RETICULOENDOTHELIOSIS VIRUSES AND DERIVED VECTORS FOR HUMAN GENE THERAPY

Ralph Dornburg

Thomas Jefferson University, Division of Infectious Diseases, 1020 Locust Street, Suite 329, Philadelphia, PA 19107

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1. ABSTRACT

The reticuloendotheliosis viruses (REV) spleen necrosis virus (SNV) and reticuloendotheliosis virus strain-A (REV-A) are amphotropic retroviruses which infect a large variety of cells of avian and some mammalian species. They normally do not infect primate or rodent 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) or rabies viruses (RV). Moreover, SNV-derived retroviral vectors, which display single chain antibodies or other targeting ligands on the viral surface enable cell-type-specific gene delivery into various human cells. My laboratory has developed genetically engineered REV vectors, which are capable of infecting non-dividing cells such as quiescent human T-cells, primary monocyte-derived macrophages, and mature neurons. Thus, REV-derived vectors appear to be very interesting candidates for the further development of vectors for human gene therapy. This article reviews the replication of REVs and vectors derived from REV-A and SNV for gene transfer into human cells.

2. INTRODUCTION

The reticuloendotheliosis viruses (REVs) are C-type retroviruses, which form a group of closely related viruses. All known members of this virus group were isolated from avian species (1-4) and include chicken syncytial virus, duck infectious anemia virus, spleen necrosis virus (SNV), reticuloendotheliosis virus strain A (REV-A), and its acutely transforming variant REV-T.

REVs are more closely related to mammalian C-type retroviruses than to other avian retroviruses belonging to the avian leukemia / sarcoma virus group (7). The genomic organization of REV proviruses is typical for that of a C-type retrovirus and similar to that of murine leukemia virus (MLV). The close relationship to mammalian retroviruses was confirmed by sequence comparisons of fragments of the REV-A and SNV genomes with those of other avian and mammalian C- and D-type retroviruses (8-10). However, the envelope proteins of REV-A and SNV are more related to mammalian D-type retroviruses than to other C-type retroviruses and REV-A and SNV appear to bind to the same receptor as some simian retroviruses (SRVs) (11,12).

REVs infect some mammalian cells (e.g., dog D17 cells) in vitro, but they do not infect or replicate in
REV-derived retroviral vectors

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

primate or rodent cells (7,13). They are considered non-pathogenic for humans. The block of infection appears to result from a low affinity of the viral envelope to the primate receptor. This conclusion is based on the observation that REVs pseudotyped with the envelope protein of other retroviruses or nonretroviruses efficiently infect and integrate their genome into mouse, primate, or human cells. However, a second post-integration block further prevents replication of REVs in human cells. This post-integration block is not mediated by the viral promoter and enhancer, which are very strong in all human cells investigated (7,14-16).

REV viruses are wide-spread in nature. Up to 25% of chicken used commercially in the United States and in Japan test positive for anti-REV-A antibodies (4,17-20). Even more, adult poultry may be viremic without visible disease symptoms. Thus, chicken and / or turkey meat contaminated with replication competent virus may occasionally make its way into the shelves of supermarkets. However, no human disease has been associated with REVs and humans do not have natural antibodies against such viruses. Thus, besides having a high potential as gene delivery agents for the generation of transgenic poultry, they also appear to be good candidates for the development of safe gene delivery tools for human gene therapy (7,21-26).

SNV and REV-A are the best characterized representatives of the REV group of retroviruses. They share a 90% sequence homology and their cis- as well as trans-genes appear to be interchangeable without impairing retrovirus replication. However, it was shown that chimeric virus particles of REV-A and SNV that contain the matrix protein of REV-A infect mammalian cells more efficiently than those containing the matrix protein of SNV (27). All REV-derived retroviral vectors and packaging lines have been derived from REV-A or SNV. Thus, this review focusses on REV-A and SNV. Helper cells and vectors derived from these viruses are described.

3. REV MORPHOLOGY AND HOST RANGE

Like all retroviruses, REVs are enveloped and contain a lipid bilayer surrounding the viral core structure. Electron micrographs of SNV-derived vectors revealed that these viruses contain a heptagonal core slightly different from that of mammalian C-type retroviruses (Figure 1).

REVs have been considered not to be 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, as briefly described above, 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 (11,12). This conclusion was based on superinfection interference assays, which revealed that the receptor for REVs is also used by simian retroviruses SRV-1 and SRV-2, Mason-Pfizer monkey virus (MPMV), Baboon endogenous virus (BaEV), and squirrel monkey retrovirus (SMRV). Thus, REV-A and SNV are members of the simian retrovirus receptor interference group (11,12).

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 (28). In contrast, similar experiments performed with vectors produced from an SNV-derived packaging cell line (termed DSH134G, ref. 29) led to opposite conclusions (16,30-32). These contradictory findings recently prompted the reevaluation of the tropism of REVs.

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 murine leukemia virus (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 (13).

