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TARTU UNIVERSITY
PRESS

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Dissertation is accepted for the commencement of the degree of Doctor of Philosophy (in Molecular Biology) on January 13th, 2000 by the Council of the Institute of Molecular and Cell Biology, Tartu University

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Commencement: March 3th, 2000

The publication of this dissertation is granted by the University of Tartu

Tartu Ülikooli Kirjastuse trükikoda
Tiigi 78, Tartu 50410
Tellimus nr. 66

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LIST OF ORIGINAL PUBLICATIONS

I. Abroi, A., **Kurg, R.** and Ustav, M. (1996) Transcriptional and replicational activation functions in the bovine papillomavirus type 1 E2 protein are encoded by different structural determinants. J. Virol. 70(9), 6169–6179.

II. **Kurg, R.**, Parik, J., Juronen, E., Sedman, T., Abroi, A., Liiv, I., Langel, Ü. and Ustav, M. (1999) Effect of bovine papillomavirus E2 protein-specific monoclonal antibodies on papillomavirus DNA replication. J. Virol. 73(6), 4670–4677.

III. **Kurg, R.**, Langel, Ü. and Ustav, M. (2000) Inhibition of the bovine papillomavirus E2 protein activity by peptide nucleic acid. Virus Res. 66(1), 39–50.

LIST OF ABBREVIATIONS

aa	—	amino acid(s)
bp	—	base pair(s)
BPV1	—	bovine papillomavirus type 1
C-	—	carboxy-(terminus of the protein)
DBD	—	DNA binding dimerization domain
dsDNA	—	double stranded DNA
DTT	—	dithiothreitol
EBNA1	—	Epstein-Barr virus nuclear antigen
E2BS	—	E2 protein binding site
E2C	—	E2 repressor
E2TA	—	E2 transactivator
HPV	—	human papillomavirus
kD	—	kilodalton(s)
LCR	—	long control region
MAb	—	monoclonal antibody
N-	—	amino-(terminus of the protein)
NLS	—	nuclear localization signal
ORF	—	open reading frame
PNA	—	peptide nucleic acid
RE	—	responsive enhancer
SDS-PAAG	—	sodium dodecyl sulphate-polyacrylamid gel
TAD	—	transactivation domain
URR	—	upstream regulatory region
wt	—	wild type

1. INTRODUCTION

The papillomaviruses are small DNA viruses that induce squamous epithelial proliferative lesions (warts) in their natural hosts. Papillomaviruses maintain latency in dividing stem cells of various epithelial tissues, replicating as extra-chromosomal plasmids. The virus life cycle is greatly regulated by the viral transcription factor E2. First, the bovine papillomavirus E2 protein regulates viral protein levels by modulating the transcription of viral genes. Second, E2 is responsible for initiation of DNA replication; it activates viral replication through co-operative binding with the viral initiator protein E1 to the origin of replication. The third function of E2 is to aid in the faithful segregation of viral DNA, the E2 protein is responsible for chromatin association of the viral genome during the mitosis.

The bovine papillomavirus (BPV1) E2 is a DNA binding protein. Proteins that recognize specific DNA sequences generally determine when and to what extent activities such as DNA transcription and replication occur. In the present thesis, I would like to give an overview how the functional activities of the E2 protein are regulated through the structure of the protein, and through its activity to bind to DNA in a sequence-specific manner.

2. REVIEW OF LITERATURE

2.1. The genome structure and organization of BPV1

Papillomaviruses (PV) are small DNA viruses that infect a wide variety of vertebrate species and induce squamous epithelial proliferative lesions (warts) in their hosts. Papillomaviruses are highly species-specific. They infect both cutaneous and mucosal epithelial tissues at different body sites: skin, mouth, throat, anogenital tract and others. Viral gene expression, replication, episomal maintenance and virus assembly are tightly linked to the differentiation status of epithelial cell. Complete viral particles are shed from the terminally differentiated cells of the outer dermis, while the undifferentiated stem cells are presumed to maintain the viral genome. Papillomaviruses are nonenveloped, icosahedral particles approximately 54 nm in diameter. Their virions contain covalently closed, circular double-stranded DNA genomes of approximately 8 kilobases. The length of the BPV1 genome is 7946 bp (Ahola *et al.*, 1983; Chen *et al.*, 1982). All papillomaviruses share a similar genome organization, their translational open reading frames (ORF-s) locate on one strand of viral DNA (Engel *et al*, 1983). The genomes of several papillomaviruses have been sequenced and found to contain three distinct regions: (1) an upstream regulatory region that controls transcription and replication; (2) early genes encoding proteins required for transcription, DNA replication, and cell transformation; and (3) two late genes encoding the major and minor capsid protein.

The upstream regulatory region (URR; also called "long control region" (LCR)) is located between the 3' end of the late region and 5' part of the early region and is approximately 1 kb long. The URR contains *cis* elements necessary for the regulation of transcription and replication. There are also two polyadenylation sites in the genome, one common for the early genes (locating between the early and late region) and the other specific for the late gene expression (in URR).

The early region of the genome comprises open reading frames for E1, E2, E3, E4, E5, E6, E7 and E8 proteins. The E1 protein is a viral replication factor, which has ATPase, DNA-helicase and DNA-unwinding activities (Section 2.3.2). The E2 protein is viral transactivator, which binds to its cognate binding sites throughout the viral genome activating viral promoters and replication origin (Section 2.4). The E5, E6 and E7 ORF-s encode for the transforming proteins of papillomaviruses. The BPV1 E5 has been found to be the major oncogene in BPV, being both necessary (DiMaio *et al.*, 1986; Schiller *et al.*, 1986; Yang *et al.*, 1985) and sufficient (Bergman *et al.*, 1988; Leptak *et al.*, 1991) for transformation. E5 protein induces cellular proliferation and abrogation of contact inhibition, resulting in oncogenic transformation. It is highly hyrdophobic protein anchored into the cell membrane and can interact with other membrane-associated proteins, the receptors for PDGF (the platelet derived growth factor) and EGF (the epidermal growth factor), resulting in receptor activation. These

interactions are probably sufficient to mediate cell transformation (Martin *et al.*, 1989; Nilson and DiMaio, 1993). BPV E6 and E7 facilitate anchorage-independent transformation and are both required for full transformation of mouse C127 cells (Neary and DiMaio, 1989). The BPV1 E6 oncoprotein has been shown to transform C127 cells and to stimulate transcription when targeted to a promoter (Lamberti *et al.*, 1990). Recently it was shown that BPV1 E6 mediates sensitization of cells to TNF (Tumor Necrosis Factor) induced apoptosis (Rapp *et al.*, 1999). ORF E4 protein is associated with late gene expression (Neary *et al.*, 1987). The late ORF-s code for the structural proteins of the virus capsid, L1 and L2, which are transcribed in the upper layers of infected epithelia or epidermis and are expressed only in late phase of the viral replication (Engel *et al.*, 1983).

2.2. Regulation of BPV1 gene expression

BPV1 is transcriptionally active in both keratinocytes and fibroblasts *in vivo* (Baker and Howley, 1987). As illustrated in Fig. 1, there are at least six BPV1 promoters that are active in fibroblasts and lower level keratinocytes *in vivo* as well as in BPV1 transformed fibroblasts in culture; these are therefore considered to correspond to early gene promoters. Several early BPV1 mRNAs start at a major promoter upstream of the E6 gene, P_{89}. In addition, there is at least one major promoter that is utilized only in the upper epidermal layer; this is thought to be the viral late gene promoter, designated P_L (Baker and Howley, 1987). The URR contains a constitutive enhancer (CE) (Vande Pol and Howley, 1990; Vande Pol and Howley, 1992), which is not dependent on the virally encoded proteins and two others (E2RE1 and E2RE2) that are E2 responsive (Spalholz *et al.*, 1987).

The E2 ORF encodes a family of proteins which form the central regulatory system of the virus, controlling directly both viral gene expression and replication. E2 acts as a transcriptional activator by interacting with E2 binding sites, thereby enhancing transcription of all early viral transcripts (Szymanski and Stenlund, 1991). The E2 proteins are expressed from several different viral promoters. The E2 ORF encodes a transactivator (E2TA) which is expressed from the P_{89}, P_{890} and P_{2443} promoters (Hermonat *et al.*, 1988 Spalholz *et al.*, 1987; Spalholz *et al.*, 1985). The E2 ORF also encodes two transcriptional repressors. Repressor protein E2C is expressed from the P_{3080} promoter and translated from an internal methionine at amino acid 162 of the E2 ORF. Another repressor E8/E2 is expressed from P_{890} and encoded by a spliced message that encodes 11 amino acids from the E8 ORF linked to the C-terminal 207 amino acids of E2 via the splice acceptor at nucleotide 3225 (Choe *et al.*, 1989; Lambert *et al.*, 1989b; Lambert *et al.*, 1987). The E2TA as well as repressors E2C ans E8/E2 bind to DNA and share capacity to dimerize with

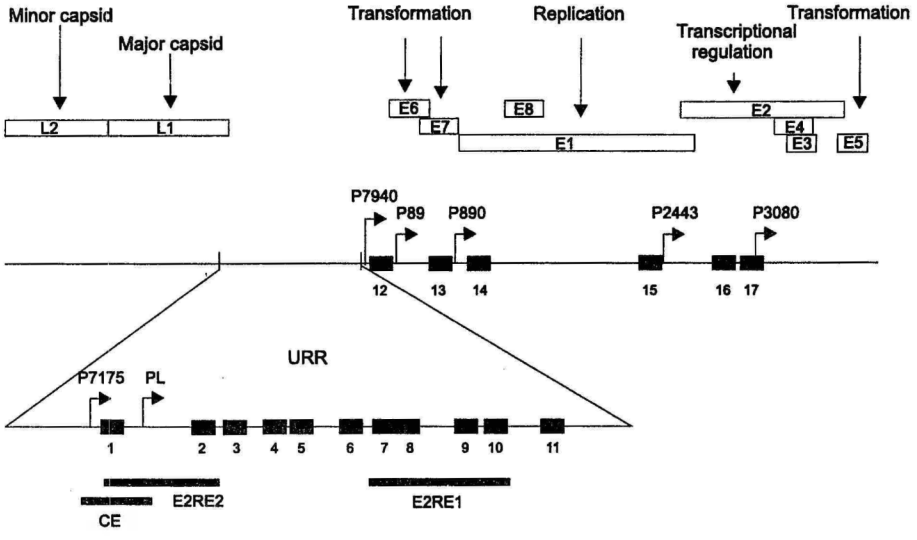

Figure 1. Map of the BPV1 genome.
Solid boxes represent the 17 E2 binding sites found in the genome. The open bars lettered E1 to E8, and L1 and L2 represent the ORFs. The upstream regulatory region (URR), which bears the E2-responsive elements (E2RE), is enlarged at the bottom.

themselves and with each other (McBride *et al.*, 1989; Monini *et al.*, 1993; Prakash *et al.*, 1992). The promoters from which the E2 gene products are expressed are themselves E2-responsive and therefore could be autoregulated by the E2 proteins (Hermonat *et al.*, 1988; Vaillancourt *et al.*, 1990, Haugen *et al.*, 1987; Szymanski and Stenlund, 1991). All three E2 polypeptides have been identified in BPV1 transformed cells (Hubbert *et al.*, 1988; Yang *et al.*, 1991b). The relative abundance of repressor over transactivator proteins in transformed C127 cells (1 E2TA: 10 E2C: 3 E8/E2) is thought to be important for the regulation of the activity of full-length E2 protein (Hubbert *et al.*, 1988). The ratio of the three E2 proteins changes throughout the cell cycle with the ratio of E2 transactivator to repressors being highest at S phase and lowest at G1 (Yang *et al.*, 1991b). By controlling the balance of viral activator and repressors, regulation of plasmid copy number is achieved. Both the competitive DNA binding at the E2 DNA binding sites and formation of heterodimers between E2TA and repressor molecules are proposed as potential mechanisms by which the repressor molecules inhibit E2TA. Repressors of the E2 protein have been shown to regulate viral transformation (Choe *et al.*, 1989; Lambert *et al.*, 1987; Riese *et al.*, 1990), transcription (Barsoum *et al.*, 1992), transient DNA replication (Lim *et al.*, 1998) and plasmid copy number (Lambert *et al.*, 1990; Riese *et al.*, 1990).

2.3. Papillomavirus DNA replication

Papillomaviruses infect basal epithelial and mucosal cells in a wide range of different hosts. Papillomavirus genome replication can be generally described as a three-step process (Howley, 1996). After the initial entry into the basal cells, the viral genomes are quickly amplified in the host cell nucleus. At the next, maintenance stage, the viral DNA replicates at a constant copy number per cell in the proliferating host cells. Papillomaviruses are able to maintain their genome extrachromosomally for prolonged periods with significant stability. The final, vegetative amplification stage, where the formation of new infectious particles occurs, takes place only after the host cells have terminally differentiated into keratinocytes.

The replication of BPV1 *in vivo* requires two viral proteins, E1 and E2, both of which bind to DNA site specifically, and a short DNA sequence called "origin of replication" (Ustav and Stenlund, 1991; Ustav *et al.*, 1991). However, the minimal origin of replication, consisting of E1 and E2 binding sites alone, is not sufficient for stable maintenance of BPV1 episomes. Additional region from URR containing at least six E2 binding sites are required for stable maintenance (Piirsoo *et al.*, 1996). Papillomavirus DNA replication is not cell type specific, despite a high degree of host and cell-type specificity for infection. BPV1 can replicate in a number of mammalian cells of epithelial and fibroblast lineage if E1 and E2 proteins are provided from heterologous expression vector (Chiang *et al.*, 1992). BPV1 DNA replication *in vitro* has been reconstituted with purified proteins and cell extracts from murine, simian and human cells (Bonne-Andrea *et al.*, 1995a; Melendy *et al.*, 1995; Muller *et al.*, 1994; Yang *et al.*, 1991a). The cellular factors essential for papillomavirus DNA replication are provided by the host cell and include replication protein A (RPA), replication factor C (RFC), proliferating-cell nuclear antigen (PCNA), and topoisomerase I and II as well as DNA polymerase α-primase complex and DNA polymerase δ (Melendy *et al.*, 1995; Muller *et al.*, 1994). In addition, the cellular factor CDE1 binding site in early coding region stimulates the replication of BPV1 *in vivo* (Pierrefite and Cuzin, 1995). The results from *in vitro* studies are generally in agreement with the *in vivo* results; however, a very modest effect of added E2 protein and dependence on the presence of an E2BS at the ori are observed *in vitro*. At a higher concentration the E1 protein alone is able to support *in vitro* BPV1 DNA replication (Bonne-Andrea *et al.*, 1995a; Bonne-Andrea *et al.*, 1997; Muller *et al.*, 1994; Yang *et al.*, 1991a; Yang *et al.*, 1993).

2.3.1. The origin of replication

The BPV1 origin of replication consists of E2-binding site (E2BS), an A+T-rich sequence and the palindromic sequence that constitutes the E1-binding site

(E1BS) (Ustav *et al.*, 1993; Ustav *et al.*, 1991). A functional replication origin has been reconstituted using oligonucleotide cassettes corresponding to three sequence subelements of BPV replication *ori* (McSahn and Wilson, 1997). This work revealed that a functional origin required at least one copy of all three subelements.

The transcription factor E2 binds to the consensus sequence ACCG(N_4)CGGT (Androphy *et al.*, 1987; Hawley-Nelson *et al.*, 1988; Li *et al.*, 1989). Results from the studies of the BPV *ori* indicate that the requirement for an E2BS is absolute, but a great deal of flexibility exists in terms of both the position and and the affinity of the E2BS. A single E2BS of very low affinity is sufficient for an active *ori*, at least under conditions where E1 and E2 are over-expressed (Ustav *et al.*, 1993; Ustav *et al.*, 1991). A relationship appears to exist between the affinity of the E2BS and the ability to function at a distance from the binding site for E1 (Ustav *et al.*, 1993) (Sedman and Stenlund, 1995). In multimerized form, the E2BSs are able to function even when placed at a distance of several kilobases from the rest of the *ori*. Gillette and Borowiec have demonstrated that the presence of both E2-binding sites (E2BS11 and E2BS12) within the origin is necessary for wild-type replication activity *in vivo*; BS11 and BS12 play separate but synergistic roles in the initiation of viral DNA replication that are dependent on their location within the origin (Gillette and Borowiec, 1998).

The binding site for the E1 replication helicase has been defined in BPV as an imperfect palindromic sequence ATTGTTGTTAACAATAAT (Holt *et al.*, 1994; Ustav *et al.*, 1991; Wilson and Ludes-Meyers, 1991; Yang *et al.*, 1991a). It has been shown that single point mutations in the BPV E1BS can reduce binding of E1, as well as replication, more than 20-fold (Sedman and Stenlund, 1995).

The A+T-rich sequence of the minimal BPV *ori* is important for replication *in vivo*, since a deletion of this sequence results in a drop in replication activity (Ustav *et al.*, 1991). While the E1BS and E2BS are sequence-specific elements, the BPV1 A+T-rich sequence can be at least partially substitute with heterologous A+T-rich sequences, suggesting that the role of this element is primarily AT content-dependent rather than sequence-dependent (McSahn and Wilson, 1997).

2.3.2. Assembly of the replication initiation complex at *ori*

The viral E1 protein is absolutely required for papillomavirus DNA replication, both *in vivo* and *in vitro* (Bonne-Andrea *et al.*, 1995a; Ustav and Stenlund, 1991; Yang *et al.*, 1991a). The E1 protein is a 72 kD nuclear phosphoprotein with a sequence-specific DNA binding activity (Blitz and Laimins, 1991; Chen and Stenlund, 1998; Lentz *et al.*, 1993; Lusky and Fontane, 1991; Santucci *et al.*, 1990; Sun *et al.*, 1990; Thorner *et al.*, 1988; Thorner *et al.*, 1993; Ustav *et*

al., 1991; Wilson and Ludes-Meyers, 1991). E1 has also DNA-dependent ATPase activity, DNA helicase activity and it can serve to unwind the origin of replication (MacPherson *et al.*, 1994; Seo *et al.*, 1993b; Yang *et al.*, 1993). In addition, the E1 protein is able to associate with the large subunit of cellular DNA polymerase α (Bonne-Andrea *et al.*, 1995b; Park *et al.*, 1994), bind to 70 kD subunit of cellular RPA complex (Han *et al.*, 1999) and interact with a key cell cycle regulator of S phase, the cyclin E-Cdk2 kinase (Cueille *et al.*, 1998). The E1 protein can interact specifically with the viral E2 protein in solution (Blitz and Laimins, 1991; Mohr *et al.*, 1990) and on the *ori* (Benson and Howley, 1995; Lusky and Fontane, 1991; Sarafi and McBride, 1995; Sedman and Stenlund, 1995). This interaction results in co-operative binding of E1 and E2 to the origin of replication (Gillette *et al.*, 1994; Gillette and Borowiec, 1998; Sedman and Stenlund, 1995; Seo *et al.*, 1993a; Spalholz *et al.*, 1993; Yang *et al.*, 1991a). E1 by itself binds to the *ori* with a low degree of sequence specificity, in the presence of E2 the sequence specificity is increased (Sedman and Stenlund, 1995; Sedman *et al.*, 1997). In this process, E2 functions transiently and "catalytically", providing sequence specificity for the formation of an E1-ori complex (Gillette and Borowiec, 1998; Lusky *et al.*, 1994; Sanders and Stenlund, 1998; Sedman and Stenlund, 1995; Sedman and Stenlund, 1996; Sedman *et al.*, 1997). The formation of an replication-competent initiation complex is a two-step process where the first step involves the binding of E1 and E2 to *ori* and the second step involves the assembly of additional E1 molecules onto this complex and the displacement of E2 in a process that requires ATP hyrdolysis (Sanders and Stenlund, 1998). In the E1-ori complex, the E1 protein forms a DNA-dependent hexameric complex (Fouts *et al.*, 1999; Sedman and Stenlund, 1998), which is able to unwind DNA bi-directionally from the *ori* site and therefore is likely to represent an early step in the initiation of DNA replication.

2.4. The papillomavirus E2 proteins

The viral E2 protein is the master regulator of the viral life cycle — this protein modulates the transcription of viral genes, is responsible for the initiation of viral DNA replication and for the stable maintenance of the viral genome. The BPV1 E2 ORF encodes three proteins: a 48 kD full-length E2 transactivator (E2TA) and two transcriptional repressors, E2C and E8/E2 with molecular masses of 30 and 28 kD, respectivaly (Haugen *et al.*, 1987; Hirochika *et al.*, 1987; Hubbert *et al.*, 1988; Spalholz *et al.*, 1985).

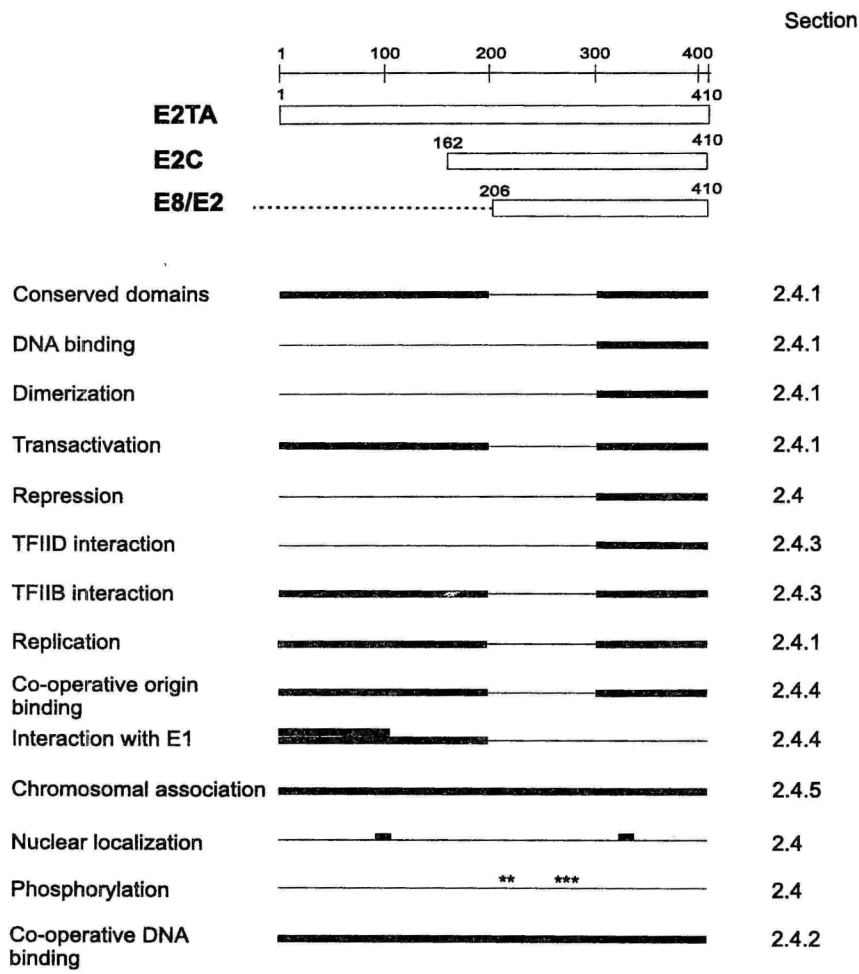

Figure 2. The structure of the three BPV1 E2 proteins are shown at the top of the figure. Below, the functions that have been mapped to different regions of the proteins are indicated.

E2 proteins localize in the nucleus of the cell (Burnett *et al.*, 1990; Hubbert *et al.*, 1988; Skiadopoulos and McBride, 1996) and a large amount of the full-length E2 protein is associated with insoluble chromatin and nuclear matrix components (Hubbert *et al.*, 1988). Two different basic regions have been suggested to function as nuclear localization signals (NLS) in the full-length E2 protein (Skiadopoulos and McBride, 1996). The NLS in C-terminus of BPV1 E2, positioned within residues 339 and 352 (BR3, KCYRFRVKKNHRHR), which contains the DNA recognition helix of the DNA binding domain has been found to mediate the transport of the protein to the nucleus (Allikas *et al.*, 2000; Skiadopoulos and McBride, 1996). The point-mutation in the second, the

amino-terminal NLS (BR2, aa 107 to 115, KRCFKKGAR) causes re-localization of the protein into the cytoplasm, but also induces the aggregation and oligomerization of E2 (Abroi *et al.*, 1996). Recent studies have revealed that in addition to diffuse nuclear localization, some part of E2 together with E1 is localized in intranuclear domains, called ND 10 or POD (promyelocytic oncogenic domains) (Swindle *et al.*, 1999) or is transported there by viral L2 protein (Day *et al.*, 1998). These foci are proposed to be DNA replication compartments and sites of papillomavirus assembly.

BPV1 E2 protein is phosphorylated in mammalian cells. Five phosphorylation sites (aa 235, 277, 290, 298, 301) in the hinge region of the protein are mapped (Lehman *et al.*, 1997; McBride *et al.*, 1989). In transient assays, phosphorylation site mutants of E2 are normal for transcriptional activation and DNA replication; the genome replicated even to higher levels than the wild-type. But phosphorylation site mutants of E2 are severly crippled for function(s) required for viral plasmid retention and oncogenic transformation (Lehman *et al.*, 1997; McBride *et al.*, 1989). Recent data show that segregation of viral plasmids during cell division is regulated by phosphorylation (Lehman and Botchan, 1998).

2.4.1. Structural and functional domains of the E2 protein

The 410 amino acid BPV1 E2 protein is composed of relatively well-defined function-specific modules. Structural and mutational analyses have revealed three distinct domains. The amino-terminal part (residues 1 to 210) is an activation domain for transcription and replication. It is followed by the unstructured hinge region and the carboxy-terminal DNA-binding-dimerization domain (DBD)(residues 310 to 410) (Giri and Yaniv, 1988; Haugen *et al.*, 1988; McBride *et al.*, 1989; McBride *et al.*, 1988).

