Gammaretroviral and Lentiviral Vectors for Gene Therapy

Stability and Inactivation Mechanisms

Marlene Carmo

Dissertation presented to obtain a Ph.D degree in Engineering and Technology Sciences, Gene Therapy at the Instituto de Tecnologia Quimica e Biológica, Universidade Nova de Lisboa

Oeiras, January 2009
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Cover
By Marlene Carmo. Front cover: schematic representation of a gammaretroviral vector and a lentiviral vector and of the possible inactivation mechanisms affecting these vectors and implications on the infection process. Back cover: schematic representation of a gammaretroviral vector and a lentiviral vector.

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Dr. Paula M. Alves, Head of the Animal Cell Technology group at ITQB, Oeiras, Portugal.
FOREWORD

The present thesis dissertation is the result of four years of research at the Animal Cell Technology Laboratory of ITQB-UNL/IBET, Oeiras, Portugal, under the supervision of Professor Manuel J.T. Carrondo and Dr. Pedro E. Cruz. It gave me the opportunity to be introduced to the challenging fields of gene therapy and retrovirology.

This thesis intends to identify the main causes for the fast inactivation of gammaretroviral and lentiviral vectors with the final goal of increasing the stability and consequently the final quality and efficacy of vector preparations to be used in clinical trials.
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ABSTRACT

Gene therapy consists in the delivery and expression of genetic information in order to treat or prevent a disease. There are several ways of delivering this genetic information, namely using viral and non-viral vectors. In this thesis two types of viral vectors, gammaretroviral and lentiviral vectors, were studied. Both vectors present the advantages of having low immunogenicity, good transduction efficiency and, most importantly, being able to insert the gene of interest; these advantages have shown great potential to definitively cure monogenic recessive diseases and to improve the outcome of other diseases. On top of these advantages, lentiviral vectors are also able to infect dividing and non dividing cells. Although both vectors infect cells with relatively high efficiency, still large amounts of vectors are necessary to undertake pre-clinical and clinical trials. A disadvantage of both these vectors is the difficulty to produce them in high infectivity titers. One of the reasons for this problem is that gammaretroviral and lentiviral vectors are very unstable and lose most of their infectivity after only a few hours at 37°C. This low stability affects not only the titers but also the quality/efficacy of vector preparations to be used in pre-clinical and clinical trials. Thus, understanding what causes this fast inactivation is of the utmost importance to increase stability and consequently the quality of gammaretroviral and lentiviral vectors preparations.

Gammaretroviral and lentiviral vectors lose infectivity in all the stages of manufacturing: production, purification, storage and also during clinical gene transfer. This inactivation can be caused by several factors inherent to the process, temperature being a determinant factor affecting vector stability in all production stages. During production temperature affects viral stability
in two ways: (i) it affects the intrinsic stability of the produced vector particles; and, (ii) causes inactivation of the particles in solution. The two effects of temperature on vector stability/inactivation were addressed in this thesis with the final goal of improving vector stability.

Following the introduction to the theme of viral inactivation in Chapter I, in Chapter II the effect of culture temperature on the stability of gammaretroviral vectors was studied. It had been already described in the literature that the production temperature affected the composition of the producer cell membrane. Thus, in order to understand what changes on the vector membrane, caused by production at different temperatures, could be related to the changes in vector stability, a thorough study on the physico-chemical properties of the vector membrane was performed. For this purpose vectors produced at 37 and at 32°C were characterized by electron paramagnetic resonance, composition of viral membrane, differential scanning calorimetry and transduction efficiency of clinically relevant cell models. The results showed that vectors produced at 32°C have a more rigid membrane with higher cholesterol to phospholipid ratio. Also the envelope proteins of the vectors produced at this temperature are present in a more folded state. The membrane properties of vectors produced at 32°C were shown to be responsible for their lower stability at storage temperatures and also for decreased transduction efficiency in gene therapy relevant cells, when compared to vectors produced at 37°C. Therefore, it was concluded that the production of gammaretroviral vectors should be performed at 37°C in order to obtain more stable vectors. Furthermore, since vector membrane physico-chemical properties are affected in response to changes in culture temperature, such changes, along with alterations in medium composition,
can be used prospectively to improve the stability and the transduction efficiency of gammaretroviral vectors for gene therapy purposes.

After verifying the effect of culture temperature on vector stability and determining 37°C as the best condition to obtain more stable vectors, the direct effect of temperature on vector particles was studied. Due to the complexity of viral particles, the fast inactivation of gammaretroviral and lentiviral vectors can be attributed to the degradation/inactivation of several viral constituents, as referred in Chapter I. Thus, in Chapter III degradation/inactivation of several viral constituents were determined in order to identify the inactivation mechanism of gammaretroviral vectors. It was possible to verify that a high correlation existed between loss of infectivity and the capacity of the virus to perform reverse transcription. It was further shown that the amount of viral RNA that entered the cells decreased slowly due to viral incubation at 37°C. These two observations led to the conclusion that the loss of the virus capacity to perform reverse transcription is the main mechanism responsible for gammaretroviral vectors inactivation. Since reverse transcription is a rather complex process which involves several viral constituents, the stability of reverse transcriptase and viral RNA were also investigated. It was observed that both DNA polymerase and RNaseH activities of reverse transcriptase suffer inactivation at 37°C, although at a much slower rate than viral infectivity loss. Also, viral RNA degradation was shown not to be implicated in the loss of the virus capacity to synthesize DNA. Thus, inactivation of some other viral constituents at 37°C, possibly nucleocapsid protein and tRNA, affect DNA synthesis.
In Chapter IV the inactivation mechanism of lentiviral vectors was studied. Lentiviral vectors pseudotyped with two different envelope proteins, amphotropic and RDpro, were used. For both pseudotyped vectors, a high correlation existed between the loss of the virus capacity to perform reverse transcription and viral infectivity loss at 37°C. As observed with gammaretroviral vectors, lentiviral entry into the host cell does not determine the rate of viral inactivation, with both pseudotypes showing similar profiles. Thus, the loss of the virus capacity to perform reverse transcription was also shown to be the main inactivation mechanism of lentiviral vectors. Reverse transcriptase inactivation and RNA degradation again showed not to be implicated in the loss of the virus capacity to perform reverse transcription. Interestingly, vectors pseudotyped with RDpro envelope showed a more stable reverse transcription process than vectors pseudotyped with amphotropic envelope that in turn were more stable in terms of infectivity loss.

The results from Chapters III and IV permit designing strategies to stabilize the reverse transcription process in order to improve the applicability of gammaretroviral and lentiviral vectors in gene therapy.

In Chapter V the knowledge that the main inactivation mechanism of gammaretroviral and lentiviral vectors is the loss of the virus capacity to perform reverse transcription was used to stabilize the vectors. By adding recombinant human albumin to the storage buffer it was possible to improve the stability of purified gammaretroviral vectors, both at 37°C and at 4°C, by increasing the stability of the reverse transcription process. Nevertheless, for lentiviral vectors it was observed that further protection was needed. This was achieved by adding to the storage buffer a mixture of
lipoproteins and recombinant human albumin. Complexes of lipoproteins and recombinant human albumin may associate with the vector membrane protecting their structure and preventing conformational changes more efficiently than recombinant human albumin alone does. The difference of stabilization between gammaretroviral and lentiviral vectors was validated by performing tests with vectors produced by different cell lines and pseudotyped with different envelope proteins.

Overall, in this thesis, the important role of temperature on gammaretroviral and lentiviral vectors stability was assessed and this knowledge was used to improve vector stability. First, it was observed that the temperature affects the rigidity, lipid composition and the conformation of proteins present in the gammaretroviral vector membrane leading to the production of vectors with a different stability; vectors produced at 37°C proved to be more stable. This knowledge raises opportunities for additional improvement of vectors stability, through changes in the culture conditions. Secondly, the knowledge that gammaretroviral and lentiviral vectors lose their infectivity, at 37°C and 4°C, by losing the capacity to perform reverse transcription, impelled the development of storage formulations that greatly improved the stability of purified vectors.

