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3 Retroviral Vectors

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3.1 INTRODUCTION

Viruses are obligate parasites and cannot replicate without the help of host cells. Thus they are, by nature, adapted to efficiently transmit genetic information to cells. Among the viruses many different strategies have evolved that allow them to enter cells, and the form of their genetic information to some extent determines the later mechanisms used for replication and production of new virus particles or virions.

Retroviruses are particularly of interest in this respect, because although they carry an RNA genome, there is a requirement that this genome be transcribed into a double-stranded DNA form in the infected cell, which can then be efficiently integrated into the host cell DNA. Once integrated, the virus DNA, known as a provirus, is transcribed like any other cellular gene and the virus-specific RNA is used to produce virus proteins and new genomic RNA, both of which are assembled into new virions. The integrated provirus is a stable part of the cell genome for the life of the cell and is passed on to all daughter cells that arise from the original infected cell. This latter property of retroviruses makes them ideal candidates for gene delivery vehicles, also known as vector systems, that give long-term gene expression.

In addition to the 'vector-friendly' properties of the retroviral life cycle, the knowledge gained from over 40 years of intensive research means that, in comparison to other viruses, the biology of these viruses and their interaction with the host cell is very well understood. It was realised very early on that these viruses can transmit cellular genes (Stehelin *et al.*, 1976) and consequently they were used as the first viral vector system. Although these early vector systems were very unsophisticated, relying on wild-type virus to transmit the recombinant genome carrying a marker gene (Shimotohno and Temin, 1982; Tabin *et al.*, 1982), improved systems have been created since and their evolution to ultimately create the perfect vector is still proceeding. Before discussing some of these systems, it is necessary to outline briefly the

salient features of the replicative cycle of retroviruses and how vectors are derived from these viruses.

3.2 THE RETROVIRAL REPLICATION CYCLE

The retroviral life cycle is summarised in Figure 3.1.

3.2.1 EARLY EVENTS

Retroviruses are enveloped viruses that carry two identical copies of a single-stranded RNA genome in the virus particle (for a review of retroviruses see Varmus and Brown, 1989). The outermost viral envelope protein (surface or SU protein) binds specifically to defined receptors that extend out of the target cell plasma membrane, triggering virus uptake by the cell (Figure 3.1). After release of the inner viral capsid from the viral envelope (uncoating), the viral genomic RNA within the capsid is converted into a double-stranded DNA form by the capsid-associated reverse transcriptase (RT) enzyme. This process initiates using a tRNA primer that is found in the capsid, specifically bound to the retroviral genomic RNA. During reverse transcription, unique sequences at either end of the viral genomic RNA are duplicated and placed at both ends of the newly synthesised DNA, generating a relatively long repeated sequence at each end of the DNA molecule, termed a long terminal repeat (LTR). The double-stranded DNA copy, flanked by the two LTRs, is then translocated to the nucleus (Figure 3.1).

3.2.2 INTEGRATION OF VIRAL DNA INTO HOST CELL GENOMIC DNA

After arriving in the nucleus, the double-stranded DNA form is integrated into the host cell chromosomal DNA by the virally associated integrase (IN) enzyme (Figure 3.1). The integrated DNA form is termed a provirus. Integration of the provirus is essentially random with respect to the host cell chromosomal DNA, although there may be some preference for actively transcribed regions. There are two major features of retroviral integration that are also relevant for gene therapy applications. First, the provirus is always found as a co-linear DNA with the structure LTR–retroviral genes–LTR. This is in sharp contrast to other integrated DNAs resulting from infection with other viruses or from naked DNA transfer, in which the transferred DNA is present in a permutated form. Second, the provirus is stably inherited by all the offspring or daughter cells of the originally infected cell, as if it were a normal cellular gene, usually without apparent deleterious effects. This contrasts with other viruses such as adenoviruses or

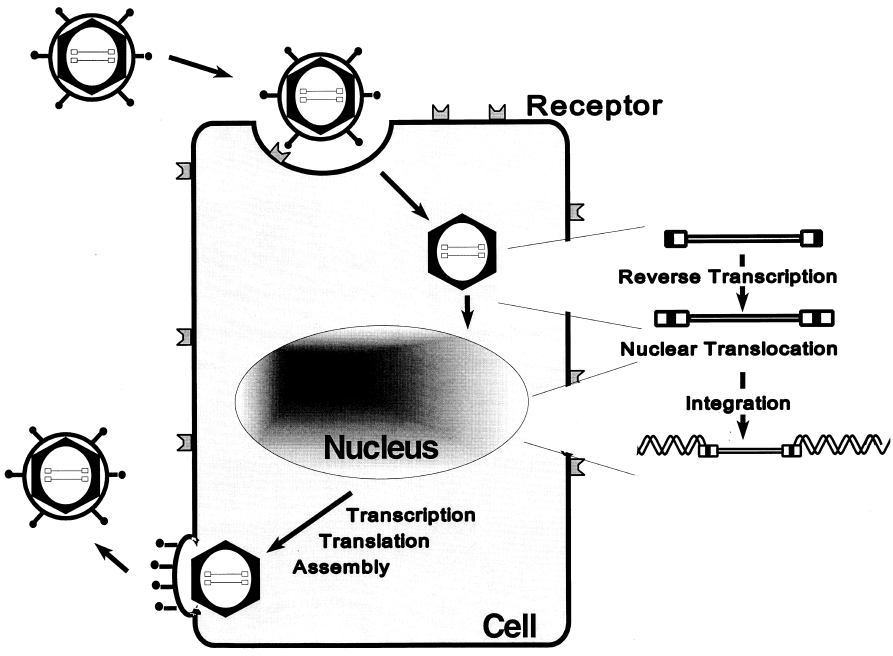

Figure 3.1. The replication cycle of a retrovirus. Retroviruses are enveloped viruses that carry two identical copies of genomic RNA. In order to infect a cell, the virus SU envelope protein (circles on stalks) interact with cell encoded receptors that are displayed on the plasma membrane. This interaction leads to the uptake and internalisation of the virus particle and the loss of the virus envelope, which occurs by membrane fusion. The viral genomic RNA is used as a template for the production of a double-stranded DNA molecule by the reverse transcriptase enzymatic activity, present in the core (shown in the upper inset). During this process, sequences at either end of the viral genome are duplicated and translocated (see also Figure 3.6). The complex then translocates to the nucleus, where the double-stranded DNA molecule is inserted into the host cell genomic DNA by the viral integrase enzyme (shown in the lower inset). The integrated DNA form of the virus genome, known as the provirus, is then transcribed like any cellular gene by the host cell transcription machinery from the viral promoter. Newly transcribed viral RNAs are used for the translation of new virus proteins and also as the genomic RNA for virus particles. The virus proteins and RNA assemble into new cores or capsids, which interact with regions of the host cell membrane that contain the newly synthesised virus envelope proteins, and newly produced virus particles bud out of the infected cell, where they undergo the final stages of maturation to form fully infectious virus particles.

pox viruses, which do not integrate their genetic information and which kill the successfully infected cell.

