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Retroviral vectors

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Retroviral vectors

Applications and aspects of biosafety

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INDEX

page

Why Virus? 1

Conventional techniques for gene transfer 1
Virus-mediated gene transfer 3

Applications of Retroviral Vectors 6

Applications in research 6
Construction of transgenic livestock 8
Use in human gene therapy 10
Industrial applications 11

General Aspects of Safety 13

Physical containment 14
Biological containment 17

Strategy for Biological Containment 19

Retroviral genome and life cycle 19
Principles of complementation
in retroviral systems 22
Principles for generation of
safer retroviral systems 23

Retroviral Vectors and Packaging cells 26

Generation of wild-type retroviruses 26
Vectors: Safety analysis 29
Packaging cells: Safety analysis 35

Conclusions and Perspectives 41

Conclusions 41
Perspectives 43

Figures 45

References 52

Introduction

The report "Retroviral Vectors: Applications and Aspects of Biosafety", represents a risk analysis of the uses of retroviral vectors in biotechnology.

These vectors are the laboratory analogues of oncogenic retroviruses which have been modified to accept foreign gene sequences. Their most common use in industry, agriculture, human and veterinary medicine is to introduce foreign genes into cells for the purpose of modifying various aspects of gene regulation and expression.

Vectors derived from retroviruses are useful for the stable introduction of genes either into animal cells in culture or for the production of transgenic animals. The advantages of retroviral vectors are the efficient infection and transformation of most kinds of target cells, the wide choice of different host/vector systems, and the ease of manipulation of the retroviral genome. Their use in experimental cancer therapy in humans has been approved in 1989 by the Food and Drug Administration and The National Institutes of Health in the USA.

Together with the increasing interest in the use of these vectors for commercial production is the need to understand the broader aspects of their application and the risk factors associated with their use. The present report represents a broad discussion of the subjects connected with the application of retroviral vectors, their safety to the human health and the environment, and choice of strategies for containment.

The report is written by Holger Riemann from the Genetic Engineering Group (Danish Technical High School, Denmark) and has been developed as a joint venture between scientific members of the Genetic Engineering Group, The National Food Agency of Denmark, and Novo-Nordisk (Denmark). Members of the steering

group who had input into the development of the project were Poul Andersson, Ph.D., Genetic Engineering Group, Nanni Din, Ph.D. and Søren Carlsen, MSc., chem.ing., Novo-Nordisk, Ib Knudsen, DVM (chairman), and Ilona K. Sørensen, Ph.D., National Food Agency of Denmark, Institute of Toxicology.

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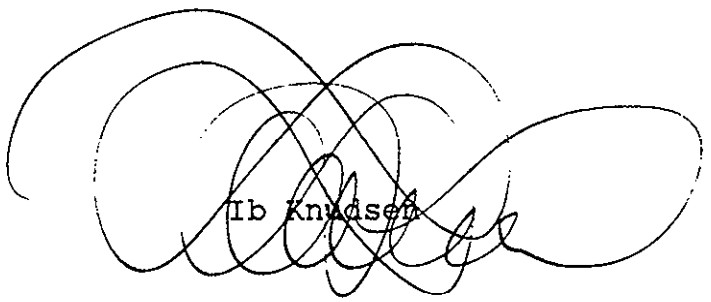


WHY VIRUS?

During the last 10-20 years there has been an explosion in the field of genetic engineering which initially only applied to manipulation of genes in bacteria.

Powerful as it is, cloning in bacteria cannot answer a range of questions related to functions of regulatory regions in eukaryotic DNA, nor questions related to posttranscriptional or post-translational modifications of eukaryotic gene products. Therefore, it is desirable to return the cloned genes into eukaryotic cells where their functions can be studied.

An efficient way to introduce and express genetic material in eukaryotic cells is to use viruses as vehicles. Firstly, the infection of target cells with virus is a highly efficient process, i.e. all cells in a population may be targetted. Secondly, the intracellular handling of introduced genetic material may be facilitated by having the novel genes in the context of a replicable virus genome.

However, the factors that make viruses attractive as tools in eukaryotic genetic engineering do at the same time raise significant safety issues: the risk of spread of the recombinant virus beyond its intended host cells. This question will be addressed in later chapters.

Conventional techniques for gene transfer

Genetic engineering of higher eukaryotic or mammalian cells requires efficient introduction and expression of genes in these

cells. Since the well-known methods used for manipulation of genes in prokaryotes cannot be transferred to eukaryotic systems, new techniques for transferring genes into eukaryotic cells, tissues, and intact animals are required.

Historically, the pursuit of effective means for gene transfer into eukaryotic cells has involved a wide variety of methods based on many different approaches.

Chemical methods, such as calcium phosphate and dextran sulphate mediated transfections, in which DNA is introduced into cells as part of a co-precipitate with the chemical in question. The precipitate facilitates transfection by enhancing the adsorption of DNA to cell membranes and also acts to limit the digestion of DNA by DNases after uptake.

Encapsulation of DNA into lipid vesicles (liposomes). Liposomes interact with DNA spontaneously, can be fused with tissue culture cells and thereby deliver functional DNA into the cell.

Physical means, such as scrape loading, sonication loading, electroporation, and microinjection. In the scrape loading technique adherent cells are scraped from a surface; in the sonication loading technique cells are exposed to ultrasound; and in the electroporation technique cells are exposed to an electric field. The three procedures facilitate uptake of extracellular DNA by inadequately understood mechanisms involving damage of the cell membrane. The underlying principle in microinjection is more straightforward, i.e. a needle is used to deliver the DNA directly to the cell nucleus.

However, all these methods result in totally random integration with respect to the organisation of the introduced DNA and suffer from one or more disadvantages related to either cellular toxicity, poor reproducibility, inconvenience, or inefficiency of DNA delivery.

Virus-mediated gene transfer

During the last ten years, a new gene transfer technology has emerged which appears to be superior to the DNA transfection and other previous techniques. This new technology exploits natural biological principles and is known as virus-mediated gene transfer in which viruses are used as vehicles to deliver genes into cells.

The techniques make use of the only potential gene transfer systems currently available for higher eukaryotic systems, namely derivatives of the genomes of DNA viruses or those RNA viruses that replicate via a double-stranded DNA intermediate. Two properties of the viruses are important in this context. Firstly, that viruses can be used to infect cells thereby delivering their genetic material at high efficiency. Secondly, the handling of the genetic information introduced into the cells in the form of a viral genome will follow predictable biological patterns. Properly engineered, these vehicles for recombinant DNAs can function as shuttle vectors for prokaryotic and eukaryotic systems.

DNA viruses

The papova group of viruses are perhaps the most thoroughly studied of all DNA viruses, and several members have been

adopted for the use as vehicles for gene transfer and proved useful in many lines of work: SV40, Polyoma, and papilloma viruses. In addition, members of the Pox virus group and the Herpes virus group are commonly used, e.g. vaccinia virus and Epstein-barr virus.

There are, however, some major limitations to the usefulness of the animal DNA viruses as vectors. Most systems suffer from problems concerning either poor capability of accepting non-viral DNA sequences, a narrow host-range limiting their use to a few cell types, an inherent transformation capability causing profound effects on almost all processes in a cell, or cytotoxic effects related to the viral life cycle.

Retroviruses

Retroviruses are RNA viruses that replicate their genome through a DNA intermediate. In recent years the exploitation of retroviruses as vehicles for gene transfer has provided an important and versatile new method of introducing and expressing cloned genes in eukaryotic cells. Many features of retroviruses favour their choice for this purpose as an alternative to DNA mediated gene transfer methods or other viral systems.

Retroviruses have a relatively large capacity for insertion of non-viral sequences (in the range of 7-15 kb) and only a small part of their genomes are essential for the characteristic biologically behaviour of this group of viruses: the copying of the RNA genome into a DNA form and the subsequent integration of this DNA into the chromosome of the infected cell.

Retroviruses have proved exceedingly useful mammalian transducing agents because of the broad host range of different

viruses and the fact that almost every cell type is susceptible to infection by some retroviruses. In addition, their well understood mechanisms of infection, replication, integration and gene expression also favours their choice as vehicles for gene transfer.

With a few exceptions retroviruses do not transform cells in vitro and are non-pathogenic to recipient cells, i.e. are able to grow to high titers in cells without cytopathic effects.

The efficiency of infection of susceptible cells is extremely high, and since high-titered virus stocks may be obatined, it is possible to infect close to 100% of the target cells in a culture. Moreover, after infection a copy of the retroviral DNA becomes stably integrated in the host cell genome in a precisely defined manner, such that virtually all the infected cells can be made to express the genes carried by the virus. In addition, retroviruses carry powerful genetic elements that make high levels of expression possible in a wide range of cell types.

APPLICATIONS OF RETROVIRAL VECTORS

Retroviral vectors are constructed from naturally occuring retroviral DNA genomes by inserting non-viral genetic information. In most cases, parts of the non-essential viral information is deleted to make space for insertion of DNA.

There are, of course, the same basic and trivial problems of recombinant techniques involved in the construction of a particular retroviral vector as is the case with any other vector. The use of retroviral vectors requires a correct insertion of the genes to be expressed in an appropriate vector but once the correct recombinant virus is constructed the subsequent handling of virus stocks and infection of target cells is technically quite simple.

