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Regulation of papillomavirus transcription and replication; insights for the design of extrachromosomal vectors

Review Article

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Summary

The papillomaviruses infect and replicate in the stratified layers of skin and mucosa and give rise to benign lesions called warts or papillomas. The virus infects basal epithelial cells and within these persistently infected cells the viral genome is maintained at low levels as extrachromosomally replicating viral DNA. The genomes of papillomaviruses can also be stably maintained as high copy number extrachromosomal elements in cell lines and within these cells the viral genomes replicate in synchrony with cellular DNA. The E1 and E2 viral proteins regulate viral transcription, initiation of replication and long term episomal maintenance of viral genomes. This review will describe the functions of the E1 and E2 proteins and discuss how these functions can be exploited in the design of extrachromosomal replicating vectors for gene therapy.

I. Introduction

Certain DNA viruses, such as papillomavirus or Epstein-Barr virus, are able to maintain their genomes as stable extrachromosomal elements in the nuclei of infected cells. The papillomaviruses are small DNA viruses that infect basal epithelial cells and replicate in terminally differentiating keratinocytes. These viruses have been isolated from a wide range of vertebrates and they exhibit both host species and tissue specificity. Viral DNA replication has been studied mostly in bovine papillomavirus type 1 (BPV-1) and the human papillomaviruses (HPV), HPV-1, -11, -16 and -18 and -31.

The viral E1 and E2 proteins are important for initiation of viral DNA replication and for regulation of viral transcription. The E1 protein is the primary viral replication initiator protein (Ustav and Stenlund, 1991a; Mohr *et al*., 1990; Yang *et al*., 1991) and E1 also functions as a transcriptional repressor (Sandler *et al*., 1993; Le Moal *et al*., 1994); the viral E2 protein(s) are transcriptional regulatory proteins that regulate the expression of the other viral gene products and, in addition, play an important role in DNA replication. The E2 transactivator protein is also required for long-term episomal maintenance of viral genomes within replicating cells (Piirsoo *et al*., 1996). Papillomavirus genomes and the E2-TA protein interact with mitotic chromosomes in dividing cells and this association is likely to be important for genome segregation (Skiadopoulos and McBride, 1998; Lehman and Botchan, 1998).

II. The papillomavirus life cycle and function of the viral proteins

Papillomaviruses infect and replicate in stratified epithelium and give rise to benign lesions called warts or papillomas. Papillomaviruses infect the lower basal layer of cells of a stratified epithelium (**Figure 1**). The α6β4 integrin protein, expressed exclusively in this cell layer, acts as a receptor for the virus (Evander *et al*., 1997). Damage to the superficial layers of the epithelium is probably necessary to allow access of virus to the basal

layer. Within basal cells the viral genome is amplified to a low copy number and maintained as an extrachromosomally replicating circle of double stranded DNA (**Figure 2**). DNA replication in these cells probably requires the viral E1 and E2 replication proteins. The viral E5 protein is also expressed in basal cells. E5

Figure 1. Diagram of differentiating cells in a stratified epithelium and expression of viral functions in a papilloma. Papillomaviruses infect basal skin cells; within these cells the viral genome is replicated extrachromosomally and early gene products are expressed. Viral DNA amplification and late gene expression only occur in differentiating cells.

Figure 2. Circular genomic map of bovine papillomavirus type 1 (BPV-1). The early ORFs (E1-E8) and late ORFs (L1 and L2 are indicated). The LCR (long control region) contains regulatory elements for transcription and DNA replication such as the origin

and minichromosome maintenance element (MME). E2 DNA binding sites are represented by red circles and promoters by arrows.