Retroviral infections in animals are often associated with tumour induction, notably leukaemias. This is a result of multiple integration events eventually leading to integration into, or in the vicinity of, cellular genes involved in growth control (proto-oncogene or tumour suppressor gene), thereby resulting in their dysfunction, a process known as insertional mutagenesis. This requires actively replicating (replication-competent) retrovirus capable of multiple successive integrations. Retroviral vectors are replication defective (see below) and only integrate once. The chance of a single retroviral integration occurring in such a gene locus is thus extremely low. Even if such a cellular gene were affected by retroviral integration, other genetic lesions would be required before a cell could be transformed to a malignant state. There is no evidence of retroviral integration causing tumours in humans. Nevertheless, it is imperative that no replication-competent virus is present in clinical stocks of retroviral vectors, and considerable effort has been devoted to this issue.

3.2.3 TRANSCRIPTION, TRANSLATION AND ASSEMBLY

Transcription of the integrated provirus is directed by the viral promoter and enhancer elements, located in the 5′ (left-hand) LTR and terminated in the 3′ (right-hand) LTR. Retroviruses carry three major genes which encode the viral core proteins (*gag* gene) forming the inner structure of the virus, the enzymes reverse transcriptase and integrase (*pol* gene) and the viral envelope proteins (*env* gene). The primary genomic length transcript carries all of the viral genetic information and is used both as the genome for new virions and also for the production of the Gag and Pol proteins. A second spliced transcript is used for the synthesis of the viral Env proteins. Some retroviruses, notably lentiviruses such as human immunodeficiency virus (HIV), produce other spliced transcripts, giving rise to accessory proteins that regulate virus protein production.

The Gag and Pol proteins are translated as polyprotein precursors from the genomic length transcripts and sequentially proteolytically cleaved into the mature proteins. Cleavage is an ongoing process that occurs as the virus assembles, buds from the host cell and matures outside of the host cell. Domains of the precursor proteins specifically interact with viral RNA (Zhang and Barklis, 1995), ensuring that viral rather than cellular RNA is packaged within the progeny virus. This interaction requires one or more specific RNA sequences, known as packaging signals, on the genomic RNA, some of which are obligatory (ψ) and located at the beginning of the Gag region, just 3′ of the splice donor (SD) for subgenomic RNA production (see below), ensuring that only full-length viral RNAs can be packaged.

The Env proteins (SU and TM) are translated from a spliced subgenomic viral RNA. These proteins are synthesised as a precursor which carries a rapidly removed signal peptide at the amino terminus, and thus enters the secretory membrane system of the host cells. During its passage through the secretory system, the precursor becomes modified by a number of glycosylation steps and is finally cleaved into the mature SU and TM proteins by a cellular protease. However, both proteins remain associated with each other by disulphide bonds and finally arrive at the host cell plasma membrane.

The final steps of assembly and release of progeny virions occur at the cell membrane, where the Gag proteins associated with the retroviral genomic RNA and viral RT and IN enzymes form new capsid structures (Figure 3.1). The capsid buds through the cell membrane in areas where the concentration of inserted viral Env proteins is relatively high. The newly released virions undergo further maturation steps associated with protein cleavage, conformational changes and reorganisation of viral proteins within the core to give the final infectious progeny virions. It should be stressed that for most retroviruses the whole process of retroviral infection and virus production does not harm the producing cell in any obvious way.

3.3 RETROVIRAL VECTOR DEVELOPMENT

The first retroviral vector systems were derived from murine leukaemia virus (MLV) and these vectors are the ones that have and are being used in clinical trials. There were (and are still) a number of reasons for choosing MLV as the basis for such gene delivery systems, including (i) the biology of this retrovirus is particularly well understood, (ii) the MLV genome was among the earliest retroviral genomes molecularly cloned and (iii) these viruses are able to infect cells efficiently. MLVs exist that are able to infect only rodent cells and these are termed ecotropic. The ecotropic viral SU protein interacts with an amino acid transporter protein that is found in the plasma membrane of target cells (Kim *et al.*, 1991; Wang *et al.*, 1991). The murine form of this transporter functions as a virus receptor whereas the human form does not due to critical amino acid differences. Thus, ecotropic MLV is not able to infect human cells unless it is engineered to do so. A different MLV variant is able to infect many cell types, including human and rodent cells. The SU protein of this amphotropic virus interacts with a cellular phosphate transporter protein (Miller and Miller, 1994; van Zeijl *et al.*, 1994). Amphotropic MLVs are used in many of the ongoing gene therapy trials. The envelope of gibbon ape leukaemia virus (GaLV) has also been used since GaLV envelope carrying retroviral vectors gives better haematopoietic cell gene transfer efficiencies (Bunnell *et al.*, 1995).

3.3.1 PRINCIPLES

Retroviral vector systems consist of two components: (i) a vector construct that carries the gene to be delivered and provides the genome for the recombinant virus, and (ii) a cell line that provides the viral proteins required to produce the recombinant virus, known as packaging cells (Figure 3.2). Two-component systems originally arose because insertion of additional genetic information into the MLV genome is detrimental to virus production, necessitating the deletion of structural gene coding sequences and the provision of these proteins *in trans* by packaging cells. Even though it may be possible to create one-component replication-competent retrovirus vectors (for example based on HIV or Rous sarcoma virus), it seems unlikey that this approach will be pursued, given the concerns about retroviral-mediated insertion mutagenesis (see Section 3.2.2).