The N-terminal transactivation domain of E2 (TAD) has a very high structural integrity, as any deletion that has been made within this domain inactivates all the protein functions. (Haugen *et al.*, 1988; Winokur and McBride, 1992; Winokur and McBride, 1996). Even a single amino-acid substitution can easily inactivate the protein (Abroi *et al.*, 1996; Brokaw *et al.*, 1996; Ferguson and Botchan, 1996; Grossel *et al.*, 1996). Computer predictions of secondary structure of the TAD of E2 reveal that this domain begins with two amphipathic α-helices, the rest of the domain is mainly composed of small hydrophobic β-sheets (Abroi *et al.*, 1996; Giri and Yaniv, 1988). In 1999, the crystal structure of the protease-resistant core (residues 66 to 215) of the HPV 18 E2 activation domain was published (Harris and Botchan, 1999). This reveals a fold creating a cashew-shaped form with a glutamine-rich α helix packed against a β-sheet framework. Residues 106–190 form a core structure of the activation domain, this is a novel β-sheet framework where is an intricate layering of β strands that are relatively bowed or briefly disrupted as they transition between alternate

sheets. Various E2 proteins average 30% amino acid sequence identity and papillomavirus E2 proteins show some functional conservation, for instance, combinations of intertypic E1 and E2 genes can complement each other for viral replication (Berg and Stenlund, 1997; Chiang *et al.*, 1992) and mutational analyses on BPV1, HPV16 and HPV11 E2 genes have shown consistent results. These results suggest that various E2 activation domains share a common fold. When joined to a DNA binding domain, the N-terminal 194 amino acids of BPV1 E2 are able to activate transcription from an E2-responsive promoter (Winokur and McBride, 1992). The results from our lab indicate that the N-terminal residues 1–192 form a structural and functional activation domain of the BPV1 E2 protein for replication, when this domain is anchored to DNA via heterologous DNA binding domain (Allikas *et al.*, 2000). The mutational analyses of the activation domain of E2 show that the determinants responsible for activation of replication and transcripition are partially separable (Abroi *et al.*, 1996; Brokaw *et al.*, 1996; Ferguson and Botchan, 1996; Grossel *et al.*, 1996). However, a single critical region required for transcription have not identified, inactivating mutants are dispersed throughout the transactivation domain. Some transcriptional activation-defective mutants (R37, I73, E74) retain the ability to cooperate with E1 in viral replication (Ferguson and Botchan, 1996; Grossel *et al.*, 1996) and to support replication (Abroi *et al.*, 1996), whereas the E2 mutant E39 reduced replication activity but left transcriptional activation intact (Ferguson and Botchan, 1996).

The carboxy-terminal 85 aa of BPV1 E2 (aa 326 to 410) are sufficient for sequence-specific DNA binding and dimerization (Corina *et al.*, 1993; Dostatni *et al.*, 1988; Lambert *et al.*, 1987; McBride *et al.*, 1989; McBride *et al.*, 1988; Prakash *et al.*, 1992). A high-resolution crystal structure for the minimal core region complexed with DNA revealed that the DNA-binding domain is folded into a dimeric β-barrel. Across the surface of the barrel, each E2 monomer contributes a short α helix (aa 336 to 344) which lies in the major groove of DNA, making specific contacts with the DNA (Hegde *et al.*, 1992; Hegde *et al.*, 1998). Later it was shown that aa 311–325 are also important for stability and integrity of C-terminal DNA-binding-dimerization domain as this region influences the DNA-binding affinity and protein stability (Pepinsky *et al.*, 1997) and that the effect is mediated through intramolecular interactions between the flanking region and the core domain (Vec.araghavan *et al.*, 1998). The residues which make direct contacts with DNA are N336, K339, C340, F343 and R344 (Hegde *et al.*, 1992). A highly conserved cysteine residue at position 340 is very sensitive to oxidation (McBride *et al.*, 1992). The DNA binding domain of the E2 proteins forms a stable dimer even in the absence of DNA (Corina *et al.*, 1993; McBride *et al.*, 1989). Dimers of E2 are highly resistant to urea denaturation (Corina *et al.*, 1993). The DNA binding and dimerization properties of this domain cannot be separated by deletion analysis; all deletions that have been tested eliminate both properties of the C-terminal domain. A highly conserved tryptophan residue at position 360 has been designated to form the

tryptophan bridge (Corina *et al.*, 1993). Mutated E2 proteins containing hydrophobic residues at this position are functional but substitution of W360 by polar residues disrupts dimerization.

The amino-terminal activation and carboxy-terminal DNA-binding-dimerization domains are separated by hinge region of approximately 100 residues. There has been found no sequence conservation in the hinge region among different papillomavirus E2 proteins, despite conservation in the DNA binding and activation domains. This may indicate that the hinge regions of the E2 proteins do not carry the conserved function. Several studies have indicated that hinge region of the BPV1 E2 may be involved in the regulation of the E2 protein activity through interaction with transcription factors (Ham *et al.*, 1994) and could be important for replication (Kurg *et al.*, 1999; Winokur and McBride, 1992). Our recent work demonstrates that some critical length and flexibility, but not the particular amino acid sequence of the hinge region is required for the ability of E2 to bind to DNA and to initiate DNA replication from the minimal-origin-containing plasmid in transient assay (Allikas *et al.*, 2000). However, the hinge region of E2 is required for stable plasmid maintenance; it is needed for chromatin attachment of the viral genome as it contains phosphorylation sites of the BPV1 E2 protein (Lehman and Botchan, 1998).

Analyses of mutations in the E2 ORF which delete various regions of the E2 protein have revealed that, in most cases, an intact transactivation domain and an intact DNA-binding-dimerization domain are required for transcription, and for E1–E2-ori complex formation as well as for the initiation of replication. Large deletions in the hinge region affect mostly DNA binding and replication, but less transcription (Allikas *et al.*, 2000; Winokur and McBride, 1992; Winokur and McBride, 1996).

2.4.2. E2 DNA-binding sites

The dimeric E2 protein binds specifically to the 12-bp palindromic sequence ACC(N_6)GGT (Androphy *et al.*, 1987; Dostatni *et al.*, 1988; Moskaluk and Bastia, 1987; Moskaluk and Bastia, 1988b). These sites are located throughout papillomavirus genome but are particularly concentrated within the URR (Harrison *et al.*, 1987; Hawley-Nelson *et al.*, 1988; Spalholz *et al.*, 1988; Spalholz *et al.*, 1987). BPV1 has 12 sites that correspond to this sequence and additional five closely related sequences that can also bind E2 (Li *et al.*, 1989). Equilibrium and kinetic studies show that the range of E2 affinities of these 17 sites vary over 300-fold (Li *et al.*, 1989). The higher affinity sites are clustered in the URR in regions called E2-dependent enhancer elements E2RE1 and E2RE2 (Spalholz *et al.*, 1987). A low level of E2-dependent activation can be obtained with one E2 binding motif, however, two E2 DNA-binding sites cooperate to constitute a strong enhancer (Lambert *et al.*, 1989a; Morrissey *et al.*, 1989; Stanway *et al.*, 1989; Thierry *et al.*, 1990; Spalholz *et al.*, 1987). Some

co-operativity in DNA binding has been observed *in vitro*, the region responsible for this co-operativity is encoded by the N-terminal part of the protein (Monini *et al.*, 1993; Monini *et al.*, 1991; Thierry *et al.*, 1990). DNA-bound E2 molecules have been demonstrated to associate and form stable DNA loops visible by electron microscopy (Knight *et al.*, 1991). E2TA binds co-operatively to two adjacent DNA binding sites with a cooperativity parameter of 8.5, while the 86 aa DNA binding domain and the E2C protein exhibit much less cooperativity (factors 1.9 and 2.9, respectively) (Monini *et al.*, 1991). Genomic footprinting have shown that *in vivo* E2 binds DNA molecules that contain one, two or three E2BS-s even in the absence of transcriptional activation (Lefebvre *et al.*, 1997).

The BPV1 E2 proteins bind to DNA with high affinities which have been measured in the range of 2×10^{-10} to 2×10^{-11}M (Li *et al.*, 1989; Monini *et al.*, 1993; Monini *et al.*, 1991). The precise contact points of the E2 protein on the $ACC(N_6)GGT$ motif have been determined (Hegde *et al.*, 1992; Hines *et al.*, 1998; Moskaluk and Bastia, 1988b; Rozenberg *et al.*, 1998). According to the crystallographic data, the base pairs GGT are the region of greatest contact with the protein recognition helix. The nucleotides in the non-conserved core and immediately outside the binding site determine the affinity for the E2 protein. The sequence-specific DNA binding is often accompanied by deformation of the DNA. The DNA within the E2/E2BS complex adopts ~43°C bend towards the minor groove at the center of the target (Hegde *et al.*, 1992; Moskaluk and Bastia, 1988a) and recently it has been shown that BPV E2 protein binding affinity depends on DNA flexibility (Hines *et al.*, 1998; Rozenberg *et al.*, 1998). E2 binding can be inhibited by CpG methylation of the $ACCGN_4CGGT$ motif (Thain *et al.*, 1996)(M. Ustav, unpublished data).

2.4.3. The E2 protein as a transcription factor

The BPV1 E2 protein, functioning through E2 binding sites, regulates the transcription of the early viral genes (McBride *et al.*, 1991; Spalholz *et al.*, 1985). E2 can also activate heterologous promoters when its binding sites are cloned either upstream or downstream of the transcription initiation site (Gauthier *et al.*, 1991; Hawley-Nelson *et al.*, 1988; Thierry *et al.*, 1990). E2 can act either as a repressor or an activator of transcription depending on the position of its binding sites relative to the promoter sequence (Dostatni *et al.*, 1991). For instance, E2TA can also repress transcription when the E2 motifs are situated close to the transcriptional start site, overlapping the binding sites for essential cellular transcription factors (Stenlund and Botchan, 1990; Vande Pol and Howley, 1990). E2 has also an ability to activate several heterologous promoters which lack E2BS in transient assay, albeit to much lesser extent (Haugen *et al.*, 1987). The E2 protein stimulates transactivation probably by direct or indirect interactions with some components of the basic transcriptional

machinery. E2 is unable to activate minimal promoters containing only a TATA box but requires additional promoter proximal elements such as Sp1 (Ham *et al.*, 1991; Ushikai *et al.*, 1994). E2 interacts with basal transcription factors, including Sp1 (Li *et al.*, 1991; Ushikai *et al.*, 1994), TBP (TATA-binding protein) (Dostatni *et al.*, 1991; Ham *et al.*, 1994; Rank and Lambert, 1995; Steger *et al.*, 1995) and TFIIB (Benson *et al.*, 1997; Rank and Lambert, 1995; Yao *et al.*, 1998). The activation domain modulating factor (AMF-1) has also been shown to associate with the BPV1 E2 (Breiding *et al.*, 1997) and E2 co-operatively stimulates transcription with cellular transcription factors USF and CTF (Ham *et al.*, 1991; Ushikai *et al.*, 1994). BPV1 E2 can also transactivate promoters containing E2 binding sites in *Saccharomyces cerevisiae* (Lambert *et al.*, 1989a; Morrissey *et al.*, 1989; Stanway *et al.*, 1989) indicating that interactions of E2 with transcription machinery are well conserved. Furthermore, the *in vitro* interaction between in *Saccharomyces cerevisiae* TFIIB and E2 has been demonstrated (Benson *et al.*, 1997). Both the N-terminal transactivation domain (Benson *et al.*, 1997; Yao *et al.*, 1998) and the C-terminal DNA-binding-dimerization domain of E2 (Rank and Lambert, 1995) interact with TFIIB. However, the E2 transactivation domain itself can stimulate transcription in eukaryotic cells when cloned onto a heterologous DNA-binding domain (Breiding *et al.*, 1996; Winokur and McBride, 1996). The BPV E2 protein can substitute for cellular enhancer binding factors in initiation of polyomavirus DNA replication, if the enhancer region is replaced by binding sites for the E2 protein (Nilsson *et al.*, 1991) (A.Abroi; unpublished results). In addition, the binding of transcriptionally competent E2 to two or three tandem binding sites but not to a single site modifies the chromatin structure around the promoter (Lefebvre *et al.*, 1997; Li and Botchan, 1994).

2.4.4. The E2 protein as a replication factor

The E2 protein is the master regulator of extrachromosomal replication of papillomaviruses (for review (Ustav and Ustav, 1998)). The E2 protein facilitates binding of the viral helicase E1 to the origin of replication (Lusky *et al.*, 1994; Sedman and Stenlund, 1995; Sedman *et al.*, 1997). In this process, E2 functions transiently and "catalytically", providing sequence specificity for the formation of an E1-ori complex (Gillette and Borowiec, 1998; Lusky *et al.*, 1994; Sanders and Stenlund, 1998; Sedman and Stenlund, 1995; Sedman and Stenlund, 1996; Sedman *et al.*, 1997). A direct interaction between the viral E2 and E1 proteins has been demonstrated in solution as well as on the *ori* (Blitz and Laimins, 1991; Lusky and Fontane, 1991; Mohr *et al.*, 1990; Sarafi and McBride, 1995), this interaction is mediated through the amino-terminus of the E2 protein (Benson and Howley, 1995; Berg and Stenlund, 1997; Winokur and McBride, 1996). In the yeast two hybrid system, the first 91 amino acids of E2 were able to interact with the E1 protein (Benson and Howley, 1995). Berg and

Stenlund have demonstrated that BPV1 E2 DNA-binding-dimerization domain is also independently capable of interacting with E1 when the E1 and E2 binding sites are adjacent to each other (Berg and Stenlund, 1997). However, the E2C protein is unable to interact with or cooperatively bind to the origin with the E1 protein (Mohr *et al.*, 1990; Winokur and McBride, 1996). The activation domain of E2, fused to the DNA-binding domain of either yeast transcription factor GCN4 or EBNA1 of Epstein-Barr virus, is shown to be sufficient for initiation of replication (Berg and Stenlund, 1997; Kivimäe *et al.*, 2000). The BPV1 and HPV-11 E2 activation domains are equally capable of interacting with BPV E1 and to support replication (Berg and Stenlund, 1997). This indicates that the replication functions of these proteins are quite well conserved. In addition, E2 has also shown to facilitate the single-stranded DNA-binding protein A (RPA) to interact with the *ori* (Li and Botchan, 1993).

2.4.5. The other activities of the E2 protein

The BPV1 E2 is necessary for long-term episomal maintenance of viral genomes within replicating cells (Piirsoo *et al.*, 1996). Recent studies have shown that the BPV genome is attached to mitotic chromosomes (Lehman and Botchan, 1998; Skiadopoulos and McBride, 1998) and that this association is mediated by the viral E2 protein (Ilves *et al.*, 1999; Lehman and Botchan, 1998; Skiadopoulos and McBride, 1998). The N-terminal transactivation domain of E2 is shown to be crucial for the chromatin attachment activity (Skiadopoulos and McBride, 1998; Lehman and Botchan, 1998; Ilves *et al.*, 1999). Lehman and Botchan suggest that the hinge region between the N- and C-terminal domains, which includes the major phoshorylation sites of the E2 protein, is also important for the attachment (Lehman and Botchan, 1998). Ilves and co-workers suggest that the E2 protein in *trans* and its multiple binding sites in *cis* are both necessary and sufficient for the chromatin attachment of the plasmid (Ilves *et al.*, 1999).

The expression of BPV1 E2 results in growth inhibition of HeLa and other HPV-positive cell lines (Desaintes *et al.*, 1997; Desaintes *et al.*, 1999; Dowhanick *et al.*, 1995; Goodwin *et al.*, 1998; Hwang *et al.*, 1993). An intact transactivation and DNA-binding-dimerization domains are required for growth suppression (Desaintes *et al.*, 1997; Dowhanick *et al.*, 1995; Goodwin *et al.*, 1998). It has been suggested that E2 represses the endogenous E6 transcription and this causes accumulation of a transcriptionally active p53 (Desaintes *et al.*, 1997; Dowhanick *et al.*, 1995; Hwang *et al.*, 1993). However, recent data suggest that E2-induced apoptosis is an early event, independent of p53 accumulation and unrelated to downstream p53-dependent transcriptional events and that E2 is able to activate also p53-independent growth-inhibitory signals (Desaintes *et al.*, 1999; Goodwin *et al.*, 1998).

3. RESULTS AND DISCUSSION

As described in review of literature, the BPV1 E2 protein is the master regulator of the papillomavirus replication and transcription, the activity of which is regulated through sequence-specific DNA binding. The E2 protein consists of three function-specific domains. The N-terminal part (residues 1 to 210) is an activation domain for transcription and replication, it is followed by the hinge region and the C-terminal DNA-binding — dimerization domain (residues 310 to 410) (Giri and Yaniv, 1988; Haugen *et al.*, 1988; McBride *et al.*, 1989; McBride *et al.*, 1988). The transactivation domain of E2 is the only part of the protein, which is specifically required for both, replication and transcription activities (Winokur and McBride, 1996, Berg and Stenlund, 1997); it is shown to interact with several replication and transcription proteins (Sections 2.4.3 and 2.4.4). More information about the structure of this domain and of the whole protein is needed to understand better how the functioning of E2 in replication and transcription is achieved. The crystal structure of the full-length BPV1 E2 protein is not yet determined and we have to rely on other methods to examine the structural organization of the whole protein and the molecular interactions that must occur to accomplish the activity of the protein. To study the structure-function relationship of the E2 protein we have used two different approaches. First, point-mutations within the transactivation domain of E2 were made to determine regions important for the transcription and replication functions and second, monoclonal antibodies were produced and used as probes and tools to study the structure and function of the protein.

The E1 and E2 proteins are required for the initiation of DNA replication from the papillomavirus origin (Ustav and Stenlund, 1991). The viral DNA replication origin contains an E1 binding site flanked by E2 binding sites (Ustav *et al.*, 1991). The E1 and E2 proteins interact to form a protein complex and bind cooperatively to the origin of replication (Gillette *et al.*, 1994; Gillette and Borowiec, 1998; Sedman and Stenlund, 1995; Seo *et al.*, 1993a; Spalholz *et al.*, 1993; Yang *et al.*, 1991a). Formation of the replication preinitiation complex requires specific protein-protein and protein-DNA interactions between the E1 and E2 proteins and their respective DNA binding motifs. We wanted to determine is it possible to inhibit papillomavirus DNA replication by preventing binding of the E2 protein to its recognition sequence. Again, two different ways were used: first, monoclonal antibody against the E2 DBD was used to inhibit E2 binding to DNA and second, E2BS was blocked by PNA to hide the E2 recognition sequence.

3.1. Mutational analysis of the BPV1 E2 protein (Reference I)

In order to determine regions within the transactivation domain of E2, which are important for the transcription and replication function, a set of mutant E2 proteins was constructed. Single conserved charged residues in the amino-terminal transactivation domain of E2 were replaced with alanine. These mutant E2 proteins were tested for expression and for transcriptional and replicational activation in functional assays. The mutational analysis of the activation domain of E2 showed that the transcription and replication activities of E2 are partially separable and are encoded by different determinants in the E2 protein. Transcription activation defective mutants, which retained their ability to support replication, were R37A, E74A, E90A, D122A and D143A/R172C. E2 mutants K111A, K112A and E176A were defective in both assays (I, Fig. 4).

Some years ago not much was known about the structure of the activation domain of E2 and point-mutations were made according to computer predictions only (Fig. 3). In 1999, the crystal structure of the protease-resistant core (residues 66 to 215) of the HPV 18 E2 activation domain was published (Harris and Botchan, 1999). According to these data, residues 106–190 form a core structure of the activation domain, this is a β-sheet framework built of antiparallel strands, flexible loops that connect the strands are generally exposed at the edges of the core of the protein (Harris and Botchan, 1999). Based on the crystallographic data of HPV18 E2, a protein model of the same fragment of BPV1 E2 (Fig. 4) was made using the SWISS-MODEL service (Peitsch, 1995; Peitsch, 1996). In the present thesis I have used this theoretical protein model to analyze the E2 mutants, which were made based on computer predictions only. Transcription-defective but replication competent E2 mutants E90A and D122A fell in the loop regions of core structure of activation domain and residue E74 is involved in the long NH_2-terminal α helix (Fig. 4D). All these residues are exposed on the surface of the core structure of the activation domain of E2. So, they can, in principle, be involved in interactions with transcription machinery of the cell. These results also showed that there is no single critical region required for transcription, inactivating mutants were dispersed throughout the transactivation domain. However, mutations I73A and Q76A by others (Ferguson and Botchan, 1996; Harris and Botchan, 1999; Sakai *et al.*, 1996) induced proteins with the same phenotype and this α helix surface is probably important in transcriptional activation. Transcription-defective but replication competent mutant proteins R37A, E90A and D122A localized in the nucleus of the cell and existed in the dimeric form, however, in the case of mutations E74A and D143A/R172C only part of the protein existed as a dimer able to form a specific E2-DNA complex. Our futher studies indicated that mutation E74A caused also formation of oligomers, including tetramers, which were stable enough to be separated from the dimeric form of E2 by the glycerol gradient centrifugation.

We have proposed that amino terminus-mediated oligomerization could also serve as a mechanism for the regulation of E2 biological activity.

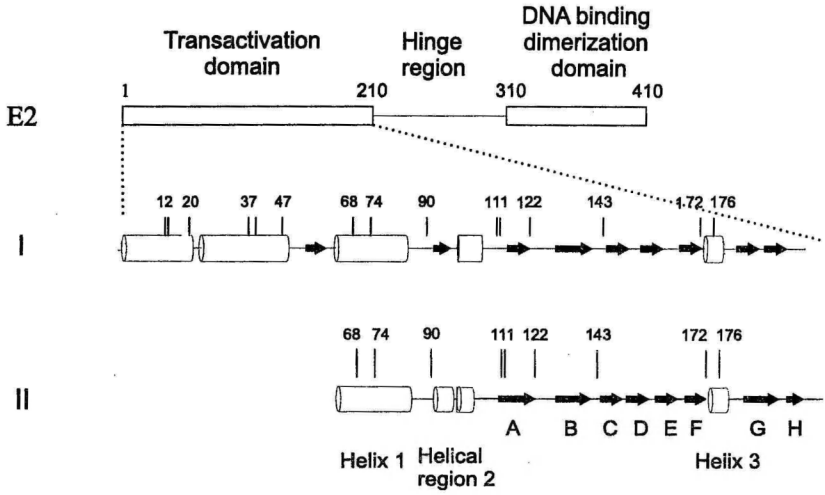

Figure 3. Positions of the point-mutations in the transactivation domain of the E2 protein. (I) The prediction of secondary structure (cylinder, α-helix; arrows, β-sheets) made by Rost and Sander (Rost and Sander, 1993). (II) The secondary structure of the protease resistant core of the BPV1 E2 based on the crystallographic data of the HPV18 E2.

Substitutions K111A, K112A and E176A in the transactivation domain of E2 resulted in inactive proteins in both, transcription and replication assays and caused re-localization of E2 from the nucleus into the cytoplasm of the cell. These mutants were also unable to form an E2-DNA complex of the usual size; however, considerable retention of the probe in wells was detected (I, Fig. 4C). Glycerol gradient sedimentation analysis showed that mutations in the positions of K111, K112 and E176 induced conformations of the proteins, which were able to oligomerize and aggregate. The pronase treatment of inactive mutant K111A, K112A and E176A proteins showed that their DNA binding abilities were readily present in the proteins, they were only masked due to aggregation. So, we concluded that these mutations probably disrupted the conformation of the transactivation domain, which lead to aggregation of the E2 protein and that the oligomerization and aggregation of the protein resulted in their inactivity in all functional assays. On the structural model of the activation domain of BPV1 E2 protein, E2 residues K111 and K112 lie within the β sheet A and are involved in the formation of the core structure of E2 activation domain. Amino acid E176 lies on the inner edge of the main cavity and is involved in a short α helix within the core structure of the activation domain. Therefore all these three amino acids are necessary for functionally active conformation of the activation domain of E2. K111A and K112A fell in the region (residues 107 to

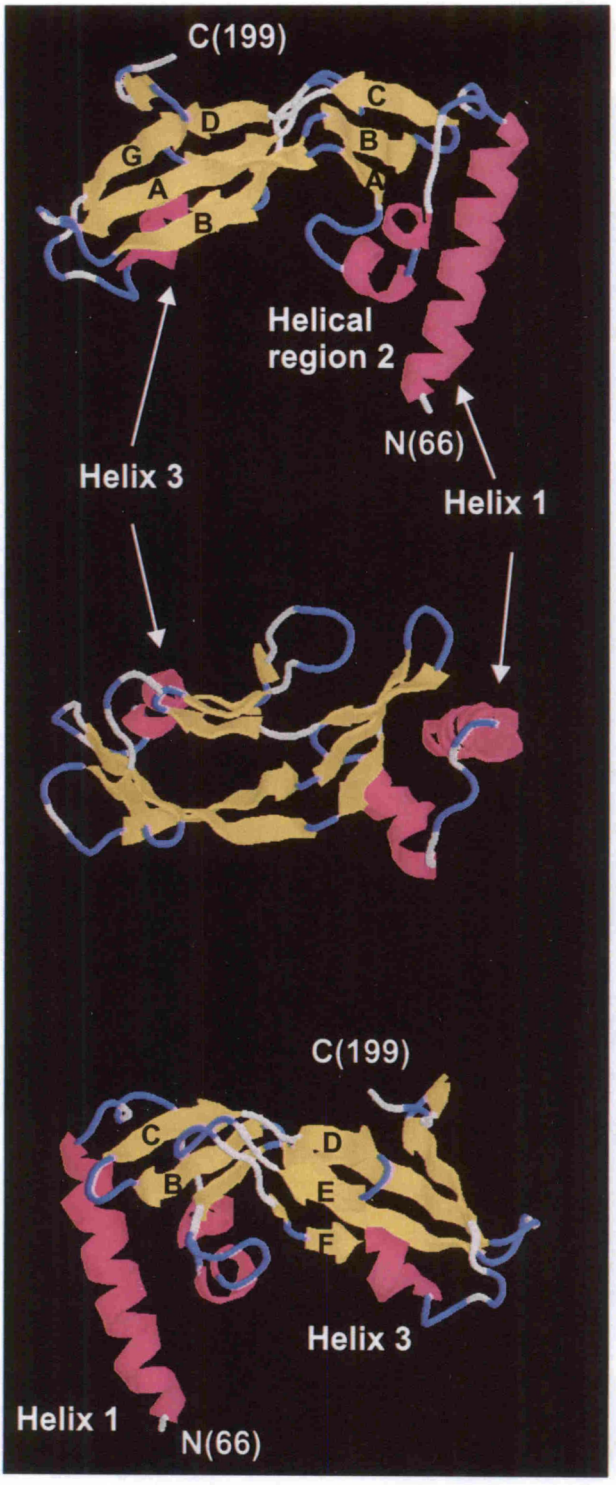
A
C(199)
D
C
G
B
A
A
B
Helical
region 2
N(66)
Helix 3
Helix 1
B
C
C(199)
C
D
B
E
F
Helix 3
Helix 1
N(66)

Figure 4. Structure of the protease-resistant core of the BPV1 E2 modelled by SWISS-MODEL service (Peitsch, 1995; Peitsch, 1996) according to the crystallographic data of the protease resistant core of the HPV18 E2 (Harris and Botchan, 1999). (A) The molecule is coloured by protein secondary structure: α-helixes are coloured red, β-sheets yellow, turns are blue and others white. (B) Ribbon diagram rotated 90 degrees around the x axis to show the pattern of β-sheets. (C) Ribbon diagram rotated 180 degrees around the y axis. (D) Location of transcription-defective but replication competent E2 mutants is shown on the ribbon diagram of protease-resistant core of E2. (E) Location of inactive E2 mutants K111A, K112A and E176A on the core structure of the activation domain of the E2 protein.