The results obtained in this thesis permit the improvement of the final vector yield on the manufacture process, as well as of the final quality and efficacy of viral preparations, essential factors to ensure the success of these vectors in gene therapy applications.
RESUMO
A terapia génica consiste na introdução de material genético no interior da célula para que o produto da sua expressão possa tratar ou prevenir uma doença. Existem várias formas de introduzir esta informação genética, nomeadamente usando vectores virais e não virais. Nesta tese foram estudados dois tipos de vectores virais, gammaretrovirais e lentivirais. Ambos os vectores apresentam as vantagens de possuírem baixa imunogenicidade, boa eficiência de transdução e, mais importante, serem capazes de inserir o gene de interesse. Estas vantagens demonstraram já grande potencial para curar definitivamente doenças monogénicas recessivas e para melhorar os sintomas de outras doenças. Para além destas vantagens, os vectores lentivirais são também capazes de infectar células que não se encontrem em divisão. Embora ambos os vectores sejam capazes de infectar células com uma eficiência relativamente elevada, são necessárias elevadas quantidades de vectores para proceder a ensaios pré-clínicos e clínicos. Uma desvantagem de ambos os vectores é o facto de ser difícil produzi-los com elevados títulos de infecciosidade. Uma das principais razões para este problema é que os vectores gammaretrovirais e lentivirais são muito instáveis e perdem a maioria da sua infecciosidade em apenas algumas horas a 37°C. Esta baixa estabilidade afecta não só os títulos virais mas também a qualidade/eficácia das preparações de vectores a serem utilizadas em ensaios pré-clínicos e clínicos. Assim, é de extrema importância perceber o que causa esta rápida inactivação de forma a poder aumentar a estabilidade e consequentemente a qualidade dos vectores.
Os vectores gammaretrovirais e lentivirais perdem infecciosidade em todas as fases de manufactura – produção, purificação e armazenamento – e
durante a aplicação clínica. Esta inactivação pode ser causada por diversos factores inerentes ao processo, sendo a temperatura um factor determinante que afecta a estabilidade dos vectores de duas formas: (i) afecta a estabilidade intrínseca das partículas virais produzidas e (ii) causa a inactivação das partículas em solução. Estes dois efeitos da temperatura na estabilidade/inactivação dos vectores foram alvo dos estudos efectuados nesta tese com o objectivo final de melhorar a estabilidade dos vectores.

Após uma introdução ao tema da inactivação viral no Capítulo I, no Capítulo II é apresentado um estudo do efeito da temperatura de cultura na estabilidade de vectores gammaretrovirais. Havia já sido descrito na literatura que a temperatura de produção afecta a composição da membrana das células produtoras de vectores retrovirais. Assim, por forma a determinar quais são as alterações na membrana dos vectores causadas pela produção a diferentes temperaturas, que podem estar relacionadas com a estabilidade dos vectores, foi realizado um estudo rigoroso sobre as propriedades físico-químicas da membrana dos vectores. Para tal, caracterizaram-se vectores produzidos a 37 e a 32°C por ressonância paramagnética electrónica e calorimetria exploratória diferencial, tendo sido ainda analisada a composição da membrana viral e a eficiência de transdução de modelos celulares clinicamente relevantes. Os resultados mostraram que os vectores produzidos a 32°C possuem uma membrana mais rígida com um racio colesterol/fosfolípidos mais elevado. Estes vectores têm também as proteínas de envelope num estado de conformação diferente (mais “folded”). Verificou-se que os vectores produzidos a 32°C adquirem membranas com propriedades diferentes dos vectores produzidos a 37°C. Estas alterações causam uma menor estabilidade a temperaturas abaixo de 4°C e uma menor
eficiência de transdução em células relevantes para terapia génica. Concluiu-se assim que a produção de vectores gammaretrovirais deve ser realizada a 37°C para obter vectores mais estáveis. Para além disso, como as propriedades físico-químicas das membranas virais são afectadas em resposta a alterações da temperatura de cultura, estas alterações em conjunto com alterações da composição do meio de cultura, podem ser usadas para melhorar a estabilidade e eficiência de transdução de vectores gammaretrovirais a serem aplicados em terapia génica.

Após a verificação do efeito da temperatura de cultura na estabilidade dos vectores e da determinação da melhor temperatura para obter vectores mais estáveis, 37°C, estudou-se o efeito directo da temperatura nos vectores. Devido à complexidade das partículas virais, a rápida inactivação dos vectores gammaretrovirais e lentivirais pode ser atribuída à degradação/inactivação de vários constituintes virais, como referido no Capítulo I. Assim, no Capítulo III determinou-se a degradação/inactivação de vários constituintes virais para identificar o mecanismo de inactivação de vectores gammaretrovirais. Foi possível verificar a existência de uma elevada correlação entre a perda de infecciosidade e a capacidade do vírus realizar a transcrição reversa. Foi também demonstrado que a quantidade de RNA viral que entra na célula decresce lentamente devido à incubação do vírus a 37°C. Estas duas observações levaram à conclusão de que a perda da capacidade do vírus realizar a transcrição reversa é o principal mecanismo responsável pela inactivação de vectores gammaretrovirais. Visto que o processo de transcrição reversa é muito complexo e envolve vários constituintes virais, investigou-se também a estabilidade do enzima transcriptase reversa e do RNA viral. Observou-se que ambas as actividades, RNaseH e DNA
polimerase, do enzima transcriptase reversa sofrem inactivação a 37°C, embora muito mais lentamente do que a perda de infecciosidade viral. Mostrou-se também que a degradação do RNA viral não tem influência na perda de capacidade do vírus sintetizar DNA. Assim, a inactivação de outro constituinte viral a 37°C deve afectar a síntese de DNA, possivelmente inactivação da nucleocapside ou do tRNA.

O mecanismo de inactivação de vectores lentivirais foi estudado no Capítulo IV. Utilizaram-se vectores lentivirais com duas proteínas de envelope diferentes, anfotrópica e RDpro. Em ambos os vectores observou-se uma elevada correlação entre a perda da capacidade do vírus realizar a transcrição reversa e a perda de infecciosidade a 37°C. Verificou-se que, tal como nos vectores gammaretrovirais, a entrada dos vectores lentivirais na célula hospedeira não determina a taxa de inactivação viral; ambos os vectores com diferentes proteínas de envelope mostraram perfis semelhantes de entrada. Concluiu-se assim que o principal mecanismo de inactivação dos vectores lentivirais é a perda da capacidade do vírus realizar a transcrição reversa. Foi também demonstrado que a inactivação do enzima transcriptase reversa e a degradação de RNA viral não possuem um papel relevante na perda da capacidade do vírus realizar o processo de transcrição reversa. Curiosamente, os vectores com a proteína de envelope RDpro possuem um processo de transcrição reversa mais estável do que os vectores com a proteína de envelope anfotrópica. Esta maior estabilidade implica que estes vectores são mais estáveis em termos de infecciosidade.

Através dos resultados apresentados nos Capítulos III e IV verifica-se que é possível desenvolver estratégias para estabilizar o processo de transcrição
reversa de forma a melhorar a aplicabilidade dos vectores gammaretrovirais e lentivirais.

No capítulo V utilizou-se o conhecimento de que o principal mecanismo de inactivação de vectores gammaretrovirais e lentivirais é a perda da capacidade do vírus realizar a transcrição reversa por forma a estabilizá-los. Foi possível melhorar a estabilidade dos vectores gammaretrovirais purificados a 37°C e a 4°C através da adição de albumina recombinante humana (rHSA) ao tampão de armazenamento. A adição de rHSA leva a uma estabilização do processo de transcrição reversa. No entanto, verificou-se que para os vectores lentivirais é necessário uma maior protecção. Esta protecção extra foi concretizada adicionando uma mistura de lipoproteínas e rHSA ao tampão de armazenamento. Provavelmente, complexos de lipoproteínas e rHSA associam-se à membrana dos vectores protegendo a sua estrutura e prevenindo alterações conformacionais mais eficientemente do que adicionando apenas albumina. A diferença de estabilização existente entre vectores gammaretrovirais e lentivirais foi validada através da realização de testes com vectores produzidos por diferentes linhas celulares e com diferentes proteínas de envelope.

Nesta tese foi estabelecido o importante papel da temperatura na estabilidade de vectores gammaretrovirais e lentivirais e este conhecimento foi utilizado para melhorar a estabilidade dos vectores. Primeiro observou-se que a temperatura de cultura afecta a rigidez, composição lipídica e a conformação das proteínas presentes na membrana viral levando à produção de vectores com diferentes estabilidades; os vectores produzidos a 37°C apresentam maior estabilidade. Este conhecimento cria oportunidades para um
melhoramento adicional da estabilidade dos vectores através de alterações nas condições de cultura. Segundo, a determinação de que os vectores gammaretrovirais e lentivirais perdem a sua infecciosidade, a 37°C e a 4°C, através da perda da capacidade de realizar a transcrição reversa, levou ao desenvolvimento de formulações de armazenamento que permitiram o melhoramento da estabilidade de vectores purificados.

Os resultados obtidos nesta tese permitiram o melhoramento do rendimento final de vectores no processo de manufactura, assim como da qualidade final e eficácia de preparações virais, factores essenciais para aumentar o sucesso destes vectores em terapia gênica.
THEESIS PUBLICATIONS


- Carmo, M.; Panet, A.; Carrondo, M. J. T.; Alves, P. M.; Cruz, P. E.; 2008; From retroviral vector production to gene transfer: spontaneous inactivation is caused by loss of reverse transcription capacity; Journal of Gene Medicine, 10 (4): 383-391.

- Carmo, M.; Dias, J. D.; Panet, A.; Coroadinha, A. S.; Carrondo, M. J. T.; Alves, P. M.; Cruz, P. E.; Thermo-sensitivity of the reverse transcription process as an inactivation mechanism of lentiviral vectors; Submitted.

