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THE HISTORY AND PRINCIPLES OF RETROVIRAL VECTORS

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TABLE OF CONTENT

1. ABSTRACT

Retrovirus-derived gene transfer systems (retroviral vectors) are the most commonly used gene transfer tools in modern biology. They have been used to study various aspects of retroviral replication, the organization and function of oncogenes and other eucaryotic genes, and, recently, to transduce therapeutic genes to cure inborn errors of metabolism, cancer, AIDS, and many other diseases in man. Highly oncogenic retroviruses served as a model for the construction of artificial retroviral gene transfer systems. These viruses carry a non-viral gene in their genome in addition or substituting for viral protein coding sequences. The replication of such defective retroviruses depends on the presence of a wild-type-virus, which supplies all proteins in trans for particle assembly and infection of a new target cell. Thus, highly oncogenic retroviruses can be considered as naturally occurring gene transfer vectors. Following this principle, cell lines have been constructed which express retroviral protein coding sequences from plasmid DNAs and which contain a viral genome in which the protein coding sequences have been replaced with a gene of interest. This article describes the history, principles, and basic building blocks of first and modern retrovirus-derived gene transfer systems.

2. INTRODUCTION

Retroviral vectors have become the most commonly used gene transfer tools in modern biology. In the past decade, they have been used to transduce therapeutic genes into humans to correct inborn errors of metabolism and many other diseases like cancer, AIDS, degenerative diseases of the brain, and more (for some reviews, see ref. 1-4). The first retroviral vector systems have been derived from genetically modified C-type retroviruses almost 20 years ago (5,6) and have been used originally to study many different aspects of the retroviral life cycle and the function of oncogenes.

Retrovirsuses are RNA viruses which convert their RNA genome into a double-stranded DNA copy (reverse transcription) in the infected cell (7). This DNA copy is stable integrated into the genome of the host. This process is very efficient and C-type retroviruses usually do not kill the infected cell. Thus, after one cell division, both daughter cells carry the retroviral genome and become factories for progeny virus production. These features made them prime candidates for the development of the first virus-based gene transfer system.

The mechanisms of retroviral replication, e.g., viral entry, the reverse transcription and integration of the viral genome, are very complex and have been reviewed in great detail elsewhere (8-13) (see also "Replication of Lentiviruses", by Acheampong, this issue). It would be beyond the scope of this article to extensively review this process. Thus, in this article, the replication cycle of retroviruses is outlined only briefly and particular attention is only paid to those aspects of retroviral replication which are important in the design of retroviral vector systems.

C-type retroviruses are simple enveloped viruses. The viral core contains two copies of the RNA genome, structural proteins, and all enzymes necessary to reverse

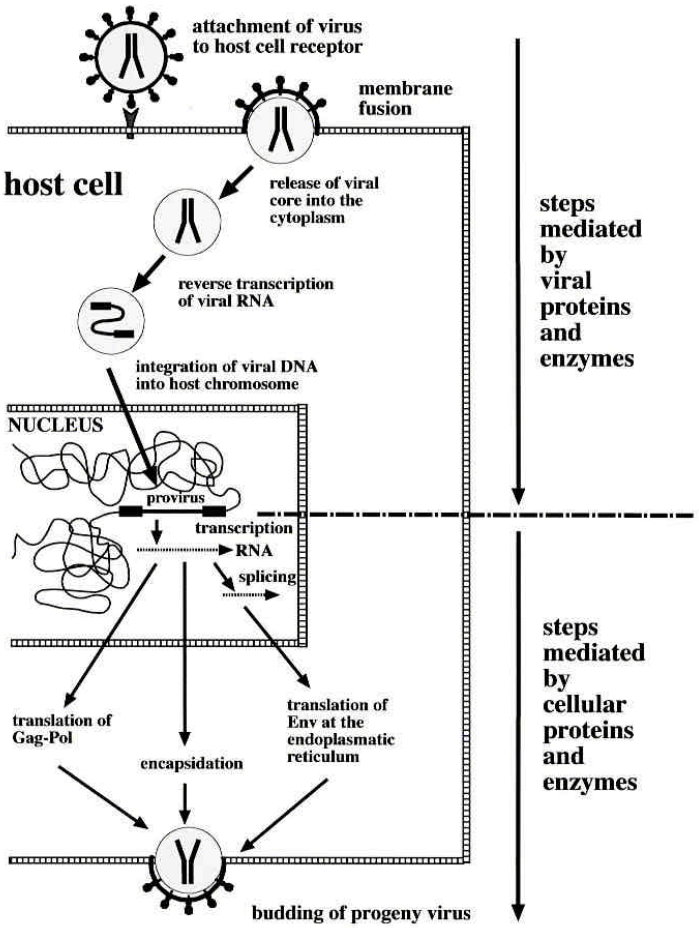

Figure 1. Replication of a C-type retrovirus. The retrovirus binds to a specific host cell receptor through its envelope protein. After viral attachment, in most retroviruses, the viral and cellular membrane fuse, and the retroviral core is released into the cytoplasm. Now, the virus has access to nucleotides and reverse transcribes its RNA genome into a double stranded DNA copy. Viral core proteins and the DNA copy remain assembled until the core reaches the host chromosome and the DNA of the virus is integrated into that of the host. Up to this step, the virus is independent from cellular proteins and enzymes. The integrated DNA copy of the retrovirus is termed a provirus. Once integrated, RNA transcription and partial splicing generate full-length viral RNA and a spliced RNA product. The full length RNA serves as template for the translation of retroviral core proteins and is encapsidated into new viral cores due to the presence of encapsidation sequences recognized by the viral core proteins. The viral core proteins are synthesized as one precursor peptide. Proteolytic cleavage mediated by a viral protease during particle maturation generates the individual viral core proteins including the reverse transcriptase and integration protein. The spliced RNA encodes the viral envelope protein, which is translated at the endoplasmatic reticulum and follows the pathway of cellular membrane proteins. The envelope is also synthesized as a precursor. Cleavage of this precursor by a cellular protease gives rise to the surface (SU) and transmembrane (TM) units. SU mediates membrane binding, TM the fusion of the viral and cellular membrane. Viral core proteins assemble at the cellular membrane, from which new virus particles bud. Most retroviruses do not kill the infected cell, but continuously use it for progeny virus production. Thus, after one cell division, both daughter cells become virus factories.

transcribe the viral RNA and to integrate the DNA copy into the chromosome of the host. The core is surrounded by a lipid bilayer which contains the retroviral envelope proteins. The envelope mediates the attachment of the virus to a specific cell membrane protein (receptor) of the host cells, and determines the host range and tropism of the virus (14). It also mediates the fusion of the viral and cellular membranes to release the core into the cytoplasm (Figure 1). To modify the host range of retroviral vectors, efforts are now being made to genetically modify the envelope protein (see below: cell-type-specific retroviral vectors).