115), which has been identified as a potentional NLS for E2, however, this sequence is not able to mediate active transport of a hybrid protein into the nucleus (Skiadopoulos and McBride, 1996). As aa K111 is not exposed on the surface of the protein and K112 is involved in charge interactions with the acidic side chain of E90 (Harris and Botchan, 1999), therefore it is unlikely that they can act in the transport of the protein to the nucleus proposed by Skiadolopous and McBride (Skiadopoulos and McBride, 1996). So, our conclusion that localization of mutant proteins to cytoplasm was caused rather by aggregation of the proteins than by a mutation in NLS, was correct and is consistent with the crystallographic data. In summary, mutations, which fell into β sheet or α helix of the core structure of the activation domain of E2 influenced the functionally active conformation of the protein and resulted in inactive proteins in sequence-specific DNA binding as well as in transcription and replication. Mutations, which fell in the loop regions of core structure, maintained the structure of the domain and were able to exist in dimeric form.

In conclusion, our work showed that the activation domain of E2 has a discrete disruptable structure, which is very sensitive to point-mutations. Later, this suggestion has been confirmed by crystallographic data (Harris and Botchan, 1999). In several studies (Abroi *et al.*, 1996; Brokaw *et al.*, 1996; Cooper *et al.*, 1998; Ferguson and Botchan, 1996; Grossel *et al.*, 1996; Sakai *et al.*, 1996), amino acid substitutions have been generated in the transactivation domains of the BPV1, HPV11 and HPV16 E2 proteins to determine which regions of this domain are important for the transcription and replication functions. Among these studies, we were the only one who showed that sometimes the point-mutation can also disrupt the conformation of the protein and how difficult it may be by simple loss-of-function assays to discern those residues of E2 that may be interacting specifically with other cellular or viral partners.

3.2. Monoclonal antibodies against the BPV1 E2 protein (Reference II)

In this study, we have produced and characterized a panel of monoclonal antibodies (MAbs) as probes and tools for studying the structure and function of the BPV1 protein.

First, the BPV1 E2 protein was expressed in *E.coli* and purified by conventional chromatography. This purified functionally active protein was used to immunize the mice. A total of 22 MAbs that were reactive to the E2 protein in an enzyme immunoassay were isolated. Seventeen of these MAbs were directed against linear epitopes and were mapped within the region between amino acids 180–309 of E2. In fact, the last part of the amino-terminal transactivation domain and the first 10 amino acids of the hinge region, residues 180 to 218, appear to constitute highly immunogenic "hot spot", since epitopes for 12 of

these 17 MAbs were found to be localized within this region. All MAbs against the linear epitopes, with the exception of 1E2, were able to recognize the DNA-bound E2 protein. Epitopes for 5 of the 22 MAbs were mapped within the C-terminal DNA-binding-dimerization domain. All of these antibodies recognized the composite epitopes of native E2 and did not react with the denatured E2 protein (II, Fig. 2). None of the epitopes for the MAbs tested were mapped to the first 180 residues of the E2 protein. When only a purified transactivation domain, containing residues 1 to 208, of E2 was used for immunization, four MAbs against the region between aa 1 and 180 of E2 were obtained; however none of them was able to recognize E2-DNA complex in a mobility shift assay (A.Abroi, unpublished results). The most antigenic regions are usually the less ordered regions of the protein, and thus the antigenicity could be, to some extent, a reflection of the structure. So, we concluded from these results that in a native context, both the transactivation domain and the DNA binding-dimerization domain of E2 have a complex and relatively rigid structure, while the central, hinge region is highly mobile and flexible. This conclusion is supported by crystallographic data of C-terminal DNA-binding-dimerization domain of BPV1 E2 (Hegde *et al.*, 1992) and of the protease-resistant core (residues 66 to 215) of the HPV 18 E2 activation domain (Harris and Botchan, 1999), which show that both of these domains are tightly packed. In addition, a study of the HPV16 E2 protein has shown that the hinge is an unstructured region (Gauthier *et al.*, 1991). Polyclonal antibodies were generated against overlapping peptides covering the entire E2 protein and it was found that only antibodies against the hinge region could recognize the native, undenatured E2 protein.

MAb 1E2 (epitope within residues 184 to 190) was able to recognize neither E2-DNA nor E1–E2-ori complex in a mobility shift assay. Curiously, deletion of the first α helix from the BPV1 E2 protein revealed the epitope for MAb 1E2, and protein in the E2-DNA complex was recognized by the antibody (II, Fig. 2C). We suggested that the epitope for this MAb is probably buried within the compact structure of the N-terminal domain and is not accessible unless the structure of the molecule is distorted in some fashion. According to crystallographic data (Harris and Botchan, 1999), epitope for Mab 1E2 (TGHYSVR) is exposed on the surface of the protease resistant core (aa 66–215) of the protein. This epitope is situating on the opposite side of the protein comparing to the first α helix of the crystallized fragment of the protein. As the first 65 amino acids are not involved in the crystal structure of the protease resistant core of the activation domain of E2, it is difficult to say how are the first two amphipatic α helixes placed and are they able to hinder the epitope for MAb 1E2 or not.

The observation that antibodies recognized their respective epitopes in the DNA-bound E2 protein raised the possibility that some of them are able to block the E2 protein functions in the initiation of BPV1 origin replication. Therefore, the purified antibodies were tested in the transient replication assay.

MAb 5H4, directed against the C-terminal DBD prevented efficiently the formation of E2-DNA as well as E1–E2-origin complexes and also dissociated preformed complexes in a concentration-dependent manner (II, Fig. 3A, 5A). Co-transfection of MAb 5H4 with BPV1 minimal origin containing plasmid pUCAlu into CHO 4.15 cells resulted in a dose-dependent inhibition of replication (II, Fig.4A). The Fab' fragment of MAb 5H4 was also capable of dissociating the preformed E2-DNA complex and able to inhibit BPV1 origin replication (II, Fig. 5). So, we were able to suppress the papillomavirus origin replication by preventing E2 binding to its recognition sequence. This is a new and very specific way to demonstrate that the BPV1 E2 protein interaction with the specific recognition sequence within an origin of replication is essential for the initiation of viral DNA replication.

MAb 3F12 and MAbs 1H10 and 1E4, directed against the hinge region, recognized their respective E2 epitopes in the E1–E2-origin complex (II, Fig. 3A). At the same time they were capable of inhibiting BPV1 origin replication in CHO 4.15 cells (II, Fig. 4). However, the Fab' fragments of 1H10 and 3F12 had no effect in the transient replication assay (II, Fig. 5). These data suggested that MAbs directed against the hinge region sterically hinder the inter- or intramolecular interactions required for the replication activity of the E2 protein. We concluded that the conformational freedom of the E2 protein is important for its role in replication, the MAbs probably would not allow E2 to assume the proper conformation required for its replication activity. It has been shown that E2 proteins with deletions of the entire hinge region ($E2_{\Delta 220-309}$ and $E2_{\Delta 212-309}$) were unable to support replication (Winokur and McBride, 1992), but could efficiently enhance the binding of E1 to the replication origin (Winokur and McBride, 1996). So, we can suggest that some sequence is required between the two conserved domains to maintain some other function of E2, in addition to E1 binding, required for the replicational activity of the protein. Further studies in our lab have indicated that some critical length and flexibility, but not the particular amino acid sequence of the hinge region, is required for the ability of E2 to bind to DNA and to support replication in transient assay (Allikas *et al.*, 2000).

3.3. Inhibition of the BPV1 E2 protein activity by PNA (Reference III)

PNA-s are oligonucleotide analogues in which the deoxyribose phosphate backbone is replaced by non-charged N-(2-aminoethyl)glycine polymer (Nielsen *et al.*, 1991). The ability of PNA to associate with high affinity to complementary ssDNA, RNA and dsDNA (Demidov *et al.*, 1995; Egholm *et al.*, 1993; Peffer *et al.*, 1993) and, additionally, very high stability of PNA polymers in biological systems (Demidov *et al.*, 1994), makes this new class of biopolymers very attractive as a potential candidate for therapeutics. In the present study we have investigated if the specific peptide nucleic acid (PNA) is able to interfere with E2 binding to DNA.

First, we demonstrate that PNA can specifically associate with the E2BS by forming the complex with dsDNA. *In vitro* binding experiments using a radiolabelled E2BS demonstrated that binding of PNA to duplex DNA was sequence specific and salt-dependent, and preceding separation of DNA strands and linearization of PNA enhanced the formation of PNA-DNA complex *in vitro* (III, Fig.3). Stable PNA-DNA complexes have so far been demonstrated mostly for homopyrimidine PNA-s binding to homopurine targets. We showed that PNA is able to form a stable and specific complex with dsDNA of mixed sequence. The PNA bound to the dsDNA with sufficient affinity to specifically prevent binding of the E2 protein to its target site (III, Fig. 4). However, PNA was able to inhibit E2 binding *in vitro* under certain conditions. We were not able to demonstrate specific activity when the PNA binding was performed in E2 binding buffer, which had a KCl concentration of 100mM. Specific activity was observed when PNA was incubated with the DNA target in low salt buffer (TE) prior to addition to the E2 binding buffer. In this case, binding of PNA to its duplex target correlated with inhibition of E2 binding.

Next we looked at the ability of PNA to block the E2 protein dependent BPV1 origin replication by inhibiting E2 binding to its target site. Our aim was to block the E2BS within the origin of replication with PNA and to inhibit in this way the formation of replication initiation complex. In a cell culture model, specific inhibition was observed when PNA was incubated with origin containing plasmids in buffer with low salt concentration prior to electroporation. BS9-specific PNA bound effectively to Ori construct Msp15BS9 and resulted in the inhibition of replication. In the case of papillomavirus minimal origin plasmid pUCAlu, which contains E2BS11 and E2BS12, a weak binding of PNA was observed. But binding of PNA to both E2BS with low efficiency resulted in strong inhibitory effect in replication assay (III, Fig. 5). So, partial blocking of both E2BS within an origin of replication resulted in synergistic response of viral DNA replication. However, we were not able to detect any specific inhibitory effect when PNA was mixed with the plasmid pUCAlu and electroporated into the cells without pre-forming PNA-plasmid complex.

In summary, in this study we were able to suppress the E2 protein dependent BPV1 origin replication by blocking the E2BS with PNA and as a result, inhibiting E2 binding to its target site. This is another way to demonstrate that E2BS within an origin of replication is necessary and the BPV1 E2 protein interaction with the specific recognition sequence within an origin of replication is essential for the initiation of viral DNA replication.

4. CONCLUSIONS

1. The activation domain of the E2 protein has a discrete disruptable structure, which is sensitive to point-mutations. Substitutions K111A, K112A and E176A in the transactivation domain of E2 disrupted the conformation of the domain and induced oligomerization and aggregation of the protein, which resulted in their inactivity in replication and transcription and caused re-localization of E2 from the nucleus into the cytoplasm of the cell.
2. In a native context, both the transactivation domain and the DNA-binding-dimerization domain of BPV1 E2 have a complex and rigid structure, while the central, hinge region, is highly mobile and flexible. This conclusion is based on the immunogenic properties of the E2 protein. 17 of the 22 monoclonal antibodies obtained were directed against linear epitopes within the hinge region. Epitopes for 5 of the 22 MAbs were mapped within the C-terminal DBD and these antibodies recognized only the composite epitopes. In addition, MAbs against the hinge region and DBD but not against the activation domain were able to recognize the native form of the BPV1 E2 protein.
3. The flexibility of the hinge region is important for the replication activity of the E2 protein. MAb 3F12 and MAbs 1H10 and 1E4, directed against the hinge region, were capable of inhibiting BPV1 origin replication, while the Fab' fragments of 1H10 and 3F12 had no effect in the transient replication assay. We suggest that MAbs directed against the hinge region sterically hinder the inter- or intramolecular interactions required for the replicational activity of the E2 protein.
4. The BPV1 E2 protein interaction with the specific recognition sequence within an origin of replication is essential for the initiation of viral DNA replication *in vivo*. We were able to suppress the E2 protein dependent BPV1 origin replication by inhibiting E2 binding to its recognition sequence. The inhibition of replication was achieved in two different ways: first by MAb and Fab' 5H4, which prevented efficiently the formation of E2-DNA as well as E1–E2-origin complexes and second, by PNA, which blocked the E2BS within the origin of replication.

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STRUCTURE-FUNCTION RELATIONSHIP OF THE BOVINE PAPILLOMAVIRUS E2 PROTEIN

Summary

The papillomaviruses are small DNA viruses that induce squamous epithelial proliferative lesions (warts) in their natural hosts. The papillomavirus life cycle is greatly regulated by the viral transcription factor E2. This protein regulates viral protein levels by modulating the transcription of viral genes, is responsible for initiation of DNA replication and for the stable maintenance of the viral genome, which is achieved through facilitiation of the association of the viral genome with chromatin. E2 is a sequence-specific DNA-binding protein; it consists of three function-specific domains. The N-terminal part is an activation domain for transcription and replication, it is followed by the hinge region and the C-terminal DNA-binding dimerization domain. In this work, we have shown that in a native context, both the transactivation domain and the DNA-binding dimerization domain of BPV1 E2 have a complex and rigid structure, while the central, hinge region, is highly mobile and flexible. The activation domain of the E2 protein has a discrete disruptable structure, which is sensitive to point-mutations. Mutations in the transactivation domain of E2 disrupted the conformation of the domain and induced oligomerization and aggregation of the protein, which resulted in their inactivity in replication and transcription and caused re-localization of E2 from the nucleus into the cytoplasm of the cell. We also showed, that the flexibility of the hinge region is important for the replication activity of the E2 protein. In addition, we demonstrate that the BPV1 E2 protein interaction with the specific recognition sequence within an origin of replication is essential for the initiation of viral DNA replication *in vivo*. We were able to suppress the E2 protein dependent BPV1 origin replication by inhibiting E2 binding to its recognition sequence. The inhibition of replication was achieved in two different ways: first by MAb and Fab' 5H4, which prevented efficiently the formation of E2-DNA as well as E1-E2-origin complexes and second, by PNA, which blocked the E2BS within the origin of replication.

VEISE PAPILLOOMIVIIRUSEVALGU E2 STRUKTUURI JA FUNKTSIOONI SEOS

Kokkuvõte

Papilloomiviirused on väikesed kaheahelalised DNA-viirused, mis tekitavad oma peremeesorganismidel soolatüükaid ja muid epiteelkoe vohandeid. Papilloomiviiruse elutsüklis etendab keskset rolli viiruse transaktivaatorvalk E2. See reguleerib transkriptsiooniks, replikatsiooniks ja transformatsiooniks vajalike viirusevalkude ekspressiooni rakus. Samuti on E2 vajalik viiruse DNA replikatsiooniks ja viiruse genoomi pikaajaliseks säilimiseks rakus. E2 on DNA-seoseline valk ja seega reguleerib tema aktiivsust võime seonduda DNA-le.

Käesolevate teeside esimeses pooles antakse ülevaade veise papilloomiviiruse genoomist, geeniekspressioonist, transkriptsioonist ja replikatsioonist. Eraldi alapeatükid on pühendatud veise papilloomiviirusevalgu E2 omadustele ja funktsioonidele. Kolmandas peatükis on lühidalt kokku võetud käesoleva töö aluseks olevate publikatsioonide sisu.

Käesoleva doktoritöö peamised tulemused on järgmised.

Viirusevalk E2 koosneb kolmest domeenist: transkriptsiooniks ja replikatsiooniks vajalik aktivatsioonidomeen, DNA-seostumis- ja dimerisatsioonidomeen ja neid lahutav "hinge"regioon. E2 aktivatsioonidomeeni suunatud punktmutatsioonanalüüs näitas, et muteerides mõnda valgu konformatsioonile olulist aminohapet, rikutakse ära valgu funktsionaalse struktuur ning selle tulemusena on täheldatav E2-valgu oligomeriseerumine ja agregeerumine. Agregeerunud valk on inaktiivne ega suuda täita oma rolli viiruse transkriptsioonil ja replikatsioonil. Meie katsed näitavad ka, et E2 aktivatsiooni- ja DNA-seostumisdomeen on mõlemad jäiga struktuuriga ja korrektselt pakitud, kuid neid lahutav "hinge"-piirkond liikuv ja struktureerimata. Kasutades monokloonseid antikehasid, näitasime, et E2 hingepiirkond on samuti vajalik valgu aktiivsuseks replikatsioonis.

Viiruse DNA replikatsiooniks rakkudes on vajalikud kaks viirusevalku: replikatsiooni initsiaatorvalk E1 ja transaktivaatorvalk E2. Replikatsiooni initsieerimiseks peavad E1 ja E2 mõlemad seostuma replikatsiooni alguskohaga, kusjuures E2 roll on kaasa aidata E1 järjestusspetsiifilisele seostumisele. Käesolevates töödes on õnnestunud inhibeerida E2-st sõltuvat DNA replikatsiooni, blokeerides E2 seostumist tema sidumisjärjestusele. Esimesel korral kasutasime selleks monokloonset antikeha, mis takistas valgu seostumist tema sidumisjärjestusele, teisel korral blokeerisime E2 sidumisjärjestuse replikatsiooni alguspunktis PNA-ga (peptiidne nukleiinhape).

ACKNOWLEDGEMENTS

First of all I want to thank my family for their support, encouragement, love and friendship. Special thanks are going to my husband, whose love and care have been essential for me to complete the thesis.

I wish to express my gratitude to my supervisor Prof. Mart Ustav, for his advice and support.

I also wish to thank all my former and present colleagues in the lab, for fruitful cooperation, discussions and help, and creating a friendly and creative atmosphere in the lab. The following people have remarkable role in my scientific career:

Aare Abroi, my nearest co-worker, has helped me in computer works. I appreciate his skepticism and helpful discussions throughout our co-operation.

Drs. Jaanus Remme, Juhan Sedman, Tanel Tenson, Margus Pooga and Jüri Parik have always supplied me with good ideas. I would like to thank them for their helpful comments and opinions.

My special thanks go to Dr. Aksel Soosaar for supporting young scientists and keeping people together and Anne Kalling, for her technical help in cell culture.

This work was partly supported by grants from Estonian Science Foundation, The Independent Estonian Cancer Research Fund, Howard Hughes Medical Institute and The International Science Foundation.

PUBLICATIONS

I

Abroi, A., **Kurg, R.** and Ustav, M. (1996)
Transcriptional and replicational activation functions in the bovine papillomavirus type 1 E2 protein are encoded by different structural determinants.
J. Virol. 70(9), 6169–6179.

JOURNAL OF VIROLOGY, Sept. 1996, p. 6169–6179
0022-538X/96/$04.00+0

Vol. 70, No. 9

Transcriptional and Replicational Activation Functions in the Bovine Papillomavirus Type 1 E2 Protein Are Encoded by Different Structural Determinants

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Received 20 February 1996/Accepted 10 June 1996

A set of E2 proteins with mutations in the amino-terminal transactivation domain was made by a scheme called clustered charged-to-alanine scan. These mutant E2 proteins were tested for expression, stability, and compartmentalization in cells and for sequence-specific DNA binding, as well as in functional assays for transcriptional and replicational activation. We identified four groups of mutants. First, mutants K111A, K112A, and E176A were unable to activate replication and transcription because of oligomerization-induced retention of oligomers in the cytoplasm. Second, although fractions of the mutant proteins E74A and D143A/R172C existed in the oligomeric form, they were localized in the nucleus. Certain fractions of these proteins existed as a dimer able to form a specific complex and activate replication; however, these proteins were inactive in transcriptional activation. Third, mutants R37A and D122A were localized in the nucleus, existed in the dimeric form, supported replication efficiently, and were severely crippled in transcriptional activation. The fourth group of mutants did not differ considerably from the wild-type protein. The activation of transcription by the wild type as well as mutant E2 proteins was dependent on the concentration of input E2 expression vector DNA and had a bell-like shape. We suggest that the reduction of transcriptional activation at higher E2 concentrations, the self-squelching activity, is caused by oligomerization of the E2 transactivator and is one of the mechanisms for the regulation of E2 activity. Our results also show that transcriptional and replicational activation activities are encoded by different determinants in the E2 protein.

Transcriptional activators modulate the activity of the basal transcription machinery in eukaryotic cells, and they function at the assembly stage of the transcription initiation complex or stimulate the formation of the elongation complex (for reviews, see references 30 and 61). In addition, transcription factors are frequently involved in the modulation of DNA replication (for a review, see reference 12). Small DNA viruses, like papillomaviruses, provide a useful model system to study the functional and structural determinants of transcription factors involved in the regulation of transcription and replication. The 48-kDa full-length E2 protein of bovine papillomavirus type 1 (BPV1) was identified as a typical eukaryotic transcription activator, with all the properties of such proteins, including modular DNA binding and activation domains and the ability to activate heterologous promoters from DNA binding sites at a distance from the transcription start site. It has been demonstrated previously that E2 binding sites function synergistically in vivo and mediate E2-dependent induction of the expression of viral early genes of papillomaviruses (19, 21, 47, 51, 52, 60). That fact served initially as an explanation for the involvement of the E2 protein in the regulation of viral DNA replication as a transcriptional activator of the expression of viral early genes (13, 38). Later, however, it was shown that E2 protein directly participates in the replication process of viral DNA during each of the three stages of the viral life cycle. First, after the initial entry of the viral genome into the nucleus of the cell, papillomavirus DNA is amplified, i.e., papillomavirus DNA replicates faster than does cellular DNA (27). The E1 and E2 proteins are the only viral factors used for the initiation of DNA replication from the papillomavirus origin at this amplification stage of the viral life cycle (9, 40, 56, 57, 59). The minimal origin of replication has been identified as the essential *cis* element in this process and consists of three sufficient and necessary elements, an AT-rich region, E1 binding site, and E2 binding site (55). The second stage of the virus life cycle, the latent replication, is established when the optimal copy number of the viral genome is achieved. The establishment of stable extrachromosomal replication of the BPV1 genome requires at least six E2 binding sites in *cis* and E1 and E2 proteins in *trans*, in addition to the minimal origin (37). During the third stage, the vegetative stage of the papillomavirus life cycle, DNA amplification is initiated in terminally differentiated keratinocytes. It has been suggested that the E2 protein is directly involved in the regulation of this process (8). Therefore, the E2 protein is the master regulator of viral gene expression as well as all the stages of viral DNA replication.

Structural and mutational analyses of the E2 protein have revealed three distinct functional domains. The N-terminal part (residues 1 to 210) is an activation domain for transcription and replication. It is followed by the unstructured hinge region (residues 210 to 324) and the carboxy-terminal DNA binding-dimerization domain (residues 325 to 410) (15, 19, 31, 32). The structure of the carboxy-terminal DNA binding-dimerization domain has been solved by X-ray analysis and has revealed a novel dimeric DNA binding-dimerization motif (20). A similar organization of a DNA binding-dimerization domain has been determined for Epstein-Barr virus nuclear antigen 1 (5). However, very little, except for computer predictions, is known about the structural organizations and properties of the hinge region and the amino-terminal transactiva-

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tion domain of the E2 protein. The dimeric E2 protein binds an interrupted palindromic sequence, ACC(N_6)GGT; dimers are stable and highly resistant to urea denaturation; and a small 85-residue protease-resistant C-terminal core domain retains the ability to bind DNA (10, 14). E2 binding sites function synergistically in vivo because of cooperativity in the binding of E2 to clustered binding sites, which is mediated via the amino-terminal transactivation domain (35, 52). E2 is able to loop DNA when E2 binding sites are widely spaced on the template (22). The E2 protein binds multiple proteins, including cellular replication protein A, papillomavirus replication protein E1, cellular transcription activator Sp1, and basal transcription factors TATA-binding protein (TBP) and TFIIB (4, 25, 26, 28, 34, 39, 50). Sp1 and E1 are known to bind the N terminus of E2, while TBP and TFIIB interact with the C-terminal part of the E2 molecule. Very little, though, is known about the structural determinants of E2 involved in the direct interactions with these or other proteins. To identify structurally and functionally important determinants within the E2 protein, a number of deletion and in-frame insertion mutations have been made (13, 19, 58). The majority of these mutations abolished the replication and transcriptional activities of the E2 protein. These studies indicate that the E2 DNA binding-dimerization domain, in addition to the intact transactivation domain, is needed for this protein to function in replication and transcriptional activation. However, it was not determined which E2 mutations disrupt its activity directly and which inhibit E2 function indirectly by interfering with the DNA binding or stability of the E2 protein.

Here we characterize 15 different mutant E2 proteins with point mutations within the transactivation domain. These mutations were designed by a scheme called clustered charged-to-alanine scan (11). Analyses of these mutants showed that the structural determinants responsible for the transcriptional activation function in the E2 protein could genetically be separated from the structural determinants responsible for the activation of replication. In addition, our data indicate that oligomerization of the E2 protein mediated by the N-terminal transactivation domain could be one of the additional mechanisms regulating the activities of this protein in transcription and replication.