- Carmo, M.; Alves, A.; Rodrigues, A. F.; Coroadinha, A. S.; Carrondo, M. J. T.; Alves, P. M.; Cruz, P. E.; Stabilization of gammaretroviral and lentiviral vectors: from production to gene transfer; Submitted.
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AAV</td>
<td>adeno-associated vectors</td>
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<tr>
<td>Ad</td>
<td>adenoviral vectors</td>
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<td>ADA</td>
<td>adenosine deaminase deficiency</td>
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<td>Ampho</td>
<td>amphotropic envelope protein</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>CA</td>
<td>capsid protein</td>
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<td>CGD</td>
<td>chronic granulomatous disease</td>
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<td>C/P</td>
<td>cholesterol to phospholipids ratio</td>
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<td>DSC</td>
<td>differential scanning calorimetry</td>
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<td>DMEM</td>
<td>dulbecco minimum essential medium</td>
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<td>EIAV</td>
<td>equine infectious anemia virus</td>
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<td>Env</td>
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<td>electron paramagnetic resonance</td>
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<td>fetal bovine serum</td>
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<td>feline immunodeficiency virus</td>
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<td>gibbon ape leukaemia virus</td>
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<td>IP</td>
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<td>long terminal repeat</td>
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<td>MA</td>
<td>matrix protein</td>
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<td>Acronym</td>
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<tr>
<td>MLV</td>
<td>murine leukemia virus</td>
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<td>recombinant competent lentivirus</td>
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<td>X-linked severe combined immunodeficiency</td>
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<tr>
<td>TM</td>
<td>transmembrane envelope protein</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>VSV-G</td>
<td>vesicular stomatitis virus G protein</td>
</tr>
<tr>
<td>γ-RV</td>
<td>gammaretroviral vectors</td>
</tr>
</tbody>
</table>
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1. GENE THERAPY

Gene therapy can be defined as the delivery and expression of genetic information in cells of an individual to restore health or to ease the consequences of a disease (Goncalves, 2005). This technology was received with great enthusiasm by the scientific community in the 80's; such a simple concept could revolutionize medicine. However, this enthusiasm has waned as a consequence of the results obtained in the first two approved gene therapy clinical trials. In these first trials only mild improvements of the diseases were directly related to gene therapy (Blaese et al., 1995; Rosenberg et al., 1994). In fact, these disappointments made clear that this was not a simple technology and that profound knowledge of human genetics, vector biology and pharmacology would be necessary to take advantage of the full potential of gene therapy (Thomas et al., 2003). Thus, over the past years, intense efforts have been carried out to make this technology feasible.

Initially gene therapy was developed for treatment of monogenic recessive disorders but, due to the high potential of this technology, it has also been adapted to other disorders, such as cancer, cardiovascular, neurological and infectious diseases (Andreadis et al., 1999; Goncalves, 2005). Figure 1a lists the diseases for which gene therapy is being tested. The number of candidate diseases is increasing every year due to the increasing knowledge of genes function that became available after the discovery of the human genome sequence and its use to identify specific disease genes (Aiuti et al., 2007; Alexander et al., 2007; McTaggart and Al-Rubeai, 2002). Alas, even with all this increasing knowledge, evidence of clinical improvement has been shown in only a few clinical trials. Nevertheless, the first gene therapy products have entered the market through China: Gencidine™ in 2003
(SiBiono GeneTech) and Oncorine™ in 2006 (Shangai Sunway Biotech); two other are almost ready to enter the market: Cerepro™ (Ark Therapeutics) and TroVax (Oxford Biomedica). Hopefully this will facilitate further research and commercialization (Raty et al., 2008).

This apparent poor outcome can be attributed to the number of challenges faced by gene therapy, such as its ability to consistently transduce a sufficient number of target cells with the therapeutic gene and to obtain a sustained and therapeutically sufficient level of the gene product. The efficient transfer of a therapeutic gene into human cells will depend upon the technology used for gene delivery (McTaggart and Al-Rubeai, 2002). Three different categories of methods exist for gene delivery: physical delivery of naked DNA; delivery by non-viral vectors; and delivery by viral vectors.

Physical delivery of naked DNA to a cell has been achieved using a number of methods such as microinjection, electroporation, or microparticle bombardment. While the thought of introducing directly the DNA to correct the gene defect of the cells would be perfect, the problems that arise from this delivery system, such as low DNA transfer and the fact that the transfection of the therapeutic gene is only transient, are major drawbacks of this technology (Kawakami et al., 2008).

The goal in designing non-viral vectors is to create pseudoviruses that mimic selected viral properties while overcoming targeting, plasmid cargo and adverse immunogenic issues. Normally, non-viral vectors consist in the use of a carrier molecule complexed with DNA to insert the gene of interest into the host cell. Several types of carrier systems exist like liposomes, polymers, lipids and polymers and peptides or proteins. Unfortunately, despite the
success in pre-clinical trial studies, non-viral vectors have not yet demonstrated consistent transfection efficiency comparable to that of viruses, regardless of gene or target cell type (Audouy et al., 2002; Liu et al., 1995).

Viruses have evolved to efficiently transfer their own genome into a host cell, thereby using the cell’s machinery for their own replication. This unique process has been exploited in the development of viral vectors for gene delivery. Ideal virus-based vectors for most gene therapy applications harness the viral infection pathway but avoid the subsequent expression of viral genes that leads to replication and toxicity (McTaggart and Al-Rubeai, 2002; Thomas et al., 2003). Figure 1b indicates present utilization of vectors in gene therapy clinical trials.

**Figure 1:** (a) Indications addressed by gene therapy clinical trials; (b) Vectors used in gene therapy clinical trials. Adapted from the Journal of Gene Medicine clinical trial database, 2008, Wiley and Sons Ltd.
1.1. Viral vectors

Given the diversity of disease targets that are potentially amenable to gene transfer, it has become clear that there can be no single vector that is suitable for all applications (Flotte, 2007; Thomas et al., 2003). Perhaps the only characteristics required by all vectors are the abilities to be reproducibly and stably propagated and purified to high titers, in order to mediate target delivery (that is, to deliver the transgene specifically to the tissue or organ of interest without widespread vector dissemination elsewhere) and to mediate gene delivery and transgene expression without inducing harmful side effects (Thomas et al., 2003). The number of different viruses that are under development as gene therapy vectors is increasing, but there are, at present, four main classes of clinically applicable viral vectors that are derived from adenovirus, adeno-associated virus, gammaretrovirus and lentivirus (Flotte, 2007; Raty et al., 2008; Thomas et al., 2003). Substantial work has also gone into developing poxvirus derived vectors (especially vaccinia) for genetic vaccines and herpes simplex virus derived vectors. A summary of the advantages, disadvantages and applications of the four main classes of viral vectors is presented in Table 1.

Adenoviral vectors (Ad) are the most commonly used vectors in gene therapy clinical trials (Edelstein et al., 2007). For a wide variety of cell types, Ad gives more-efficient gene transfer compared with other systems, especially for *in vivo* applications. Ad vector can transfer genes to both proliferating and quiescent cells. Immediately after delivery transgene expression is at a high level but, being transient, it becomes almost undetectable in most tissues after two weeks. This is because Ad vectors
have very low frequency of integration (Harui et al., 1999) and, for safety reasons, are disabled for replication (Mountain, 2000).

**Table 1**: Gene therapy viral vectors: main advantages and disadvantages (Flotte, 2007; McTaggart and Al-Rubeai, 2002; Mountain, 2000).

<table>
<thead>
<tr>
<th>Vector</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Examples of applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gamma-retrovirus</td>
<td>- High transfection efficiency <em>ex vivo</em></td>
<td>- Low transfection efficiency <em>in vivo</em></td>
<td>- ADA-SCID</td>
</tr>
<tr>
<td></td>
<td>- Long-term expression</td>
<td>- 8 kb insert-size limit</td>
<td>- SCID-X1</td>
</tr>
<tr>
<td></td>
<td>- Substantial clinical experience <em>ex vivo</em></td>
<td>- Only transfects proliferating cells</td>
<td>- CGD</td>
</tr>
<tr>
<td></td>
<td>- Low immunogenicity</td>
<td>- Safety concerns due to insertional mutagenesis</td>
<td>- familial hyperlipidemia</td>
</tr>
<tr>
<td></td>
<td>- Proved good results</td>
<td>- Extremely difficult manufacture</td>
<td>- tumour vaccine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Low stability</td>
<td>- HIV</td>
</tr>
<tr>
<td>Lentivirus</td>
<td>- Long-term expression</td>
<td>- 8 kb insert-size limit</td>
<td>- HIV</td>
</tr>
<tr>
<td></td>
<td>- Transfects proliferating and non-proliferating cells</td>
<td>- Safety concerns due to insertional mutagenesis</td>
<td>- β-thalassemia</td>
</tr>
<tr>
<td></td>
<td>- Transfects hematopoietic stem cells</td>
<td>- Extremely difficult manufacture</td>
<td>- melanoma</td>
</tr>
<tr>
<td></td>
<td>- Low immunogenicity</td>
<td>- Low stability</td>
<td>- leukaemia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Reduced clinical experience</td>
<td>- lymphoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- mucopolysaccharidoses</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>type VII</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>- Very high transfection efficiency</td>
<td>- 7.5 kb insert-size limit</td>
<td>- cystic fibrosis</td>
</tr>
<tr>
<td></td>
<td>- Substantial clinical experience</td>
<td>- Strong immune responses (repeat dosing ineffective)</td>
<td>- ornithine transcarbamylase deficiency</td>
</tr>
<tr>
<td></td>
<td>- Transfects proliferating and non-proliferating cells</td>
<td>- Moderately difficult manufacture</td>
<td>- mesothelioma</td>
</tr>
<tr>
<td></td>
<td>- Efficient retargeted transfection</td>
<td>- Short-term expression</td>
<td>- colon cancer</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AAV</td>
<td>- Transfects efficiently a wide variety of cells <em>in vivo</em></td>
<td>- 4.5 kb insert-size limit</td>
<td>- cystic fibrosis</td>
</tr>
<tr>
<td></td>
<td>- Prolonged expression <em>in vivo</em></td>
<td>- Safety concerns due to insertional mutagenesis</td>
<td>- haemophilia B</td>
</tr>
<tr>
<td></td>
<td>- Low immunogenicity</td>
<td>- Extremely difficult manufacture</td>
<td>- Leber congenital amaurosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Low clinical experience</td>
<td>- canavan disease</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Repeat dosing affected by neutralising antibody responses</td>
<td>- alpha-antitrypsin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>deficiency</td>
</tr>
</tbody>
</table>
Introduction