To produce recombinant retroviral vector virions, the vector construct carrying the gene(s) to be delivered is introduced by physical gene transfer methods (such as transfection, electroporation etc.) into a retroviral packaging cell line. These packaging cells produce the viral structural (Gag and Env) proteins and enzymes (*pol*-encoded RT, IN), but are not able to package the viral RNA encoding these proteins since the ψ region required for encapsidation has been deleted. Instead the proteins recognise and associate with genomic length RNA from the introduced vector construct, which carries an intact ψ region, to form recombinant virus particles. The recombinant virus particles carrying the retroviral vector genome bud out of the packaging cell line into the cell culture medium. The virus-containing medium is either directly filtered to remove cells and cellular debris and then used to infect the target cell, or virus is purified and concentrated before infecting target cells. After the virus has bound to the receptor on the cell surface, the viral capsid is delivered into the cell and the viral RNA is reverse transcribed into a DNA form which integrates into the host cell DNA. The integrated viral DNA (provirus) functions essentially as any other cellular gene and directs the synthesis of the products of the delivered gene(s). However, unlike in the case of normal wild-type replication-competent virus (described in Section 3.2), no further infectious virus can be produced by the infected cell since the genetic information encoding the viral proteins is not present in this cell (Figure 3.2).

The major problem with two-component retroviral vector systems arises as a result of the naturally occurring phenomenon of homologous recombination. If the vector provirus and the provirus providing the structural proteins in the packaging cells recombine, there is a possibility that replication-competent retrovirus will arise (Figure 3.3; Miller and Buttimore, 1986; Muenchau *et al.*, 1990). Such virus is essentially a wild-type retrovirus and no longer carries the delivered gene(s). Replication-competent virus rapidly infects many cells and may eventually cause insertional mutagenesis. Conse-

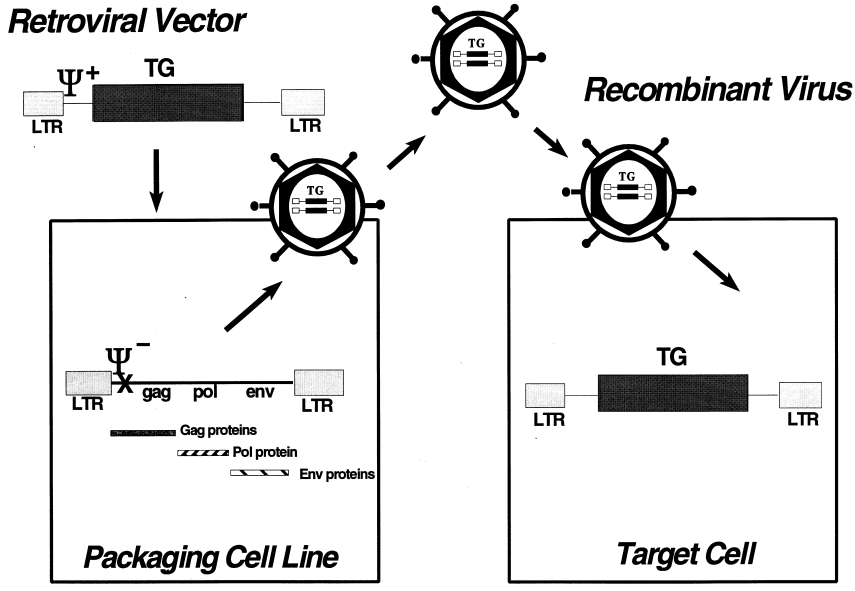

Figure 3.2. Principle of retroviral vector production. The vector construct consists of a provirus that carries a gene(s), for example a therapeutic gene (TG), in place of viral genetic information. The gene is placed under the transcriptional control of, for example, the viral promoter located in the long terminal repeat (LTR). The vector is introduced into a cell line (packaging cell line) which carries a modified retroviral provirus. This provirus produces the virus proteins that the vector cannot produce, but because it lacks the appropriate ψ packaging signal ((Ψ^-), cannot insert its own genome into the virus particles produced. The vector construct carries the ψ packaging signal, and thus RNA transcribed from this construct is preferentially inserted into the newly formed virus particles that are constantly being released from the packaging cells. The virus particles carrying the vector genome are then used to infect target cells, leading to reverse transcription and eventual integration of the vector genome carrying the therapeutic gene (TG). This gene is then expressed, giving therapeutic protein production in the target cell. No further virus can be produced since no viral structural proteins are present in these cells.

quently, considerable effort has been devoted to the design of superior packaging systems that drastically reduce the possibility of recombination occurring, as well as to produce improved, safer vectors that cannot replicate even if recombination occurs.

3.3.2 IMPROVEMENTS

Improvements to packaging cells have involved removing as much of the retroviral information as possible to reduce the possibility of homologous recombination occurring (Figure 3.4). The retroviral promoter and termina-

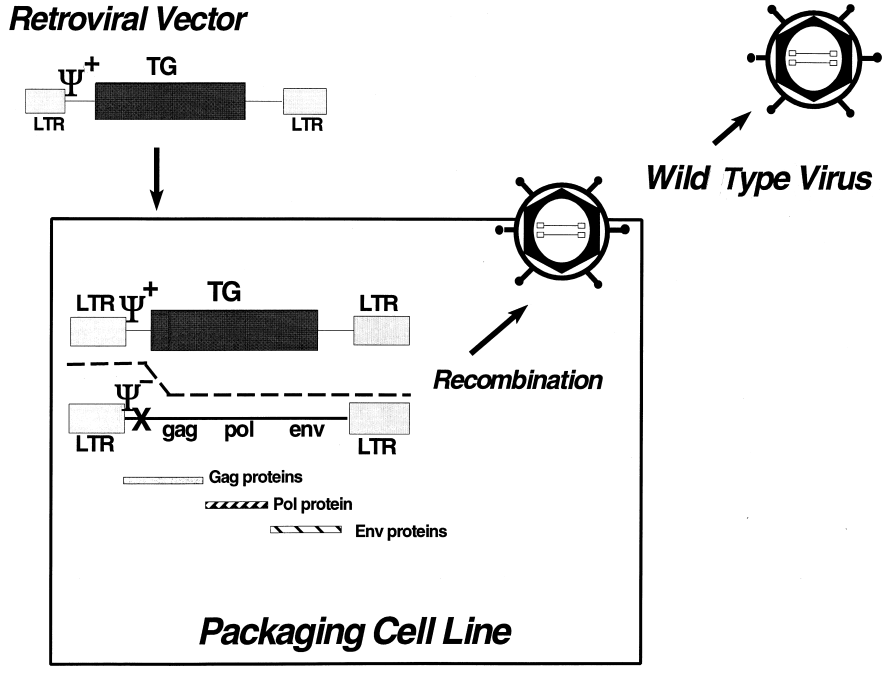

Figure 3.3. Generation of replication competent (wild-type) virus by recombination. Initial retroviral vector systems often rapidly became contaminated with replication-competent virus due to at least one homologous recombination event (dotted line) occuring between the vector construct and the packaging construct in the packaging cells. The probability of this happening depends on the degree of homology shared between the two constructs.