MATERIALS AND METHODS

Plasmids. E1 expression vector pCGEag, E2 expression vector pCGE2, and replication reporter pUCAlu have been described previously (56). Reporter plasmids pP2CAT and pSV3BS9CAT were kind gifts of Paul Szymanski. Plasmid pSV3BS9CAT contains three E2 high-affinity binding sites (binding site 9 [BS9]), three 21-bp GC-rich repeats, and an enhancerless simian virus 40 (SV40) early promoter (nucleotides 5172 to 103 from SV40) in front of the chloramphenicol acetyltransferase (CAT) gene. Plasmid pP2CAT contains BPV1 nucleotides 7476 to 94 (including the P2 promoter) in front of the CAT gene. Plasmid p53:E2 was generated by substituting the E2 transactivation domain for the p53 transactivation domain (amino acids 1 to 58) by using *Xba*I and *Sca*I restriction sites in pCGE2epi (55) (see Fig. 1A). Plasmid VP16:E2 has been described previously (25) and contains 80 C-terminal amino acids from VP16 fused to the C terminus of E2 (starting from amino acid 250) in the context of pCG.

To generate mutant E2 proteins, the *Xba*I-*Kpn*I fragment from pCGE2 was cloned into pUC19. E2 protein mutants were created by the PCR-based method of Mikaelian and Sergeant and then inserted back into pCGE2 at the *Xba*I-*Kpn*I sites (33). All mutants were verified by sequencing. The introduced mutations at the nucleic acid and protein levels are presented in Table 1.

Cells and transfections. Transient-replication assays were carried out as described by Ustav and Stenlund (56). CHO cells were electroporated with 100 ng of pUCAlu DNA, 500 ng of pCGEag, and 250 ng of wild-type or mutant pCGE2. Replication assays were quantitated with a PhosphorImager SI (Molecular Dynamics). For transcription assays, CHO cells were electroporated with 250 ng of the respective reporter and various amounts of pCGE2 or derivatives. Forty hours later, cells were harvested and lysed by freezing-thawing, and CAT assays were performed by the thin-layer chromatography method in the linear range (43). The acetylated form of chloramphenicol was quantitated with a liquid scintillation counter. Activities were normalized to the total amount of protein in the lysate, as determined by the Bradford assay (6). The values shown are the averages of at least three independent transfection experiments.

TABLE 1. Generated point mutations in the E2 coding sequence

Starting position	Codon		Change at the amino acid level	Name of mutant
	Wild type	Mutated[a]		
2641	CAA	**GCA**	Gln-12→Ala	Q12A
2644	GAA	**GCA**	Glu-13→Ala	E13A
2665	GAG	**GCG**	Glu-20→Ala	E20A
2716	AGA	**GCA**	Arg-37→Ala	R37A
2722	GAG	**GCG**	Glu-39→Ala	E39A
2746	AGG	**GCG**	Arg-47→Ala	R47A
2809	AGA	**GCA**	Arg-68→Ala	R68A
2827	GAA	**GCA**	Glu-74→Ala	E74A
2875	GAA	**GCA**	Glu-90→Ala	E90A
2938	AAG	**GCG**	Lys-111→Ala	K111A
2941	AAA	**GCA**	Lys-112→Ala	K112A
2971	GAT	**GCT**	Asp-122→Ala	D122A
3034	GAC	**GCC**	Asp-143→Ala	D143A
3133	GAG	**GCG**	Glu-176→Ala	E176A
3121	CGC	**TGC**	Arg-172→Cys	R172C

[a] Mutated nucleotides are in bold letters.

DNA binding assay. For the preparation of COS-7 extracts, cells transfected by electroporation with expression plasmids were removed from the semiconfluent 100-mm-diameter plates with a rubber policeman, washed, and lysed in 100 μl of lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.1 mM EDTA, 0.35% Nonidet P-40, 10 mM dithiothreitol, and protease inhibitors) on ice for 30 min. The cell debris was removed by centrifugation, glycerol was added to a final concentration of 20%, and the extracts were aliquoted and stored at −70°C. The amounts of E2 protein in cell extracts were determined by enzyme-linked immunosorbent assay (ELISA) using bacterially expressed E2 protein as a standard. Equal amounts of lysates containing E2 mutant proteins were used in DNA binding assays. For gel shifts, respective amounts of cell extracts were incubated in 10 μl of binding buffer (10 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 15% glycerol, 5 mg of bovine serum albumin [BSA] per ml, 1 μg of leupeptin per ml, 1 μg of aprotinin per ml) at room temperature for 15 min in the presence of 1 μg of sonicated salmon sperm DNA and 0.2 ng of end-labeled probe. The double-stranded high-affinity BS9 of BPV1 (5′-ACAAAGTACCGTTGCCGGTCGAA-3′) was used as a probe. Protein-DNA complexes were separated from unbound DNA on 6% PAAG (80:1) in 0.25× Tris-borate-EDTA. Gels were dried and exposed to X-ray film. Quantitative analysis was done with a PhosphorImager SI (Molecular Dynamics). For pronase digestion experiments, increasing amounts of pronase were added; the reaction mixtures were incubated for an additional 10 min at room temperature before or after DNA binding, loaded onto a polyacrylamide gel, and processed as described above.

Immunoblotting of E2. Comparisons of the expression levels and estimations of the intactness of E2 mutant proteins in COS-7 and CHO cells were done by Western blot (immunoblot) analysis using rabbit anti-E2 polyclonal antibody. Cells from a semiconfluent 60-mm-diameter dish were lysed 36 h after electroporation in 200 μl of Laemmli sample buffer and boiled for 10 min, and Western blotting was done as previously described (18). In addition, COS-7 cell extracts prepared for the DNA binding assay were also tested for the concentration of E2 protein by immunoblotting.

Glycerol density-gradient centrifugation. Extracts (100 μl) of COS-7 cells transfected with E2 or mutants were layered on the top of 5-ml linear glycerol gradients (10 to 30%; 10 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 100 mM NaCl, 1 mM dithiothreitol, and protease inhibitors). The samples were centrifuged in a Beckman SW55 rotor at 45,000 rpm for 15 h at 4°C. Marker proteins, alcohol dehydrogenase (150 kDa), and BSA (66 kDa) were run in parallel gradients. The gradient was dripped into 14 fractions, and the bottom of the tube was washed with 250 μl of buffer containing 10% glycerol. Collected fractions were subjected to quantitative ELISA analysis and sequence-specific gel shift assay. The final wash was done with 50 μl of Laemmli buffer in order to solubilize the precipitated protein, which was then subjected to sodium dodecyl sulfate (SDS)-PAAG and Western blot analysis with monoclonal antibody 1E2 (epitope, amino acids 180 to 190).

Immunofluorescence. For immunofluorescence analysis, COS-7 cells were transfected by electroporation with 150 ng of E2 expression vector pCGE2. After that, cells were allowed to adhere to coverslips, grown for 48 h, and fixed in cold (−20°C) acetone-methanol (1:1) for 10 min. Coverslips were washed three times with phosphate-buffered saline (PBS), and primary antibody was added at a concentration of 10 ng/μl (diluted in PBS containing 1 μg of BSA per μl). Coverslips were incubated for 1 h at room temperature and washed three times with PBS, and fluorescein isothiocyanate-conjugated goat anti-mouse secondary

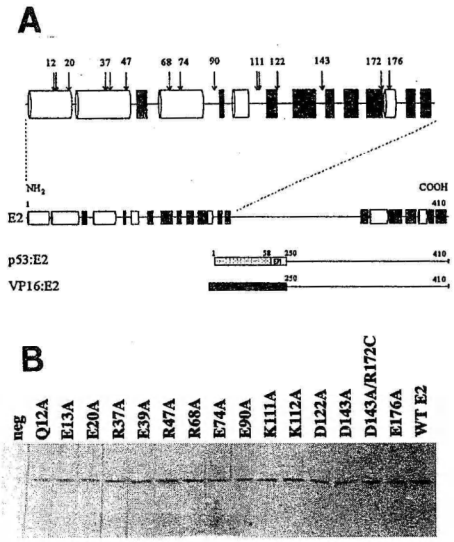

FIG. 1. (A) Schematic representations of designed E2 point mutations and chimeric E2 proteins. The prediction of the secondary structure (cylinder, alpha-helix; shaded box, β-sheet) was made by the method of Rost and Sander for the N-terminal part of the E2 molecule (41). The structure of the C-terminal part of the E2 protein is presented according to the X-ray structure (20). The locations of mutations are indicated by arrows. (B) Immunoblot analysis of the expression of E2 proteins in CHO cells. Cell lysates were prepared from cells transfected with a wild-type (WT) or mutant E2 expression plasmid. Thirty microliters of cell lysate was loaded in each lane. The negative (neg) control lysate was prepared from cells electroporated with carrier DNA only. The mutant used is indicated above each lane.

antibody (Sigma) was added at the concentration recommended by the manufacturer. After 1 h, coverslips were washed three times with PBS and mounted on glass slides in 50% glycerol. Cells were examined on a Olympus Vanox-S AH2 microscope. Monoclonal antibody 3F12 (epitope, amino acids 199 to 207) was used as the primary antibody.

RESULTS

Expression of mutant proteins. To identify the structural and functional determinants of the E2 protein involved in the activation of transcription and/or replication functions, a set of mutant E2 proteins was constructed. Single conserved charged residues in the amino-terminal transactivation domain were replaced with alanine. The introduced point mutations are described in Fig. 1A on the putative secondary structure of the E2 protein and summarized in Table 1 (41). We used two different cell lines (CHO and COS-7) to test the stabilities and expression levels of mutant proteins in vivo. The expression vector was delivered into cells by a standard electroporation procedure (56), cells were lysed, and the expression level and stability of each mutant protein were tested by ELISA and Western blot analysis. All mutant E2 proteins were expressed at similar levels compared with the wild-type E2 protein in CHO (Fig. 1B) and COS-7 (data not shown) cells. No degradation of mutant proteins in the cell lysate was observed. These data indicated that the constructed mutant proteins were stable and expressed at approximately the same level.

The activities of mutants in transient-replication assays. We used two different origin-containing plasmids (pUCAlu and pP2CAT) in transient-replication assays with CHO cells to study the abilities of E2 mutants to activate replication. The expression plasmid for mutant E2 proteins was cotransfected with the origin plasmid and E1 expression vector pCGEag into CHO cells by electroporation, and episomal DNA was extracted by alkaline lysis, purified, digested with *Dpn*I and linearizing enzyme, and analyzed by Southern blotting as described earlier (56) (Fig. 2). All mutant E2 proteins, except for K111A, K112A, and E176A (Fig. 2, lanes 21 to 24, 31, and 32), supported the replication of origin-containing plasmid pUCAlu in CHO cells in the transient-replication assay. Similar results were obtained with origin-containing plasmid pP2CAT (data not shown). Eight independent replication assays were quantitated and normalized to wild-type E2 protein activities (see Fig. 4B). Two mutants, R37A and E90A, seem to be up-mutations and activate the replication of the BPV1 origin 1.6 and 2.2 times better, respectively, than does the wild-type protein.

The activities of E2 mutants in transcriptional activation of promoters. It has been shown that different promoters can be activated to different extents by the BPV1 E2 protein, depending on the promoter content (17, 54). The activities of mutants were tested in CAT assays using two different E2-dependent reporter plasmids. Plasmid pP2CAT contains the BPV1 URR and the native P2 promoter, including the transcription initiation site (nucleotide 89). Reporter plasmid pSV3BS9CAT contains an SV40 early promoter in which the 72-bp enhancer is replaced by three E2 binding sites (BS9) in front of the basal promoter driving the CAT coding sequence (Fig. 3A). The reporter plasmid either alone or with increasing amounts of the E2 expression vector was transfected by electroporation into CHO cells. Forty hours later, the extracts were prepared for CAT assays (43). The CAT activities in the extracts of cells transfected with mutant E2 proteins were normalized to the basal activity without added E2 expression plasmid and to the total amount of protein. We tested the transactivation function of all mutants at various concentrations of transfected expression vectors (0.0004 to 3 μg of DNA per 7×10^6 cells). We observed that the level of activation of promoters by wild-type E2 and E2 mutants was dependent on the amount of the input DNA of the E2 expression vector (Fig. 3B to E). The extracts of cells transfected with expression constructs for E2 mutants E74A, K111A, K112A, E176A, and D143A/R172C showed

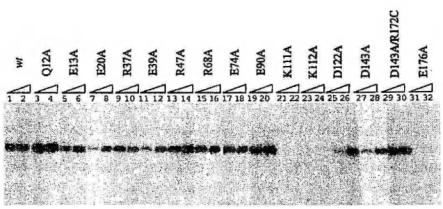

FIG. 2. The abilities of mutant E2 proteins to activate the replication of the BPV1 origin in CHO cells. Cells were electroporated with 100 ng of reporter plasmid pUCAlu, 500 ng of pCGEag, and 250 ng of pCGE2, which expresses wild-type (*wt*) E2 or derivatives. Cells were harvested either 36 or 48 h (◿) after electroporation. Episomal DNA was digested with *Dpn*I and linearizing enzyme *Hin*dIII and analyzed by Southern blotting.

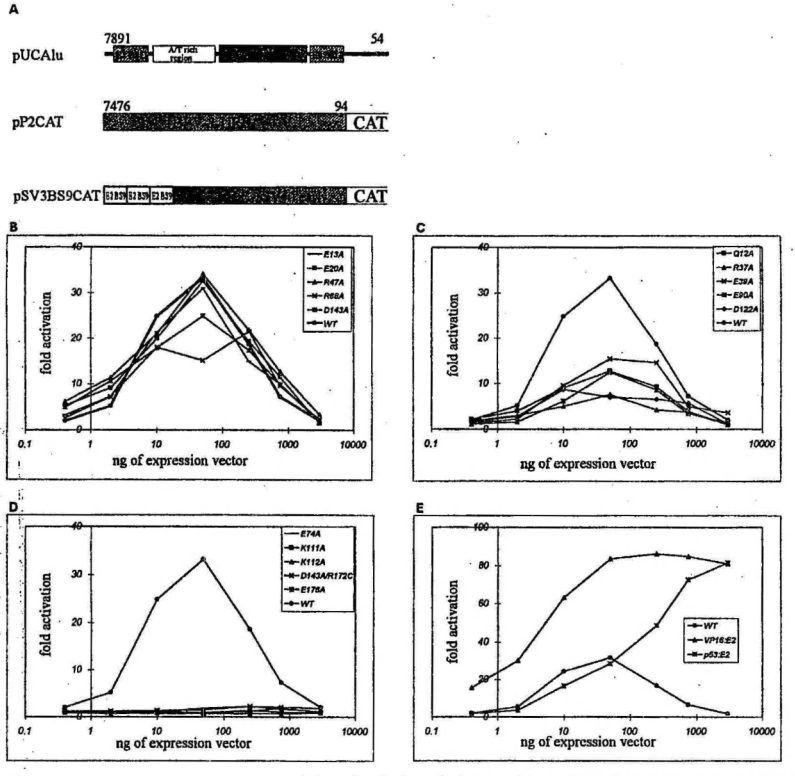

FIG. 3. (A) Structures of the reporter plasmids used in transcription and replication activation assays. The numbers indicate positions in the BPV1 URR sequences. (B to E) Transcription activation assays with E2 protein mutants. CHO cells were transfected with increasing amounts of the expression vector for E2 mutants or chimeric E2 proteins, as indicated, and with 250 ng of reporter plasmid pSV3BS9CAT. Normalized CAT activities were determined 40 h after transfection. The symbol for each mutant proteins is indicated in the corresponding panel. (B) Mutants with nearly wild-type protein properties in transcription activation assays. (C) Mutant proteins whose transcriptional activities have decreased to approximately 50% of that of the wild-type protein. (D) Inactive mutant proteins. (E) Transcriptional activation properties of chimeric proteins p53:E2 and VP16:E2.

essentially basal levels of CAT activity, independent of the amount of input E2 expression vector, with pSV3BS9CAT (Fig. 3D) or pP2CAT reporters (data not shown). In the case of active mutants, the optimal concentration of E2 expression vector was within 80 to 100 ng of pCGE2 DNA per transfection and resulted in activation of more than 30 times the basal level (Fig. 3B to E). In all cases, higher-than-optimal E2 concentrations resulted in a reduction in transactivation (Fig. 3B and C), most likely caused by the self-squelching activity of E2. This activity for the E2 protein has been described previously, and the mechanism for this is unknown (39). The suppression of activation at higher E2 concentrations is specific for the E2 transactivation domain because it is not observed for p53:E2 and VP16:E2 chimerical proteins, in which the E2 transactivation domain is replaced by the activation domains of p53 and VP16, respectively (Fig. 3E). The expression levels of wild-type and hybrid p53:E2 and VP16:E2 proteins in CHO cells were comparable (data not shown).

The activities of R37A, D122A, and D143A were dependent on the promoter context of the reporter plasmid used. Point mutations R37A and D122A (Fig. 3C and 4B) resulted in a sharp decrease in transactivation to 20% of that of the wild-type protein from reporter pSV3BS9CAT; however, with pP2CAT as the reporter, these mutants were moderately active (Fig. 4B). In the case of D143A, the same tendency was observed; however, the corresponding relative activities were higher. The third group of mutants (Q12A, R68A, and E90A) were 50% active with both reporters compared with wild-type E2.

The results described above show that point mutations K111A, K112A, and E176A create proteins inactive for both functions, transcriptional and replicational activation. E2 pro-

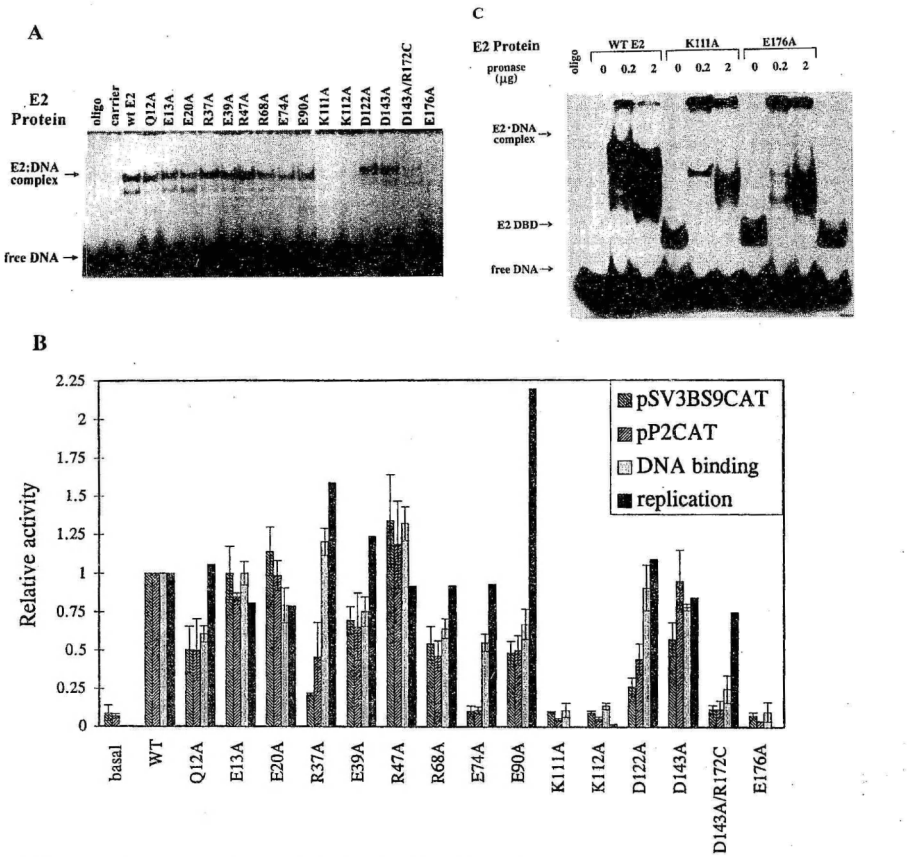

FIG. 4. (A) DNA binding assay with mutated E2 proteins. The sequence-specific binding of E2 to one DNA palindromic target was determined by gel retardation assays. The E2 proteins were expressed in COS-7 cells. Band shift assays were performed with 2 μl of cell extract and 0.5 ng of radiolabeled BS9 for 15 min at room temperature. Protein-DNA complexes were resolved on 6% PAAG in 0.25× Tris-borate-EDTA. oligo, oligonucleotide; wt, wild type. (B) Comparison of the activation of transcription from two reporter plasmids, replication, and DNA binding abilities of E2 protein mutants. The radioactive signals of gel shift and replication assays were quantitated with a PhosphorImager. The wild-type (WT) E2-specific signal in all assays was set at 1.0. For transcription activation assays, CHO cells were electroporated with 250 ng of the indicated reporter and 250 ng of pCGE2 or a derivative. Normalized CAT activities were determined 40 h after transfection. In all cases, the values shown are the results (± standard errors) of at least three independent transfection experiments. (C) Pronase treatment of lysates containing inactive E2 mutant proteins. The reaction mixtures were incubated first with 0.2 or 2 μg of pronase for 10 min at room temperature and then with labeled BS9 for an additional 10 min. Protein-DNA complexes were resolved on 8% PAAG in 0.25× Tris-borate-EDTA. DBD, DNA binding domain.

tein mutants E74A and D143A/R172C were crippled in the transcriptional activation function but were competent to support replication in the transient-replication assay. E2 protein mutants R37A and D122A were also defective in transcriptional activation, though this defect was less obvious in the case of the P2 promoter. These data may indicate that the replication and transcriptional transactivation functions of the BPV1 E2 protein are encoded at least in part by separate determinants.

The activities of mutant E2 proteins in sequence-specific DNA binding. Sequence-specific DNA binding is essential for the expression of most of the functional activities of the E2 protein. Therefore, we decided to study the activities of mutant E2 proteins in sequence-specific DNA binding assays, even though mutations were made in the N-terminal transactivation domain of this protein. The expression vector pCG allows high-level expression of the cloned cDNA in COS cells, which makes the quantitation of the concentrations of mutant E2

proteins in lysates by ELISA more accurate. The expression levels of the E2 protein and mutants in CHO cells were sufficient for the detection of specific gel shift; however, they were difficult to measure by quantitative ELISA using the standard curve obtained with bacterially expressed E2 protein. In addition, the expression levels of wild-type and mutant E2 proteins were sufficient for the separation of lysates in the glycerol gradient to establish the physical oligomerization status of E2 mutants. The lysates of transfected COS-7 cells were tested for the ability to form an E2-specific complex with double-stranded high-affinity BS9 (5′-ACAAAGTACCGTTGCCGG TCGAA-3′) of the BPV1 E2 responsive element (E2RE1) by quantitative gel shift assay. Lysates obtained by the sonication of cells or by the use of 0.35% nonionic detergent Nonidet P-40 gave essentially the same results in gel shift assays. The shifted double band was specific for E2, as it was supershifted by anti-E2 monoclonal antibody 3F12 (epitope, residues 199 to 207) and was inhibited by an excess of the nonlabeled specific oligonucleotide (data not shown) (23). The cloned E2 open reading frame carried a mutation from the initiating methionine (codon ATG) to isoleucine (codon ATC) in the E2 transcription repressor and was unable to express the repressor form of E2. Therefore, the appearance of the lower band is not caused by the shorter repressor form of the E2 protein. A similar gel shift pattern has been observed in several instances for baculovirus-, yeast-, or bacterium-expressed E2 protein and probably reflects a different conformation of the complex (23, 35, 50). Equal amounts of lysates of cells containing wild-type and mutant E2 proteins were used in the binding reaction mixtures, and complexes were separated in a standard gel shift assay. Our data indicate that most of the E2 mutants had approximately the same or, in some cases, even better ability to bind DNA (Fig. 4A); however, E2 mutants with point mutations K111A, K112A, and E176A, which were inactive for both replication and transcription, failed to form the specific protein-DNA complex able to enter the gel. These data indicate that mutations K111A, K112A, and E176A seem to abolish the DNA binding abilities of these proteins and therefore the functioning of the E2 protein in transcription and replication, or to induce the formation of E2 protein oligomers or aggregates unable to enter the gel. Mutants E74A and D143A/R172C showed reduced affinity in the DNA binding assay (50 and 30%, respectively, from the wild-type level). The carboxy-terminal part of the E2 protein is responsible for DNA binding and contains a protease-resistant core (14). In order to test whether introduced mutations K111A, K112A, and E176A destroyed the total folding of the E2 protein, including the DNA binding domain, induced a protein conformation inactive for interaction with DNA, or resulted in a protein with high-level oligomerization-aggregation properties, we subjected the lysates to pronase treatment. Pronase treatment of the lysates of cells transfected with K111A, K112A, or E176A revealed the E2-specific DNA binding activities of these mutants (Fig. 4C). The same results were obtained when the extracts were treated with protease either before or after the addition of DNA to the binding reaction mixture. These data indicate that the point mutations introduced into the amino-terminal part of the molecule did not destroy overall folding of the E2 protein, but some other mechanism prevented the formation of an E2-DNA complex of the usual size. We also observed that the trapping of a specific DNA probe in a gel well, especially in the case of K111A, K112A, and E176A mutants, disappeared upon treatment with pronase.

One interesting group consisted of E2 mutants which were able to support DNA replication at the wild-type level in the transient-replication assay but had severely reduced transcriptional activities. E2 mutants with point mutations R37A and D122A bound DNA as well as wild-type E2 did; however, E74A and specifically D143A/R172C bound DNA at reduced levels (Fig. 4A). Again, considerable entrapment of the specific probe could be detected in wells.