Cells that have integrated a copy of the vector DNA can be identified if the vector carries a gene coding for a selectable property such as resistence to a toxic compound (e.g the antibiotic G418 (Emerman and Temin, 1984)). Alternatively, identification of the cells may rely on a vector-born property which can be easily monitored such as enzymatic activities (e.g. beta-galactosidase (Sanes et. al., 1986)).

Application in research

The most widespread use of retroviral vectors is as general purpose expression vectors for experimentation in eukaryotic cell culture. Selectable genes may be used to study non-selectable genes. The two genes can be introduced into the same viral genome and the nonselectable gene can be traced by this genetic tag.

In addition to their use as general purpose expression vectors, the retroviral vectors harbour a range of special features enabling several more specialized applications:

Unique markers for chromosomes:
Retroviral infections can be adjusted to result in integration of a single copy of viral DNA within the host genome which may be assigned to a specific chromosome by standard methods. Retroviral vectors can therefore be used as unique genetic markers for individual chromosomes in somatic cell hybrids (Lugo et al., 1987), chromosome-mediated gene transfer experiments, and recombination studies.

Unique markers for cells:
The ability of retroviral vectors to provide easily identifiable markers can be exploited in cell lineage studies (Price et al., 1987). Pluripotential stem cells can be infected so that individual differentiated cell lineages can be traced, e.g. in bone marrow reconstitutions.

Transgenic animals:
Retroviral vectors can be used to introduce new, heritable traits into animals, i.e. to generate transgenic animals, by infecting fertilized ova in vitro with subsequent implantation into a foster animal (Stewart et al., 1987).

Generation of cDNA:
Retroviral genomic RNA can undergo splicing. This means that retroviral vectors can be used to remove intron sequences from inserted DNA thereby generating full

length cDNA copies of moderately sized genes (Dornburg and Temin, 1990).

Insertional mutagenesis:
Retroviral vectors can be used as insertional mutagens in cultured cells and transgenic animals (King et al., 1985). Incorporation of a marker gene in the vector, which is easily selectable in E. coli, may greatly facilitate subsequent recovery of sequences from the mutated locus.

Construction of transgenic livestock

Fertilized ova can be infected with recombinant retroviruses, either in vitro with subsequent implantation to a foster animal or in situ, and animals developing from such infected ova may carry the desired new trait.

Breeding better animals

One important goal for development of transgenic animals in different model systems is to facilitate breeding of livestock with specific, economically significant traits, e.g. to create animals that will be more efficient at utilizing feed, have leaner meat, grow to marketable size more rapidly, or will be immune to specific diseases.

The pioneering work on mice transgenic for rat growth hormone has turned out to be quite misleading for experiments on many other species. The mice model system only revealed the positive aspects of the procedure, namely that the amount of growth hormone to some extent could be controlled at will by changing the

expression of the rat gene (Palmiter et al., 1982). However, the experiments did not reveal the adverse physiological effects as seen in subsequent experiments with larger animals transgenic for growth hormone (Pursel et al., 1989). In almost all cases such animals suffer from a reduced ability to fight infections; they tend to die young; the females are infertile; and, furthermore, there is no consistent increase in growth rate.

As almost all transgenic animals are produced from microinjected ova and because the overall success rate for producing live transgenic animals by this method is still only one percent (Pursel et al., 1989), the discouraging results mentioned above might at least to some extent, represent a case of sampling errors.

The retroviral systems might be used to increase the success rate for production of live transgenic animals, as infection as well as integration occurs with high frequency. Yet another advantage of retroviral systems is the simple handling of these systems in contrast to the laborious work associated with microinjection into the pronuclei of fertilized ova. Especially in the case of the domestic fowl, introduction of DNA into pronuclei of chicken eggs is very complicated and most researchers agree that retroviral vectors provide an important tool (Salter et al., 1987).

Live bioreactors

Another important goal for development of transgenic animals is their possible use as production systems for such valuable recombinant proteins as pharmaceutical products. In this application, genetic information for the desired trait (e.g., production of a human pharmaceutical) is introduced into all cells of

the animal but expression of the genetic information is limited to certain tissues by using proper expression control systems.

In contrast to the development of improved livestock, the use of transgenic animals as production systems may not result in harmful physiological imbalances similarly to those reported in animals transgenic for growth hormone. An important strategy to avoid deleterious effects is to achieve compartmentalization of the products. Examples of possible avenues are the use of transgenic constructs that instruct mammary glands to secrete human pharmaceuticals (Pittius et al., 1988) or result in excretion of the product into the urine; and use of constructs in domestic fowl that lead to accumulation of the product in the eggs.

Since the transgenic animals used as live bioreactors are conventionally made by microinjection of single cell ova, the retroviral vectors may also in this case prove a better choice.

Use in human gene therapy

Several important human diseases are due to defects in single genes and with access to technology that allows introduction of new genetic information into cells, it might be within reach to cure some of these diseases (Friedmann, 1989).

The principle behind the gene therapy is that the required genetic information is introduced into somatic cells from the patient with subsequent engraftment of the cells back to the patient.

The haemopoetic system is particularly amenable as a source of

cells for gene transfer and a great amount of experimentation has been performed in mice models (Chang and Johnson, 1989). By retroviral infection of hemopoetic stem cells in bone marrow cultures and subsequent transplantation into mice in which the bone marrow has been depleted by irradiation, the entire haemopoetic tissue can be reconstituted with cells containing a retrovirus vector. Techniques of this sort suggests that it might be possible to treat certain genetic disorders in humans.

Genetic disorders affecting the haemopoietic tissue might be cured by such gene transfer but a major limitation is the difficulty in obtaining lineage-specific gene expression. Such lineage-specific expression of certain genes can only be obtained if the retroviral construct includes DNA sequences located at a considerable distance from the coding region. Apart from this, a general problem seems to be the maintenance of continued expression of the introduced gene (Chang and Johnson, 1989).

Genetic disorders of non-blood cells can only be cured by gene therapy employing haemopoietic cells in cases where the essential product normally is released into the blood in a non-regulated manner. Gene therapy of non-haemopoietic cells is still at a very early stage of experimentation (Eglitis and Anderson, 1988).

Industrial applications

Several important therapeutics for human use have to be produced in cells from higher animals due to protein modifications that are specific for such cells, e.g. glycosylation (Parekh, 1989).

The extensive knowledge of retroviral systems and their multiple

application possibilities make these systems extremely amenable for construction of industrial production systems.

The important characteristics which make retroviruses attractive in industrial production are manyfold. Especially the high efficiency of infection and the high level of expression make the application of retroviral vectors a striking alternative even to already existing and well-known systems. The retroviral vectors may be used as general expression vectors in two separate ways. Firstly, as vehicles for the genetic information encoding the protein of interest and, secondly, as vehicles for the genetic information necessary for immortalization of a given cell having some desired property in relation to production.

Furthermore, the well-understood mechanisms of infection, replication and integration, most certainly will contribute by making the characterization of the infected cells quite simple. This is of special importance for documentation of the production system and the products as required by regulatory agencies.

The fact that most companies are reluctant to release any information concerning their production makes it quite difficult to assess to which extent retroviral systems are already in use. Nevertheless, the commercial interest can be clearly seen from the fact that retroviral systems are being patented all over the world.

GENERAL ASPECTS OF SAFETY

All new techniques may impose hazards and stresses on the health and safety of those who exploit them and seek for new applications and approaches. The pursuit, in the laboratory and elsewhere, of the diverse potential applications of retroviruses is no exception.

To identify precautions to be taken, it is necessary to assess realistically the hazards that might be imposed on the laboratory workers and the community during and as a result of work with any particular retroviral system. The precautions should be appropriate to the organism being investigated, the techniques used, and the possible effects of the final construction.

Two different categories of precautions are considered:

Physical Containment — Refers to characteristics of the laboratory facilities, the equipment, and the work procedures that are aimed at reducing release to the environment and to minimize risk of worker exposure.

Biological Containment — Refers to properties of the biological system (the vector and the host) that are aimed at reducing survival and multiplication of the vector and host in the environment.

Physical Containment.

The level of physical containment required depends upon the host organism, the vector, and the nature of genetic information being introduced.

Human and animal pathogens, bacteria as well as viruses, have been classified into groups according to the risks they represent to the health of laboratory workers and to the human and animal community should they escape from the laboratory (Collins, 1983). The use of various bacteria and viruses for recombinant DNA work is regulated in the US by Guidelines for Research involving Recombinant DNA Molecules and the framework developed by NIH has been accepted with minor modifications in most European countries.

General guidelines

The Guidelines suggests four safety levels, Biosafety level 1-4, with an increasing stringency of physical containment. These levels and the appropriate conditions can be summarized as follows:

Biosafety level 1 is for agents of no known or of minimal potential hazard to laboratory workers and the environment. No special facilities nor equipment is required, but the laboratory personnel should receive specific training and supervision by a scientist with general training in microbiology or a related science.

Biosafety level 2 is suitable for work with agents of moderate potential hazard to personnel and the environment. Laboratory workers should receive specific

training in handling of pathogenic agents and supervision by competent scientists. Facilities with restricted access and the use of cabinets are prescribed.