There are also safety issues regarding Ad vectors, the main one being the possibility of provoking a severe immune and/or inflammatory response (Edelstein et al., 2007; Raty et al., 2008). However, recent improvements that reduce the immunogenicity of Ad vectors have enhanced the prospects for long-term gene transfer in a wide range of different tissues (Alba et al., 2005; Flotte, 2007; Mountain, 2000; Raty et al., 2008; Romanczuk et al., 1999). Although long-term expression is a problem for these vectors, they continue to be widely used for their usefulness for direct cell killing, many immunotherapy strategies and some acute diseases. It is worth mentioning that the two gene therapy products commercially available are based on adenovirus (Raty et al., 2008).

Recombinant adeno-associated vectors (AAV) are one of the most promising vector systems for safe, long-term gene transfer and expression in non-proliferating tissues. AAV is unique among viruses that are being developed for gene therapy in that the wild-type virus is not implicated in any human disease. The small size and simplicity of the vector particle makes it possible to administer high doses of vector systemically without eliciting acute inflammatory responses or toxic effects (Thomas et al., 2003). The main disadvantages of AAV concern insert size, manufacturing and cell specificity. The virus can only accommodate inserts up to 4.5 kb and manufacturing processes require the use of helper viruses, raising issues of low titer, contamination and costly purification procedures (Mountain, 2000). Improved manufacturing processes as well as infection specificity of the vectors are under way to circumvent these problems (Blouin et al., 2004). Another concern with AAV vector is the possibility of causing
insertional mutagenesis; this vector led to the development of cancer in mouse models (Donsante et al., 2007).

Gammaretroviral vectors (γ-RV) also referred to as oncoretroviral vectors were the first class of viral vectors to be developed and have been one of the most widely studied and used vector in clinical trials, specifically murine leukaemia virus (MLV)-derived vectors (Blaese et al., 1995; Markowitz et al., 1988; Rosen et al., 1986). They target dividing cells with a high degree of efficiency and provide stable gene transfer as they integrate into the chromosomes of the target cells (Andreadis et al., 1999; McTaggart and Al-Rubeai, 2002; Palu et al., 2000). Both these properties constitute also the setbacks of these vectors, they have to be used ex vivo and cannot be used to infect all types of cells, since they only infect non-dividing cells and additionally the integration of the gene of interest may pose a problem of insertional mutagenesis (Raty et al., 2008). Sadly, this last problem of gammaretroviral vectors has already caused four cases of leukaemia in a clinical trial, where one of the patients died. Nevertheless, all other patients of this clinical trial and of other similar clinical trials have benefited from the gene therapy, with correction of their condition (Hacein-Bey-Abina et al., 2008). So far these vectors present the highest success in curing monogenic diseases.

Lentiviral vectors (LV) offer similar advantages to the gammaretroviral vectors, in that they mediate long-term integration of the therapeutic gene, but unlike gammaretroviral vectors, they do not require cellular mitosis in order to gain access to the host genome for integration. They also are thought to share the potential for insertional mutagenesis with eventual carcinogenesis, although this has not been observed in the pre-clinical trials
performed so far (Flotte, 2007). These vectors are considered the solution for some of the problems of gammaretroviral vectors. In most instances, the nature of the disease may direct the vector to be used, since it may dictate a specific cell type, expression level, and duration of expression that will be required for therapeutic effect. Accordingly, there is no universal vector for all diseases, nor can a vector be chosen as the best one.

2. GAMMARETROVIRAL AND LENTIVIRAL VECTORS

2.1. Historical perspective and clinical applications

As indicated above gammaretroviral and lentiviral vectors are the most promising vectors for the treatment of monogenic disorders, since they integrate the gene of interest in the host-cell chromosome, resulting in prolonged gene expression. Gammaretroviral vectors are one of the most studied and most widely used classes of viral vectors in gene therapy. Traditionally, gammaretroviruses have been the vectors of choice for ex vivo transduction of hematopoietic stem cells. The first clinical trial where gammaretroviral vectors were applied occurred in 1990 to correct adenosine deaminase deficiency (ADA) (Blaese et al., 1995). The treatment improved the physical conditions of the patients and the ADA-containing provirus was stable in the blood for several years. It was concluded that gene therapy could be a safe and effective addition to the treatment of some patients with this severe immunodeficiency disease. Since the first studies, this vector has become the focus of an ever-expanding research activity. The demonstration of full correction of X-linked severe combined immunodeficiency (SCID-X1)
Chapter I

phenotype in infants (Cavazzana-Calvo et al., 2000) and more recently of ADA-SCID (Gaspar et al., 2006) confirmed the efficacy of gammaretroviral vectors. Other monogenic diseases have been tested for treatment with this vector ex vivo, such as the Gaucher disease (Dunbar et al., 1998), chronic granulomatous disease (Ott et al., 2006) and hypercholesterolemia (Gough and Raines, 2003). Gammaretroviral vectors have also demonstrated some promising results in cancer therapy, bone marrow transplantation and HIV. The capacity of lentiviral vectors to infect non-dividing cells is an attribute that significantly broadens the utility of the vectors to numerous target tissues and cell types. Several lentiviral vectors exhibit proficient transduction of non-dividing cells in target organs in vivo, including the central nervous system, liver, eye, heart, hematopoietic stem cells, and pancreas (Cockrell and Kafri, 2003; Wiznerowicz and Trono, 2005). In the beginning lentiviral vectors were looked with great suspicion; the fact that they derive from highly pathogenic viruses, like HIV, was not looked upon favourably. Motivated by safety issues, prevailing efforts have finally guided lentiviral vector development toward corrective gene therapy for a number of incurable infections and genetic diseases. Throughout the course of this maturation stage, lentiviral vectors have also facilitated advances in transgenic animal development and stable delivery of molecules (shRNA and miRNA) for RNA interference (RNAi) to establish post-transcriptional control of gene expression (Rubinson et al., 2003; Tiscornia et al., 2003). The pre-clinical studies using lentiviral vectors comprise diseases of the CNS, such as Alzheimer’s, Parkinson’s and Huntington’s, correction of genetic ocular diseases and correction of genetic disorders including immunodeficiencies and haemoglobin disorders. With respect to clinical
trials this vector is being used to treat HIV infection, β-thalassemia, malignant melanoma, leukaemia/lymphoma and mucopolysaccharidoses type VII (Cockrell and Kafri, 2007; Flotte, 2007).

2.2. Biology of retroviruses

Retroviruses comprise a large and diverse family of enveloped RNA viruses defined by common taxonomic denominators that include structure, composition, and replicative properties (Vogt, 1997). The hallmark of the Retroviridae family is its replicative strategy which includes as essential steps reverse transcription of the virion RNA into linear double-stranded DNA and the subsequent integration of this DNA into the genome of the cell.

Retroviruses are broadly divided in two categories – simple and complex – distinguishable by the organization of their genomes and are further divided into seven genus defined by evolutionary relatedness, each with the taxonomic rank of genus. Five of these groups represent retroviruses with oncogenic potential, also referred to as oncoviruses: Alpharetrovirus (e.g. Avian Leukosis Virus - ASLV); Betaretrovirus (e.g. Mouse Mammary Tumour Virus - MMTV); Gammaretrovirus (e.g. Murine Leukemia Virus - MLV); Deltaretrovirus (e.g. Human T-cell Leukemia Virus 1 – HTLV-1); Epsilonretrovirus (e.g. Walleye Dermal Sarcoma Virus - WDSV). The other two genus are the Lentivirus (e.g. Human Immunodeficiency Virus 1 – HIV-1) and the Spumavirus (e.g. Simian Foamy Virus - SFV) (Overbaugh et al., 2001).

