		+++		-		++	
M12(del194-197)		+++		++++		-	
M7(L26P/I73P)		-		-		-	
M8(I42P/I73P)		-		-		-	

Table 7: P indicates data from Piccini *et al.*, 1997; S indicates data from Sakai *et al.*, 1996.

Interestingly, mutant M4(Ile-Pro73) had wild-type E1 binding activity, but nonetheless failed to stimulate transient DNA replication. In general, enhancer proteins that act as auxiliary factors in DNA replication, directly contact replication factors to stabilize the preinitiation complex. The result obtained with mutant M4 supports the notion that interaction with E1 is not the only critical activity provided by E2 for DNA replication. E2 has been reported to physically interact *in vitro* with RPA (Li and Botchan, 1993) and with cellular factors necessary for transcriptional activation (Breidin *et al.*, 1997), therefore the mutation at the level of amino acid 73 might disturb a molecular interaction different from the association with E1.

The GST pull-down approach used to monitor the formation of the E1-E2 complex cannot rule out an E2 defect in tethering E1 to the *ori*; however, computer prediction analysis but also studies based on chimeric E2 proteins have demonstrated that E2 is a modular protein, containing an activation domain clearly separated from the DNA binding domain by a hinge region (Giri and Yaniv, 1988; Li and Botchan, 1993; Berg and Stenlund, 1997). Chimeric E2 proteins, in addition, have always remained functionally active for DNA replication. All the mutations introduced in the E2 protein in this analysis map to the transactivation domain and are believed not to interfere with E2 ability to bind DNA. Obviously in *vivo* biochemical analysis is required to confirm this supposition.

The novel mutants in this analysis have mutations which fall in the latter half of the transactivation domain of the protein. Of these, the most interesting are represented by mutants M11(Val-Pro186), M12(Δ194-197) and M14(Glu-Ala100/Tyr-Ala102). Firstly, the Pro186 mutation defines an additional region of the E2 protein which is essential for DNA replication, although further

mutational analysis will be required to determine the extent of this region. This mutant has wild-type levels of E1 binding activity and almost wild-type levels of transcriptional activity.

In contrast, mutant 14 displays wild-type levels of DNA replication and E1 binding activity, yet has weak transcriptional activity. Therefore, both the mutations at Ala100/102 and Pro186 allow the transcriptional activity of E2 to be separated from its DNA replication activity. This conclusion is further supported by the 194-197 deletion since this mutant displays extremely efficient activity in the replication assay but it is defective in the transcriptional assay.

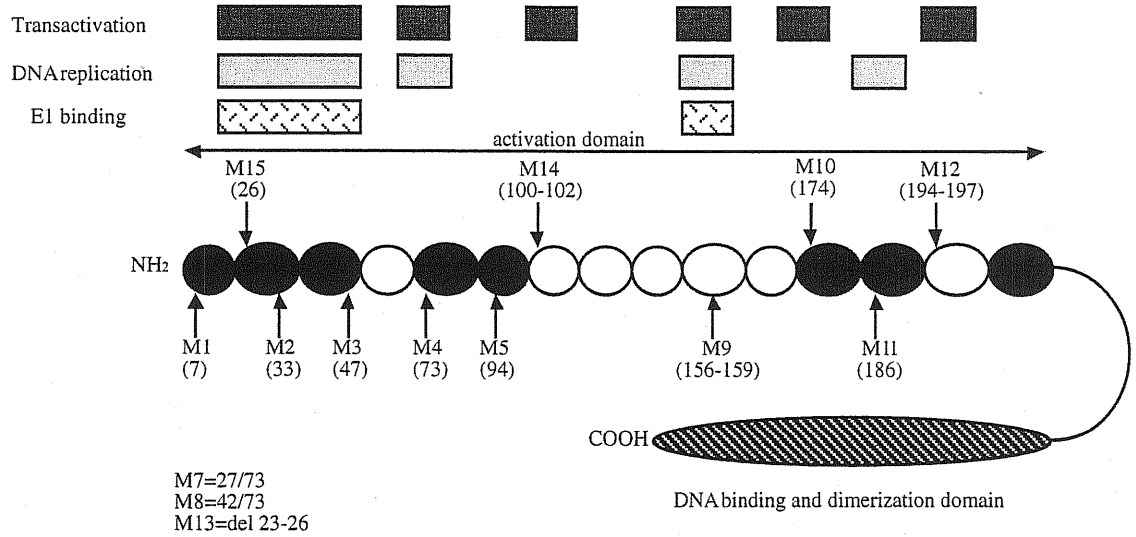


Fig.16: summary of the relative ability of the different E2 mutants to bind to the HPV-16E1 protein, stimulate HPV-16 enhancer activity and stimulate HPV-16 DNA replication.

