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Tissue- and Tumour-Selective Targeting of Murine Leukemia Virus-Based Replication-Competent Retroviral Vectors

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Abstract and aim (English)

Replication-competent retroviral (RCR) vectors are very useful for cancer gene therapy, as they exhibit high infection efficiency and a certain degree of tumour selectivity. Improving tumour selectivity is nonetheless desirable, and this can be achieved by transcriptional targeting, whereby the promiscuous viral promoter is replaced by a tumour-selective one.

It was the aim of this study to characterise one or several promoters which confer the desired cell selectivity and high levels of transgene expression onto an RCR vector, while retaining adequate genomic stability.

Based on an extensive literature search, seven promoters were chosen, namely the cancer-selective cellular Midkine, c-erbB-2 and AFP promoters, the cancer-selective synthetic CTP4 promoter, the liver tissue-selective hybrid EII-Pa1AT promoter, as well as the promiscuous c-fos promoter, whose usefulness as a promoter for retroviral vectors was to be evaluated, and the promiscuous viral mCMV promoter, which was used as a ubiquitously active control. In a preliminary experiment, the strength and cell selectivity of these promoters was evaluated in the context of an eGFP expression plasmid. The most promising of them, namely the CTP4, EII-Pa1AT and c-fos promoters, as well as the mCMV promoter, were then incorporated into a Murine Leukemia Virus - based RCR vector in two different ways: In one design, the entire MLV promoter was replaced by the heterologous promoters, whereas in the other design, the portion of the MLV promoter upstream of the TATA box was removed and replaced with the heterologous promoters lacking their TATA box. While a vector bearing the c-fos promoter replicated only poorly in cell culture, vectors containing the CTP4 and EII-Pa1AT promoters performed considerably better - but only in the latter design, in which the MLV TATA box was retained. These vectors exhibited transgene expression levels and replication kinetics similar to the parental vector, but in a cell-selective manner. Also, their genomic stability was on par with the parental vector, and their cell selectivity was retained over several repeated infection cycles.

The results of this research project were published in the Journal of Virology in 2006 (see chapter 3), with the focus on vectors bearing the CTP4 or EII-Pa1AT promoters. This thesis expands on this publication by addition of, amongst others, a more extensive introduction, presentation of preliminary results, and a description of further developments.

Zusammenfassung und Zielsetzung (Deutsch)

Replikationskompetente retrovirale (RCR) Vektoren sind für die Gentherapie sehr interessant, da sie eine hohe Infektionseffizienz und ein gewisses Maß an Tumorselektivität aufweisen. Dennoch ist es wünschenswert, die Tumorselektivität noch zu steigern, und das kann unter anderem durch *transcriptional targeting* erreicht werden. Hierbei wird der promiskuitive, d.h. in einer Vielzahl von Zelltypen aktive, virale Promoter durch einen tumorselektiven ersetzt.

Das Ziel der vorliegenden Studie war es, einen oder mehrere Promotoren zu charakterisieren, die einem RCR Vektor starke Transgen-Expression und zellselektive Replikation verleihen, dabei aber die genomische Stabilität des Vektors nicht vermindern. Ausgehend von einer umfangreichen Literaturrecherche wurden sieben Promotoren ausgewählt, nämlich die krebsselektiven, zellulären Midkine, c-erbB-2 und AFP Promotoren, der krebsselektive, synthetische CTP4 Promotor, der Lebergewebe-selektive EII-Pa1AT Promotor, sowie der promiskuitive c-fos Promotor, dessen Nutzen als Promoter für retrovirale Vektoren evaluiert werden sollte, und der promiskuitive, virale mCMV Promotor, welcher als Kontrolle diente. Zuallererst wurde die Stärke der Genexpression und die Zellselektivität dieser Promotoren im Kontext eines eGFP-Expressionsplasmids evaluiert. Die vielversprechendsten Promotoren, nämlich die CTP4, EII-Pa1AT und c-fos Promotoren, sowie der mCMV Promotor, wurden danach in einen auf dem murinen Leukämievirus (MLV) basierenden RCR-Vektor inkorporiert, und zwar auf zwei verschiedene Arten: Entweder wurde der gesamte MLV Promotor durch den gesamten heterologen Promoter ersetzt, oder

es wurde nur ein Teil des MLV Promoters - bis zum 5' Ende der TATA Box - entfernt und durch den heterologen Promoter, dessen TATA Box entfernt worden war, ersetzt. Während sich ein Vektor unter Kontrolle des c-fos Promotors in Zellkultur nur kaum replizierte, lieferten Vektoren unter der Kontrolle des CTP4 oder EII-Pa1AT Promoters weit bessere Ergebnisse, allerdings nur, wenn die Promotoren auf die zuletztgenannte Weise, bei der die MLV TATA Box erhalten blieb, inkorporiert wurden. Diese Vektoren wiesen starke Transgen-Expression auf und eine Replikationskinetik, die dem parentalen Vektor entspricht, allerdings auf eine zellselektive Art und Weise. Auch ihre genomische Stabilität war der des parentalen Vektors ebenbürtig, und ihre Zellselektivität blieb auch nach mehreren Replikationsrunden erhalten.

Die Ergebnisse dieses Forschungsprojekts wurden 2006 im Journal of Virology veröffentlicht (siehe Kapitel 3), wobei das Hauptaugenmerk auf den Vektoren lag, die den CTP4 oder den EII-Pa1AT Promotor enthielten. Diese Diplomarbeit erweitert diese Publikation um u.a. eine ausführlichere Einleitung, die Präsentation der Vorergebnisse, und eine Beschreibung der weiteren Entwicklung, nachdem dieses Projekt abgeschlossen war.

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1 Introduction

1.1 Gene therapy

1.1.1 Basics of gene therapy

Gene therapy is defined as the introduction of foreign genetic material into a patient's cells in order to prevent or treat a disease. There are several types of gene therapy:

- Gene therapy may be targeted to a selection of an individual's somatic cells only (somatic gene therapy). This approach leads to mosaicism, in which the germline cells remain unaltered, and the newly inserted genes are not inherited by the patient's offspring. The actual introduction of genetic material can be performed either *ex vivo* or *in vivo*. In the former case, target cells are first extracted from the patient, the genetic material is introduced, and the altered cells are then transplanted back into the target tissue. There, the altered cells either proliferate and outgrow their unaltered counterparts, or they secrete a desired gene product (Fig. 1a). This strategy can be employed to treat monogenetic, congenital diseases of the hematopoietic system (Gaspar HB, 2004) (Aiuti A, 2009). In an *in vivo* approach, gene therapeutical vectors are directly administered to resident cells in the patient's target tissue (Fig. 1b).
- Gene therapy may also be employed to alter an individual as a whole, in which case the treatment must be administered very early in life: Vectors may be injected into zygotes or early embryonic stages, or into sperm or egg cells. These strategies could be used to treat all kinds of monogenetic, hereditary diseases. Although such methods have already been employed to successfully generate transgenic primates (Smith KR, 2004), germline gene therapy in humans remains highly controversial and is in fact banned in many jurisdictions (Kimmelman J, 2008).

Although naked DNA may be injected into target cells, usually a vector is employed. The most commonly used vectors are viruses, which, by their very nature, are very apt to introduce genes into cells.

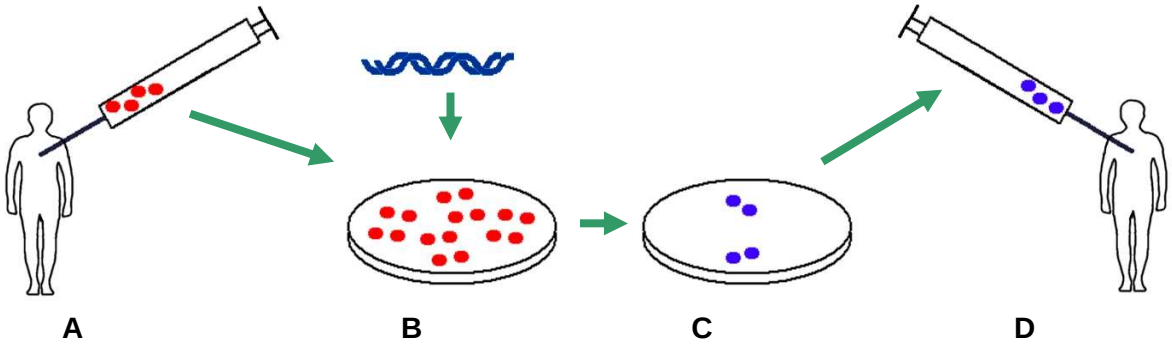


Fig. 1a *Ex vivo* approach to gene therapy. (A) Cells to be modified are extracted from patient. (B) Cells in culture are genetically altered. (C) Modified cells are selected for. (D) Modified cells are administered to patient.

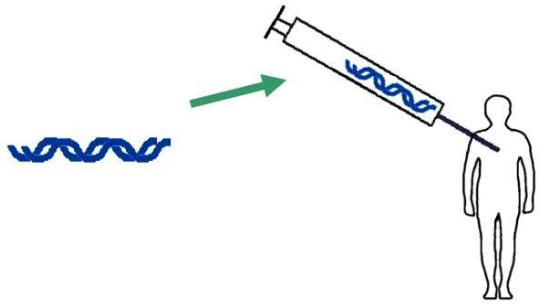


Fig. 1b *In vivo* approach to gene therapy. Foreign genetic material (most often contained in a vector) is administered to a patient.

1.1.2 Milestones of gene therapy development

Although the concept of gene therapy was proposed as early as 1963 (Lederberg, 1963), it was not until 1989 that the first clinical trial involving gene therapy was performed (Rosenberg SA, 1990). Although clinical success remained elusive and there was already an ongoing debate about the ethical implications of gene therapy, there were no major setbacks (Gillet JP, 2009).

Then, in a 1999 study, a patient died of a systemic inflammatory response, triggered by the administration of large quantities of an adenoviral vector (Marshall E, 1999). In 2000, a

widely publicised international gene therapy trial on X-linked severe combined immunodeficiency syndrome (SCID-X1) began (Cavazzana-Calvo M, 2000). SCID-X1 is a lethal immune deficiency affecting T, B, and natural killer (NK) cell development, and it is caused by mutations in the gene encoding for the common γ-chain of several interleukin receptors. The therapy involved transfer of the γ-chain gene into autologous, γ-chain deficient bone marrow cells, using an MLV vector in an *ex-vivo* approach. The therapy was successful, resulting in the sustained restoration of a functional adaptive immune system in 7 out of the 10 involved patients. Another clinical trial in 2004 had similar results (Gaspar HB, 2004). Two to six years after the procedure, however, 5 out of the total of 20 patients enrolled in these trials developed acute leukemia. One of these 5 patients died of this severe side effect (Hacein-Bey-Abina S, 2010). Investigation of these cases revealed that, in 4 out of 5 patients, the vector integrated close to the *LMO2* proto-oncogene, causing aberrant expression of *LMO2* via retroviral enhancer activity (Hacein-Bey-Abina S, 2003). It was established that gammaretroviral vectors integrate preferentially near transcription start sites (Deichmann A, 2007), and a so-called high incidence region of vector integration was identified near exon 1 of *LMO2* in T cells (Yamada K, 2009). However, other genetic abnormalities may need to be present for leukemia to arise (Howe SJ, 2008).

Recently, an HIV-based self-inactivating vector for treatment for SCID-X1 has been developed, which was shown not to induce *LMO2* expression in a mouse model (Zhou S, 2010).

Gene therapy was also employed to treat another type of SCID, namely SCID due to adenosine deaminase deficiency (ADA-SCID). ADA is an enzyme involved in purine metabolism, and its defectiveness leads primarily to impaired lymphozyte proliferation. As of 2010, over 30 ADA-SCID patients had been treated by gene therapy, in most cases successfully (Ferrua F, 2010). In one trial, ten patients were treated using a protocol similar to the one employed in the aforementioned SCID-X1 trial, with a retroviral vector bearing the *ADA* gene. In eight of these patients, a functional immune system was restored. In contrast to the SCID-X1 trials, no patient developed a leukemia-like disorder (Aiuti A, 2002) (Aiuti A,

2009). It is hypothesised that the proliferation signal provided by the common γ-chain, in cooperation with deregulated expression of a proto-oncogene, favours the establishment of malignant cells. ADA, in contrast, confers no such proliferation signal (Kohn DB, 2009).

Another study in 2006 was concerned with the treatment of X-linked chronic granulomatous disease (X-CGD), an immunodeficiency caused by mutations in gp91(phox), which leads to deficient antimicrobial activity in phagocytes. Two patients were treated with gammaretroviral vectors expressing gp91(phox), and the condition of these patients improved significantly during therapy. Similar to the SCID-X1 trials, the vectors often integrated next to one of three proto-oncogenes, namely the *MDS1-EVI1, PRDM16* or *SETBP1* genes, which led to a – beneficial – expansion of gene-corrected cells (Ott MG, 2006). After two years, however, a silencing of gp91(phox) expression occurred, as well as chromosomal rearrangements and myodysplasia due to oncogene expression. Eventually, one patient received a hematopoietic stem cell (HSC) transplant, whereas the other patient died of a severe sepsis, which was most likely a consequence of his underlying disease (European Society of Gene Therapy (ESGT), 2006) (Stein S, 2010).

The world's first commercial gene therapy product, Gendicine, was approved in China in 2003. Gendicine is a recombinant, oncolytic adenovirus whose E1B transcriptional unit has been replaced by a human p53 expression cassette, and it is used against head- and neck tumours. English language literature on Gendicine appears to be scarce, but in 2005 it was reported that it has been administered to over 4000 patients, with encouraging results (Peng Z, 2005). Also in 2005, China approved a second gene therapy product, Ocorine (Shi J, 2009). Also known as H101, this is an oncolytic adenovirus similar to Gendicine, but of lower toxicity (Huang PI, 2009).

1.1.3 Cancer gene therapy

Initially, gene therapy was directed at monogenetic hereditary diseases. Soon, however, it became clear that gene therapy can also be employed to combat vascular diseases,

infectious diseases such as AIDS and, most importantly, cancer. Up to 2007, over sixty percent of all gene therapy clinical trials performed were related to cancer (Edelstein ML, 2007). The following list provides an overview over therapeutic genes and strategies investigated for their use in cancer gene therapy.

- Tumour-suppressor genes can replace tumour suppressors no longer active in malignant cells. For instance, the re-establishment of p53 function has been considered a promising strategy (Kumar S, 2007).
- Suicide genes lead to the death of tumour cells in which they are expressed, after the administration of a prodrug. The bystander effect, whereby nearby cells are also affected, constitutes an added advantage.
- Some therapeutic genes, such as TNFα, are radiation-inducible. Others can be expressed under the control of a radiation-inducible promoter element. Ionising radiation can then be used to induce transgene expression at the right time and in the right tissue.
- Drug resistance genes, transferred to certain types of normal cells such as HSCs, can protect them from the toxic effects of anticancer drugs.
- RNA interference (RNAi) can be used to downregulate genes involved in the development or progression of cancer.
- Angiogenic inhibitors impede the growth and dissemination of tumours. For example, several approaches are being investigated to block vascular endothelial growth factor (VEGF) signaling (Mae M, 2005) (Drevs J, 2000).
- Genes expressing tumour antigens can be used for cancer vaccination. In animal models, success has been achieved using adenoviruses expressing tumour-associated antigens (Akbulut H, 2006).
- Autologous T cells can be engineered *ex vivo* and then transfused back into the patient. Most notably, such T cells can be transduced with a T cell receptor which confers specificity to a tumour antigen (Thomas S, 2007).

