BRIEF COMMUNICATION

Development of human papillomavirus plasmids capable of episomal replication in human cell lines

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Gene delivery into human cells has been facilitated by the development of viral vector systems. These vectors have shown great potential for the efficient delivery of therapeutic genes into human cells. A problem with many of the existing systems, however, is the integration of these vectors into the chromosome which affects the length of gene expression and may promote oncogenic transformation. In an effort to develop viral vectors that can replicate extrachromosomally in human cells, we have generated human papillomavirus (HPV) plasmids containing all the elements required for replication on a single DNA molecule. HPV

plasmids containing the viral E1 and E2 genes (or the E1 gene alone) and an origin of replication were shown to replicate to significant levels in the transfected human cervical carcinoma C-33A cell line. Since approaches towards the possible gene therapy of cystic fibrosis (CF) are currently under intensive investigation, we have also tested short-term replication of HPV plasmids in the IB3 cell line derived from a CF patient. Our results demonstrate that HPV plasmids are capable of extrachromosomal replication in these cell lines and may potentially be important vectors for the delivery of therapeutic genes into human cells.

Keywords: human papillomavirus vectors; autonomous replication; cystic fibrosis

Viral vectors have become important tools for the delivery of therapeutic genes into cells for human gene therapy. Many viral vectors have been developed such as those based on retrovirus, simian virus 40 (SV40), Epstein-Barr virus, adenovirus, vaccinia virus, adeno-associated virus (AAV) and herpesvirus.^{1–5} While many of the above viral vectors have proven to be of immense value in initial attempts to treat human genetic diseases, it is desirable to develop additional vector systems that replicate extrachromosomally and may potentially provide stable, long-term expression of cloned genes in human cells. We decided to develop human papillomavirus (HPV)-based vectors because of their potential for autonomous replication in human cells.

HPVs are small circular double-stranded DNA viruses of approximately 8-kb that infect squamous epithelial cells and produce benign warts and papillomas, as well as cancer of the cervix.^{6–8} The viral genome exists in an extrachromosomal form as a chromatin and is maintained in the basal cells of the squamous epithelium in its latent state in a stable copy number estimated to be approximately 50.⁹ In the upper differentiating cells of the epithelium, vegetative replication of HPV occurs resulting in a high copy number.⁹ HPV types such as 1a and 2 infect cutaneous epithelium whereas types such as 6, 11, 16 and 18 infect the mucosal epithelium.⁶ HPVs require the viral E1 and E2 proteins for their replication, and a *cis*-acting sequence termed the long control region (LCR) contains the promoter and enhancer elements as

well as the origin of replication (*ori*).¹⁰ The *ori* includes one E1 and multiple E2 binding sites. Previous studies using three-plasmid systems, one expressing the HPV E1 protein, another the E2 protein and a plasmid containing the HPV *ori* have demonstrated HPV DNA replication in transfected fibroblast and epithelial cell lines.^{10–14} In addition, in the case of HPV type 1a, the E1 protein alone was shown to be sufficient for replication of *ori* plasmids.¹⁵ The E6 and E7 proteins of high-risk HPVs such as types 16 and 18 are involved in cellular transformation and target the cellular p53 and RB proteins, respectively.¹⁶ However, it is important to note that the transforming E6 and E7 genes of HPVs are not required for HPV DNA replication, at least in short-term assays.^{10–14} Also, the vegetative replication of HPV DNA is not necessary for their potential use as vectors for gene therapy since their DNA is maintained as a nuclear plasmid in latently infected cells. Bovine papillomavirus type 1 (BPV-1) vectors have been used to produce stable cell lines expressing foreign proteins.^{17–19} The BPV-1 vectors used in these studies contained both replication and transforming genes, and in most cases, extrachromosomal replication of these vectors was accompanied by transformation of the target cells. Recent advances in our understanding of the replication and transforming genes of HPVs has made it feasible to attempt to develop HPV vectors that can be established as extrachromosomal plasmids that express foreign proteins without the oncogenic transformation of the host cell.

