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The baculovirus vector system for gene delivery into hepatocytes

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Summary

Gene therapy in the liver requires powerful vectors capable of mediating sufficient gene delivery and expression in affected hepatocytes. Viral vectors are amongst the most efficient tools for gene delivery, and the search for tissue-specific infecting viruses is important for the development of *in vivo* gene therapy strategies. We have recently shown for the first time that a genetically modified baculovirus *Autographa californica* can efficiently and specifically transfer genes into cultured liver cells from various origin. The efficiency of baculovirus-mediated gene transduction into hepatocytes was determined to approach 100% using appropriate virus titers. Apart from these features, potential advantages of baculovirus vectors are the nearly unlimited capacity for insertion of foreign DNA, a supposed restriction of viral promoters to the arthropod host and the ease of generating high vector titers. Uptake of the virus occurs via the endosomal pathway, most likely via a receptor that is currently under investigation. Baculovirus-mediated gene expression is transient in dividing cells, but prolonged expression can be achieved in non-dividing primary hepatocytes. Baculovirus-mediated gene transfer is feasible into *ex vivo* perfused human liver tissue. Systemic application of baculovirus vectors *in vivo* is hampered by the complement (C) system. Current attempts to facilitate baculovirus-mediated gene transfer *in vivo* include strategies for both, blocking or avoiding the C system and generation of new baculovirus vectors that are not affected by the C system. Alternatively, direct injection of baculovirus vectors was successful into normal mouse liver and into induced human hepatocarcinomas in nude mice. The potential of baculovirus vectors *in vitro* and the feasibility of vector application *in vivo* provide the basis for gene therapy strategies for metabolic diseases and tumors of the liver.

I. Introduction

Gene therapy in the liver is a promising approach for the treatment of various inherited and malignant diseases affecting this organ. In order do realize this concept, powerful tools capable of transferring therapeutic genes into affected hepatocytes at sufficient efficacy are required. The principle of an *ex vivo* approach for liver gene therapy does presently not allow for sufficient rates of genetically corrected hepatocytes (Grossman et al., 1995). Therefore, tremendous efforts are made to develop potent gene transfer vectors for *in vivo* application. Viral vectors, such as retroviruses and adenoviruses, are generally considered superior to non-viral vectors with regard to gene transfer efficiency. However, retroviruses for example are not able to integrate their genome into non-dividing cells so that hepatic gene transfer by retroviral vectors requires stimulation of liver cell division (Ferry et al., 1991; Cardoso et al., 1993; Rettinger et al., 1993). Adenoviruses can deliver genes at a high frequency into the liver (Li et

al., 1993), but induce a strong immunological response *in vivo* (Yang et al., 1994). An important drawback of all existing viral vectors is, in addition, the lack of liver cell specific targeting. Since the mainly used viral gene transfer vectors are derived from mammalian species, general problems have to be considered, such as emergence of replication competent vectors, preexisting or induced immune response and undesired gene expression from the viral backbone.

Baculoviruses comprise a large group of viral pathogens of arthropods particularly of insects. The best studied member of this family, *Autographa californica* nuclear polyhedrosis virus (*Ac*NPV), is a large, enveloped virus with a double-stranded, circular, completely sequenced DNA genome of about 130 kilobase pairs (Ayres et al., 1994). Baculoviruses are normally used for the overproduction of recombinant proteins under control of strong baculoviral promoters in insect cells (Luckow and Summers, 1988; Fraser *et al*., 1992; Kidd and Emery, 1993; Miller, 1993) or as biopesticides (Cory *et al.*, 1994). Although the ability of *Ac*NPV to infect mammalian cells was studied in the past (Doller et al., 1983; Tjia et al., 1983; Carbonell and Miller, 1987; Hartig et al., 1992), neither gene expression nor DNA replication could be observed. Since these studies did not include hepatocytes, a block of infection of mammalian cells was assumed.