In most of these cases, the tumour cells are the actual targets of gene therapy. In contrast to gene therapy of hereditary enzymopathies, where it may be sufficient to transduce only a fraction of target cells, in cancer gene therapy it is desirable to transduce as high a proportion of tumour cells as possible (Vile RG, 2000). By necessity, the mode of application is *in vivo*, although local application may be possible, i.e. injecting the vector directly into the tumour. However, precautions to make sure that only tumour cells are transduced are advisable, especially when the therapeutic gene is a suicide gene. Vectors suitable for cancer gene therapy should therefore (1) allow for highly efficient transduction of target cells in a physiological (*in vivo*) environment, and (2) be selectively targeted to tumour cells.

1.1.4 Main gene delivery systems

The most commonly used vectors are adenoviruses and retroviruses. Other viruses employed include adeno-associated virus, simian virus 40, Herpes Simplex, vaccinia and poxvirus, as well as oncolytic viruses. In addition, there are delivery systems based on bacteria, lipofection or transfer of naked plasmid DNA (Edelstein ML, 2007).

Adenoviral vectors

The main advantages of adenoviral vectors are their high efficiency of transduction and high levels of - transient - gene expression. They infect cycling as well as quiescent cells. During the development of these vectors, more and more of the early transcription units have been deleted to render the viruses unable to replicate, to reduce immunogenicity and also to increase the transgene capacity. The latest generation of adenoviral vectors retains no more adenoviral coding sequences, and is produced using a helper virus (Kay MA, 2011). Aside from their high immunogenicity, one major problem associated with these vectors, especially in the field of cancer gene therapy, is their tropism: The most commonly used serotypes use the coxsackie-adenovirus receptor (CAR) for cell entry, which is not present on tumour cells. This problem could be circumvented by modifying the fiber proteins of the virus so that it can utilise other receptors (Sharma A, 2009).

Retroviral vectors

Simple retroviruses, such as murine leukemia virus (MLV), are very well characterised. Their genomes contain only a small number of genes, none of which needs to be expressed for a virus to successfully infect a target cell. Therefore, a large proportion of the viral genome can be replaced with foreign DNA without compromising infectivity. The most prominent feature of retroviruses, however, is the ability to integrate into the chromosomal DNA of the host cell, where they can remain indefinitely in the form of proviruses. Because of this, retroviruses are suitable vectors when long-term expression of therapeutic genes needs to be achieved. In the case of most retroviruses, including MLV, integration into host DNA is only possible when the nuclear membrane is disassembled during mitosis (Yamashita M, 2006). Therefore, only proliferating cells can be transduced, which confers a certain degree of tumour selectivity to those retroviruses. Furthermore, retroviruses exhibit lower immunogenicity than other viral vectors (Xu L, 2007).

Retroviral vectors can be used either in a replication-defective or replication-competent form. Replication-defective retroviruses (RDR) were the first to be developed because there was less chance that genes would be inserted in nontarget cells (Weber E, 2001). It has been shown, however, that the transduction efficiency of defective vectors is too low for widespread application in cancer gene therapy (Rainov NG, 2003). Replication-competent retroviral (RCR) vectors, which multiply in the infected tumour, could be capable of transducing a great number of tumour cells after an initial application of only a moderate amount of virus (Wang WJ, 2003). This could also alleviate the fact that retroviruses cannot be produced at high titres when compared with, for instance, adenoviruses (Rodrigues T, 2007). Furthermore, their relatively short genome restricts the amount of transferable genetic material, and they exhibit poor diffusion in tissue. Because of the danger of insertional mutagenesis, it is imperative that RCR vector replication be as tightly restricted to tumour cells as possible. RCR vectors are also prone to losing the foreign genetic material following

extended replication cycles, since it is not essential for viral replication. Therefore, stability of the transgene is an issue which must be addressed.

Lentiviral vectors

Amongst the *Retroviridae*, lentiviruses possess the unique ability to transduce not only dividing, but also quiescent cells. This has facilitated their use as vectors for targeting cells of the nervous system, muscles, lungs, and liver (Gillet JP, 2009). The long period of lentiviral genome expression reduces the need for repeated injections. Another advantage is their relatively low immunogenicity (Han JJ, 1999). As with other retroviruses, however, their packaging capacity is comparatively small, and insertional mutagenesis also poses a potential problem (Mátrai J, 2010).

A further development are “self-inactivating” (SIN) vectors, whereby a large portion of the 3’ U3 region, including the TATA box, is deleted. After reverse transcription, the LTRs of these vectors exhibit only little promoter or enhancer activity, and the expression of the transgene is driven by an internal promoter (Pauwels K, 2009). The reduced influence of these vectors on neighbouring cellular genes, such as proto-oncogenes, constitutes a key advantage (Modlich U, 2006).

Nonviral delivery systems

Although viral vectors are highly efficient gene vehicles, they suffer from certain disadvantages, such as toxicity or immunogenicity. Nonviral systems, in comparison, are less problematic in these respects, and also easier to prepare.

The simplest nonviral delivery system is the transfer of naked plasmid DNA. Successful transfer of DNA has been reported for, amongst other organs, muscle, epidermis and liver, as well as solid tumours. However, because naked DNA is quickly degraded, low transfection efficiency has often been observed. (Gillet JP, 2009).

To facilitate cell entry, DNA can be complexed with cationic lipids. The positively charged portion of these compounds binds to DNA, while the hydrophobic part interacts with the cell

membrane. Cationic lipids may also form micellar structures around DNA. These complexes are often internalised by endocytosis, and the DNA may be released into the cytoplasm by a rupturing endosome (Gillet JP, 2009).

The main drawback of these chemical delivery systems is the lack of long-term transgene expression. This issue may be circumvented by addition of the SV40 or Epstein-Barr virus replicon to the vector, which turns the vector into a stable episome (Shibata MA, 2005).

Various bacteria strains have been employed as gene delivery systems. For instance, an attenuated *Salmonella* strain expressing the E. coli cytosine deaminase gene has been used to treat solid tumours in a pilot clinical trial. The bacteria preferentially colonised the tumour site, and significant conversion of 5-fluorocytosin to 5-fluorouracil at the tumour site was observed (Nemunaitis J, 2003).

Oncolytic viruses

Not a gene delivery system *per se*, oncolytic viruses nevertheless hold promise for cancer therapy. They selectively replicate in and lyse tumour cells. Some viruses, such as Newcastle disease virus, naturally have this ability, whereas others, such as the adenovirus ONYX-15 (on which Gendicine is based), are engineered to target tumour cells (Gillet JP, 2009).

1.2 Retroviruses

1.2.1 Structure

Retroviruses are enveloped, with the lipid envelope taken from the producing cell upon budding. The envelope carries virus-encoded trans-membrane and surface glycoproteins, which are arranged as trimers and form spikes in the membrane. Beneath the envelope is a roughly spherical matrix, which surrounds the capsid. The capsid contains two copies of the

RNA genome, complexed with nucleocapsid proteins and smaller numbers of reverse transcriptase and integrase. Protease molecules are also present in the virion, although their location is uncertain (Fig. 2) (Coffin JM, 1997) (Knipe DM, 2007).

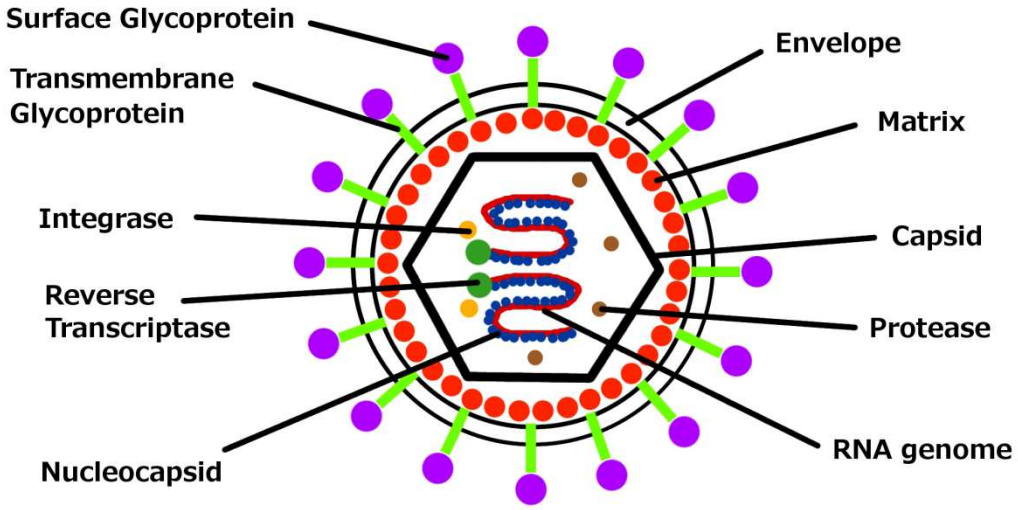


Fig. 2 Retroviral structure.

1.2.2 Genome

The genomic organisation of retroviruses is in many ways unique. Most notably, they are the only truly diploid viruses, with the two RNA copies being held together by a "dimer linkage structure" at their 5' ends. The two genome copies, each one 7 to 13 kb in size, are positive-stranded and thus identical to the (full length) viral mRNA. Indeed, it has been shown that Rous Sarcoma Virus (RSV) packages RNAs that have previously been translated (LeBlanc JJ, 2004). In MLV, however, there is evidence for the existence of two distinct populations of full length RNA, one destined for protein synthesis, the other one for packaging (Butsch M, 2002). Genomic RNA alone is not productively infectious, since, upon infection, the genome needs to undergo reverse transcription with the help of proteins present in the nucleocapsid. All retroviral genomes have at least 3 large coding domains, each one encoding a polyprotein:

- *gag* (group specific antigen): structural proteins of the matrix, capsid and nucleocapsid.
- *pol* (polymerase): reverse transcriptase and integrase
- *env* (envelope): envelope glycoproteins

In addition, all retroviruses contain the small *pro* domain between *gag* and *pol*, coding for the viral protease. Complex retroviruses such as HIV also encode many "accessory genes" in addition to *gag, pro, pol* and *env*. Another hallmark of retroviruses are the long terminal repeats (LTRs), direct repeats flanking the DNA form of the viral genome. Each LTR consists of the regions U3 (unique to the 3' end), R (repeated), and U5 (unique to the 5' end). They are thus called because in the RNA genome, U3 is missing at the 5' end and U5 at the 3' end, while R is present at both termini (Fig. 3) (Knipe DM, 2007).

Fig. 3 Genomic organisation of simple retroviruses (Regions not to scale).

1.2.3 Life cycle

Cell entry

The retroviral life cycle (Fig. 4) begins with the attachment of the virus to the cellular membrane. Initial binding is largely unspecific and has been shown to occur even with viral particles lacking Env proteins (Pizzato M, 1999). For virus entry, however, binding of viral surface proteins to one or several cellular receptors is necessary. Ecotropic MLV, for example, binds to the amino acid transporter mCAT-1. Most retroviruses then enter the cell by direct fusion of the envelope with the plasma membrane. MLV, however, appears to enter via an endocytic pathway (Katen LJ, 2001). Immediately after its release into the cytoplasm, the viral core commences a progressive disassembly known as uncoating.

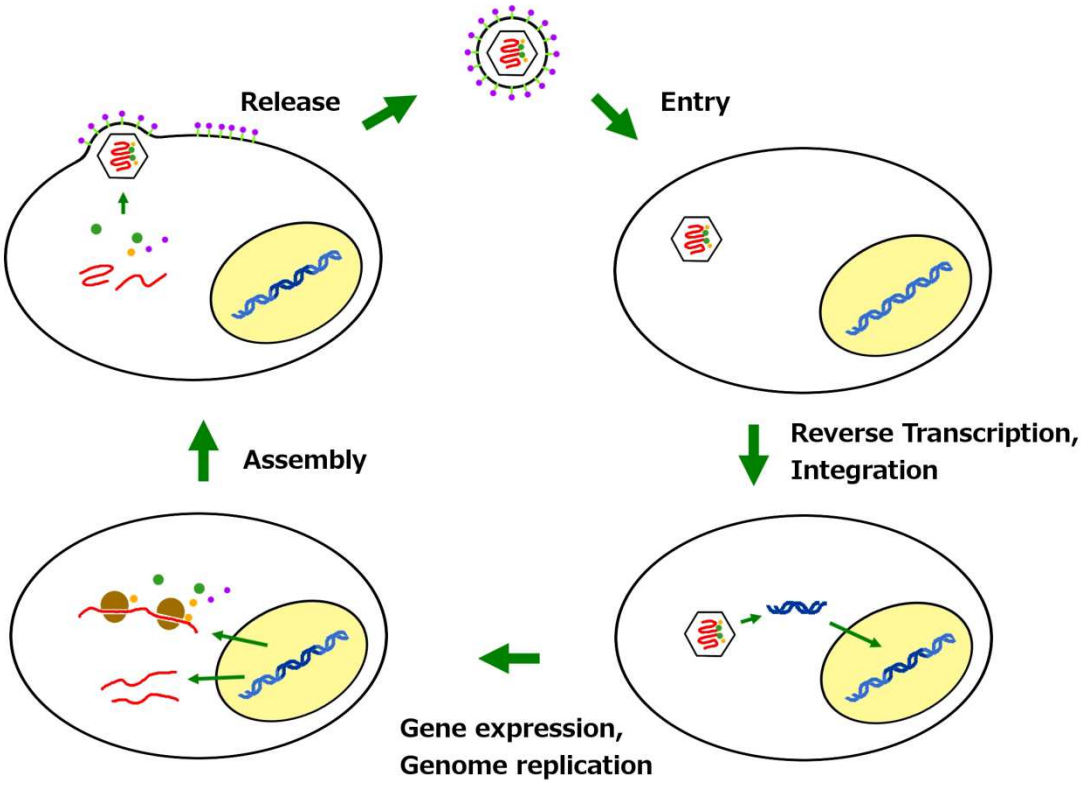


Fig. 4 Retroviral life cycle.

Reverse transcription

With access to dNTPs, reverse transcription of the viral RNA genome into its DNA form takes place. It is noteworthy that reverse transcription already starts in free viral particles, using dNTPs which were taken along from the producing cell upon budding (Zhang H, 1993).

Reverse transcription is initiated at a cellular tRNA primer, which was taken from the producing cell and is bound to a primer binding site (PBS) close to the 5' end of the viral genome. MLV generally utilises a $tRNA^{Pro}$ molecule, although a certain degree of flexibility in the choice of primer has been observed (Lund AH, 2000). Reverse transcriptase elongates the primer up to the 5' end of the viral RNA, creating the so-called minus strand strong stop DNA. Because reverse transcriptase has RNaseH activity, the RNA strand complementary to the newly synthesised DNA strand is degraded. During the first translocation, the minus strand strong stop DNA is transferred to the 3' end of the viral genome, where it binds with its R region to the complementary R region of the RNA genome. This first translocation can be

intra- or intermolecular. In the latter case, both RNA copies are used to generate the reverse transcribed genome; if these copies are differing in sequence, a mutated virus can arise. Following the first translocation, elongation of the DNA strand then proceeds to the end of the RNA genome, which is simultaneously degraded. A short stretch of RNA, the polypurine tract (PPT), survives degradation and its 3' end serves to prime the synthesis of the plus strand strong stop DNA. During this process, the sequence complementary to the PBS in the tRNA primer is also transcribed and the tRNA is subsequently removed. In some retroviruses, such as lentiviruses, a second copy of the PPT near the centre of the genome is also used with high efficiency (Charneau P, 1992). In the second translocation, the PBS in the plus strand pairs with the complementary sequence in the minus strand to form a circular structure. Then, both strands are elongated to create a double-stranded DNA genome with complete LTRs (Fig. 5).