Biosafety level 3 is suggested for handling agents of high potential risks to workers and the environment. Personnel should receive specific training in work with these agents and supervision by scientists experienced in this kind of microbiology. Under-pressured laboratories and the use of safety cabinets are prescribed and access is strictly controlled.

Biosafety level 4 is for experiments with agents that are extremely hazardous to laboratory personnel and the community. Strict training and supervision is required and the work is done in specially designed laboratories under stringent safety conditions and access is strictly limited.

An important element of physical containment is strict adherence to good microbiological practices. Consequently, all personnel directly or indirectly involved in experiments on recombinant DNA must receive adequate instruction concerning good laboratory practices, i.e. proper collection, transport, receipt, and handling of infectious material; proper containment of aerosols; appropriate decontamination during and after work; and application of protective clothing.

Safety of retroviruses

The Guidelines suggests that the physical containment of Biosafety level 1 is appropriate for experiments with most retro-

viral systems, the only exceptions being the cases of retroviral vectors which carry oncogenes or genes coding for molecules toxic for vertebrates and which at the same time have a host range that covers human cells as well as the ability to replicate in these cells.

Substantial amounts of data concerning the biohazards involved in the work with different retroviruses has accumulated over many years of laboratory work with the naturally occuring retroviruses of animal origin that are relevant in recombinant DNA work.

A large number of animal retroviruses infect human cells in culture (retroviruses from cat, monkey, and a few exotic viruses from mice) but there has never been reported any serological evidence of laboratory-acquired infections in humans. A great number of investigations has been performed in which blood samples from laboratory personnel have been tested for the presence of antibodies against retroviral antigens and none of these investigations has shown positive reactions (Andersson, 1988). This would indicate that the viruses can not infect human cells in situ or that no replication of the viruses take place after infection.

As some of the animal retroviruses are capable of inducing tumors in infected animals, studies have also been carried out to trace possible oncogenic effects in laboratory personnel. but no retrovirally related tumors has been reported (Andersson, 1988).

Throughout the years, the work with these retroviruses has been performed under the assumption that viruses which do not infect human cells represent a minimal risk and viruses with ability to infect human cells represent a moderate risk. Experiments with

retroviruses belonging to the moderate risk group have been performed under physical containment restrictions comparable to a level between Biosafety level 1 and 2 (Andersson, 1988).

Summarizing, the published data seem to justify the choice of Biosafety level 1 for work with most retroviral systems at least for small scale laboratory experiments and experiments which do not involve oncogenes in connection with a system that replicates in human cells. Thus research with the aid of retroviral system can be performed in most cases with no precaution other than level 1 physical containment.

Biological containment

Some applications of retroviral vectors may present safety problems that can not be solved by physical containment alone, i.e. by preventing escape of infectious particles from the contained environment. In these cases, it is appropriate to consider use of retroviral vectors that are specifically debilitated.

Large scale use of retroviruses in the pharmaceutical industry, i.e. growing large volumes of infected cells, increases the risk of accidental exposure of workers and the level of incidental release to the environment during daily operations as well the level in case of major accidents. This increased risk merely reflects the larger number of cells harbouring the retroviral vector in the industrial context.

Similarly, use of retroviral vectors in larger transgenic animals raises certain safety issues to be considered. Firstly, the facilities for raising the animals can not be made to correspond to physically contained facility according to the NIH

criteria. Secondly, many operations will unavoidably involve aerosol-generating procedures; and, thirdly, consumers will come in contact with the meat, milk, or of the animals. It is therefore essential that no infectious particles containing the retroviral vector are produced in the transgenic animals.

Also in case of use of retroviral vectors for human gene therapy, it is of obvious importance that the treated patients do not produce infectious particles containing the retroviral vector which might infect other cells in the patient or spread to other people by contact.

The important issue in biological containment of retroviral vectors is therefore to prevent generation of infectious particles which might be able to spread the genetic information contained in the retroviral vector. The ultimate, safe retroviral vector system is thus characterized by being a **ONE-TIME-ONLY DELIVERY SYSTEM** where the infection process is halted in the first infected cell.

STRATEGY FOR BIOLOGICAL CONTAINMENT

Biological containment is a wellknown concept in relation to prokaryotic systems where the containment is accomplished by genetic manipulations limiting the transmissibility of the recombinant vector or the growth properties of the host cell (Molin et al., 1987). Several attempts have been made to construct retroviral systems with a high degree of biosafety using the principles of biological containment. The containment strategies is solidly founded on the knowledge of the retroviral genome and life cycle.

Retroviral genome and life cycle

In order to explain how retroviruses can be utilized for gene transfer it is important to describe some of the salient features of their genomes and their mode of replication. For a review see Varmus and Swanstrom, 1984.

The retroviruses comprise a family of RNA viruses whose replication cycle is distinguished by the conversion of their RNA genomes into a DNA form, i.e. the viral genes are encoded in an RNA molecule rather than in a DNA molecule. The virus replicates through formation of a DNA copy of the RNA genome, and this process is catalysed by the virus-encoded reverse transcriptase. The life cycle of a typical retrovirus is illustrated in simplified form in Fig. 1.

Infection and establishment of the provirus.

A virus particle contains two copies of a single stranded RNA molecule, the viral genome. This diploid structure is embedded in a central protein core which is surrounded by a membrane coat carrying virally specified envelope glycoproteins on its outer surface. Infection of target cells is initiated by the interaction of these glycoproteins with specific cell surface receptors.

Upon penetration of the virus into a cell, the viral RNA is copied into a linear double stranded DNA form by reverse transcriptase molecules present in the viral particles. This involves a complex series of steps that includes site-specific binding of primers for the DNA synthesis and serial transfers of the nascent DNA strands between templates.

The DNA copy of the viral genome enters the nucleus where it integrates into the host cell genome to become indistinguishable from any other cellular gene. Most importantly, the integration is random with respect to chromosomal location but is extremely specific with respect to the structure of the viral genome.

The precursor for integration is a linear form of the viral DNA in some cases (Brown et al., 1989) and a circular form in other cases (Panganiban and Temin, 1984).

Expression of viral genes and production of viral particles.

All the viral genes are expressed from the integrated linear DNA form of the retrovirus genome which is known as a provirus. The provirus has a characteristic structure with a long terminal repeat (LTR) at both ends (Fig. 2C), and this form of the viral

DNA is the basis for construction of all recombinant retroviral vectors.

A provirus of a wild-type retrovirus, i.e. a virus capable of replication, contains three coding regions (Fig. 2C): gag, encoding virion capsid proteins; pol, encoding the virion reverse transcriptase and integrase; and env, encoding the virion envelope proteins responsible for recognition of cellular receptors which in turn determines the host range of the virus.

Non-coding regions found at the ends of the genome are important for genome replication, control of expression, and packaging of viral genomes into virus particles (Fig. 2C):

LTR The repeat structure found at both ends of the proviral DNA; is composed of sequences derived from different parts of the viral RNA genome during replication (Fig 2A): U_3 (from 3' end), R which are present at both ends of the RNA genome, and U_5 (from 5' end). The LTRs contain enhancer and promoter elements required for tissue specific viral transcription after infection.

phi Sequence that directs encapsidation of RNA genomes into virions (Ψ), Fig. 2A.

PB Sequences involved in replication of the viral genome (Fig. 2C). Primers for initiation of DNA synthesis binds to the PB- and PB+ sequences.

The promoter in the 5' LTR drives transcription of the integrated provirus while the 3'LTR provides a signal for polyadenylation of the transcript. Most retroviruses produce two

distinct RNAs in approximately equal proportions: a full-length genomic transcript and a smaller subgenomic mRNA which has undergone splicing and serves as a template for translation of the env gene products (see Fig. 2B).

The genomic length transcripts direct translation of the gag and pol gene products, but a proportion of them also serves as RNA genomes and is packaged into viral particles representing the next generation of infective viruses.

Progeny RNA, reverse transcriptase, and viral coat proteins assemble into virions and the nascent viral particles form by budding from the plasma membrane (Fig. 1). In most cases, this scenario has no apparent effect on the viability of the infected cell.

Principles of complementation in retroviral systems

In deciding on strategies for development of biologically contained retroviral systems, we can take advantage of naturally occuring examples of replication-defective retroviruses.

Retroviruses are natural vectors for transmission and expression of non-viral genes, the best known cases being viruses that induces tumors in their host species due to the presence of a cellular gene within the viral genome. Most natural recombinant viruses are replication defective, i.e. they are unable to complete a normal life cycle. This is due to the fact that important viral genetic information has been replaced by the non-viral gene (Weiss, 1984).

Such replication-defective viruses may spread to other cells if the essential functions are supplied by replication-competent

helper viruses infecting the same cell. This type of cooperation is called trans-complementation and the genes coding for the complementing factors are called **trans-acting** viral sequences. The trans-acting viral sequences are dispensable as their loss can be complemented in trans. These sequences comprise the viral structural genes gag, pol and env.

In contrast, the sequences involved in genome replication, control of gene expression, and packaging of viral genomes are indispensable and are termed **cis-acting** viral sequences. These comprise the two LTRs, the priming sites for reverse transcription, and the packaging signal.