II. Baculovirus-mediated gene transfer *in vitro*

We have recently shown that the baculovirus, *Ac*NPV, can efficiently deliver genes into hepatocytes (Hofmann et al., 1995). This unexpected property of baculovirus was confirmed by others (Boyce and Bucher, 1996). Further applications of baculovirus vectors were recently presented for recovery of an infectious virus from cDNA by means of a hybrid baculovirus-T7 RNA polymerase system (Yap et al., 1997). This study highlighted the lack of replication and toxicity after baculovirus-mediated gene transfer into mammalian cells in contrast to the vaccinia-T7 polymerase system, which is widely used for that purpose. The major prerequisit for the expression of a baculovirally transferred gene in either application is its control by a functionally active promoter in mammalian cells. Recombinant baculoviruses are generated in insect cells via homologous recombination after cotransfection of a linearized *Ac*NPV-genome and a baculovirus transfer vector bearing the mammalian expresssion unit. The expandability of the capsid structure of baculoviruses allows for packaging and expression of very large genes with an until now not challenged upper size limit.

A. Cell-type specificity of baculovirus

In order to investigate the cell-type tropism of baculovirus vectors, we constructed recombinant baculoviruses bearing the luciferase reporter gene under control of the immediate early promoter of cytomegalovirus. After incubation of this virus with a large panel of cells, high levels of gene expression could be detected in hepatocytes, including primary cultures derived from various species (**Table 1**). In contrast, no or very low levels of gene expression could be detected in more than 40 tested non-hepatic cell lines. Relative gene transfer efficiencies among hepatocytes seem to decline in a species specific manner by a maximal factor of 40 from human > rabbit > to mouse. Therefore, a human non-hepatic cell line (T47-D) was just as susceptible to baculovirus infection as primary mouse hepatocytes. In other studies using baculovirus vectors, high levels of gene expression were also achieved only in hepatocytes (Boyce and Bucher, 1996). Thus, the present *in vitro* data show baculovirus to be liver cell specific, which would be a highly advantageous feature of the vector, if it could be confirmed *in vivo*.

B. Efficiency of gene transfer by baculovirus

In the first report on baculovirus-mediated gene transfer into hepatocytes, we described a baculovirus vector coding for a C-terminally truncated simian virus 40 large tumor antigen under control of the cytomegalovirus (CMV) immediate early promoter. With this vector, we demonstrated the ability of baculovirus vectors to approach a transduction efficiency of 100% in human hepatocytes (Hofmann *et al.*, 1995). A dose-response analysis was performed in the hepatocarcinoma cell line Huh7 by using a baculoviral vector (*Ac*NPV-ß-gal) with a nuclear localised ß-galactosidase gene under control of the Rous-Sarcoma-Virus long terminal repeat (RSV-LTR). The gene transfer efficiency increased gradually with the respective multiplicity of infection (moi). After infection at a moi of 750, almost all cells were positive as determined by histochemical staining for ß-galactosidase (Sandig et al., 1996). However, the histochemical ß-gal staining method often underestimates the percentage of actually transduced cells and does not allow for an analysis of gene transfer events on living cells. Therefore, we constructed a baculovirus vector bearing the green fluorescent protein under control of the human CMV-promoter (*Ac*NPV-GFP). We found that all Huh7 cells were successfully transduced after infection at a moi of only 100 (**Figure 1**). Analogous to previous reports, no signs of cell toxicity were observed even if very high doses were applied.