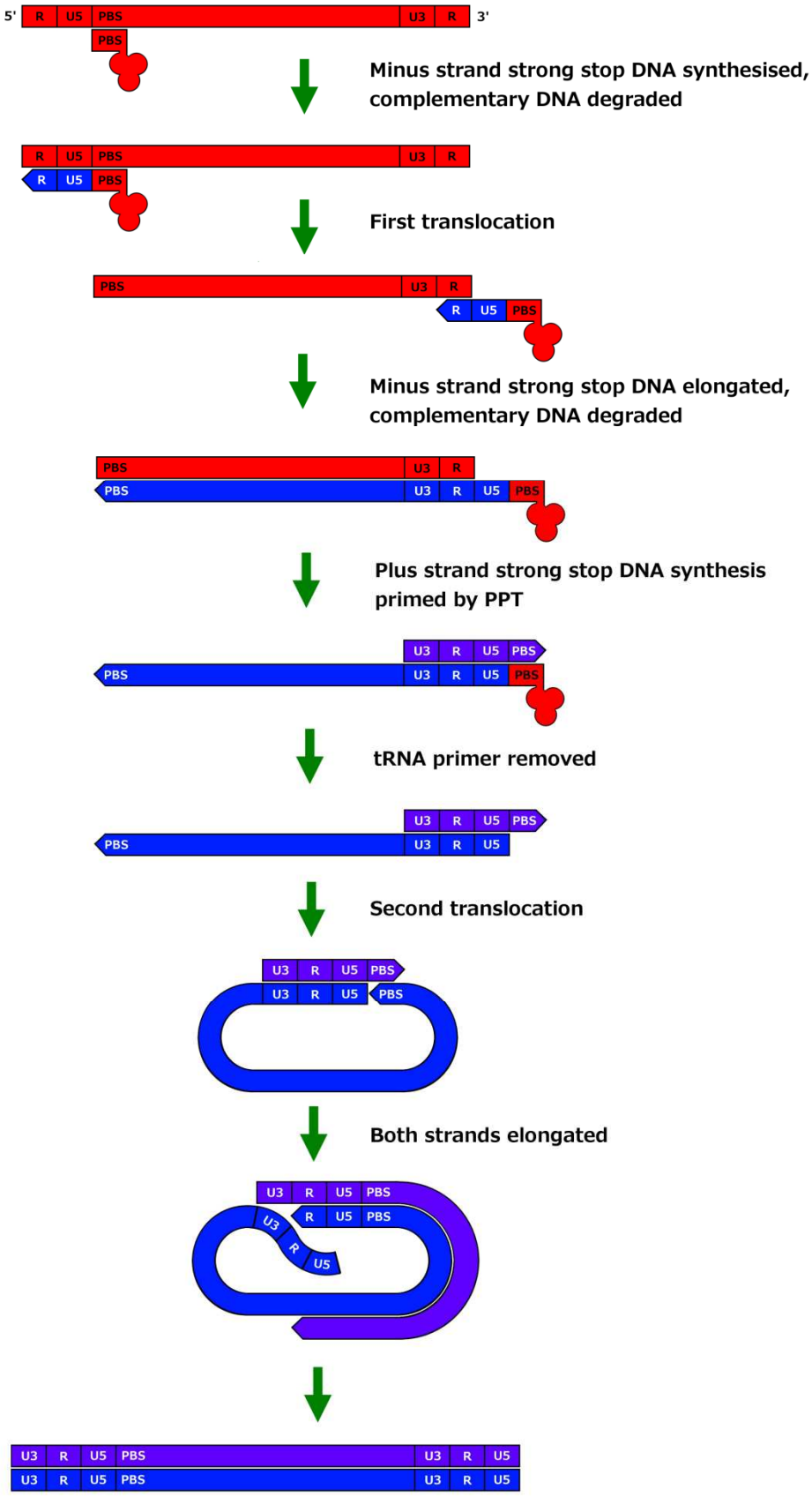


Fig. 5 Reverse transcription.

Integration

The progressive uncoating eventually leads to the formation of a pre-integration complex (PIC), a fully reverse transcribed viral genome associated with viral proteins. The PIC is then translocated into the nucleus by various means. By an as yet uncertain route, lentiviral PICs are able to enter intact nuclei and thus infect non-dividing cells. However, the efficiency of transduction is comparatively low in G_1 and especially G_0 cells, presumably because the concentration of dNTPs in these cells is too low for reverse transcription to take place (Zack JA, 1990). Most other retroviruses, including MLV, have to wait for the disassembly of the nuclear membrane during cell division to gain access to the host genome. Catalysed by the viral integrase, and probably aided by other viral and even host factors (Suzuki Y, 2004), the viral genome is then integrated into host chromosomal DNA. Although integration is not sequence-specific, distribution of integration sites is not entirely random. HIV has a tendency to integrate in active transcription units, while MLV preferentially integrates in the vicinity of transcription start sites (Lewinski MK, 2006). The integrated provirus, which is physically indistinguishable from the surrounding DNA, is the only part of the virus necessary for the continuation of the viral life cycle.

Gene expression

Expression of viral genes is carried out entirely by the cellular genetic machinery. In most retroviruses, all viral coding regions are transcribed from a single promoter, situated in the U3 region of the 5' LTR. Transcription gives rise to a large primary transcript, encompassing the entire provirus from the transcription start site (TSS) at the 5' end of the 5' R region, to the 3' end of the 3' R region. This transcript can be processed to become a full length mRNA, or spliced to give rise to shorter, subgenomic RNAs, from which the various viral proteins are translated. Complex retroviruses form several subgenomic RNAs from which their accessory proteins are translated. In simple retroviruses, however, there are only 2 species of mature RNAs:

- Full-length RNA: This RNA is used for the synthesis of the Gag, Pro and Pol proteins. The 5' UTR is relatively long and often contains additional AUG codons. In MLV, it is being suggested that an IRES is present near the start of the *gag* ORF, eliminating the need for the ribosome to scan the entire 5'UTR (Berlioz C, 1995) (Bolinger C, 2009). The amount of Gag proteins produced is 10-20 times higher than the amount of Pro and Pol proteins. This is achieved by various means. In MLV, *gag* and *pro-pol* are in the same reading frame, but separated by a UAG codon. While this stop codon is utilised in most cases and translation termination occurs at the end of *gag*, in some cases termination suppression leads to the synthesis of the longer Gag-Pro-Pol polyprotein. This RNA also contains a packaging signal (**Ψ)** in its 5' portion, necessary for assembly of nucleocapsid proteins with genomic RNA.
- Sub-genomic RNA: In this RNA species, a large portion of the genome encompassing the *gag-pro-pol* coding region is removed by splicing. This RNA serves for the translation of the Env precursor protein, which is synthesised at the rough endoplasmatic reticulum (RER), cleaved to form TM and SU proteins, and eventually incorporated into the plasma membrane. (Fig. 6)

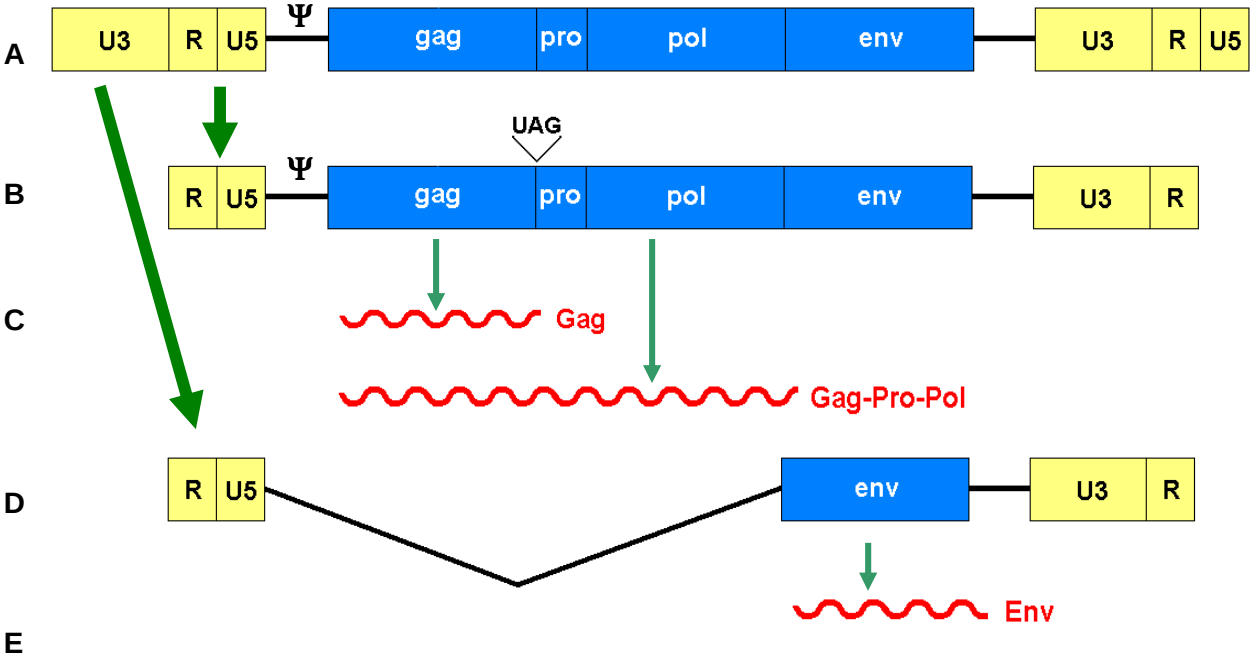


Fig. 6 Gene expression in MLV. (A) Integrated provirus. (B) Full-length RNA. (C) Gag and Gag-Pro-Pol polyproteins translated from full-length RNA. (D) Subgenomic RNA. (E) Env polyprotein translated from subgenomic RNA.

Assembly and budding

In most retroviruses, including MLV, assembly of nascent virions is mediated by Gag. Gag proteins aggregate at the plasma membrane and associate with genomic RNA. Curvature is induced in the plasma membrane and the nascent virion buds out from the cell, taking with it a part of the membrane containing viral Env proteins. Nascent virions also contain a number of tRNA molecules, most importantly those which are used to prime reverse transcription. After release, maturation of the virions occurs by proteolytic cleavage of the Gag-Pro-Pol polyprotein, catalysed by Pro.

1.3 Strategies, vectors and promoters used in this study

1.3.1 Transcriptional targeting and the ProCon vector system

As mentioned above, retroviral vectors should ideally be targeted to tumour cells for use in cancer gene therapy. This can be achieved in two ways: In infection targeting, the virus is modified to preferentially infect the intended target cells, by modification or replacement of the viral surface glycoproteins. In transcriptional targeting, on the other hand, the spectrum of cell types which can be infected remains unaltered, but viral gene expression is restricted to target cells by using a cell-selective promoter. The choice of promoter is crucial. Cell-selective mammalian promoters are usually several times weaker than their viral counterparts, while viral promoters usually do not offer the desired cell type selectivity. Of late, hybrid or synthetic promoters have been developed, which ideally combine high levels of expression with the desired cell selectivity (Hashimoto T, 2007) (Logg CR, 2002) (Kramer MG, 2003). Also, candidate promoters should be as small as possible because the capacity of the vector to carry foreign DNA is limited.

The usage of a highly cell-selective promoter raises the problem that the promoter is most likely inactive in standard producing cells. This problem can be circumvented very elegantly by utilising the "promoter conversion" (ProCon) vector system (Fig. 7). An integrated provirus possesses two promoters: One in each U3 region of both LTRs. While both promoters may be active, only the one in the 5' U3 region drives expression of the viral genes. However, it is always the promoter in the 3' U3 region which is incorporated into nascent virions as part of their genomes. During reverse transcription, this promoter is copied to the 5' end and thus drives expression in newly infected cells. Barring mutations, the two promoters are identical in a normal viral life cycle. In ProCon vectors, on the other hand, two different promoters are used in the provirus that is to be expressed in producing cells:

- The promoter in the 5' U3 region does not have to exhibit any cell selectivity, but it should offer high level expression in the intended producing cells.

Promiscuous viral promoters, such as the human cytomegalovirus (hCMV) immediate early promoter, may be used.

- The promoter in the 3' LTR is the one which will be active in target cells, therefore it should be cell-selective. Activity of this promoter in producing cells is not necessary.

Therefore, high levels of production can be achieved in any type of producing cell, of a vector that is nonetheless highly cell-selective (Saller RM, 1998).

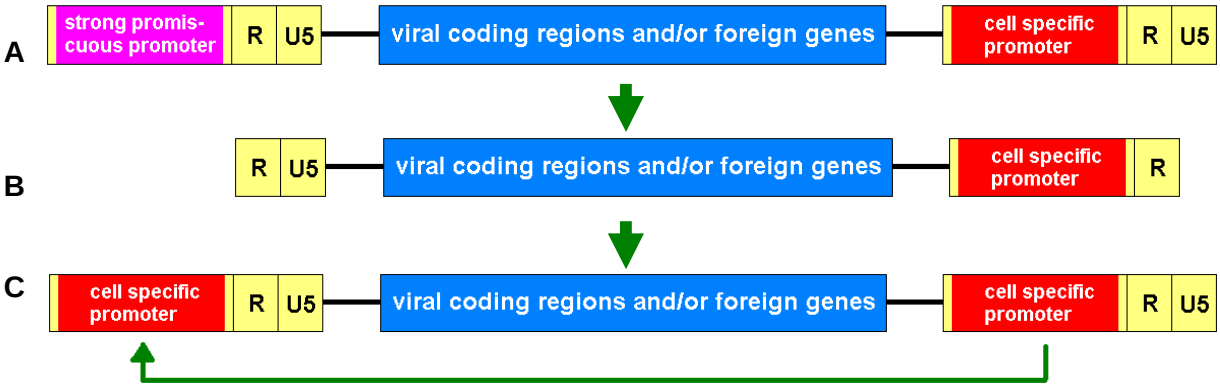


Fig. 7 The ProCon vector system. (A) Provirus which gives rise to virions in producing cells. (B) RNA genome of nascent virions. (C) Integrated provirus in target cells.

1.3.2 The retroviral vector *ACE-GFP*

All vectors used in this study are derivatives of *ACE-GFP*, a replication competent, Moloney-MLV based retroviral vector created by the group around Noriyuki Kasahara. A precursor of this vector was designed in 2000, using a novel approach to harbour the transgene. In most earlier studies, the transgene had been placed in the 3' U3 region, but this resulted in frequent recombination events and poor stability. Kasahara and coworkers fused the transgene to the 3' end of the *env* gene instead, preceded by the encephalomyocarditis virus internal ribosome entry site (IRES). This design also allowed for the translation of the transgene from both full-length and subgenomic RNAs. The group then demonstrated that

this vector replicated efficiently in mammalian cell culture (Smith E, 2000). In 2001, using the enhanced green fluorescent protein (eGFP) as the transgene, they used this type of vector to efficiently transduce solid tumours in mice, and also demonstrated the genomic stability of this design over multiple serial infection cycles. Although the vector was not targeted in any way, there was no apparent spread outside the tumours. Non-tumour cells within the tumour mass were transduced, though, and the group also suspected that there might have been spread outside the tumours which has escaped detection (Logg CR, 2001). In 2002, therefore, they transcriptionally targeted this vector with a prostate cell-selective rat probasin (PB) promoter inserted in the 3' LTR, while the hCMV promoter in the 5' LTR drove expression in producing cells. In order to allow for the infection of non-murine cells, they pseudotyped the virus with the amphotropic 4070A Env protein, thus creating the vector *ACE-GFP* (Fig. 8). In an *in vitro* study, the group demonstrated that this vector allowed for high level expression of eGFP in a prostate cell selective manner. Their results also suggested that a hybrid promoter design was superior to a total replacement of the viral promoter: Vectors in which the heterologous promoter from its 5' end to the beginning if its TATA box was fused to the MLV TATA box exhibited greatly enhanced expression levels over vectors in which the entire viral promoter, up to the TSS, was removed. Their results also demonstrated that a strong promoter is needed to drive efficient virus replication (Logg CR, 2002).

Fig. 8 The retroviral vector *ACE-GFP*, outfitted with a heterologous promoter.