tion sequences can be replaced by heterologous promoters and termination sequences. This has the additional advantage of allowing the use of promoters that are more strongly active than the retroviral promoter, thereby giving rise to higher levels of viral protein production. The coding information for the viral proteins cannot be removed by necessity, but these proteins can be made from separate constructs so that additional recombination events are required to recreate a complete replication-competent retrovirus. This has been achieved by expressing the Gag and Pol proteins from one construct and the Env proteins from a second construct (Markowitz *et al.*, 1988a,b).

In addition to the improvements to packaging cells, safer retroviral vector constructs also have been produced that carry an artificially inserted stop codon in the Gag reading frame. This ensures that even if replication-competent virus is generated, it will not be able to express its Gag and Pol proteins and thus virus assembly and release will be inhibited (Bender *et al.*, 1987; Morgenstern and Land, 1990; Scarpa *et al.*, 1991). Another strategy for

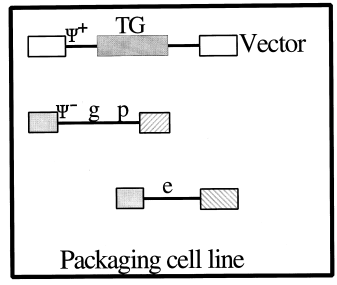

Figure 3.4. Improved retroviral vector system. In order to reduce the possibility of homologous recombination leading to the production of replication-competent retrovirus, systems have been constructed in which only those viral sequences that are absolutely required are maintained in the vector and packaging constructs. This has been facilitated by the use of heterologous promoters (open and shaded boxes) and termination signals (striped boxes). In addition, such systems consist of three components, for example two independent constructs, one of which expresses the Gag (g) and Pol (p) gene products and a second that is used for expression of the Env (e) proteins. At least three recombination events between the vector and the two packaging constructs are required for the production of replication-competent virus.

creating a fail-safe mechanism to prevent the infectivity of any potential replication-competent virus is to ensure that the packaging sequence becomes deleted. This can be achieved by flanking the ψ sequences with direct repeats, facilitating their removal. The majority of vector proviruses in cells infected with such vectors were shown to have deleted the packaging sequences, presumably due to template switching during reverse transcription (Julias *et al.*, 1995). The Cre/loxP recombinase system has also been incorporated into retroviral vectors and used to specifically excise viral vector sequences in the infected target cell (Cholika *et al.*, 1996; Russ *et al.*, 1996). Vectors have also been constructed that carry a modified, artificial primer binding site (PBS) (Lund *et al.*, 1997). Normally a cellular tRNA is bound to this region of the genomic RNA and it is used as the primer for initiation of reverse transcription. In combination with a packaging cell line which also synthesises the artificial tRNA, recombinant virus can be produced and used to infect cells. However, any replication-competent virus that may be produced from this system as a result of homologous recombination will not be able to replicate after the initial infection of a cell since the infected cells do not synthesise the required artificial tRNA (Lund *et al.*, 1997).

3.4 INFECTION TARGETING

The receptor for the amphotropic (and GaLV) SU are expressed on many cell types and thus confer a promiscuous infection spectrum upon MLV-based

retroviral vectors. For reasons of safety and efficacy, it would often be desirable to redirect or limit the infection spectrum of retroviral vectors, so that only the correct target cell type is infected. This requires the modification or alteration of the MLV envelope proteins so that they interact with more exclusive cell receptors. A number of strategies have been employed to achieve this goal (reviewed in Salmons and Günzburg, 1993). In many of these studies, ecotropic MLV vectors were modified to demonstrate that infection targeting had indeed taken place since these retroviral vectors cannot normally infect human or other non-rodent cells.

Infection targeting has been attempted using antibodies, directed against known proteins that are expressed on the surface of the target cell, linked via streptavidin to antibodies specific for the virus Env protein. In this system antibodies directed against class I and class II major histocompatibility antigens (Roux *et al.*, 1989) or against the receptor for epidermal growth factor (Etienne-Julan *et al.*, 1992) gave targeted infection of cells expressing these molecules on their surface, but with low efficiencies. Another drawback with this, and indeed other, strategies is that not all receptors are competent for virus uptake or allow later steps in the viral life cycle to occur (Goud *et al.*, 1988).

In the past few years the most popular strategy for modification of the infection spectrum of retroviral vectors has involved the genetic engineering of the viral *env* gene carried in the retroviral packaging cell line. This has been facilitated by the identification of the regions of the SU Env protein involved in receptor recognition (Battini *et al.*, 1992, 1995; Morgan *et al.*, 1993; Ott and Rein, 1992). These regions have been replaced with gene segments encoding epitopes that would recognise other receptors such as erythropoeitin (Kasahara *et al.*, 1994) or heregulin (Han *et al.*, 1995), thereby allowing the selective control of receptor targeting of the resultant chimeric Env protein. Alternatively, targeting ligands have been inserted at the amino terminus of the SU protein between amino acids 6 and 7 (Cosset *et al.*, 1995b; Marin *et al.*, 1996; Russell *et al.*, 1993). In these approaches, the ligand–Env protein is produced in the packaging cell line *in addition* to the normal non-modified retrovirus envelope protein, which is presumably required for stability since Env proteins are presented as trimers on the surface of the virus. The variable domain of single-chain antibodies specific for a defined receptor/cell surface protein have also been used to target retroviral vector infection (Chu and Dornburg, 1995; Marin *et al.*, 1996; Russell *et al.*, 1993; Somia *et al.*, 1995). Even though retargeting of the infection event by modification of the retroviral envelope protein has been successfully achieved by a number of groups, it is invariably associated with reduced titres, probably for similar reasons to those mentioned above. Such manipulations of the viral SU protein are also of limited use because of the relatively complex synthesis and processing of this protein, ensuring both its functionality and its ability to become incor-

porated into newly synthesised virus particles. These requirements place constraints on replacement or modification since the conformation of certain domains is likely to be critical. Clearly, more knowledge is required about the mechanisms that govern the normal functioning of retroviral envelope proteins before chimeric envelopes can be constructed that retain the ability of these proteins to recognise the receptor *and* initiate infection efficiently.