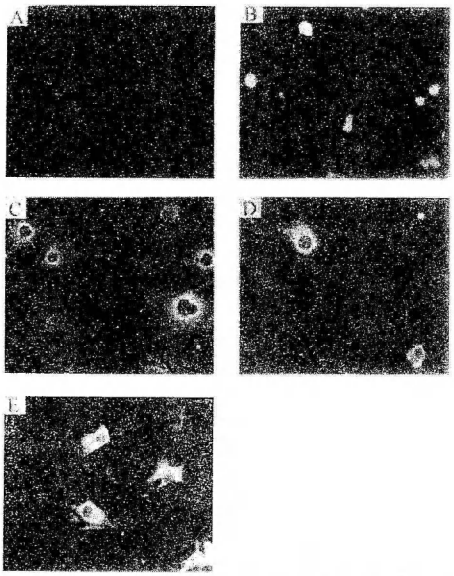

FIG. 5. The localization of E2 mutant proteins in COS-7 cells by immunofluorescence analysis. COS-7 cells were transfected with carrier DNA (A), pCGE2-wt (B), pCGE2 K111A (C), pCGE2 K112A (D), and pCGE2 E176A (E). Cells were probed with monoclonal antibody against the E2 protein and subsequently with fluorescein isothiocyanate-conjugated anti-mouse antibody as described in Materials and Methods.

DNA binding activities were quantitated with a PhosphorImager and normalized to the binding activity level of expressed wild-type E2 protein. The results, DNA binding together with the activities of mutant proteins to activate transcription from the two different reporter constructs and quantitation of the replication data, are shown in Fig. 4B. These results confirm earlier observations that the DNA binding ability of E2 is absolutely required for specific transcriptional activation. Furthermore, these data indicate that the transcription activation domain of the E2 protein has considerable impact on the regulation of the functioning of this protein.

Compartmentalization of mutant proteins in COS-7 cells. The E2 transactivator contains three regions which can constitute a potential nuclear localization signal for this protein, BR1 (residues 47 to 49), BR2 (residues 107 to 115), and BR3 (residues 340 to 353). It has been shown that only BR3 is able to mediate active transport of a hybrid protein (β-galactosidase) into the nucleus; however, BR2 has been suggested to work as the major nuclear localization signal of E2TA. Partial or complete deletion of this sequence or a proline-to-glycine mutation at residue 106 causes mutant proteins to accumulate in the cytoplasm (46). Two such mutations, K111A and K112A, were

made in the BR2 region, therefore raising the possibility that the inactivity of these mutants in biological assays is caused by the failure of this protein to enter the nucleus. We studied the nuclear localization of all mutant proteins in COS-7 and C127 cells. The localization of E2 mutants in COS-7 cells is presented in Fig. 5. We used mouse monoclonal antibody 3F12, directed against the epitope within residues 199 to 207, for the detection of the E2 protein. Essentially, the same results were obtained when another monoclonal antibody, 3C1 (residues 280 to 310), or polyclonal rabbit immunoglobulin G against the E2 protein was used in these experiments (data not shown). Figure 5A and B show the immunostaining of COS-7 cells transfected with a mock and wild-type E2 expression construct, respectively. We detected specific exclusive nuclear immunostaining in cells expressing wild-type E2 protein compared with mock-transfected cells. Contrary to wild-type E2, mutant proteins K111A (Fig. 5C), K112A (Fig. 5D), and E176A (Fig. 5E) are preferentially localized in the cytoplasm, with very little, if any, detectable specific immunostaining in the nucleus. All the remaining mutant proteins were localized in the cell nucleus (data not shown).

Oligomerization of mutant proteins. The expression levels of all mutant proteins in CHO (Fig. 1B) and COS-7 cells were comparable, as estimated by Western blotting of the total cell extract and soluble cell extract used for gel shift analysis. In the case of mutants K111A, K112A, and E176A, we were unable to detect the formation of a specific E2-DNA complex with mobility similar to that of wild-type E2 protein; however, we observed considerable retention of the probe in the wells of the gel, indicating that mutant E2 proteins may form larger oligomers which are unable to enter the gel. Pronase digestion of the lysate revealed protease-resistant cores within the mutant E2 proteins (Fig. 4C) as well as the disappearance of the signal in wells, supporting the possibility that these mutant proteins exist as oligomers or larger aggregates which are still able to bind DNA but are unable to enter the gel or nucleus. We studied the oligomerization status of wild-type and mutant E2 proteins in COS-7 lysates by using the sedimentation of this protein in 10 to 30% glycerol gradients. Five-milliliter gradients were dripped into 14 fractions after centrifugation at 45,000 rpm for 15 h at 4°C with a Beckman SW55 rotor. The fractions were analyzed for E2 protein content by a specific monoclonal antibody 3F12-based sensitive ELISA and for E2 protein sequence-specific DNA binding. We used PhosphorImager quantitation of the gel shifts in the region of the unit-sized E2-DNA complex and in the region near wells for the detection of the larger protein-DNA complex, which has difficulties in entering the gel. The results are presented in Fig. 6. The distribution of the E2 protein from the lysate of COS-7 cells transfected with the wild-type E2 expression construct is shown in Fig. 6A. The E2 protein moves as a single peak in the gradient and could be found mostly in fractions 7 and 8. These fractions also show the maximal levels of E2 protein DNA binding activity. We did not detect any considerable entrapment of the probe in the wells of this gel. For marker proteins, alcohol dehydrogenase (150 kDa) peaked in fraction 2 and BSA (66 kDa) peaked in fraction 9. We also did not detect any wild-type protein precipitated onto the bottom of the centrifuge tube (Fig. 6B). From these results, we concluded that the wild-type protein exists in COS-7 lysates as a soluble dimeric protein. Completely different results were obtained with inactive E2 protein mutants K111A, K112A, and E176A. Very little, if any, E2 protein was detected in the fractions in which the dimeric wild-type protein was found. Instead, we detected the E2 protein by ELISA in the near-bottom fractions, fractions 1 to 5 (Fig. 6A), and in the precipitated form (Fig. 6B). An inspection of the gel shifts indicated that there was very little specific E2-DNA complex with the mobility of the wild-type protein detected in this gel; however, considerable amounts of signal could be detected in the regions of this gel near wells. An analysis of mutants E74A and D143A/R172C, which display weaker DNA binding (Fig. 4A), showed that fractions of these mutants exist in the dimeric form, which allows some of the specific complex to enter the gel, as well as in the oligomeric form, which is capable of binding DNA (Fig. 6). Some of the oligomers which are stable enough to be separated by the glycerol gradient from the dimeric form of E2 in the case of E74A, were dissociated under the conditions for the formation of the E2-DNA complex, and an E2-DNA complex of the size of the wild-type could be detected in the bottom fractions of the gradient (Fig. 6A). From these results, we concluded that the inactivity of mutants K111A, K112A, and E176A may be caused by the oligomerization of the protein, which inactivates the transport of mutant proteins to the nucleus. Mutants E74A and D143A/R172C create proteins with oligomerization capabilities greater than that of the wild-type protein; however, this process is reversible and a considerable fraction of the protein still exists in the dimeric and active form.

DISCUSSION

We constructed a set of BPV1 E2 protein mutants by a scheme called clustered charged-to-alanine scan. This approach allows the identification of functionally important side chains involved in interaction with other proteins. Alanine is the most abundant amino acid in proteins and was selected as the replacement residue because it is found in all types of secondary structure and because this substitution does not impose new structural effects related to hydrogen bonding, unusual hydrophobicity, or steric bulk. Charged-to-alanine substitution generally does not interfere with the packing of buried residues nor disrupt the structural integrity or expression of the protein (11). It is highly likely that clustered charged residues are exposed on the protein surface. It is reasonable to assume that the functioning of the protein in the regulation of different biological activities takes place through direct interactions and is mediated through the surfaces of components of the system (2). Therefore, clustered charged-to-alanine scan is a good approach for identifying the structural determinants of E2 interacting with factors of transcription and replication machinery. Our initial analysis of the clustered charged residues of the BPV1 E2 protein identified about 30 epitopes (determinants) which were located presumably on the surface of the protein and therefore could be identified as potential candidates for interactions with basal transcription and replication machinery. In the current study, we decided to include only the conserved charged residues of the BPV1 E2 protein. Altogether, 14 E2 mutants with alanine substituted for charged residues were constructed by PCR-mediated mutagenesis. In the process of construction of the 13th mutation, Asp-143→Ala, we picked up a double mutation, Asp-143→Ala and Arg-172→Cys, which was also included in the analysis. Altogether, we tested 15 E2 mutants in transient-replication, transcriptional activation, and sequence-specific DNA binding assays. We found that all E2 mutants, except for K111A, K112A, and E176A, were able to activate the replication of the BPV1 origin. In addition to these three mutants, four E2 mutants, E37A, E74A, D122A, and D143A/R172C, were crippled for the activation of transcription. We concluded that only determinants containing residues E-37, E-74, D-122, and R-172 are involved in the activation of tran-

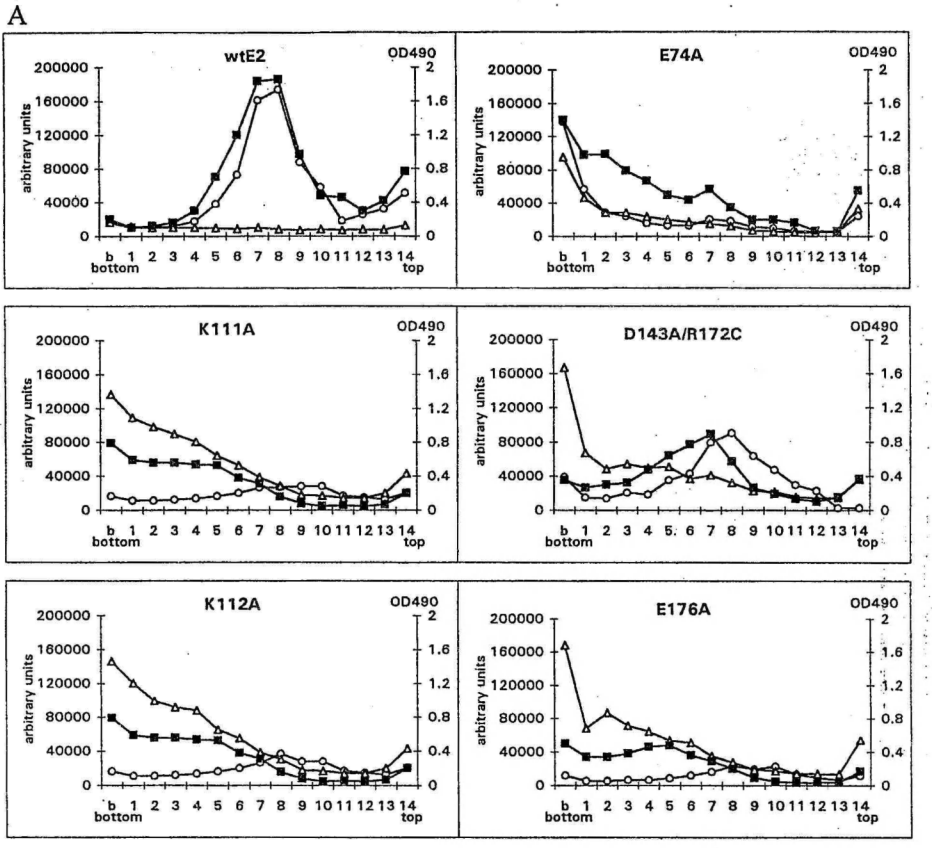

FIG. 6. (A) Sedimentation analysis of wild-type and mutant E2 proteins expressed in COS-7 cells. COS-7 cells were transfected with the respective expression vectors for wild-type and mutant E2 proteins, and extracts of transfected cells were loaded onto the top and sedimented through a 10 to 30% glycerol gradient. Fourteen fractions were collected, the bottom of the tube was washed with 250 μl of buffer, and all fractions were subjected to ELISA analysis (optical density at 490 nm [OD490]) (■) and tested for a sequence-specific gel shift of the size (in arbitrary units) of the wild type (○) and for a signal (in arbitrary units) near wells (△). DNA binding activities were quantitated with a PhosphorImager, and radioactivity values are expressed in arbitrary units. Marker proteins, alcohol dehydrogenase (150 kDa) and BSA (66 kDa), were centrifuged in the parallel gradient and peaked in fractions 2 and 9, respectively. b, bottom fraction. (B) Immunoblot analysis of wild-type and mutant E2 proteins precipitated upon centrifugation in 10 to 30% glycerol gradients. The precipitated protein at the bottom of the tube was solubilized with 50 μl of SDS sample buffer and subjected to Western blot analysis using an E2-specific monoclonal antibody. The purified E2 protein expressed in bacteria was used as a control. wtE2, wild-type E2.

scription and therefore presumably interact with components of the transcription machinery. It has been shown that the E2 protein is able to interact with transcription factor Sp1 in solution and cooperate with Sp1 in the transcriptional activation of promoters (17, 26). In addition, E2 has a capacity to interact with TBP and with TFIIB (39). Although biochemical experiments have demonstrated that major determinants of interaction between E2 and TBP and TFIIB lie within the carboxy-terminal domain of the E2 protein (39, 50), these data do not exclude the involvement of certain determinants within the transactivation domain in interaction with TBP (39, 50). We believe that mutants R37A, E74A, D122A, and D143A/R172C are inefficient in transcription activation because of weakened interaction with Sp1, TBP, TFIIB, or other general transcription factors. Further biochemical studies are in progress to identify the interactions of E2 with these factors.

The amino-terminal domain of the E2 protein has remarkable structural integrity (7, 15, 19, 23), and very little is known about the structural and functional determinants located in this part of the E2 molecule. The computer prediction of the structure of the N-terminal domain of the BPV1 E2 protein was made by the PredictProtein method (41) and is presented in Fig. 1A. In addition to our results, two recently published studies have analyzed E2 protein functional and structural determinants in the N-terminal domain. Human papillomavirus type 16 (HPV16) E2 protein was analyzed by alanine substitution of conserved residues (42), as in our study, and BPV1 E2 protein was analyzed by using highly conservative amino acid substitutions (7) in the activation of replication and transcription, as well as in interaction with the E1 protein. A comparison of these studies allows us to reach interesting conclusions about the structural and functional determinants of the E2 protein. The replacement of conserved residues in the first alpha-helix (Fig. 1A) with alanine or a conservative amino acid does not change the biological or biochemical properties of the HPV16 or BPV1 E2 protein. Thus, as in all three studies good care in design and characterization of the mutant protein was made to avoid gross changes of the structural organization of the protein, we may conclude that mutations in positions Q-12, E-13, and E-20 of the first alpha-helix have a phenotype essentially similar to that of the wild-type protein. At the same time, deletion of the first alpha-helix from the BPV1 E2 protein inactivates E2 completely in all biological assays and reveals denatured protein-specific epitopes within the N-terminal part of E2 (23). This allows us to speculate that the first alpha-helix is an important structural organizer of the N-terminal part of the E2 protein and determines the integrity of the structure of the transactivation domain. This may be achieved through electrostatic interaction of the negatively charged first alpha helix-surface, consisting of glutamic acid residues.

In the second alpha-helix of the E2 protein (Fig. 1A), substitutions for four conserved residues, W-33, R-37, E-39, and R-47, have been studied. Conservative changes at two positions, W-33 and E-39, of BPV1 E2 rendered the protein inactive in replication and transcription (7), while alanine substitutions for W-33 and R-47 in HPV16 E2 had no inactivating effect and that for E-39 abolished the replicational activation ability of E2 (42). In BPV1 E2, the replacement of E-39 and R-47 with alanine had little effect on protein activity. The replacement of arginine by alanine at position 37 impaired HPV16 E2 (42) and BPV1 E2 for transcriptional activation, leaving the replication function of the protein intact. The replacement of this residue in BPV1 E2 by a conservative amino acid, lysine, produced a mutant with an essentially wild-type phenotype (7). Although the E2 proteins of different papillomaviruses have the same functions, activation of transcription and replication, they are not readily interchangeable in replication assays (9). For example, HPV11 E2 and BPV1 E2 are very inefficient in the activation of replication with heterologous E1 protein and in interaction with heterologous E1 protein (9, 44). This incompatibility of functionally identical proteins in replication assays may be a reflection of differences in the structural determinants responsible for the activation of replication and may explain the differences in replicational activation by E39A mutants of HPV16 and BPV1 E2 proteins.

In the third alpha-helix (Fig. 1A), the effects of mutations at three positions, R-68, I-73, and E-74, can be compared. Mutation R68A did not induce very large changes in the properties of E2 proteins (42), while E74A was not studied by others. In our study, we found that this protein was transcriptionally inactive but moderately functioned in the activation of replication. Interestingly, mutation I73A in HPV16 E2 induced a protein with essentially the same phenotype, while conservative change I73L produced a BPV1 E2 protein with reduced transcriptional activation ability (7). In addition to being impaired in transcriptional activation, the same protein with mutation E74A showed another biochemical property. Although localized in the nucleus, this mutant protein exhibited an increased ability to form oligomers. These oligomers were still able to dissociate into the dimeric form and were able to bind DNA and form an E2-DNA complex of normal size. From these three studies, we may conclude that the third alpha-helix carries the capacity to interact, presumably through the conserved determinant between residues 72 and 74 (alanine-isoleucine-glutamic acid), with the transcription machinery of the cell. Hydrophobic and aromatic residues may be critical for and participate directly in the interaction of the transcription activation domain with components of the basal transcription complex (16, 24). We may speculate that the relatively hydrophobic isoleucine at position 73 is probably critical for this interaction, while a charged residue, E-74, is responsible for the presentation of this determinant for interaction.

We found that substitutions K111A, K112A, and E176A in the E2 transactivation domain completely abolished the ability of these expressed mutant proteins to form an E2-DNA complex of the usual size; however, considerable retention of the probe in wells was detected. The pronase treatment of extracts of COS-7 cells containing inactive mutant K111A, K112A, and E176A proteins showed that the DNA binding abilities of these mutants were readily present in the proteins; however, only large complexes were formed. The formation of oligomers and aggregates with these mutants was detected by glycerol gradient centrifugation. We also studied the cellular localization of mutant E2 proteins and observed that three mutants, K111A, K112A, and E176A, were exclusively localized in the cytoplasm. In a study by Skiadopolus and McBride, the BR2 region (residues 107 to 115) was identified as a potential nuclear localization signal for the E2 transactivator protein E2TA (46). Two of the mutations, K111A and K112A, fell in the same region. Conservative change K111R did not disrupt nuclear transport (7, 46); however, this protein was inactive in transcription but retained replicational activity at a very low level. This indicates that the lysine residue at position 111 is invariant and is important for the active conformation of this protein; the replacement of lysine by arginine creates an inactive nuclear E2 protein, while a replacement by alanine induces a conformation of this protein which is able to oligomerize and even to aggregate, as estimated by glycerol gradient centrifugation. In our study, the replacement of lysine by alanine at position 112 resulted in a mutant protein with the same phenotype as that of K111A. However, the same mutation in HPV16 E2 (42), as well as replacement K112R in BPV1 E2

(7), had little effect on the activity of the protein. This discrepancy may be a reflection of the differences in the structures of the HPV16 and BPV1 E2 proteins. The BR2 region of the BPV1 E2 protein can be responsible for directing E2TA into the nucleus (46); however, in our experiments, oligomerization and aggregation are probably responsible for the retention of mutants K111A, K112A, and E176A in the cytoplasm. The amino-terminal parts of E2 dimeric molecules mediate the cooperative binding of E2 on the clustered E2 binding sites in DNA (35). This interaction may also take place in solution and could serve as a mechanism for the regulation of E2 biological activity. Amino terminus-mediated oligomerization could explain the self-squelching of E2 described by Rank and Lambert (39) and by us here. Self-squelching is specific to the E2 transactivation domain, as its replacement with the p53 or VP16 transactivation domain abolished self-squelching, although the expression levels of E2 and hybrid proteins were comparable. We may conclude that introduced mutations K111A, K112A, and E176A remove certain constraints in the interactions of E2 molecules in solution and induce the formation of inactive configurations of E2 oligomers.

Several studies have presented data consistent with the finding that interaction between BPV1 E2 and E1 facilitates the binding of E1 to its cognate site (29, 44, 45, 48, 53, 55). This cooperative binding has been suggested to play an important role in the initiation of BPV1 DNA replication and is very specific for E1 and E2 proteins. We tested a number of transactivation domains, p53, VP16, c-jun, and Epstein-Barr virus BZLF-1, linked to the E2 DNA binding domain and found all of them to be unable to support BPV1 origin replication in vivo (1). The first 91 residues of E2 participate in interaction with the E1 protein, as shown in biochemical assays and in a two-hybrid system (3). Eight of our designed mutations fell in this region, and curiously none affected the activation of BPV1 origin replication, though two of them were inactive in transcription. These data indicate that the determinants for interaction with the E1 protein are not encoded by the clustered conserved charged residues, but some other sequences within this region may be involved in the specific interaction between E2 and E1. This conclusion is supported by the results of in vivo and in vitro replication assays using mixed and matched combinations of the E1 and E2 proteins from BPV1 and HPV11 (9, 44). Certain combinations of the E1 and E2 proteins of BPV1 and HPV11 were inactive for the activation of replication, indicating that the interaction between E1 and E2 is virus type specific. In contrast, BPV and HPV E2 proteins are able to transactivate in different mammalian cell lines and in yeasts (36, 49, 54). This indicates that the interactions with the transcription machinery are well conserved. Mutations at conserved positions lead to the disruption of interactions with the transcription machinery but not with E1.

As shown by in vitro replication experiments, transcription per se is not required for replication of the BPV1 origin (58). Our data also show that the ability of E2 to activate transcription is not required at all for the activation of BPV1 replication in vivo and that the determinants responsible for the activation of transcription and replication only partly overlap.

ACKNOWLEDGMENTS

We thank J. Sedman, J. Remme, M. Piirsoo, R. Villems, and M. Remm for comments on the manuscript and Anne Kalling for excellent technical assistance.

This work was supported in part by grants 1134, 287, and 295 from the Estonian Science Foundation, a grant from The Independent Estonian Cancer Research Fund, and grants LDL 6000 and LKL 100 from The International Science Foundation.

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Kurg, R., Parik, J., Juronen, E., Sedman, T., Abroi, A., Liiv, I., Langel, Ü. and Ustav, M. (1999) Effect of bovine papillomavirus E2 protein-specific monoclonal antibodies on papillomavirus DNA replication. J. Virol. 73(6), 4670–4677.

JOURNAL OF VIROLOGY, June 1999, p. 4670–4677 Vol. 73, No. 6
0022-538X/99/$04.00+0

Effect of Bovine Papillomavirus E2 Protein-Specific Monoclonal Antibodies on Papillomavirus DNA Replication

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Received 5 October 1998/Accepted 23 February 1999

The bovine papillomavirus type 1 (BPV-1) E2 protein is the master regulator of papillomavirus replication and transcription. We have raised a panel of monoclonal antibodies (MAbs) against the BPV-1 E2 protein and used them to probe the structure and function of the protein. Five MAbs reacted with linear epitopes, and four MAbs recognized conformation-dependent epitopes which mapped within the C-terminal DNA-binding and dimerization domain. MAb 1E2 was able to recognize the replication- and transactivation-defective but not the competent conformation of the transactivation domain of the E2 protein. MAb 5H4 prevented efficiently the formation of E2-DNA as well as E2-dependent E1-E2-origin complexes and also dissociated preformed complexes in a concentration-dependent manner. Cotransfection of several MAbs with the BPV-1 minimal origin plasmid pUCAlu into CHO4.15 cells resulted in a dose-dependent inhibition of replication. Inhibition of replication by MAb 5H4 and the Fab′ fragment of 5H4 correlated with their ability to dissociate the E2 protein from the DNA. MAb 3F12 and MAbs 1H10 and 1E4, directed against the hinge region, were also capable of inhibiting BPV-1 origin replication in CHO4.15 cells. However, the Fab′ fragments of 1H10 and 3F12 had no effect in the transient replication assay. These data suggest that MAbs directed against the hinge region sterically hinder the inter- or intramolecular interactions required for the replication activity of the E2 protein.

Bovine papillomavirus type 1 (BPV-1) has been studied extensively as a model for papillomavirus replication and transcription. The viral E2 protein is the master regulator of the viral life cycle—this protein modulates the transcription of viral genes (41) and is responsible for the initiation of DNA replication (43, 44, 48) and for the stable maintenance of the viral genome (31), which is achieved presumably through facilitation of the association of the viral genome with chromatin (19a, 23, 40). E2 is a sequence-specific DNA-binding protein, and it interacts with the components of the cellular transcription (33, 49) and replication (24) machinery. The viral E2 and E1 proteins interact with each other (2, 4, 30, 35) during the initiation of replication, resulting in cooperative binding of E1 and E2 on the BPV-1 replication origin (25–27, 35–39).

The BPV-1 E2 protein, like other transcription factors, is composed of relatively well-defined function-specific modules. Structural and mutational analyses have revealed three distinct domains. The amino-terminal part (residues 1 to 210) is an activation domain for transcription (12, 28) and replication (43). It is followed by the unstructured hinge region and the carboxy-terminal DNA-binding and dimerization domain (residues 310 to 410) (29). Deletion analysis of the E2 protein has shown that the transactivation domain and the DNA-binding and dimerization domains are necessary for both replication and transcription, while large deletions in the hinge region affect replication preferentially and transcription less (46). The structure of the carboxy-terminal DNA-binding and dimerization domain has been solved by X-ray analysis and has revealed a dimeric DNA-binding and dimerization motif (15, 16). Most of the information about structural and functional determinants in the amino-terminal activation domain of the E2 protein has been obtained by mutational analysis (7, 12, 14, 46). These data confirm that the E2 amino-terminal domain, like the C-terminal domain, has a highly organized structure and that even a single point mutation can inactivate the function of the E2 protein in the activation of transcription, replication, or both (1, 5, 9, 13, 34).

Antibodies are efficient and highly specific tools for identifying the structural determinants of macromolecules and/or for studying the role of a protein in functional assays (18, 19, 21, 42, 45). Antibodies have been used for the characterization of the human papillomavirus (HPV) E2 protein. For example, polyclonal antibodies against overlapping synthetic peptides that cover the HPV-type 16 (HPV-16) E2 protein have been used to test the structure of this protein (10), and the interaction of the HPV-16 E2 protein with the E1 protein could be blocked by a monoclonal antibody (MAb) that bound E2 in the region of amino acids 18 to 41 (17).