Since then, *ACE-GFP* - in its non-targeted form - has been the subject of further research. It was successfully used to transduce gliomas in athymic mice (Wang WJ, 2003) and hepatic metastases of colorectal tumours in immunocompetent mice (Hiraoka K, 2006), as well as murine and human orthotopic bladder tumours in mice (Kikuchi E, 2007). The vector was also used to express several suicide genes. Replacement of the eGFP gene with the yeast

cytosine deaminase (CD) gene gave rise to a vector which mediated efficient cell killing in gliomas of athymic mice (Tai CK, 2005) and the aforementioned bladder tumours. Similarly, the *E. coli* purine nucleoside phosphorylase (PNP) was successfully employed, also against bladder tumours (Kikuchi E, 2007 (2)) and gliomas (Tai CK, 2010), both in athymic mice.

In a particularly interesting study, a predecessor to *ACE-GFP* was outfitted with an IgG binding site in the Env protein, and virions were complexed with anti-HER2 antibodies to target HER2 expressing cells *in vitro*. Although binding to target cells was efficient, cell entry was attenuated unless wild-type Env was coexpressed in the virions. This technique could be used to target an initial vector inoculum to the tumour, whereupon other targeting methods take over. Furthermore, this vector uses the relatively short VEGF IRES instead of the EMCV IRES (Tai CK, 2003).

1.3.3 The mCMV IE promoter

One of the most often utilised promoters in expression vectors is the human cytomegalovirus (hCMV) immediate early (IE) gene promoter. The strength of this promoter, though, varies greatly between species (Addison CL, 1997), which is reflected by the fact that hCMV infects only humans. Murine cytomegalovirus (mCMV), on the other hand, has a much broader host range and infects human as well as murine cells (Lafemina RL, 1988). Although there is no detectable sequence homology between hCMV and mCMV (Knipe DM, 2007), the organisation of their genomes in general and their IE promoters in particular is similar. The mCMV IE promoter contains several imperfect repeat elements, which are putative transcription factor binding sites (Dorsch-Häsler K, 1985). The two genes transcribed from this promoter, *ie-1* and *ie-3*, encode transcriptional regulatory proteins (Liu XF, 2008).

A comparison of the hCVM and the mCMV promoter in the context of a replication defective adenoviral vector was carried out by Addison and coworkers in 1997. They demonstrated that in human cells, the activity of the hCMV IE promoter is on par with the activity of the mCMV IE promoter, while in murine cells, the activity of the hCMV IE promoter is up to 100-fold lower. They suggest that, when high levels of transgene expression need to be

achieved, which are ideally comparable between murine and human cells, the mCMV IE rather than the hCMV IE promoter should be employed (Addison CL, 1997).

1.3.4 The EII-Pa1AT promoter

This chimeric promoter, created by Gabriela Kramer and collaborators, consists of two liver cell-selective, rather than tumour-selective, elements:

- The human α1-antitrypsin promoter (Pa1AT). α1-antitrypsin (AAT) is one of the major protease inhibitors in serum and expressed mainly in liver cells, but also in macrophages during inflammation (Tardiff J, 1998). In macrophages, however, the AAT gene is transcribed from a promoter other than Pa1AT.
- The enhancer II (EII) of human hepatitis B virus (HBV). The EII is responsible for liver-selective transcription of the pregenomic 3.5 kb long HBV RNA that codes for the core proteins and serves as template for viral DNA synthesis (Yee JK, 1989)

Kramer and coworkers created several constructs using liver-selective cellular promoters alone, or in combination with liver-selective enhancers (Kramer MG, 2003). *In vitro* assays showed that the promoters alone exhibited significant cell selectivity, but poor expression levels. Constructs which combine these promoters with liver-selective enhancers exhibited varying strength; the highest activity, while retaining liver selectivity, was observed with the 460 bp long EII-Pa1AT construct. The group then evaluated the *in vivo* performance of the enhancer-promoter combination constructs. In liver tissue, the EII-Pa1AT construct showed the highest activity of all liver-selective constructs tested, while in extrahepatic tissues, activity of EII-Pa1AT was much lower and similar to the other constructs.

1.3.5 The CTP4 promoter

This synthetic promoter consists of 10 T cell factor (TCF) binding sites fused to the TATA box of the adenovirus 5 E1B promoter (Ad5 E1B). It is selective for cells which are deregulated for β-catenin, which is the case in many tumours, especially those of colorectal origin. β-

catenin is an important factor of the Wnt signaling pathway, which plays a fundamental role in embryonic development and adult homeostasis (Logan CY, 2004). In the absence of Wnt signaling, free cytoplasmatic β-catenin binds to a complex consisting of Axin, glycogen synthase kinase 3 (GSK3), casein kinase 1 (CK1) and the adenomatous polyposis coli (APC) protein. This complex phosporylates β-catenin and targets it for degradation. Binding of Wnt to its cellular receptor leads to inhibition of the aforementioned complex, which in turn causes free β-catenin to accumulate. Eventually it translocates into the nucleus and, together with the TCF/LEF family of proteins, activates Wnt target genes (MacDonald BT, 2009). β-catenin deregulation can be caused by a mutation in several of the involved proteins. Stabilising mutations in β-catenin are fairly frequent, but the most common cause of β-catenin accumulation, especially in colorectal cancers, are inactivating mutations in the APC protein (Waltzer L, 1999) (MacDonald BT, 2009).

The group around Kai Lipinski constructed a promoter which contained 5 TCF binding sites, upstream of the minimal SV40 promoter (Lipinski KS, 2001). The promoter was then extensively optimised, especially with respect to the number of TCF binding sites, their distance from each other and to the TATA box, and the minimal promoter used. The resulting design, termed CTP4, was 380 bp in length and had a total of 10 TCF binding sites fused to a fragment encompassing the Ad5 E1B TATA box (Lipinski KS, 2004). In the context of an adenoviral vector, the CTP4 promoter exhibited equal or higher activity than the CMV promoter in β-catenin deregulated cells, but was virtually inactive in cells which are not β-catenin deregulated. The group even suggests using this promoter in conjunction with directly toxic suicide genes such as diphtheria toxin A (DTA).

1.3.6 The Midkine promoter

Midkine (MK) is a heparin-binding growth factor, whose diverse functions include the promotion of growth, survival and migration of cells. It is normally expressed in a controlled manner during embryogenesis, while expression in adult normal tissues is severely limited.

Aberrant expression of MK is frequently observed in tumours, including hepatocellular carcinoma, colon carcinoma, neuroblastoma and breast cancers. The exact role of MK in tumorigenesis is not well understood, but it has been shown that the inhibition of MK expression in MK-positive tumours also inhibits their growth (Muramatsu T, 2010).

Masatoshi Tagawa and coworkers explored the potential of the MK promoter for cancer-selective expression of a transgene, employing it in the context of an adenoviral (Yu L, 2004) and an RDR (Miyauchi M, 2001) vector, both in *in vitro* and in mouse studies. The minimal promoter appears to be a 0.3 kb fragment, but it was a 0.6 kb fragment which conferred the highest difference of transgene expression between normal and immortalised cells (Sakiyama S, 2003). It was this 0.6 kb MK promoter that was used in the current study.

1.3.7 The c-erbB-2 promoter

The c-*erbB-2* gene, also known as HER-2, encodes a tyrosine kinase receptor which is a member of the epidermal growth factor receptor family. c-*erbB-2* is normally expressed at low levels in certain epithelial cells, but is overexpressed in about 30% of breast and ovarian cancers (Slamon DJ, 1989). Overexpression of c-*erbB-2* leads to elevated tumorigenicity, enhanced metastatic potential, and a generally worse prognosis of the patients (Wang SC, 2000).

In their 2001 and 2002 studies, Tagawa and coworkers tested the ability of several c-*erbB-2* promoter fragments to drive the expression of a transgene in normal and breast cancer cell lines. They found that the minimal promoter responsible for tumour-selective transcription was contained in a 251 bp (-213/+38) fragment, while a 533 bp fragment conferred lower transcriptional activity and poorer cell selectivity, and a 124 bp fragment conferred no transgene transcription at all. The 22 base pairs at the 5´end of the 251 bp minimal promoter were shown to contain an element necessary for preferential activity in breast cancer cells (Maeda T, 2001) (Yu L, 2002).

1.3.8 The AFP promoter

Alpha-fetoprotein (AFP) is an important component of fetal serum. It is produced in the fetal liver and yolk sac. AFP synthesis drops sharply immediately after birth, but is resumed in liver tumours and certain teratoblastomas, which is why AFP blood levels have become a widely used diagnostic marker for liver tumours. The expression of the AFP gene is regulated primarily at the level of transcription, by an upstream regulatory region consisting of a tissue-selective promoter and several enhancers and silencers (Lazarevich, 2000). The AFP regulatory region thus holds great promise as a tool to target transgene expression to liver tumours. Since the proximal AFP promoter has been shown to be comparatively weak (Ido A, 1995), the large regulatory unit has been used in combination with stronger promoters in some studies (Cao G, 2000) (Cao G, 2001). As promoter length is critical in the context of a retroviral vector, the 0.3 kb proximal promoter, which has already been employed successfully in an adenoviral vector (Shi YJ, 2004), is used in the current study.

1.3.9 The c-fos promoter

c-*fos* belongs to the family of immediate early genes, which are activated quickly but transiently in response to intracellular signaling (Soloaga A, 2003). As a component of the AP-1 transcription factor, the c-*fos* gene product plays a role in cell differentiation and proliferation (Shaulian E, 2001), but also in tumorigenesis (Bakin AV, 1999) (Hu E, 1994). Although c-*fos* overexpression was reported to be associated with cancer (Gamberi G, 1998), it may also have tumour-suppressor function (Teng CS, 2000), and the c-*fos* promoter is not generally regarded a cancer-selective one. It is, however, highly active and inducible by mitogenic stimuli or stress factors, which is why Manfred Wirth and coworkers investigated its usefulness as a promoter for mammalian expression vectors. They found that the 755 bp long c-*fos* promoter drove stronger transgene expression than the hCMV promoter in the majority of cell lines tested, and it can be induced up to 8-fold by addition of fetal calf serum (Bi JX, 2003).

2 Preliminary results

2.1 Transcriptional activity of all heterologous promoters in various cell lines

The tissue- or tumour-selective EII-Pa1AT (E2), AFP, CTP4, Midkine (MK) and c-erbB-2 promoters were chosen as candidate promoters for targeted RCR vectors. In addition, RCR vectors under the control of the promiscuous mCMV and c-fos promoters were also generated, the former as a control, the latter because its utility as a strong, ubiquitously active promoter was to be investigated.

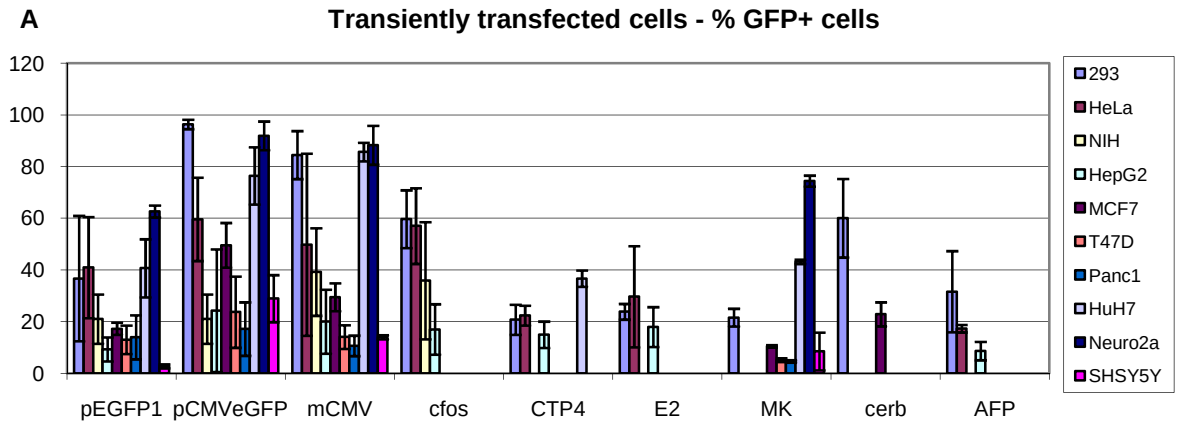
Before the heterologous promoters were tested in the context of an RCR vector, their strength and cell selectivity was evaluated in a preliminary experiment. To this end, the promoters were inserted into the promoterless expression vector pEGFP1, upstream of the eGFP gene. Because expression of the retroviral vector is driven by the hCMV promoter in its plasmid backbone in producer cells, a plasmid expressing eGFP under the control of the hCMV promoter was also included in this experiment. Also, the promoterless plasmid pEGFP1 was included, to assess any background fluorescence. The resulting constructs were subsequently used to transfect a variety of tumour and non-tumour cell lines. Generally, cell lines were chosen according to published results, i.e. when a promoter has previously been tested in a particular cell line, this cell line, if available, was used. The used cell lines were HEK-293 (293), HeLa, NIH-3T3 (NIH), the hepatic cancer cell lines HepG2 and HuH-7, the breast cancer cell lines MCF7 and T47D, the neuroblastoma cell lines Neuro2a and SHSY5Y, and the pancreatic cancer cell line Panc1. All cell lines are of human origin, except for the murine cell lines NIH and Neuro2a. Calcium phosphate coprecipitation was the preferred transfection technique for most cell lines (see chapter 3), only Neuro2a and SHSY5Y cells were transfected using the Lipofectamine 2000 kit (Invitrogen), according to the manufacturer's recommendations. See table 1 for an overview of the promoters, their cell selectivity, the expression vectors containing them and the cell lines they were employed in.

Promoter	Expression vector	Cell selectivity	Employed cell lines
hCMV	pCMVeGFP	ubiquitously active	☑ 293, HeLa, NIH, HepG2, MCF7, T47D, Panc1, HuH-7, Neuro2a, SHSY5Y
mCMV	pEGFP1-mCMV	ubiquitously active	☑ 293, HeLa, NIH, HepG2, MCF7, T47D, Panc1, HuH-7, Neuro2a, SHSY5Y
c-fos	pEGFP1-cfos	ubiquitously active	☑ 293, HeLa, NIH, HepG2
EII-Pa1AT	pEGFP1-E2	liver tissue	☑ HepG2 ☒ 293, HeLa
AFP	pEGFP1-AFP	liver cancer	☑ HepG2 ☒ 293, HeLa
CTP4	pEGFP1-CTP4	various cancers	☑ HepG2, HuH-7 ☒ 293, HeLa
Midkine	pEGFP1-MK	various cancers	☑ MCF7, T47D, Panc1, HuH7, Neuro2a, SHSY5Y ☒ 293
c-erbB-2	pEGFP1-cerb	breast and ovarian cancer	☑ MCF7 ☒ 293

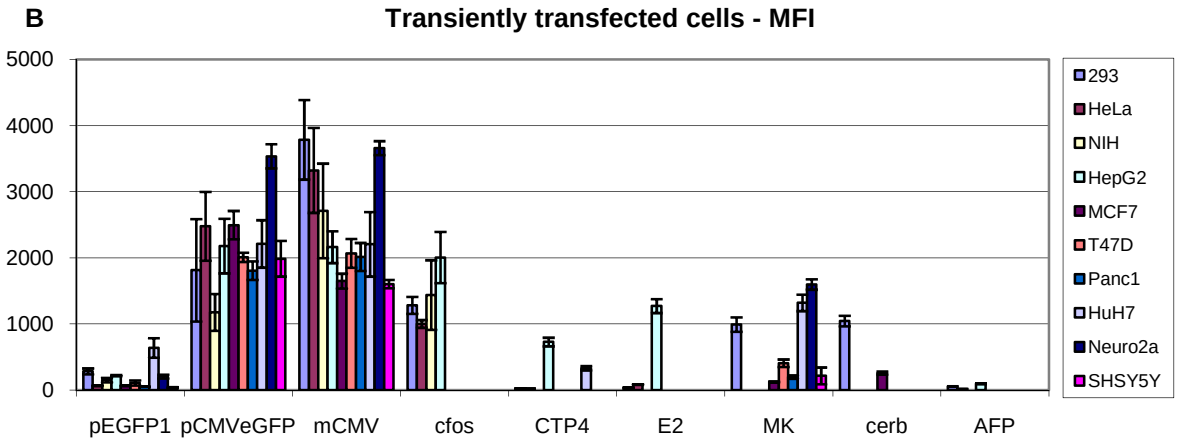
Table 1 Overview of heterologous promoters, the expression vectors outfitted with them, their expected cell selectivity and the cell lines they were tested in (expected promoter activity: ☑ active, ☒ inactive).

