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Plasmoviruses: Nonviral/viral vectors for gene therapy

(retroviral vector/nonviral vector/thymidine kinase)

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ABSTRACT **We have generated a chimeric gene transfer vector that combines the simplicity of plasmids with the infectivity and long-term expression of retroviruses. We replaced the *env* gene of a Moloney murine leukemia virus-derived provirus by a foreign gene, generating a plasmid that upon transfer to tumor cells generates noninfectious retroviral particles carrying the transgene. We added to this plasmid an independent expression cassette comprising a cytomegalovirus promoter, an amphotropic retroviral envelope, and a polyadenylylation signal from simian virus 40. These constructs were designed to minimize the risk of recombination generating replication-competent retroviruses. Their only region of homology is a 157-bp sequence with 53% identity. We show that the sole transfection of this plasmid in various cell lines generates infectious but defective retroviral particles capable of efficiently infecting and expressing the transgene. The formation of infectious particles allows the transgene propagation *in vitro*. Eight days after transfection *in vitro*, the proportion of cells expressing the transgene is increased by 10–60 times. There was no evidence of replication-competent retrovirus generation in these experiments. The intratumoral injection of this plasmid, but not of the control vector lacking the *env* gene, led to foci of transgene-expressing cells, suggesting that the transgene had propagated *in situ*. Altogether, these "plasmoviruses" combine advantages of viral and nonviral vectors. They should be easy to produce in large quantity as clinical grade materials and should allow efficient and safe *in situ* targeting of tumor cells.**

Numerous anticancer gene therapy strategies have proved their efficacy in experimental animal models, and some of them are already being tested in clinical trials (1, 2). The clinical success of these treatments is likely to rely on efficient gene transfer into a significant proportion of the cells of the tumor mass. Viral and nonviral vectors are being developed for this purpose (2, 3). They all have advantages and limits. Among the viral vectors, adenoviruses allow an efficient transfer both *in vitro* and *in vivo* (4, 5). However, adenoviruses can infect and express their genes in both dividing and nondividing cells, and therefore there is always a risk of transgene dissemination outside of the tumor. In addition, although improvements have been made in the design of safer adenoviral vectors, the production of recombinant adenoviruses still suffers from the generation of recombinant replication-competent adenoviruses (2).

Retroviruses have important advantages for transducing tumor cells, especially regarding safety. Gene expression after retroviral infection depends on viral integration and, except for lentivirus, cell division (6). When the tumor is growing in an organ mostly made of quiescent cells, this property limits the expression of the transferred gene to the dividing tumor cells, preserving the surrounding normal cells. In addition, the present generation of retroviral vectors and packaging cell lines has been used in many patients without any evidence of toxicity or generation of recombinant replication-competent retroviruses (2).

However, current retrovirus vectors have several limitations. First, the titer of infectious particles produced by packaging cells are low, although it is now possible to obtain a titer exceeding 10^7 infectious particles per ml (7).

Another drawback of murine retrovirus vectors is that in human serum they are rapidly inactivated by complement and natural antibodies recognizing an α-galactosyl epitope. Human serum also lyses murine packaging cells (8, 9). Nevertheless, the relevance of these *in vitro* experiments to *in vivo* applications of these strategies in cancer patients is not known and is difficult to assess. Indeed, similar antibodies are present only in Old World primates (10, 11) for which there is no model of transplantable tumor. This potential problem might be overcome by producing recombinant retrovirus in cells of human origin (8), although this raises questions regarding the safety of using materials of human origin for injection into patients. Clinical trials in progress, using direct intratumoral injections of murine packaging cells, will help in clarifying this important issue (12–14).

Whatever the viral vectors used, their production will require mass cell cultures, making clinical grade production difficult. In this regard, nonviral vectors have obvious advantages (15). Recently, important progress has been achieved in the formulation of molecules that allow efficient gene transfer of plasmid DNA into cells with an increase of *in vitro* transduction efficiency to above 50% (16, 17). However, nonviral vectors still achieve a low transduction efficiency *in vivo*– e.g., when injected directly into tumors (2).

To overcome problems associated with viral and nonviral vectors, we designed a new vector system combining the advantages of both types of vectors. The central idea was to generate plasmids that could transform the transduced cell into a local packaging cell producing recombinant defective retroviruses capable of one round of transduction of neighboring cells. Following the initial transduction step, the local production of viruses ought to achieve an overall high efficiency gene transfer within a tumor, for example. Such a strategy will have the advantage of relying on an initial vector that will be easy and safe to produce, combined with the advantages of retroviruses that efficiently infect tumor cells and have a restricted expression for dividing cells. In addition, since the viruses will be locally synthesized by human cells, they are not likely to be neutralized by human sera.