The most logical approach to retrovirus design is to follow the precedence set by nature and to employ replication defective vectors which require trans complementation of viral functions.

The fact that the trans- and cis-acting sequences are nicely separated in the provirus (Fig. 2C) makes it easy to insert non-viral sequences in such regions of the viral genome that can be complemented in trans.

The principle of trans-complementation constitutes the basis of almost all retroviral systems. In relation to biosafety precautions, the exploitation of the principle of trans-complementation is the only approach contributing to the innovation and development in biological containment of retroviral vector systems.

Principles for generation of safer retroviral systems

The all-important issue in creating biologically contained retroviral systems is, as mentioned above, the use of

replication-defective systems as replication-competent vectors have the potential to perpetuate virus spread.

In order to generate infectious virus particles from a replication-defective retroviral vector, vector DNA is introduced into a cell containing all the necessary factors required for trans-complementation.

Introduction of the vector DNA into a cell, is performed by conventional DNA transfection procedures. This inconvenient step is unavoidable since the proper and effective encapsidation of viral RNA genomes cannot at present be executed in vitro but only in vivo.

The host cell receiving the DNA must harbour all the genes coding for the necessary trans-acting factors required for packaging of viral RNA into virions and subsequent release of infectious virus particles. In the most primitive form these trans-acting factors are supplied by a wild-type replication-competent retrovirus introduced into the cell by infectious virus particles or by DNA transfection of the provirus.

The use of replication-competent helper viruses as a source of trans-acting factors is, however, inconsistent with biosafety, and, therefore, various helper viruses which are defective in the cis-acting sequence for packaging (Fig. 2A) have been developed. Such defective helper viruses are unfortunately less effective than packaging-proficient helper viruses (Mann et al., 1983).

The packaging-defective helper viruses are used as follows. The helper genome is introduced into a cell by DNA transfection to become stably integrated into the cellular chromosome. Such

cells, termed **packaging cells**, thus harbours a mutant provirus that cannot itself be packaged into mature virions but which codes for all trans-acting factors required for replication. Fig. 3 exemplifies the basic principles for the procedures necessary when using a packaging cell line. Standard packaging cell lines can be used with a multitude of different retroviral vectors.

The RNA transcribed from the helper genome in the packaging cell codes for the trans-acting factors but lack the cis-sequences needed for encapsidation. As a result, the virus particles released from a packaging cell line transfected with DNA of a retroviral vector contains only the recombinant vector RNA together with helper-virus encoded trans-acting factors. Since the recombinant viral RNA is defective for some or all of the sequences coding for trans-acting factors, the particles can infect only once.

Thus, the combination of a defective retroviral vector and a packaging cell line offers significant biological containment of the infectious virus particles as they constitute a **ONE-TIME-ONLY delivery system.**

RETROVIRAL VECTORS AND PACKAGING CELLS

A retroviral system based on the principles of trans-complementation should in theory be fully biologically contained i.e. function as a ONE-TIME-ONLY delivery system. In practice, however, this is not the case as most retroviral systems suffer from varying degrees of "leakiness".

The leakiness of a particular system can be observed as a very slow spreading of replication-competent wild-type viruses, an even slower spreading of the trans-defective vector, and rare phenotypic changes of the target cells (insertional mutagenesis), Danos and Mulligan, 1988.

Unfortunately, the ongoing research on retroviral vectors and packaging cell lines has revealed that the two main goals, efficiency and biological containment, are more or less incompatible phenomena. This means that the sole act of imposing extreme stringent biosafty precautions to a retroviral vector system, e.g. reducing the leakiness of a ONE-TIME-ONLY delivery system to nearly zero, might reduce the efficiency of the system and render is useless.

Generation of wild-type retroviruses

One of the aspects of leakiness of retroviral systems is the generation of replication-competent viruses in the packaging cell line or the target cell.

The generation of wild-type virus in a packaging cell containing a retroviral vector is primarily caused by recombinational events (homologous or non-homologous) between the three sources of retroviral elements of the system: (1) the trans-defective retroviral vector supplying cis-required sequences; (2) the cis-defective helper genome present in the packaging cells supplying the structural genes coding for the trans-factors; and (3) retroviral elements inherent to the normal genomes of the packaging cells, i.e. endogenous viruses supplying various parts of the retroviral genome.

In the target cell, the same processes and retroviral genetic elements can be involved in generating wild-type retrovirus: (1) the trans-defective retroviral vector is transcribed in the cell; (2) at low frequency the helper viral genome is encapsidated into an infectious particle and will undergo retroviral replication and integration into the target cell chromosome; and (3) the target cell, irrespective of the species, will contain endogenous retroviral sequences (Coffin, 1984).

The safety problem posed by the generation of replication-competent, packageable wild-type viruses arises from the fact that the recombinant vector may then spread between cells by rescue.

In addition, wild-type viruses capable of rescuing the recombinant retroviral vector can be exogenously derived. By superinfection of either packaging or target cells spread of the viral vector may occur. The criterion is, however, that the superinfecting virus encodes trans-acting factors that are capable of complementing the defective retroviral vector in question. The risk of superinfection can be easily controlled in the laboratories, whereas the problem is difficult to deal with in connection with transgenic animals or human patients.

As endogenous viruses are extremely widespread in eukaryotic cells, it is almost impossible to exclude the possibility of recombination events involving these retroviral genetic elements.

However, the main source of retroviral elements responsible for the generation of wild-type virus are the genomes of the trans-defective vector and the cis-defective helper, and they can be manipulated in such a way that the risk of recombination becomes negligible. The engineering of these genomes has focused on two things.

a. The removal of overlapping sequences responsible for homologous recombination thereby reducing the risk of de novo generation of replication-competent viruses. The non-homologous events are extremely rare and difficult to handle as they are poorly understood. Because helper genomes only supply transfactors and are not involved in later steps as are the trans-defective vector, their retroviral parts can be reduced to no more than the coding sequences for these trans-factors.

b. The construction of vectors resulting in proviruses (in the target cell) from which genome-like transcripts can not be produced. The absence of genome-like transcripts minimizes the risk of rescue and spread of the vector by endogenously generated or exogenously derived replication-competent viruses.

The attempts to avoid generation of wild-type virus and genome-like proviral transcripts will be discussed when describing the different types of vectors and packaging cells.

Vectors: Safety analysis

Almost all replication-defective retroviral vectors are derivatives of either murine or avian retroviruses. These vectors are replication-defective because the majority of the sequences coding for the structural genes have been replaced by the genes of interest. Most vectors have no special safety features other than the loss of trans-sequences and the level of biological containment for systems involving such vectors therefore depends on the design of the packaging cells.

The principally different types of replication-defective vectors and general methods of incorporation of new genes into the proviral backbone will be discussed.

Single gene vectors.

In the simplest retroviral vectors the structural genes of the virus are replaced by a single gene which is then transcribed under control of the viral regulatory sequences contained in the LTR (Pastan et al., 1988). Such single gene vectors have the advantage of simplicity over other types of vectors, and these vectors generally produce high titre virus stocks and the resultant proviruses do express the inserted gene successfully.

The main disadvantage of single gene vectors is the lack of a suitable selection for the presence and expression of the recombinant virus if the inserted gene is not selectable. The production of high titre stocks can often compensate for this but, in general, this type of vector is best suited for transmission of genes that elicit a distinguishable phenotype.

Double gene vectors.

Genes for which no convenient selection nor easily scored phenotype exists pose the problem of identification of infected cells harbouring the recombinant provirus. The solution has been to insert a second gene which encodes a selectable phenotype. In most examples of double gene vectors, the gene proximal to the 5'LTR is expressed from the genomic length viral RNA while two different strategies have been adopted to express the more distal gene:

a) either the gene is expressed from a separate subgenomic mRNA formed by splicing of the viral RNA i.e. **splicing vectors** (Cepko et al., 1984).
b) or the gene is expressed from an internal promoter inserted within the vector i.e. **vectors with internal promoters** (Kantoff et al., 1986)

SPLICING VECTORS mimic the mechanism employed by the parental retroviruses by using the viral splicing donor and acceptor sites to form a separate subgenomic RNA analogous to env RNA. Hereafter this becomes the template for translation of the distal gene while the proximal gene is translated from unspliced RNA analogous to the gag/pol mRNA (see section on Retroviral life cycle and genome).

There are two major drawbacks when using the splicing vectors. First, the expression of the two genes is dependent on the formation of the two mentioned viral RNA species which in turn depend on a properly regulated splicing process. In splicing vectors, the efficiency of viral splicing is sometimes severely altered leading to poor expression of one or another of the two genes carried. As the problems are caused by the inserted heterologous sequences, a particular vector may behave quite differently when different sequences are inserted.

The second drawback is the fact that the transduced genes are expressed from the viral promoter. This means that the usefulness of the vector is limited to the target cells where the viral promoter is active and to the specific promoter/enhancer properties of the viral LTR.

VECTORS WITH INTERNAL PROMOTERS *were designed to circumvent some of the limitations of* splicing vectors. By introducing an internal promoter, each gene is transcribed from its own independent promoter thus abolishing the need for efficient splicing. Nevertheless, the proximal gene is still expressed from the viral LTR. These vectors possess the flexibility of expressing the gene of interest in a manner most appropriate for a particular target cell.