Two days after transfection, FACS analysis was performed on a portion of the cells, while another portion was used to create populations of stably transfected cells. The pEGFP1 backbone contains the *neo* resistance marker, and cells were subjected to selection with 400 µg/ml G418 for three to five weeks. FACS analysis was performed again thereafter (Fig. 9). The entire population of SHSY5Y cells died within one day of selection, hence they are either very sensitive to this dose of G418, or no transfection took place.

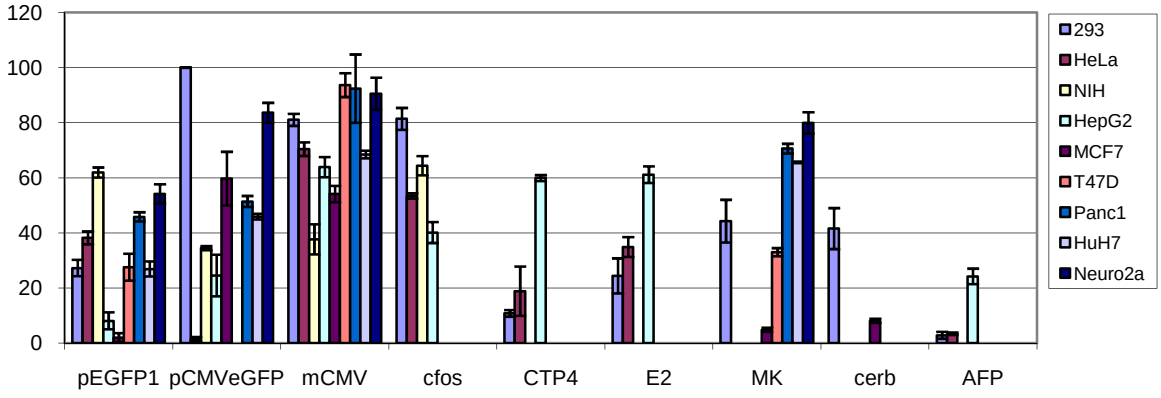
A


Transiently transfected cells - % GFP+ cells
120
100
80
60
40
20
0
pEGFP1
pCMVeGFP
mCMV
cfos
CTP4
E2
MK
cerb
AFP
293
HeLa
NIH
HepG2
MCF7
T47D
Panc1
HuH7
Neuro2a
SHSY5Y

B


Transiently transfected cells - MFI
5000
4000
3000
2000
1000
0
pEGFP1
pCMVeGFP
mCMV
cfos
CTP4
E2
MK
cerb
AFP
293
HeLa
NIH
HepG2
MCF7
T47D
Panc1
HuH7
Neuro2a
SHSY5Y

C


Stably transfected cells - % GFP+ cells
120
100
80
60
40
20
0
pEGFP1
pCMVeGFP
mCMV
cfos
CTP4
E2
MK
cerb
AFP
293
HeLa
NIH
HepG2
MCF7
T47D
Panc1
HuH7
Neuro2a

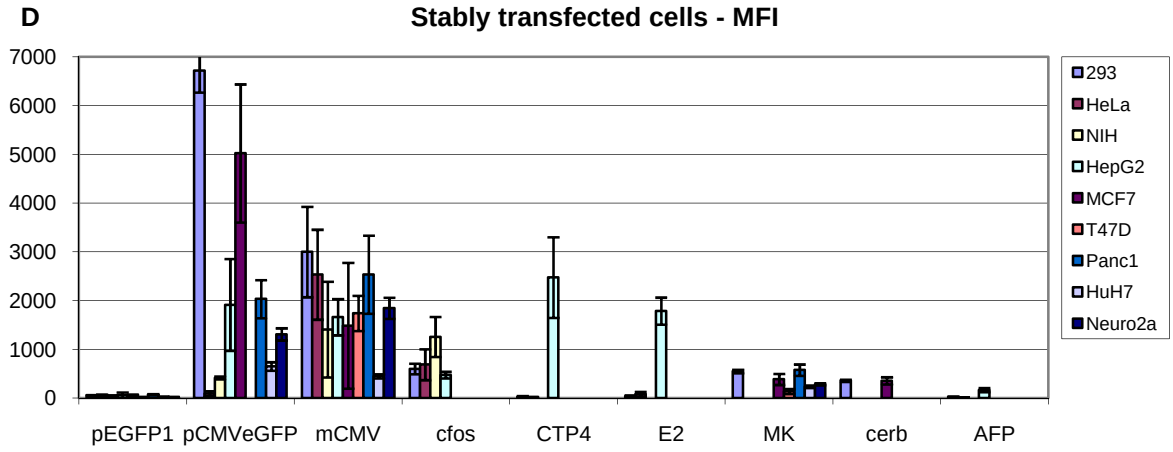


Fig. 9 Transcriptional activity of heterologous promoters. A number of cancer and non-cancer cell lines of human and murine origin were transfected with the eGFP expression vector pEGFP1 under the control of the heterologous promoters. (A) Percentage of eGFP-positive cells and (B) MFI of cells after transient transfection. (C) Percentage of eGFP-positive cells and (D) MFI of cells after selection of stable populations.

In most cases, the MFI was higher in transiently transfected than in stably transfected cells, which is to be expected as only a fraction of the plasmids brought into a cell by transfection are eventually integrated into the genome.

Significant background fluorescence by the promoterless plasmid pEGFP1 could only be observed in transiently transfected cells.

The promiscuous hCMV and mCMV promoters mediated considerable transgene expression in all cell lines. The hCMV promoter - which has been described to be predominantly active in human cells - exhibited especially low activity in stably transfected NIH cells, much less so than the mCMV promoter. That being said, the activity of the hCMV promoter in the human cell line HeLa was even lower.

The c-fos promoter drove robust gene expression in all cell lines tested, although it did not reach the expression levels of the mCMV promoter.

Both the CTP4 and EII-Pa1AT promoters exhibited the desired strength and cell selectivity. In the liver-derived, β-catenin deregulated cell line HepG2, they achieved expression levels on par with the mCMV promoter, while being virtually inactive in 293 and HeLa cells. The

CTP4 promoter also showed some activity in HuH-7 cells, which are not known to be deregulated for β-catenin, but, again, only in transient assays.

Both the MK and the c-erbB-2 promoters, which have been described to be tumour-selective, appeared to be equally active in 293 as in tumour cells, and the transgene expression levels they mediated in stably transfected cells were generally four times lower than those of the CTP4 or EII-Pa1AT promoter in permissive cell lines.

The AFP promoter was more active in HepG2 than in 293 or HeLa cells, but the absolute expression levels were very low: In HepG2 cells, the activity of the AFP promoter was about one order of magnitude lower than that of the CTP4 or E2-Pa1AT promoter.

Based on these results, the c-fos, CTP4 and EII-Pa1AT promoters, along with the mCMV promoter, were chosen as the subjects of further research, to be evaluated in the context of an RCR vector.

2.2 Replication kinetics of an RCR vector under the control of the c-fos promoter

For the evaluation of vectors with the mCMV, EII-Pa1AT and CTP4 promoters, as well as the parental vector ACE-GFP, see chapter 3.

The c-fos promoter was inserted into the 3' U3 region of the replication-competent, MLV based vector ACE-GFP. Replication of this vector in producer cells is driven by the hCMV promoter in its plasmid backbone, but during reverse transcription, the promoter in the 3' LTR is copied to the 5' LTR and drives viral gene expression in infected cells. Instead of replacing the entire MLV promoter in the 3' LTR by the heterologous promoter, however, the so-called TATA fusion (TF) design was employed, whereby only a portion of the wild-type promoter, up to the 5' border of the TATA box, was removed and replaced with the corresponding sequence of the c-fos promoter, form its 5' end to the 5' border of its TATA box. The resulting

vector, termed cfos-TF, retained the MLV TATA box, a design which has proven superior to a full replacement of the wild-type promoter (Logg CR, 2002) (Fig. 10).

Fig. 10 Design of the RCR vector cfos-TF. A large portion of the 3`U3 region of the MLV vector ACE-GFP was replaced by the corresponding sequence of the c-fos promoter.

In order to evaluate the replication kinetics and cell selectivity of cfos-TF, 293 cells were transiently transfected with the vector construct and virus-containing supernatant was harvested two days later. The supernatant was subsequently used to infect 293, HeLa, NIH and HepG2 cells. The cells were passaged every 3 to 4 days and FACS analysis was performed at each passage. Over a course of 19 days, cfos-TF spread very little in any cell line, infecting 20% of the cell population at the most, and exhibited only low eGFP expression levels (Fig. 11).

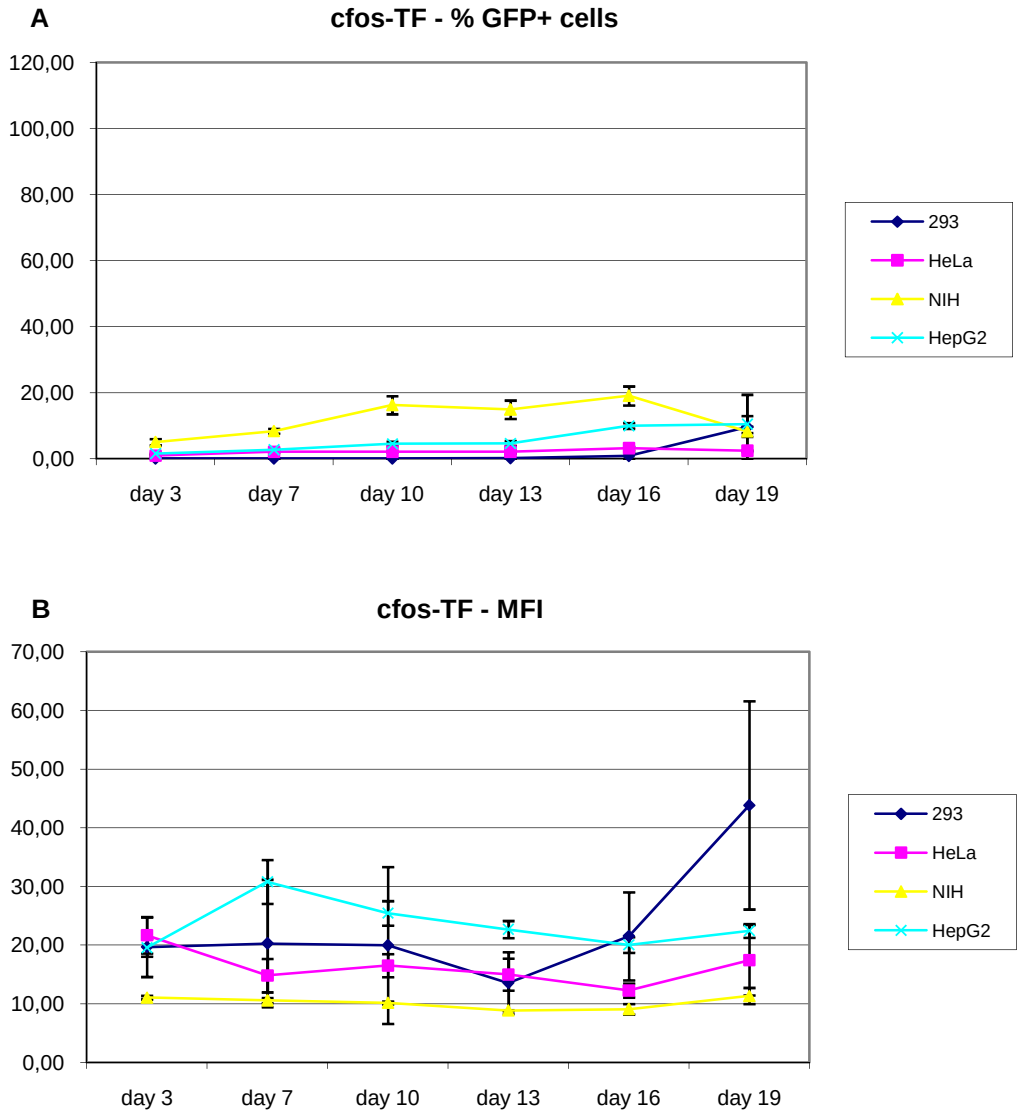


Fig. 11 Replication kinetics of cfos-TF. 293, HeLa, NIH and HepG2 cells were infected with virus harvested from transiently transfected 293 cells. The infected cells were passaged every 3 to 4 days and FACS analysis was performed at each passage. (A) Percentage of eGPF-positive cells and (B) MFI of infected cells over a course of 19 days.

It was decided to concentrate any further research on the CTP4, EII-Pa1AT and mCMV promoters.

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Tissue- and Tumor-Specific Targeting of Murine Leukemia Virus-Based Replication-Competent Retroviral Vectors

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Replication-competent retrovirus vectors based on murine leukemia virus (MLV) have been shown to effectively transfer therapeutic genes over multiple serial infections in cell culture and through solid tumors in vivo with a high degree of genomic stability. While simple retroviruses possess a natural tumor selectivity in that they can transduce only actively dividing cells, additional tumor-targeting strategies would nevertheless be advantageous, since tumor cells are not the only actively dividing cells. In this study, we used the promiscuous murine cytomegalovirus promoter, a chimeric regulatory sequence consisting of the hepatitis B virus enhancer II and the human α1-antitrypsin (EII-Pa1AT) promoter, and a synthetic regulatory sequence consisting of a series of T-cell factor binding sites named the CTP4 promoter to generate replicating MLV vectors, whereby the last two are transcriptionally restricted to liver- and β-catenin/T-cell factor-deregulated cells, respectively. When the heterologous promoters were used to replace almost the entire MLV U3 region, including the MLV TATA box, vector replication was inefficient since nascent virus particle production from infected cells was greatly decreased. Fusion of the heterologous promoters lacking the TATA box to the MLV TATA box, however, generated vectors which replicated with almost-wild-type kinetics throughout permissive cells while exhibiting low or negligible spread in nonpermissive cells. The genomic stability of the vectors was shown to be comparable to that of a similar vector containing wild-type MLV long terminal repeats, and tropism analysis over repeated infection cycles showed that the targeted vectors retained their original specificity.

Because simple retroviruses can transduce only actively dividing cells (3, 26, 36, 44), their use in cancer gene therapy has been extensively investigated, and over the last decade, numerous preclinical in vivo studies and clinical trials have been carried out using replication-defective retroviral (RDR) vectors (13). Although promising results have been obtained with animal models, therapeutic benefit in clinical settings has remained elusive, especially for cancer gene therapy, since the infection efficiency of solid tumors is too low (34). Of late, therefore, the use of replication-competent retroviral (RCR) vectors has been advocated, and it has been demonstrated by various groups that these are much more efficacious than their RDR counterparts (15, 23, 26–29, 40–42, 45). Mitotic cells, of course, are not unique to tumors, and although it may be expected that RCR vectors would not replicate efficiently outside of the immune-privileged environment of a solid tumor in a healthy individual, the possibility of spread occurring in dividing cells outside of the tumor mass must nevertheless be considered (7, 33, 35). Moreover, not least due to recent events demonstrating that retroviral vectors are capable, albeit in rare circumstances, of inducing oncogenesis in humans (19, 30), safety is a primary concern in retroviral vector design (46).