Once the core is released into the cytoplasm, the virus gets access to nucleotides for reverse transcription. The resulting DNA copy stays associated with viral proteins until it is integrated into the chromosome of the host. Virus entry, the reverse transcription, and the

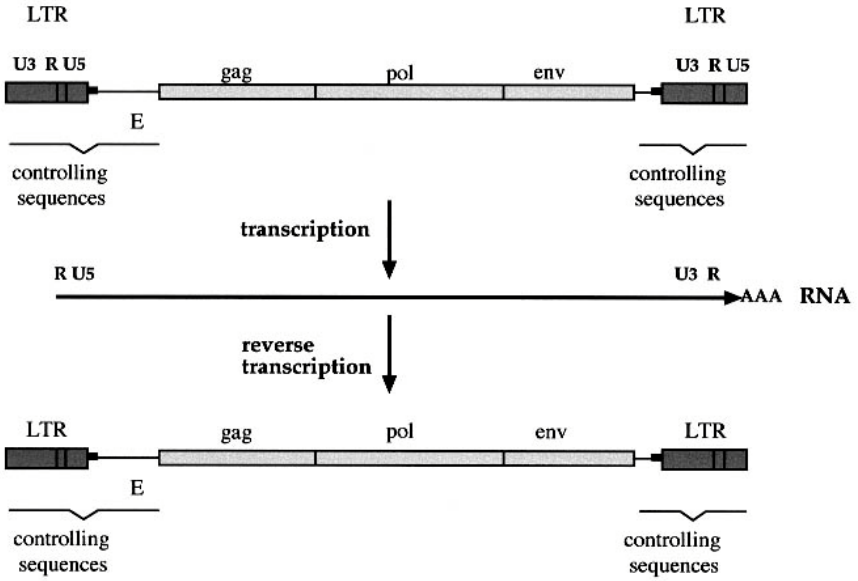

Figure 2. Replication of the retrovirus genome. A provirus is shown at the top. Transcription from the viral long terminal repeat (LTR) promoter (located in the U3 region of the LTR) generates viral RNA which is polyadenylated like cellular mRNAs. The presence of a polyadenylation signal sequence in the downstream (3') LTR (located in the U5 region of the LTR) mediates precise addition of the poly(A) tail downstream of a region which is sequence identical (repeated) at both ends of the RNA genome (termed R). In an infected target cell, the RNA genome is reverse transcribed by the retroviral reverse transcriptase. During this complicated process, the U3 region, now only present at the 3' end of the RNA genome, is duplicated and attached to the 5' end. The U5 region, present as single copy at the 5' end of the RNA genome, is also copied and attached to the 3' end of the DNA copy. This mechanism of replication guarantees that no sequences are lost during retroviral replication. A specific encapsidation sequence (E) mediates the incorporation of the viral RNA into viral particles.

integration of the DNA copy is mediated solely by viral proteins present in the virus particle. Once integrated into the host genome the viral DNA copy is termed a provirus (15). Now, further retrovirus replication relies on the transcription / translation machinery of the host cell (Figure 1). Briefly, full-length viral transcripts are used to translate core proteins and are finally encapsidated into viral cores. Some of the viral RNA is spliced for the translation of the envelope protein, which takes place at the endoplasmatic reticulum and follows the pathway of cellular membrane proteins. At the cell membrane, viral particles are assembled and bud from the infected target cell (Figure 1).

Thus, the retroviral life cycle is separated into two parts: (i) the first part comprises all steps leading to the establishment of a DNA copy in the host chromosome. These steps are solely mediated by retroviral enzymes present in the viral particle; (ii) the second part includes all steps to generate new progeny virus, which are dependent on cellular proteins and enzymes. The first part enables the construction of virus particles which contain a genome carrying a gene of interest, the second part enables the construction of cells continuously producing retroviral vector particles, which can be harvested to infect new target cells. This article first reviews natural retroviral vectors which served as model for the construction of retroviral vectors. Next, the construction of first and modern retroviral vector systems is described, followed by the description of recent vector developments, problems, and efforts to overcome shortcomings.

3. NATURAL RETROVIRAL VECTORS

Retroviruses have been studied in extensive detail since almost one hundred years, because they can cause the malignant transformation of the host cell. The malignant transformation can be caused by two different mechanisms (for recent reviews, see references 16-18):

First, during retroviral replication, the retroviral promoter and enhancer, which are located in the 3' end of the viral RNA genome are duplicated during reverse transcription and flank the viral DNA copy (Figure 2). Thus, the provirus contains two promoters / enhancers, which are part of long terminal repeats (LTRs). The left LTR drives transcription of the viral genome. The promoter in the right LTR can drive or enhance transcription of cellular genes, which are located downstream of the promoter. In addition, it has been shown that termination of viral RNA transcription in the right LTR at the R-U5 boundary is not completely efficient and read-through transcripts are generated (19). Such read-through transcripts are spliced and the downstream gene is basically expressed from the viral LTR promoter (see also article "Encapsidation and Transduction of cellular Genes by Retroviruses" by Muriaux and Rein, this issue). Occasionally, retroviruses integrate upstream or within a

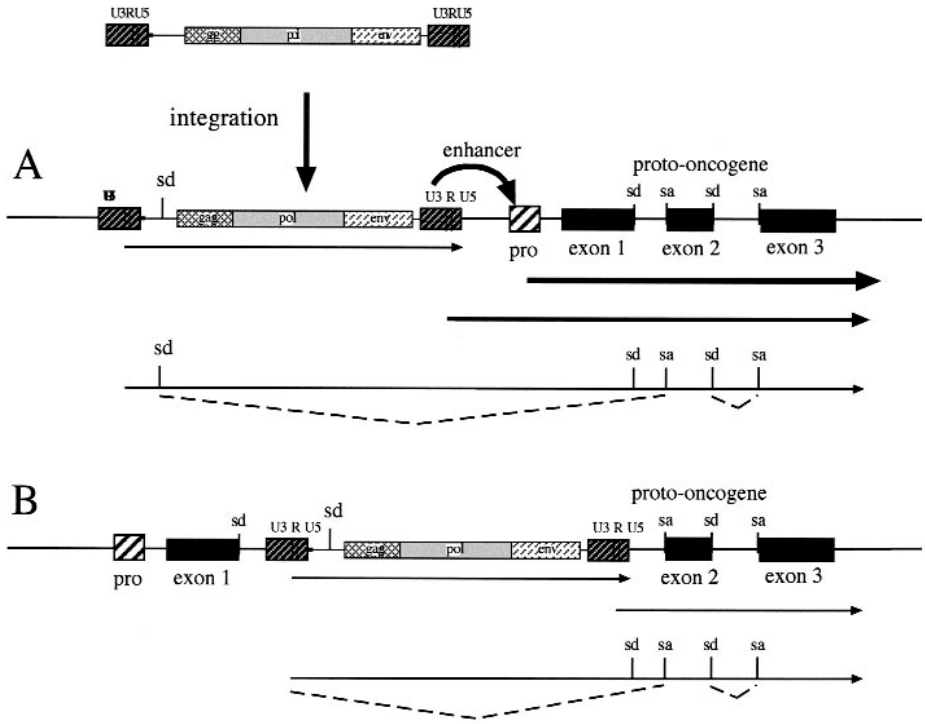

Figure 3. Transformation of a cell by retroviruses. A: The insertion of a retroviral DNA upstream of a proto-oncogene leads to the increased transcription of that gene stimulating cell division. Further, RNA read-through transcription and RNA splicing can generate abnormal products of the downstream gene B) The integrated provirus disrupts a proto-oncogene resulting in the transcription of an aberrant (truncated) proto-oncogene protein. This abnormal proto-oncogene may also lead to the disruption of a controlled cell-cycle mechanism of the host cell and, consequently, to continuous cell divisions. Arrows indicate RNAs, the dashed lines splicing events.

cellular gene involved in the regulation of the cell-cycle (proto-oncogene). The presence of the viral LTR can upregulate the expression of the downstream gene or result in the transcription / translation of an abbreviated, abnormal gene product. In either case, the altered expression of a proto-oncogene can lead to the malignant transformation of the host cell (Figure 3a and 3b) (16-18).