stimulates the activity of growth factor receptors expressed by the cell and induces cellular proliferation (reviewed in Howley, 1995). Enhanced proliferation of basal cells may be important to increase the population of infected cells and to provide a suitable environment for establishment of a productive viral lesion. As basal cells differentiate and migrate up to the stratum spinosum, expression of the E2 proteins is greatly increased and vegetative DNA replication begins (Burnett *et al*., 1990; Howley, 1995). The cells in this layer do not normally divide nor express cellular proteins necessary for DNA replication. Therefore, the viral E7 protein is required to induce the differentiated keratinocytes to enter S-phase and synthesize cellular replication proteins by binding to and inactivating the cellular retinoblastoma protein, Rb (reviewed in Jones and Munger, 1996). However, the conflicting signals of cell cycle progression and differentiation induce the p53 protein, which in turn signals cells to undergo apoptosis or growth arrest. The viral E6 protein can inactivate this function of p53 by targeting it for degradation by the ubiquitin-proteasome pathway (reviewed in Kubbutat and Vousden, 1996). The viral E4 protein is also abundant in the more differentiated layers of a papilloma. It has been hypothesized that E4 may function as a nuclear structural protein, an RNA splicing and transport factor, or in release of viral particles from the papilloma (reviewed in Howley, 1995). In the upper differentiated layers of the papilloma, the viral capsid proteins L1 and L2 are synthesized and virions are assembled (reviewed in Howley, 1995).

III. Different modes of DNA replication in the papillomavirus life cycle

Three modes of DNA replication take place in the papillomavirus life cycle: initial DNA amplification, maintenance replication and vegetative replication. After initial uptake of the virus, the virion particle is uncoated and the genome transported to the nucleus of the basal cell where it is presumed to be amplified to a low copy number (Zhou *et al*., 1995). Presumably, a low level of the E1 and E2 proteins must be expressed early after infection since there is no evidence that they are in the viral particle. Most experimental studies have examined transient DNA replication in cultured cells, a system that is probably most analogous to this initial amplification stage and which requires the E1 and E2 proteins and the viral replication origin (Ustav and Stenlund, 1991a; Ustav and Stenlund, 1991a).

Stable episomal maintenance is the second stage of papillomavirus DNA replication. In a papilloma, the infected basal cells proliferate and maintain low levels of extrachromosomal viral DNA. The genomes of papillomaviruses can also be stably maintained as high copy number extrachromosomal elements in cell lines (Law *et al*., 1981) and within these lines the viral genomes replicate in synchrony with cellular DNA. The viral genome copy number remains constant overall but the genomes are replicated by a random choice mechanism (Gilbert and Cohen, 1987; Ravnan *et al*., 1992). Long term, stable maintenance of papillomavirus-derived plasmids requires expression of the E1 and E2 proteins, the replication origin and a region from the LCR, that has been designated a minichromosome maintenance element (MME) (Piirsoo *et al*., 1996). This element contains multiple high affinity E2 binding sites. Recent studies have shown that both the BPV1 E2 transactivator protein and BPV genomes are associated with cellular chromosomes at mitosis (Skiadopoulos and McBride, 1998; Lehman and Botchan, 1998). This could be the mechanism by which approximately equal numbers of viral genomes are segregated to daughter cells at cell division to ensure that all basal cells of a papilloma contain viral DNA .

The third stage of viral replication is vegetative DNA replication, which is required to generate progeny virus. Vegetative DNA replication only occurs as the basal cells of a papilloma migrate upwards and differentiate in the stratum spinosum layer. Increased expression of the E2 proteins also occurs in the stratum spinosum and may be important for amplification of viral DNA (Burnett *et al*., 1990). The E2 protein is important for initiation of viral DNA replication but it has also been shown that HPV-31 E2 can arrest cells in S phase (Frattini *et al*., 1997). Clearly this could be important for vegetative replication by allowing sustained synthesis of viral DNA. There appears to be a switch from bidirectional theta replication in the maintenance stage of replication to a rolling circle mode in the vegetative stage (Flores and Lambert, 1997). Little else is known about vegetative viral DNA replication because of the requirement for terminally differentiating keratinocytes and difficulties in reproducing these conditions in a culture system. However, great advances are being made by replicating papillomaviruses in organotypic raft cultures and in xenografts of mice and these systems are proving to be very useful in studying the entire viral life cycle (reviewed in Meyers and Laimins, 1994).

IV. Transcriptional regulation by the viral E1 and E2 proteins

Papillomavirus transcription is regulated primarily by the viral E2 gene products. These proteins regulate transcription by binding to specific DNA sites located in the viral genomes (see **Figure 2**). In bovine papillomavirus type 1 several gene products are expressed from the E2 ORF and they have been shown to function as transcriptional activators and repressors (**Figure 3**). cDNA species that could potentially encode truncated human papillomavirus E2 repressor proteins have been cloned but, as yet, no such proteins have been identified. Some HPVs may have evolved a mechanism to both activate and repress viral transcription with the full-length E2 protein (see **Figure 5**, reviewed in McBride and Myers, 1997; Fuchs and Pfister, 1994).