To date, the most effective targeting strategies for RDR vectors are transcriptional targeting (11, 14, 18, 31), whereby promiscuous viral promoter elements are exchanged for more-tightly regulated cellular, viral, or synthetic promoter elements, and, to a lesser extent, modifications of the envelope protein such that infection is restricted to certain cell types (infection targeting) (21). It has recently been demonstrated that, by using a highly active synthetic variant of the probasin promoter, the expression targeting strategy can also be applied to RCR vectors such that both transgene expression and vector replication are strictly confined to prostate cells (27). Although transcriptional targeting of RCR vectors, in contrast to infection targeting, is not designed to prevent infection of nontarget cells, it should prevent subsequent spread following an initial infection event. Moreover, transcriptional targeting should minimize the risk of oncogenesis via insertional mutagenesis since the promoter/enhancer elements may not be able to activate cellular genes (37).

In this study, we have investigated whether a transcriptional targeting strategy could be applied to RCR vectors to target specific cell types. In contrast to the transcriptional targeting of replication-deficient retroviral vectors, in which only expression of the transgene is required, in RCR vectors, sufficient amounts of Gag, Pol, and Env proteins must also be made in order to facilitate efficient virus spread, necessitating a promoter which drives high levels of transcription (27). Moreover, since the promoter is inserted into such vectors in addition to the full complement of viral genes and it is known that lengthening of the viral genome can lead to large decreases in replication efficiency (38), suitable candidate promoters should be of similar size to or smaller than the murine leukemia virus

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(MLV) elements which they replace and should mediate strong transcription in permissive cells. Following an extensive literature search, we therefore selected the liver-specific chimeric promoter EII-Pa1AT (24), the synthetic, beta-catenin/T-cell factor (TCF)-dependent promoter CTP4 (25), and the promiscuous murine cytomegalovirus (mCMV) promoter (1), all of which are relatively short and have been shown to drive high levels of transcription in permissive cells (1, 24, 25). The 380-bp CTP4 promoter consists of a minimal TATA box preceded by 10 TCF binding sites and has been shown to allow strict expression targeting of adenoviral vectors to cells deregulated for β-catenin, including those derived from prostate, ovarian, liver, and colorectal cancers, and to be highly active in biopsy specimens from primary human colon and colorectal cancers (25). The 460-bp EII-Pa1AT promoter, which consists of enhancer II (EII) of the human hepatitis B virus (HBV) fused to the human α1-antitrypsin promoter (Pa1AT), has been described as having strong transcriptional activity in a liver-selective manner, both in vitro and in vivo (24).

The above-described regulatory sequences were used to create two types of RCR vectors. TATA fusion (TF) vectors were constructed by fusing the heterologous promoters lacking the TATA box precisely to the TATA box in the MLV U3 region, such that transcription initiation should occur at the 5′ end of the MLV R region, as is the case for wild-type MLV. TATA replacement (TR) vectors, on the other hand, were designed such that the entire heterologous promoter region, including the TATA box and transcriptional start site (TSS), was used to replace almost the entire MLV U3 region, including the TATA box.

Our results show that although both TF and TR vectors restricted transgene expression to permissive cell lines, only the TF design allowed efficient replication of targeted vectors within these cells. TF vectors replicate in a wide range of permissive cells, with kinetics similar to that of the parental nonspecific RCR vectors, while retaining their genomic stability and transcriptional specificity over multiple infection cycles.

MATERIALS AND METHODS

Construction of plasmids. To generate nonviral expression constructs harboring the heterologous regulatory sequences to be tested, the mCMV, EII-Pa1AT, and CTP4 promoters were cloned into the promoterless expression vector pEGFP1 (Clontech), upstream of the enhanced green fluorescent protein (eGFP) gene. To generate targeted RCR vectors in which the MLV TATA box was retained (TF), heterologous promoters were PCR amplified using forward primers binding to their 5′ ends and reverse primers binding to the 5′ borders of their TATA boxes. For the generation of vectors in which the MLV TATA box was replaced by the heterologous TATA box (TR), the sequence encompassing most of the 3′ long terminal repeat (LTR) and part of the downstream plasmid backbone was excised from pACE-GFP, using NheI and SspI, and replaced with the corresponding NheI-SspI fragment from vector pPCEWmCMVΔSNori (22), which contains a unique MluI site 21 bp downstream of the MLV TATA box in the 3′ LTR, generating plasmid pACE-GFP-ProCon, into which the heterologous promoters were cloned.

Cell lines, transfections, and virus production. 293 (ATCC CRL-1573), 293T (ATCC CRL-11268), NIH 3T3 (ATCC CRL-1658), HeLa (ATCC CCL-2), HepG2 (ATCC HB-8065), HuH-7 (ATCC CCL-185), and SW480 (ATCC CCL-228) cells, as well as cell lines AKH12 and AKH13 which were obtained from human primary liver tumors, were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). DLD-1 cells (ATCC CCL-221) were maintained in RPMI medium supplemented with 10% FCS.

A total of 8×10^5 293 or 293T cells, 2×10^5 HeLa cells, or 3×10^5 HepG2 cells were seeded per well of six-well plates and transfected 24 h later with 3 μg plasmid DNA per well, using the calcium phosphate coprecipitation technique (17). Stably transfected cell populations were generated by selection in DMEM with 10% FCS supplemented with 400 μg/ml G418 for a minimum of 3 weeks. Subsequently, fluorescence-activated cell sorting (FACS) analysis to measure eGFP expression was performed three times within an interval of 2 to 3 days. Viral stocks were generated by transient transfection of 293 or 293T cells, from which the supernatant was harvested at 48 h posttransfection, filtered through a 0.45-μm filter, and stored at −80°C.

β-Catenin/TCF activity assay for AKH12 and AKH13 cells. A total of 2×10^6 cells of cell lines AKH12 and AKH13 were transfected with 3 μg of plasmids pEGFP1, pEGFP1-CTP4, pEGFP1-E2, and pEGFP1-mCMV, followed by selection with 400 μg/ml G418 and expansion to T75 flasks. Stably transfected cells were analyzed by FACS at 3 weeks posttransfection.

Infections and determination of viral titers. Infections were carried out in a total volume of 1 ml medium per well in the presence of 8 μg/ml polybrene, followed by the addition of DMEM with 10% FCS or RPMI medium with 10% FCS after 6 h. The infected cells were passaged at regular intervals, and FACS analysis was performed at each passage. For titer determination, cells were infected with serially diluted virus stocks and 50 μM azidothymidine was added 24 h after infection to avoid secondary spread of the vector. Two days after infection, the number of infected cells was measured by FACS analysis.

Flow cytometry (FACS) analysis. Cells transfected with expression constructs were washed with phosphate-buffered saline (PBS) and resuspended in a solution of 10% FCS in PBS. Cells transfected or infected with RCR vectors were fixed by being incubated in a solution of 4% formaldehyde in PBS for 30 min, and then the cells were washed and resuspended in PBS. Resuspended cells were subjected to FACS analysis, which was carried out with a FACSCalibur apparatus (Becton Dickinson).

Quantification of viral RNA and proviral DNA. Viral RNA from the supernatant of infected cells and genomic DNA from infected cells were extracted using the QIAGEN viral RNA kit and the QIAGEN DNeasy kit (QIAGEN), respectively, according to the manufacturer's instructions. Real-time PCR and real-time reverse transcription (RT)-PCR were performed using primers 5′-GC AGTGCTTCAGCCGCTAC-3′ and 5′-AAGAAGATGGTGCGCTCCTG-3′ and probe 5′–6-carboxyfluorescein–ACCACATGAAGCAGCACGACTT–6-ca rboxytetramethylrhodamine–3′, all binding within the eGFP gene, or primers 5′-GTAGCGGTCGTGGGCACTTATA-3′ and 5′-CTTATGTTGGGAAGTG GCCGTA-3′ and probe 5′–6-carboxyfluorescein–CATTCCACCGCTCCGGCC AACT–6-carboxytetramethylrhodamine–3′, which bind specifically in *env* RNA.

PCR analysis and sequencing. For molecular analysis of the genomic stability of the vectors, DNA was extracted from infected cells of each infection cycle by using the QIAGEN DNeasy kit. A fragment encompassing the transgenic cassette of the integrated vector genome was PCR amplified using forward primer 5′-GGCCAAGGATGGTTCGAAGG-3′, binding to the MLV *env* gene, and reverse primer 5′-GCGAGACCACAAGTCGGATG-3′, binding to the MLV 3′ LTR. PCR was carried out using Advantage genomic PCR polymerase mix (BD Biosciences) according to the manufacturer's recommendations. The PCR products were subjected to agarose gel electrophoresis to determine length variations. To analyze the promoters of integrated proviruses, the promoter region in the 3′ LTR was PCR amplified using forward primer 5′-GGCCGCGCCATAGATAA AAT-3′, binding just downstream of the eGFP gene, and reverse primer 5′-CG GGTAGTCAATCACTCAGA-3′, binding in the 3′ LTR U5 region, or forward primer 5′-CTCGGCATGGACGAGCTGTA-3′, binding in the eGFP gene, and reverse primer 5′-AAGGAACAGCGAGACCACAA-3′, binding in the 3′ LTR U5 region. The PCR products were subjected to agarose gel electrophoresis and sequencing, using the same primers as those used for the PCR, to detect length variations and mutations, respectively.

RESULTS

Transcriptional activity of mCMV, EII-Pa1AT, and CTP4 promoters in human cell lines. Based on an extensive literature search for short, highly active, and preferably tissue- or tumor-specific promoters which would be promising candidates for the generation of transcriptionally targeted RCR vectors, the 380-bp CTP4 promoter fragment, the 460-bp HBV enhancer II/human α1-antitrypsin promoter (E2), and the 300-bp murine CMV immediate-early enhancer/promoter region (mCMV) were inserted into the promoterless eGFP expression vector pEGFP1 and the resulting vectors pEGFP1-

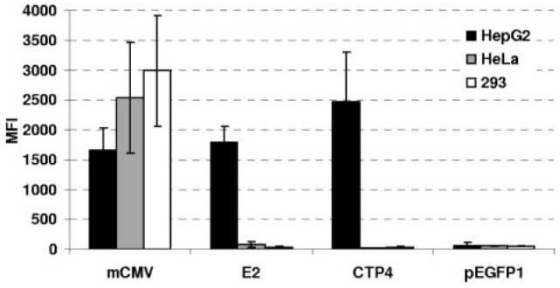

FIG. 1. Transcriptional activities of mCMV, EII-Pa1AT, and CTP4 promoters in human cell lines. HepG2, HeLa, and 293 cells were stably transfected with plasmids driving eGFP expression under the control of the mCMV, EII-Pa1AT (E2), and CTP4 promoters, and expression levels were quantified by FACS analysis. The MFI values depicted are the means from three independent FACS measurements. Plasmid pEGFP1 is the promoterless plasmid control.

CTP4, pEGFP1-E2, and pEGFP1-mCMV were used to generate populations of stably transfected HepG2, HeLa, and 293 cells. The hepatic carcinoma cell line HepG2 is highly deregulated for β-catenin (4, 10) and has been shown to facilitate high-level HBV enhancer II activity (2); hence, both CTP4 and EII-Pa1AT promoters, as well as the promiscuous mCMV promoter, were expected to be active in these cells. FACS analysis of the stably transfected cell populations revealed that while the mCMV promoter was highly active in all cell lines, EII-Pa1AT or CTP4 promoter activity was restricted to HepG2 cells and was similar to or even higher than that of the mCMV promoter, respectively (Fig. 1).

Replication kinetics of targeted retroviral vectors. The mCMV, EII-Pa1AT, and CTP4 promoters were then inserted into the 3′ LTR U3 region of the MLV-based replication-competent retroviral vector ACE-GFP (27). Upon infection and reverse transcription, these promoters are duplicated to the 5′ LTR U3 region, thereby driving expression of the viral genes as well as of the heterologous transgene inserted following a heterologous internal ribosome entry site element downstream of the *env* gene (Fig. 2). Two different designs of promoter insertions were applied. On the one hand, in so-called TF vectors, most of the 3′ LTR U3 region up to the 5′ border of the MLV TATA box is removed and replaced with the mCMV, EII-Pa1AT, and CTP4 promoter sequences from the 5′ end to the 5′ border of their respective TATA boxes, such that transcription initiation in the respective vectors mCMV-TF, E2-TF, and CTP4-TF should occur at the 5′ end of the MLV R region, as is the case in wild-type MLV (Fig. 2). On the other hand, in TR vectors, almost the entire MLV U3 region, including the TATA box, is deleted and replaced by the full-length heterologous promoter, including its TATA box and TSS, generating vectors mCMV-TR, E2-TR, and CTP4-TR (Fig. 2).

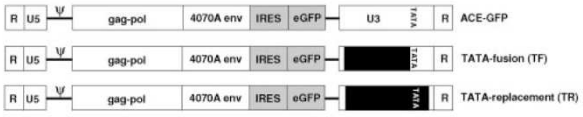

FIG. 2. Vector construction. All constructs are based on the parental vector ACE-GFP, which is comprised of Moloney MLV containing the amphotropic 4070A *env* gene and from which eGFP expression is mediated by the encephalomyocarditis virus internal ribosome entry site (IRES) fused to the 3′ end of the 4070A *env* gene. The position of the inserted heterologous promoters is depicted by a black box, and its insertion is designed such that either the heterologous promoter lacking its TATA box is fused to the MLV TATA box (TF) or almost the entire MLV U3 region is deleted and replaced by the heterologous promoter, including its TATA box and transcriptional start site (TR). As a result of these modifications, the original 448-bp MLV U3 region in the parental vector ACE-GFP is either shortened to 299 bp and 402 bp in the case of vectors CTP4-TF and CTP4-TR, respectively, or lengthened to 492 bp and 489 bp, in the case of vectors E2-TF and E2-TR, respectively, and to 538 bp and 615 bp, in the case of vectors mCMV-TF and mCMV-TR, respectively.

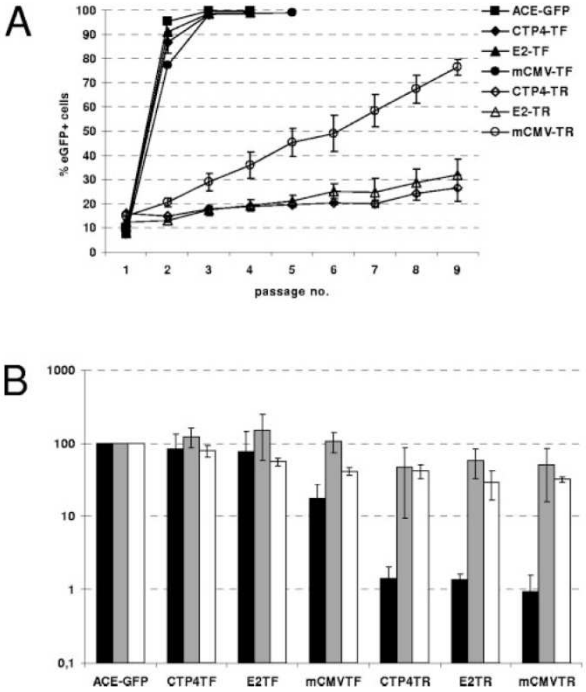

FIG. 3. Replication kinetics, virus production, infectivity, and transgene expression of targeted vectors in HepG2 cells. (A) HepG2 cells were infected with RCR vectors, and spread of virus was monitored by serial passaging and FACS analysis at each passage. The mean values from three independent experiments of the percentage of eGFP-positive cells at each passage are depicted. (B) Virus-containing supernatant was harvested from infected HepG2 cells and used to infect new HepG2 cells, and 50 μM azidothymidine was applied at 24 h postinfection to prevent secondary infection events. The quantity of integrated proviral copies per infected producer cell and the quantity of nascent virus released into the supernatant from each infected producer cell were determined by real-time PCR and real-time RT-PCR, respectively, using primers and probes binding in the rRNA genes and eGFP genes. Values shown indicate the number of nascent virions released per proviral copy in infected producer cells relative to the value obtained for ACE-GFP (black bars). The infectivity of nascent virus particles was quantified by FACS analysis at 2 days postinfection of HepG2 cells. The quantity of eGFP-positive cells generated per virus particle was calculated for each vector relative to the value obtained for ACE-GFP (gray bars). White bars represent the MFI of cells at 48 h postinfection relative to the values obtained for ACE-GFP.