2.2.1. The virus particle

Retroviruses contain two identical copies of single-stranded, positive-sense RNA (typically 7-10 kb pairs in length) complexed with nucleocapsid
proteins. Also contained in the nucleocapsid are the proteins reverse transcriptase, integrase and protease (Palu et al., 2000). This structure is enclosed by a protein shell formed by the capsid proteins (core). Matrix proteins form a layer outside the core and interact with an envelope consisting of a lipid bilayer, which surrounds the viral core particle. The lipidic envelope is originary from the cellular membrane where, during budding of the newly formed particles, viral envelope glycoproteins (Env) are incorporated (Cruz et al., 2008; Palu et al., 2000). The envelope glycoprotein, responsible for the virus interaction with specific receptors, is the only viral protein on the surface of the particle. The diameter of a typical retrovirus particle has been reported to range from 90 nm to 140nm (McTaggart and Al-Rubeai, 2002). Figure 2 depicts the structure of a retrovirus particle.

![Figure 2: Structure of a retrovirus.](image)

### 2.2.2. The genome organization

On the basis of their genome structure, retroviruses can be classified into simple (e.g. MLV) or complex retroviruses (e.g. HIV). Both encode four gene families, *gag*, *pro*, *pol* and *env* (Cruz et al., 2008; Vogt, 1997). The *gag* sequence encodes the three main structural proteins: matrix (MA), capsid (CA) and nucleocapsid (NC). The *pro* gene encodes the proteases responsible
for Gag and Gag-Pol cleavage and the viral particle maturation after budding. The \textit{pol} sequence encodes the enzyme reverse transcriptase (RT) which carries out reverse transcription of the viral RNA to DNA during the infection process, and integrase (IN), which catalyses the integration of the proviral DNA into the host genome. The \textit{env} sequence encodes the two subunits of the envelope glycoprotein.

The complex lentivirus has two additional groups of genes that have essential functions during the life cycle and pathogenesis: the regulatory genes (\textit{tat} and \textit{rev}) and the accessory genes (\textit{vpr, vpu, vif} and \textit{nef}). The Tat protein regulates the promoter activity of the 5'LTR and is necessary for the transcription from the 5'LTR. The Rev protein binds to the rev response element (RRE) within the viral RNA, allows the transport of unspliced RNA out of the nucleus, and is necessary for efficient \textit{gag} and \textit{pol} expression. The accessory genes are essential virulence factors for HIV and have functions in nuclear transport, virion assembly and release, growth arrest of infected cells and infectivity (Blesch, 2004; Vogt, 1997).

Efficient gene transduction and integration depends on a number of \textit{cis}-acting sequences present in the retroviral genome: two long-terminal repeats (LTRs), a viral packaging signal (ψ), signals for reverse transcription such as the primer bidding site (PBS) and the polypurine tract (PPT). The LTRs are formed during reverse transcription and contain elements required to drive gene expression, reverse transcription and integration into the host chromosome. The packaging signal is required for specific packaging of the RNA into the newly formed virions. PBS and PPT are required for reverse transcription (Cruz et al., 2008; Palu et al., 2000; Vogt, 1997).
2.2.3. The life cycle

The life cycle of retrovirus follows a series of complex necessary stages that can be summarized in four main steps: (i) interaction and fusion of the viral particles with the cell surface, leading to viral entry into the cell; (ii) reverse transcription; (iii) integration; and (iv) synthesis, assembly and budding of newly formed virus.

2.2.3.1. Viral entry

The process of retroviral entry into a target cell represents the first step in the viral infection cycle. It is characterized by a complex series of events that are initiated through the binding of the viral surface glycoproteins to specific receptor molecules on the cells’ outer membrane which leads to the fusion of the lipid bilayers of the cell and viral membranes and allows the virus to be introduced into the host cell cytoplasm (Hunter, 1997).

The envelope glycoprotein complex of retrovirus includes two polypeptides, an external, glycosylated hydrophilic polypeptide (SU) and a transmembrane spanning protein (TM), that are connected by a disulfite bond and together form an oligomeric spike on the surface of the virion (Hunter, 1997). The SU protein initiates entry by binding to a specific cell surface protein; SU is therefore the primary determinant of the range of cells susceptible to infection by a retrovirus. The specificity of the SU/receptor interaction defines the host range and tissue tropism of a retrovirus; viral particles lacking envelope glycoproteins are non infectious, and cells lacking a receptor are non permissive for viral entry. The interaction between the SU and the TM proteins has been considered a weak point of these viruses, with the possible occurrence of envelope shedding. The loss of the molecule responsible for recognition of cell receptors, SU, is considered by some
Introduction

authors the cause for the virus loss of infectivity (Layne et al., 1992; Le Doux et al., 1999; Merten, 2004).
The interaction of the SU portion of the retroviral envelope to its receptor induces a conformational change that exposes a viral fusion peptide, present in the ectodomain of TM, allowing the viral membrane to fuse with the cell membrane. After fusion of the viral and cell membranes, the nucleocapsid is transported to the cytoplasmic side of the cell membrane (Nisole and Saib, 2004; Russell and Cosset, 1999).

2.2.3.2. Reverse transcription
The fusion of the viral and cellular membranes delivers the viral core into the cytoplasm, where the viral RNA is converted into a double stranded DNA. Immediately after its release into the cytoplasm, the viral core undergoes a partial and progressive disassembly, known as uncoating, that leads to the generation of sub-viral particles called reverse transcription complexes (RTC) (Wacharapornin et al., 2007). The composition of the reverse transcription complexes has been rather difficult to define but it is known that different retroviruses have different RTC compositions. The RTCs of MLV contain at least the viral genome, nucleocapsid proteins, capsid proteins, integrase, and RT proteins, while the RTCs of HIV contains the same constituents except the capsid protein. These complexes are of utmost importance for the protection and correct completion of the reverse transcription process (Fassati and Goff, 1999, 2001; Nermut and Fassati, 2003).
A graphic description of the major steps involved in the reverse transcription process is depicted in Figure 3.
Figure 3: The process of reverse transcription. (a) Minus strand DNA synthesis initiates from the tRNA primer, annealed to the PBS (primer binding site) region of the plus sense RNA genome, and proceeds to the 5’ end of the RNA. (b) After RNase H degradation of the R and U5 sequences of the resulting RNA-DNA hybrid, 1st strand transfer occurs (c) with the annealing of the (-)DNA R sequence to the complementary 3’RNA R sequence. Minus strand DNA synthesis R-U3 sequence resumes, accompanied by further RNase H digestion of the template RNA, only the RNA PPT sequence is not digested (d). The tRNA is subsequently removed by RNase H (e) and the second strand transfer occurs through annealing of the PBS regions (f). Reverse transcription proceeds via strand displacement and in the end the proviral DNA possesses two long terminal repeats (g). The grey boxes represent viral RNA and the white boxes represent the cDNA.
As in the case of other DNA polymerases, RT needs a primer carrying a free 3'-OH group to initiate DNA synthesis; in the retrovirus reverse transcription this molecule is a tRNA. Annealing of the tRNA primer to the viral plus-strand RNA takes place in a location termed as the primer binding site (PBS). This region is complementary to the 3' terminal nucleotides of tRNA. RT recognizes the binary tRNA/RNA complex and initiates reverse transcription by extending the 3'-end of the annealed primer, while the recessed template strand guides DNA synthesis. Synthesis of minus-strand DNA proceeds towards the 5'-end of the RNA template (Figure 3a). This intermediate is called the (-) strand strong stop DNA or (-) ssDNA. The RNase H domain of RT degrades the RNA template annealed to the (-) ssDNA (Figure 3b). Consequently, the (-) ssDNA is released and anneals to the 3'-R region located at the 3'-end of the genome. This step is referred to as the first strand transfer reaction (Abbink and Berkhout, 2008; Gotte et al., 1999; Telesnitsky and Goff, 1997) (Figures 3 b and c).

The (-)ssDNA subsequently serves as primer for (-) strand DNA synthesis. Reverse transcription proceeds and generates a full-length (-) strand cDNA that serves as a template for (+) strand DNA synthesis. RNase H degrades the RNA template, except for two fragments that resist the cleavage: polypurine tracts in the U3 region (3'-PPT) and the center of the template (central PPT, cPPT). The resistant RNA sequence serves as primer for (+) strand synthesis, which terminates at the first modified base in the tRNA (Figure 3d). The tRNA is subsequently removed from the (+) strand DNA by RNase H (Figure 3e). A second strand transfer reaction results in the annealing of the (+) strand DNA to the 3' end of the full-length (-) strand DNA (Figure 3f). Reverse transcription proceeds over the (-) strand DNA until it encounters
the cPPT-extended (+) strand. Elongation occurs through the cPPT via a mechanism called strand displacement until RT reaches a nearby site (80-100 nucleotides downstream of the cPPT), the central termination sequence. The consequence of the two strand transfer reactions is that the provirus acquires a duplicated U3-R-U5 sequence at both of its ends (Figure 3g); these are termed long terminal repeats (LTRs) (Abbink and Berkhout, 2008; Basu et al., 2008; Telesnitsky and Goff, 1997).

Macromolecules participating in reverse transcription

Several viral constituents have major roles in the reverse transcription process. The most important ones are the viral genome, the reverse transcriptase, the primer tRNA and the nucleocapsid proteins. The retroviral genome consists of two molecules of single stranded RNA which contains sequence elements important for reverse transcription. These include: (i) the direct repeats, termed R, that lie at the 5’ and 3’ ends of the RNA immediately adjacent to the 5’ cap and 3’ poly(A) tail; (ii) sequences that are unique to each end of the viral RNA and are adjacent and internal to the R sequences, U5 and U3; and (iii) the PBS and the PPT. Any loss of these sequences due to RNA degradation leads to the inability to synthesize DNA or to the formation of deficient DNA incompetent for integration.