Figure 3. A structural and functional map of the BPV-1 E2 proteins.

Figure 4. A structural and functional map of the BPV-1 E1 proteins.

The full-length E2 protein from all papillomaviruses consists of a 200 amino acid N-terminal transactivation domain linked to a 100 amino acid C-terminal DNA binding and dimerization domain by a flexible hinge region of variable length and sequence (reviewed in McBride and Myers, 1997; McBride *et al*., 1989; Dostatni

et al., 1988). The E2-TA protein activates transcription by binding to specific DNA binding sites that are located within enhancer elements in the viral genome (reviewed in McBride and Myers, 1997). There are seventeen different E2 binding sites in the BPV-1 genome that vary in affinity for the E2 protein over two orders of magnitude (Li *et al*., 1989) (**Figure 2**). The well-studied genital-associated HPV genomes contain only four E2 sites in the LCR (**Figure 5**).

The C-terminal domain of E2 binds specifically to DNA as a dimer. The X-ray crystal structure of the C-terminal 85 amino acids of E2 bound to DNA was the first example of an anti-parallel β-barrel DNA binding structure (Hegde *et al*., 1992). The DNA binding domain forms an eight-stranded anti-parallel β-barrel made up of four strands from each subunit. A pair of α-helices symmetrically positioned on the outside of the barrel contain the amino acids residues that are required for specific DNA interaction. The DNA binding domain of the Epstein Barr virus EBNA1 protein has a very similar structure to the E2 DNA binding domain despite no sequence similarity (Bochkarev *et al*., 1995).

The 200 amino acid E2 transactivation domain, unlike many other transactivation domains, appears to have a very constrained structure that is easily disrupted by deletion or certain non-conservative point mutations (reviewed in McBride and Myers, 1997). The transactivation domain is also critical for the replication function of the E2 protein and for interaction with the E1 protein (reviewed in McBride and Myers, 1997). The exact mechanism of transactivation has not been elucidated but probably involves interaction with components of the basic transcriptional machinery. BPV1 E2 has been shown to interact with SP1, TBP, TFIIB and a novel cellular protein, AMF-1 (Li *et al*., 1991; Steger *et al*., 1995; Rank and Lambert, 1995; Breiding *et al*., 1997).

In BPV-1, the E2 ORF encodes three different polypeptides; the E2-TA transactivator protein is encoded by the entire ORF and two smaller polypeptides, E2-TR and E8/E2, are encoded by the 3' half of the ORF. The shorter polypetides function as transcriptional repressors by antagonizing the function of E2-TA (Hubbert *et al*., 1988; Lambert *et al*., 1987; Spalholz *et al*., 1985; Lambert *et al*., 1989; Choe *et al*., 1989). The repressors contain only the DNA binding/dimerization domain and function both by direct competition with E2-TA for binding to the E2 DNA binding sites and by the formation of inactive heterodimers with the full-length E2-TA protein (Lim *et al*., 1998; Barsoum *et al*., 1992) (**Figure 5**).

In several HPVs associated with the anogenital tract, the full length E2 protein appears to repress the promoter located upstream from the E6 gene (reviewed in McBride and Myers, 1996, 1997; Fuchs and Pfister, 1994). This probably occurs when the E2 dimer binds to E2 DNA binding sites that overlap binding sites for the cellular SP1 and TFIID transcription factors. Recent studies have indicated that these proximal E2 binding sites have lower affinity for the E2 protein than the E2 binding sites are located further upstream from the promoter start site. This has led to a model in which low levels of E2 bind to the higher affinity upstream E2 sites and activate transcription, but at high levels of E2 protein the lower affinity proximal E2 sites are occupied leading to transcriptional repression (**Figure 5**). The situation is probably even more complex in a papilloma as the levels and activities of the E2 proteins and cellular transcription factors are likely modulated by cell cycle and epithelial differentiation.

Figure 5. Mechanisms of transcriptional regulation by the papillomavirus E2 proteins. **A**. BPV1 expresses a transcriptional transactivator with a transactivation domain and DNA binding/dimerization domain. Two shorter repressor proteins contain only the DNA binding/dimerization domain and repress E2 transactivation by forming heterodimers with the transactivator and by competing for the binding to the E2 sites in the viral

genome. **B**. In many HPVs the full-length E2 protein can activate transcription by interacting with higher affinity E2 binding sites upstream from the transcriptional start site. At higher levels of E2, the lower affinity sites close to the promoter become occupied. This displaces essential cellular factors, SP1 and TFIID and results in repression of basal promoter activity.

