

Reticuloendotheliosis virus-derived vectors for human gene therapy

Review Article

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Abbreviations: 3'-azido-3'-deoxythymidine, (AZT); American Type Culture Collection, (ATCC); avian leukemia virus, (ALV); colony units per ml, (cfu/ml); deoxyribonucleoside triphosphate, (dNTP); gibbon ape leukemia virus, (GaLV); internal ribosomal entry site, (IRES); long terminal repeats, (LTRs); murine leukemia virus, (MLV); nuclear localization sequence, (NLS); nucleocapsid, (NC); replication competent retroviruses, (RCR); reticuloendotheliosis virus strain-A, (REV-A); reticuloendotheliosis viruses, (REV); simian retroviruses, (SRVs); single chain antibody, (scA); Spleen necrosis virus, (SNV); vesicular stomatitis virus, (VSV); vesicular stomatitis virus, (VSV)

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Summary

Spleen necrosis virus (SNV) and reticuloendotheliosis virus strain-A (REV-A) belong to the family of avian reticuloendotheliosis viruses (REV). These amphotropic retroviruses infect a large variety of cells of avian and some mammalian species. SNV or REV-A with wild-type envelope does not infect human cells. However, they efficiently infect and integrate their genome into that of human cells when they are pseudotyped with the envelope protein of other mammalian retroviruses or the G protein of vesicular stomatitis virus (VSV). Moreover, SNV-derived retroviral vectors, which display single chain antibodies on the viral surface, enable cell-type-specific gene delivery into various human cells. In particular, the SNV cell-type-specific gene delivery vector system appears to be very well suited to transduce genes into cells of the human hematopoietic system. Moreover, my laboratory has developed genetically engineered SNV vectors, which are capable of infecting non-dividing cells such as quiescent human T-cells and primary monocyte-derived macrophages. Thus, REV-derived vectors appear to be very interesting candidates for the development of vectors for human gene therapy. The biology, genomic organization, and replication of these viruses have been reviewed in detail previously (Dornburg, 1995). Thus, this review focuses on the recent progress in the developments of REV-derived vectors for gene transfer into human cells.

I. REV morphology and host range

Reticuloendotheliosis viruses are avian C-type retroviruses which are more closely related to mammalian C-type retroviruses than to other avian retroviruses belonging to the avian leukemia / sarcoma virus group (Dornburg, 1995). E.g., the genomic organization of REV proviruses is simple and similar to that of murine leukemia virus (MLV). However, electron micrographs of SNV-derived vectors reveal that REVs contain a hexameric core different from that of mammalian C-type retroviruses (Figure 1).

REVs have been considered to be not infectious in

human cells for many years. In fact, the finding that REVs are unable to infect human cells led to the vigorous development of MLV-derived vectors for gene transfer into human cells. However, protein sequence comparisons revealed that the envelope proteins of REVs are more closely related to that of D-type retroviruses such as simian retroviruses (SRVs) than to that of MLVs. Further it has been suggested that SNV uses the same receptor for viral entry as SRVs (Kewalramani et al, 1992; Koo et al, 1992). Moreover, Koo et al. reported that vectors produced by an REV-A derived packaging cell line (termed D17.2G) are able to efficiently infect human cells

(Koo et al, 1991). In contrast, similar experiments performed with vectors produced from an SNV-derived packaging cell line (termed DSH134G, ref (Martinez and Dornburg, 1995)) led to opposite conclusions (Chu and Dornburg, 1995, 1997; Jiang et al, 1998; Engelstadter et al, 2000). These contradictory findings recently prompted the reevaluation of the tropism of REV_s. A systematic comparison of the tropism of REV vectors produced by two REV-A derived and two SNV derived packaging cell lines revealed that only vectors produced from the D17.2G packaging line displayed the capacity to infect 15 different human cell lines or primary cultures. However, none of these human cells could be infected by vector viruses harvested from the three other packaging lines. FACS analysis and immunocytochemical approaches revealed that D17.2G cells used in these studies express and produce an amphotropic MLV envelope. Moreover, fresh D17.2G helper cells obtained from the American Type Culture Collection (ATCC) and which had been deposited soon after D17.2G cells had been constructed did not produce vector virus capable of infecting human cells (Gautier et al, 2000).