We recently showed that it is possible to replace the *env* gene of a Moloney murine leukemia virus (Mo-MuLV)-derived provirus by a therapeutic gene, thus generating a plasmid that upon transfer to tumor cells generates noninfectious retroviral particles carrying the transfene (18). This plasmid can be

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Abbreviations: Mo-MuLV, Moloney murine leukemia virus; PEI, polyethylenimine; GCV, ganciclovir; IDC, iododeoxycytidine; TK, thymidine kinase.

trans-complemented by an independent *env*-expressing vector. Likewise, when the defective recombinant provirus was introduced in *env*-expressing cells, when an *env* gene was introduced in a cell expressing this provirus, or when two plasmids were introduced simultaneously in a tumor cell, infectious defective retroviral particles were produced (18).

To further simplify this approach, and increase transduction efficacy, we engineered a plasmid containing both the defective recombinant proviral genome and a transcription unit coding for *env*, which we refer to as "plasmovirus." We demonstrate that, upon transfection into a tumor cell, plasmoviruses generate infectious recombinant defective retroviruses carrying the therapeutic gene. These viruses then go on to infect neighboring tumor cells, allowing for propagation and expression of the transgene.

MATERIALS AND METHODS

Plasmids. pNCA plasmid is an infectious provirus containing the entire genome of the ecotropic Mo-MuLV [kindly provided by S. Goff (19)]. pMTK is a herpes simplex virus type 1 thymidine kinase (HSV1 TK)-expressing retroviral vector and was described previously (20). pV-TK and p-env, expressing an *env* gene from the 4070A amphotropic Mo-MuLV under the control of the cytomegalovirus promoter, have been described previously (18). All plasmids were constructed by standard techniques. All enzymes were obtained from Boehringer Mannheim and oligonucleotides from Eurogentec, Brussels. pV-TK-env was generated by cloning the p-env expression cassette into a unique *Eco*RI restriction site lying outside of the proviral sequences of pV-TK. Sequences of the generated PCR fragments were verified. pV-lacZ and pV-lacZ-env are similar constructs to pV-TK and pV-TK-env, respectively. pV-lacZ and pV-lacZ-env were designed by replacing the HSV1 TK gene with the *nls-lacZ* gene construct from the pLLZ plasmid (kindly provided by O. Schwartz, Paris).

Cells, Cell Culture, and DNA Transfection. Four cell lines that are deficient in cellular TK activity were used. NIH 3T3 TK^- cell line (kindly provided by J. Ghysdael, Gif sur Yvette, France) and the murine connective tissue L-M(TK^-) cell line (ATCC number CCL-1.3) were grown in Dulbecco's modified Eagle's medium (GIBCO/BRL) containing 10% (vol/vol) newborn calf serum (HyClone). The 143B TK^- human osteosarcoma cell line (ATCC number CRL 8303) was grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (HyClone) with 10 mg of bromodeoxyuridine per ml. tk^-ts13 Syrian hamster cell line (ATCC number CRL-1632) and the ΨCRIP cell line (21) were grown in Dulbecco's modified Eagle's medium containing 10% fetal or newborn calf serum, respectively. MC26SC31 primitive colon carcinoma cell line (kindly provided by A. Kinsella, Liverpool, England) was grown in RPMI 1640 medium (GIBCO/BRL) containing 10% fetal calf serum. Cell lines were transfected by using polyethylenimine (PEI, 25 kDa, Aldrich) as described (17).

Sensitivity to Ganciclovir (GCV). The effect of GCV (Syntex, France) on DNA replication was measured by a [^{3}H]thymidine incorporation proliferation assay. Cells were cultured in media containing different concentrations of GCV for 4–5 days. Then, 1.5 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine (Amersham, 5 Ci/mmol) was added for 24 h, cells were washed twice, and incorporated radioactivity was counted in a liquid scintillation counter Micro β-plus counter (Wallac).

Viral Titers. Measurement of viral titers was performed as described previously (18).