8. Effect of dexamethasone upon transient DNA replication

It has been suggested that, in addition to HPVs, other agents such as hormones act as cofactors in cervical neoplasia (Pet and zur Hausen, 1986). Gloss *et al.* (1987) identified a potential glucocorticoid receptor-binding

element (GRE) in the HPV-16 genome and glucocorticoid-dependent regulation of viral gene expression was later demonstrated by several groups (Pater *et al.*, 1988; Lees, *et al.*, 1990; Cantley *et al.*, 1991). Since the *ori*-plasmid used in this study contains the GRE, the levels of DNA replication in 293 cells was assessed following addition of the synthetic glucocorticoid dexamethasone. These experiments demonstrated an increase in the level of viral DNA replication in the presence of dexamethasone. Surprisingly this effect was still present when the GRE was deleted from the ori plasmid, indicating that the effects of GR was not due to binding on the HPVURR.

This study raised some questions about the possible modulation of the E1 and E2 expression by glucocorticoids, however no evidence for such a possibilty was found in previous studies. Cellular growth was not significantly affected by addition of dexamethasone. Therefore, the much likely explanation for these results is that dexamethasone is affecting a component of the cellular DNA replication machinery which in turn indirectly results in an increase in viral replication. Obviously, extensive additional studies would need to be performed to identify the precise nature of this regulation.

9. Modulation of HPV-18 DNA replication by cellular transcription factors

Transcription factors can positively or negatively influence transcription by binding on regulatory elements on the DNA, however, it has become clear that several transcription factors are multifunctional and also directly influence initiation of DNA replication. Most, if not all, of the eukaryotic origins of DNA replication characterized so far consist of two principal components: the core origin and auxiliary regions that stimulate replication (Guo and

DePhamphilis, 1992). These auxiliary regions contain transcription factor binding sites. In the case of the closely related polyomavirus group, presence of the transcriptional enhancer stimulated DNA replication up to 1000-fold, and presence of the enhancer increased SV40 DNA replication approximately 100-fold (van der Vliet, 1996). Based on these observations and on the presence of several cellular transcription factor binding sites on the papillomavirus URR, it seemed reasonable to investigate whether this potential modulation is realized also in the papillomavirus group, and whether other proteins, in addition to the viral factor E2 are required for optimal viral DNA replication. The approach to study the effect of different mutations in the URR is based on cotransfection of two *ori*-plasmids into the same cell, a reference plasmid and a test plasmid. In this type of assay, the test and the reference plasmid compete for the cellular replication machinery. Replication efficiency of each mutant (test) plasmid can be calculated as a ratio of the amount of replication of the mutant template to that of the reference template. The presence of an internal control in each sample renders the replication assay quantitative and independent of experimental variations.

A systematic analysis of the *cis*-regulatory elements in the physiological contex of the HPV-18 URR, failed to identify mutations which can substantially affect transient viral DNA replication, only minor differences (2-fold) were detected. This would be in contrast to the auxiliary transcription factors required for replication of other papovaviruses. The present findings, however, do not rule out modulation by another, yet uncharacterized cellular factor or cooperation amongst multiple cellular factors at the initiation stage.

An earlier *in vivo* replication study indicated that an Sp1 binding site, important for early transcription plays a role also in replication (Demeret *et al.*, 1995). A similar modest difference was detected in the present competition

experiments, however, the final effect was opposite. A mutation which inactivated the same Sp1 element in the URR produced a plasmid replicating slightly better than the wild-type. Since in the previous work the largest difference in relative replication of the two *ori* plasmids was noticed in the presence of excess reference *ori*, the same conditions were reproduced, but the discrepancy was confirmed. One possible explanation for these conflicting results could be the difference in the nature and extent of the mutations inactivating the Sp1 element.

The most likely explanation for the lack of effect of cellular transcription factors on papillomavirus DNA replication is that the auxiliary element for DNA replication is largely provided by the viral transcription factor E2. The absolute requirement of E2 for DNA replication is the generation of the specific E1/E2/*ori* complex. Various other transcription factors that have been tested are inactive (Ustav *et al.*, 1991; Li and Botchan, 1993). Although, the effects of cellular transcription might be more pronounced at limiting E2 concentration, for this reason, further studies will be required to understand the role of cellular transcription upon DNA replication.

However, the mutational analysis described in this study shows that there are regions of E2 other than those involved in binding E1 which are essential for efficient viral DNA replication. This supports the notion that E2 represent an auxiliary element for papillomavirus DNA replication.

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