These recent results clearly demonstrate that REV-A or SNV are not capable of infecting human cells and that D17.2G helper cells used in infectivity studies of human cells were contaminated with an amphi-MLV of unknown origin (Gautier et al, 2000). However, these data also show that REV_s can be pseudotyped with the envelope protein of other retroviruses such as MLV. My laboratory has found that SNV-derived vectors can not only be efficiently

pseudotyped with the envelope of MLV, but also that of gibbon ape leukemia virus (GaLV) or the G protein of vesicular stomatitis virus (VSV) (Parveen et al, manuscript in preparation).

Recently, a cDNA has been identified, which appears to code for a cell surface protein, which is used as a receptor for feline endogenous retrovirus RD114 and all strains of simian immunosuppressive type D retroviruses (Rasko et al, 1999). The cloned cDNA, which has been denoted RDR, is an allele of a previously cloned neutral amino acid transporter termed ATB0. Both RDR and ATB0 serve as retrovirus receptors and both act as transporters of neutral amino acids. However, in the light that REV_s are not infectious in human cells, it will be interesting to test whether either one of these receptors is also utilized by SNV or whether the failure of SNV to infect human cells is due to the lack of an additional co-receptor.

II. Genome and replication

The molecular biology and replication of REV_s has been reviewed in detail previously (Dornburg, 1995; Witter, 1997; Fadly, 1997). Briefly, the genomes of REV_s code only for structural proteins which are necessary for retroviral particle formation and replication and no genes coding for accessory or regulatory proteins have been described (**Figure 2**). However, recent investigations indicate that SNV contains several unique cis-acting

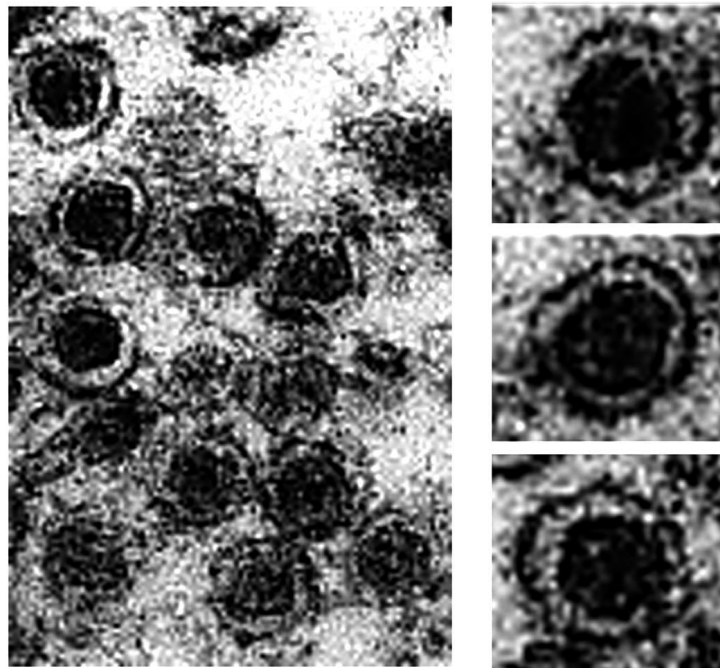


Figure 1. Electron micrograph of retroviral vector particles derived from spleen necrosis virus, SNV

elements, which compensate for the lack of accessory proteins involved in RNA nuclear export and/or translation. E.g., it has been reported that SNV long terminal repeats (LTRs) are associated with Rex/Rex-responsive element-independent expression of bovine leukemia virus RNA and it has been hypothesized that SNV RNA contains a cis-acting element that interacts with cellular Rex-like proteins (Boris-Lawrie and Temin, 1995). Recent data indicate that sequences located in the 5' RU3 region contain a cis-acting posttranscriptional control element that interacts with hypothetical REV-like proteins to facilitate RNA nuclear export and efficient translation (Butsch et al, 1999). It has also been reported that an internal ribosomal entry site (IRES) is present within the 5' leader of avian reticuloendotheliosis virus type A (REV-A) genomic RNA. This IRES element was located downstream of the packaging / dimerization (E/DLS) sequence and the minimal IRES sequence appears to be within a 129 nt fragment (nucleotides 452-580) immediately upstream of the gag AUG codon (Lopez-Lastra et al, 1997). The REV-A IRES has been successfully used in the construction of novel high titer MLV-based retroviral vectors, containing IRES elements. Sequences downstream of the envelope gene appear to be evolved in the regulation of Env translation (Yin and Hu, 1999). However, the SNV Env protein can be efficiently expressed without such downstream sequences in standard eucaryotic gene expression vectors (Martinez and Dornburg, 1995).