Since HPVs show a strong tropism for epithelial cells, we tested whether the HPV *ori* plasmids can replicate in IB3, a bronchial epithelial cell line derived from a cystic fibrosis patient,²⁰ since CF is a common genetic disease and an important target for gene therapy.²¹ In the pres-

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ence of the HPV E1 and E2 proteins, the pUCLCR-18 plasmid containing the HPV-18 ori replicated efficiently in IB3 cells in short-term assays as demonstrated by the *DpnI*-resistance of the ori band (Figure 1). Optimal replication of the ori plasmid was observed when 5 μ g of the E1 expressing plasmid and 0.5 μ g of the E2 expressing plasmid were used (Figure 1). Addition of higher amounts of the E1-expressing plasmid did not result in a further increase in replication (data not shown). The copy number of the ori plasmid was approximately 500 per cell. This was calculated by running known quantities of the pUC19 plasmid on the same gel and measuring the radioactivity in various bands by an AMBIS Radioanalytic detector (Ambis, San Diego, CA, USA) (not shown). In these experiments, the E1 and E2 expressing plasmids also serve as negative controls since they lack an origin of replication and do not show replication as shown by the lack of *DpnI*-resistance in these bands (Figure 1). There was no further increase in the level of replication of the ori plasmid with increasing levels of the E2-expressing plasmid. This is consistent with our published data demonstrating that low levels of E2 are sufficient for maximal replication and high levels of E2 may negatively regulate replication.¹⁴

Next, we constructed HPV plasmids containing all the elements required for replication in human cell lines. The HPV-18 based plasmids (pFS101, pFS102, pFS103, pFS105 and pFS106) include the E1 and E2 genes driven from various promoters while the HPV-1a based plasmid (pori80-2-E1) contains only the E1 gene and the ori (Figure 2). Plasmid pFS101 contains the E1 gene downstream of the RSV 3' LTR and the E2 gene under the control of the HPV-18 LCR which also contains the origin of replication. This plasmid also contains the gene for hygromycin resistance driven from the HSVtk promoter. Plasmid pFS102 contains both the E1 and E2 genes under the control of the RSV 3' LTR along with the LCR and the gene for hygromycin resistance. In pFS103, the E1 gene is transcribed from the RSV 3' LTR while the E2 gene is expressed from the HSVtk promoter. This plasmid con-

tains a region of the HPV-18 LCR (nt 7714-119) encompassing the origin of replication. Both the pFS105 and pFS106 plasmids contain the E1 gene under the control of the SV40 early promoter, the E2 gene driven by the HSVtk promoter, and the HPV-18 ori (nt 7714-119). The difference in these two plasmids lies only in the direction of transcription of the E1 genes. The various HPV-18 constructs were cotransfected into C-33A cells along with pUC19 DNA as an internal control for transfection efficiency. Three different EBV-derived vectors⁴ (p291, p220.2 and pREP4) were also included in these experiments for comparison purposes. The presence of a band in the *DpnI*-treated samples indicates plasmid replication. Transient replication analysis showed that plasmids pFS101 and pFS106 replicated to robust levels, pFS103 replicated to modest levels and pFS102 and pFS105 replicated to very low, but detectable levels (Figure 3 and Table 1). The copy number of pFS101 and pFS106 in transient replication assays was approximately 50 per cell, while those of pFS102, pFS103 and pFS105 was several-fold lower. Plasmid pFS101 replicated to much higher levels than pFS102. The E2 gene in the pFS102 plasmid is expressed from the strong RSV 3' LTR whereas in pFS101 it is driven from the weak HPV-18 LCR promoter. The low level replication of pFS102 may be due to high levels of E2 expression from the RSV LTR since high levels of E2 inhibit HPV replication in C-33A cells.¹⁴ Plasmid pFS106 replicated to much higher levels than pFS105 (Figure 3), indicating that the orientation of the E1 expression cassette is an important factor in replication. It is likely that convergent transcription of the E1 and E2 genes inhibits their expression, severely affecting plasmid replication. However, it is also possible that juxtaposition of various sequences to the ori region in the above plasmids may affect ori function, and therefore, the level of replication. Plasmid p18E1E2, a derivative of the pFS series of vectors that lacks the ori, did not give a signal indicating that replication was ori-specific (Figure 3).