Internal promoters have been inserted in retroviral vectors in either orientation relative to transcription from the viral LTR. When the internal promoter is oriented opposite to the viral promoter virus production is sometimes impaired as a result of transcriptional interference and possibly by an effect of antisense RNA. The advantage of this configuration is that the inserted gene can retain its own polyadenylation signal. When the internal promoter directs transcription in the same orientation as the viral promoter the presence of a second polyadenylation signal may severely impair virus production via premature polyadenylation of the viral genomic transcript.

The major drawback of vectors with internal promoters is the interaction of the two promoters that may result in poor expression of one or other of the genes (Emerman and Temin, 1984) . This sort of transcriptional interference applies to vectors with promoters in either the same or opposite direction, and the effect can be monitored as a poor expression of the nonselected gene.

Double copy vectors.

The unique feature of these vectors is that the transduced genes are inserted outside the retroviral transcriptional unit. These vectors were designed to circumvent the main drawback of the double gene vectors with internal promoters and thereby improve the expression of the transduced genes. The engineering strategy for double copy vectors exploits a curiosity in the retroviral mode of replication. During retroviral replication the 3'LTR of the viral RNA genome constitutes the template for the synthesis of both the 3' and the 5' LTR of the viral DNA. This means that any change introduced into the 3' LTR of the retroviral vector will be duplicated in the resultant provirus, hence the name double copy vectors (Hantzopopoulus et al.,1989).

In the double copy vector the translational unit of interest is placed 5' to the promoter in the U3 region of the 3' LTR (see section on retroviral life cycle and genome). After one round of viral replication the inserted sequences will be situated at both the original location and in the 5' LTR. While the copy in the 3' LTR are still within the retroviral transcriptional unit the new copy in the 5' LTR is outside since it is placed 5' to the viral promoter.

At this new position the negative effects of the retroviral transcriptional unit (promoter interference) are eliminated or at least reduced. This means that a proper expression of the transduced gene can be ensured in spite of selection for a marker gene expressed via the retroviral promoter.

Shuttle vectors.

It is often desirable to be able to recover recombinant proviruses from infected cells by molecular cloning, e.g. the cloning of a provirus is often required to examine the genetic structure of a retroviral construction after it has been through a cycle of viral replication. To avoid the laborious work associated with cloning by conventional methods (construction and screening of genomic libraries), retroviral vectors with the

ability to replicate in both bacteria and mammalian cells have been constructed (Cepko et al., 1984). The design of these so-called shuttle vectors can be transferred to any of the vectors described in this section.

Apart from the retroviral backbone and inserted genes a typical shuttle vector contains three things. A eukaryotic origin of replication from a DNA virus (e.g. SV40); a bacterial plasmid replication origin (e.g. from pBR322); and a selectable marker useful in eukaryotic as well as bacterial cells, typically the neo gene that confers resistance to G418 in animal cells and to kanamycin or neomycin in bacteria.

Self-inactivating vectors.

All the above mentioned retroviral vectors have as the sole containment feature that they depend upon the availability of trans-acting factors for replication, i.e. they are replication-defective.

The only successful attempts to create retroviral vectors with an additional degree of biological containment are the construction of the self-inactivating vectors (SIN vectors). As for other replication-defective vectors the self-inactivating vectors have no genes coding for trans-acting factors. In addition, the SIN vectors share the property that the retroviral promoter and enhancer elements are deleted during the viral life cycle. The proviral LTRs in infected cells are thus transcriptionally inactive (Yu et al., 1986).

The SIN vectors vectors were originally designed to eliminate the potentially undesirable effects of the viral promoter and enhancer sequences on regulation and expression of a gene under control of an internal promoter. The vectors are constructed with a deletion in the U3 region of the 3'LTR encompassing the promoter and enhancer sequences. Due to the nature of the retroviral replication this deletion is transferred to the 5' LTR of the integrated provirus (see Fig. 4).

Apart from circumventing the problem of promoter interference, the inactivation of the LTR accomplishes two things. First, it minimizes the possibility of activating cellular genes adjacent to the integrated provirus and, second, it improves the degree of biological containment by minimizing the risk of rescuing the defective provirus by recombination or by a superinfecting, replication-competent virus (phenotypic mixing).

The risk of successful recombination involving the provirus is minimized as the proviral cis-sequences cannot donate a functional LTR. The risk of rescuing by phenotypic mixing is minimized as the transcriptional products of the provirus are expressed from the internal promoter and thus have no packaging sequences. Should the RNA expressed from the provirus be packaged into a viral particle, however, the lack of all 5'cis-required sequences, including the 5' primer binding site for DNA synthesis, will inhibit the crippled genome from entering a second round of replication.

Although the titer of virus generated so far using these vectors has been low, SIN vectors are the leading candidates for potential use in human gene therapy.

Phi+ vectors.

In attempts to increase the efficiency of retroviral vectors, a key improvement in the design of murine virus-based retroviral vectors has been the discovery that the signal for packaging of viral RNA into virions extends into the gag gene. In early generation vectors the packaging signal (phi) did not contain any gag sequences and the inclusion of parts of gag in newer vectors provided a 10-fold increase in titer of vector virus (Bender et al., 1987). This extended packaging signal (phi+) yielded a corresponding increase in gene transfer efficiency.

The Phi+ vectors have a larger region of homology to sequences in the cis-defective virus genome present in the packaging cells, namely the gag region. This increases the risk of recombination leading to generation and spreading of replication-competent viral genomes and, hence, spreading of the vector.

This problem has been solved elegantly for some vectors. The approach has been to design a retroviral vector to a specific packaging cell so that the regions of homology are minimized and thus the risk of generating wild type virus by recombination between these specific defective genomes (Miller and Rosman, 1989). The main limitation to this approach is that such vectors can only be considered safe when used in connection with the particular packaging cell line.

Future improvements

In creating safer and more efficient retroviral vectors several of the above principles for vector design must be combined as two different safety issues need to be addressed: rescue of vector by phenotypic mixing and inadvertent activation of cellular genes by the proviral LTRs.

The combination of the principles of SIN vectors with the principles of the phi+ vectors designed to eliminate generation of wild-type virus in connection with a particular packaging cell line might improve the low titers of SIN vectors and at the same time increase the level of biological containment.

The future improvements in the development of safety vectors will be built on the accumulating knowledge of the details of the retroviral life cycle. One possibility would be to design retroviral vectors that inactivate themselves when integrating into the target cell chromosome, integrational inactivating vectors (ININ vectors), due to altered integration of the pro-

virus (Fig. 5). Furthermore, by combining SIN vectors and ININ vectors it should be possible to obtain a system in which the proviral transcript contained neither a 5' nor a 3' LTR. This combination would confer strong limitations to the ability of the vector to complete a second round of replication and, therefore, bring the ONE-TIME-ONLY delivery situation within reach.

We have attempted to develop an ININ-type vector based on the Moloney murine leukemia virus genome.

The design of MLV ININ vectors was built on the observation that spleen necrosis virus (SNV) appeared to use the circular form of the viral DNA as precursor for the provirus as shown by its ability to use a secondary viral attachment site for integration (Panganiban and Temin, 1984). In the circular viral DNA molecules the viral attachment site is the sequence created by the covalent joining of the ends of the two LTR's of the linear viral DNA (circle junction).

The concept of the MLV ININ vectors was based on using an attachment site located within the vector genome which, when used for integration, would result in non-genomic structure of the 'proviral' DNA as shown in Fig. 5. Integration through an internally inserted circle junction sequence would create a provirus linearized at this internal site. The effect would be that any transcript expressed from the proviral LTR has no 3' LTR and thus would be unable to replicate. The inactivation of the normal attachment site (Colicelli and Goff, 1985) should ensure that only the desired proviruses were generated.

In order to test the efficiency of the secondary site from the murine leukemia virus (MLV), vectors containing also the natural site was constructed. The two LTRs with the native site was used to express the neo marker gene and this transcriptional unit was placed inside the intron of a functional lacZ marker gene. The secondary site was placed outside the transcriptional units of the two marker genes.

Proviruses resulting from integrations via either the native attachment site or the secondary site should express the neo gene and confer resistance to G418. In contrast, only cells with proviruses resulting from integrations via the secondary site should express the lacZ gene and at the same time be positive for beta-galactosidase (see Fig. 6). Unfortunately, none of more than 2000 G418 resistant clones of infected cells were positive for beta-galactosidase suggesting that the secondary site was not used for integration.

Subsequent reports from other laboratories using detecting by Southern blotting and sequencing of DNA from infected cells (Lobel et al., 1989; Ellis and Bernstein, 1989; Fujiwara and Mizuuchi, 1988) have confirmed this result. It appears that the linear DNA of the viral genome comprises the main, if not the sole, substrate for integration of murine leukemia virus and that the apparently frequent use of secondary attachment sites in SNV based vectors might be attributable to experimental artifacts or misinterpretations.