III. Retroviral packaging lines and vectors derived from REVs

REV-A and SNV were the first retroviruses from which a retroviral vector system has been derived (Watanabe and Temin, 1983). Due to the fact that REV-A and SNV are closely related (90% sequence homology) and that parts of their genome are interchangeable, the first vector systems consisted of parts derived from both viruses. [Earlier REV-derived vector systems are described in detail elsewhere (Dornburg, 1995)]. However, due to earlier findings that REVs do not infect human cells, REV-derived vectors have been used mainly to study various aspects of retroviral replication, such as retroviral recombination, mutation rates, transduction of cellular genes (pseudogene formation), the generation of transgenic chicken, and much more. As MLV-based vectors became the standard retroviral vectors for human gene therapy, no specific vectors for human gene therapy applications have been developed from REVs.

The first generation of REV-derived packaging cells spontaneously released replication competent retroviruses (RCR), which arose by recombination between the retroviral vector and DNA constructs expressing the retroviral structural protein (Hu et al, 1987). In such early packaging lines, there were considerable stretches of sequence homology among the different plasmid

constructs allowing homologous recombinations, which ultimately led to the generation of RCR. However, this problem has been addressed by the construction of gag-pol and env gene expression vectors which have no homology to the retroviral gene transduction vector (Martinez and Dornburg, 1995, 1996). In fact, the retroviral packaging line DSH134G (Martinez and Dornburg, 1995) has now been kept in tissue culture since 1994 and remained free of RCR over the past six years (Figure 2, Martinez and Dornburg, 1996, and unpublished data).

Retroviral vector titers obtained from the DSH134G helper cells, which produced a vector transducing the bacterial beta-galactosidase gene were about 10⁶ to 5 x 10⁶ colony units per ml supernatant medium (cfu/ml) and could be increased by concentration through ultrafiltration to up to 10⁸ cfu/ml (Chu and Dornburg, 1997). My laboratory found that the level of gag-pol expression in the packaging cell was the major limiting step in reaching high vector virus titers (Martinez and Dornburg, 1995). Further, it has been shown that chimeric SNV viruses which contained the gag region of REV-A infected mammalian cells at least ten times more efficiently than wild-type SNV (Casella and Panganiban, 1993). We recently constructed novel gag-pol gene expression vectors, which contain gag of REV-A and pol of SNV. Preliminary data indicate that vector virus titers can be increased up to 100-fold using such constructs (Krupetski et al, unpublished observation).

It has been reported earlier that SNV can efficiently encapsidate MLV-based vectors, but not vice versa (Embretson and Temin, 1987). Using chimeric gag-pol expression constructs and a competitive packaging system, it has been shown recently that Gag is solely responsible for the selection of viral RNAs. Furthermore, the nucleocapsid (NC) domain in the SNV Gag is responsible for its ability to interact with both the SNV encapsidation sequence (E) and that of MLV (termed Psi). However, MLV proteins cannot efficiently package SNV-based vector RNA. Replacement of the SNV NC with the MLV NC generated a chimeric Gag that could not package SNV RNA but retained its ability to package MLV RNA. Moreover, a construct combining the SNV gag and the MLV pol gene supported the replication of SNV gag and the MLV pol gene supported the replication of both MLV and SNV vectors, indicating that the gag and pol gene products from these two different retroviruses can functionally cooperate. However, viral titer data suggest that SNV cis-acting elements are not ideal substrates for MLV pol gene products since infectious viruses were generated at a lower efficiency (Yin and Hu, 1997; Certo et al, 1998, 1999).