Iododeoxycytidine (IDC) Incorporation and *in Vitro* Staining for *lacZ* Expression. Cells were plated on six-well plates at a density of 5×10^4 cells per well. At different times after transfection, 1 μCi of IDC (ICN, 2000 Ci/mmol) was added per well, and 24 h later cells were centrifuged on slides. The incorporated radioactivity was revealed by microautoradiography using the LM-1 emulsion (Amersham). To detect *lacZ* expression, 24 h after transfection, cells were rinsed twice with phosphate-buffered saline (PBS, pH 7.3), fixed with a solution composed of 1% formaldehyde and 0.2% glutaraldehyde for 5

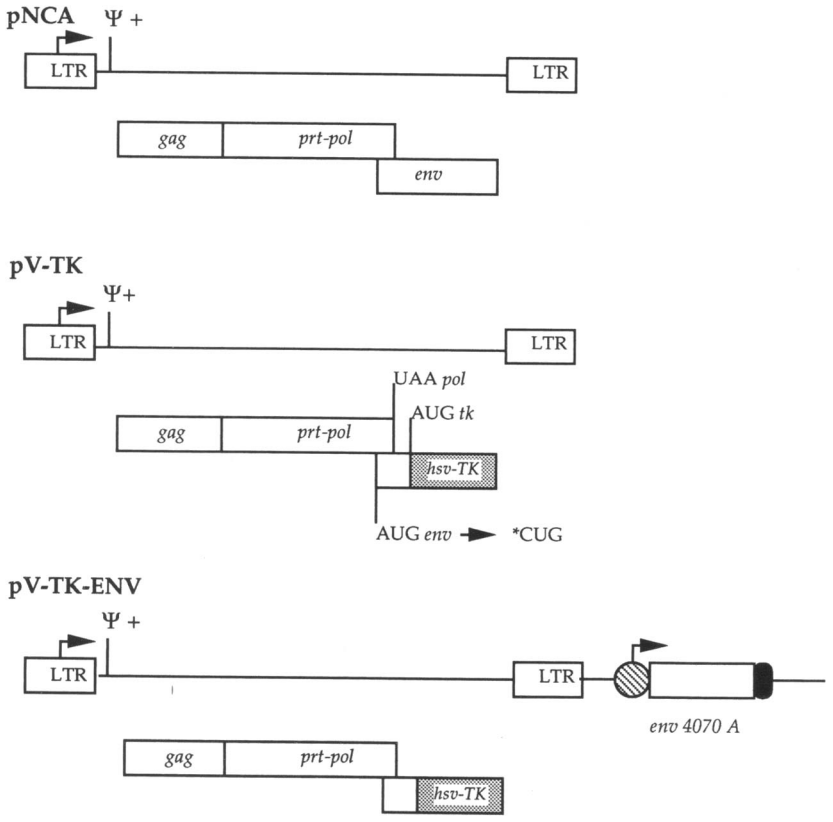

FIG. 1. Representation of pNCA, pV-TK, and pV-TK-env plasmids. □, Genome from Mo-MuLV; ▨, HSV1 TK gene. The *env* 4070A gene is under the control of the cytomegalovirus promoter (◎) and is followed by the poly(A) sequence of simian virus 40 (●). LTR, long terminal repeat.

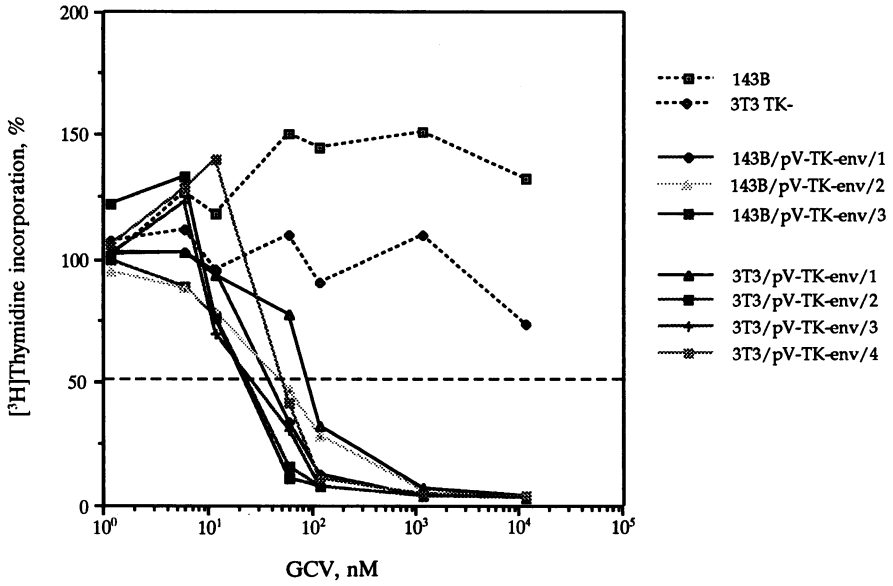

FIG. 2. Functional expression of an HSV1 TK transgene carried by a plasmovirus. The functional expression of an HSV1 TK gene carried by a plasmovirus is assessed by a GCV toxicity assay. Clones of murine 3T3, or human 143B, cells expressing HSV1 TK after transfection with pV-TK-env, or parental cells, were cultured with increasing concentrations of GCV. The toxicity of GCV was measured by a [³H]thymidine proliferation assay.

min, washed three times with PBS, and incubated for 8 h at 37°C in a solution containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride, and 1 mg of 5-bromo-4-chloro-3-indolyl β-D-galactoside per ml. After staining, cells were rinsed twice with PBS.