Second, the molecular investigation of acute transforming retrovirsuses (in particular, avian leukosis and mammalian C-type retroviruses) revealed that these viruses carry a gene in their genome, which is not required for retroviral replication. Expression of this gene (oncogene) leads to the malignant transformation of the infected cells. Such retroviruses are termed highly oncogenic retroviruses. They arose by recombination of the viral genome with cellular protooncogenes (19,20). As a result of this process, in most oncogenic retroviruses the viral oncogene substitutes for parts of the protein coding regions of the viral genome (for some examples of highly oncogenic retroviruses, see Figure 4). Thus, the oncogene present in the viral genome is a recombination product, e.g., a fusion protein of viral and cellular sequences (see also article "Retroviral Recombination: Review of Genetic Analysis" by Hu *et al*., this issue). Retroviral oncogenes are always expressed from transcripts originating and terminating in the viral LTRs.

Almost all highly oncogenic retroviruses have deletions in their protein coding genes, and, therefore, cannot synthesize all proteins necessary for retroviral replication. Thus, they are replication defective. They can only further spread and be transferred to new target cells, if the infected cell also carries a complete genome of a replication-competent retrovirus. The replication-competent virus supplies all proteins which are necessary to assemble a complete infectious virus particle (Figure 5).

Detailed molecular investigation of the retrovirus life cycle and that of highly oncogenic retroviruses revealed that the retroviral genome contains several sequences which do not code for viral proteins but which are essential for virus replication. These cis-acting sequences are located at the 5' and 3' end of the viral genome and flank the protein coding sequences, also termed trans-acting sequences (Figure 2). One cis-acting sequence, termed E in avian retroviruses, (psi) in murine retrovirsuses, which is located downstream of the left LTR, mediates the encapsidation of the viral RNA genome into viral particles (21,22). Since the genome of an oncogenic retrovirus still contains functional encapsidation sequences, the genome of an oncogenic retrovirus is packaged into infectious retroviral particles. Further, oncogenic retrovirus also still contain all other cis-acting sequences needed for replication. Thus, the oncogenic retroviral RNA genome is

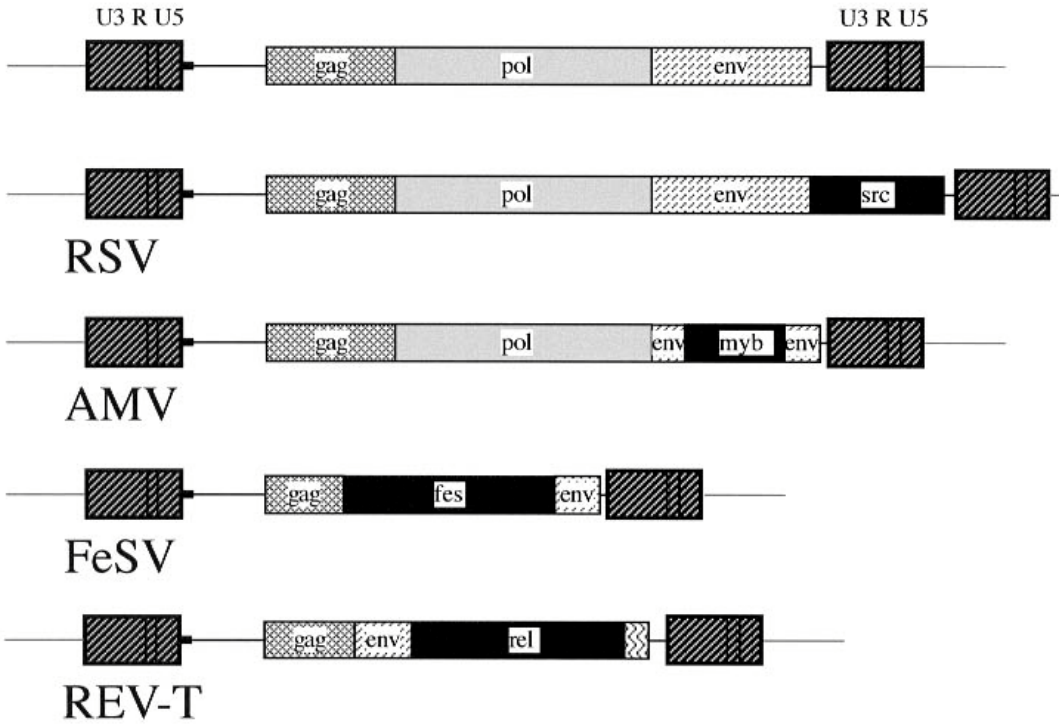

Figure 4. Examples of highly oncogenic retroviruses. In almost all highly oncogenic retroviruses, some protein coding of the virus are deleted and substituted with a gene the expression of which leads to the malignant transformation of the host cell. Such viruses are replication deficient. However, they still contain encapsidation sequences and all other cis-acting sequences required for the replication of the RNA genome. Thus, the genome of such defective retroviruses can still be encapsidated and replicated when a wild-type virus is co-present and supplies the missing viral proteins in trans. RSV: Rous sarcoma virus; AMV: avian myeloblastosis virus; FeSV: feline sarcoma virus; REV-T: T-strain of reticuloendotheliosis virus. src, myb, fes, and rel are oncogenes which recombined into the viral genome.

reverse transcribed and integrated into that of a fresh target cell.

Highly oncogenic retroviruses can be considered as natural occurring gene transfer vehicles (vectors) which carry a non-viral gene (23). The wild type virus, which supplies the proteins missing in the genome of the oncogenic retrovirus is called a "helper virus". The helper virus provides those viral proteins the defective retrovirus cannot synthesize as a result of the deletion and substitution of DNA sequences in its genome.

4. RETROVIRAL HELPER CELLS

Oncogenic retroviruses served as a model in the construction of the first artificial retroviral gene transfer systems. Like in cells infected with a highly oncogenic retrovirus and a wild-type virus, such systems comprise two components: the retroviral vector which contains the gene of interest replacing retroviral protein coding sequences and plasmids encoding the retroviral structural genes, which express and supply the retroviral proteins for the encapsidation of the vector genome. Such plasmids are stable transfected into cells, which now express retroviral proteins and a defective retroviral genome. Such cells are called helper cells (also termed packaging cells) (24-29).

4.1. First generation of retroviral helper cells

The first artificial retroviral gene transfer systems were reported in 1983. One system has been derived from the closely related avian reticuloendotheliosis viruses strain A (REV-A) and spleen necrosis virus (SNV) (5), the other from ecotropic murine leukemia virus (eco-MLV) (6). REVs and MLV are C-type retroviruses which, like all C-type retroviruses, express the inner core proteins (gag-pol) from full-length viral RNA and the envelope protein from a spliced RNA (as outlined in Figure 1) (26,30). The first packaging systems closely mimicked a natural retroviral gene transfer system: It consisted of cells, which contained a retrovirus provirus and a retroviral genome carrying a gene of interest. However, the provirus had a deletion of the encapsidation sequence, and, therefore was defective. Further, the provirus constitutively expressed retroviral proteins, but could not encapsidate its own genomic RNA due to the lack of the encapsidation sequence. Since the deletion of the encapsidation sequence in the REV-derived system also eliminated the splice donor site essential for the splicing of viral RNA to express the envelope gene, a second plasmid contained a viral genome in which most of the gag-pol coding region was deleted in order to express Env.