IV. Stability and recombination of REV-derived vectors

Retroviral particles contain two identical RNA genomes, which recombine with rather high frequencies. Moreover, the retroviral enzyme reverse transcriptase

appears to lack faithful proof-reading functions and mutations are introduced into the retroviral genome at each cycle of replication with a rather high frequency. SNV-based vectors served as the first system to study retroviral mutation rates and recombination (Hu and Temin, 1990a, 1990b; Pathak and Temin, 1990; Dougherty and Temin, 1991). In the past years, SNV vector systems have been used extensively to further quantitatively determine these processes in vitro and in vivo. Using SNV-based vector systems, the mutation rate of SNV has been investigated in great detail. It has been found that the SNV reverse transcriptase incorporates approximately one wrong nucleotide per 10,000 bases (Dougherty and Temin, 1986, 1988; Pathak and Temin, 1990). Similar mutation rate studies have been expanded recently and various factors influencing the mutation rate, e.g., deoxyribonucleoside triphosphate (dNTP) pool imbalances, and the presence of nucleotide analogs, have been studied in detail. It has been found that deoxyribonucleoside triphosphate (dNTP) pool imbalances are associated with an increase in the rate of misincorporation and hypermutation during in vitro reverse transcription reactions approximately 4-fold. In addition, 3'-azido-3'-deoxythymidine (AZT) also increases the retroviral mutation rates by a mechanism not involving

alterations in dNTP pools (Pathak and Temin, 1992; Kim et al, 1996; Julias et al, 1997; Julias and Pathak, 1998).

Reverse transcription involves at least two cDNA transfer reactions to produce a full-length DNA copy of the retroviral RNA genome. Since one retrovirus particle contains two identical RNA genomes, the transfers can occur in an intramolecular or intermolecular manner. The mechanism of the first transfer step (minus-strand strong-stop cDNA transfer) has been studied previously in detail with spleen necrosis virus vectors containing genetic markers. Different results have been reported with respect to the type of strand transfer mechanism (Hu and Temin, 1990b). Overall the homologous recombination rate in one retroviral replication cycle has been determined to be 4% for markers 1.0 kb apart. These results led to the calculation that approximately 30 to 40% of the replication-competent viruses with 7- to 10-kb genomes undergo one recombination event. However, these estimates were based on the assumption that recombination occurs randomly in a linear manner. Recent similar studies indicate that the recombination rate increases when the marker distance increases from 1.0 to 1.9 kb. However, the recombination rates with marker distances of 1.9 and 7.1 kb appear not to be significantly different. Thus, retroviral recombination appears not to be