The ability of the pFS103 plasmid to replicate in the bronchial epithelial cell line IB3 was also tested. Transient replication analysis showed that the pFS103 plasmid replicated to moderate levels similar to those obtained with the EBV-based pREP4 plasmid (Figure 4).

We have previously shown that the E1 protein alone is sufficient to support replication of HPV-1a ori plasmids and multimerization of the E1 binding site greatly stimulates replication.^{15,24} We have also constructed an HPV-1a based plasmid (pori80-2-E1) that expresses the E1 gene from the SV40 early promoter and contains two copies of an 80-bp origin region that is known to support efficient replication (Figure 2). This plasmid was found to replicate to significant levels in transiently transfected C-33A cells (Figure). The plasmid 80-2 which lacks the E1 gene and pUC19 which lacks the HPV-1a ori did not replicate. These data demonstrate that replication is both E1- and origin-dependent, and establish that an HPV-1a based plasmid can replicate episomally in transfected cells.

Results described in this study demonstrate that plasmids containing HPV-1a or HPV-18 sequences can replicate episomally in human cell lines to various extents in short-term assays. We have incorporated the hygromycin resistance gene in the pFS101, pFS102 and pori80-2-E1 vectors that will be used to study the stable, long term

Figure 1 Transient replication analysis of the pUCLCR-18 plasmid in human bronchial epithelial cell line IB3. Five hundred nanograms of the pUCLCR-18 plasmid (pori177) was transfected into IB3 cells along with the indicated amounts of the pSGE1 and pSGE2 plasmids expressing the HPV-18 E1 and E2 proteins, respectively.¹⁴ Four days after transfection, low molecular weight Hirt DNA was harvested²² and digested with *EcoRI*. Treatment with this enzyme linearizes the pSGE2 and pori177 plasmids and cleaves the pSGE1 plasmid into two fragments, only one of which is complementary to the probe used. One-half of each sample was digested with *DpnI* to remove unreplicated DNA. The DNA was subjected to agarose gel electrophoresis and the Southern blot was probed with ³²P-labeled pUC19 DNA.²³ Positions of the HPV-18 origin-containing plasmid (pori177), pSGE1 (E1), and pSGE2 (E2) DNA are indicated.

Figure 2 Vectors constructed from HPV-18 (pFS101, pFS102, pFS103, pFS105 and pFS106) and HPV-1a (pori80-2-E1). The plasmids are shown in a linearized form and are present on a pUC19 backbone. The E1 gene is indicated by solid arrows and its expression is directed by either the RSV 3' LTR (RSV) or the SV40 early promoter (SV40). The E2 gene is indicated by shaded arrows and its expression is directed by either the HPV-18 LCR p105 (LCR), the RSV LTR, or the HSV thymidine kinase (TK) promoters are indicated by open arrows. Amp, ampicillin-resistance gene; Hyg, hygromycin-resistance gene for selection in mammalian cells; LCR, long control region of HPV-18 (nt 6929–7857/1–119) containing the origin of replication; ori, a region of HPV-18 LCR (nt 7714–7857/1–119) containing the ori; pori80-2-E1, HPV-1a based vector containing two copies of a 80-bp ori sequence (nt 7767–7815/1–31) that includes one E1 and one E2 binding site.²⁴