The retroviral vector genome containing the gene of interest retained all sequences for encapsidation and viral

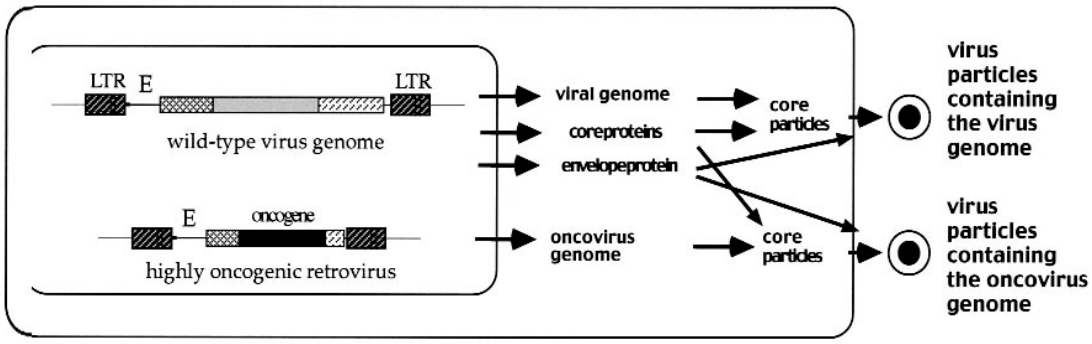

Figure 5. Replication of natural retroviral vectors. A cell is infected with a wild-type virus and a highly oncogenic retrovirus. The wild-type virus (also termed helper virus) supplies retroviral proteins for the encapsidation of the genome of the highly oncogenic retrovirus. Cells shed two different types of virus particles: wild-type-virus and a highly oncogenic retrovirus which contains a defective RNA genome. However, due to the presence of all viral proteins in the particle, the defective virus still can establish a provirus in an infected target cell (see also Figure 1). A highly oncogenic retrovirus can be considered a natural occurring gene transfer vector, as it carries a gene which is of cellular origin. The wt virus is called "helper virus", because it helps the defective virus to replicate.

replication, and, therefore was encapsidated into retroviral particles. Retroviral vector particles were shed from the helper cells and could be used to infect fresh target cells. As outlined above, such particles contain all viral proteins for the reverse transcription and integration of the vector virus genome into that of the target cell. However, since no retroviral proteins are expressed in the target cell, no further retroviral replication and spread of the vector genome takes place. This retroviral gene transfer system was called "helper-free", because it does not produce retroviral particles, which contained the genome of a replication-competent (helper) retrovirus (RCR) (Figure 6A). REV-derived helper cells were termed C3A2 (5), the MLV-derived system ψ2 (6).

Although the first retroviral vector systems were very efficient (more than 1,000 fold more efficient than DNA transfection protocols at that time, virus titers up to 107 colony forming particles per ml supernatant medium), it had a major shortcoming: In spite of the missing encapsidation sequence, the block of encapsidation of the RNA encoding viral proteins was not complete. Thus, helper virus genomes were still encapsidated into viral particles and transferred to fresh target cells, albeit at a very low efficiency (31). Further, it was found that recombination of the genome containing the protein coding sequences with the vector genome, which contains a full-length encapsidation sequence, led to the sporadic generation of fully replication-competent retroviruses (RCR) after relatively short time periods (32). Only one recombination event was necessary to restore a RCR, because the vector and the helper cell genomes contained overlapping regions of homology at their 5' end (Figure 7A). Detailed investigation of retroviral recombination rates later revealed that this process is very efficient and occurs during reverse transcription (the reverse transcriptase switches RNA templates at least once per replication cycle) (33,34) (see article "Retroviral Recombination: Review of Genetic Analyses" by Hu *et al*., this issue).

To reduce the possibility of generating RCR, in the next generation of helper cells, more deletions were introduced into the helper virus genome. For example, helper cells termed PA317, which had been derived from amphotropic murine leukemia virus (ampho-MLV), contained a helper virus genome in which not only the packaging sequence had been deleted. The complete 3' LTR had been substituted for the polyadenylation signal sequence of simian virus 40 (SV40) (Figure 6B). In addition the 5' end of the 5'LTR was also deleted. Thus, two recombination steps were necessary to restore a RCR (Figure 7B). As expected, this retroviral vector system was much less prone to recombination resulting in RCR (35). However, the occasional production of RCR from such packaging cells has also been reported (36-38).

In the next approach, further improvements were made by deleting more sequences from constructs designed to express gag-pol from one plasmid and env from another. For example, in the MLV-derived retroviral packaging lines termed CRE and CRIP (39), mutations and more deletions were introduced into the gene units used to express Gag-Pol and Env. E.g., most of the 3' LTRs in both constructs to express Gag-pol and Env have been replaced with the polyadenylation signal sequence of SV40. Thus, in these retroviral vector systems, three recombination events between the gene units used to express Gag-Pol and Env and the vector genome are required to generate RCR (39) (Figure 7C).

4.2. Modern helper cells

The elegance and the high efficiency of stable transfer of genes of interest spurred the further development of other retroviral helper cell systems derived from other retroviruses like avian leukosis viruses (ALV) (40-43), mouse mammary tumor virus (MMTV) (44), gibbon ape leukemia virus (45,46), bovine leukemia virus (BLV) (47) and, more recently, from lentiviruses (1,48), like human immunodeficiency virus type 1 (HIV-1) (25,48-50), simian immunodeficiency virus (SIV) (51-53), feline immunodeficiency virus (FIV) (54,55), and equine infectious anemia virus (EAV) (56). Moreover, the need of safe and highly efficient gene transfer vectors for human