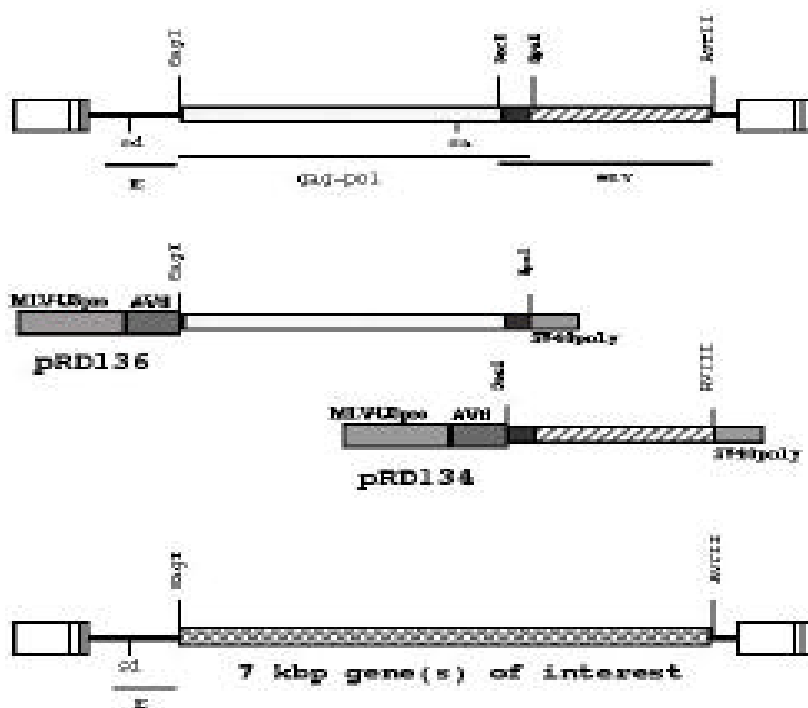


Figure 2. Constructs to build a SNV-derived retroviral packaging cell line. A SNV provirus is shown at the top. SNV (and REV-A) express gag-pol proteins from genomic RNA and Env from a spliced mRNA (sd: splice donor site; sa: splice acceptor site). The reading frames of gag-pol and env overlap by about 160 bases. The encapsidation sequence (E) does not extend into gag and the env gene does not overlap with 3' regulatory sequences. Thus, packaging cells can be constructed which express retroviral gag-pol and env proteins without sequence homology to retroviral vectors transducing non-retroviral genes (bottom) (Martinez and Dornburg, 1995). pRD136 and pRD134 are plasmid constructs to express gag-pol or env proteins, respectively, and have been used to make the retroviral packaging line DSH134G (Martinez and Dornburg, 1995). In both constructs, the retroviral proteins are expressed from the murine leukemia virus promoter (MLV-U3pro) followed by the adenovirus tripartite leader sequence (AVT1) for enhanced translation. SV40poly(A): polyadenylation signal sequence of simian virus 40.

proportional to marker distance. Additional studies revealed that the recombination rate of SNV is very similar to that of MLV. In another recent study, an SNV vector-based recombination system was used to investigate whether a known hot spot for mutation was also a hot spot for retroviral recombination. PCR and restriction enzyme analysis of 228 proviral sequences revealed a higher frequency of recombination in the regions immediately following the hot spot of mutation. Moreover, the overall pattern of recombination appears to be nonrandom and one region was recombination-prone. More recent studies suggest that retroviral recombination *in vivo* is similar to that determined in *in vitro* experiments (Bowman et al, 1996, 1998; Hu et al, 1997; Anderson et al, 1998; Wooley et al, 1998).

V. Cell-type-specific retroviral vectors

The host range of retroviruses is determined by the nature of the retroviral envelope protein (Hunter and Swanstrom, 1990). The envelope protein of all retroviruses studied until today consists of two peptides, which are derived from a single precursor protein by proteolytic cleavage (for a detailed review, see (Hunter and Swanstrom, 1990)). The larger peptide, termed SU (surface unit) binds to a specific cellular receptor, and, therefore, determines the host range of the virus. Like in mammalian retroviruses, SU of REVs are non-covalently bound to the second peptide, termed TM. TM is a transmembrane peptide, which anchors the envelope protein in the viral lipid membrane. The amino terminus of TM is involved in the membrane fusion of the viral and cellular membranes. The carboxy terminal and cytoplasmic part of TM appears to be involved in interactions with the retroviral core at various stages of the retroviral life cycle. Although the protein sequences of envelopes of different retrovirus species differ markedly, the functional organization of the envelope protein into a SU and TM unit with these defined functions has been conserved among all retroviruses investigated. The SU domains of most retroviruses utilize house-keeping cell surface proteins as receptors. Thus, the host-range and of most retroviruses is very broad and many different cell-types of one or many species can be infected by a particular retrovirus. However, for most, if not all future *in vivo* gene therapy applications, it will be necessary to have vectors available which infect only one particular cell type (or a very few selected cell-types).

To make vectors specific for one particular human cell type, several groups have modified the SU domain of the envelope protein of ecotropic Moloney MLV, which is infectious only on mouse cells, or the envelope of avian leukemia virus (ALV) (Roux et al, 1989; Young et al, 1990; Etienne-Julan et al, 1992; Russell et al, 1993; Kasahara et al, 1994; Cosset et al, 1995a, 1995b; Han et al, 1995; Somia et al, 1995; Cosset and Russell, 1996; Marin et al, 1996; Nilson et al, 1996; Valsesia-Wittmann