These recent results clearly demonstrate that REV-A or SNV are not capable of infecting human cells and that some D17.2G helper cells stocks were contaminated with an amphoto-MLV of unknown origin (13). However, these data also show that REVs can be pseudotyped with the envelope protein of other retroviruses such as MLV. We recently showed that SNV-derived vectors can also be efficiently pseudotyped with envelopes
REV-derived retroviral vectors

Figure 2. SNV-derived retroviral packaging lines. A SNV provirus is shown at the top. The black box indicates the overlapping reading frame (160 bp) of the gag-pol and env genes. Restriction enzyme sites used for cloning are indicated. The EagI site is located 5 bp upstream of the Gag-ATG codon. The HpaI site downstream of the pol-gene was introduced by site directed mutagenesis. The Avr2 site is located at the end of the Env gene. pRD136 and pRD134 express the SNV protein coding sequences. They contain the U3 promoter of MLV (MLV-U3pro) followed by the adenovirus tripartite leader sequence for enhanced gene expression. Polyadenylation is mediated by the SV40 poly(A) site. A retroviral vector, in which all SNV protein coding sequences have been deleted is shown below. Please note that SNV-derived gene transfer vectors do not contain gag-sequences and do not contain sequences upstream of the Avr2 site. Thus, there is no overlap between vector and helper cell sequences to allow homologous recombination resulting in replication-competent retroviruses.

of gibbon ape leukemia virus (GaLV) or the G protein of vesicular stomatitis virus (VSV), or the envelope protein of rabies viruses (unpublished observation, for more details see below).

The finding that REVs do not infect human cells still seems to contradict the observation that these viruses are members of the simian retrovirus superinfection interference group. These simian viruses efficiently infect human cells. However, it is possible that REVs do use the same cell surface protein as receptor for virus entry, but bind to a different peptide domain. The human receptor may contain a distinct amino acid sequence which does not allow high affinity binding of REVs.

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 (33,34). 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 REVs 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.

4. RETROVIRAL GENOME

The genomic organization of REVs is typical for that of mammalian C-type retroviruses. The protein coding regions are flanked by cis-acting sequences involved in the encapsidation and replication of the viral genome (see also below and Figure 2). REV viruses encode two polyproteins, gag-pol and env, which are expressed from genomic and a spliced viral RNA, respectively (7). Northern blot analysis of RNAs isolated from REV-A or SNV infected D17 cells revealed that about 50% of the
REV-derived retroviral vectors

genomic RNA is spliced (29). In addition, my laboratory found that such cells also contain at least two more spliced viral RNA species (about 7 and 4.5 kb respectively) which account for less than 5% of the total viral RNA. However, it is unknown whether such RNAs code for other proteins or whether they are just cryptic splicing products. Of note, the SNV genome does not reveal open reading frames of significant length frame-shifted to those of gag-pol and env. Since expression of gag-pol and env is sufficient to generate high levels of infectious vector virus (see below), such putative proteins, if they exist, are most probably not essential for virus replication (29).

The encapsidation sequences of REV-A and SNV are well characterized. They are located between map units 268 to 452 (KpnI and SalI sites, ref. (35-38)), located about 100 base pairs upstream of the ATG codon of gag (see also below and Figure 2). Unlike in the case of MLV, in which sequences in the gag region increase the efficiency of encapsidation up to two orders of magnitude, the addition of more sequences downstream of this region had only a moderate effect on encapsidation and increased the efficiency of encapsidation only up to fivefold (35). The maximum length of an RNA that can be encapsidated into SNV virions was determined to be about 10 kb (39).

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. The REV-A IRES has been successfully used in the construction of novel high titer MLV-based retroviral vectors containing IRES elements (40).

5. VIRUS REPLICATION

The life cycle of REV s is typical for that of C-type retroviruses, although REV s reveal some unique features. In fact, much of our knowledge about the retrovirus life cycle in general, in particular reverse transcription, integration, recombination, etc. has been obtained by studying the life cycle of REV-A and SNV.

5.1. Virus entry

The entry of the reticuloendotheliosis viruses is mediated by the envelope glycoprotein, which consists of a 90 kd SU and a 25 kd TM peptide. SU is heavily glycosylated and mediates receptor binding (41,42). TM mediates membrane fusion. Like in other mammalian retroviruses, SU and TM appear not to be covalently linked by a disulfide bridge. As described above, the REV envelope is closely related to that of SRVs. E.g., amino acid comparison of the SNV and REV-A envelopes to that of simian D-type retroviruses revealed significant sequence and structural homologies (43,44). For example, 21 of the 23 cysteine residues of the SNV glycoprotein are also found in the corresponding position in MPMV. This also applies to seven of eight potential glycosylation sites.

Data obtained in my laboratory suggest that the SNV SU contains several domains involved in receptor binding, which are dispersed in discontinuous stretches of SU. This conclusion was based on the finding that truncated envelope proteins or various deletion mutants are transported to the cell surface, incorporated into virions, and still confer a low level of infectivity (about 0.1% of that of wild-type envelope) (43,44). Amino acid 192 (asp), located in the middle of SU, appears to form a hydrogen bond to the cellular receptor (44). Although the complete mechanism of virus entry of REV-A and SNV have not been investigated in great detail, it has been reported that the entry of SNV is pH independent: ammonium chloride as well as chloroquine did not inhibit SNV infection. Thus, SNV appears to enter cells by direct membrane fusion (45).

The SNV envelope appears to be rather stable and does not undergo (tolerate?) frequent mutations to escape the immune system like HIV. E.g., SNV Env inserted in fowlpox virus (FPV) recombinants was used to immunize one day old chicken. Vaccinated chicken produced neutralizing antibodies against wild-type virus more rapidly than control animals and were protected against both viremia and the SNV induced runting syndrome (46-48).