Packaging cells: Safety analysis

Helper or packaging cell lines were developed for the propagation of replication-defective retrovirus vectors in the absence of wild type helper viruses.The aim was to generate cell

lines with stable expression of all the necessary trans-factors resulting in the production of virus particles containing the desired vector genome. Such cell lines are part of the biological containment systems developed for retroviral vectors, and improvements in retrovirus packaging cells have been focused on reducing the potential of these cell lines to produce replication-competent helper virus while at the same time maintaining high-titer production of retroviral vector virus.

The main safety issues considered in construction of novel packaging cell lines are: (1) reduction of the risk of recombination between the vector and the helper virus genome so as to prevent formation of replication-competent viruses; and (2) reduction of the risk that co-package of the helper virus genome with the vector due to phenotypic mixing will result in transmission of a replicative helper genome to the target cells.

The major strategy for reducing the risk of recombination is therefore to separate physically the components of the helper virus genome and thereby increase the number of recombinational events required to generate a replication-competent virus. This will at the same time make transfer of the packaging genome by co-packaging dependent upon recombinational events.

Single events.

Early packaging cell lines were based on retroviral genomes with deletion of the packaging sequence (phi), Mann et al., 1983. These helper genomes retain all other viral cis-sequences as well as all viral sequences coding for trans-acting factors. Deletion of the cis-acting packaging sequence decreases the assembly of the helper RNA genome into virions by a factor of 1.000 (Mann and Baltimore, 1985). Unfortunately, wild type

retroviruses can be generated by only a single homologous recombinational event between most vectors and these early packaging genomes (see Fig. 7). In addition, such packaging cell lines will produce a genome-like, replicative RNA molecule and co-packaging is a major problem (Danos and Mulligan, 1988).

Double events.

In order to reduce the possibility of homologous recombinational events leading to generation of wild type virus, several different packaging genomes with deletions of other cis-required sequences in addition to the phi-sequences were developed. As an example, the PA317 cell line harbours a e packaging genome which was crippled by deletion of the phi-sequences, minor deletion of the 5'LTR, and deletion of almost the entire 3'LTR (Miller and Buttimore, 1986). In spite of these changes only two recombinational events are required to generate wild type virus in connection with most retroviral vectors (Danos and Mulligan, 1988; see Fig. 8). However, the lack of the 3'LTR means that co-packaging of the helper virus genome will not result in transmission of the helper genome as this can not replicate.

Triple events.

One strategy for development of packaging cell lines that are almost completely refractory to generation of wild-type virus by homologous recombination is based upon complete separation of the genetic unit for gag/pol expression from the genetic unit expressing env (Watanabe and Temin, 1983). Rather than using the normal spliced and unspliced transcripts for translation of the viral proteins (see Fig. 2A,B), the gag/pol and the env

protein products are produced from separate transcripts under individual transcriptional control.

Such packaging cell lines have been developed based on helper genomes of murine leukemia virus origin. In the three cases described below, the two genetic units each consist of the entire MLV coding region but in each unit one component (gag/pol or env) has been inactivated. In addition, one of the LTRs is deleted which further increases their safety as discussed above.

As will be evident, no genome-like helper virus RNA is expressed and, hence, the risk of transmission by co-packaging is negligible.

In two of the packaging systems, the retroviral structural genes in both the genetic units remain under control of the viral LTR (Danos and Mulligan, 1988; Markowitz et al., 1988). In the third example a metallothionein (MT) promoter is used for expression (Bosselman et al., 1987).

One approach to inactivation of the non-required structural gene within a genetic unit has been deletion, i.e. the env gene is deleted in the gag/pol expressing genome while the gag/pol sequences are deleted in the env expressing genome. An alternative solution has been to eliminate translation of one of the structural genes within a genetic unit by point mutations.

All vectors contain additional safety features. In all vectors the packaging signal (phi) has been deleted. The two LTR-driven systems furthermore lack the 3'LTR and the MT-driven system the 5'LTR.

For all systems a total of three homologous recombinatorial events between the two packaging genomes and most retroviral vectors are required to produce a replication competent virus (see Fig. 9). In addition, none of the packaging cell lines express a genome-like helper RNA and no transmission by co-packaging is therefore possible.

Multiple events.

The latest approach to avoid generation of wild type virus has been the construction of retrovirus helper genomes with almost no nucleotide sequence homology to specific retrovirus vectors (Dougherty et al., 1989).

These packaging systems were constructed as above by employing expression of gag/pol and env from physically separated genetic units lacking all cis-acting retroviral sequences.

The range of cell types that can be infected by the particles produced in the packaging cell lines is determined by the nature of the env gene expressed. In these new packaging lines the gag/pol genes from the avian spleen necrosis virus (SNV) were co-expressed with either an env gene from an amphotrophic (broad host range) MLV or an env gene from SNV thus allowing propagation of MLV-based vectors and of vectors based on the avian reticuloendotheliosis virus (REV) without any risk of homologous genetic exchange between the vectors and helper sequences in the packaging cells.

The risk of generating replication-competent virus in these new packaging cell lines would appear to require multiple, non-homologous recombinational events. In addition, no genome-like helper RNA molecules are expressed and the risk of transmission by co-packaging therefore minimized.

Nevertheless the rare occurrence of replication competent virus in these packaging systems indicates that even with these helper cells with almost no sequence homology to vector sequences unwanted genetic events can occur. These genetic events might be caused by some nonhomologous process or by homologous events including noncontrollable viral elements such as endogenous viruses or superinfecting viruses.

The safety/efficiency issue revisited

At present there has been reports of packaging cell lines allowing efficient propagation of almost any type of retroviral

vector with targetting of the vector virus particles to almost all types of cells. The price to be paid for increased safety is, however, a reduction in efficiency.

As expected the packaging systems with the highest degree of biological containment are also the least effective and even these systems are not fully contained (Dougherty et al., 1989; Danos and Mulligan, 1988). Together the helper and vector genomes still retain all essential viral functions and apparently generate replicationcompetent virus through a complex and unlikely chain of events. In fact none of the available systems can be expected to be 100% biologically contained as endogenous viruses of the cells in the system (packaging cells as well as target cells) might contribute essential retroviral sequences in a non-predictable manner. For all practical purposes, however, the latest packaging systems with almost no homology between helper and vector genomes have a satisfying level of biological containment as the probability of unwanted genetic events is extremely low.

CONCLUSIONS AND PERSPECTIVES

Conclusions

Retroviruses have provided an attractive means of introducing exogenous DNA into eukaryotic cells. While it has been relatively easy to construct efficient gene transfer systems based on retroviral genetic elements, the development of an efficient retroviral system with a high degree of biological containment has proven quite elusive.

As no all-purpose system has been developed, different applications of retrovirus require differently designed systems and any safety issue, by implication, must be resolved on a case-to-case basis. The choice of the right retroviral system for a certain purpose must be based on the specific knowledge of the safety property of the specific system and on a realistic assessment of the level of biological containment required in the particular situation. The important decisional variables will be: (1) host range of recombinant retrovirus, (2) probability of generating replication-competent helper virus, and (3) the effects of the genetic material to be carried by the retroviral vector. A general problem to be addressed is the probability that insertion of functional LTRs in the chromosome of the infected cell might activate host cell genes.

In deciding on the type and level of bio-safety, the crucial point is to determine for a given application which steps in the procedure have to be contained. In case that physical containment is not considered sufficient, the strategy for biological containment should aim at reducing the probability of spread of infectious vector virus and, if appropriate, the probability of unwanted gene activation in the infected cells.

Research applications

In most cases of basic research it will be possible to use retroviral systems (vector/packaging cell line) with some degree of leakiness as containment can be ensured by physical means. The principal question in research applications is therefore the effects of the genetic information to be carried by the retroviral vector and the host range of the infectious particles, cfr NIH Guidelines Section III-B-3 'Caution'.

Industrial applications.

The main issue in industrial applications is that physical containment may not provide sufficient protection of the employees due to the large quantity of biological material handled in productions. It is therefore essential that the level of generation of replication-competent helper virus is kept at a minimum.

This can be ensured either by using the (ineffective) highly contained retroviral systems or, if more effective systems with a lesser degree of containment are used, by extensive screening of production cell lines for release of replication-competent virus.

Transgenic animals.

In construction of transgenic animals using retroviral vectors, two safety issues should be addressed: spread of the vector genome within the animal and to the surroundings; and inadvertent activation of cellular genes in the animal.

The natural approach would be to apply the non-leaky packaging cell lines in connection with SIN vectors. Alternatively, retroviral systems with some degree of leakiness might be used followed by extensive screening of the transgenic animals to ensure that only animals which do not transmit the vector or show peculiar phenotypic traits are used.

Gene therapy.

The safety issues are as above the risk of spread of the vector genome and the risk of activation of cellular genes by the viral LTRs.

Retroviral systems with some degree of leakiness might be used in cases where the target cells can be infected, selected, and screened for production of replication-competent retrovirus outside the organism, i.e. procedures involving the isolation of target cells from the patient with in vitro infection and subsequent reintroduction into the organism.

It is crucial, however, that the vectors employed do not allow inadvertent gene activation by introducing active LTR sequences nor formation of genome-like transcripts of the vector genome. Vectors based on principles like the SIN vectors should therefore be used.