Intratumoral Gene Transfer and *in Vivo lacZ* Expression. Male BALB/c mice were obtained from Iffa-Credo, St. Germain sur l'Arbresle, France. MC26SC31 cells (2×10^6) were subcutaneously injected at the tail base. Eleven days later, when tumors reached a volume of 0.7–1 cm^3, mice were anesthetized by an intraperitoneal injection of 0.3 ml of 2,2,2-tribromoethanol at 25 mg/ml in 150 mM NaCl. A mixture of 3 μg of pV-lacZ-env or pV-lacZ in 100 μl of 150 mM NaCl and 5.45 μl of PEI, prepared according to Boussif *et al.* (17), were injected into tumors. After 10 days, mice were sacrificed, and tumors were dissected and placed for 2 h in 10 ml of 4% formaldehyde in PBS and then rinsed in PBS. For staining, tumor fragments were incubated for 6 h at 32°C in the solution described above. Tumor fragments were placed in 4% formaldehyde overnight and then embedded in paraffin. Five-micrometer-thick slices were cut and counterstained with nuclear red.

RESULTS

Plasmovirus Generation. The initial step in the generation of plasmoviruses was the construction of a recombinant defective provirus harboring the transgene. The plasmid pV-TK has been described previously (18). In this plasmid, the first ATG codon of the *env* gene of a Moloney-derived provirus has been point-mutated to keep the sense of the corresponding codon in the overlapping *pol* gene (Fig. 1). A few base pairs downstream of the *pol* gene stop codon, an HSV1 TK gene has been cloned to remove any *env* viral sequence between the stop codon of this transgene and the polypurine tract of the 3′ long terminal repeat. In such a construct, the *gag* and *pol* genes are synthesized from the unspliced genomic RNA, while the transgene is synthesized from a spliced RNA utilizing splice donor and acceptor sites from the wild-type provirus. We then constructed an *env* expression cassette such as to reduce the possibility of recombinations generating replicative viruses.

Table 1. Titers of infectious recombinant retroviruses in the supernatant from cells 72 h after tranfection

Transfected cells	Vector	Titer (particules/ml)72 h
ΨCRIP	/pMTK	100
3T3	/pV-TK	0
3T3	/pV-TK-env	70

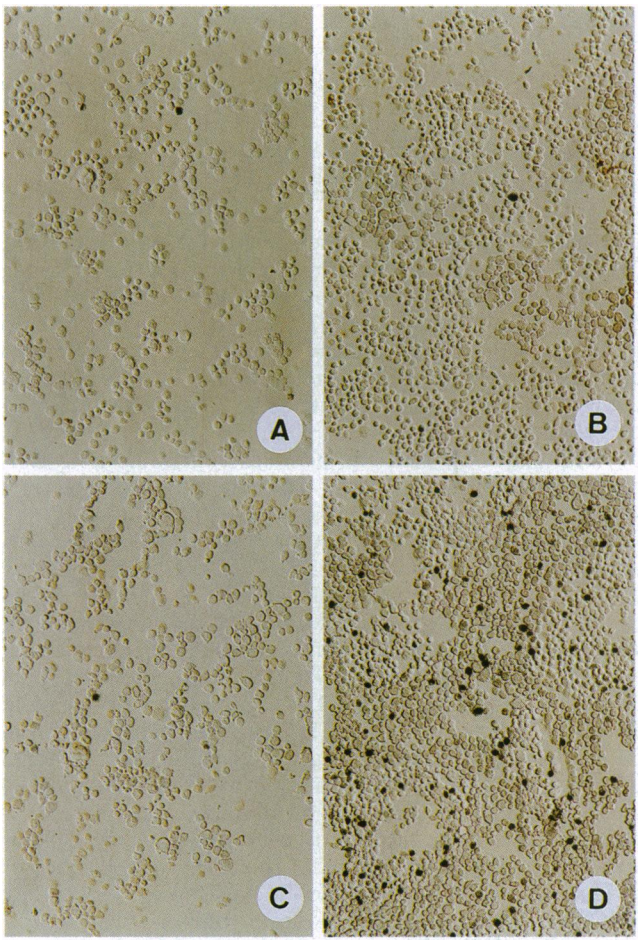

FIG. 3. *In vitro* propagation of plasmoviruses expressing an HSV1 TK gene revealed by IDC incorporation in 3T3 TK⁻ cells. *A* and *B* represent transfections with the control plasmid pV-TK lacking an *env* gene; *C* and *D* represent tranfections with pV-TK-env. IDC assays were revealed either 2 days (*A* and *C*) or 8 days after transfections (*B* and *D*).