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Table 1. Cell-typ specificity of baculovirus vectors

Tissue	Species	Name	Relative gene transfer efficiency (arbitrary units)
liver	human	Huh7	1000
	human	HepG2	100
	human	primary hepatocytes	100
	rabbit	primary hepatocytes	200
	mouse	primary hepatocytes	30
colon	human	LS 174T	5
pancreas	human	Capan-2	0
kidney	monkey	CV-1	0
	dog	MDCK	2
lung	human	A549	5
bladder	human	Ej28	5
gastric	human	MKN-45	1
breast	human	MCF7	0
	human	T-47D	25
blood	human	HL60	0
	human	MOLT-4	0
	human	UC729-6	0
	human	SKW6.4	0
CNS	human	SW1088	0
	mouse	Neuro-2a	2
skin	human	HaCat	4

Cells were infected with a baculovirus vector (*Ac*NPV-CMVL) bearing the luciferase gene under control of the immediate early promoter of cytomegalovirus at a multiplicity of infection (moi) of 100. Relative gene transfer efficiency was determined from luciferase values measured 36 hours after infection.

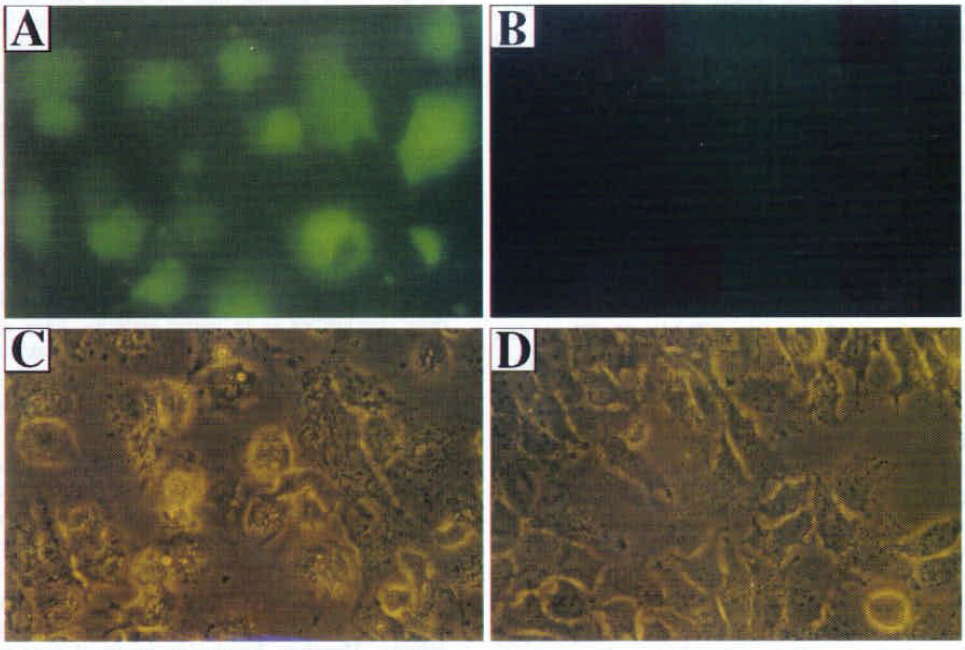

Figure 1. Baculovirus-mediated expression of the green fluorescent protein in Huh7 cells.

Human hepatocarcima cells (Huh7) were infected with recombinant baculoviruses bearing the green fluorescent protein (GFP) under control of the CMV immediate early promoter (*Ac*NPV-GFP) at a moi of 100. (**A**) Expression of GFP was detected 42 hours after infection by direct immunofluorescence of living cells. (**C**) Corresponding phase-contrast micrograph. (**B**) Immunofluorescence of non-infected Huh7 cells and corresponding phase-contrast micrograph (**D**).

Baculovirus-mediated gene transfer is most likely independent of the cycling status of the cell, since non-dividing primary hepatocytes from different origin could be efficiently transduced. Boyce and Bucher reported a gene transfer efficieny > 70% in primary cultures of rat hepatocytes using a moi of 430 (Boyce and Bucher, 1996).