et al, 1996). My group has performed extensive studies using the envelope of SNV (Chu et al, 1994; Chu and Dornburg, 1995, 1997; Jiang et al, 1998, 1999). Stephen Russell in Greg Winter's laboratory and my laboratory were the first groups which developed retroviral vector particles that display the antigen binding site of an antibody on the viral surface (Russell et al, 1993; Chu et al, 1994). This has been achieved using single chain antibody (scA) technology (**Figure 3**). First, using hapten model systems, Dr. Russell and our group were able to show that such particles are competent for infection (Russell et al, 1993; Chu et al, 1994). Using SNV retroviral vectors and a scA directed against a human CEA-related cell surface protein (B6.2), we were able to show that such scA-displaying particles are infectious as well (Chu and Dornburg, 1995, 1997; Chu et al, 1995). We found that the presence of wild-type Env was necessary to confer infection of such targeting vectors in human cells (Chu and Dornburg, 1995; Chu and Dornburg, 1997) (**Figure 4**). However, many studies with single chain antibodies directed against various other human cell surface proteins indicate that most scA-displaying vectors derived from eco-MLV are not or only minimally infectious (Marin et al, 1996; Nilson et al, 1996; Schnierle et al, 1996; Valsesia-Wittmann et al, 1996).

To further test, whether other scAs displayed on SNV-derived retroviral vector particles are competent for infection, we developed vector particles that displayed three other scAs derived from monoclonal antibodies. These were: an scA directed against the Her2neu antigen, an scA against the stem-cell antigen CD34, and an scA against the transferrin receptor. The results with vectors displaying the anti-Her2neu scA can be summarized as follows (**Table 1**): Stable packaging lines produced more than 10⁵ infectious particles per ml supernatant medium titered on human cells expressing Her2neu (e.g., COLO320DM cells, BRK-SK cells, HeLa, etc.). Her2neu-negative cells (HT1080 or A431 cells) could not be infected. The level of Her2neu expression on the target cells did not play a role in the level of infectivity. Particles displaying both, the chimeric and the wild-type Env, were more infectious in human cells than particles displaying the chimeric Env alone. Furthermore, they were more stable than vector particles containing wild-type Env alone. The infectivity on human cells could be inhibited by pre-incubating the target cells with the original monoclonal antibodies or by saturating the vector particle with soluble antigen recognized by the scA (e.g., soluble Her2neu) (Jiang et al, 1998).

Retroviral particles that displayed a scA against the human stem cell marker CD34 or the transferrin receptor were competent for infection as well (**Table 1**). The efficiency of infection of particles displaying anti-CD34-scAs (harvested from stable packaging lines) was determined in various human cell lines. The virus titer in human KG-1a cells (a CD34-positive hematopoietic cell

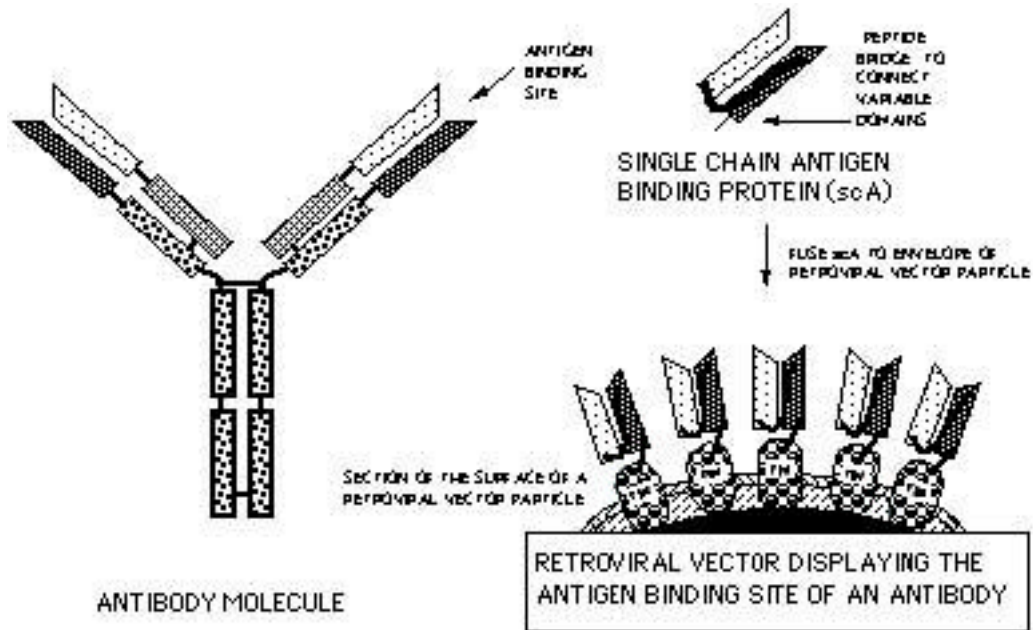


Figure 3. Retroviral vector particles displaying a single chain antibody.