Figure 6. Model of initiation of viral DNA replication. The E1 and E2 proteins initiate DNA replication by cooperatively binding to specific sites in the viral origin of replication. It has been proposed that an E1-alone complex then assembles in a ring-like hexamer structure around the DNA and the helicase activity of E1 unwinds the origin to allow access of the cellular replication machinery.

The viral E1 replication protein can also function as a transcriptional repressor. Inactivation of E1 increases the immortalizing or growth transforming potential of HPV-16 and BPV-1, respectively (Schiller *et al*., 1989; Lambert and Howley, 1988; Romanczuk and Howley, 1992) and this correlates well with the frequent disruption of E1 and/or E2 expression found in HPV-associated carcinomas. The E1 protein of BPV-1 can repress E2-mediated transactivation of the viral P89 promoter, which expresses the E6 and E7 gene products (Sandler *et al*., 1993; Le Moal *et al*., 1994). This is probably a consequence of binding of an E1/E2 complex to the replication origin, which is located just upstream from P89.

V. Initiation of viral DNA replication by the E1 and E2 proteins

In addition to the cell's replication machinery, papillomavirus DNA replication requires the full-length E2 transactivator protein, the viral E1 protein and the replication origin (Ustav and Stenlund, 1991a; Ustav *et al*., 1991b; Ustav and Stenlund, 1991a). The minimal origin of replication consists of an E1 binding site, an E2 binding site and an AT rich region that may facilitate origin unwinding. (Ustav *et al*., 1991b). The E1 protein has several replication-associated activities such as origin-specific binding (Wilson and Ludes-Meyers, 1991) and helicase activities (Yang *et al*., 1993) and forms a complex with the E2 transactivator (Mohr *et al*., 1990; Blitz and Laimins, 1991; Seo *et al*., 1993; Spalholz *et al*., 1993; Sedman and Stenlund, 1995) (**Figure 4**). The E1 and E2 sites have relatively low affinity for their respective proteins but together they cooperatively bind to the origin with high affinity (**Figure 6**). After the initial binding of an E1/E2 complex to the origin, the E1 protein oligomerizes to form a trimer or hexamer that encircles the DNA and E2 dissociates from the origin (Sedman and

Stenlund, 1996, 1998). The E1 helicase function of the hexamer then unwinds the DNA at the origin to allow DNA synthesis to begin (Sedman and Stenlund, 1998) (**Figure 6**).

The E2-TA transactivator plays an auxiliary role in replication by enhancing and regulating the functions of the E1 protein. In addition to cooperatively binding to the origin with the E1 protein, E2 alleviates repression of replication by nucleosomes (Li and Botchan, 1994) and interacts with cellular replication proteins such as RPA (Li and Botchan, 1993).

VI. Viral genome plasmid maintenance and genome segregation

Rodent cells transformed by BPV-1 maintain approximately 50 to 200 copies of the viral genome indefinitely as extrachromosomal nuclear plasmids (Law *et al*., 1981). Cell lines derived from cervical carcinomas can also maintain human papillomavirus genomes as extrachromosomal elements (Bedell *et al*., 1991). Plasmids containing the minimal viral replication origin replicate transiently in cells expressing the E1 and E2 proteins but the replicated DNA is lost with time. Long-term stable maintenance of origin-containing plasmids also requires regions from the LCR that contain multiple high affinity E2 DNA binding sites in addition to the replication origin (Piirsoo *et al*., 1996). This region has been designated the minichromosome maintenance element (MME) and can be substituted by ten tandem copies of E2 DNA binding sites, suggesting that the E2 protein and the E2 DNA binding sites are important for genome segregation (**Figure 7**). This finding is supported by the observation that the E2-TA protein and BPV-1 genomes are associated with condensed mitotic chromosomes in dividing cells (Skiadopoulos and McBride, 1998; Lehman and Botchan, 1998) (**Figure 8**) and supports a model in which viral genomes are attached to mitotic chromatin indirectly via the E2 protein and E2 DNA binding sites (**Figure 9**). This interaction would ensure that approximately equal numbers of viral genomes are segregated to daughter cells. Viral genomes that replicate as extrachromosomal plasmids may also require a mechanism to ensure that they are not lost from the nucleus during cell division. Association with cellular chromosomes would ensure that viral genomes are enclosed in the nuclear membrane during telophase. The genomes may also interact with some cellular component that ensures that they are in a transcriptionally active region of the nucleus as the cells move into the G1 stage of the cell cycle.