Recently, Steven Russell and colleagues (Nilson *et al.*, 1996) have described a new two-step strategy that promises to revolutionalise targeting of retroviral vectors to predefined receptors. The viral envelope protein is modified by the linear addition of a protease cleavage site and the selected receptor ligand domain at the amino terminus of the SU protein. After binding the appropriate receptor on the target cell, the receptor/ligand can be cleaved off by expression of the protease (Nilson *et al.*, 1996). The retrovirus then attaches to its usual receptor on the same target cell, allowing the retrovirus to enter the cell by the natural route, circumventing the entry problems that often occur when retroviruses are targeted to use non-retroviral receptors (Etienne-Julan *et al.*, 1992). Titres of up to 10^6 cfu/ml (colony forming units per millilitre cell culture medium) have been achieved using the EGF binding domain as a means to target in this two-step system (Nilson *et al.*, 1996). The use of phage display libraries also promises to reveal ligands that will be useful for targeting purposes. Such libraries facilitate the rapid screening of peptides for their ability to bind to cell receptors present on specific cell types. Peptides identified using this screening system could be incorporated into any gene delivery system, including retroviral vectors, to achieve infection targeting (Barry *et al.*, 1996). The same system can also be utilised to achieve targeted infection by blocking retroviral infection of non-desired target cells. In this approach, the terminal end of the SU protein has been linked to a cleavage site, and in addition, to a peptide that masks the normal SU binding domain as well as to a specific ligand present on all non-target cells.

3.5 GENE EXPRESSION FROM RETROVIRAL VECTORS

3.5.1 GENERAL CONSIDERATIONS

The heterologous genes delivered by retroviral vectors can be expressed in a number of different ways (Figure 3.5). The retroviral promoter within the LTR can drive heterologous gene expression when such genes are cloned into the position formerly occupied by *gag*. Indeed, in many applications where it is advantageous to transfer and express two genes, for instance a therapeutic gene and a marker gene, the second gene can be inserted into the *env* position and expressed from the subgenomic viral RNA. Unfortunately the retroviral promoter is not particularly powerful and also suffers the disadvantage of

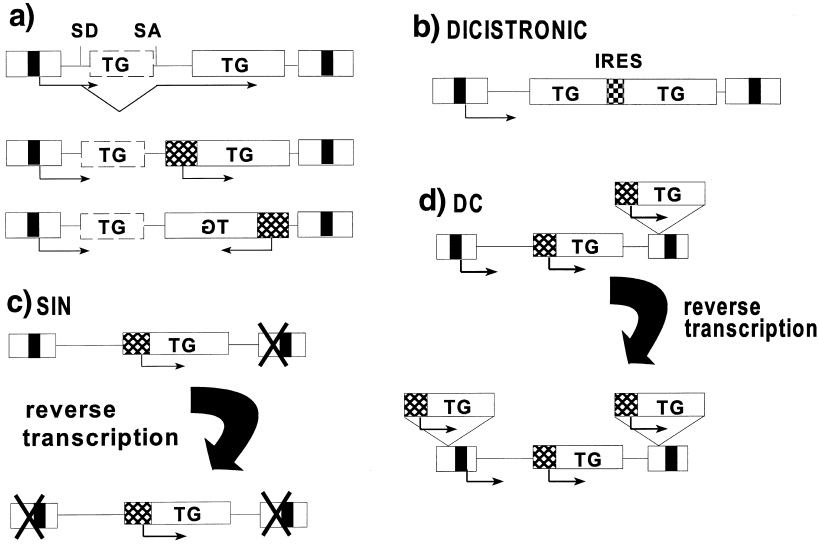

Figure 3.5. Expression configurations of retroviral vector constructs. Therapeutic genes (TG) can be expressed in a number of ways from the vector construct. In **(a)** and **(b)** the TG is expressed, either directly from the retroviral promoter as a genomic length transcript or (shown in (a)) as a spliced transcript. Retroviral vectors can accommodate one or two genes. The insertion of an internal ribosome entry site (IRES) allows a second gene to be expressed from the same genomic RNA, as shown in **(b)**. The TG can also be expressed from a heterologous promoter inserted into the retrovirus vector (hatched box in (a)), either in the sense or antisense orientation. Self-inactivating (SIN) and double-copy (DC) vectors as shown in **(c)** and **(d)** carry modified 3′ LTRs that lead to either loss of the retroviral promoter (SIN) or a duplication of an expression cassette (heterologous promoter linked to the TG) in the infected cell.

being shut down or silenced after a variable period in many cell types (Lund *et al.*, 1996; Xu *et al.*, 1989) or in transgenic animals (Richards and Huber, 1993; Vernet and Cebrian, 1996). Methylation and host factors have been identified as culprits for this shut-off, and efforts have been made to alter retroviral LTR and downstream sequences to prevent transcriptional silencing in certain cell types (reviewed in Lund *et al.*, 1996).

Expression of genes delivered by retroviral vectors also depends on the site of integration in the host cell genome. Tissue-specific regulation from heterologous promoters (see below) may be overridden or compromised by strong cellular regulatory elements located in the vicinity of the integration site. Locus control regions (LCRs), regulatory elements that have been shown to confer position-independent, high-level expression upon genes (Dillon and Grosveld, 1993), can be included in retroviral vectors to try to overcome

silencing problems. The inclusion of a 36 base pair LCR core sub-sequence from the human β-globin gene in a retroviral vector carrying a human β-globin gene was shown to enhance the expression of human β-globin in mouse erythroleukaemia cells, although expression levels were still therapeutically suboptimal (Chang *et al.*, 1992). However, a similar retroviral vector construct was shown to give widely varying levels of expression (4–146%) in different infected cell clones (Sadelain *et al.*, 1995). Thus, either more of the LCR region is necessary to obtain position-independent expression or LCR sequences are generally not able to function in the context of retroviral vectors. An alternative strategy to ensure position-independent expression of genes in retroviral vectors is to shield them from the effect of enhancers or repressors located in the vicinity of the integration site (Duch *et al.*, 1994). A number of such insulators have been identified in drosophila (Gerasimova and Corces, 1996) and mammalian cells (Felsenfeld *et al.*, 1996), and these could be incorporated into future retroviral vectors.