C. Mode of baculovirus uptake by hepatocytes

A obvious assumption as to the striking preference of baculovirus-mediated gene transfer for hepatocytes would be the existence of a specific receptor on hepatocytes. Although the desialiated baculoviral envelope proteins represent putative ligands for the hepatocyte-specific asialoglycoprotein receptor, various experiments excluded this initially postulated candidate for baculoviral entry into hepatocytes (Hofmann et al., 1995). Indications for a receptor on hepatocytes became apparent, however, within the same study by both, competition experiments and a clear dose-response curve of baculovirus-mediated gene transfer into hepatocytes. We started investigating the mechanism of baculovirus uptake by hepatocytes by following data available from its natural arthropod host. Baculovirus enters insect cells by adsorptive endocytosis (Volkman and Goldsmith, 1985). A receptor on insect cells has not yet been identified, but it was proven by the use of neutralizating monoclonal antibodies (mAb) that the main baculoviral envelope protein gp64 is responsible for entry of baculovirus into insect cells (Volkman et al., 1984). Therefore, we treated luciferase expressing baculovirus with mAbs against gp64 prior to infection of hepatocytes and compared subsequently measured luciferase levels with those obtained with untreated vector. No influence on baculoviral gene transduction was observed after virus preincubation with mAb, AcV_5 or AcN_9. In contrast, AcV_1 completely blocked baculovirus-mediated gene expression in hepatocytes (**Table 2**). These data reflect exactly the ability of these mAb to block baculovirus infection of insect cells (Hohmann and Faulkner, 1983; Withford et al., 1989). In order to determine if the AcV_1-mediated block of baculovirus infection of hepatocytes is due to a block of receptor binding or due to later fusion events during endocytosis, we investigated the ability of AcV_1-treated baculovirus to bind to hepatocytes. We observed that binding of baculovirus to hepatocytes is not affected by the neutralizing mAb, AcV_1 (**Figure 2**). This result indicates that AcV_1 blocks baculovirus penetration into hepatocytes or plays a role in low pH-dependent fusion. The necessity of endosomal maturation for the transport of baculovirus was demonstrated for both, insect cells (Volkman and Goldsmith, 1985; Charlton and Volkman, 1993) and hepatocytes (Hofmann et al., 1995; Boyce and Bucher, 1996).

Table 3: Inactivation of baculovirus by serum

Source of serum	Baculovirus survival
human	4 %
rat	1 %
guinea-pig	25 %
[a]C3d guinea-pig	70 %
[a]C4d guinea-pig	84 %

Baculovirus survival in sera of different species was recorded as the remaining ability of a luciferase expressing baculovirus vector (AcNPV-CMVL) to mediate gene expression in Huh7 cells after virus-pretreatment with native serum compared to treatment with the corresponding heat-inactivated serum.
[a]Cd: deficient in indicated complement components.

D. Kinetics of baculoviral gene expression

The stability of gene expression is an important aspect of the use of a vector for treatment of disorders, which require permanent provision of a missing gene product. Retroviruses and adeno-associated viruses are able to integrate their genome into the target cell which should allow for long term gene expression. However, integration of an expression cassette into the target cell does not preclude that other events, such as a promoter shut-off (Löser et al., in press) or elimination of the transduced cell by the immune system, prevent from stable gene expression.

We compared the duration of gene expression after baculovirus-mediated gene transfer into the hepatic cell line Huh7 and into non-dividing primary mouse hepatocytes using a luciferase expressing virus (Sandig et al., 1996). The instability of luciferase RNA and protein allowed to draw conclusions as to vector stability from expression data obtained with this reporter gene. We observed transient gene expression in the dividing hepatocarcinoma cell line Huh7, peaking at 42 hours and decreasing continously over four orders of magnitude within 19 days. Baculovirus shares short-term expression of genes transferred into dividing cells with other non-replicating and also with non-integrating vector systems due to the manifold arthropod-specific requirements for replication (Pearson *et al.*, 1992; Kool *et al.*, 1994; Lu and Miller, 1995) and due to the lack of an integration machinery. The liver consists, however, of cells with low regenerative activity. Therefore, baculovirus-mediated gene expression in primary cultures of hepatocytes declines more slowly and the kinectic is almost equal to that recorded from a stably transducing retroviral vector (Sandig, et al., 1996). These results support the idea that the baculoviral genome