HepG2 cells were infected with these vectors generated from transiently transfected 293 cells, and replication kinetics

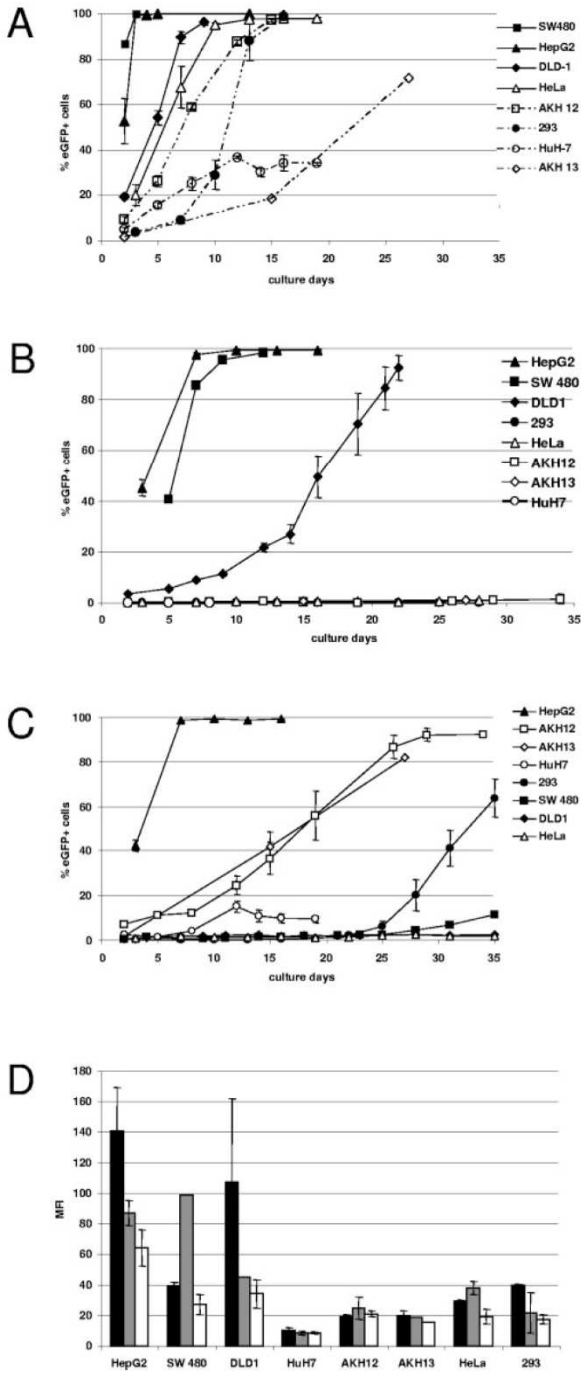

FIG. 4. Replication kinetics and transgene expression of targeted vectors. HepG2, SW480, DLD-1, AKH12, AKH13, HuH-7, HeLa, and 293 cells were infected with vectors (A) ACE-GFP, (B) CTP4-TF, and (C) E2-TF. Virus-containing supernatant (500 μl) was used to infect each cell line. Infected cells were passaged at regular intervals, and FACS analysis was performed at each passage. The values shown are the percentage of eGFP-positive cells at each passage. (D) The MFIs of eGFP-positive cells infected with ACE-GFP (black bars), CTP4-TF (gray bars), and E2-TF (white bars) are shown as the means from three independent experiments. Error bars represent the standard deviations between experiments.

were monitored by passaging the cells every 3 to 4 days and performing FACS analysis at each passage. The parental vector ACE-GFP as well as the TATA fusion vectors mCMV-TF, E2-TF, and CTP4-TF spread rapidly and infected 100% of the target cells after two to three passages (Fig. 3A). The respective TATA replacement vectors, mCMV-TR, E2-TR, and CTP4-TR, on the other hand, spread much more slowly and in a linear, rather than exponential, manner (Fig. 3A). These results cannot simply be explained by differences in promoter activity between TF and TR vectors, since the differences in replication kinetics did not correspond to the differences in intensity of eGFP expression of HepG2 cells infected with TF and TR vectors (Fig. 3B).

We thus compared the levels of virus production from infected cells and the infectivities of these viruses. To this end, infected HepG2 cell populations and the supernatants thereof were harvested, and the number of proviral copies in the infected cells and the number of virions released into the supernatant were quantified by real-time PCR and real-time RT-PCR, respectively. Compared to vector ACE-GFP, TF vectors produced between 2-fold and 10-fold fewer genome-containing virus particles per proviral copy in the producer cells (Fig. 3B). TR vectors, on the other hand, produced about 100-fold fewer genome-containing virus particles per provirus copy (Fig. 3B). Subsequent infection of HepG2 cells with the same amount of vector particles revealed roughly equal infectivities of vector ACE-GFP and of the TF and TR vectors. Thus, transcription of vector RNA from a heterologous promoter in the TR design does not lead to a decrease in the infectivity of such vectors per se. However, the amount of nascent virus particles produced from cells infected with TR vectors is severely retarded in comparison to the amount produced from ACE-GFP- or TF vector-infected cells, indicating a possible reason for the differences in replication kinetics between the TR and TF vectors.

Cell-specific replication of targeted TF vectors. TF vectors were subsequently used to infect a wider range of human cell lines. HeLa and 293 cells, the liver carcinoma-derived cell lines HepG2 and HuH-7, the primary liver tumor-derived cells AKH12 and AKH13, as well as the colon carcinoma cell lines SW480 and DLD-1, were infected with vectors CTP4-TF, E2-TF, and ACE-GFP, and vector spread was followed by serial passaging and FACS analysis of the infected cells every few days. As expected, vector ACE-GFP spread in all cells, although replication kinetics differed considerably among cell lines and apparent initial spread in HuH-7 cells over the first few passages did not continue (Fig. 4A).

Vector CTP4-TF replicated with kinetics similar to those of ACE-GFP in HepG2 and SW480 cells, which are known to be deregulated for β-catenin, infecting nearly all HepG2 cells within two passages and all SW40 cells within four passages. Considerable spread of CTP4-TF could also be observed in DLD-1 cells, also deregulated in the β-catenin pathway, although at a much lower rate than for ACE-GFP, taking eight

passages to reach over 80% of the cells. The CTP4-TF vector did not spread at all in cell lines which are not known to be deregulated for β-catenin, such as HuH-7, 293, and HeLa cells (Fig. 4B). The β-catenin status of the AKH12 and AKH13 cell lines, in which vector CTP4-TF also did not spread, was determined according to a commonly employed assay (6). To this end, cells were stably transfected with plasmids pEGFP1, pEGFP1-CTP4, pEGFP1-E2, and pEGFP1-mCMV, and FACS analysis of stably transfected cell populations at 3 weeks post-transfection revealed that they were not deregulated in the β-catenin/TCF pathway (data not shown). Vector E2-TF replicated very efficiently in HepG2 cells, exhibiting kinetics similar to that of ACE-GFP and rapidly infecting almost 100% of the cells within two passages (Fig. 4C). Considerable, albeit slower, spread was also observed in AKH12 and AKH13 cells (Fig. 4C) and was also comparable to the rate of spread of ACE-GFP in these cells (Fig. 4A). In the liver-derived HuH-7 cells, however, only very little spread could be observed and only for the first few passages. Little or no spread could be detected in the nonliver-derived cell lines SW480, DLD-1, 293, and HeLa, at least during the first 20 days of passaging (Fig. 4C). To a certain extent, there is a relationship between the mean fluorescence intensity (MFI) of the infected cells (Fig. 4D) and the ability of the respective vector to spread in these cells; in cell lines where very rapid spread is observed, the MFI of the infected cells is usually much higher than in cell lines where no or only slow spread is observed. In several cases, however, there is no discernible difference in MFI between cells infected with a vector which replicates moderately well and those infected with the same vector which does not spread at all. Moreover, vector CTP4-TF does not replicate at all in AKH12 or AKH13 cells, even though the MFI of the infected cells is the same as that when the cells are infected with vector ACE-GFP or E2-TF, which replicate in both cell lines.

In 293 and SW480 cells, spread of vector E2-TF could not be observed for the first 20 days of passaging but was thereafter quite robust in spite of the fact that these cell lines are not liver derived (Fig. 4B). Analysis of the 3′ LTR of integrated proviruses in genomic DNA isolated from a late passage of the 293 cells in which the E2-TF vector had begun to spread showed that mutant vectors had emerged which contained sequences derived from the human CMV promoter which was originally present in the 5′ LTR of the vector on the plasmid DNA, indicating that a rare recombination event had taken place at the plasmid DNA level during production of vector stocks by transient transfection (data not shown). The recombination junction occurred between sequences 85 bp downstream of the 5′ end of the human CMV promoter and 248 bp downstream of the 5′ end of the EII-Pa1AT promoter.

Genomic stability of targeted RCR vectors. The parental vector, ACE-GFP, has been shown to allow stable transgene propagation over multiple serial infection cycles in several different cell lines (28; M. Paar, personal communication). To investigate the potential effect on genomic stability of the presence of heterologous promoters in the transcriptionally targeted vectors, we followed the replication kinetics of the vectors E2-TF, CTP4-TF, and ACE-GFP through multiple serial-diluting infection cycles and analyzed vector stability at different time points.

HepG2 cells were initially infected at a multiplicity of infection of 0.01 and subsequently passaged and subjected to FACS analysis every 3 days, until a maximum percentage of cells in the population was expressing eGFP. For every second passage, the supernatant from infected cells was harvested, diluted 100-fold, and used to infect fresh HepG2 cells. This was repeated for 13 consecutive infection cycles (Fig. 5A).

Virions which have lost the transgene cassette do not express eGFP but can still infect cells and prevent superinfection of

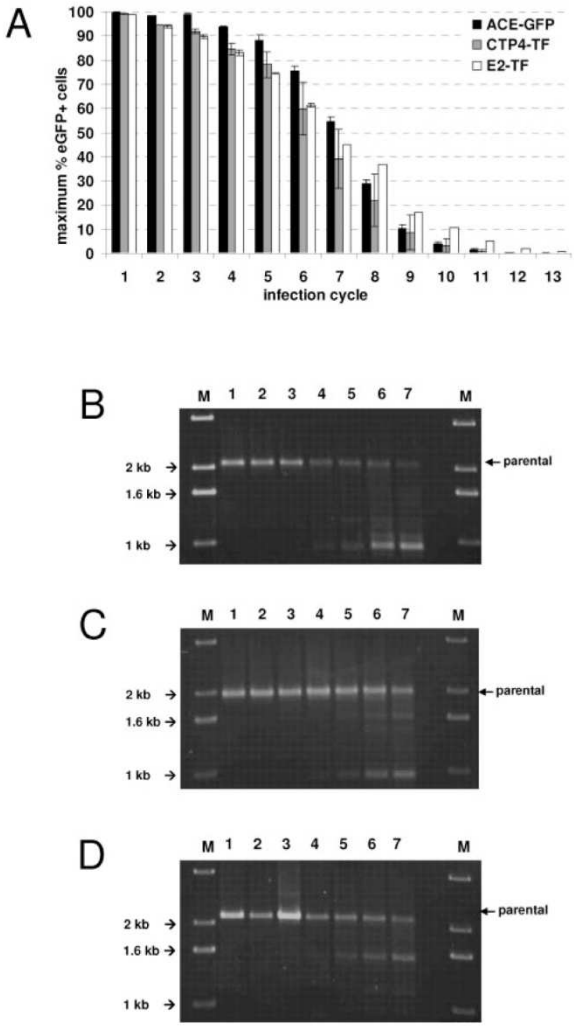

FIG. 5. Genomic stability of targeted vectors. HepG2 cells were infected with vectors ACE-GFP, CTP4-TF, and E2-TF at a multiplicity of infection of 0.01 and passaged until a maximum percentage of eGFP-positive cells was reached. The supernatant was harvested from infected cells at the second passage, diluted 100-fold, and used to initiate a new infection cycle. This was repeated for 13 serial infection cycles. (A) The values shown indicate the maximum percentage of eGFP-expressing cells reached in each infection cycle. (B to D) Genomic DNA was extracted from HepG2 cells infected with ACE-GFP (B), CTP4-TF (C), and E2-TF (D) at cycles 1 to 7, and PCR was performed using primers binding in the 3′ end of the *env* gene and 3′ U3 regions flanking the transgene cassette. Lane M, marker.

these cells by other virions via superinfection resistance (32). Therefore, when the vector becomes unstable, it is expected that the maximum number of eGFP-positive cells in the population should gradually decrease with each infection cycle. This process had already become evident by the second cycle, and by the 13th cycle, almost all virions had lost their insert. However, the stability of the vectors containing heterologous promoters was not less than that of the parental vector ACE-GFP.

In addition, the genomic stability of the vectors was monitored on a molecular level by performing PCR on genomic DNA extracted from infected HepG2 cells at cycles 1 to 7, using primers designed to bind to sequences flanking the transgene cassette in the integrated proviruses. Intact proviral genomes of ACE-GFP (Fig. 5B), CTP4-TF (Fig. 5C), or E2-TF (Fig. 5D) give rise to PCR products of 2,093, 1,944, or 2,137 bp in length, respectively. For the first three infection cycles, only PCR products corresponding to intact genomes were detectable, but from cycle 4 onward, several shorter products, representing deletion mutants, could be observed (Fig. 5B to D), which is in accordance with the FACS data showing that the maximum percentage of eGFP-positive cells began to decrease from the fourth cycle. The main mutant vector arising in cells infected with vector ACE-GFP carries a deletion of about 1,100 bp, while the major mutant vectors arising in CTP-TF- and E2-TF-infected cells carry deletions of about 900 bp and 600 bp, respectively (Fig. 5B to D). It is most likely that these deletions confine themselves to the transgene cassette and confer a replicative advantage on the vector mutants such that they become increasingly dominant in the viral population. Supporting this hypothesis, real-time RT-PCR performed on viral RNA extracted from filtered supernatants from infected cells from infection cycles 1, 5, 9, and 13, using primers and probes which bind in envelope-specific RNA, demonstrated that the production of infectious retrovirus particles did not decrease over successive infection cycles, even though they no longer led to eGFP expression in infected cells (data not shown).

Analysis of promoter sequence and specificity over multiple infection cycles. To monitor the genetic integrity of the promoter following several serial diluting infection cycles, the 3′ promoter regions of integrated proviruses from the first and seventh infection cycles were PCR amplified using primers binding to sequences flanking the U3 region. No change in length of promoters between the first and seventh cycles could be observed by gel electrophoretic analysis of the PCR products (Fig. 6A), and subsequent sequence analyses of the obtained PCR fragments revealed that no mutations occurred in the CTP4 promoter, while in a fraction of the virus populations, two point mutations occurred in the EII-Pa1AT promoter of vector E2-TF and the U3 region of vector ACE-GFP. Neither of the point mutations in the EII-Pa1AT promoter is located in any known transcription factor binding site.