Reverse transcription is mediated by the viral enzyme reverse transcriptase (RT). This unique multifunctional enzyme possesses both RNA-dependent and DNA-dependent DNA polymerase activities, as well as ribonuclease H (RNase H) activity that specifically degrades the RNA strand of RNA/DNA hybrids. The subunit composition and organization of various retroviral RTs are markedly different. MLV RT is a monomeric enzyme that contains both
DNA polymerase and RNase H domain in a single polypeptide. HIV-1 RT is a heterodimer of 66 and 51 kD subunits, whereas the larger HIV-1 RT subunit, p66, contains both the DNA polymerase and RNase H domain, and the smaller HIV-1 subunit is a carboxy-terminal truncation of the larger subunit that lacks essentially the entire RNase H domain (Gotte et al., 1999). All DNA polymerases, including RT have an absolute requirement for the 3'OH group of primers during DNA polymerization. However, retroviral RTs are unusual in that they all utilize host cell-derived tRNAs as their primer, that are uptaken during virus encapsidation. Different RTs employ different tRNA molecules as primers. In the case of MLV, this role is played by tRNA\text{PRO}, in the case of HIV-1 is tRNA\text{LYS} (Abbink and Berkhout, 2008).

*In vivo*, the template for reverse transcription is actually a complex of RNA and NC protein. In general NC is considered to be a nucleic acid chaperone that catalyses structural rearrangement in RNA or DNA to form thermodynamically stable conformations. A number of functional activities have been ascribed to the NC that are important for the reverse transcription process: unwinding of tRNA; stimulation of reverse transcription; stimulation of strand transfer; and increasing the processivity of DNA synthesis (Gotte et al., 1999; Thomas and Gorelick, 2008).

**2.2.3.3. Integration**

After its complete synthesis, viral DNA has to be carried into the nucleus where integration is going to occur. The linear viral DNA is the proximal precursor to the integrated provirus and is contained in a specific nucleoprotein complex called the pre-integration complex (PIC). This complex varies in composition among different retroviruses, but all include the newly synthesized DNA and integrase. PICs from most retroviruses are
unable to enter intact nuclei and must therefore wait for the breakdown of the nuclear membrane occurring during mitosis. Consequently, these retroviruses, such as MLV, are dependent on cell cycle and cannot replicate in non-dividing cells. In contrast, lentiviruses, such as HIV-1, are able to productively infect non-dividing cells, such as macrophages or quiescent T lymphocytes, indicating that their PICs are able to actively cross the nuclear membrane. Four different viral components have been identified to contribute to the nuclear import of HIV-1: integrase, matrix protein (MA), Vpr and the viral DNA, although the exact function of each remains to be fully understood (Cullen, 2001; Nisole and Saib, 2004).

Upon entry into the nucleus, the PIC encounters the host DNA and IN starts the unique process of integration. First the 3’-terminal bases at either end are removed by cleavage reaction of IN, leaving a 3’-OH end (Brown, 1997; Fields et al., 1996). Binding of host DNA by the integrase-viral DNA complex is followed by a concerted, integrase-catalysed reaction. Once integrated the provirus is stable and is inherited by daughter cells as any other autossomal gene.

### 2.2.3.4. Replication – synthesis, assembly and budding

Transcription of proviral DNA recreates the full-length viral RNA genome and subgenomic-sized RNA molecules are generated by RNA processing. All RNA products serve as templates for the production of viral proteins. Full-length RNA transcripts serve two functions: they encode the *gag* and *pol* gene products, and they are packaged into the progeny virion particles as genomic RNA. Subgenomic RNA molecules provide mRNAs for the remainder of the viral gene products. The processing of viral transcripts is performed by host-cell machinery; to exploit this machinery, the virus
contains the necessary *cis*-acting regulatory elements (Swanstrom and Wills, 1997).

After RNA transcription and viral proteins production assembly of viral particles proceeds. The polyprotein precursors used to initiate the process of assembly are encoded by the *gag*, *pro*, *pol* and *env* genes common to all replication competent retroviruses. Gag has the central role in assembly. In particular, Gag has the ability to direct the budding of virus-like particles from the cell, even when expressed in the absence of all the other virus-encoded components. The Gag protein is also involved in packaging of most of the other components of the virion, including the two copies of genomic RNA (Swanstrom and Wills, 1997).

The Env polyprotein contains the sequences for SU and TM that are synthesized from a spliced transcript and glycosylated. The viral glycoproteins are carried through the stacks of the Golgi apparatus and then to the plasma membrane, where they are exposed outside of the cell. Lateral movements bring them to the site of budding where they are assembled onto particles containing Gag, Gag-Pro-Pol, and viral RNA as they emerge and bud out of the cell surface with the release of newly formed viruses (Demirov and Freed, 2004). These viruses further undergo a maturation step, where the protease processes the Gag and Gag-Pro-Pol precursors. This step is obligatory in the formation of an infectious particle (Buchschacher and Wong-Staal, 2000).

### 2.3. Turning retroviruses into vectors

As retroviruses are potentially pathogenic, their clinical use implies the development of vectors carrying the therapeutic sequences, but unable to replicate or to transfer viral functions. By deleting the viral helper genes
(gag, pol and env) from the retroviral genome and replacing them with a therapeutic foreign gene, the modified virus can still enter a target cell and insert its genome. However, further particles cannot be produced due to the lack of the essential gene functions. Hence, the virus is rendered replication incompetent (McTaggart and Al-Rubeai, 2002; Palu et al., 2000). As already mentioned, gammaretroviral and lentiviral vectors derive from simple and complex retroviruses, respectively. Thus, it was necessary to follow different development strategies for each vector type. Most recombinant retroviral systems designed for gene delivery are divided into a transfer vector, expressing the therapeutic gene and helper constructs that encode the viral proteins.

2.3.1. Development of gammaretroviral vectors
For the production of gammaretroviral vectors, the majority of the gag coding region and the complete coding region for the pol and env genes are removed leaving a backbone of the 5’ and 3’ LTRs, the packaging signal (ψ), and a small part of the gag coding region that enhances the packaging of the viral RNA (ψ+). The transgene is placed between the LTRs, and the resulting RNA transcript can be packaged into a virus if all the helper functions (gag, pol, and env proteins) are provided in trans within a cell. The production of gag, pol and env genes in trans was initially accomplished using a gammaretroviral genome lacking the sequences necessary for packaging (ψ), replacing the 5’ LTR with a different enhancer/promoter and the 3’ LTR with a polyadenylated signal (Cone and Mulligan, 1984). More recently, gag-pol and env coding regions have been separated on different transcriptional units, diminishing the risks of wild-type virus production through recombination (Danos and Mulligan, 1988). Gammaretroviral vectors can be
produced transiently by transfection of plasmids coding for the helper functions and transfer plasmid into a suitable cell line, mostly derived from 293 cells (Naviaux et al., 1996). Alternatively, a variety of producer cell lines are available that contain some or all the helper functions stably integrated into the producer cell line. The latter has the advantage of generating higher virus titers, reproducible batches and higher safety (Blesch, 2004; Cruz et al., 2008).

Building on this basic concept of gammaretrovirus production, gammaretroviral vectors with additional modifications in the LTRs have been developed. Production of high titers of gammaretroviral vectors is not only dependent on sufficient amounts of packaging proteins but also on high levels of viral RNA. To increase the production of viral RNA in the producer cell line, the U3 region in the proviral DNA containing the enhancer promoter can be replaced by a stronger enhancer resulting in substantial increases in viral titer. This change has no influence on the structure of the viral RNA because the enhancer replacing the U3 region of the 5’ LTR is not present in the viral transcript (Rigg et al., 1996). The introduction of deletions of the 3’ LTR has led to the development of self-inactivating (SIN) vectors. Removal of the enhancer in the U3 region of the 3’ LTR in the provirus/transfer plasmid leads to a deleted U3 region after reverse transcription and target cell transduction. Thereby, the enhancer/promoter of the LTRs and potential influences on neighbouring genes are removed. To express the transgene of interest, an internal promoter needs to be included in SIN vectors as the promoter and enhancer activity of the 5’ LTR is lost upon reverse transcription and viral integration (Yu et al., 1986).
2.3.2. Development of lentiviral vectors

Generally, lentiviral vectors are produced in the same way as gammaretroviral vectors. The gene of interest is placed between the 5’ and 3’ LTRs and helper functions are provided in *trans*. To completely prevent contamination of vector preparations with wild-type or helper virus, a major concern with HIV-based lentiviral vectors, a number of changes and modifications were made over time leading to the safe production of high titer lentiviral vector preparations (Blesch, 2004). Vectors derived from HIV-1 are the most commonly used and studied; nevertheless, vectors based upon Feline Immunodeficiency Virus (FIV) (Poeschla et al., 1998), Equine Infectious Anemia Virus (EIAV) (Olsen, 1998) and Simian Immunodeficiency Virus (SIV) (Mangeot et al., 2000) are also frequently used.