In this study, we describe the production of a set of MAbs against the BPV-1 E2 protein and characterize their ability to interfere with the functions of the E2 protein in vivo and in vitro in biochemical and functional assays.

MATERIALS AND METHODS

Production of the BPV-1 E2 protein. E2 protein was expressed in the pET11c-based system in *Escherichia coli* and was purified to homogeneity by conventional methods (37) with modifications. First, we precipitated nucleic acids from clarified cell lysates by the slow addition of polyethylenimine (Polymin P; Sigma) to a final concentration of 0.6%. Precipitation was carried out on ice for 30 min, and the pellet was collected by centrifugation. Proteins were recovered from the supernatant by precipitation with 35% ammonium sulfate and purified to homogeneity by conventional chromatography.

Production of MAbs. Female BALB/C mice were injected with 50 μg of purified BPV-1 E2 protein five times at 3- to 4-week intervals. The injections were intraperitoneal, with E2 suspended initially in Freund's complete adjuvant

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and subsequently in phosphate-buffered saline (PBS). Following the final injection, mice were allowed to rest for 5 weeks and then were injected with 100 μg of antigen. One week later, final boosts with 100, 200, and 200 μg of protein in PBS at 4, 3, and 2 days before fusion, respectively, were performed. Sp2/0 myeloma cells and cells from one third of the spleen were washed three times with sterile PBS. The final pellet was mixed by tapping the tube, and 1 ml of 50% polyethylene glycol (PEG) 4000 (Merck) was added over 1 min with gentle shaking. The cells were centrifuged at 100 × *g* for 5 min, the PEG solution was removed, and the resuspended cells were plated on five 96-well microtiter plates containing hypoxanthine-aminopterin-thymidine medium. Supernatants were tested 10 days after fusion as described below by a direct enzyme-linked immunosorbent assay (ELISA).

Screening of hybridomas. Wells of ELISA plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with 100 μl of E2 (2 μg/ml in PBS) overnight at 4°C. After the coating step, the plates were washed three times with PBS containing 0.05% Tween 20 (PBS/T) and blocked with PBS/T containing 0.05% casein for 30 min. Then, 80 μl of PBS/T and 20 μl of hybridoma supernatants were added to the wells, and the plates were incubated for 60 min on a shaker at room temperature. The plates were washed with PBS/T, followed by the addition of 100 μl of peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (LabAS Ltd., Tartu, Estonia) diluted 1:2,500 in PBS/T supplemented with 2% PEG 6000 (Merck). The plates were incubated for 15 min and then washed with PBS/T. Then, 100 μl of TMB substrate solution (3,3',5,5'-tetramethylbenzidine–H_2O_2 in 0.1 M acetate-citrate buffer [pH 4.5]) was added.

The antibody subclasses were determined by an ELISA as described above with peroxidase-labelled goat anti-mouse isotype antibodies (LabAS).

Purification of MAbs. The MAbs were purified from ascitic fluid by ammonium sulfate precipitation and ion-exchange chromatography on Blue DEAE-Toyopearl 650S with a Pharmacia standard fast protein liquid chromatography system (20). The IgG concentration was estimated at 280 nm by use of an extinction coefficient of 14. The purified MAbs were stored in PBS containing 50% glycerol at −20°C.

Preparation of Fab' fragments. Fab' fragments were prepared from the MAbs as described by Porter (32) with modifications. The MAbs were dialyzed against 0.1 M sodium acetate buffer (pH 5.5) containing 1 mM EDTA and 25 mM 2-mercaptoethanol. The antibodies were digested with papain at an enzyme/antibody ratio of 1:10 (wt/wt) for 24 h at 37°C. The reaction was stopped by the addition of iodoacetamide to a final concentration of 30 mM. The digested antibodies were dialyzed against 20 mM Tris-HCl buffer (pH 7.2), and Fab' fragments were purified on a Mono Q column.

Peptide synthesis. Peptides were assembled in a stepwise manner on a solid support with a model 431A peptide synthesizer (Applied Biosystems) by the standard NMP/HOBt solvent activation strategy on a 0.1-mmol scale (22).

ELISA with peptides. The surfaces of the microtiter wells were activated with 0.25% glutaraldehyde in PBS for 30 min at 60°C. The plates were washed three times with PBS, and a peptide solution at a concentration of 20 μg/ml in PBS was added to the wells. The plates were incubated overnight at room temperature, washed with PBS/T, and blocked with 1% nonfat dry milk in PBS/T for 2 h. The plates were washed with PBS/T, and antibodies diluted in PBS/T were added to the wells, incubated, and processed as described above.

Plasmids. The pET-E2 vector used for the expression of E2 in *E. coli* was generated by PCR amplification with specific primers and was cloned between the *Nde*I and *Bam*HI sites of plasmid pET11c. The E2 expression constructs pCGE2, pCGE2C, pCGE8/E2, and pCGE2(D92-161) have been described previously (43, 44). Plasmid VP16:E2 (24) contains 80 C-terminal amino acids from VP16 fused to the C terminus of E2 (starting from amino acid 250) in the context of pCG. The E2 N-terminal deletion mutants E2(D1-23), E2(D1-85), E2(D1-112), and E2(D1-183) were generated by PCR with appropriate oligonucleotide primers containing an initiation methionine codon in the optimal Kozak context and were cloned into the pCGE2 expression vector at the *Xba*I-*Bam*HI sites. For the E2 C-terminal deletion mutants E2(D219-410), E2(D284-410), and E2(D310-410), PCR primer pairs were designed with terminal recognition sequences for *Kpn*I-*Bcl*I and were cloned into the corresponding sites in pCGE2. The replication reporter plasmid pUCAlu has been described previously (43). pHookAlu was made by cloning the *Alu* fragment from pUCAlu at the *Hin*dIII-*Bam*HI sites of pHook-2 (Invitrogen) and deleting the cytomegalovirus promoter with restriction enzymes *Hin*dIII and *Bss*HI.

Cells and transfections. E2 expression constructs (100 ng) were electroporated at 180 V into COS-7 cells (2×10^6 to 6×10^6) in 250 μl of Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (FCS) and 50 μg of denatured salmon sperm DNA at room temperature (43). For replication assays, CHO4.15 cells were trypsinized, centrifuged, and resuspended in F12 medium containing 10% FCS at a density of 10^7 cells/ml. The cell suspension (250 μl) was mixed with 100 ng of pUCAlu DNA, 50 μg of salmon sperm DNA, and various concentrations of MAbs or Fab' fragments in a disposable electroporation cuvette and subjected to an electric discharge of 230 V from an Invitrogen Gene Pulser. After the discharge, the cell suspension was left at room temperature for 15 min, and then the cells were washed and plated in F12 medium supplemented with 10% FCS. The extraction of episomal DNA from cells and its analysis by Southern blotting were performed as described previously (43). For Western blot analysis, 500 ng of pHookAlu was cotransfected with MAbs (80 μg/ml), and transiently transfected cells were separated from the total population of CHO4.15 cells with magnetic beads (Invitrogen) according to the manufacturer's recommendations.

Immunoblotting of E2. COS-7 cells transfected with E2 proteins in 60-mm diameter dishes were lysed 36 h after electroporation in 200 μl of Laemmli sample buffer. Transfected CHO4.15 cells were separated from the magnetic beads by boiling in 100 μl of Laemmli sample buffer. Proteins were separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (PAGE). After transfer, the nitrocellulose membranes were incubated with mouse E2-specific MAbs and a secondary horseradish peroxidase-conjugated antibody by use of an ECL detection kit (Amersham) according to the manufacturer's recommendations. To analyze the E2 protein level in CHO4.15 cells transfected with MAbs, rabbit anti-E2 polyclonal antibody was used.

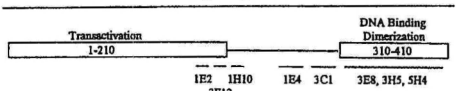
Mobility shift assays. For preparation of COS-7 cell extracts, transfected cells were removed from semiconfluent growth on 100-mm-diameter plates with a rubber policeman, washed, and lysed in 100 μl of lysis buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 30 mM KCl, 0.1 mM EDTA, 0.35% Nonidet P-40, 10 mM dithiothreitol, protease inhibitors) on ice for 30 min. Cell debris was removed by centrifugation, glycerol was added to a final concentration of 20%, and the extracts were divided into aliquots and stored at −70°C. For gel shift assays, 2 μl of cell extract was incubated in 10 μl of binding buffer (10 mM Tris-HCl [pH 7.5], 100 mM KCl, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 15% glycerol, 5 mg of bovine serum albumin per ml, 1 μg of leupeptin per ml, 1 μg of aprotinin per ml) at room temperature for 15 min in the presence of 1 μg of sonicated salmon sperm DNA and 0.2 ng of ^{32}P-labelled oligonucleotide containing the E2 binding site. For bacterially expressed E2, 2 ng of protein was used per reaction. Double-stranded high-affinity binding site 9 of BPV-1 (5'-A CAAAGTACCGTTGCCGGTCGAA-3') was used as a probe. Protein-DNA complexes were resolved by 6% PAGE (acrylamide-*N*,*N*-methylene-bisacrylamide, 80:1) with 0.25× Tris-borate-EDTA. Gels were dried and exposed to X-ray film. MAbs (1 to 10 ng/μl) were added either before or after DNA binding and were incubated for an additional 20 min. For protease digestion experiments, bacterially expressed E2 protein was incubated with 2 μg of pronase for 5 min. The E1-E2-origin complex formation assay was performed as described by Sedman and Stenlund (35). The effect of antibodies on E1-E2-origin complex formation was tested either before or after the assembly of the complex.

RESULTS

Generation of E2-specific MAbs. The soluble E2 protein was purified to apparent homogeneity from lysates of isopropyl-β-D-thiogalactopyranoside (IPTG)-induced *E. coli* overexpressing BPV-1 E2 from the pET11c expression vector by conventional chromatography (37). BALB/c mice were immunized with the purified functionally active E2 protein as described in Materials and Methods. We obtained nearly 200 hybridoma cell lines, 17 of which were positive for the E2 protein in both ELISAs and immunoblot assays, while 5 hybridomas were positive in ELISAs only. Nine MAbs that were deemed most useful were purified from the ascitic fluids of the respective hybridoma cell lines and studied in various assays as described below (Table 1). All studied antibodies belonged to the IgG1 subtype, with the exception of 3C1, which belonged to the IgG2a subtype. All of these MAbs had high affinities for and fast kinetics of binding to their respective epitopes, as found by the concentration dependence of antibody binding in ELISAs (data not shown).

Epitope mapping. To define the continuous epitopes recognized by the antibodies, the reactivity of each MAb to full-length and truncated E2 proteins expressed in COS-7 cells was determined by Western blot analysis. The linear epitopes for the initially isolated 17 MAbs were mapped in the region between amino acids 184 and 309; for 12, the epitopes were found within the region between residues 184 and 218 (data not shown). We concluded from these results that the sequence of the 34 amino acids within the region from residues 184 to 218 is the major immunodominant determinant of the BPV-1 E2 protein. The results of immunoblot analysis for five selected purified antibodies with the linear epitopes are shown in Fig. 1A. To map the epitopes for the 1E2, 3F12, and 1H10 antibodies more precisely, we synthesized four overlapping peptides covering the region between amino acids 162 and 210. The sequences of the synthesized peptides and the ability of the MAbs to bind to these peptides in an ELISA are shown in

TABLE 1. MAbs recognizing the BPV-1 E2 protein[a]



Antibody	Location of epitope (residues)	Type of epitope	Binding to E2-DNA complex	Inhibition of E2 binding to oligo	Binding to E1-E2-origin complex
1E2	184–190	Linear	–	–	–
3F12	199–206	Linear	+	–	+
1H10	208–218	Linear	+	–	+
1E4	250–280	Linear	+	–	+
3C1	280–309	Linear	+	–	+
3E8	310–410	Conformational	+	–	+
3H5	310–410	Conformational	+	–	+
5F10	162–410	Conformational	+	–	+
5H4	310–410	Conformational	–	+	–

[a] +, positive result; –, negative result.

Fig. 1B. Peptides P2 (residues 171 to 192) and P3 (residues 184 to 201) were recognized by 1E2, while peptide P4 was recognized by 3F12. To narrow down the sizes of the epitopes, two additional peptides, P5 (residues 179 to 190) and P6 (residues 197 to 208), were synthesized and confirmed by an ELISA to contain the recognition sequences for the 1E2 and 3F12 antibodies, respectively. 1H10 did not recognize any of the synthesized peptides and was therefore mapped by the Western blot analysis to the region between amino acids 208 and 218.

To test the ability of the antibodies to recognize their respective linear epitopes on the E2 protein in the E2-DNA complex, a mobility shift assay was used. All MAbs against the linear epitopes, with the exception of 1E2, were able to induce a supershift (Fig. 2A), indicating that their epitopes are exposed on the surface of the DNA-bound E2 molecule.

Accessibility of the MAb 1E2 epitope in the E2 protein. The epitope for MAb 3F12 (residues 199 to 206) is efficiently exposed in the DNA-bound E2 protein, while the epitope for MAb 1E2, located 8 residues upstream, is poorly recognized by the respective antibody in the full-length E2 protein in complex with DNA (Fig. 2A, compare lanes 2 and 3 with lanes 4 and 5). However, the epitope for MAb 1E2 was readily exposed in truncated E2 proteins bound to DNA—E2(D1-23), E2(D1-85), E2(D1-112), E2(D1-183), and E2C (Fig. 2C)—as well as in the internal deletion mutant E2(D92-161) (data not shown). All of these deletion mutants were inactive for the activation of transcription and replication (21a). Therefore, the epitope for MAb 1E2 could be identified as a transactivation domain denaturation-specific epitope of the E2 protein; the exposure of the 1E2 epitope indicates that the E2 protein transactivation domain has an inactive conformation for transcription and replication.

Antibodies against the C-terminal domain of the E2 protein. MAbs 3E8, 3H5, 5F10, and 5H4 did not react with the E2 protein on immunoblots, indicating that the epitopes of these MAbs are sensitive to denaturation. To define further the

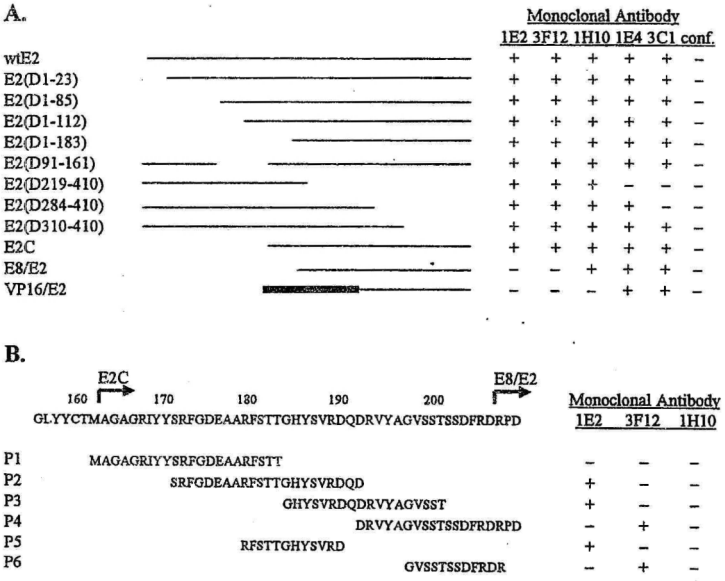


FIG. 1. Epitope mapping of E2-specific MAbs. (A) Schematic representation of the truncated E2 proteins used and the results of the immunoblot analysis of the E2 proteins (diagram at right). wt, wild type; conf., MAbs with discontinuous epitopes. (B) Reactivity of MAbs to synthetic peptides (P1 to P6) covering the region from amino acids 162 to 210 of E2 in an ELISA. +, positive result; –, negative result.

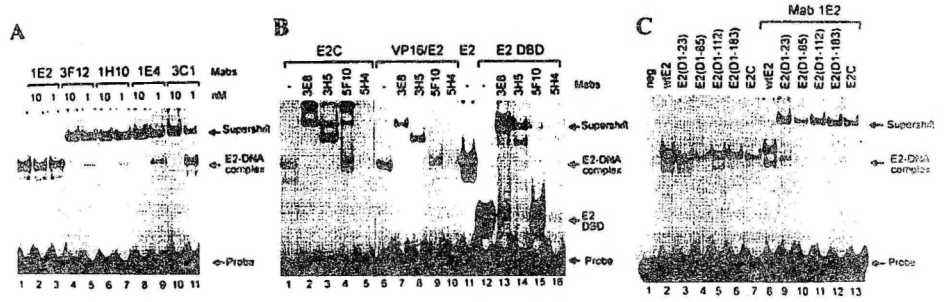


FIG. 2. Characterization of E2-specific MAbs. (A) Reactivity of E2-specific MAbs with the native E2 protein. The mobility shift assay was carried out with 2 ng of bacterially expressed and purified E2 protein and 0.2 ng of radiolabelled E2 binding site for 15 min at room temperature. (B) Lanes 1 to 10 show reactivity of MAbs with discontinuous epitopes with truncated E2 proteins expressed in COS-7 cells. Band shift assays were performed with 2 μl of cell extract. Lanes 11 to 16 show reactivity of MAbs to the E2 DNA-binding domain (DBD). Bacterially expressed E2 protein was treated with 2 μg of pronase for 10 min at room temperature, and then reactivity was determined. (C) Reactivity of MAb 1E2 with truncated E2 proteins expressed in COS-7 cells. MAbs were added after E2 was mixed with its DNA target. neg., cells transfected with carrior only; wt, wild type. Protein-DNA complexes were resolved by 6% PAGE with 0.25× Tris-borate-EDTA.

epitopes for these MAbs, the reactivity of each MAb to the full-length or truncated E2 protein expressed in COS-7 cells was determined by a gel shift assay. All four studied antibodies were able to react with both the full-length E2 protein (data not shown) and E2C expressed in COS-7 cells (Fig. 2B, lanes 1 to 5). MAbs 3E8, 3H5, and 5F10 were able to induce a supershift, and MAb 5H4 prevented the formation of the E2-DNA or E2C-DNA complex (Fig. 2B, lane 5; see also Fig. 5A, lanes 2 to 5). MAb 5H4 not only prevented the formation of the E2-DNA complex but also dissociated the preformed E2-DNA complex, and this effect was dependent on the concentration of the antibody. The dissociation of the preformed complex required concentrations of MAb 5H4 higher than those required to block complex formation. The chimeric protein VP16-E2, which contains amino acids 250 to 410 of the E2 protein, was recognized by MAbs 3E8, 3H5, and 5H4 but poorly, if at all, by MAb 5F10 (Fig. 2B, lanes 6 to 10).

The carboxy-terminal DNA-binding and dimerization domain of the E2 protein forms a protease-resistant core (8). When the E2 protein was incubated with pronase prior to the addition of antibodies, the DNA-binding domain of the E2 protein was still able to interact with MAbs 3E8, 3H5, and 5H4 and weakly with MAb 5F10 (Fig. 2B, lanes 11 to 16), resulting in a supershift or dissociation of the E2-DNA complex. These data allowed us to map the epitopes for the conformational MAbs 3E8, 3H5, 5F10, and 5H4 within the carboxy-terminal ~100 residues of the E2 protein. Immunoprecipitation studies with truncated E2 proteins mapped the epitopes for these MAbs within amino acids 310 to 410 (data not shown). The epitope mapping for MAb 5F10 was less definitive because the accessibility of the epitope for this MAb was dependent on the context of the protein. Although this MAb recognized an epitope in the E2-DNA and E2C-DNA complexes, the same epitope in VP16-E2 and the E2 DNA-binding and dimerization domain (Fig. 2B, compare lanes 4, 9, and 15) was poorly recognized.

Effect of MAbs on E1-E2-origin complex formation. The BPV-1 minimal origin of replication comprises the E1 binding site, the A/T-rich region, and the E2 binding site (44). The E1 and E2 proteins bind cooperatively to the origin and form an E1-E2-origin complex (27, 35, 39). It has been shown that the ability of the E2 protein to form a complex with the E1 protein on DNA correlates with the efficiency of initiation of replication in vivo (11, 36). We studied the effect of the antibodies on E2-dependent E1-E2-origin complex formation. Antibodies 3F12, 1H10, 1E4, 3C1, 3E8, 3H5, and 5F10 all recognized their respective E2 epitopes in the E1-E2-origin complex and supershifted this complex (Fig. 3A, lanes 3 to 9). Interestingly, MAbs which recognize the C-terminal part of the E2 molecule resulted in an E1-E2-origin MAb complex which migrated much more slowly than complexes in which the MAbs bound to epitopes closer to the center of the E2 molecule. The epitope for MAb 1E2 is masked in the DNA-bound E2 protein and

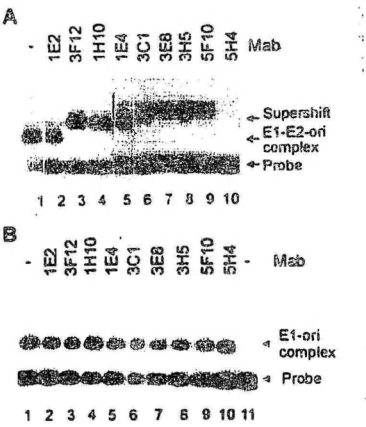


FIG. 3. Effect of E2-specific MAbs on the formation of the E1-E2-origin (ori) complex. (A) A gel mobility shift assay was performed with 2 ng of E1 protein and 5 ng of E2 protein for 20 min. MAbs were added to a final concentration of 10 ng/μl and incubated for an additional 20 min. (B) A gel mobility shift assay was used to analyze the complex formed in the presence of E1 only. The resulting complexes were treated with 0.4% glutaraldehyde and separated on agarose gels.

remains nonaccessible to this antibody in the E1-E2-DNA complex (Fig. 3A, lane 2). MAb 5H4, which specifically dissociated E2 from DNA, prevented the formation of the E1-E2-origin complex (Fig. 3A, lane 10). None of the antibodies had any effect on the mobility of the E1-origin complex formed at a high E1 protein concentration (Fig. 3B). These results showed that the epitopes for the most studied MAbs, with the exception of 1E2, are exposed in the E1-E2-origin complex and that MAb 5H4 is the only antibody which interferes with E2-dependent E1-E2-origin complex formation.

Effect of anti-E2 MAbs on BPV-1 origin replication in cells. The observation that antibodies recognized their respective epitopes in the DNA-bound E2 protein raised the possibility that some of these antibodies could interfere with some functions of the E2 protein in the initiation of DNA replication in vivo. Therefore, the purified antibodies were tested in a transient replication assay in vivo for their ability to block E2 protein functions in BPV-1 origin replication. Transfer of the MAbs into mammalian cells was carried out by electroporation (6). The transfection conditions were optimized to a level which allowed the uptake of both DNA and protein into CHO4.15 cells, which constitutively express viral E1 and E2 proteins (31) (see Materials and Methods). The presence of the relatively high concentrations of antibodies in the transfection mixture had no effect on CHO4.15 cell growth. We estimated that 1 to 2% of the input antibody was taken up by the cells under these conditions, as determined by Western blot analysis. Analysis of the cells immediately and 24 and 48 h after transfection showed structurally intact immunoglobulin heavy chains within the cells, indicating that no active degradation of the transfected antibodies took place in the cells (data not shown).

Different amounts of E2-specific MAbs were cotransfected with 100 ng of origin-containing plasmid pUCAlu by electroporation into CHO4.15 cells. Episomal DNA was extracted by alkaline lysis at 2 or 3 days after transfection, purified, digested with *Dpn*I and linearizing enzyme *Hin*dIII, and analyzed by Southern blotting as described earlier (43). The effect of the E2-specific antibodies on the replication of the BPV-1 origin was dependent on the antibody concentration used (Fig. 4A). At a low concentration (20 μg/ml), MAb 1H10 strongly inhibited and MAbs 3F12 and 1E4 moderately inhibited the replication of origin-containing plasmid pUCAlu in CHO4.15 cells. The inhibition of replication by MAbs 3F12, 1H10, and 1E4 became almost complete when the antibody concentration in the cell suspension was increased to 80 μg/ml (Fig. 4A and B, lanes 3 to 5). MAb 5H4, which efficiently inhibited the formation of both E2-DNA and E1-E2-origin complexes in vitro, exhibited only a weak inhibitory effect in the transient replication assay at a low concentration. However, at a higher concentration (80 μg/ml), strong inhibition of replication was achieved with MAb 5H4 (Fig. 4A and B, lane 10). MAbs 1E2, 3C1, 3E8, 3H5, and 5F10 exhibited only weak inhibition, and a nonrelated anti-β-galactosidase MAb had no effect on origin-containing plasmid pUCAlu replication at all concentrations tested. The differences in the abilities of the MAbs to inhibit replication were not caused by differential uptake of MAbs by cells, since equivalent concentrations of intracellular MAbs were used in the cell suspension during electroporation and comparable amounts of the antibodies were detected in the cells by Western blotting (data not shown). The affinities of all of these antibodies were similar, as indicated by the concentration-dependent binding of the antibodies in the ELISA (data not shown).