5.2. Reverse transcription and integration

Reverse transcription and integration of REV s have been studied in great detail. In fact, much of our knowledge of the mechanism of reverse transcription and integration of retroviruses in general has been gained using REV-A / SNV based retroviral vector systems. Retroviruses carry two identical RNA molecules which are believed to be both necessary to form one double stranded DNA copy. Reverse transcription is initiated from the tRNA primer, which generates the first cDNA segment, termed the strong stop cDNA (49). Using two slightly different vector genomes, which were expressed in the same helper cell, Panganiban's laboratory found that the strong stop cDNA derived from the first RNA template is mainly transferred to the 3' end of the second RNA molecule to serve as a primer for cDNA synthesis of the complete genome (50). Using a different system, Temin's laboratory reported that intramolecular strand transfer is also possible (51). Since both groups confirmed each other's results, it appears that cDNA strand transfer is dependent on the length and nature of the RNA genome.

Further investigations revealed that the enzyme reverse transcriptase can frequently switch the RNA template molecules, mainly during minus but also during plus strand DNA synthesis. The rate of template switch (recombination) during reverse transcription of the complete genome was estimated to be about 2-4% per 1000 bases per replication cycle. 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
REV-derived retroviral vectors

rates with marker distances of 1.9 and 7.1 kb appear not to be significantly different. Thus, retroviral recombination appears not to be proportional to marker distance. Additional studies revealed that the recombination rate of SNV is very similar to that of MLV (52-61).

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 non-random and one region was recombination-prone. More recent studies suggest that retroviral recombination in vivo is similar to that determined in vitro experiments (55,62,63).

Reverse transcriptases 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 also served as the first system to study retroviral mutation rates (62,64,65). In the past years, such systems have been used extensively to further quantitatively determine these processes in vitro and in vivo. It has been found that the SNV reverse transcriptase incorporates approximately one wrong nucleotide per 10,000 bases. Long homogenous stretches of one nucleotide appear to be specifically prone for short, one to three bp insertions and deletions. Moreover, various factors influencing the mutation rate have been studied in detail, e.g., deoxyribonucleoside triphosphate (dNTP) pool imbalances, and the presence of nucleotide analogs. It has been found that deoxyribonucleoside triphosphate (dNTP) pool imbalances are associated with and increase in the rate of misincorporation and hypermutation during in vitro reverse transcription 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 (62-70).

After reverse transcription, linear as well as circular DNA copies were found in the cytoplasm and nuclei of infected cells. It was believed that the circular form serves as immediate precursor for integration. Using a SNV genome containing an internal, covalently ligated LTR-LTR junction sequence (attachment site), Panganiban and Temin found that this site was enzymatically cleaved in infected chicken embryo fibroblasts. Thus, they concluded that the circular DNA form with two complete copies of the long terminal repeats serves as a precursor for the integration of the viral DNA into the host genome (71). However, this finding could not be reproduced by many other investigators working with other retrovirus systems: their data strongly suggested that the linear DNA serves as the immediate precursor of retroviral integration (reviewed in ref. 7). In a recent study, Panganiban repeated his original experiments. He found that integration of SNV-DNAs from the internal LTR-LTR junction took only place in chicken embryo fibroblasts, but not in mammalian D17 cells. Further, proviruses formed from the internal attachment site lacked the molecular features found after normal retroviral integration (72). Thus, it appears that, like all other retroviruses investigated, REVs may also use the linear DNA form for integration.

Like all C-type retroviruses investigated, REVs can only integrate their genome into that of the target cell when the nuclear membrane of the infected cells is dissolved during mitosis (73). Thus, the application of retroviral vectors derived from C-type retroviruses for human gene therapy is limited to introducing genes into dividing target cells. In contrast, lentiviruses have developed mechanisms to actively penetrate the nucleus of quiescent cells. It has been postulated that the presence of a nuclear localization sequence (NLS) in the matrix protein or the action of accessory proteins (e.g., the vpr protein in HIV-1) or a combination of both endow lentiviruses with this ability (74-78).

Recently, my laboratory further genetically engineered SNV-derived retroviral vectors to enable them to infect non-dividing cells. This has been achieved by introducing a nuclear localization sequence (NLS) into MA of SNV or REV-A by site-directed mutagenesis. The amino acid sequence of SNV and REV-A resemble that of HIV at the position at which the HIV-1-NLS is located. Thus, only two amino acid exchanges were necessary to endow SNV or REV-A with a NLS consensus sequence at the homologous position in MA. The introduction of the NLS increased the efficiency of infection of dividing cells and was sufficient to endow the virus with the capability to infect non-dividing radiated or non-radiated cells, which were growth arrested with four different inhibitors of mitosis. Wild-type SNV or SNV with single point mutations towards an NLS in MA could not transduce a marker gene into such growth arrested cells. Moreover, SNV-derived particles, which displayed single chain antibodies (see below) efficiently infected human growth arrested T-lymphocytes and primary monocyte-derived macrophages (79).

5.3. Transcription and splicing
The SNV and REV-A long terminal repeats contain typical RNA polymerase II promoters and enhancers which are very strong in various cell types of different species including human cells (15,80,81). However, the REV promoters and enhancers are rather weak in rodent (e.g., rat and mouse) cells (82). The enhancer of the REV-A LTR, two 46 and 23 base-pair repeats appear to be promoter specific: transcription is about fifty-fold less when these elements are placed upstream of the SV40 early promoter (81). The start site of transcription is determined by an initiator element which has a weak homology to that of HIV (83). However, REV-A and SNV appear not to encode a transcription factor which regulates and / or enhances the level of transcription efficiency (like Tat in HIV-1). This also applies to REV-T which contains the v-rel oncogene. V-rel was first shown by Gelinas and Temin to code for a transcription enhancer of various promoters (84). However, the v-rel protein does not have a regulatory effect on the REV-LTR transcription control elements.