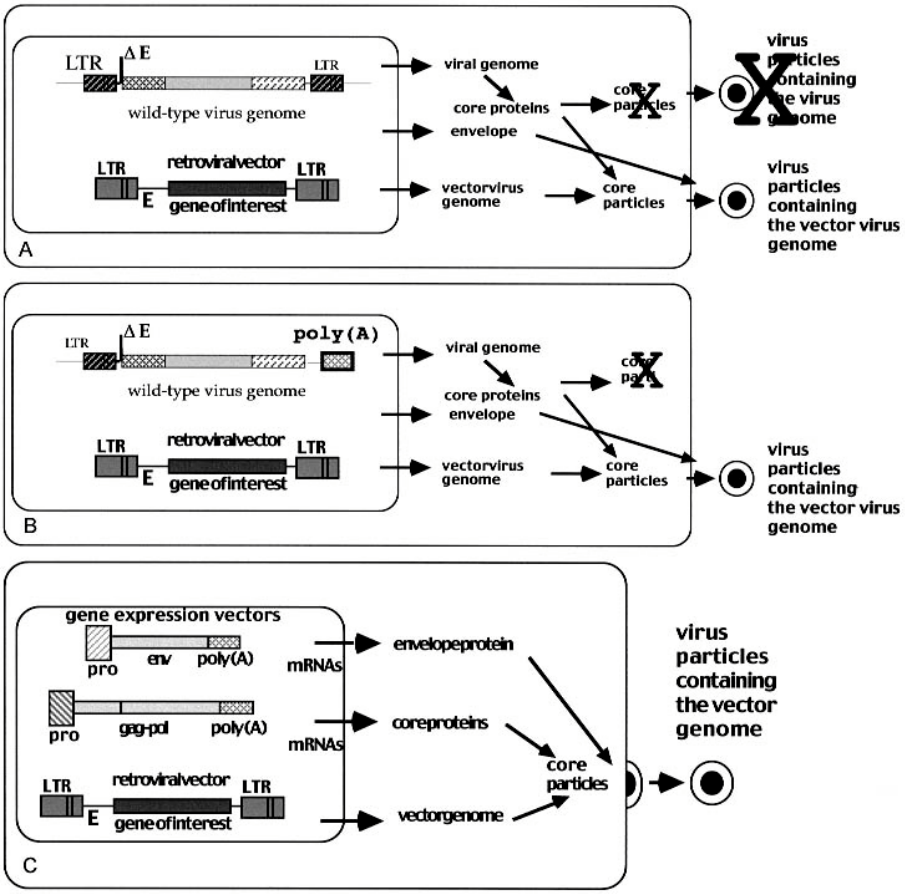

Figure 6. Retroviral packaging cells. A) In the first generation of helper cells, tissue culture cells were transfected with plasmids containing a provirus in which the encapsidation sequence (E) has been deleted. Helper cells are next transfected with a plasmid containing a retroviral vector DNA construct, which carries functional encapsidation sequences and a gene(s) of interest replacing the viral protein coding sequences. Thus, RNA transcripts of such vectors are packaged into virions provided by the helper cell. The virus produced from the helper cell is called "helper-free", since it contains no replication competent helper virus. However, only one recombination step is necessary to regenerate a replication competent retrovirus (RCR) (see also Figure 7A) B) In the next generation of helper cells, the downstream LTR has been replaced with a polyadenylation signal sequence, poly(A). Here, two recombination steps are necessary to generate a RCR (Figure 7B). C) In modern helper cells, Gag-Pol and Env are split into different expression cassettes and expressed from heterologous promoters. Three recombination events are necessary to generate a RCR (Figure 7C).

gene therapy has now initiated the systematic improvement and further development of retroviral vector systems in many laboratories.

Considering observations with first and second generation of packaging cells, further improvement in the design of such vectors systems have been made. [For a detailed review on first generation packaging cells, see ref. (29)]. In most modern helper cells today, retroviral protein coding sequences are expressed from separate gene expression units, in which most or all non-essential sequences have been deleted (Figure 6C) (57-61). It has been found that smaller regions of sequence homology greatly reduce the possibility of recombination, and, consequently, RCR formation (31). The LTRs have been completely removed and Gag-pol and Env are expressed from heterologous promoters. However, since the Gag-pol and Env coding regions usually overlap in C-type retroviruses, it is basically impossible to eliminate this region of homology. To bypass this shortcoming and to widen or narrow the host range of the vector, packaging lines have been developed which express Gag-pol of one retrovirus and the envelope of another. E.g., retroviruses can utilize not only the envelope protein of other retroviruses in the absence of their own envelope, but also envelope proteins of non-retroviruses (see below).

Another problem in some retroviral vector systems (e.g., MLV) is the finding that the encapsidation sequence extends into the gag gene (62). Thus, some regions of sequence homology still remain and there is the possibility of homologous recombination. In other retroviruses, like reticuloendotheliosis viruses, the encapsidation sequence appears not to extend into the gag region. Thus, in modern REV-derived packaging lines, eg., in helper cells termed DSH134G (61), there is no region of

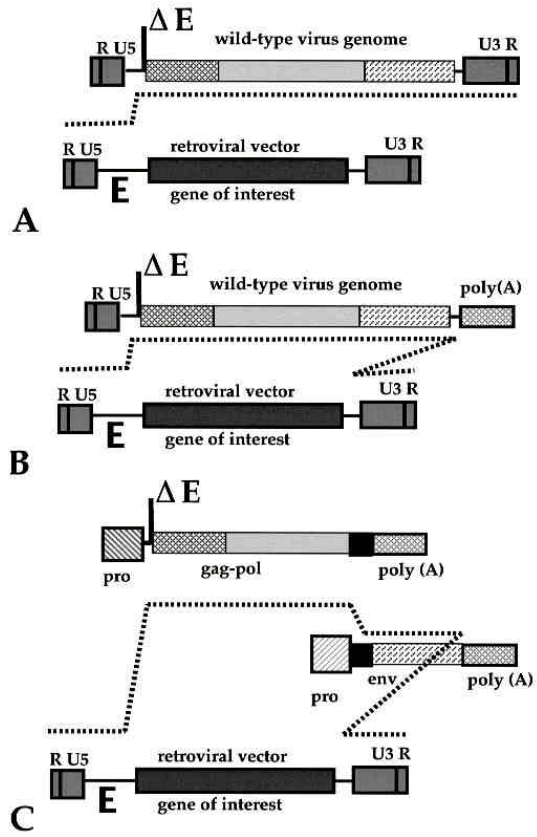

Figure 7. Generation of replication competent viruses. Overlapping regions of homology between the viral protein coding sequences and the retroviral vector genome can lead to the generation of a replication competent retrovirus (RCR). In the first retroviral vector system (A), one recombination event was sufficient to generate a RCR. Deleting viral controlling sequences and splitting the components of a helper cell into different plasmids reduces the regions of sequence overlap and increases the number of recombination events to generate a RCR. Consequently, in modern helper cells, the risk of RCR formation is greatly reduced (B and C).

homology between the retroviral vector genome and the gene units expressing Gag-pol and Env. Thus, the risk of sporadic generation of RCR has been further reduced and this helper cell system has remained free of RCR for more than six years (31) (see article in this issue "Reticuloendotheliosis Viruses and Derived Vectors for Human Gene Therapy").

Retroviral vectors derived from C-type retroviruses are very efficient gene delivery tools to stable introduce a gene of interest into the genome of a target cell. However, they normally do not infect quiescent cells and can only get access to the host genome during cell-division, when the nuclear membrane is dissolved (63). In contrast, lentiviruses (e.g., human immunodeficiency virus I, HIV-1, simian immunodeficiency virus, SIV, and feline immunodeficiency virus, FIV) are capable of actively penetrating the nucleus, and, therefore, inserting their genome into that of non-dividing cells (64-68). Thus, efforts began in several laboratories to develop retroviral vector systems derived from lentiviruses (48-50,69) (see also "Vectors Derived from the Human Immunodeficiency Virus, HIV-1" by Barker and Planelles, this issue). Lentiviruses carry additional genes (accessory genes) involved in replication. Thus, the construction of retroviral gene transfer systems derived from lentiviruses is more complicated. Further, the safety of HIV-1-derived vectors for human gene therapy is being questioned by many scientists. To bypass this problem, my laboratory recently developed SNV-derived vectors which contain a nuclear translocalization sequence in the viral matrix (MA) protein. We found that such vectors infected quiescent human T-cells, macrophages, and neurons (70).

5. HOST RANGE OF RETROVIRAL VECTORS

In the first generation of retroviral vector systems, the envelope protein of the original virus has been used to construct helper cells. Consequently, the host range of the vector virus particle was restricted to the host range mediated by the retroviral envelope protein of the virus from which the vector has been derived (14). For example, vector virus particles derived from REVs infect a large variety of avian and some mammalian cells, but do not infect rodent, primate, or human cells (71). Thus, although the first retroviral vector system has been derived from REVs, further retroviral vector research focussed on the development of vectors derived from ampho-MLV, which can infect a very large variety of mammalian including human cells. In particular, extensive research and vector development in the laboratory of Dr. Dusty Miller led to the first MLV-derived retroviral vector system, which has been used in first human gene therapy trials (72-75).