Self-inactivating (SIN) (Yu *et al.*, 1986) and double-copy (DC) vectors (Figure 3.5; Hantzopoulos *et al.*, 1989) utilise a unique feature of the retroviral life cycle, the reverse transcription of viral genomic RNA into a double-stranded form. During this process sequences from the 5′ end of the RNA (U5) are duplicated and placed additionally at the 3′ end of the DNA, while sequences from the 3′ end of the RNA (U3) are copied onto the 5′ end of the DNA. The process generates the identical LTR structures which flank the viral genome. The retroviral promoter is located within the U3 region, which means that the promoter that is used in the infected cell is derived from the U3 region at the 3′ end of the viral RNA. In SIN vectors, this U3 has been deleted, leading to an integrated provirus that lacks a retroviral promoter in the infected cell. Inclusion of an internal heterologous promoter means that this will be the only promoter present to drive the expression of the delivered gene. In DC vectors, a cassette consisting of a heterologous promoter linked to the gene to be delivered is inserted in place of the U3 region in the 3′ LTR of the vector, and becomes duplicated after reverse transcription, ensuring that the promoter–gene cassette is present twice (i.e. in double copy) in the target cell. The R region can also be used as a site for insertion of genes since cDNAs encoding MyoD and purine nucleoside phosphorylase (PNP) have been successfully inserted into this region of the LTR (Adam *et al.*, 1995).

The use of internal ribosome entry sites (IRES) in retroviral vectors allows two or more genes to be expressed from the same transcript expressed from a single promoter (Figure 3.5; Koo *et al.*, 1992; Levine *et al.*, 1991). These vectors are reported to give higher titre, permit the insertion of larger heterologous gene segments, and show more stable expression of transferred genes compared to two-gene, two-promoter vectors (see above), and may overcome the reported interference between multiple promoters present in the same retroviral vector (Li *et al.*, 1992; McLachlin *et al.*, 1993; Xu *et al.*, 1989).

3.5.2 EXPRESSION TARGETING

As well as infection targeting, through redirection of infection specificity (see above), it is possible to limit the expression of therapeutic genes using promoters from genes that are expressed specifically or preferentially in defined cell types. Thus, a retroviral vector may deliver a gene to many cell types (if infection targeting is not possible) but the gene will be expressed only in the required cell type. Strict specificity should combine both infection and expression targeting.

A plethora of promoters that are preferentially active in particular cell types have been utilised in retroviral vectors. These include the hepatocyte- (and hepatoma-) specific promoters from the phosphoenolpyruvate carboxylase (Hafenrichter *et al.*, 1994; Hatzoglou *et al.*, 1990; McGrane *et al.*, 1988), α-fetoprotein (Huber *et al.*, 1991), α_1-antitrypsin (Hafenrichter *et al.*, 1994; Rettinger *et al.*, 1994) and albumin genes (Hafenrichter *et al.*, 1994) and promoters that are preferentially active in tumours such as from the tyrosinase gene for expression targeting to melanomas (Vile *et al.*, 1995) and *erb* B-2 for mammary tumours (Harris *et al.*, 1994).

Early vectors expressed the delivered gene from tissue-specific or inducible heterologous promoters inserted into the body of the vector, i.e. in addition to the retroviral promoter. However, this configuration is often associated with transcriptional interference effects due to the presence of both the retroviral and the heterologous promoters. These interference effects can either be manifested as loss of expression from one or both promoters or as a loss of tissue specificitity or inducibility of expression from the heterologous promoter. More recent vectors carry heterologous promoter/enhancer elements in the LTR in place of the retroviral promoter/enhancer (Ferrari *et al.*, 1995; Günzburg *et al.*, 1995; Salmons *et al.*, 1995; Vile *et al.*, 1995). Such vectors in which the promoter is converted from that of MLV in the packaging cell line to the introduced heterologous promoter in the infected target cell have been termed promoter conversion (ProCon) vectors (Figure 3.6; Günzburg *et al.*, 1995; Salmons *et al.*, 1995). It remains to be seen whether these vectors may be safer but (i) the lack of viral promoter sequences is expected to reduce the frequency of recombination with viral sequences in the producer or target cell and (ii) it has yet to be shown that a promoter from a cellular gene can activate or inactivate cellular genes in the context of a retrovirus.

3.5.3 INDUCIBLE PROMOTERS

The glucocorticoid inducible promoter of mouse mammary tumour virus (Günzburg and Salmons, 1992) has been successfully used in ProCon vectors to give regulatable gene expression in cell culture (Günzburg *et al.*, 1997;

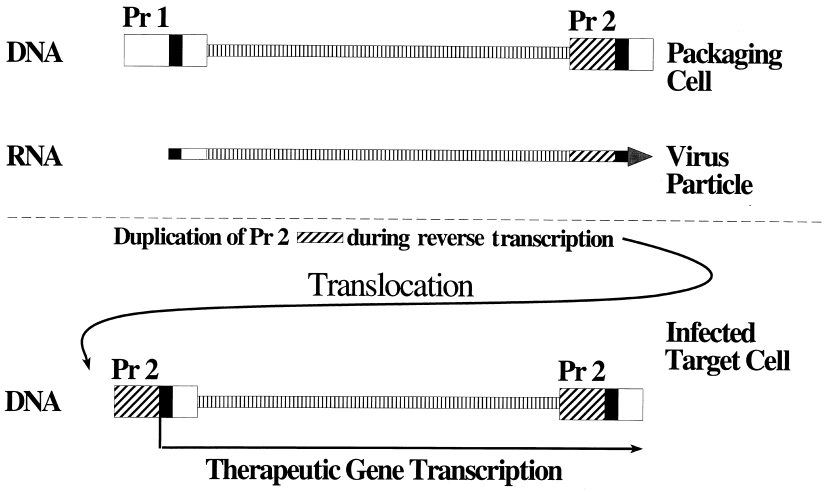

Figure 3.6. Retroviral vectors that undergo promoter conversion. The vector construct carries the retroviral promoter (Pr 1; promoter 1), in the U3 region of the 5′ LTR, that directs expression of the viral genomic RNA in the packaging cell line. The 3′ LTR promoter sequence of the virus has been replaced by the heterologous promoter chosen to drive gene expression in the infected cell (Pr 2). Note that this sequence (shaded) is the only promoter present in the genomic RNA that is packaged into the virus particle. Upon infection of the target cell, the RNA is converted to a double-stranded DNA molecule (as in Figure 3.1) by reverse transcriptase. This process results in the duplication of Pr 2 and the translocation of one copy to the 5′ end of the proviral DNA, where it is the exclusive promoter used to drive gene expression in the infected cell.