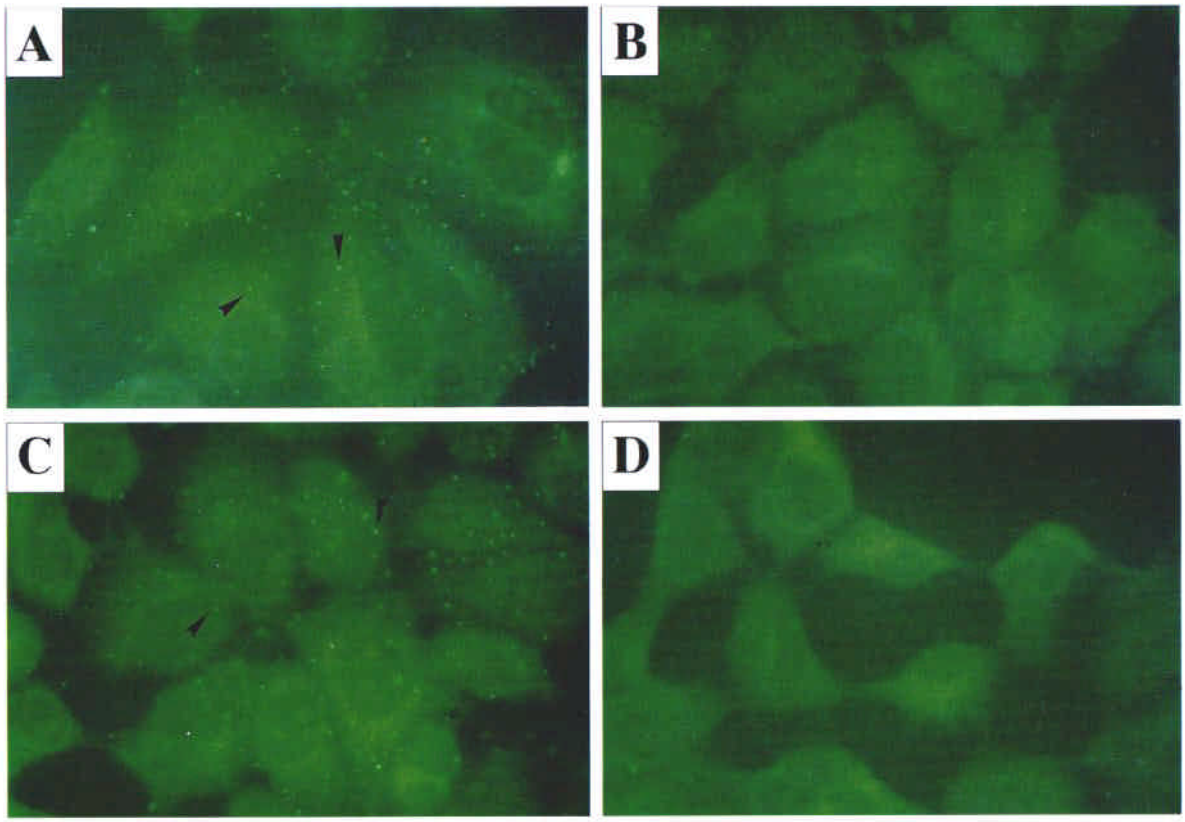

Figure 2. Role of AcV_1 mAb in inhibition of baculovirus uptake by Huh7 cells.