However, MLV-derived vectors were also found to have some other limitations. For example, they poorly infect cells of the human hematopoietic system, because such cells do not express the receptor recognized by the MLV envelope protein (76). Some shortcomings imposed by the nature of the envelope have been overcome by the construction of systems in which the viral envelope protein has been replaced with that of other viruses. E.g., in MLV-derived vector systems, the envelope of gibbon ape leukemia virus (GaLV) or the envelope (G protein) of vesicular stomatitis virus (VSV), a rhabdovirus, have been used (77-81). This process is termed pseudotyping. It has been known that retroviruses can utilize envelope proteins of most other retroviruses or non-retroviruses if the own envelope protein is not expressed (77).

In recent years, considerable efforts have been made to further widen or restrict the host range of retroviruses by pseudotyping. For example, MLV- or HIV-1-derived vectors have also been pseudotyped with envelope proteins of other murine retroviruses like neurotropic Friend murine leukemia virus (82) or Mus dumni endogenous retrovirus (83), human foamy virus (84), the feline endogenous retrovirus RD114 (85), jaagsiekte sheep retrovirus (86), lymphocytic choriomeningitis virus (87), fowl plaque virus

hemagglutinin (88), or even the envelope of ebola virus (89). Pseudotyping of REV-derived vectors with the envelope of a neurotropic rabies virus enabled cell-type-specific gene delivery into neuronal cell (Dornburg, unpublished). However, pseudotyping is not completely unlimited. E.g., the envelope of HIV-1 cannot be incorporated into MLV-derived particles. However, it was found that truncation of the carboxy terminus of the envelope bypassed this shortcoming (90-92).

Pseudotyping retroviral vectors with VSV-G protein enabled concentration of the viral particles by ultracentrifugation. Vector virus titers of more than 10^9 infectious particles per ml supernatant have been reported after concentration. This concentration protocol cannot be performed with vector particles containing retroviral envelope proteins, because retroviral envelopes are rather fragile and get damaged during this process resulting in low amounts of infectious particles in the viral pellet (81).

VSV-G pseudotyped retroviral vectors have been proven to be very useful in a large variety of applications. Moreover, modern vectors derived from lentiviruses, such as the human immunodeficiency virus HIV-1, are almost always pseudotyped with the VSV-G protein, because of the advantage of virus concentration and because the HIV-1 envelope is cytotoxic and enables only the efficient infection of CD4-positive cells (50,65). However, VSV-G pseudotyped vectors also have some shortcomings: The VSV-G protein is cytotoxic as well, and, therefore, no stable packaging lines can be generated which continuously express this envelope. Thus, VSV-G pseudotyped vectors are generated by transfecting all plasmids necessary to form a retroviral vector particle into cells, which are highly permissive for transient transfection like human 293T cells. Such cells then efficiently transcribe and translate genes encoded in plasmid DNAs leading to relatively high amounts of vector virus production for a short time period. Virus is harvested from supernatant tissue culture medium 48 hours after transfection, concentrated by ultracentrifugation, and used to infect fresh target cells.

Transient transfection / infection protocols work well in the laboratory, but they are not practical for large scale production of vector viruses. Further, it is known that homologous and non-homologous recombination takes place in cells simultaneously transfected with several plasmids with rather high efficiency (6). This may lead to the generation of unusual gene transfer constructs. To bypass this problem, inducible systems have been developed. E.g., VSV-G has been expressed from the tetracycline controlled gene expression system (93-97). However, no inducible packaging cell lines have been obtained so far, which produce high titre vector particles.

Another shortcoming is the finding that VSV-G pseudotyped vectors are neutralized very rapidly by human serum (98). Thus, they cannot be used for direct *in vivo* human gene therapy. Further, VSV-G pseudotyped vectors do not allow cell-type-specific gene delivery, which will be another major prerequisite for future *in vivo* human application.

In general, neutralization of retroviral vectors by serum of a heterologous species had posed another problem for the use of retroviral vectors for *in vivo* gene therapy. It has been known for more than two decades that retrovirus particles produced in one species are rapidly inactivated by the serum of another (99-101). However, this problem can be bypassed by the construction of packaging cells derived from the same species (102-105). E.g., MLV-derived vectors produced from mouse packaging cells are rapidly inactivated by human serum, while MLV-vectors produced by human packaging cells are not (105). Another problem reported was the finding that proteoglycans produced by the packaging cell decrease the efficiency of infection of retroviral vectors (106,107).

6. CELL-TYPE-SPECIFIC RETROVIRAL VECTORS

All retroviral vectors currently used in human gene therapy trials have been derived from amphotropic (ampho) murine leukemia virus (MLV) containing wild-type MLV envelope or that of VSV. As outlined above, these vectors have a very broad host range and infect cells of various tissues of many species. Thus, if injected directly into the blood stream of a patient, the chances that the vector particles would infect their actual target cells are extremely low. Thus, the gene delivery has to be performed *ex vivo*: the target cells of interest are removed from the patient and cultivated in tissue culture. After infection with the retroviral vector, the cells are re-injected into the patient. However, *ex vivo* gene therapy has several limitations and cannot be applied on a broad clinical basis: besides extremely high costs (about $50,000 to $100,000 per patient!), the cultivation of cells in a non-physiological environment alters their natural long-term behavior (termed "homing problem"). Thus, even autologous cells cultivated *ex vivo* for several days have a short life term when they are reintroduced into a patient.

To overcome these problems, retroviral vector particles have been further modified to create cell type-specific gene delivery systems which would allow the injection of the gene delivery vehicle directly into the patient's blood stream or tissue of interest (26,108-110).

One of the first attempts to specifically deliver genes into distinct target cells has been performed in the laboratory of Dr. Varmus in 1990. Using retroviral vectors derived from avian leukosis virus (ALV), these authors incorporated the human CD4 molecule into virions to specifically transduce genes into HIV-1 infected cells (111). However, such particles were not infectious, probably due to the lack of a co-receptor, which HIV-1 needs to infect CD4+ cells.

In another attempt to target retroviral particles to specific cells, Roux *et al*., have shown that human cells could be infected with ecotropic-MLV, which is only infectious in mouse cells, if they added two different antibodies to the virus particle solution. The antibodies were connected at their carboxy termini by streptavidine. One antibody was directed against a cell surface protein,

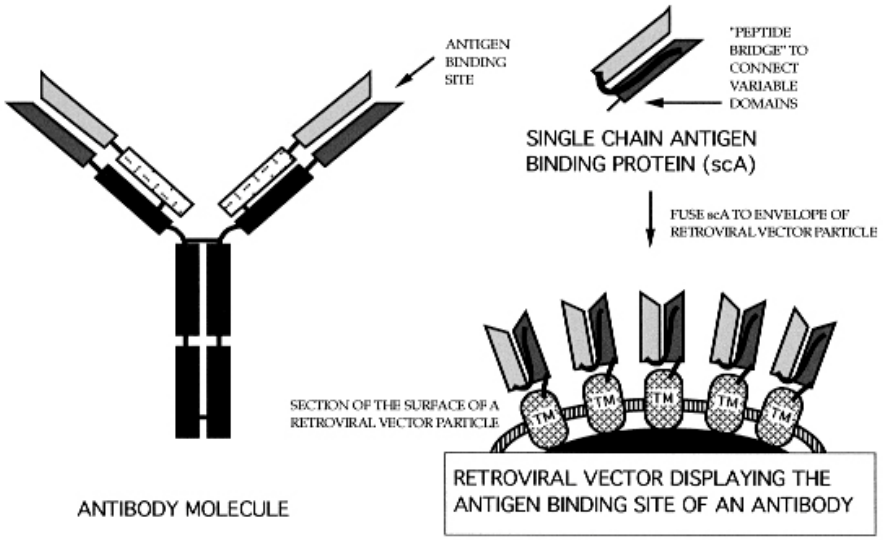

Figure 8. Display of the antigen binding site on retroviral vector particles. The coding region of a single chain antibody is fused to the envelope (e.g., transmembrane unit, TM) of a retroviral envelope gene. The fusion protein is incorporated into and displayed on retroviral vector particles.

the other antibody was directed against the retroviral envelope protein (112,113). Although this approach was rather inefficient (and was performed at 4°C), these data showed that cells that do not have an appropriate receptor for a particular virus can be infected with that virus, if binding to the cell surface had been facilitated. These data also indicated that an antibody mediated cell targeting with retroviral vectors was possible.