SNV-based vectors have also been used to address the enigma of retroviral RNA polyadenylation.
REV-derived retroviral vectors

E.g., both R-regions in the viral LTRs contain polyadenylation signal sequences, but only the downstream site appears to be used. Detailed analysis revealed that the distance between the AATAAA site and initiation of RNA transcription is the major factor which determines that only the poly(A) site in the 3' LTR but not in the 5' LTR is used. Moreover, it was found that if the distance between the poly(A) site in the 5' LTR and the 5' cap site was shorter than 500 bp, less than 9% of SNV vector RNAs were polyadenylated at this site (85-87).

5.4. Nuclear export and translation

RNA splicing is believed to be a major part of exporting cellular mRNAs from the nucleus into the cytoplasm. Some retroviruses, like HIV-1 or HTLV code for proteins (Rev or Rex) involved in the regulation of RNA splicing and nuclear export of unspliced full-length viral RNA. SNV does not code for such a protein. However, recent investigations indicate that SNV contains several unique cis-acting elements, which compensate for the lack of accessory proteins involved in RNA nuclear export and/or splicing. 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 that it has been hypothesized that SNV RNA contains a cis-acting element that interacts with cellular Rex-like proteins (88). 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 (89). More detailed analysis revealed that the SNV RU5 region accesses a nuclear export pathway distinct from the CRM1 pathway, which is used by the HIV-1 RRE (90). Moreover, the replacement of simian immunodeficiency virus, SIV, LTR with that of SNV was sufficient to restore virus titers of Rev- /RRE-negative SIV-derived vectors (91). However, it still is not clear, what regulatory mechanisms are involved that about 50% of the genomic SNV RNA is spliced to generate mRNAs coding for Env (29).

As described above, gag and pol proteins are translated from full-length genomic RNA. The gag and pol gene are in the same reading frame and separated by an in-frame amber stop codon. Expression of pol is dependent on stop codon suppression. Mutation of the stop codon does not abolish proteolytic cleavage of the precursor proteins but did abolish particle assembly (92). Unlike in other avian retroviruses, the gag precursor protein is myristylated further underlining the close relationship of REVs to mammalian retroviruses (93).

Translation of the envelope protein follows the pathway of cellular transmembrane glycoproteins. Viral sequences downstream of the envelope gene appear to be evolved in the regulation of Env translation (94). However, the SNV Env protein can be efficiently expressed without such downstream sequences in standard eucaryotic gene expression vectors (29). Like in all retroviruses investigated, Env is translated as a single precursor peptide. The precursor protein is cleaved by a cellular protease in the endoplasmatic reticulum (ER) (41). N-linked glycosylation is mediated by cellular enzymes and begins with the transport of the nascent envelope polypeptide through the ER. Asparagine N-linked glycosylation is essential for intracellular transport and further envelope processing, and, consequently, for the formation of infectious virus particles (42). The mature, proteolytically processed envelope is highly glycosylated and about one half of the molecular weight is contributed by N-linked carbohydrate groups.

After protein synthesis, the envelope protein can interact with the cellular receptor within the ER. This interaction can prevent the transport of the viral receptor to the cell surface. Thus, expression of retroviral envelopes in general results in superinfection interference. Federspiel and colleagues found that expression of the REV-A or SNV envelope may be sufficient to establish efficient superinfection interference (47). However, only two D17 cell clones transfected with a plasmid expressing high levels of env revealed superinfection interference in the range observed with cells infected by wild-type virus. Other cell clones were only partially resistant (47). Similar results have been obtained by Delwart and Panganiban (45). Using highly efficient gene expression vectors to express the SNV Env in D17 cells, we also found only a moderate superinfection interference (43). These data indicate that other viral or cellular factors may also be necessary for the establishment of an efficient superinfection interference.

5.5. Encapsidation

As described above, the encapsidation sequence of REV-A and SNV is located mainly between map units 268 to 452 of the RNA genome. The region necessary for encapsidation co-maps with the region required for RNA dimerization. SNV-based vectors in which the dimer linkage structure has been inserted at the 3' end of the vector genome were found to replicate almost as efficiently as wild-type vectors. Thus, the position of E has little effect on the efficiency of virus propagation. Further, it was shown that two defined regions, a double hairpin structure and a region downstream of this structure are necessary for efficient SNV replication. These regions can be efficiently replaced with the encapsidation sequences of Moloney murine sarcoma virus (M-MSV) or Mo-MLV which show no sequence homology to that of SNV (35-38, 39-95).

Using chimeric gag-pol expression constructs and a competitive packaging system, it has been shown 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). Overall, REV proteins can package and transfer MLV-based vectors 50% as efficiently as SNV-derived vectors in the absence of a competing SNV vector (35,38). Recent data obtained in my laboratory indicate that SNV particles can also encapsidate and transduce HIV-1 based vector genomes but not vice versa (Parveen and Dornburg, unpublished).