Perspectives

A main problem to be addressed in future research is the development of standardized methods to measure the rather elusive phenomenon 'Biological containment'. At present novel retroviral systems are assayed for safety in many different ways

which do not allow direct comparison and which do not readily provide quantitative guidelines. The level of biological containment of retroviral systems is therefore expressed in probabilistic terms rather than in actual performance terms.

An important improvement in terms of safety would be the development of in vitro packaging of RNA which would eliminate the need for one of the component of the retroviral system, namely the packaging cell line.

The development of the SIN and the double copy vectors exemplifies the interaction between the basis research in retroviral biology and applicative aspects which will need to be further strengthened in the future so that more efficient, safe retroviral systems can be developed.

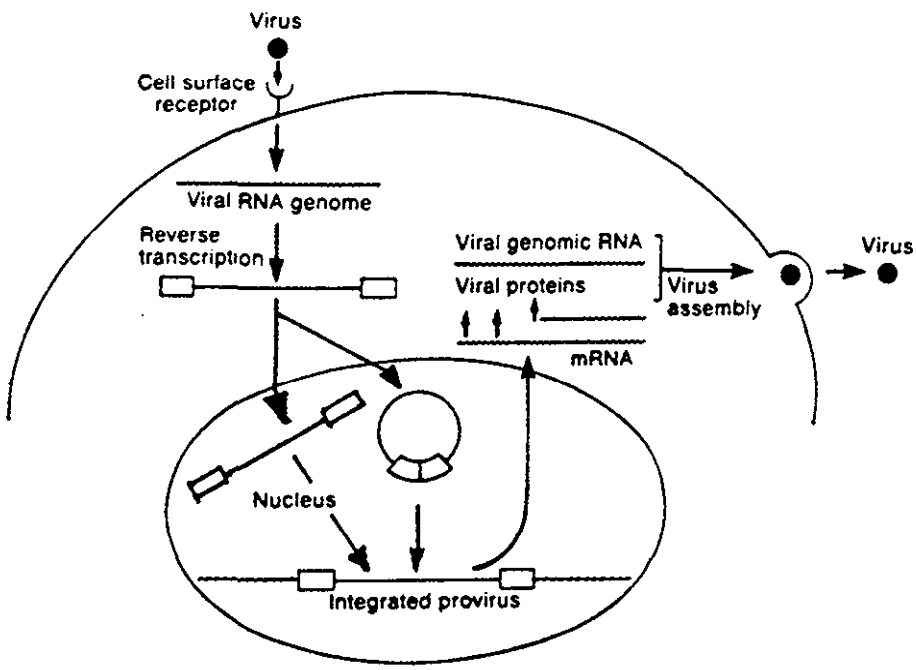
A possible efficient **ONE-TIME-ONLY delivery system** might be based upon combination of the SIN vector and the double copy vector with the additional design feature of having minimal sequence homology to the helper genomes of a packaging cell line. The packaging cell line might in turn be based on the principle of expression of the gag/pol and env genes from different genetic units.

The number of future applications of retroviral vectors would appear to be large from the literature on the topic. Possible novel applications include: targetting of the infectious particles to specific tissues which might lead to the possibility of in situ infection of animals or humans; the use of retrovirus for gene correction by homologous recombination (Ellis and Bernstein, 1989); and the development of new ways to infect cells without an appropriate receptor (Innes et al., 1990; Albritton et al., 1989).

Fig. 1

Modified from:

DNA cloning Vol. III
Ed. D.M. Glover (1987)
page 191

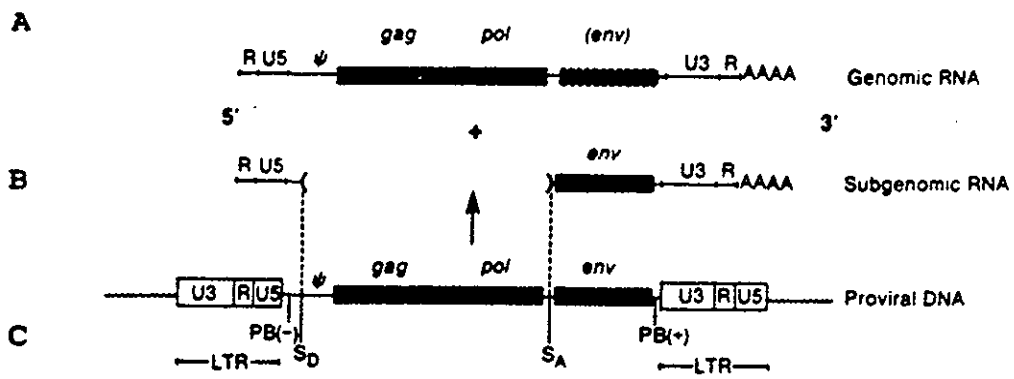


Retroviral life cycle. The precursor for the integrated provirus is either a linear or a circular form of the viral DNA. For further explanation see text.

Fig. 2

From:

DNA cloning Vol. III
Ed. D.M. Glover (1987)
page 192

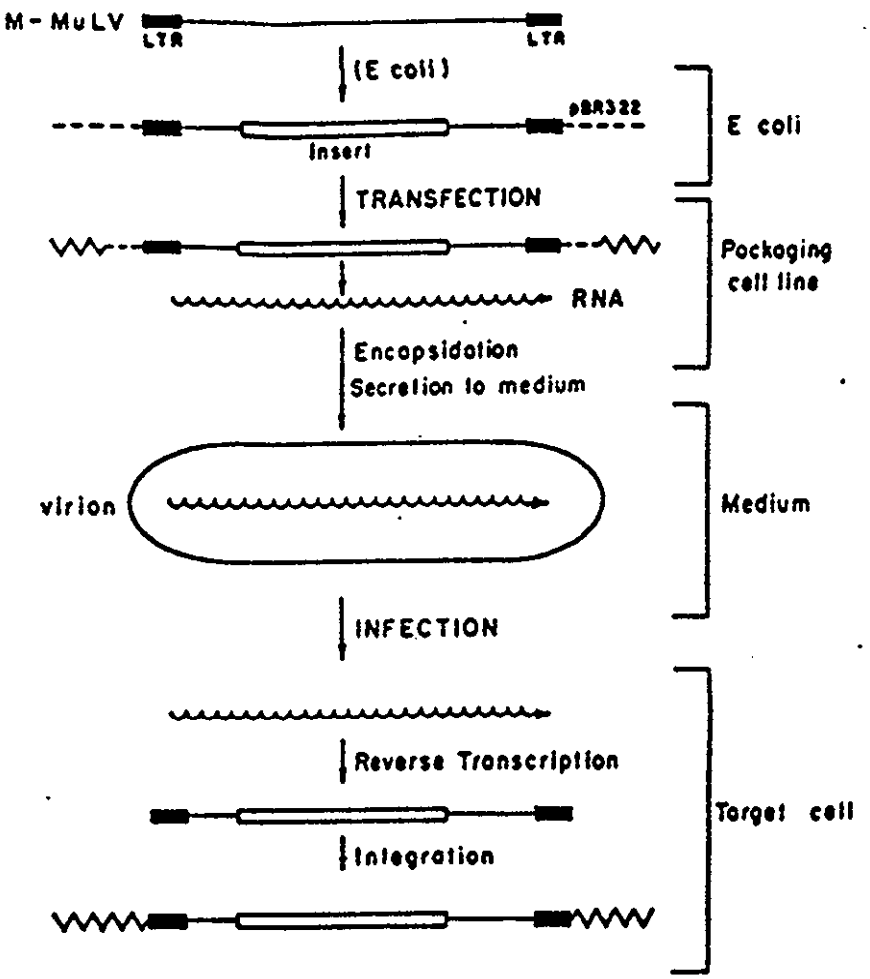


Structure of a retroviral provirus and its relationship to viral transcripts. For further information see text.

Fig. 3

From:

Bio Techniques Vol. 4, No. 6 (1986)
page 506.

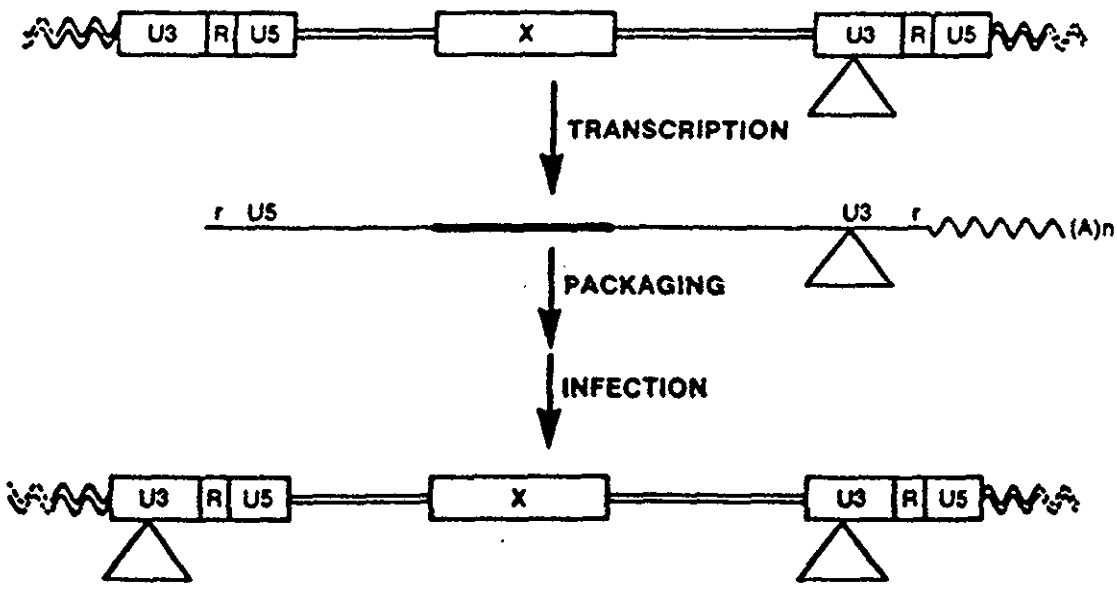


Principles for generation and use of recombinant virus. The necessary part of the native provirus is engineered and the resultant recombinant retroviral vector is manipulated as a bacterial plasmid in E. coli. The vector DNA is then transfected into the packaging cell line. For further information see text.