To overcome the technical problems of creating an antibody bridge, it was logical to incorporate the antibody into the virus particle. However, complete antibodies are very bulky and are not suitable for this approach. The problem has been solved independently by Dr. S. Russell in Dr. G. Winter's laboratory and my group using single chain antibody technology (114,115). Single chain antibodies (scA) have been developed for E.coli expression to bypass the costly production of monoclonal antibodies in tissue culture or mice (116). 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 (Figure 8).

Using hapten model systems, Dr. Russell and our group have shown that retroviral vectors that contained scAs fused to the envelope are competent for infection (114,115). In these experiments haptens were conjugated to the cell surface of cells, which are not permissive for infection with a particular retrovirus. However, after hapten conjugation to the cell surface, these cells could be infected with vector particles which displayed a scA directed against the hapten. My laboratory has also shown that SNV vector particles that display another ligand (we used SU of ecotropic (eco) MLV) were infectious on non-permissive hamster CHO cells, which were transfected with a plasmid expressing the eco-MLV receptor. However, infectivity was only observed when the wild-type envelope was co-present in the virus particle (114). Particles containing wild-type envelope alone were not infectious in these cells. This finding was verified by the group of Dr. Kan. This group showed that eco-MLV particles that displayed the hormone erythropoietin (epo) on the viral surface were infectious on human cells that express the epo receptor, but only, if the wild-type envelope was also present (117,118).

My laboratory then first showed that retroviral particles that display a scA directed against a human cell surface protein are infectious as well (119). Again, the presence of wild-type envelope greatly enhanced infectivity. These findings have been confirmed using MLV retroviral particles that display an scA directed against the LDL receptor (120). However, no or only minimal infectivity was observed with other scAs or targeting ligands displayed on MLV-derived vectors (121-124). In contrast, SNV vectors displaying various other scAs (e.g., anti-Her2neu or anti-CD34 scAs were highly infectious (23,125) (for a detailed description of SNV-derived targeting vectors, see article "Reticuloendotheliosis Viruses and Derived Vectors for Human Gene Therapy, this issue). Moreover, utilizing scA phage display technology it is now possible to screen for and identify novel scAs for display on SNV-derived retroviral vectors. E.g., using this technology, a scA has been identified and displayed on SNV vectors, which enabled highly-efficient infection of human T-lymphocytes (126).

Following these original studies, extensive research has been initiated in several laboratories to further develop cell-type-specific vectors, not only derived from retroviruses, but also from other viral vector systems like adenoviruses with various success (108,110). My

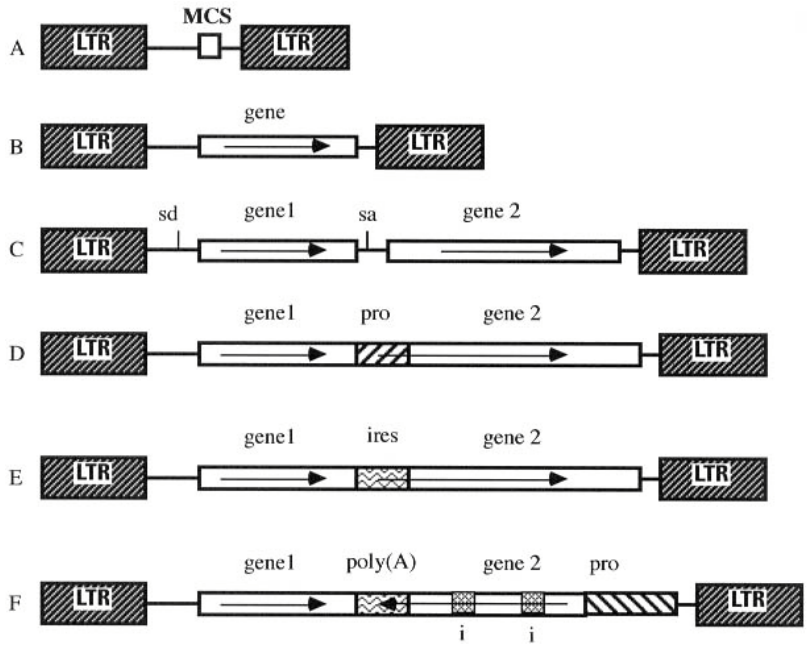

Figure 9. Retroviral vector genomes. In modern retroviral vectors almost all or all retroviral protein coding sequences have been deleted and replaced with the gene(s) of interest. A universal retroviral cloning vector is shown at the top (A). It only contains a multiple cloning site for the insertion of the gene of interest. (B): A retroviral vector containing one gene. (C-F): retroviral vectors containing two genes. The second gene can be expressed from a spliced RNA (C), an internal promoter (D), or an internal ribosomal entry site (E). (F): Vector that contain the gene of interest in the opposite vector orientation can contain introns (i).

laboratory gave first proof of principle that retroviral vectors displaying a scA enable cell-type-specific gene delivery *in vivo* as well (127).

The further development of cell type specific gene delivery systems will not only pave the way for *in vivo* human gene therapy, but will also have a major impact on biomedicine: for example, it would be possible to create animal model systems for specific diseases for which no animal model systems exist (e.g., by targeting viral or toxic genes into distinct organs). However, many questions regarding specificity and efficiency still remain to be answered and more research is still necessary to bring this technology into the clinic.

7. RETROVIRAL VECTOR GENOMES

In modern retroviral vector genomes, most or all viral protein coding sequences are removed and replaced by the gene(s) of interest (2,128-130). Such vectors only retain all cis-acting sequences required for the encapsidation of the vector genome into retroviral core particles and all sequences necessary for the reverse transcription and integration into that of the target cell (e.g., primer binding site, polypurine tract, attachment sites, etc). For many vectors systems, "universal cloning vectors are now available which only contain a multiple cloning site (a short DNA sequence containing several restriction enzyme sites) for the easy insertion of the gene of interest (Figure 9A).

7. 1. Standard retroviral vectors

In the past two decades, hundreds of different retroviral vectors have been constructed containing various genes of interest. Although more than 3,000 pier reviewed papers have been published reporting the use of retroviral vectors for gene transfer, there is no report that a particular gene cannot be transferred and expressed in a retroviral vector (recently reviewed in references 2,128-130). However, some genes appear to be transferred more efficiently than others. Figure 9 illustrates some examples of retroviral vectors. In most retroviral vectors, genes are inserted in the same orientation to the vector and expressed from the long terminal repeat (LTR) promoter (Figure 9B). Such genes are always cDNA genes, because introns present in a vector genome would be spliced after transcription, and, therefore, not present in a provirus formed after retroviral replication. In vectors containing more than one gene of interest, the second gene can be expressed from either spliced RNA (Figure 9C), or using an additional internal promoter (Figure 9D), or using an internal ribosomal entry site (e.g., derived from a picornavirus) (Figure 9E) .