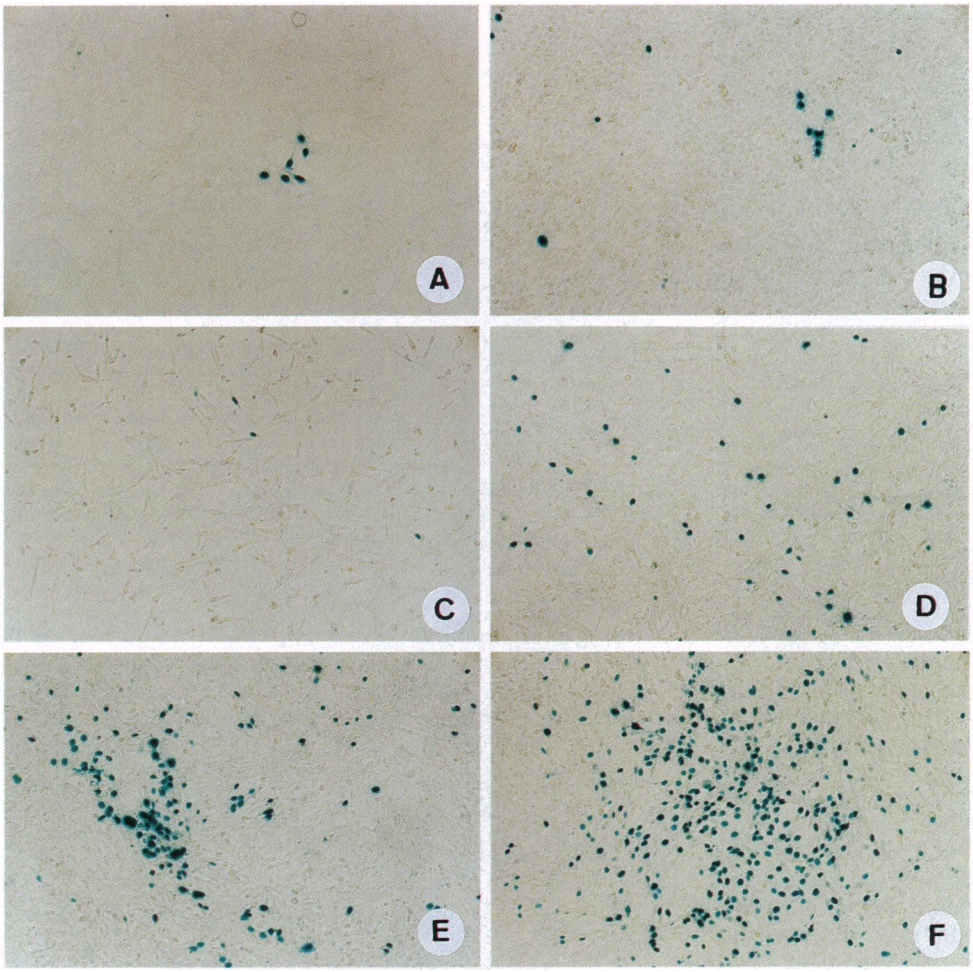

FIG. 4. *In vitro* propagation of plasmoviruses expressing the *nls-lacZ* gene construct revealed by staining in 3T3 TK$^-$ cells. *A* and *B* represent tranfections with the pV-lacZ control plasmid lacking an *env* gene, and *C*, *D*, *E*, and *F* represent transfections with pV-lacZ-env. Plates were stained either 2 days (*A* and *C*), 6 days (*D*), 8 days (*E*), or 10 days (*B* and *F*) after transfections.

Likewise, we placed an amphotropic envelope from 4070A Mo-MuLV under the control of a cytomegalovirus strong promoter and polyadenylylation sequences from SV40. The *env* gene was cloned as a PCR fragment from the first ATG to the stop codon. This *env* expression cassette was then subcloned into a unique *Eco*RI site of the pV-TK plasmid, both the provirus and the *env* cassette being in the same transcription orientation, generating the plasmid pV-TK-env (Fig. 1). A pV-lacZ and a pV-lacZ-env plasmids containing a *nls-lacZ* gene in the place of the HSV1 TK gene were generated in a similar way.