(**A, B**) Baculovirus and (**C**) baculovirus, pretreated with infectivity neutralizing amounts of AcV_1 mAb were allowed to adsorb onto Huh7 cells for 1 h at 4°C. (**D**) Huh7 cells were preincubated with AcV_1 without baculovirus as control. After washing, cells were fixed and (**A**) incubated with AcV_1 mAb. Bound virus (exemplary marked by arrowheads) was visualized using a fluorescein-conjugated goat anti-mouse antibody (**A-D**).

may persist in hepatocytes *in vivo* for some time leading to longer periods of expression as has also been observed with adenoviral vectors in immunodeficient animals (Dai *et al.*, 1995). In contrast to first generation adenovirus vectors (Yang et al., 1994), an advantageous feature of baculovirus could be the evasion of a cellular immunitiy to viral antigens because of the strong restriction of baculoviral promoters even within different arthropod species (Morris and Miller, 1992; Bilimoria *et al.*, 1993).

III. Baculovirus-mediated gene transfer *in vivo*

We have undertaken a number of attempts for systemical and intraportal application of baculovirus vectors in rodents. The absence of a significant number of positively transduced cells in these *in vivo* experiments indicated that the virus is somehow inhibited in transferring genes to the liver. Clues as to the reasons for the inefficiency of baculovirus vectors *in vivo* derived from the observation that baculoviral gene transduction into hepatocytes is dramatically reduced by heat-labile serum components.

A. Inactivation of baculovirus by serum

Incubation of baculovirus with native serum from different species prior to infection, causes a marked decrease in its ability to mediate gene expression in hepatocytes. In contrast, complete survival of baculovirus vectors was observed upon preincubation with the corresonding heat-treated sera. Since most of the components of the complement (C) cascades are heat-labile, we used sera deficient in different C components and determined baculovirus survival. The C component C4 is involved in triggering the classical complement cascade, whereas C3 is a component of both, the classical and the alternative pathway. Neither C3-deficient nor C4-deficient guinea-pig serum had a significant influence on baculovirus survival (**Table 3**). These data indicate that

activation of the classical pathway of the C system has an impact on baculovirus survival *in vivo*. Triggering of the C cascade is also a major cause for the inactivation of a variety of currently used gene delivery vectors and contributes to inefficient gene transfer after *in vivo* application. C activation has been shown for liposomes (Szebeni et al., 1994), for various synthetic DNA complexes (Plank et al., 1996) based on polylysins, dendrimers or polyethyleneimine and for murine retrovirus vectors in primate serum (Takeuchi et al., 1996). However, inactivation of baculovirus in the presence of C can be prevented by treatment with complement blocking agents, such as cobra venom factor (CVF) or anti C5 antibodies (Hofmann and Strauss, 1998). The usefulness of CVF or anti-C mAb has already been demonstrated to protect murine retroviruses from C inactivation (Rother et al., 1995).

B. Gene transfer into *ex vivo* perfused liver tissue

Another possibility to circumvent complement-mediated neutralization of baculovirus seems to be likely by *in situ* perfusion methods, which have already been used for retrovirus-mediated gene transfer into the liver (Ferry et al., 1991; Cardoso et al., 1993; Rettinger et al., 1993). We established an *ex vivo* perfusion model of human liver segments (**Figure 3**). Human liver tissue was chosen because of the high levels of baculovirus-mediated gene expression obtained in human hepatocytes *in vitro* (**Table 1**). The liver segments were perfused with culture medium