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
REV-derived retroviral vectors

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 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 (98-100). These findings suggest that SNV mainly recognizes its E-sequences by their secondary or tertiary structure (38). More detailed analysis revealed that the double hairpin structure of the SNV E can be utilized by MLV for RNA packaging and that sequences 5’ of these hairpin structures enable MLV to discriminate between MLV and SNV RNAs (98).

REV-A particles can also encapsidate non-viral RNAs without encapsidation sequences in the absence of viral RNA. Furthermore, such non-viral RNAs are reverse transcribed and integrated into the genome of infected target cells to form pseudogenes. However, such cDNA genes lack the hallmarks of naturally occurring processed pseudogenes (101-103).

5.6. Particle assembly and maturation

The life cycle of REVs is completed by the budding of C-type particles from infected cells. Little is known about particle maturation of REVs and interactions of env with core proteins. The presence of envelope appears not to be essential for budding: reverse transcriptase activity in supernatants from envelope negative cells is almost as high as in supernatants of cells after the supplement of an env gene (29). Further, env negative virus particles can unspecifically infect several mammalian cell types although at extremely low efficiencies (titers are less than 10 cfu/ml supernatant medium). Although syncytia formation of SNV infected cells has been described (42), REV-A and SNV usually do not kill or lyse their host cells.

6. REV-DERIVED GENE TRANSFER SYSTEMS

REV-A and SNV were the first retroviruses from which a retroviral vector system has been derived (104). [See also introduction article "The History and Principles of Retrovirus-based Gene Transfer Systems", this issue.] REV-A and SNV are closely related (90% sequence homology) and all genes of their genomes appear to be interchangeable. Thus, the first vector systems consisted of parts derived from both viruses. Earlier REV-derived vector systems have been reviewed in detail previously (7). However, due to earlier findings that REVs do not infect human cells, no efforts have been made for many years to further develop REV-derived vectors for application in human gene therapy. They have been used mainly to study various aspects of retroviral replication, such as retroviral recombination, mutation rates, transduction of cellular genes (pseudogene formation), studies of the rel oncogene, the generation of transgenic chicken, and more.

6.1. REV-derived packaging cells

The first generation of REV-derived packaging cells (termed C3A2, ref. 104)) proved to be a very efficient helper cell system and vector virus titers up to 10^7 colony forming units per ml (cfu/ml) supernatant medium have been obtained. However, C3A2 cells spontaneously released replication competent retroviruses (RCR), which arose by recombination between the retroviral vector and DNA constructs expressing retroviral structural proteins (105). 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 (29,106). In fact, the retroviral packaging line DSH134G (29) developed in my laboratory has now been kept in tissue culture since 1995 and remained free of RCR over the past seven years (Figure 2, ref. (106) and Dornburg, unpublished data).

Retroviral vector titers obtained from the DSH134G helper cells, which produced a vector transducing the bacterial beta-galactosidase gene were about 10^6 to 10^7 cfu/ml supernatant medium. Vector titers could be increased by concentration through ultrafiltration to up to 10^8 cfu/ml (31). 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 (29).

Earlier, 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 that wild-type SNV (27). My laboratory recently constructed novel gag-pol gene expression vectors, which contain gag of REV-A and pol of SNV. In addition, in this new generation of Gag-pol expression plasmids, gene expression is driven from the cytomegalovirus immediate early promoter. Preliminary data obtained in transient transfection / infection assays indicate that vector virus titers can be increased up to 100-fold using such constructs (Krupetsky et al., unpublished observation).

All current REV-derived helper cells have been constructed using dog D17 cells, which are highly permissive for REV replication. We recently found that mouse NIH3T3 cells or human 293T cells are also very well suited to make packaging cells for REVs (unpublished) and the development of packaging lines derived from such cells is currently in progress in my laboratory.

6.2. Retroviral vector genomes

All current retroviral vectors transducing gene(s) of interest contain all cis-acting sequences of SNV (for some examples, see Figure 3). As outlined above, the encapsidation sequence of SNV does not extend into the gag coding region and all modern vector genomes do not contain overlapping regions of homology with viral protein coding sequences present in plasmids to express REV proteins. Various SNV vectors containing one or two genes have been constructed in several laboratories. In most vectors the first gene was expressed from the LTR
REV-derived retroviral vectors

Figure 3. Retroviral vector genomes. Vector genomes shown at the left are universal constructs and contain a multiple cloning site for the insertion of gene(s) of interest. Vectors shown at the right are examples of vectors derived from such universal cloning vectors and contain the bacterial beta-galactosidase (lacZ) gene. All vectors contain all cis-acting sequences required for retroviral replication. pAK3 is a standard vector containing two full-length SNV LTRs. In pZP35, the SNV promoter and enhancer (U3 region) in both LTRs have been replaced with that of the cytomegalovirus (CMV) immediate early promoter and enhancers. pAK2 is a self-inactivating retroviral vectors, in which the U3-region of the right LTR has been deleted with the exception of the attachment site, which is required for integration of the vector genome. After one round of replication the deletion is copied to both LTRs. Thus, the gene(s) of interest have to be expressed from an internal promoter (e.g., that of the simian virus 40, SV40, as in pAK8).

My laboratory has recently constructed a series of novel universal standard and self-inactivating vectors for the construction of vectors containing any gene of interest (Figure 3). As described above, the LTR promoter is very strong in avian, dog, and most human cells. However, it is rather weak in rodent cells. To achieve high levels of gene expression from SNV vectors in rodent cells, my laboratory has recently constructed vector genomes in which the SNV promoter and enhancer in both LTRs has been replaced with the cytomegalovirus immediate early promoter and enhancer (Figure 3).