Figure 3 Transient replication of HPV-18 derived vectors. Five micrograms of the plasmid p291 or an equimolar amount of the other indicated plasmids were transfected into C-33A cells. Plasmid pUC19 (200 ng) was cotransfected with each sample (except for the p18E1E2 plasmid) as an internal control for transfection efficiency and sample recovery. Four days after transfection, Hirt samples were isolated and the samples analyzed as described in the legend to Figure 1. The various plasmids generate a common 2.5 kb band (vector) upon treatment with EcoRI which is complementary to the probe. This band is partially resistant to DpnI-treatment, indicating plasmid replication. The first two lanes represent a control experiment in which the E1 and E2 expressing plasmids were cotransfected with an HPV-18 origin (LCR) plasmid as well as the internal control pUC19. The p18E1E2 plasmid contains the E1 and E2 genes but lacks the origin, and generates a single band upon treatment with EcoRI.

Table 1 Relative replication levels of the HPV plasmids

HPV plasmid	Relative replication
pFS101	+++
pFS102	+
pFS103	++
pFS105	+
pFS106	+++
pREP4	++++

Figure 4 Transient replication of plasmid pFS103 in IB3 cells. Five micrograms of the pFS103 or the EBV-based pREP4 plasmid along with 200 ng of pUC19 were cotransfected into IB3 cells. Four days after transfection, Hirt fractions were analyzed as described in the legend to Figure 1. ori corresponds to the pREP4 or pFS103 bands.

Figure 5 Transient replication analysis of the HPV-1a based por180-2-E1 vector (80-2E1) containing the E1 gene and a duplicated origin subregion. Plasmid 80-2 contains the duplicated origin in pUC19 background but lacks the E1 gene, while pUC19 lacks both the E1 gene and the origin. The plasmids were transfected into C-33A cells and the experiment was done as described in the legend for Figure 1. Treatment of 80-2E1 with EcoRI generates two bands both of which contain sequences complementary to the pUC19 probe used.

episomal replication of these plasmids in human cell lines. We have also cloned DNA of up to 6 kb in some of the HPV vectors and shown that they replicate efficiently in transfected cells (data not shown), and we are currently testing the maximal size of insert DNA that can be cloned into these vectors without affecting their ability to replicate episomally. The LCRs of HPVs include the origin of replication as well as skin-specific promoter-enhancer elements.^{25,26} Thus, this region can also be used simultaneously to drive the expression of therapeutic genes for the gene therapy of human skin diseases. It should be possible to introduce reporter genes such as luciferase or β -galactosidase into such vectors to determine the expression levels of the cloned genes. Since HPV plasmids replicated episomally in the bronchial epithelial cell line IB3, in the future HPV vectors may potentially be useful for the expression of therapeutic proteins such as CFTR in the lung epithelium.

The HPV vector systems may provide several desirable features such as extrachromosomal replication, ability to manipulate their copy number based on the promoters used for the expression of the E1 and E2 genes, their ability to replicate in epithelial and fibroblastic cells and potential for high level expression of cloned genes due to their accessibility to the cellular transcriptional machinery when present in an episomal state. Furthermore, the HPV E1 and E2 proteins have very low immunogenicity^{27,28} that may allow repeated introduction of such vectors containing therapeutic genes into individuals.

Although a high efficiency system for HPV virion production using transfected DNA has not been reported, raft culture systems have been established which allow viral DNA replication and low-level virion production using HPV-infected primary cells or cell lines.^{29,30} Recently, HPV-like particles consisting of the HPV-16 major capsid protein L1 have been generated that package plasmid DNA and can deliver and express foreign genes into infected cells.³¹ The use of the above two systems as well as the development of high-efficiency HPV packaging systems in the future would facilitate the use of HPV vectors for gene therapy. The self-replicating HPV plasmids can also be used to generate hybrid viral vectors³²⁻³⁵ such as HPV/HSV-1 amplicon hybrids that may allow efficient packaging and delivery as well as long-term expression of cloned genes. Currently, the use of physical delivery methods such as calcium phosphate precipitation, electroporation, lipofection and bioballistics would appear to provide the best means for introducing HPV vectors containing desired genes into cells.

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