Figure 7. Requirements for long-term episomal maintenance of papillomavirus genomes.

The BPV-1 E2-TA protein interacts with mitotic chromatin in the absence of viral genomes. Conversely, the E2-TR and E8/E2 proteins are dispersed throughout the cell during mitosis and are excluded from mitotic chromatin. This indicates that the DNA binding domain of the E2 protein is not sufficient for the interaction with mitotic chromosomes and suggests that the interaction is not mediated by binding to cellular DNA sequences. The finding that a DNA-binding defective E2-TA protein retains the ability to interact with mitotic chromatin also supports this. Furthermore, deletions within the N-terminal domain abrogate the ability of E2 to interact with mitotic chromosomes. These findings indicate that the N-terminal

transactivation domain of E2-TA is necessary for the interaction (Skiadopoulos and McBride, 1998).

As yet, it is not known what component of mitotic chromatin is important for interaction of the E2 protein with mitotic chromatin. One possibility is that E2 interacts with some constituent of the chromosomal scaffold or chromosomal periphery. The chromosomal periphery is a region around the condensed chromatids that contains many proteins, some of which form a network of fibrils and granules (Hernandez-Verdun and Gautier, 1994). Several components of the nuclear matrix are found in the perichromosomal region as well as a number of "passenger" proteins from the nucleus and nucleoli. The E2-TA protein (but not the E2-TR or E8/E2 proteins) has been shown to be associated with the nuclear matrix (Hubbert *et al*., 1988) and it will be interesting to determine whether the same interactions are important for the association with mitotic chromosomes. Nuclear matrix attachment sites have also been identified in the BPV-1 genome (Adom and Richard-Foy, 1991; Adom *et al*., 1992; Tan *et al*., 1998) and it is possible that these sites are also important for interaction of the genomes with mitotic chromatin instead of, or in addition to, E2 DNA binding sites.

Although the overall viral copy number in a population of BPV-1 transformed cells remains relatively constant, several studies have shown that individual cells contain a wide range of copy numbers (Roberts and Weintraub, 1988; Ravnan *et al*., 1992; Ravnan and Cohen, 1997). Stewart *et al*. (1994) also demonstrated that there is significant randomization in replication and/or partitioning. This suggests that segregation does not occur by a very precise mechanism and is consistent with the model that the E2 proteins and viral genomes randomly associate with mitotic chromatin as passenger molecules. This model would also predict that the viral copy number depends on the levels of the E2-TA protein.

A similar phenomenon has been observed for Epstein-Barr virus (EBV). EBV infects and immortalizes B-lymphocytes and the viral genome is maintained indefinitely as an extrachromosomal element. The EBNA-1

Figure 8. Papillomavirus genomes and the E2-TA transactivator protein are associated with cellular chromosomes in mitotic cells. E2 proteins were detected in COS7 cells expressing the E2-TA protein by indirect immunofluorescence using an E2-specific antibody. Panels **A** and **B** show COS-7 cells as a control. Panels **C** and **D** show COS-7 cells expressing E2-TA. In panels A and C, cellular DNA was detected by propidium iodide staining. In panels B and D, FITC-labeled E2 protein is detected in the same field of cells. BPV DNA was detected by fluorescent in situ hybridization in C127 cells (**E** and **F**) and 137 cells (that contain BPV-1) (**G** and **H**). In panels E and G cellular DNA was detected by the propidium iodide signal. In panels F and H the same field of cells are shown with the FITC-labeled BPV DNA signal. Mitotic cells are indicated by white arrows.