Self-inactivating retroviral vectors (also termed SIN vectors) of SNV work with high efficiency (102,103,107). SIN vectors contain a deletion of the U3 region in the right long terminal repeat (LTR). The U3 region contains the viral promoter and enhancer. As a result of the mechanism of retroviral replication, this deletion is copied to the left LTR after one round of retroviral vector replication. Thus, genes have to be expressed from an internal promoter which can be cell-type-specific. Besides this advantage, SIN vectors also reduce the risk of downstream activation of cellular proto-oncogenes upon integration into the host genome (for details, see "The History and Principles of Retrovirus Based Gene Transfer Systems").

Some early SIN vectors of SNV revealed problems of recombination. We found that the U3 deletion was frequently repaired using vector or helper cell LTRs as template (108). The recombination appeared to be sequence-specific. Based on these studies, we constructed a new generation of highly-efficient, recombination-free SIN vectors (Figure 3) (83). Furthermore, using a SIN-vector-based experimental system, my laboratory built a recombination activator trap. We succeeded to identify a specific 7 bp long DNA sequence (CCCACCC) which triggered the LTR repair and generally activates DNA recombination in mammalian cells (109). Such a recombination activator may be useful in future experiments to approach gene repair therapy.

In another approach to develop SIN vectors, the laboratory of Dr. Pathak constructed retroviral vectors containing direct repeats within the vector genome. It had been shown that sequences between direct repeats are eliminated with a greater than 90% efficiency after one round of retroviral replication (62). Thus, this principle can be applied in retroviral vector development (110).

7. Cell-type-specific retroviral vectors

In 1990, my laboratory initiated the first experiments to develop cell-type-specific gene delivery vectors derived from SNV. Since SNV containing wild-type envelope does not infect human cells, we hypothesized that this vector system would be ideal for the development of cell-type-specific vectors for future human gene therapy. As described above, the SU peptide of Env mediates the binding of the virus to the cell surface of the target cell. Thus, we further hypothesized that replacing SU or parts of SU with a non-viral targeting ligand may alter the host range of the vector virus.

Our intention was to develop a universal vector system, which would allow cell-type-specific gene delivery into a large variety of different cells. The ideal molecules that can be directed basically against any cell surface
REV-derived retroviral vectors

Table 1. Virus titers of SNV-derived retroviral targeting vectors that display the antigen binding site of an antibody

<table>
<thead>
<tr>
<th>Target Antigen</th>
<th>scA</th>
<th>Virus Titer (1 \times \text{cfu/ml} )</th>
<th>Antigen-Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEA-RELATED</td>
<td>B6.2</td>
<td>(10^4)</td>
<td>Yes</td>
</tr>
<tr>
<td>HER2NEU</td>
<td>N29</td>
<td>(&gt; 10^5)</td>
<td>Yes</td>
</tr>
<tr>
<td>CD34</td>
<td>scA9069</td>
<td>(&gt; 10^5)</td>
<td>Yes</td>
</tr>
<tr>
<td>Tf-receptor</td>
<td>anti-Tfr</td>
<td>(&gt; 10^5)</td>
<td>Not Done</td>
</tr>
<tr>
<td>unknown T-cell antigen</td>
<td>7A5</td>
<td>(&gt; 10^6)</td>
<td>Yes</td>
</tr>
</tbody>
</table>

\(^1\) titers determined on human cells expressing the corresponding antigen

protein are antibodies. However, antibody molecules are too bulky to be displayed on viral particles and contain several domains and features not desired to be present on a vector, e.g., complement binding and activation sites.

In 1988, Bird and colleagues had described the development of single chain antibodies (scAs) (111). ScAs have been developed for E. coli expression to bypass the costly production of monoclonal antibodies in tissue culture or mice (111,112). They comprise the variable domains of both the heavy and light chain of an antibody molecule connected by a peptide bridge. This peptide bridge is encoded in a spacer region inserted between the coding regions of the two v-domains. Since this peptide only comprises the antigen binding domains of an antibody, we considered scAs ideal candidates for the construction of targeting vectors (see also "The History and Principles of Retroviral Vectors", this issue).

In our first approach, we constructed a single chain antibody gene from an antibody directed against the hapten dinitrophenol (DNP). We chose this antibody for the following reasons: First, this was one of the very few antibodies for which the heavy and light chain cDNA genes had been cloned and sequenced. Second, this antibody had been very well characterized. Third, DNP can be easily conjugated to the surface of cells. Thus, we could conjugate DNP to target cells which were not permissive for infection of SNV and test and compare infectivity of targeting vectors in such cells before and after DNP conjugation. In our first experiments, the scA gene was fused to various parts of SU or directly to the transmembrane coding region of the SNV envelope gene for incorporation and display on retroviral vector particles. To allow flexibility the scA and the envelope part of the fusion protein were also separated by a short peptide bridge. In another set of control experiments, we fused the complete SU unit of ecotropic MLV to the SNV TM.

We found that vector particles displaying the scA or the eco-MLV SU were infectious in several target cells which could not be infected with vectors displaying the wild-type SNV Env. Of note, infectivity was greatly enhanced or only possible when the wild-type Env was also present in the virus particle. We hypothesized that the targeting envelope anchors the virus particle to the cell-surface while the wild-type Env mediates membrane fusion.