The first generation of lentiviral vectors was produced using transient transfection of three different plasmids into 293T cells. To provide the necessary helper functions, the ψ sequences necessary for packaging and the gene encoding the envelope (*env*) were removed from the wild-type HIV genome, the 5’ LTR was replaced by a heterologous promoter, and the 3’ LTR was replaced by a polyadenylation signal. The envelope protein was supplied by a second helper plasmid coding for the vesicular stomatitis virus G protein (VSV-G). The transfer vector for gene delivery contained the wild-type 5’ and 3’ LTRs, the packaging signal, and an expression cassette. Co-transfection of these three plasmids into 293T cells resulted in the production of VSV-G pseudotyped lentiviral vectors (Naldini et al., 1996). However, high titer stock had a considerable risk of containing some pseudotyped HIV-1 due to recombination during virus production. To
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Further reduce the risk, the accessory genes \textit{vpr}, \textit{vpu}, \textit{nef} and \textit{vif}, crucial virulence factors of HIV, were deleted from the helper vector in the second generation lentiviral vector production systems. These deletions had no negative effect on the \textit{in vitro} production of vectors (Zufferey et al., 1997). In the third generation of lentiviral helper systems, the U3 region in the 5’ LTR was replaced by a heterologous enhancer. Thus, the activity of the 5’ LTR during production of the vectors became independent of the \textit{tat} gene, which could be completely removed from the helper construct. The \textit{rev} gene necessary for efficient expression of \textit{gag} and \textit{pol} was cloned into a separate expression plasmid further diminishing the likelihood of recombination (Dull et al., 1998). In addition, a deletion introduced into the transfer plasmid in the U3 region of the 3’ LTR originated a self-inactivating vector (see above), eliminating the possibility of replication competent virus production (Zufferey et al., 1998). Thus, the current generation of lentiviral vectors is produced using four separate plasmids with minimal sequence homology comprising (1) the \textit{gag-pol} genes, (2) the \textit{rev} gene, (3) the \textit{env} gene and (4) the transfer plasmid containing the expression cassette of interest (Cockrell and Kafri, 2007).

To enhance the infectivity of lentiviral vectors, the central polypurine tract (cPPT) is often included in the lentiviral transfer vector (Van Maele et al., 2003), and post-transcriptional response elements such as the Woodchuck hepatitis virus post-transcriptional response (WPRE) element further enhance the transgene expression in the target cell (Mitta et al., 2005).

\textbf{2.3.3. Envelope protein and vector tropism} 

The separation of the three retroviral components, \textit{gag-pol}, viral genome, and envelope, in different constructs allowed recombinant gammaretroviral
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Vectors to be pseudotyped with surface proteins from different viruses (Cruz et al., 2008). This technique can be useful to determine the tropism of a virus that can be either expanded to an ensemble of target cells or restricted to a specific cell type (Verhoeyen and Cosset, 2004). Pseudotyping of gammaretroviral vectors with the vesicular stomatitis virus G protein (VSV-G) was considered a breakthrough in retroviral vector development since it increases vector tropism, stabilizes vector particles from shear forces during centrifugation and directs viral entry to an endocytic pathway. Although VSV-G pseudotyped vectors were found efficient transducing cells in various animal models, results have indicated that systemic administration to humans can be hampered by complement and antibody mediated immune responses directed against the VSV-G envelope (DePolo et al., 2000). Furthermore, VSV-G protein can be toxic to cells, which constrains its constitutive expression in stable packaging cell lines (Strang et al., 2004). Gammaretroviral vectors have been pseudotyped with a plethora of envelope proteins other than VSV-G like, amphotropic MLV envelopes 4070A or 10A1, gibbon ape leukaemia virus envelope (GALV), or the envelope proteins of cat endogenous virus RD114, among others (Palu et al., 2000).

Lentiviral vectors are normally produced carrying the VSV-G envelope but other pseudotypes can be used, like those mentioned above for retroviral vectors. Lentiviral vectors cannot be directly pseudotyped with all these envelope proteins, but a small mutation on the cytoplasmic tail can correct this problem (Sandrin et al., 2004). Each pseudotype presents different cell tropism and allows a higher or lower transduction of a certain cell type; furthermore it can confer different protection to the viral particles.
Genetically or chemically modified envelope glycoproteins have also been developed for cell or tissue specific targeting (Russell and Cosset, 1999).

2.3.4. Safety concerns/issues associated with gammaretroviral and lentiviral vectors

2.3.4.1. Accidental formation of newly recombined viruses

Depending on the construction of helper plasmids/cell lines, gammaretroviral and lentiviral vectors production or gene transfer can give rise to newly recombined viruses. As a consequence, infected cells obtain undesired properties or even become producer cells themselves, a process which renders the transfer incontrollable. The generation of replication competent virus has, from the very beginning, been seen as a major safety issue for gammaretroviral and lentiviral vectors and this has led to a prolonged effort to develop means of minimizing the probability of its arising (Raty et al., 2008).

Replication competent virus can be produced through recombination of the constituent parts of the vectors system (i.e. vector and helper function plasmids), either with themselves or with endogenous proviral sequence in the cell lines used for virus production or by activation of an endogenous proviral sequence. These issues have been addressed by: (i) breakdown of helper functions onto different plasmids; (ii) manipulation of codon usage in helper plasmids; (iii) removal, or mutagenesis, of unnecessary cis sequences present in the vectors; (iv) the development of SIN vectors; (v) the minimization of homology between the separate plasmids that make up the system, and (vi) the use of cell lines that do not contain endogenous viral sequences with homology to the vectors system used (Anson, 2004).
2.3.4.2. Insertional mutagenesis

The development of T cell leukaemia in 4 patients from a clinical trial that employed gammaretroviral vectors to treat eleven patients for SCID-XI disease shifted conventional concerns regarding insertional mutagenesis from a potential threat to a real outcome to be averted when considering retroviral vectors for gene therapy protocols (Hacein-Bey-Abina et al., 2008). Insertional mutagenesis can lead to genotoxicity as a result of insertion of a gammaretroviral vector into, or near, genes that confer a survival/growth advantage upon the transduced target cells. Moving to the use of lentiviral vectors has been one way to temper concerns regarding insertional mutagenesis, as it is evident from recent reports describing pre-clinical studies correcting ADA-SCID mice through HIV-1 vectors mediated deliver of adenosine deaminase (Mortellaro et al., 2006). These pre-clinical studies represent a transition away from the use of gammaretroviral vectors, which were previously demonstrated to correct ADA-SCID in humans (Blaese et al., 1995). Recently EIAV vectors were shown to be associated with the formation of tumours in the livers of mice following in utero and neonatal vectors administration. A causal relationship between EIAV vectors and tumorigenesis has yet to be established; however it is most important to note that in the same study the use of various HIV-1 based vectors were not associated with the formation of any detectable tumours (Themis et al., 2005). Despite the evidence from this study and the lack of any precedent for HIV-1 to be associated with tumour formation, lentiviral vectors that would obviate insertional mutagenesis are most desired.

Three strategies to modify the lentiviral packaging and transgene are being pursued to advert insertional mutagenesis: (1) directing lentiviral vectors
insertion to specific sites within the host genome (Tan et al., 2006); (2) use of chromatin insulators that are derived from chromatin boundaries and are inherently able to protect adjacent chromatin regions from influencing each others’ promoters/enhancers, and from silencing effects that may spread from region to region (Burgess-Beusse et al., 2002); and (3) exploiting non-integrative HIV-1 vectors episomes to deliver therapeutic transgenes (Apolonia et al., 2007; Philpott and Thrasher, 2007). These two first strategies have also been applied to gammaretroviral vectors to prevent insertional mutagenesis.

2.4. Production of gammaretroviral and lentiviral vectors

One of the major disadvantages of gammaretroviral and lentiviral vectors concerns the difficulty in manufacturing; the production of these vectors yield relatively low titers as a result of low producer cell productivity and short vector half-life (Cruz et al., 2008; Mountain, 2000). Moreover, gene transfer clinical protocols often require large amounts of biologically active vectors. Thus, successful implementation of gammaretroviral and lentiviral vectors in such trials depends on the production of high amounts of vectors and on their protection from inactivation. The manufacture of these vectors may be divided in 3 steps: production, purification and storage.

2.4.1. Production

Gammaretroviral and lentiviral vectors present some differences in terms of production. Gammaretroviral vectors are generally produced using packaging cell lines. In these, the sequences expressing the viral genes, gag, pro, pol and env are supplied in trans and the therapeutic gene is supplied into a transfer vector. Historically, most packaging cell lines used to produce recombinant retrovirus have been derived from murine cell lines, e. g. NIH
3T3. These are being replaced by human cell lines which are safer in terms of recombination; furthermore, the vectors produced by these cell lines are more resistant to human complement and higher titers can be obtained (Anson, 2004; Cosset et al., 1995; Takeuchi et al., 1994). The viral titer produced by packaging cell lines limits the use of gammaretroviral vectors in gene therapy. Nevertheless, several optimizations have been performed to packaging cell lines to increase the titer (Amaral et al., 2008; Carrondo et al., 2008; Cosset et al., 1995; Yap et al., 2000) and the safety of gammaretroviral vectors (Coroadinha et al., 2006c; Schucht et al., 2006; Xu et al., 2004).