In many vectors containing two genes, one of the genes is a selectable marker gene (e.g., an antibiotic resistance gene). This design has two advantages: First, after transfection of the vector plasmid into the packaging cells, specific helper cell lines can be selected and established from single cell colonies, which transfer the gene of interest with high efficiency. Second, infected

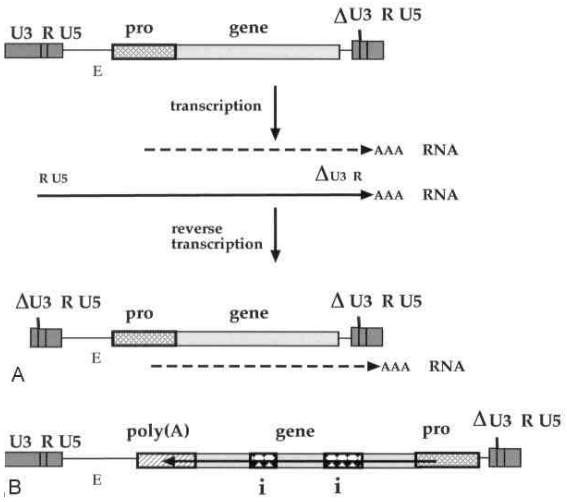

Figure 10. Self-inactivating (SIN) vectors. (A) SIN vectors contain a deletion of U3 sequences in the right LTR. As a result of retroviral replication, the provirus formed in the target cell does not contain U3 sequences. Thus the gene(s) of interest have to be expressed from internal promoters (pro). (B) example of a SIN vector containing a gene with introns (i).

target cells can be selected for antibiotic resistance. Consequently, all antibiotic resistant target cells contain the integrated retroviral vector provirus and, therefore, also express the gene of interest.

In all standard retroviral vectors, the gene(s) of interest is / are expressed from the retroviral LTR promoter. Thus, it is basically impossible to achieve regulated expression of the genes of interest. However, many eucaryotic genes contain a variety of transcriptional control elements, which are present in introns or which are dispersed over wide regions upstream or downstream of the protein coding sequences. To bypass this problems, retroviral vectors have been constructed, in which the gene of interest is inserted in the opposite direction to the vector genome and expressed from its own (cell-type-specific) promoter (Figure 9F). Genes which are inserted in the reverse orientation can contain introns. Such retroviral vectors can contain two genes. The first gene is usually in the same orientation as the vector and expressed from the LTR promoter, the second gene is in the reverse orientation and expressed from the internal promoter (Figure 9F).

Vectors, which contain a second gene in the opposite direction to the vector genome expressed from an internal promoter are not without problems. First, eucaryotic enhancers work in a orientation- and position-independent manner. Thus, the viral enhancers present in both LTRs can still interfere with the controlled transcription of the gene of interest from the internal promoter. Second, the internal promoter can interfere with and down-regulate the efficient transcription of the vector genome from the left LTR. As a result, the amount of retroviral vector particles produced from the helper cell is greatly reduced (131,132).

7.2. Self-inactivating retroviral vectors

As outlined above, in standard retroviral vectors, the gene(s) of interest are expressed from the LTR promoter or from a (usually very strong) internal promoter. This set-up is usually sufficient to investigate the biological activity and function of the gene of interest. In addition, in regard to human gene therapy of many genetic diseases and other applications, the level of expression of the therapeutic gene should be as high as possible and controlled gene expression is not essential.

However, in many applications, e.g., gene therapy of globin sickle cell anemia, it will be essential to achieve controlled expression of the gene of interest without any interference of the retroviral LTR promoter and enhancer. To bypass such problems, in 1986/87 the first generation of self-inactivating (SIN) vectors has been constructed (133-136). Moreover, SIN vectors have also advantages in human gene therapy, e., g., there is still some concern that vectors with two wild-type LTRs may occasionally lead to the malignant transformation of the gene transduced cell in some patients (see above), although this has not been observed so far.

As described above, in the course of retrovirus replication, the U3 region at the 3' end of the viral RNA is duplicated and attached to the 5' end of the DNA copy of the genome. This feature has been taken in advantage to construct vector plasmids, in which the U3 region of the right LTR has been partially or completely deleted, except a short region which interacts with the viral integration protein and which is essential for the insertion of the vector genome into that of the host. Transcription of the vector plasmid DNA in the helper cell results in an RNA, which does not contain U3, and, therefore no viral promoter an enhancer. Thus, after one round of retroviral replication, a vector provirus is formed, which is completely depleted of such sequences (Figure 10). Genes have to be expressed from internal promoters, which can be cell-type-specific. Thus, self-inactivating vectors bypass the problem of viral promoter / enhancer interference to achieve controlled gene expression in a cell-type-specific manner. E.g., vectors can be constructed in which the gene of interest is only expressed in very distinct cell types which express the transcription factors specific for the promoter in the vector.

Unfortunately, self-inactivating vectors derived from murine leukemia virus did not yield high vector virus titers for unknown reasons. Self-inactivating vectors derived from REVs have been shown to work with high efficiency (135). Lentivirus-derived vectors containing U3 deletions have also been shown to be very useful in many applications and are on the way to become standard in lentiviral vector mediated gene transfer, because they are considered safer than standard vectors for human gene therapy.

Regarding the overall level of gene expression, it also has to be considered that the integration site in the host genome plays a significant role in the efficiency of gene expression. Since retroviruses integrate their genome into that of the host in a rather random fashion, in some cells the

gene of interest may be inserted in a transcriptionally very active site resulting in high levels of gene expression. In other cells, the vector may integrate into rather inactive site of the host chromosome and gene expression may be rather low, independent from the promoter used.

8. LIMITATIONS OF RETROVIRAL VECTORS

Although there is no report that a particular gene can not be transduced in a retroviral vector, there are some limitations other than those already outlined above. For example, the average retroviral genome of C-type retroviruses is about 9,000 bases, about 1,500 of which are cis-acting sequences required for the replication of the viral genome. Thus, the length of foreign DNA that can be inserted into a retroviral vector measures about 7 to 8 kbp. Some cDNA genes exceed this length and, therefore, cannot be transduced in retroviral vectors.

Another problem in standard retroviral vectors can be the viral promoter. LTR promoters are usually very strong in many cell types. Eg., the LTR promoter of reticuloendotheliosis viruses is very strong in avian and many human cells. However, it is very weak in rodent cell, which makes it difficult to use standard REV vectors in mouse model systems (137). To bypass this problem, my laboratory has recently constructed retroviral vectors, in which the original viral promoter and enhancer in both LTRs have been replaced with the very strong immediate early promoter of cytomegalovirus (CMV).

Another problem with retroviral vectors expressing the gene from the LTR promoter is promoter shutdown. E.g., it has been reported that the promoter of murine leukemia virus is shut down in some host cells after some time due to methylation of vector sequences (138). This problem can be bypassed with vectors containing an internal cellular promoter as described above.

9. PERSPECTIVE

Retroviral vector systems are very efficient gene transfer tools. In the past decade, much progress has been made to overcome shortcomings observed in early systems (2,130). More and more sophisticated vector design now enables cell-type-specific gene delivery using vector particles that display genetically engineered envelope proteins. Vectors with internal promoters enable (therapeutic) gene expression only in distinct cell types. Moreover, the growing number of human gene therapy trials using retroviral vectors as gene delivery vehicles has now led to efforts to manufacture retroviral vector particle preparations in large bioreactors (129,139-141).

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