Mrochen *et al.*, 1997). This promoter may be less useful as an inducible promoter *in vivo*, although it may enable the targeting of gene expression to the mammary gland and B lymphocytes (Günzburg and Salmons, 1992). Cannon and co-workers have used the ProCon principle to create an MLV-derived retroviral viral vector that uses the Tat inducible HIV promoter after infection of cells. This vector may be useful for targeting the expression of therapeutic genes to HIV-infected cells. Expression will be activated in and limited to these cells since only HIV-infected cells express Tat (Cannon *et al.*, 1996). Retroviral vectors carrying a tetracyclin inducible promoter to drive expression of genes have also been constructed (Paulus *et al.*, 1996).

3.6 RETROVIRAL VECTOR TITRES AND STABILITY

Virus and viral vector titres are usually measured by virtue of an effect that the virus has on target cells. Often in the case of viral vectors this is the

number of cells that receive and express an enzymatic marker gene enabling a colour reaction such as β-galactosidase, or alternatively an antibiotic resistance gene which allows infected cells to be identified on the basis of their ability to survive in antibiotic-containing medium. Thus, the titre represents functional units of virus and not the absolute number of virus particles. One commonly cited disadvantage of retroviral vectors is that the titres obtainable seem low when compared to other viral vectors such as adenovirus and adeno-associated virus vectors, even though there are reports of retroviral titres up to 10^7 cfu/ml. It has recently been shown that retroviral titres are actually much higher (up to one order of magnitude) than previously thought since culture medium containing vector virus can be shown to contain significant amounts of infectious vector virus when serially transferred to fresh, non-infected cells (Tavoloni, 1997). It is highly questionable whether functional titres determined on a limited number of established cell lines (such as NIH3T3 cells) in culture truly reflect the amount of vector virus capable of delivering a gene to primary cells or to cells *in vivo* (Forestell *et al.*, 1995). Further, it follows that optimisation of the virus titre on such established cells may be inappropriate since such cells do not necessarily reflect the biological properties of the relevant target cells for gene therapy. A number of physical and chemical methods have, however, been used to increase the apparent titre measured *in vitro*. This includes concentration (Kotani *et al.*, 1994; Paul *et al.*, 1993), flow-through infection, in which cells are grown on a porous filter and virus is passed over them (Chuck and Palsson, 1996), and treatment of vector-producing cells with sodium butyrate (Olsen and Sechelski, 1995; Pages *et al.*, 1995; Soneoka *et al.*, 1995). Considerable effort is also being put into gaining an understanding of the physico-chemical and biological factors affecting virus stability and consequently virus titre. The half-life of MLV retroviral vectors in cell culture medium has been measured to be somewhere in the order of 3.5–9 hours at 37 °C (Chuck *et al.*, 1996; Kaptein *et al.*, 1997; Paul *et al.*, 1993; Russel *et al.*, 1995; Sanes *et al.*, 1986; Tavoloni, 1997). Polycations such as polybrene (hexadimethrinebromide) and protamine are included in vector virus preparations to increase the efficiency of infection of cells. These positively charged molecules are thought to act as 'adaptor molecules' in alleviating the electrostatic repulsion between the negatively charged virus and negatively charged cell membrane (Figure 3.7; Coelen *et al.*, 1983; Hornsby and Salmons, 1994). Polybrene has, however, also been shown to affect the kinetics of retroviral decay and may cause aggregation of vector virus particles, thus preventing them from infecting cells (Andreadis and Palsson, 1997). Temperature also has an effect on virus titres. Surprisingly, production of retroviral vector virus at 32 °C results in better titres (Bunnell *et al.*, 1995; Kotani *et al.*, 1994). This has been shown to be due to a four-fold increase in the half-life of the recombinant virus at 32 °C as compared to 37 °C (Kaptein *et al.*, 1997).

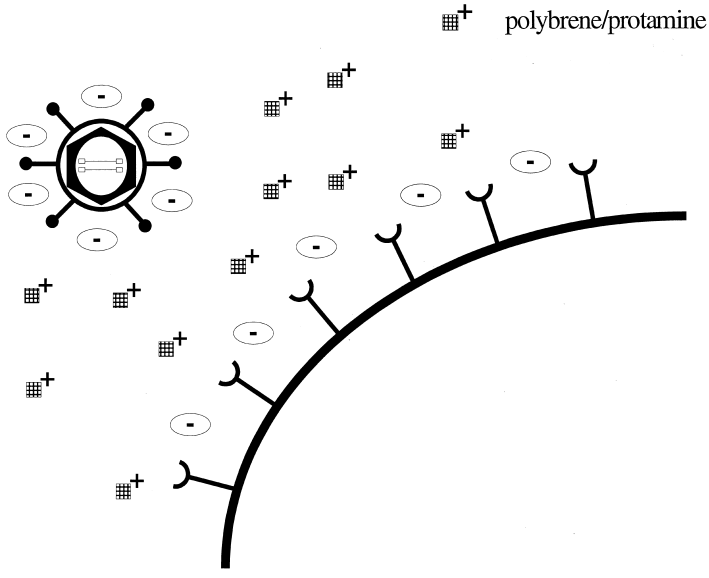

Figure 3.7. Polycations facilitate infection. The negatively charged virus and cell are shown. Polycations such as polybrene neutralise electrostatic repulsion between these two structures, thereby facilitating infection.

Titre is also dependent on the presence and abundance of cell-encoded receptor molecules on the surface of the target cell. The amphotropic receptor is expressed on many but not all cells. Non-dividing or slowly dividing hepatocytes express little amphotropic receptor, and haematopoietic stem cells may express less of this receptor than the receptor for GaLV (Bunnell *et al.*, 1995) (see also Section 3.3). In contrast, the receptor for another enveloped virus, vesicular stomatitis virus (VSV), is abundantly present on many different cell types (Coll, 1995). The VSV envelope (G) protein can be incorporated into retroviral particles, a process known as pseudotyping (Weiss, 1993), resulting in both increased titres as well as more stable virus particles than those carrying MLV envelope proteins (Burns *et al.*, 1993). These MLV/VSV pseudotyped particles additionally show a greatly expanded host range and cell type spectrum (reviewed in Friedmann and Yee, 1995).