We next studied the effect of the E2-specific MAbs on the steady-state level of the E2 protein and on the localization of this protein in transfected cells. To select and isolate transiently transfected cells from the total population of CHO4.15 cells, a Capture-Tec Hook-2 kit (Invitrogen) was used. Briefly, MAbs were cotransfected with 500 ng of the origin-containing

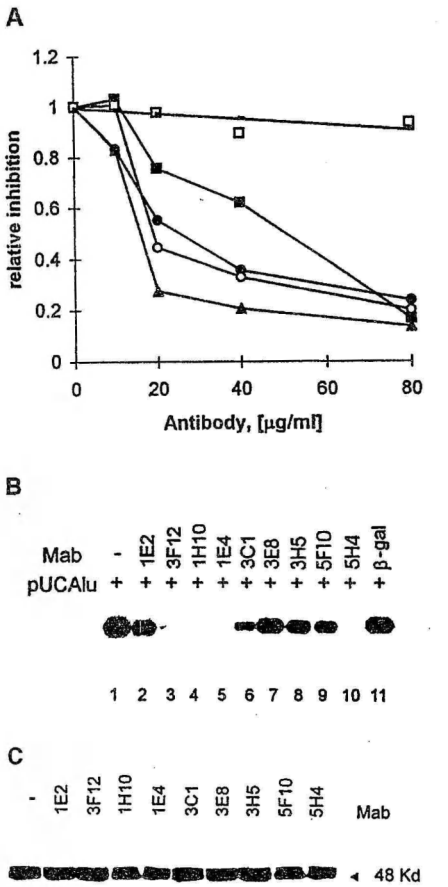


FIG. 4. Effect of E2-specific MAbs on papillomavirus replication. (A) CHO4.15 cells constitutively expressing BPV-1 E1 and E2 proteins were electroporated with 100 ng of reporter plasmid pUCAlu and various concentrations of MAbs. Cells were harvested 72 h after electroporation. Episomal DNA was digested with *Dpn*I and linearizing enzyme *Hin*dIII and analyzed by Southern blotting. The replication signals of three independent experiments were quantified with a PhosphorImager, and signals from cells transfected with the origin-containing plasmid only were used as a control to normalize the results. Symbols: ●, MAb 3F12; ○, MAb 1E4; ▲, MAb 1H10; ■, MAb 5H4; □, nonspecific anti-β-galactosidase (β-gal) MAb. (B) Southern blot analysis of transient replication of the BPV-1 origin-containing plasmid pUCAlu in the CHO4.15 cell line in the presence of MAbs at a concentration of 80 μg/ml. Episomal DNA was extracted from cells 72 h after transfection. Filters were probed with radiolabelled plasmid pUCAlu. (C) Western blot analysis of E2 protein levels in transfected CHO4.15 cells with rabbit anti-E2 polyclonal antibody.

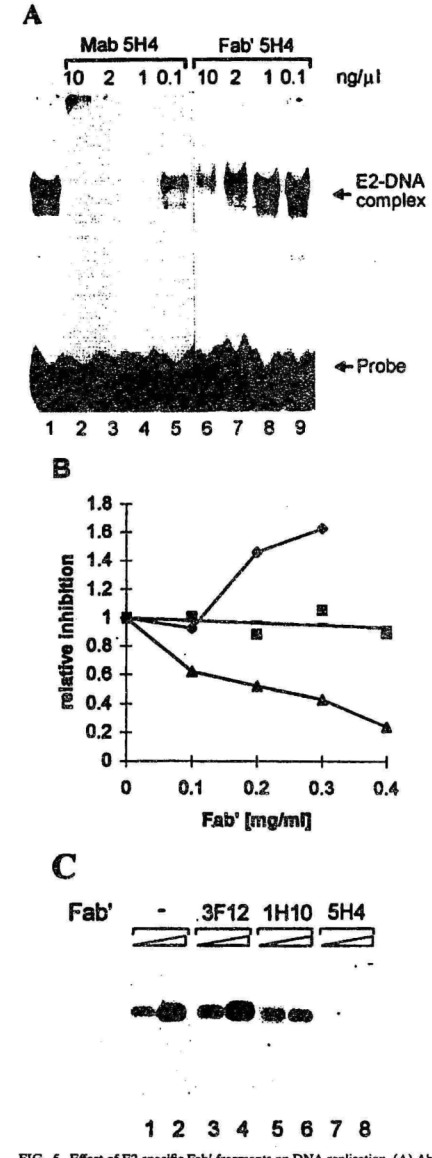


FIG. 5. Effect of E2-specific Fab' fragments on DNA replication. (A) Ability of MAb 5H4 and its Fab' fragment to inhibit the formation of the E2-DNA complex at various antibody concentrations. The mobility shift assay was carried out with 2 ng of bacterially expressed and purified E2 protein and 0.2 ng of radiolabelled E2 binding site for 15 min at room temperature. MAb 5H4 or its Fab' fragment was added after E2 was mixed with its DNA target, and incubation was carried out for an additional 20 min. (B) Inhibition of DNA replication by E2-specific Fab' fragments at various concentrations. Reporter plasmid pUCAlu (100 ng) was cotransfected together with the Fab' fragment of 3F12 (◆), the Fab' fragment of 1H10 (■), or the Fab' fragment of 5H4 (▲) into cell line CHO4.15. At 72 h after electroporation, cells were harvested, and episomal DNA was digested with *Dpn*I and linearizing enzyme *Hin*dIII and analyzed by Southern blotting. The replication signals of three independent experiments were quantified with a PhosphorImager, and signals from cells transfected with the origin-containing plasmid only were used as a control to normalize the results. (C) Southern blot analysis of transient replication of the BPV-1 origin-containing plasmid pUCAlu in the CHO4.15 cell line in the presence of Fab' fragments at a concentration of 0.3 mg/ml. Episomal DNA was extracted from cells either 48 or 72 h (⟷) after transfection. Filters were probed with radiolabelled plasmid pUCAlu.

plasmid pHookAlu, which expresses a fusion protein comprised of the PDGFR transmembrane domain fused to the variable region of the antibody capable of recognizing phOx (4-etoxymethylene-2-phenyl-2-oxazolin-5-one), into CHO4.15 cells. At 24 h after electroporation, transfected cells were selected with magnetic beads carrying immobilized phOx and analyzed for the level of the E2 protein by Western blotting as well as for the localization of the E2 protein by direct immunofluorescence analysis with rabbit anti-E2 polyclonal antibody. We did not find any effect of the cotransfected E2-specific MAbs on the localization (data not shown) or steady-state level of the E2 protein in CHO4.15 cells (Fig. 4C).

Effect of Fab' fragments on DNA replication. Our results showed that MAbs 1H10, 1E4, 3F12, and 5H4 suppressed BPV-1 origin replication in a dose-dependent fashion (Fig. 4A). At the same time, MAbs 1H10, 3F12, and 1E4 did not influence the formation of the E1-E2-origin complex and supershifted this complex efficiently (Fig. 3, lanes 3 to 5). These data suggest that MAb 3F12 (epitope at residues 199 to 206) and MAbs 1H10 and 1E4, directed against the hinge region of the E2 protein, would not interfere directly with E1 and E2 interactions with DNA; however, these antibodies can sterically interfere with the inter- or intramolecular interactions required for the replication activity of the E2 protein. In order to study the possibility that antibodies would have an effect on replication due to steric hindrance of the formation of the replication initiation complexes, the Fab' fragments of MAbs 3F12, 1H10, and 5H4 were prepared by a modified procedure (see Materials and Methods). An ELISA with E2-coated microtiter plates showed that all of the Fab' fragments were active in binding to the E2 protein. The affinities of the Fab' fragments of 1H10 and 5H4 were similar, while the Fab' fragment of 3F12 had a lower affinity, as determined by titration on the ELISA plates (data not shown).

The produced Fab' fragments were tested in biochemical assays as well. The ability of the MAb 5H4 Fab' fragment to inhibit the formation of the E2-DNA complex is shown in Fig. 5A. Concentrations of MAb 5H4 and its Fab' fragment of 1 ng/μl and at least 10 ng/μl, respectively, were required to prevent the formation of the E2-DNA complex (Fig. 5A). The MAb 5H4 Fab' fragment was also capable of dissociating the preformed E2-DNA complex (data not shown).

We transfected 100 ng of origin-containing plasmid pUCAlu in the presence of increasing concentrations of Fab' fragments into CHO4.15 cells by electroporation. A representative replication assay is shown in Fig. 5B and C. The Fab' fragment of MAb 1H10 had no significant inhibitory effect on DNA replication at any concentration tested (Fig. 5B and 5C, lanes 5 and 6). The Fab' fragment of MAb 3F12 activated rather than inhibited replication (Fig. 5B and C, lanes 3 and 4), and the

Fab' fragment of MAb 5H4 inhibited BPV-1 origin replication (Fig. 5B and 5C, lanes 7 and 8) in a dose-dependent fashion.

DISCUSSION

The crystal structure of the DNA-binding domain of the E2 protein with and without DNA has been solved (15, 16). Until the crystal structure of the full-length E2 protein is determined, we will have to rely on other methods to examine the structural organization of the whole protein and the molecular interactions that must occur to accomplish the replication and/or transcription activity of the protein. Even if the crystal structure were known, information about possible interactions should be gathered by other methods. In this study, we have produced and characterized a panel of MAbs as probes and tools for studying the structure and function of the BPV-1 E2 protein.

A total of 22 MAbs that were reactive to the E2 protein in an enzyme immunoassay were isolated. Seventeen of these MAbs were directed against linear epitopes that were mapped within the region between amino acids 180 and 309 of E2. In fact, the last part of the amino-terminal transactivation domain and the first 10 amino acids of the hinge region, residues 180 to 218, appear to constitute a highly immunogenic "hot spot," since epitopes for 12 of these 17 MAbs were found to be localized within this region. The reason for the highly immunogenic properties of the region between residues 180 and 218 is unknown. Epitopes for 5 of the 22 MAbs were mapped within the C-terminal DNA-binding and dimerization domain. Interestingly, all of these antibodies recognized the composite epitopes and did not react with the denatured E2 protein. None of the epitopes for the MAbs tested were mapped to the first 180 residues of the E2 protein.

When only a purified transactivation domain, residues 1 to 218, of E2 was used for immunization, four MAbs against the region between amino acids 1 and 180 of E2 were obtained; however, none of them was able to recognize the E2-DNA complex in a mobility shift assay (21a). In contrast, Hibma and coworkers (17) raised antibodies against the N-terminal part of the HPV-16 E2 protein, indicating that the HPV-16 and BPV-1 E2 proteins are considerably different in terms of structure and epitope presentation. The most antigenic regions are usually the less ordered regions of the protein without packed internal side chains. From this point of view, the differential antigenicity may be a reflection of the differences in the structures of the HPV-16 and BPV-1 E2 proteins. Gauthier and coworkers (10) probed the structure of HPV-16 E2 with polyclonal antibodies raised against synthetic peptides that cover the whole region of the HPV-16 E2 protein. They found that antipeptide antibodies against the hinge region but not against the transactivation domain or the DNA-binding and dimerization domain were able to recognize the native form of the HPV-16 E2 protein.

In our study, MAb 1E2 (epitope within residues 184 to 190) was able to recognize neither E2-DNA nor E1-E2-origin complexes in a mobility shift assay. Curiously, deletion of the first alpha helix from the BPV-1 E2 protein revealed the epitope for MAb 1E2, and the protein in the protein-DNA complex was recognized by the antibody. Thus, the epitope for this MAb is probably buried within the compact structure of the N-terminal domain and is not accessible unless the structure of the molecule is distorted in some fashion. These data suggest that the transactivation domain of the E2 protein, unlike many other transactivation domains, has remarkable structural integrity. As shown by X-ray analysis, the C-terminal DNA-binding and dimerization domain has a compact structure (15). Deletion of the last 13 C-terminal residues of E2 resulted in an inactive protein unable to bind DNA and support replication (21a). So, our data confirm that in a native context, both the transactivation domain and the DNA-binding and dimerization domain of BPV-1 E2 have a complex and relatively rigid structure, while the central, hinge region is highly mobile and flexible.

MAb 5H4 and its Fab' fragment efficiently inhibited the formation of both E2-DNA and E1-E2-origin complexes. They not only competed with DNA for binding but also were able to dissociate the preformed E2-DNA complex. In a transient replication assay, MAb 5H4 and its Fab' fragment efficiently suppressed BPV-1 DNA replication. This assay is another way to demonstrate that the BPV-1 E2 protein interaction with the specific recognition sequence within an origin of replication is essential for the initiation of viral DNA replication. In addition, the results indicate that it is possible to target the E2 protein interaction with DNA for therapeutic purposes by using this specific MAb or Fab' fragment to block the replication of papillomaviruses.

In our study, MAb 3F12 (epitope at residues 199 to 206), directed against the last 10 amino acids of the transactivation domain, and MAbs that bind the hinge region, 1H10 (epitope at residues 208 to 218) and 1E4 (epitope at residues 250 to 280), efficiently suppressed BPV-1 DNA replication. However, the Fab' fragments of 1H10 and 3F12 had no inhibitory effect on and even activated replication. None of these antibodies interfered with the formation of the E1-E2-origin complex. These data suggest that antibodies 3F12 and 1H10 sterically hindered the inter- or intramolecular interactions required for the replication activity of the E2 protein. On the other hand, our results demonstrate the importance of the hinge region for the replication activity of the E2 protein. This hypothesis is based on results from several laboratories. E2 proteins containing large internal in-frame deletions of the hinge region (from amino acids 195 to 309, 213 to 309, and 220 to 309) were not able to support DNA replication (or had a decreased efficiency) but could efficiently enhance the binding of E1 to the replication origin (46, 47). A fusion protein which contained the transactivation domain together with the hinge region of BPV-1 E2 linked to the GCN4 DNA-binding domain supported replication much more efficiently than a fusion protein in which only the transactivation domain of E2 was linked to the GCN4 DNA-binding domain (3). These data suggest that the conformational freedom of the E2 protein is important for its role in replication and that the inhibitory effect of the antibodies against the epitopes in the hinge region as well as in the very last part of the transactivation domain may be explained by interference with conformational freedom, which would not allow E2 to assume the proper conformation required for its replication activity. However, another possibility is that this region is important for interactions with replication factors, so that antibodies that bind to the first part of the hinge region can but Fab' fragments cannot prevent the binding of replication factors to the same region of the E2 protein.

ACKNOWLEDGMENTS

We are grateful to Aire Allikas and Saul Kivimae for providing the E2 deletion mutants, Marko Piirsoo for providing the pHookAlu construct, and Anne Kalling for technical assistance.

This work was supported by grants 2496 and 2497 from the Estonian Science Foundation, grant HHMI 75195-541301 from the Howard Hughes Medical Institute, grant CIPA-CT94-0154 from the EU, and a grant from the Citrina Foundation.

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Kurg, R., Langel, Ü. and Ustav, M. (2000)
Inhibition of the bovine papillomavirus E2 protein activity by peptide nucleic acid.
Virus Res. 66(1), 39–50.

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Virus Research 00 (1999) 000–000

Virus Research

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Inhibition of the bovine papillomavirus E2 protein activity by peptide nucleic acid

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Received 28 June 1999; received in revised form 4 October 1999; accepted 4 October 1999

Abstract

The bovine papillomavirus type-1 E2 protein is the master regulator of the papillomavirus transcription and replication, the activity of which is regulated through sequence-specific DNA binding. Peptide nucleic acid (PNA) is a nucleic acid analogue, which associates with high affinity to complementary DNA, RNA or PNA, yielding in formation of stable complexes. The potential use of PNA as a sequence-specific inhibitor of the E2 protein activity is studied in this report. We demonstrate that replacement of one or both DNA strands with the complementary PNA reduced drastically the affinity of the BPV-1 E2 protein to its target site in the direct as well as in competitive binding as shown by in vitro gel-shift assays. We demonstrate that PNA could specifically bind to the double stranded E2 binding site by forming the complex with DNA oligonucleotide. In addition, PNA was able to bind specifically to the E2 binding site within the supercoiled plasmid DNA. Such binding of PNA to the E2 binding site within the origin of replication specifically abolished the activity of the E2 protein in the initiation of DNA replication in vivo. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Papillomavirus; E2 protein; Peptide nucleic acid (PNA); Replication

1. Introduction

Bovine papillomavirus type 1 (BPV-1) has been studied extensively as a model for papillomavirus replication and transcription. The viral E2 protein is the master regulator of the viral life cycle, it modulates the transcription of viral genes (Spalholz et al., 1985), is responsible for initiation of DNA replication (Ustav and Stenlund, 1991; Yang et al., 1991), for stable maintenance of the viral genome (Piirsoo et al., 1996) and for chromatin association of the viral genome in the nucleus (Lehman and Botchan, 1998; Skiadopoulus and McBride, 1998; Ilves et al., 1999). For the initiation of papillomavirus DNA replication, two virus-encoded proteins, E1 and E2 are required (Ustav and Stenlund, 1991). The papillomavirus E1 protein is the viral helicase that initiates viral

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0168-1702/99/$ - see front matter
PII: S0168-1702(99)00124-0

DNA replication. The E2 protein is required for recruitment of the E1 replication protein to the papillomavirus origin of replication (Mohr et al., 1990; Lusky et al., 1994; Seo et al., 1993; Sedman and Stenlund, 1995). E2 is the sequence-specific DNA-binding protein which binds as a dimer to the 12-bp palindromic sequence ($ACCN_6GGT$), several copies of which are found in the viral enhancer sequences (Androphy et al., 1987). The protein consists of an N-terminal transcription activation domain and a C-terminal DNA binding-dimerization domain, connected by a central hinge region (Giri and Yaniv, 1988). The crystal structure of the DNA binding-dimerization domain of E2 has been determined and has revealed a dimeric DNA binding-dimerization motif (Hegde et al., 1992).

Peptide nucleic acids (PNAs) are oligonucleotide analogues in which the deoxyribose phosphate backbone has been replaced by non-charged N-(2-aminoethyl)glycine polymer (Nielsen et al., 1991). PNAs can bind to single-stranded DNA or RNA via Watson–Crick base-pairing, with binding affinities significantly higher than those of the corresponding DNA oligomers (Egholm et al., 1993). With duplex DNA, PNA interacts to form strand-invasion complexes, which has been described in terms of the following 'binding rules': (1) homopyrimidine PNAs invade dsDNA to form PNA_2–DNA triplex structures; (2) purine rich PNAs can invade dsDNA via PNA–DNA duplexes; and (3) cytosine rich pyrimidine PNAs can interact directly with dsDNA to form PNA–DNA_2 triplexes (Peffer et al., 1993; Demidov et al., 1995; Wittung et al., 1997). These high affinity interactions of PNA and, additionally, very high stability of PNA polymers in biological systems (Demidov et al., 1994), makes this new class of biopolymers very attractive as a potential candidate for reagents suitable for regulation of gene expression. Binding of PNA to dsDNA target interferes with the binding of proteins which also recognise this target. For instance, cleavage of DNA by restriction enzymes is inhibited if the restriction enzyme recognition sequence is occupied by PNA (Hanvey et al., 1992; Nielsen et al., 1993). In vitro studies have demonstrated that PNA–dsDNA complexes are efficient inhibitors of transcription elongation and also completely abolish binding of transcription factors to their recognition sequence and thereby inhibit transcription initiation (Nielsen et al. 1994; Vickers et al., 1995).

In the viral context the activity of viral genes as well as the initiation of replication are regulated through regulation of E2 activity (for review Ustav and Ustav, 1998). For example, the truncated forms of E2 containing the DNA-binding domain only can act as repressors. Both competitive DNA binding at the E2BS and formation of heterodimers have been proposed as potential mechanisms by which the repressor molecules inhibit E2 (Lim et al., 1998). Recently, we have shown that monoclonal antiboby and its Fab' fragment directed against the C-terminal part of the protein, capable of inhibiting the formation of E2-DNA complex efficiently suppressed the BPV-1 DNA replication in vivo (Kurg et al., 1999). These data suggest that the E2 protein can serve as a viable target for the development of therapeutics against papillomaviruses and compounds, which interfere with E2 binding to DNA are promising candidates for that. Using an NMR-based screening method, a chemical compound [5-(3'-(3'',5''-dichlorophenoxy)-phenyl)-2,4-pentadienoic acid] was found to inhibit the binding of E2 to DNA (Hajduk et al., 1997), however, it is not known whether this compound is able to interfere with the E2 protein activity in vivo, too. In the present study, we have assessed the ability of PNA to specifically interfere with the E2 protein binding to its recognition sequence. We demonstrate that specific complementary PNA was able to form a sequence-specific complex with the E2 binding site (E2BS) and that occupying of E2 target site by PNA prevented the E2 protein binding to its target site. In addition, binding of PNA to the E2 binding site within the origin of replication specifically abolished the activity of the E2 protein in the initiation of DNA replication in vivo.

2. Materials and methods

2.1. Synthesis of PNA

Three PNA polymers, PNA1 (TCGACCG-

GCAACGGTACT amide), PNA2 (AGCTGGC-CGTTGCCATGA amide) and PNA3 (CC-CTCGCTGAGGTTC amide) were synthesised from the following monomers obtained from Millipore, USA: *t*-Boc-PNA-T, *t*-Boc-PNA-(Z)A, *t*-Boc-PNA-(Z)C, *t*-Boc-PNA-(Z)G. The PNAs were synthesised in a stepwise manner in a 0.03 mmol scale manually on solid support (*p*-methylbenzhydrylamine resin to obtain C-terminal amides) using 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) activation strategy. The PNA polymers were finally cleaved from the resin with liquid HF at 0°C for 30 min as described earlier (Langel et al., 1992). The purity of the PNA polymers was > 99% as demonstrated with HPLC on an analytical Nucleosil 120-3 C_{18} reverse-phase HPLC-column (0.4 cm × 10 cm). Molecular mass of each synthetic PNA polymer was determined with a Plasma Desorption Mass Spectrometer (Bioion 20, Applied Biosystems), and the calculated values were obtained in each case.

2.2. Plasmids

BPV-1 E1 expression vector pCGEag and E2 expression vector pCGE2 have been described in (Ustav and Stenlund, 1991) and replication reporter plasmids pUCAlu, Msp(7914-27), Msp15 and Msp15BS9 in (Ustav et al., 1991, 1993).

2.3. PNA binding assays

The buffer used throughout this study was TE (10 mM Tris–HCl and 1 mM EDTA pH 7.6). The duplexes between PNA–PNA and PNA–DNA were formed by mixing equimolar amounts or 1 to 10 for DNA–PNA hybrid of the strands and annealing at 37°C for 1 h. To form DNA–PNA hybrid duplex DNA oligo 5'GATTCC GTACCGTTGCCGGTCG 3' complementary to PNA1 sequence was used. When required, the DNA oligo was end-labelled by using T4 polynucleotide kinase and [γ-^{32}P]ATP.

PNA binding to the dsDNA was measured by using the gel mobility shift assay. A fixed concentration of the end-labelled duplex DNA (1.4 nM) was incubated with increasing concentrations of the PNA in TE with or without 150 mM NaCl in the final volume of 10 µl. Following the incubation at 37°C for 2 h or at 70°C for 10 min and at 37°C for 30 min, PNA bound duplex DNA was separated from free target by electrophoresis through an 8% native polyacrylamide gel (80:1 acrylamide:N,N-methylenebisacrylamide) in 0.25 × Tris-borate-EDTA (TBE) for 2 h at 100V. Gels were fixed in acetic acid/ethanol, dried and exposed to X-ray film. Quantitative analysis was carried out with a PhosphoImager SI (Molecular Dynamics).

To examine the inhibition of binding of the E2 protein, PNA was first complexed with target DNA in TE as described above, then the buffer conditions were adjusted to E2 binding buffer (10 mM Tris–HCl, pH 7.5, 100 mM KCl, 2 mM DTT, 0.5 mM PMSF, 15% glycerol, 5 mg/ml BSA, 1 µg/ml leupeptine, 1 µg/ml aprotinine) and 1 ng of the purified E2 protein and 1 µg of non-specific competitor salmon sperm DNA were added. After a 15 min incubation at room temperature, E2–DNA complex and PNA–DNA complex were separated from free target by electrophoresis through a 6% native polyacrylamide gel as described above.

For the replication assay, 100 ng of reporter plasmid was incubated overnight with increasing concentrations of PNA in 10 µl TE at 37°C.

2.4. Detection of PNA-plasmid complexes in vitro

Biotinylated-PNA1 (300 µM) was allowed to bind to different replication reporter plasmids (100ng in each case) in 15 µl TE at 37°C overnight or at 80°C for 10 min and at 37°C for 1 h. The reactions were analyzed by 1% agarose gel electrophoresis. Blotting of gels was done by the semidry-blotting method using the Trans-blot^{R} SD electrophoretic transfer unit (Bio-Rad) at a fixed current (mA) of 2 × the gel surface area (cm^2) for 1 h. For transfer, polyvinylidene difluoride (PVDF) membrane (Boehringer Mannheim) was activated in methanol and then wetted in water, filter papers (Whatman 3MM) were equilibrated in transfer buffer (48 mM Tris/39 mM glycine/20% methanol, pH 8.5) (Demczuk

et al., 1993). For biotin detection, membranes were first blocked for 30 min in Tris-buffered saline (TBS) supplemented with 0.2% Tween 20. Antibody, streptavidin–horseradish peroxidase conjugate (Amersham) was applied at a dilution of 1:2500, and enhanced chemiluminescence detection was done using the ECL + Plus detection kit (Amersham) according to the manufacturer's recommendations.

2.5. Cell culture and replication assay

CHO cells were cultured in F12 medium supplemented with 10% fetal bovine serum. For transfection CHO cells were trypsinized, harvested by centrifugation and resuspended in F12 medium containing 10% FCS at a density of $1x10^7$ cells/ml. 200 µl of cell suspension was mixed with pre-formed plasmid–PNA complex, 100 ng of plasmid pCGE2, 250 ng of plasmid pCGEag and 50 µg of salmon sperm DNA in a disposable electroporation cuvette and was subjected to an electric discharge of 230V using a BioRad Gene Pulser at 970 µF capacity. After the discharge cell/PNA/DNA mixture was left at room temperature for 15 min, then cells were washed, divided between two 60 mm plates and plated in F12 supplemented with 10% FCS. Cells were harvested either 48 or 72 h after electroporation.

Transient replication assay was carried out as described by Ustav and Stenlund (Ustav and Stenlund, 1991). Briefly, an episomal DNA was extracted by alkaline lysis, purified, digested with DpnI and linearizing enzyme *Hin*dIII and analyzed by Southern blotting.

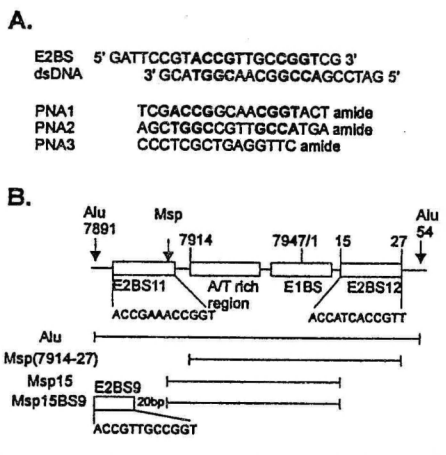

Fig. 1. Schematic representation of E2 binding site, PNAs and papillomavirus Ori constructs used in this study. (A) Oligonucleotide and PNA sequences. The E2 binding site is shown in block letters. The PNA-s are oriented from the N to the C terminus (the C terminus is a carboxy-amide) such that when the N-terminal end of the PNA faces the 5' end of the oligonucleotide the complex is termed parallel. (B) Schematic representation of the BPV-1 replication origin inserts used. Numbers indicate positions on the BPV-1 nucleotide sequence.