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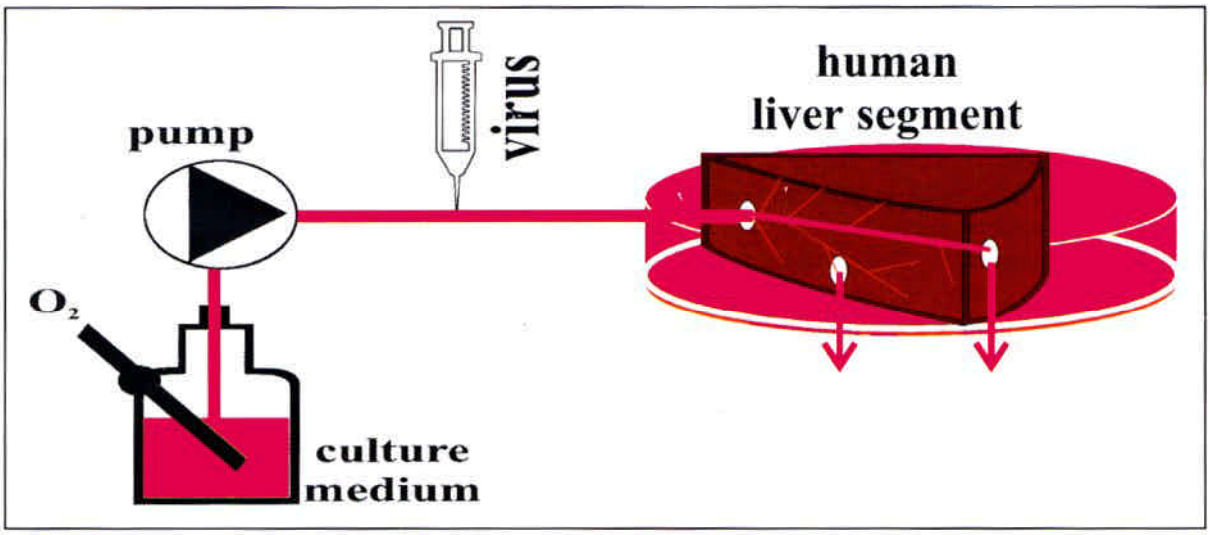

Figure 3. *Ex vivo* perfusion model of human liver tissue.

Human liver segments were perfused with conditioned culture medium through a main vessel. After introduction of luciferase expressing baculoviruses into this system, perfusion was maintained for an additional period of time (22-42h), following analysis of gene expression. Each experiment using baculovirus vectors within this model system resulted in substantial gene expression distributed in all perfused parts of the liver segments.

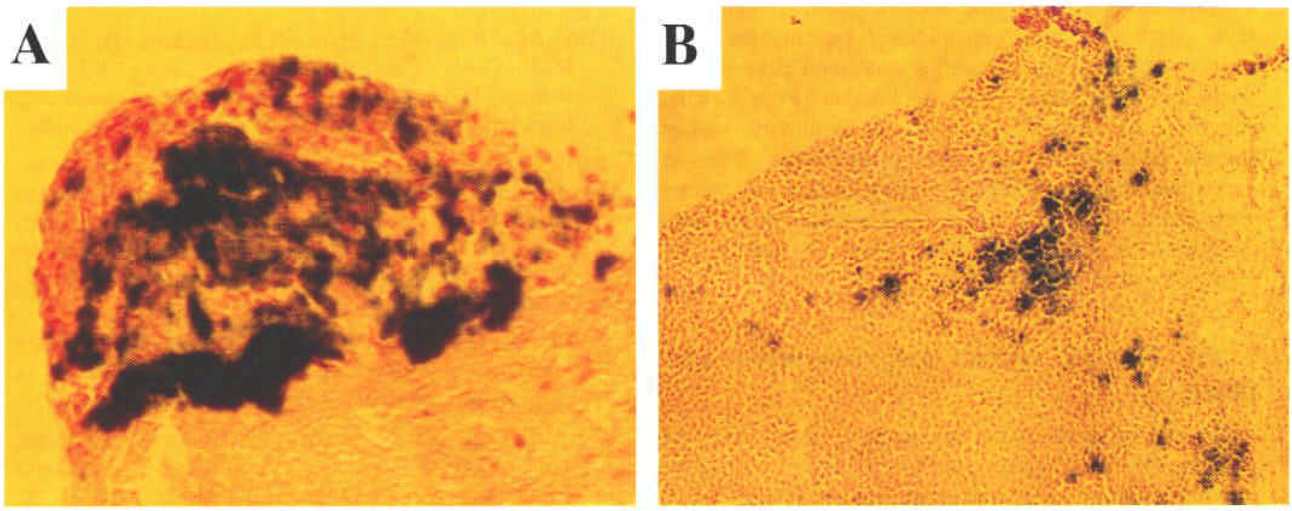

Figure 4. Baculovirus-mediated gene transfer *in vivo*.