Figure 9. This diagram shows a model in which papillomavirus genomes are linked via the E2-TA protein to condensed mitotic chromosomes.

protein of EBV is a transcriptional transactivator and a replication protein and it is the only viral protein required for replication and maintenance of plasmids containing the oriP origin of replication (which contains a number of repeated EBNA DNA binding sites) (Yates *et al*., 1985). The EBNA 1 protein and EBV genomes have also been shown to be randomly associated with mitotic chromatin (Grogan *et al*., 1983; Harris *et al*., 1985) and it has been suggested that these properties might be important for the genome segregation and nuclear retention function of EBNA-1. The EBNA-1 protein also promotes prolonged nuclear retention of plasmids containing EBNA-1 DNA binding sites but no origin of replication (Krysan *et al*., 1989; Middleton and Sugden, 1994) and it has been proposed that this is due to the interaction of plasmids with mitotic chromosomes.

VII. Similarities between the papillomavirus E2 and Epstein-Barr virus EBNA-1 protein

The Epstein-Barr virus EBNA-1 protein and the papillomavirus E2-TA protein have common roles in the life cycles of their respective viruses (Grossman and Laimins, 1996). Both proteins are transcriptional transactivators that activate transcription by binding to specific binding sites within the viral genomes. Notably, both proteins have dimeric DNA binding. domains with almost identical anti-parallel β-barrel structures, despite no amino acid homology (Bochkarev *et al*., 1995). Both viruses replicate and maintain their genomes as extrachromosomal elements in persistently infected cells. This maintenance requires both E2-TA and the multiple E2 binding sites in the MME element of papillomavirus (Piirsoo *et al*., 1996) or EBNA-1 and the multiple EBNA-1 binding sites in the oriP element of Epstein-Barr virus (Yates *et al*., 1985). In both cases the viral proteins and

genomes are associated with condensed cellular chromosomes during mitosis (Skiadopoulos and McBride, 1998; Lehman and Botchan, 1998; Grogan *et al*., 1983, Harris *et al*., 1985). The EBNA-1 protein promotes prolonged nuclear retention of plasmids containing EBNA-1 DNA binding sites even in the absence of replication (Krysan *et al*., 1989; Middleton and Sugden, 1994) and it has been proposed that this is due to the association with mitotic chromosomes.

VIII. Papillomavirus-derived gene therapy vectors

Gene therapy vectors that replicate and are retained extrachromosomally have several advantages over those that integrate in a random fashion into the host genome. These vectors will persist in proliferating cells and should not generate mutations by insertion into the cellular chromosomes. Such vectors can be maintained at a high copy number and are not susceptible to positional effects, such as inactivation, that are dependent on the integration site. EBV-based vectors that contain the EBNA-1 gene and the oriP replication origin have been developed and can express foreign gene products in primate and human cells (reviewed in Calos, 1996). Another class of EBV vectors have been developed that only contain the EBNA-1 gene and repeats of the EBNA-1 binding site required for nuclear retention. In these vectors the oriP origin has been replaced with a cellular replication origin and the resulting vectors are able to replicate in a wider range of mammalian cells (Krysan *et al*., 1989). The EBNA-1 protein also has the advantage that it is not recognized by the cell-mediated immune system (Levitskaya *et al*., 1995) as it is resistant to the proteasome-mediated degradation that is required for antigen presentation (Levitskaya *et al*., 1997). However, there is a report that the EBNA-1 protein can cause lymphomas in transgenic mice expressing this protein in B-cells (Wilson *et al*., 1996).

Sarver *et al*. (1981) first described the use of papillomaviruses as vectors in 1981. In general, these vectors comprised the 69% transforming region of the virus (the genome minus the late region) and the foreign gene to be expressed. A newer vector only contains the LCR and the E1 and E2 genes (Ohe *et al*., 1995). However, these vectors have a limited host range and, in some cases, insertion of a foreign transcriptionally active foreign gene causes the plasmid to integrate (Waldenstrom *et al*., 1992). This is probably because the small papillomavirus genomes are very compact and contain multiple overlapping genes and regulatory signals that can be inadvertently disrupted. The presence of an active heterologous enhancer and promoter could interfere with viral replication by transcriptional interference. Using the detailed knowledge of the mechanisms of papillomavirus replication and genome maintenance, it should be possible to generate a new class of papillomavirus vectors. These vectors could express the E1 and/or E2 genes from different promoters suitable for a specific cell type and either viral or cellular replication origins could be incorporated, as has been described for EBV-based vectors (Krysan *et al*., 1989). The addition of repeated E2 binding sites may be sufficient to maintain the vector as an episome when either a viral or cellular replication origin is used.

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