Transgene Functionality Within Plasmoviruses. To determine the functionality of the HSV1 TK transgene carried by pV-TK-env, 3T3-TK$^-$, and 143B cell lines defective in endogenous TK activity, were transfected and cultured in a hypoxanthine/aminopterin/thymidine-containing medium to select cells with a TK activity. Clones were obtained with both cell types, demonstrating the HSV1 TK transgene expression. To further assess the HSV1 TK transgene functionality, the HSV1 TK$^+$ colonies that emerged from selection were tested for their sensitivity to the toxicity of the nucleoside analog GCV. Indeed, HSV1 TK can metabolize GCV into monophosphated GCV, which is in turn converted by cellular enzymes to triphosphated GCV, which is highly toxic for dividing cells (22). All of the seven clones tested, originating from two different cell lines, were highly sensitive to GCV toxicity. The 50% inhibitory dose ranged from 60 to 200 nM, while parental cells are not sensitive to GCV at the concentration tested (Fig. 2).

Generation of Infectious Defective Recombinant Retroviruses by Plasmovirus Transfection. We then analyzed the generation of infectious particles upon transfection of these constructs in cell lines *in vitro*. Plasmoviruses should be mostly used for obtaining the transient formation of retroviral particles after *in situ* transduction. Therefore, we compared the formation of infectious defective particles after transfecting 3T3 TK$^-$ cells with pV-TK-env, and 3T3-derived packaging cells (ΨCRIP) (21) with a classical retroviral vector, pMTK, (20). Both pV-TK-env and pMTK plasmids encode a packageable HSV1 TK gene. 3T3 TK$^-$ and ΨCRIP cells were transfected by using PEI-formulated plasmids with the same ratio of DNA to PEI. A representative experiment is shown in Table 1. Transfection efficiency was shown to be similar in both transfections, analyzing HSV1 TK expression by the incorporation of IDC (data not shown). Seventy-two hours after transfection, cell culture supernatants were collected. Infectious particle titers were measured by infection of L-M(TK$^-$) cells lacking an endogenous TK, followed by culture in selective hypoxanthine/aminopterin/thymidine-containing media. Similar numbers of hypoxanthine/aminopterin/thymidine-resistant colonies were obtained from the two supernatants (Table 1). As a control, transfection of the pV-TK plasmid lacking an *env* gene did not yield any infectious viral particles. These results demonstrate that the sole transfection of the pV-TK-env plasmid results in the formation of infectious particles efficiently transducing and expressing the transgene.

***In Vitro* Propagation of the Transgene After Plasmovirus Transfection.** We next analyzed whether the transfection of pV-TK-env or pV-lacZ-env within cell culture would show evidence for the propagation of the transgene due to the formation of infectious defective particles. Transfections were done in tk$^-$ts13 and 3T3 TK$^-$ cells. As controls, we used the pV-TK and pV-lacZ plasmids lacking the *env* expression cassette and tk$^-$ts13 hamster cell line, which cannot support replication of amphotropic retrovirus (23).