To analyze whether the replicative ability of the vectors in different cell lines was maintained following several serial diluting infection cycles in HepG2 cells, vectors ACE-GFP, E2-TF, and CTP4-TF were harvested from cells of the seventh infection cycle, diluted 10-fold, and used to inoculate fresh HepG2, HeLa, and 293 cells. Newly infected cells were passaged every 3 days, and FACS analysis was performed at each passage (Fig. 6B). Vector ACE-GFP spread in all cell lines, whereas replication of vectors CTP4-TF and E2-TF remained restricted to the permissive HepG2 cells. Despite virus spread, the maximum percentage of eGFP-expressing cells did not reach more than 50% for any of the vectors, which correlates with data from the serial infection cycles (Fig. 5A) and is explained by the fact that by infection cycle 7, mutant vectors with deletions in the transgene cassette are becoming dominant in the virus population. Moreover, analysis of the MFI of infected cells over the course of the experiment demonstrated that transgene expression in nonpermissive cells remained low (data not shown).

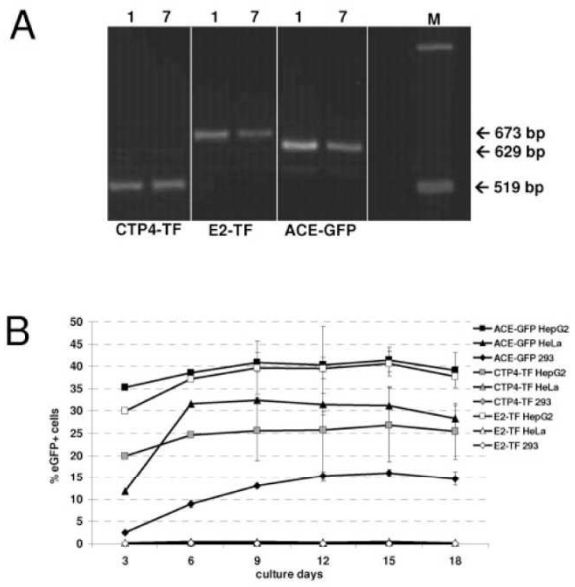

FIG. 6. Promoter sequence and specificity over multiple infection cycles. (A) PCR was performed on genomic DNA extracted from HepG2 cells infected with ACE-GFP, CTP4-TF, and E2-TF at cycles 1 and 7, using primers flanking the heterologous promoter in the MLV LTR. The expected fragment sizes are indicated. Lane M, marker. (B) Vector tropism remains unchanged following multiple serial infection cycles. HepG2, 293, and HeLa cells were infected with the supernatant from the seventh infection cycle of HepG2 cells infected with ACE-GFP, CTP4-TF, and E2-TF. The cells were passaged every 3 days, and spread of virus was monitored by FACS analysis. The percentage of eGFP-positive cells at each passage is shown.

Thus, in summary, we have generated replication-competent MLV-based vectors which can be used to specifically target either liver-derived cells or cells deregulated in the β-catenin pathway. The targeted vectors display wild-type replication kinetics in permissive cells, are genomically stable, and maintain their transcriptional targeting profile over serial infection cycles.

DISCUSSION

Through the generation of a series of MLV-based RCR vectors whose replication and transgene expression are driven by a heterologous promoter which replaces viral promoter/enhancer elements in the U3 region of the viral LTR, we

demonstrated that the expression of such vectors can be targeted in a liver-specific manner or to cells which are deregulated for β-catenin by using the chimeric EII-Pa1AT promoter or the synthetic CTP4 promoter, respectively. TF vectors, in which the EII-Pa1AT or CTP4 promoter is inserted into the U3 region such that transcription is controlled by the MLV TATA box and transcription initiation at the 5′ end of the R region is maintained, replicated with wild-type kinetics in a largely liver-specific (Fig. 3A and 4C) or strictly β-catenin deregulation-dependent (Fig. 3A and 4B) manner, respectively. Insertion of the mCMV promoter in this fashion gave rise to a vector which also demonstrated near-wild-type replication kinetics (Fig. 3A). TR vectors, on the other hand, in which promoters were inserted into the MLV U3 such that transcription was controlled by the heterologous TATA box and in which transcription should be initiated at the TSS of the heterologous promoter, demonstrated greatly attenuated replication kinetics in spite of having transgene expression levels similar to those of the TF vectors (Fig. 3A and B).

Detailed analyses revealed that particle production but not the infectivity of TR vectors was greatly reduced compared to that of ACE-GFP and TF vectors (Fig. 3B). The similar MFIs of cells infected with TR and TF vectors indicate that transcription levels are equal in the two vector designs (Fig. 3B). Since virus particles which are produced from cells infected with TR vectors are just as infectious as those produced from cells infected with ACE-GFP or TF vectors (Fig. 3B), the TR design does not seem to impinge on reverse transcription or integration, despite the fact that transcription initiation from the heterologous transcriptional start sites in TR vectors would lead to an elongation in the R region as the TSS defines the borders of the R region in retroviruses (9). Our data were not unanticipated, however, since there appears to be no particular upper size constraint on the retroviral R region, the length of which varies widely among different retroviruses, up to 247 nucleotides in the case of human T-cell leukemia virus type 2 (39). Moreover, previous work has shown that the precise sequence of the R region is of no importance during reverse transcription, provided that there are repeated sequences available for minus-strand DNA transfer (5). The MLV R region has also been proposed to act as a constitutive transport element (43). This function is based on the formation of a stem-loop structure, and disruption of this structure can greatly decrease viral gene expression (8). It is possible therefore that 5′ elongation of the R region could influence the structure of the natural R region. This would also account for the fact that only virus particle production but not eGFP expression is reduced, since eGFP expression is not dependent on full-length viral RNA as the transgene cassette is also present on the spliced *env* message which is exported efficiently from the nucleus in the absence of the proposed constitutive transport element.

Logg and colleagues (27) also made a series of transcriptionally targeted vectors in which the TATA box of the heterologous promoter is used but in which the TSS of the heterologous promoter is fused to the MLV TSS, such that the sequence between the heterologous TATA box and its TSS is maintained and transcription is initiated from the 5′ border of the MLV R region and, thus, the sequence and size of the R region do not change during virus replication (27). However, these vectors also replicate as poorly as our TR vectors.

Subsequent infection of a wider range of cells with the TF vectors revealed that vectors CTP4-TF and E2-TF are tightly restricted to β-catenin-deregulated cells and preferentially active in liver cells, respectively. Although there are some correlations between the MFI of cells infected with a particular vector and the ability of the vector to spread in these cells, in many instances, such a correlation does not exist. Clearly then, it is not always possible to make predictions about the ability of transcriptionally targeted retroviral vectors to replicate in a particular cell line based solely upon the expression of the transgene in these cells. One explanation could be that since eGFP expression can occur from both full-length and subgenomic viral RNAs in our vectors, the MFI of infected cells does not always mirror the expression level of Gag proteins, which could be a limiting factor for vector production.

In the case of HuH-7 cells, in which spread of both ACE-GFP and E2-TF was detectable for the first few passages but then ceased, it is possible that the vector becomes rapidly unstable in this cell line and loses the ability to express eGFP in infected cells. Alternatively, it is possible that due to the extremely low MFI of the infected cells, vectors did actually spread in these cells but could not be detected in all of the cells because the level of eGFP expression was below the FACS detection threshold.

In the case of the E2-TF vector, which showed spreading in 293 cells after about 25 days, it seems that a rare recombination event which took place at the plasmid DNA level during production of vector stocks by transient transfection created a vector whose transcription was driven by the human CMV promoter and that this vector emerged only following extensive passaging. Although we did not analyze the genomic DNA of SW480 cells infected with E2-TF which also showed spreading after about 25 days, it is possible that a similar event occurred here, too. This problem could be easily avoided by generating virus stocks from a characterized, stably transfected producer clone instead of by transient transfection.

The efficacies of transgene propagation of vectors E2-TF and CTP4-TF were not reduced compared to that of vector ACE-GFP (Fig. 5A), and molecular analysis by PCR amplification of the transgenic cassette revealed that deletions of the transgene region of vectors E2-TF and CTP4-TF are not detectable any earlier than in ACE-GFP (Fig. 5B to D), confirming that replacing the MLV promoter with these heterologous sequences was not detrimental to genomic stability. Real-time RT-PCR performed on viral RNA extracted from filtered supernatants from infected cells demonstrated that the production of infectious retrovirus particles did not decrease over successive infection cycles, formally ruling out the possibility that the decrease in eGFP-expressing cells was due to the vectors somehow becoming nonfunctional. Molecular analysis of the promoter regions of the targeted vectors detected mutations only in the EII-Pa1AT promoter of E2-TF, and these were not in any known transcription factor binding sites (data not shown). Moreover, the targeting specificities of vectors CTP4-TF and E2-TF were maintained over time (Fig. 6B). In accordance with FACS results from the stability analysis (Fig. 5A), no more than 50% of permissive cells infected with vectors from the seventh infection cycle expressed eGFP following

passaging. However, this is expected since by this stage, deletion mutants have already arisen (Fig. 5B to D). Interestingly, the stability of the vectors in HepG2 cells seems to be much reduced compared to that in NIH 3T3 and 293 cells (28; M. Paar, personal communication). One possible explanation is that the difference in genomic stability of the RCR vectors in different cell lines stems from rearrangements and recombination of integrated proviruses due to intrinsic differences in the genetic stability of the cell lines (12, 16, 20). The stability profiles of the targeted vectors imply, however, that even in HepG2 cells, they should be able to transduce upwards of 10^{11} cells following an initial transduction of only 5,000 cells, which should be sufficient to allow complete transduction of any solid tumor before becoming unstable.

These vectors therefore combine high replicative potential with the ability of targeted replication and expression of the inserted transgene, providing a valuable tool for specific, highly efficient cancer gene therapy.

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4 Discussion and further development

In this study, it was demonstrated that RCR vectors in which the wild-type promoter was replaced with the CTP4 or the EII-Pa1AT promoter exhibit robust transgene expression levels and rapid spread in cell culture, all in a tumour- or tissue-selective manner. But these promoters are only two of a larger set of candidate promoters, which also included the cancer-selective c-erbB-2, MK, and AFP promoters, as well as the promiscuous c-fos and mCMV promoters.

In order to assess their strength and cell selectivity, the promoters were incorporated into the promoterless expression vector pEGFP1 and the resulting constructs were used to transfect a range of cell lines both transiently and stably (Fig. 9).

Compared to the hCMV promoter, the mCMV promoter appears to be more evenly active in the cell lines used here, which may suggest that the mCMV promoter is better suited as a ubiquitously active promoter for comparative studies. However, such a conclusion probably cannot be drawn firmly from just this one experiment. In any case, the mCMV promoter was more active than the hCMV promoter in the murine cell line NIH, especially in stably transfected cells, which is in accordance with published data (Addison CL, 1997).

Both the c-erbB-2 and the MK promoter appear to be comparatively weak and, most notably, as active in 293 cells as in cancer cells. An explanation for the unexpected activity of the MK promoter in 293 cells could be that the 600 bp fragment of this promoter used in the transfected construct lacks distal regulatory sequences of the full-length promoter. These regulatory elements may normally suppress the activity of the MK promoter in non-permissive cells. On the other hand, although the MK promoter is generally described as only weakly active in adult normal tissues, considerable MK expression in kidney cells has been reported (Kubo S, 2010) (Muramatsu T., 1993).

The proximal AFP promoter has already been shown to be relatively weak (Ido A, 1995) and the results of the current study do not prove otherwise. Although the AFP promoter appears

to be liver-selective, the expression levels it mediates remain low. Therefore, it was planned to fuse the HBV enhancer II to the AFP promoter, an enhancer which - as a part of the EII-Pa1AT hybrid promoter - boosted the strength of the Pa1AT promoter considerably while retaining its liver selectivity (Kramer MG, 2003). Unfortunately, the cloning procedures were unsuccessful and this project was eventually abandoned due to time constraints.

The c-fos promoter conferred considerable levels of transgene expression to pEGFP1 in transfected cells, yet as the promoter of the RCR vector cfos-TF, the transgene expression levels and vector spread it mediated were negligible (Fig. 11). The c-fos promoter is known to be an inducible one, and has been shown to be fully induced by cell culture medium supplemented with 10% FCS (Johansen FE, 1994). The cells transfected or infected with pEGFP1-cfos or cfos-TF, respectively, were all maintained in such conditions.
By the time cfos-TF was tested, it was already apparent that TR vectors, as a rule, performed worse than TF vectors, hence no cfos-TR vector was ever created. It is not unthinkable, though, that in the case of the c-fos promoter the removal of its TATA box and the portion downstream of it deprived the promoter of a sequence vital for proper function.

The activity of the CTP4, EII-Pa1AT and MK promoters was later evaluated in a similar, but semi-replicative vector (sRCR) by Andrea Wolkerstorfer (Wolkerstorfer A, 2007). This vector was based on Moloney-MLV and lacked the *gag* and *pol* genes. A cassette consisting of an eGFP-neomycin gene preceded by the Food and mouth disease virus (FMDV) 2A protease sequence was fused to the end of the *env* gene. Utilisation of the 2A sequence led to an up to three-fold increase in infectivity compared to vectors using the (longer) EMCV IRES. Furthermore, insertion of the Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) downstream of the transgene cassette doubled the transgene expression levels in transfected cells.
In addition to creating TF and TR sRCR vectors with the heterologous promoters, Wolkerstorfer also developed an optimised TR (TRopt) design, whereby the TSS of the

heterologous promoter was placed in the same position as the TSS in wild-type MLV. This design is reminiscent of similar vectors created by Kasahara' s workgroup (Logg CR, 2002), which, unfortunately, offered no improvement compared to "traditional" TR vectors.

Promoter strength and selectivity was tested by infection of HepG2 and NIH cells with the sRCR vectors. In HepG2 cells, the MFI mediated by the vectors containing the CTP4 or the EII-Pa1AT promoter was about six to ten times higher than in NIH cells. The sRCR vectors containing the MK promoter showed no clear tissue selectivity, and generally low expression levels. They were therefore excluded from further investigation.

Replication kinetics of the sRCR vectors were evaluated by infection of Gag-Pol expressing 293 (293gp) and HepG2 (HepG2gp) cells. The vectors replicated markedly faster in HepG2gp than in 293gp cells, but there was no clear difference in performance between TF, TR and TRopt vectors: Amongst the vectors under the control of the CTP4 promoter, the TF vector spread the fastest, while amongst the vectors with the EII-Pa1AT promoter, the TRopt vector spread the fastest. Generally, these semi-replicative TR and TRopt vectors performed better than replication-competent TR vectors , and it was hypothesised that the excess quantities of Gag and Pol proteins provided in trans in the semi-packaging cells compensated for the weakness of the promoters. This would be in accordance with the results of the present study, which demonstrated that production of nascent virions, which can easily be affected by Gag and Pol levels, is reduced in TR as compared to TF vectors, while eGFP expression levels and infectivity are not. Wolkerstorfer showed that, in fact, the RCR vector E2-TR exhibited more robust spread in HepG2gp cells than in HepG2 cells, while the RCR vector CTP4-TR, on the other hand, spread as little in HepG2gp cells as in HepG2 cells.

Retroviral vectors were the first vectors to be used in gene therapy, but now their use is declining, not least due to safety concerns. Their most striking advantage, the ability to stably integrate into the host's chromosome, can lead to insertional mutagenesis (Edelstein ML, 2007). And yet the utility of retroviral vectors should not be dismissed. Further research on

the mechanisms of integration might give us clues as to how to improve their safety. Regarding replication-competent retroviral vectors, they could easily find their place in cancer gene therapy. Reliable targeting of the vector to cancer cells, which are subsequently destroyed, could make insertional mutagenesis less of an issue. The present study, as well as the works of Wolkerstorfer, Kasahara and many others, show that RCR vectors are still powerful tools for gene therapy.

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