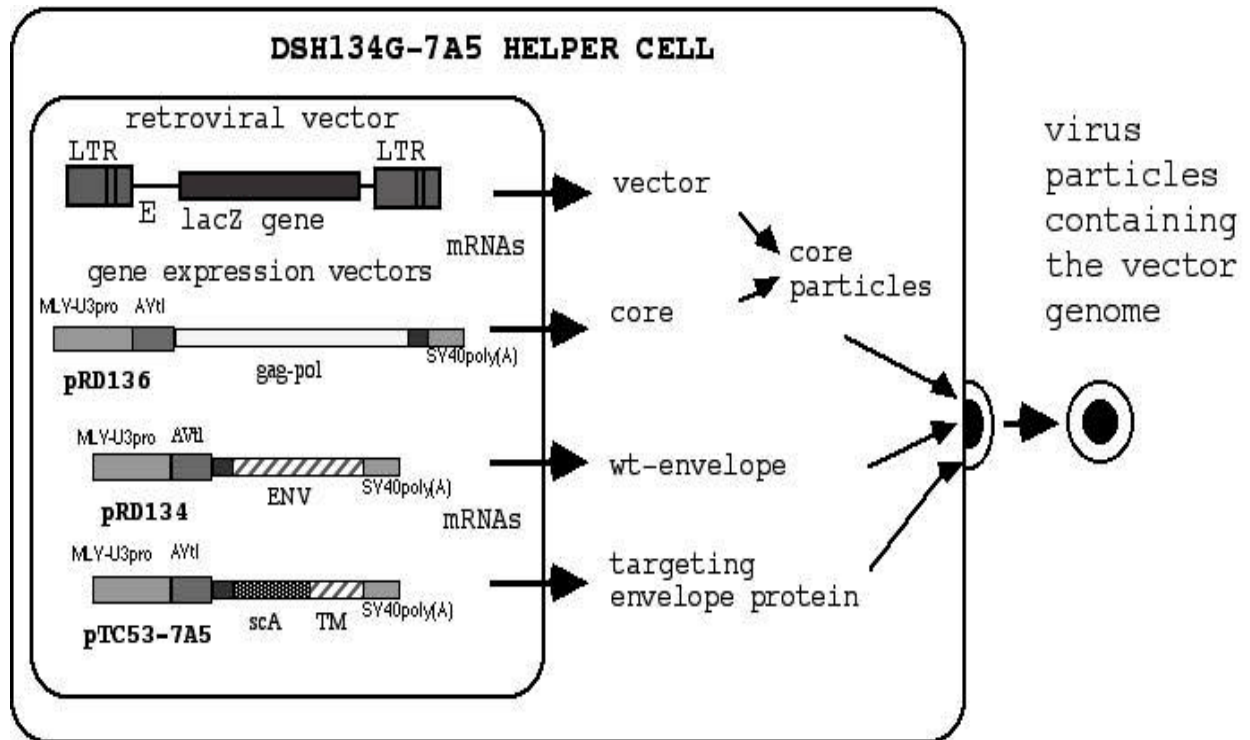


Figure 4. Example of a retroviral packaging cell to target specific human cells. Such packaging cells contain a plasmid expressing the chimeric (targeting) envelope in addition to the plasmids for the expression of retroviral core and envelope proteins (see Figure 2). pTC53-7A5 is a gene expression vector, which contains a scA directed against a T-cell surface antigen fused to TM of SNV.

line) was above 10⁵ cfu/ml. This titer was about 1,000 fold higher than that obtained with SNV vector particles pseudotyped with the envelope of amphi-MLV. However, SNV vector particles pseudotyped with amphi-MLV

envelopes infected HeLa cells with titers up to 10⁶ cfu/ml. This data show that SNV targeting vectors are excellent candidates for gene delivery into human hematopoietic cells. Surprisingly several other tissue culture cell lines

(e.g., Daudi-, HeLa-, and COLO320DM cells), which we expected to be negative for CD34 could also be infected efficiently. FACS analysis did not indicate the presence of CD34 on the cell surface of such cells. However, RT-PCR revealed that such cells do express CD34, although at extremely low levels (Jiang et al, 1998). At this point, it is unclear, whether low level antigen expression would be sufficient to obtain infection in vivo. Thus, thorough in vivo studies will be necessary to address such issues.