3. Results

3.1. Interaction of the BPV-1 E2 protein with PNA–DNA hybrid

The BPV-1 E2 protein is a transactivator, which binds specifically an interrupted palindromic sequence ACCG(N_4)CGGT (Androphy et al., 1987). PNA has been found to be a good DNA mimic in terms of its ability to form Watson–Crick base-paired helical duplexes with complementary oligonucleotides (Egholm et al., 1993). In PNA only the nucleobases of DNA are retained and the backbone of PNA is noncharged. Therefore, the study of interaction of DNA binding protein with PNA containing duplex could provide valuable information about the relative contributions of the nucleobases by excluding the electrostatic interaction with backbone. To test whether the E2 protein is able to bind specifically to PNA–DNA hybrid or PNA–PNA duplex, two PNA 18-mers (PNA1 and PNA2) were designed to mimic the E2 protein binding site (E2BS) (Fig. 1A). The duplexes between PNA1–PNA2 and ssDNA–PNA1 were formed by mixing equimolar amounts (or 1 to 10 for DNA–PNA hybrid) of the strands and annealing for 1 h in 10 mM Tris–HCl 1mM EDTA (TE). In order to deter-

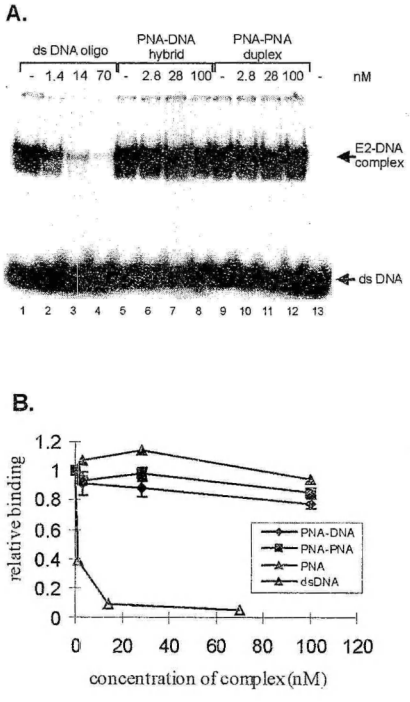

Fig. 2. The ability of the BPV-1 E2 protein to bind PNA-DNA hybrid and PNA–PNA duplex. (A) The mobility shift assay was carried out with 1 ng of E2, 1 nM of end-labelled E2BS and various concentrations of pre-formed, non-labelled ds-DNA, PNA–DNA hybrid or PNA–PNA complex for 1 h at room temperature. Protein–DNA complex was resolved on 6% PAGE in 0.25 × Tris-borate-EDTA. (B) Quantitation of data from Fig. 2A. The values shown are the results of three independent experiments.

mine the interaction between the E2 protein and PNA containing hybrids, a competition assay was used. The BPV-1 E2 protein forms a specific complex with E2BS which could be detected in the gel-shift assay (Fig. 2A, line 1). Adding of pre-formed non-labelled dsDNA, PNA–DNA hybrid or PNA–PNA duplex to the reaction mixture containing the E2 protein and labelled E2BS would interfere with the E2–E2BS complex formation in case, if these compounds would be able to compete for the binding to E2 with the labelled E2BS. As shown in Fig. 2A, lines 2–4, the formation of the E2-DNA complex was efficiently inhibited by an excess of the nonlabelled specific oligonucleotide. Addition of 1.4 nM non-labelled oligonucleotide reduced the detectable E2 binding considerably, which was virtually undetectable at 70 nM of non-labelled E2BS. Replacing one strand in dsDNA target with PNA, or in the case of PNA–PNA duplex, the considerable decrease in the formation of specific E2–DNA complex was not observed even at the highest concentrations studied (100 nM) (Fig. 2A, lanes 5–12, Fig. 2B).

We also studied the ability of the E2 protein to interact with the PNA-DNA hybrid (directly) by gel-shift assay. In this case, the end-labelled ss-DNA oligo was first incubated with complementary PNA1 (molar ratio 1:10) in TE for 1 h at 37°C. Following pre-incubation the buffer conditions were adjusted to E2–DNA binding buffer and increasing concentrations of purified E2 were added. After 45 min at 37°C the reaction was visualised by electrophoresis. We were not able to detect any specific E2:PNA–DNA band under our assay conditions used in this case as well (data not shown). Thus, the E2 protein was unable to form specific and stable complex with either PNA–DNA hybrid or PNA–PNA duplex.

3.2. PNA binding to E2 binding site

PNA molecules can bind duplex DNA to form specific and stable PNA–DNA strand-invasion complexes (Peffer et al., 1993; Demidov et al., 1995; Wittung et al., 1997). To study the PNA binding to E2 binding site (E2BS), the radiolabelled dsDNA was complexed with PNA and the resulting complex was separated from free DNA by electrophoresis in 8% polyacrylamide gel, and the intensities of both bands were quantified. First, E2BS was incubated at various concentrations of specific PNA1, PNA2 or non-specific PNA3 in TE for 10 min at 70°C and for 30 min at 37°C. Incubation at 70°C was performed in order to melt the dsDNA E2BS and to stretch out the PNA, as PNAs used in this study contain interrupted palindromic sequences that might form

relatively stable intramolecular hairpin structures. As shown on Fig. 3A (lanes 2–6) and Fig. 3B, binding of specific PNA1 to target dsE2BS was first detectable at the concentration of 0.2 μM and reached 83% at 2 μM and 93% at 6 μM. Binding of PNA2, which had a parallel orientation relative to the target was less efficient reaching only 51% at 6 μM (Fig. 3A, lanes 7–11, and Fig. 3B). Binding of non-specific PNA3 to E2BS was not observed at these concentrations (Fig. 3A, lanes 12–16, and Fig. 3B). Fig. 3C shows the data obtained when the binding reaction was carried out for 2 h at 37°C without preceding incubation at 70°C as the formation of a complex between PNA and DNA should also open the hairpin structure of the PNA (Armitage et al., 1998). Under these conditions, binding of PNA1 to E2BS started at the concentration of 0.6 μM and reached 91% at 6 μM, whereas binding of PNA2 reached only 20% at 6 μM. So, we conclude from these results that PNA is able to specifically bind to E2BS and that separation of strands at higher temperature enhances the complex formation. The binding of PNA to DNA is highly dependent on salt concentration (Peffer et al., 1993). The increase of the salt concentration would make a DNA structure more compact, which would make it more difficult for PNA to invade the dsDNA structure and form sequence-specific complex. In our experiments a decrease in binding of PNA to DNA at higher NaCl concentrations was also observed. At physiological salt concentrations (150 mM NaCl) the binding of PNA1 was reduced approximately twice-only 45% binding (6 μM PNA1, 2h incubation at 37°C) was observed to E2BS (Fig. 3C).

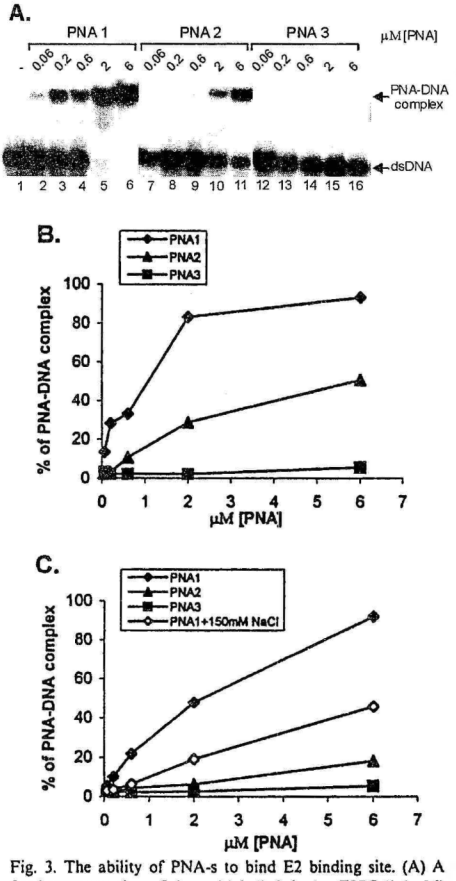

Fig. 3. The ability of PNA-s to bind E2 binding site. (A) A fixed concentration of the end-labelled duplex E2BS (1.4 nM) was incubated with increasing concentrations of each PNA in TE for 10 min at 70°C and after that for 30 min at 37°C. The samples were analysed by electrophoresis in a 8% polyacrylamide gel, followed by autoradiography. PNA binding generates a complex of reduced mobility relative to the duplex. (B) Quantitative analysis of Fig. 3A. (C) The ability of PNA-s to bind E2BS for 2 h at 37°C. The values shown are the results of three independent experiments.

Sequence-specific DNA binding is essential for the expression of most of the functional activities of the E2 protein. Therefore we have studied the ability of the E2 protein to bind to E2BS which is in complex with PNA. First, specific PNA1 was pre-incubated with E2BS in TE due to the slow kinetics of invasion in the higher salt buffer. After a 2 h pre-incubation at 37°C the buffer conditions were adjusted to E2 binding buffer and the purified E2 protein was added. The amount of E2 bound in the presence of increasing concentrations of PNA was visualized by gel-shift (Fig. 4). The BPV-1 E2 protein specifically binds E2BS (lane 7). PNA1, which is complementary to the upper strand of E2BS, effectively inhibited the binding of E2 to its target site at the concentra-

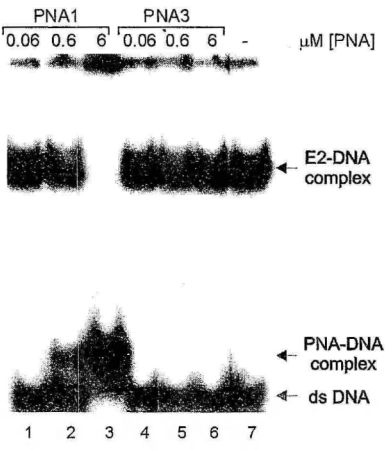

Fig. 4. Effect of PNA-s on the E2 protein binding in vitro. E2BS was pre-incubated in TE buffer with PNA at the indicated concentrations for 2 h at 37°C. Following the pre-incubation, buffer conditions were adjusted to E2 binding buffer and 1 ng of the purified E2 protein was added. After 15 min incubation, E2–DNA complex and PNA–DNA complex were separated from free target by electrophoresis through a 6% native polyacrylamide gel.

tion of 6 µM of PNA (lane 3). As a control, non-specific PNA3 had no effect on E2 binding at any concentration tested (lanes 4–6). These data demonstrate that complementary PNA is able to specifically bind to the E2BS and that this complex results in the specific disruption of the E2 binding to its target site.

3.3. Inhibition of BPV-1 ori replication by PNA in vivo

The viral E2 protein is required for the initiation of papillomavirus DNA replication in vivo (Ustav and Stenlund, 1991). The function of E2 in replication is to serve as a specificity factor enhancing binding of the viral helicase E1 to the origin of replication (Yang et al., 1991; Seo et al., 1993; Ustav et al., 1993; Sedman and Stenlund, 1995). So, next we looked at the ability of PNA to block the E2 protein dependent BPV-1 origin replication by inhibiting E2 binding to its target site. First we investigated PNA binding to different replication reporter plasmids. Ori constructs used in this study are shown on Fig. 1B and described elsewhere (Ustav et al., 1991, 1993). Biotinylated-PNA1 was incubated with various reporter plasmids in TE and the resulting complexes were separated in a standard agarose gel-electrophoresis (Fig. 5A, left image) and then transferred by electroblotting to the PVDF membrane. Immunodetection of biotin revealed the specific PNA–plasmid complexes as well as unbound PNA, appearing at the top of the lanes (Fig. 5A, right image). The best binding was observed to artificial Ori construct Msp15BS9, where a strong E2 binding site BS9 is linked to A/T rich region and E1BS (Fig. 5A, lane 10). PNA1 has a perfect match with E2BS9, whereas there are 3 mismatches out of 12 in the middle of the target sequence in the case of E2BS11 and 4 mismatches out of 12 in the case of E2BS12. Under our assay conditions, PNA1 binding to Alu fragment, which contains E2BS11 and E2BS12 (Fig. 5A, lane 8) and a slight binding to Msp(7914-27) containing E2BS12 only (Fig. 5A, lane 9) was observed. A weak binding to a negative control Msp15, which does not contain any E2BS near the E1 binding site (lane 7), was probably due to E2 binding motif present in the plasmid pUC19 backbone.

In the following experiments different replication reporter plasmids (100 ng of each) were pre-incubated at various concentrations of PNA in TE buffer at 37°C overnight. After that the PNA–plasmid complexes were co-transfected together with expression plasmids for E2 (pCGE2) and for E1 (pCGEag) into CHO cells as described in Section 2. Fortyeight and 72 hours after electroporation the episomal DNA was extracted by alkaline lysis, purified, digested with *Dpn*I and linearizing enzyme *Hin*dIII, and analysed by Southern blotting as described earlier (Ustav and Stenlund, 1991). In all cases, inhibition of the replication was dependent on the concentration of specific PNA (Fig. 5B). When tested at concentrations of 6 µM or less the E2BS specific PNA1 failed to inhibit replication of the BPV-1 origins in the cells. However, at a concentration of 300 µM specific PNA1 inhibited the replication of

artificial origin Msp15BS9 and papillomavirus minimal origin pUCAlu completely (Fig. 5B and C, lanes 11–12). At the same time the control PNA, non-specific PNA3, had no effect on pUCAlu ori replication (Fig. 5C, lanes 21–22 and Fig. 5B). A slight inhibition of replication of Ori construct Msp(7914-27) with 300 μM of PNA1 was also observed (Fig. 5B). The concentration of PNA of 300 μM in the reaction mixture (which was diluted 20 × during the electroporation) was not toxic to the cells and had no effect on the cell growth (data not shown). We also tested the

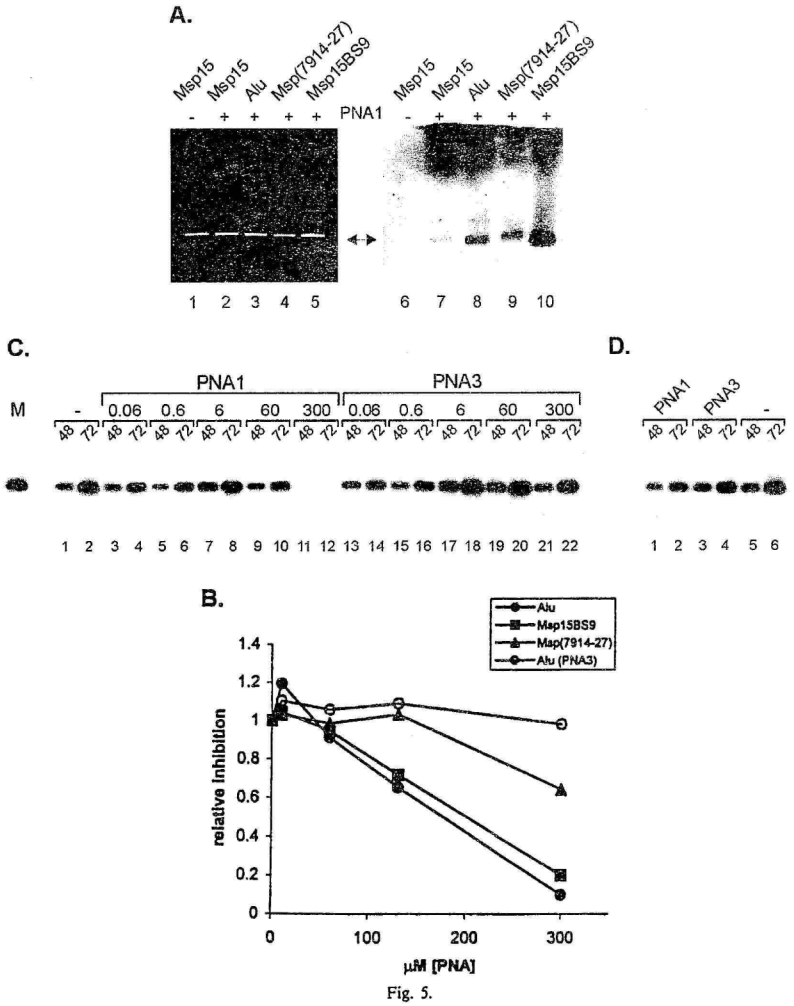

Fig. 5.

ability of PNA to inhibit the replication of papillomavirus origin in the cells without pre-forming the complex of PNA with pUCAlu plasmid. In this case, plasmids pUCAlu, pCGE2, pCGEag and PNA were mixed in the electroporation cuvette and immediately co-transfected into CHO cells. However, in this case we could not observe any inhibition of replication of papillomavirus origin plasmid pUCAlu (Fig. 5D).

4. Discussion

Molecular interaction between proteins and DNA constitute the basis for specific regulation of key biological functions such as gene expression, transcription and replication. Therefore molecules that bind DNA in a sequence-specific manner are attractive candidates as tools for modulation of these activities and have the potential, at a later stage, to be exploited as pharmaceuticals. In the present study we have investigated the potential application of the specific peptide nucleic acid (PNA) as anti-viral agent against papillomaviruses.

In this study, we show that the BPV-1 E2 protein is not able to bind specifically either PNA–DNA hybrid or PNA–PNA duplex. The interaction of double stranded DNA with protein is often divided into contributions from the negatively charged desoxyribose phosphate backbone and the nucleobases themselves. In the PNA, only the nucleobases of DNA are retained, the backbone is replaced by uncharged polyamide chain. As phosphate contacts largely contribute to the DNA-binding affinity of E2 protein (there are 20 direct and 28 water-mediated phosphate interactions per complex) (Hegde et al., 1992), it is not surprising that the E2 protein binds the PNA–DNA hybrid with dramatically reduced efficiency. The sequence-specific DNA binding is often accompanied by deformation of the DNA. The DNA within the E2/E2BS complex adopts $\approx$ 43°C bend towards the minor groove at the centre of the target (Hegde et al., 1992) and recently it has been shown that BPV E2 protein binding affinity depends on DNA flexibility (Hines et al. 1998; Rozenberg et al., 1998). So, the origin of the reduced binding of E2 to PNA–DNA hybrid may also result from the decreased flexibility of the pseudopeptide backbone. Therefore, although structure determinations of PNA–DNA duplexes have suggested that PNA adopts to its nucleic acid partner without contributing significantly to the final conformation of the structure (Eriksson and Nielsen, 1996) we can not exclude the possibility that some conformational rigidity of the structure of PNA–DNA hybrid does not allow protein binding.

In vitro binding experiments using a radiolabelled E2BS demonstrated that binding of PNA to duplex DNA was sequence specific and that the orientation of the PNA relative to the DNA was important. Our results also show that preceding separation of DNA strands and linearization of PNA enhanced the formation of PNA–DNA complex in vitro. Stable PNA–DNA complexes have so far been demonstrated for homopyrim-

Fig. 5. Effect of PNA on BPV-1 origin replication. (A) Detection of the PNA and DNA components of the PNA–plasmid complex. Biotinylated-PNA1 (12 μg) was incubated with 100 ng of various papillomavirus replication origins in TE at 37°C overnight. The resulting complexes were separated in a standard agarose gel-electrophoresis (lanes 1–5) and then transferred by electroblotting. (Lanes 6–7) Immunodetection of biotinylated-PNA on PVDF membrane. (B) Effect of PNA-s on different BPV-1 origin replication. Reporter plasmids were pre-incubated with indicated concentrations of PNA in TE buffer overnight at 37°C. The plasmid-PNA complexes were then electroporated into CHO cells as described in Section 2. Episomal DNA was extracted from cells 48 h after transfection, digested with *Dpn*I and linearizing enzyme *Hin*dIII and analyzed by Southern blotting. The replication signals of three independent experiments were quantified with a Phosphoimager, and signals from cells transfected with the origin-containing plasmids only were used as a control to normalize the results. (C) Southern blot analysis of transient replication of the BPV-1 origin plasmid pUCAlu in the presence of various concentrations of PNA. Reporter plasmid pUCAlu was pre-incubated with indicated concentrations of PNA in TE buffer overnight at 37°C. The plasmid–PNA complex was then electroporated into CHO cells, episomal DNA was extracted from cells either 48 or 72 h after transfection. Filters were probed with radiolabelled pUCAlu plasmid. M- marker, 100 pg of reporter plasmid pUCAlu. (D) Southern blot analysis of transient replication of the BPV-1 origin plasmid pUCAlu in the presence of 12 μg of PNA. In this case, DNA and PNA were mixed before co-transfection into CHO cells.

idine PNA-s binding to homopurine targets, however, PNA binding to DNA with only 66% A + G content has also been shown (Gambacorti-Passerini et al., 1996). There are distinct mechanistic variations how PNA interacts with the dsDNA target depending on nucleobases (Wittung et al., 1997). For example, cytosine-rich PNA oligomers are added to the double-stranded target polynucleotides as Hoogsteen strands, forming PNA–DNA_2 triplex structures, but the homopurine PNAs, as well as alternating T/G PNA oligomer, are instead found to invade their DNA targets, forming new PNA–DNA complexes. In our study, the specific PNA1 had a mixed sequence with 50% T + C content and it is difficult to say what kind of PNA–DNA complex was formed. However, PNA binding to E2BS was temperature and salt dependent, which shows that this reaction requires DNA 'breathing', we suggest that PNA binding caused at least partial strand exchange resulting in PNA–DNA–DNA triplex. The fact that PNA1 and PNA2, but not a control PNA3, form the complex of the same size without releasing single strand oligonucleotide suggests that in our cases PNA forms a complex with the dsDNA. Whatever the precise structure, the PNA binds to the dsDNA with sufficient affinity to specifically prevent binding of the E2 protein to its target site. However, PNA was able to inhibit E2 binding in vitro under certain conditions. We were not able to demonstrate specific activity when the PNA binding was performed in E2 binding buffer, which had a KCl concentration of 100 mM. Specific activity was observed when PNA was incubated with the DNA target in low salt buffer prior to addition to the E2 binding buffer. In this case, binding of PNA to its duplex target correlated with inhibition of E2 binding. As binding to its target site is required for expression of all the activities of E2, these findings open the possibility to use PNA as a tool in research.

The main objective of this work was to determine whether PNA binding to E2BS results in specific inhibition of papillomavirus replication in a cell culture model. Specific inhibition was observed when PNA was incubated with origin containing plasmids in buffer with low salt concentration prior to electroporation. Specific PNA bound effectively to Ori construct Msp15BS9 and poorly to BS12 within origin Msp(7914-27). PNA binding resulted in strong or weak inhibition of replication, respectively. In the case of reporter plasmid pUCAlu, which contains E2BS11 and E2BS12, a weak binding of PNA1 was observed. This result was not unexpected as both binding sites have a considerable homology, although contain mismatches comparing with PNA1. Suprisingly, binding of PNA to both E2BS with low efficiency resulted in strong inhibitory effect in replication assay. Recently it was shown that E2BS11 and E2BS12 play separate but synergetic roles in the initiation of BPV DNA replication that are dependent on their location within the origin (Gillette and Borowiec, 1998). Our present results support this hypothesis as partial blocking of both sites by PNA resulted in synergistic response of viral DNA replication.

We were not able to detect any specific inhibitory effect when PNA was mixed with the plasmid pUCAlu and electroporated into the cells without pre-forming PNA–plasmid complex. Probably this was due to decreased binding of PNA to DNA at physiological salt concentrations, which was observed in our in vitro binding experiments, and has been shown earlier, as well (Peffer et al., 1993; Vickers et al., 1995). However, once formed, the PNA–DNA complexes are all very stable at physiological salt concentrations (Cherny et al., 1993; Wittung et al., 1997). In our experiments, once formed, the PNA-plasmid complex was sufficiently stable to produce an effect even 120 h after electroporation. On the other hand, compartmentalisation of PNA to the nucleus (if it is not bound to plasmid prior to electroporation) could not be sufficient for formation of the PNA–DNA complex and thereby achieving the inhibitory effect. In addition, the accessibility of the E2 binding site to the specific PNA in vivo may also reduce its efficiency of binding.

In the present study, we have targeted the E2 protein for anti-viral agent against papillomaviruses. PNA-s have a potentional as antiviral chemotherapeutic agent, especially in view of the fact that PNA-s are extremely stable to digestion by nucleases and proteases (Demidov et al., 1994)

and can act at the specific sequence on the DNA. Under our assay conditions sequence-specific PNA was capable of specifically inhibiting E2 binding to its target site and also inhibit the replication of the papillomavirus origin in the cells when PNA was pre-bound to the origin plasmid, but it was not able to inhibit replication without pre-forming PNA–plasmid complex. In conclusion, our data suggest that PNA-s can be used as tools in research applications, however they have to be modified so that the kinetics of complex-formation with the recognition sequence under physiological salt conditions could be increased considerably in order to be used as a antiviral drug.

Acknowledgements

We thank Margus Pooga and Aare Abroi for helpful discussions throughout the work. This work was supported by Estonian Science Foundation grants No. 2496 and 2497, Howard Hughes Medical Institute grant 75195-541301 and grants from the Swedish Research Council for Natural Sciences (NFR).

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Scientific work

In 1993 I started to work in the group of Prof. Mart Ustav. My interest was to study the structure and functions of the E2 protein of bovine papillomavirus. Most of my scientific achievements are described in the publications gathered within these thesis.

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Alates 1993. aastast olen töötanud prof. Mart Ustavi töögrupis. Olen uurinud veise papilloomiviirusevalgu E2 struktuuri ja funktsioneerimist. Minu peamised teadussaavutused on kirjeldatud käesolevas dissertatsioonis.

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ISSN 1024–6479
ISBN 9985–56–469–3