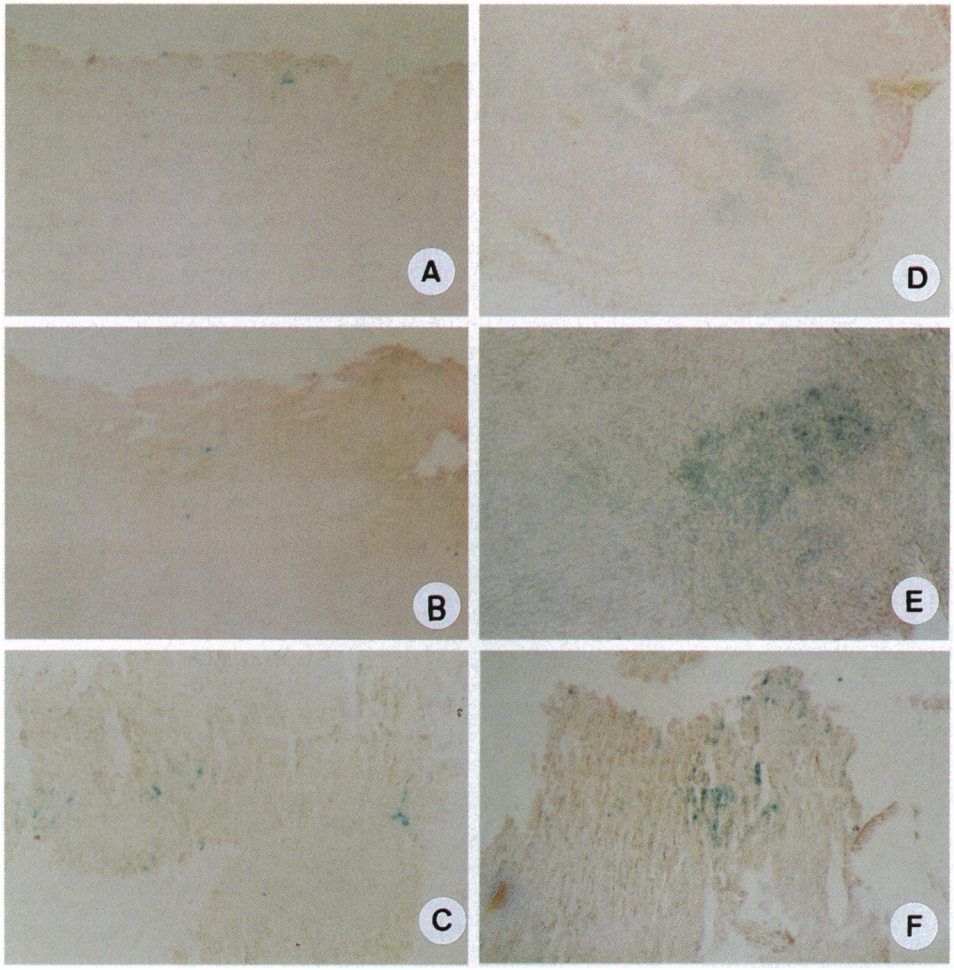

FIG. 5. *In vivo* transgene expression after transfection with plasmoviruses. MC26SC31 tumors were inoculated with PEI-formulated pV-lacZ-env or pV-lacZ as control. Ten days after injection, animals were sacrificed and tumors were analyzed for *lacZ* expression. Tumors injected with pV-lacZ contained very few isolated *lacZ* expressing cells (*A* and *B*, ×35; *C*, ×140). Foci of stained cells (*D*, ×50; *E* and *F*, ×200) in tumors transfected with pV-lacZ-env.

Expression of HSV1 TK in this setting can be revealed by incorporation of IDC that reveals individual cells expressing HSV1 TK. Transfections were performed under conditions in which only few cells were initially transfected. In 3T3 TK$^-$ cells, we clearly observed the propagation of the transgene within the culture after transfection with pV-TK-env as shown in a representative experiment (Fig. 3). At 72 h postinfection, only few transgene-expressing cells could be detected in cultures transfected with pV-TK or pV-TK-env (Fig. 3 *A* and *B*). After 8 days of culture, the proportion of cells expressing the HSV1 TK transgene was increased by 10- to 60-fold in cell cultures transfected with pV-TK-env (Fig. 3*D*) and remained stable in cells transfected with pV-TK (Fig. 3*C*). At later time points, the proportion of pV-TK-expressing cells decreased because of the transient nature of plasmid expression in this system. Similar results were obtained when using the pV-lacZ-env and pV-lacZ vectors (Fig. 4). Interestingly, in these experiments where transgene detection is performed *in situ*, we observed foci of transduced cells, which are likely to have arisen from the local infection of neighboring cells by a transfected cell (Fig. 4 *E* and *F*). No transgene propagation could be detected in tk$^-$ts13 hamster cells with either pV-TK-env or pV-lacZ-env (data not shown).

The analysis of *env* expression in these experiments revealed that the proportion of positive cells remained unchanged throughout the culture period (data not shown). This rules out the possibility that the transgene propagation could have been the result of its carryover by replicative retroviruses. Altogether, these results demonstrate the ability of plasmoviruses to propagate a transgene *in vitro*.

***In Vivo* Transgene Expression After Plasmovirus Transfection.** To analyze the ability of plasmoviruses to propagate transgene *in vivo*, we used a model of subcutaneaous experimental tumors generated from a colon carcinoma cell line (MC26SC31) in BALB/c mice. Each vector, formulated in PEI, was injected into tumors of approximately 0.7 cm^3. Ten days later, animals were sacrificed, and the tumor was analyzed for *lacZ* expression. The *in situ* gene transfer efficiency under these experimental conditions was extremely low, and only rare positive cells could be detected in animals that received the pV-lacZ construct (Fig 5 *A* and *C*). In contrast, the number of *lacZ*-positive cells was significantly higher in animals that received the pV-lacZ-env construct (Fig. 5 *D* and *F*). Although only one-fourth of the analyzed sections contained *lacZ*-expressing cells, when present they usually formed foci, suggesting that a propagation of *lacZ* from a transfected cell had occurred (Fig. 5 *D* and *F*). When foci of positive cells could be detected in one tumor section, a similar positivity was also detected in the adjacent section.