While our first manuscript describing these findings was bounced for almost 18 months by major journals before it finally got accepted for publication (113), we started to repeat our experiments using a single chain antibody directed against a cell surface protein. In the meantime, Stephen Russell in Greg Winter’s laboratory published a very similar approach using MLV-derived vectors displaying a scA directed against another hapten (114).

In our next approach, we constructed vectors displaying a scA directed against a human CEA-related cell surface protein. The single chain antibody gene (termed B6.2) was among the very first scA genes constructed and had been very well characterized. It was kindly supplied to my laboratory by the company Enzon. We were then the first group to show that such scA-displaying particles are infectious as well (30). We again found that the presence of wild-type Env was necessary to confer efficient infection of such targeting vectors in human cells (30,31). Following this report, many studies with scAs displayed on MLV vectors and directed against various other human cell surface proteins showed that scA-displaying MLV vectors were not or only minimally infectious (115-118).

To further test, whether other scAs displayed on SNV-derived retroviral vector particles are competent for infection, we next 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. We found that particles displaying these scAs were infectious as well (Table 1). In the course of these studies, we also found that the level of expression of the target antigen (e.g., Her2neu or CD34) on the target cells did not play a role in the level of infectivity in vitro. Particles displaying both, the chimeric and the wild-type Env, were again more infectious in human cells than particles displaying the chimeric Env alone (Figure 4). Furthermore, such particles 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) (16).

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 (32). 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 spleen cells and a library of scA display phages was prepared. Only those phages
REV-derived retroviral vectors

Figure 4. SNV-derived retroviral vector particles displaying wild-type and / or targeting envelopes. SNV-derived vector particles with no envelopes are not infectious in all cell lines tested so far. Particles displaying the wild-type envelope are infectious in many avian or dog D17 cells, but do not infect human cells. Particles displaying a chimeric envelope in which a single chain antibody (scA) is fused to TM reveal low levels of infectivity in cells that express a cell surface protein recognized by the scA. Particles that express the wild-type and chimeric envelopes are highly infectious in cells permissive for wild-type SNV and in cells that express the antigen recognized by the scA.

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 of 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 (119).

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. The HIV-1 SU had been derived from a CXCR4-tropic strain. Such particles allowed efficient infection of CD4-positive human T-lymphocytes, and, at a lower efficiency, also CD4+ /CXCR4+, or CD4+/CXCR4− cells (120). 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.

Retroviruses such as MLV or HIV can be pseudotyped with envelope proteins of other retroviruses or non-retroviruses, such as the envelope (G-protein) of vesicular stomatitis virus (VSV) (121,122). Recently, my laboratory found that REV-derived vectors can also be pseudotyped with the G-protein of VSV or rabies viruses. Rabies viruses (RV) are enveloped, non-segmented, negative-strand RNA viruses. RVs are highly neurotropic and usually cause a fatal infection in all warm-blooded species, with virus replication primarily occurring in neurons. The single transmembrane envelope (G) protein is responsible for both the attachment to host cells receptor and the fusion and release of the viral core into the cytoplasm. Different RV strains greatly differ in their preference for infecting neuronal cells. Recently, two RV variants, termed CVS-N2c and CVS-B2c that differ genotypically and phenotypically, were isolated from the mouse-adapted rabies virus strain CVS-24 (123-125).

The RV strain CSV-N2c is highly specific for neuronal cells and has a low affinity to other cell-types, whereas the RV strain CVS-B2c does not have a preference for neuronal cells. Pseudotyping of REV-derived vectors with the envelope protein of the neurotropic rabies virus strain N2C enabled highly-efficient cell-type-specific gene transduction into mouse and human neuronal cells (Parveen et al., manuscript submitted). The envelope of the rabies virus strain B2C conferred a host range similar to that of VSV-G. Experiments with new-born mice showed that vectors pseudotyped with the N2C envelope also enabled cell-type-specific gene delivery into neurons in vivo (see below).

Recently, it was shown that MLV-based vectors can also be pseudotyped with SNV wild-type and targeting envelopes. Thus, MLV-based vectors can be made cell-type-specific using SNV envelopes (126).

8. IN VIVO CELL-TYPE-SPECIFIC GENE DELIVERY

The ultimate goal of using cell-type-specific retroviral vectors is their application for in vivo human gene therapy. In the past years, my laboratory performed several experiments to test cell-type-specific gene delivery in vivo using mouse model systems. As described above, REV-derived vectors are not infectious in mouse or human cells, unless they are endowed with a targeting envelope specific for a receptor expressed on a certain cell-type. Thus, mouse model systems are very well suited for cell-type-specific gene delivery experiments in vivo and mimic future application in humans, because human cells and primary tissues are also non-permissive for infection with REV-derived vectors.
REV-derived retroviral vectors

In first experiments, we tested in vivo cell-type-specific gene delivery using retroviral vectors which displayed an scA directed against the Her2neu protein. Her2neu is over-expressed in many breast cancer cells, but also expressed in many other cell-lines (e.g., HeLa or COLO-320DM cells) at low levels. Some cell-lines, like A321 cells do not express Her2neu. Antibiotic-resistant target and non-target cells (or a mixture of both) were injected into the peritoneum of SCID mice, followed by the injection of vector virus stocks. The mice were sacrificed and the human cells recovered (antibiotic selection). The results of these studies can be summarized as follows. Up to 5% of the target cells (Her2neu-positive cells) were infected in vivo, while no infectivity was observed in recovered non-target cells (Her2neu-negative cells). Normal mouse tissue was not infected. These data gave first proof of principle that a cell-type-specific gene delivery can be obtained in vivo and that SCID mice are suitable model systems to study in vivo gene delivery (127).