Baculoviruses bearing the lacZ gene under control of the Rous sarcoma virus long terminal repeat (*Ac*NPV-ß-gal, 10^8 plaque forming units) were directly injected into (**A**) the big liver lobe of AKR-mice or into (**B**) Huh7 cell derived human hepatocarcinomas generated in nude mice. Histochemical staining for ß-galactosidase of the injection sites was performed 48 hours after infection. The number of successfully transduced cells decreases with increasing distance to the injection sites. Uninfected liver or tumor stained negative (data not shown).

through a main vessel immediately after resection from patients with liver metastases of colon carcinoma. After application of a luciferase expressing baculovirus vector to this model system and subsequent analysis of small regions of the liver segments for gene expression, we found varying levels of luciferase activity distributed in all perfused parts of the liver segments (Sandig et al., 1996). These experiments demonstrated on the one hand that baculovirus-mediated gene transfer is not restricted to cells in culture and on the other hand that *in situ* perfusion methods represent an attractive means to facilitate gene transfer into the liver *in vivo* using baculovirus vectors.

C. Gene transfer into normal liver tissue of mice

Based on the knowledge that the complement system poses so far a major hurdle for the success of baculovirus vectors *in vivo*, we evaluated the ability of baculovirus vectors to transfer genes into the livers of C-deficient mice (Lynch and Kay, 1995). In these pilot experiments, we injected a ß-galactosidase expressing baculovirus directly into the liver parenchyma of AKR-mice (C5-deficient). After histochemical staining for ß-galactosidase, we could detect a convincing amount of successfully transduced hepatocytes around the injection site (**Figure 4A**). The number of positive staining cells decreased with increasing distance to the injection site. These results demonstrate for the first time that baculovirus-mediated gene transfer in the liver is feasible *in vivo*. Just as important is the availability of a model, which is useful to evaluate important requirements on baculovirus vectors *in vivo*, such as duration of gene expression and interactions of the cellular immune system with the successfully transduced hepatocytes. These aspects of baculovirus-mediated gene transfer are currently under investigation with respect to the treatment of liver diseases, where already expression of low levels of the therapeutic gene product results in a therapeutic effect (Wilson´s diseases and heamophilias).

D. Gene transfer into liver tumors *in vivo*

The treatment of liver tumors by gene transfer is highly dependent on the quality of the vector as well as on the gene-therapeutic concept. Although, the development of baculoviral vectors is not nearly ready, the usefulness of this vector system for the treatment of liver tumors is conceivable. In preliminary experiments, we generated human liver cell tumors derived from the cell line Huh7 in nude mice and injected the ß-galactosidase expressing baculovirus vector into the tumors. Even though nude mice possess an intact complement system, the ß-gal staining of the tumor revealed a successful gene transfer using this intratumoral vector application (**Figure 4B**). A definite answer for the usefulness of baculovirus vectors for the treatment of liver tumors will result from an experiment that combines the features of this new vector with an established concept for treating tumors with complementing tumorsuppressor genes (Sandig et al., 1997; Strauss et al., 1997).

IV. Future vector improvements and prospects

The investigation of the baculovirus vector system for gene transfer into hepatocytes has, since its discovery, revealed a variety of advantegeous features of the vector, but there are still hurdles to overcome. Even if evasion or inactivation of the C system *in vivo* seems to be feasible, the ultimate goal will be generation of C-resistant viruses. We are currently approaching this goal by screening of baculovirus vector mutants as well as by insertion of C regulating molecules, such as decay accelerating factor (Lublin and Atkinson, 1989), into the viral envelope. Preclinical experiments using existing and improved baculovirus vectors have to be carried out for the treatment of inherited and malignant diseases of the liver. The outcome of those studies will provide clues as to the most promising application of baculovirus vectors in the field of liver gene therapy.

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