Fig. 4

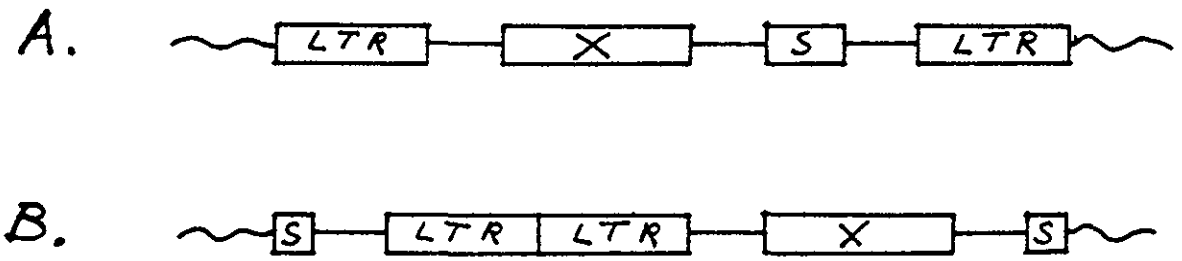
From:

Cold Spring Harbour Symposia on Quantitative Biology, Vol. L1 (1986), page 1023



Scheme illustrating the transfer of 3' LTR deletions (triangle) to the 5' LTR. Double lines represent double-stranded DNA, while the X represents the transduced gene(s). Cellular sequences flanking the provirus are indicated by wavy lines. The vector DNA is transcribed from the functional 5'LTR in the packaging cell. The resultant provirus in the target cell has no functional LTR. For further explanation see text.

Fig. 5

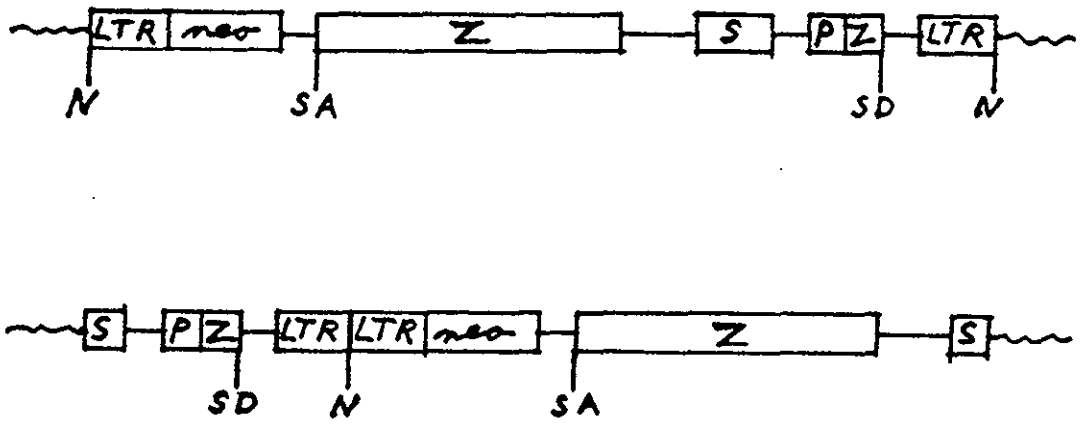


Integrational inactivation.

A. Packaging cell. The genomic structure vector DNA (both 5' and 3' LTR) is transcribed in the packaging cell.

B. Target cell. Integration through an internally inserted secondary retroviral attachment site (S) creates a provirus linearized at this internal site. The non-genomic structure proviral DNA has no 3' LTR. Wavy lines represent cellular sequences flanking the provirus, while the X represents the transduced gene. For further information see text.

Fig. 6

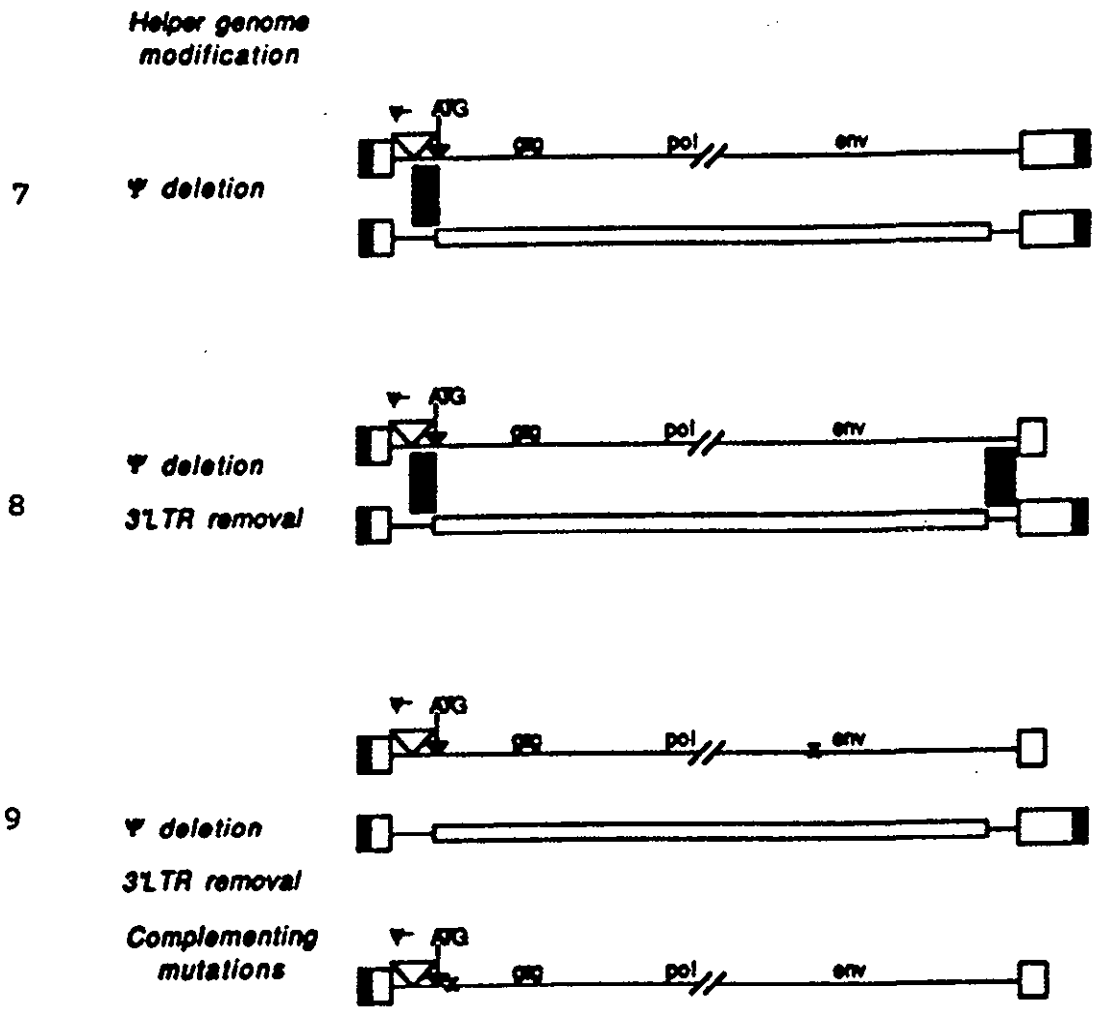


The two types of provirus resulting from integrations via either the natural (N) or the secondary attachment site(S). P represents the promoter of the LacZ marker gene (Z). SD and SA represent the splice donor and splice accepter sites flanking the intron of Z. Wavy lines represent cellular sequences flanking the provirus. For further information see text.

Fig. 7,8, and 9.

From:

Prox. Natl. Acad. Sci. USA (1988),
page 6461



Generation of helper virus by recombination between helper and vector genomes. The RNA genomes are presented. The helper genomes carry the gag/pol and env genes and the defective vector carry the transduced sequence depicted as an open box. Solid and open boxes at the extremities represent the R-U5 and U3-R terminal sequences. The point mutations are indicated by an X. The shaded areas indicate regions on the genomes through which homologous recombination can occur. In fig. 9 these regions are not indicated, but as an example a single crossover between the to helper genomes followed by a double crossover involving the trans-defective vector might generate helper virus. For further information see text.

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