In a second in vivo gene delivery approach, we injected concentrated retroviral vectors, which displayed VSV-G or rabies virus envelope proteins into the lateral lobes or cerebellum of new-born mice. Two month after the injection the mice were sacrificed and the complete brain investigated for infected cells by X-gal staining. No difference in the efficiency of infection was observed between viruses expressing rabies virus or VSV envelope proteins. Control mice were infected with tissue culture medium harvested from cells expressing a retroviral vector transducing the lacZ gene and the envelope protein of SNV, but no gag-pol proteins. No blue cells were detected in the brains of the control group. These data indicate that SNV vector particles displaying the rabies virus or the VSV envelope are infectious in vivo as well.

To further investigate cell-type-specificity of vectors pseudotyped with the RV N2C envelope, thin sections of the brains of infected mice were prepared and stained with an antibody against the bacterial beta-galactosidase or with the NeuN antibody. In the case of mice brains infected with vectors displaying the N2C envelope, the expression of the lacZ gene was confined to cells which also stained positive with an antibody against neuronal cells: for example, hippocampi pyramidal neurons as well as mature neurons towards the cortex region, but no surrounding non-neuronal cells expressed the bacterial beta-galactosidase protein. However, in the case of brains infected with vectors displaying the RV B2C or VSV envelope, many different cell-types appeared to be infected and no specific infection of neuronal cells was observed. These data showed that REV-derived vectors pseudotyped with the rabies virus N2C envelope enable cell-type-specific gene delivery into neurons in vivo (Parveen et al., submitted).

In another approach, my laboratory tested in vivo gene delivery using Theracyte immunoisolation devices, which can be implanted subcutaneously into a patient. To solve many of the problems associated with the delivery of biological therapeutics such as insulin, the company Baxter Co. has developed an immunoisolation device called TheraCyte (for a detailed description, see article Krupetsky et al., in this issue). The system encapsulates cells, which produce a therapeutic agent, and prevents direct contact with the tissue of the patient. The inner membrane of the device partially restricts entrance for the cells of the immune system, thus minimizing destruction of allografts, but not xenografts by the immune system. The outer membrane of the device allows vascularization around the system promoting molecular and nutritional flow to and from the encapsulated cells (128-131).

Recently, TheraCyte implantation bags were used in vivo for delivery of Human Factor IX, antitumor antigen, recombinant human growth hormone and insulin (128-130,132,133). We initiated a series of in vitro experiments to test Theracyte implantation bags as a tool for gene delivery by retroviral vectors. Retroviral helper cells were encapsulated in such devices and tested for the release of retroviral vectors. In vitro experiments show that such devices release infectious retroviral vectors into the tissue culture medium for up to 4 months. In vivo gene delivery was tested in a mouse model system. Tumors were induced in SCID mice by subcutaneous injection of dog D17 osteosarcoma cells. Immunoisolation devices were filled with helper cells releasing spleen necrosis virus-derived vectors, which are infectious in D17 cells, but not in normal mouse tissues. When such devices were implanted subcutaneously in SCID mice, infectious virus was released, transported to, and only infected the induced tumors. This novel concept of a continuous, long-term gene delivery may constitute an attractive approach for future in vivo human gene therapy, e.g., of genetic diseases or slow growing tumors, or gene therapy of HIV-1 infection (see Krupetsky et al., this issue).

9. PERSPECTIVE

REV-derived vectors have been used in several laboratories for about two decades. They have been used to study many aspects of the retroviral life cycle. Further they have been applied to generate transgenic chicken. They are not pathogenic in humans and do not infect human tissue culture or primary cells. We have shown that an SNV-derived retroviral vector system is very well suitable for the development of cell type-specific agents to deliver therapeutic genes into various cell types and, most importantly, into quiescent cells such as human T-cells, macrophages, and neurons. SNV vector cores can be efficiently pseudotyped with envelope proteins of VSV and various rabies viruses. Such virus particles enable a highly-efficient cell-type-specific gene transfer into mouse or human neuronal cells. In regard to future human gene therapy, the REV-A/SNV vectors system has the following further advantages over other retrovirus-based gene transfer systems:

(i) Highly-efficient retroviral packaging lines can be constructed that do not contain stretches of homology to retroviral vector genomes. Thus, there is no risk of homologous recombination that could result in the generation of replication competent virus. (ii) The SNV LTR promoter is very strong in human cells. (ii) SNV-
REV-derived retroviral vectors

derived self-inactivating vectors work with high efficiency. Thus, vectors can be made that express genes under the control of a cell type-specific or inducible promoter without any interference from the SNV-LTR promoter and enhancer. (iv) Experiments in mice indicate that an in vivo cell-type-specific gene transfer with SNV targeting vectors can be obtained.

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**Send correspondence to:** Dr Ralph Dornburg, Thomas Jefferson University, Division of Infectious Diseases, 1020 Locust Street, Suite 329, Philadelphia, PA 19107, Tel:215-503-31117, Fax: 215-923-1956, E-mail: ralph.dornburg@mail.tju.edu