Fast selection of successfully infected primary cells has not been practicable to date because the assays used for currently available marker genes often affect cell viability. Even the sorting of cells for the expression of β-galactosidase using the fluorescence-activated cell sorter (FACS) is stressful for cells due to cell permeabilisation required to allow the substrate to enter cells. Antibiotic resistance selection requires cultivation of cells for a number of days before non-transduced cells are killed off, although a quick

assay has now been developed for G418 (Byun *et al.*, 1996). Recently, a new marker gene has been used in retroviral vectors that finally allows facile selection in the FACS without any significant cell toxicity. This gene encodes the green fluorescent protein (GFP), which emits green light under ultraviolet (reviewed by Chalfie *et al.*, 1994). GFP genes have been engineered for better expression in mammalian cells, and these hGFP and EGFP genes have been inserted into retroviral vectors (Klein *et al.*, 1997; Muldoon *et al.*, 1997) and used to rapidly identify successfully infected cells.

3.7 LENTIVIRAL VECTORS

MLV-derived vectors, like MLV, are limited to infecting dividing cells because the pre-integration complex consisting of the viral DNA and integrase cannot be transported across the nuclear membrane (Lewis and Emerman, 1994; Roe *et al.*, 1993). During cell division, this membrane breaks down, allowing the viral DNA complex to reach, and integrate in, the genomic DNA of the target cell. In contrast, lentiviruses, such as HIV and simian immunodeficiency virus (SIV), are able to infect non-dividing cells (reviewed by Stevenson, 1996). This appears to be due to the presence of redundant nuclear localisation signals both in the HIV MA protein (Bukrinsky *et al.*, 1993) as well as in the HIV accessory gene product Vpr associated with the MA protein (Heinzinger *et al.*, 1994).

Due to this property of lentiviruses, attention has turned to the construction of lentivirus-based vector systems for gene transfer to quiescent and/or differentiated cells. At first glance, members of this subfamily of retroviruses, such as HIV, do not appear to be acceptable gene transfer vehicles because of their association with immunodeficiency. However, assuming that vector systems can be developed that are highly unlikely to lead to the production of replication-competent HIV and assuming that the viral components necessary to make vector particles are not themselves involved in causing the immunodeficiency, for example by stimulating an autoimmune response, these systems may be ideal for this purpose. A possible second advantage for creating vector systems based on lentiviruses is that there is no evidence to date for insertional mutagenesis by these viruses, although there is no known reason why this should not occur.

A number of groups have constructed vectors based upon HIV and, invariably, these systems have relied on the use of envelope proteins from other viruses and not from HIV, reducing the probability of recombination giving rise to infectious virus. The VSV G protein and the amphotropic Env of MLV have both been used in such pseudotyped lentiviral vectors.

It has been reported that HIV vectors pseudotyped with the envelope proteins of amphotropic MLV or VSV can give titres of up to 10^7 cfu/ml (Naldini

et al., 1996; Page *et al.*, 1990; Reiser *et al.*, 1996) or ~105 cfu/ml when pseudotyped with the envelope of human T-cell leukaemia virus (HTLV) (Landau *et al.*, 1991). The HIV accessory genes (such as *vpr, vpu* and *nef*) have been inactivated in the HIV vector system described by Reiser and co-workers and this is believed to contribute to the safety of such vectors (Reiser *et al.*, 1996). On the other hand, some of these gene products, for example Nef, have been postulated to enhance virion infectivity, and thus might be beneficial in the vector (Naldini *et al.*, 1996).

It has long been known that retroviruses (and thus retroviral vectors) are directly inactivated by human serum complement (Welsh *et al.*, 1975). It has been shown that complement-mediated inactivation is non-lytic and depends both on the retrovirus and on the cells from which the virus has been produced. For example, MLV produced from mouse cells is much more readily inactivated than MLV produced from human or mink cells (Takeuchi *et al.*, 1994). This is because human serum contains antibodies to the Gal(α1-3)Gal modification of carbohydrates. Such modified carbohydrates are found on proteins of most mammals but not in humans since the enzyme that performs this modification ((αl-3) galactosyltransferase) is not present in humans (Takeuchi *et al.*, 1996). Virus produced from non-human cell lines displays modified carbohydrates as part of its outer lipid bilayer, allowing it to be recognised by antibodies which recruit complement and cause inactivation by a non-lytic process. The use of established human cell lines, such as 293 (Pear *et al.*, 1993) or HT1080 (Cosset *et al.*, 1995a) for the construction of packaging cells has reduced this problem. Alternatively, the complement pathway can be inhibited by administration of monoclonal antibodies to specific complement components (Rother *et al.*, 1995). Lentiviral vectors based upon HIV would be less susceptible to complement – mediated inactivation than those derived from MLV.

3.8 CONCLUSIONS AND PERSPECTIVES

Retroviral vectors are the most commonly used gene delivery vehicles in clinical gene therapy trials and, as of June 1996, 969 patients have been treated with these vectors (Marcel and Grausz, 1996). Nevertheless, retroviral vectors have in the past few years lost ground, particularily to adenovirus vectors, due to their higher titres and infectivity spectrum. However, recent advances, some of which are mentioned in this review, have renewed interested in retroviral vectors. These advances have included (i) general design improvements, (ii) vectors based on lentiviruses, (iii) greater insight into retroviral stability and production, giving the hope of higher titres, and (iv) safer systems to ensure that replication-competent virus is not produced. The quest for the perfect retroviral vector is not yet over and is probably not

achievable. Synthetic vector systems will probably solve many of the problems associated with current gene delivery systems in general, but it is a safe bet that components of retroviruses will be included in the artificial vector systems of the future.

*NOTE ADDED IN PROOF

Climaeric vector systems from which retroviral vectors are produced after delivery of the necessary components into target cells using other virus delivery systems (so-called ''launching pad'' systems) have been successfully developed based on adenoviruses, alphaviruses, herpes viruses and poxviruses (Reynolds, P.N., Feng, M., and Curiel, D.T. (1999) Chimeric viral vectors – the best of both worlds? *Mol. Med. Today* 5, 25–31.

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