DISCUSSION

Our main aim was to generate a prototypic gene transfer vehicle combining the advantages of plasmid vectors introduced by nonviral methods into target cells, together with the infectious property of retroviruses. The results presented in this paper demonstrate that plasmoviruses fulfill such requirements. However, their usefulness as gene transfer vehicle will depend on qualitative and quantitative aspects of their life cycle. First, the limits of the transgene size that can be accommodated in such vectors, and the levels of expression that can be achieved, remain to be determined. Like retroviral vectors, plasmoviruses must have a genome size compatible with an efficient packaging. Although no precise limit can be

established now, it is noticeable that plasmoviruses can at least accommodate and express a 3.5-kb *nls-lacZ* transgene, which is longer than the replaced *env* gene fragment. In any case, size limitation is mostly a problem for gene therapy of genetic diseases where the transgene to be expressed is often large. On the contrary, most of the transgenes used for anticancer gene therapy (24) like those coding for suicide genes, cytokines, p53, or B7 are cDNAs that have a size ranging from 0.5 to 2 kb. Given the possible synergy between different gene therapy strategies, it should even be possible to express two of these genes in the same plasmovirus vector. We have shown that the level of HSV1 TK gene expression by plasmoviruses is sufficient to make target cells sensitive to GCV, with 50% lethal doses similar to those obtained with classical retroviral vectors carrying the HSV1 TK gene (20). More precise quantitative experiments assessing the amounts and kinetics of the different transcripts and proteins generated during a plasmovirus expression and replication cycle remain to be performed.

In their expected use, plasmoviruses could be introduced into cells through various physical methods, like cationic lipid formulation or particle bombardment. These methods do not lead to efficient transgene integration but allow transient transgene expression. Therefore, we monitored the efficacy of viral particle formation in an *in vitro* experiment mimicking the *in vivo* use. We showed that the efficiency of infectious viral particle formation after plasmovirus transfection into 3T3 cells is similar to that obtained with a classical retroviral vector transfected in 3T3-derived packaging cells. Therefore, particle formation seems to be efficient in our system. *In vitro*, within 8–10 days, the proportion of cells expressing the transgene was increased by a factor of 10–60. Our *in vivo* experiments in mice suggest that plasmoviruses could also significantly improve gene transfer efficiency. Although the initial plasmid transfection efficiency was poor, the number of transduced cells increased significantly when *env*-expressing plasmoviruses were used. Moreover, these cells were forming foci, suggesting that a propagation of the transgene had occurred. However, the overall gene transfer efficiency achieved so far is still low. For instance, for the HSV1 TK suicide gene, it would be necessary to obtain the transduction of 10–20% of tumor cells to have a significant therapeutic effect, a level that is not yet achieved (12, 20, 25, 26). On the contrary, therapeutic efficacy might be obtained with a local low level expression of certain cytokines (27), which could possibly be achieved with plasmoviruses. In any case, increasing the efficacy of the initial transfection step remains the major challenge for the use of plasmoviruses.

Plasmoviruses were designed to minimize the possibility of recombination between the *env* cassette and the provirus. The only region of homology between the pV-TK and the *env* expression cassette is a 157-bp sequence downstream the mutated ATG of the former *env* gene of the provirus. The homology in this region is only 53%, since the envelope of the original provirus is ecotropic. In addition, recombination in this homology region should leave the mutated ATG and a 5′ polyadenylylation signal from simian virus 40. Therefore, in human cells that do not harbor endogenous murine retroviral genome, the risk of recombination leading to appearance of the replication competent retroviruses should be extremely low. Although there was no detectable replication competent virus in the experiments we performed with small cultures, the detection of replication-competent retroviruses by sensitive methods remains to be performed in large cultures of transfected human cells. If necessary, the level of homologies in the *env* region can be further reduced by using different envelope proteins, including envelope from viruses other than retroviruses. Besides, it has been reported that the *pol* reading frame can be terminated just before the first initiator of the *env* gene, without affecting infectivity (28). Such a construction will delete any remaining *env* sequences in the plasmoviruses.

Finally, the plasmoviruses presented here offer numerous additional possibilities for improvement. For example, it would be interesting to perform similar constructs utilizing the backbone of lentiviruses that are capable of infecting quiescent cells. Such vectors might be useful to target the fraction of slowly dividing cells that are present within a tumor mass. In addition, it is also possible to design plasmoviruses in which only the transgene would be packaged and transferred by the recombinant particles.

Plasmoviruses thus represent a new generation of vectors for gene therapy. They are easy to produce and allow an efficient gene transfer to tumor cells. The assessment of plasmovirus efficacy in experimental tumor models in animals is the next step to assess their therapeutic potential.

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