Recently, the group of Dr.Cichutek at the Paul Ehrlich Institute has made the Pharmacia scA phage-display library system compatible with the SNV targeting system (Engelstadter et al, 2000). Now, a large variety of scAs created with this phage display system can be easily transferred from the phage genome for SNV vector display. Using this scA phage display system, a scA library directed against human T-cell surface antigens has been generated. In the first step, mice were immunized with a human T-cell line. Next, mRNAs were isolated from immune cells and a library of scA display phages was prepared. Only those phages binding to the cell surface of the human T-cells used for immunization were selected by panning. This first screening led to the identification of about 150 scAs capable of binding to membrane proteins of human T-cells. Next, all such scAs were displayed on SNV retroviral vector particles. Transient transfection and infection protocols as described (Chu and Dornburg, 1995) led to the identification of six scAs capable of transducing a marker gene into human T-cells. Using such six scA-SNV-Env expression vectors, stable packaging lines were made and high-producer clones were selected. Virus particles produced from one packaging line were not only able to infect human tissue culture T-cells, but also primary cells with titers up to 2×10^6 cfu/ml.

These data show that SNV-derived retroviral vector particles which display a scA on the viral surface are a valuable tool to deliver genes into specific target cells. In all experiments, the co-presence a fully functional envelope was necessary to act as an efficient helper for targeting vector virus entry. We hypothesize, that the targeting envelope binds the virus to the cell surface receptor. Human cells may still contain a receptor for the wild-type SNV envelope, to which, however, the wild-type envelope does not have sufficient affinity to trigger all events required for virus entry. High affinity to the cell surface is restored by the targeting envelope. Now the wild-type envelope can interact with its natural receptor and trigger membrane fusion (Dornburg, 1997).

To further test this hypothesis, my laboratory constructed retroviral vector particles which display chimeric HIV-1-SU-SNV-TM proteins plus wild-type SNV envelope on the viral surface. Such particles allowed efficient infection of CD4-positive human T-lymphocytes, and, at a lower efficiency, also cells expressing CXCR4 without CD4 (Jiang et al, 1999). These data coincide with the hypothesis that the chimeric envelope is only required

to bind the vector particle to a cell surface receptor of the target cell, while membrane fusion is mediated by wild-type Env, which alone is not sufficient to enable infection of human cells.

VI. Cell-type-specific gene delivery in vivo

To test cell-type-specific gene delivery in vivo, my laboratory recently developed a SCID mouse model system (Jiang and Dornburg, 1999). Antibiotic resistant human target and non-target cells were injected into the peritoneum of SCID mice. Subsequently, a vector solution containing 106 infectious particles, which display scAs against the human her2neu cell surface protein, was injected. Cells were recovered from the peritoneum, subjected to antibiotic selection, and tested for the expression of a lacZ gene transduced by the retroviral vector. We found that human target cells, which express her2neu, were infected in vivo. However, neither human cells that do not express her2neu nor normal mouse cells were infected by such viral particles. These data give proof of principle that retroviral vector-mediated, cell-type-specific gene delivery can be obtained in vivo.

VII. Vectors for non-dividing cells

The application of retroviral vectors derived from C-type retroviruses for human gene therapy has been limited to introducing genes into dividing target cells. Recently, we developed genetically engineered C-type retroviral vectors, derived from spleen necrosis virus, SNV, which are capable of infecting non-dividing cells. This has been achieved by introducing a nuclear localization sequence (NLS) into the matrix protein (MA) of SNV by site-directed mutagenesis. The introduction of the NLS increased the efficiency of infection of non-dividing cells and was sufficient to endow the virus with the capability to efficiently infect growth arrested human T-lymphocytes and quiescent primary monocyte-derived macrophages. This is the first report that a genetically engineered C-type retroviral vector can actively penetrate the nucleus of a target cell and can be used as a gene therapeutic vector to transduce genes into non-dividing cells (Parveen et al, 2000).

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