Normally, lentiviral vectors are produced by transient transfection of HEK 293 cell line with 3 or 4 plasmids depending on the generation of the vectors (see above). This process allows rapid flexibility in testing different vectors in the laboratory setting; however, as applications shift to pre-clinical testing in multiple animal models, and eventually human gene therapy trials, so will the requirement for large and reproducible stocks of lentiviral vector that are safe, and for this the use of packaging cell lines is indispensable (Cockrell and Kafri, 2007). A number of packaging cell lines have been developed for the production of lentiviral vectors. As for gammaretroviral vectors a stable packaging cell line for lentiviral vector production contains the envelope, helper, and transfer vectors’ cassettes stably incorporated into the genome of a cell line. However, the construction of a packaging cell line passed through some difficulties since the constitutive expression of the protease, Vpr and VSV-G proteins had cytotoxic and cytostatic effects (Loewen and Poeschla, 2005). Therefore, it was necessary to develop systems with inducible expression of the packaging and envelope components in the initial packaging cell lines (Dull et al., 1998; Kafri et al., 2000; Klages et al., 2000;
Reiser et al., 2000). Lentiviral vectors packaging cell lines passed through a
great number of optimizations accompanying the evolutions obtained in
vector constructs. Packaging cell lines derived from codon optimized gag-pol
packaging cassettes facilitate the elimination of the RRE, thus reducing the
risk of recombinant competent lentivirus (RCL) formation through
recombination with the vector (Cockrell and Kafri, 2007). The STAR
packaging cell line described by Ikeda conferred high plasticity with regard
to pseudotyping HIV-1 vectors with envelope proteins other than VSV-G
(Ikeda et al., 2003; Strang et al., 2004). Recently, a cell line was developed
for production of lentiviral vectors in serum-free suspension cell systems
(Broussau et al., 2008).

2.4.1.1. Culture conditions
As with all other animal cells, mammalian cells used for the production of
gammaretroviral and lentiviral vectors are also affected by nutrient
limitations (oxygen and glucose). As a consequence, producer cell
metabolism has been studied in order to better understand the factors
influencing both vector productivity and vector stability. It has already been
shown that the use of alternative carbon sources like fructose increased both
retroviral vector productivity and stability (Coroadinha et al., 2006a;
Coroadinha et al., 2006b; Coroadinha et al., 2006d).

Also the presence of culture by-products, such as producer cell contaminant
proteins and DNA, and some medium additives, like serum, can affect the
quality of vector preparations. Gammaretroviral and lentiviral vectors are
usually produced by adherent cell lines that secrete variable quantities of
extracellular matrix proteins, partially consisting of proteoglycans. These
macromolecules are negatively charged and influence the transduction
efficiency of gammaretroviral vectors (Le Doux et al., 1996). Cell lines grown in suspension produce less extracellular matrix and, combined with high process scalability potential, they offer attractive advantages in large scale vector production (Merten, 2004). The development of production processes that use serum-free media is also an important step towards prevention of immunological responses to viral preparations and simplification of the downstream strategy (Cruz et al., 2008; Rodrigues et al., 2007a).

Culture temperature has been shown to affect the final gammaretroviral titer in the large majority of the reports. In fact, culture temperature assumes a particularly important role as it influences both the rates of retroviral vector production and degradation (McTaggart and Al-Rubeai, 2002). Some authors reported increases in vector production at lower temperatures (Kaptein et al., 1997; Kotani et al., 1994; Le Doux et al., 1999; Lee et al., 1996). However, the increments are not always very significant as the temperature affects the cell yield negatively. Unfortunately, the viral vector inherent stability was also demonstrated to be lower when the vectors were produced at 32°C instead of 37°C (Beer et al., 2003). Regarding lentiviral vectors, only one study indicates that the production of vectors at 32°C or 37°C have no significant difference in terms of production and stability (Reiser, 2000).

2.4.1.2. Culture system and operation mode
The production systems used to date to produce gammaretroviral and lentiviral vectors for clinical trials are considered for small scale, preferably disposable systems. These include cell factories, large T-Flasks and roller bottles. Although these systems are considered as not being the state of the
art for mass production, they perfectly fit the current need for the production of clinical batches of vectors. There are, nonetheless, a number of alternative culture systems for retroviral production, these include a number of systems of greater scalability such as fixed bed bioreactors and stirred tanks that allow improving the titers by 10 to 100-fold (Merten, 2004).

Although the production of gammaretroviral and lentiviral vectors is usually performed in batch cultures, due to the low stability of these vectors at 37°C, several harvests can be performed (Higashikawa and Chang, 2001; Le Doux et al., 1999; Pizzato et al., 2001). The use of this stepwise perfusion mode has the advantage of recovering vectors from the culture batch at shorter residence times with visible benefits upon gammaretroviral vector quality, derived from reduced degradation and increased final infectious particle number per batch. The collection of culture supernatants in cell factories can be performed every 24 hours for up to 4 days (Eckert et al., 2000). The same interval can be used in continuous perfusion mode in the cell cube with equivalent gain in total infectious particle yields per batch for gammaretroviral vectors. These several harvests are then stored at -80°C after clarification before they are thawed and pooled for quality control and release.

2.4.2. Purification

Purification of gammaretroviral and lentiviral vectors from cell culture contaminants (e.g., producer cells, DNA, proteins from culture media or released by the producer cells) is mandatory to prevent toxicity, inflammation, or immune response in the individuals undergoing therapy (Tuschong et al., 2002). Until recently, downstream processing (i.e. the
process by which the vectors are separated from contaminants) of
gammaretroviral and lentiviral vectors for phase I clinical trials has been
based only on the separation of producer cells and debris through
centrifugation and ultracentrifugation techniques. This is insufficient
regarding the increasingly stringent quality standards set by the regulatory
agencies (FDA and EMEA). The removal of the contaminants requires the
use of separation technology usually based on chromatography and
membrane processes.
Membrane separations, like microfiltration and ultrafiltration, can be used to
clarify and concentrate viral stocks, respectively. Also, chromatographic
methods like affinity chromatography and anion exchange chromatography
(AEX) have been used to purify gammaretroviral and lentiviral vectors with
good recovery yields (Cruz et al., 2008; Rodrigues et al., 2007a). Recently a
scheme of purification using microfiltration, ultrafiltration and AEX was
developed for gammaretroviral vectors showing good recovery yields and
scalability (Rodrigues et al., 2007b). Similar purification developments for
lentiviral vectors have also been performed (Segura et al., 2007).

2.4.3. Storage
The efficacy of gammaretroviral and lentiviral vectors as gene delivery
agents depends on the assurance of efficient and stable transfer of the
transgene into the target tissue. Therefore, the maintenance of viral vector
potency until administration is of the utmost importance for the success of
pre-clinical and clinical studies. The rapid loss of vector infectivity during
storage and shipment has been reported, but the mechanisms of vector
degradation are still poorly understood to permit improved technologies to
be proposed and tested.
Historically, viral vector formulations have consisted of glycerol (10-50% v/v) in phosphate or Tris buffer with addition of other excipients such as salts, sugars and bovine serum albumin (Croyle et al., 2001). Long-term storage of viral preparations usually require cryopreservation where small volume aliquots of viral stocks are stored at -80°C until use or, alternatively, kept in a lyophilized form at 4°C. It has already been shown that the sensitivity of gammaretroviral vectors during these processes is high. Similar to proteins, viral structures depend on hydrogen bonds, hydrophobic forces, and electrostatic interactions to keep their proper function. These non-covalent interactions are sensitive to variations in pH, temperature, and composition of the surrounding environment, particularly to osmotic pressure, all factors that are affected during freezing and lyophilisation (Croyle et al., 2001; Cruz et al., 2006). These factors can be counteracted by compatible solutes, a strategy undertaken by several organisms (Lentzen and Schwarz, 2006; Santos and da Costa, 2002). Some of these compounds were proven to protect retroviral vectors upon freezing and lyophilisation, increasing their storage time capacity (Cruz et al., 2006).

Since lentiviral vectors are a recent technology regarding its use in a clinical setting, not many studies have been performed as yet on the storage of these vectors, but given their similarity in terms of stability at physiological temperatures with gammaretroviral vectors it is probable that they will behave similarly when it comes to storage conditions.

2.5. Stability of gammaretroviral and lentiviral vectors

Gammaretroviral and lentiviral vectors are known to be very sensitive to environmental conditions, losing infectivity relatively fast. Their half-life ranges from 5 to 8 hours in the cell culture supernatant (Andreadis et al.,
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1999; Beer et al., 2003; Higashikawa and Chang, 2001) and from 1 to 3 hours after purification (Strang et al., 2004), at physiological temperature; this fast infectivity loss affects not only the titers but also the quality and efficacy of vectors preparations to be used in clinical settings. The low stability of these vectors has consequences in all the steps of manufacturing and application.

2.5.1. Stability during manufacture

During production vectors bud out from the producer cell line and start to lose infectivity immediately due to their labile nature at 37°C. As a consequence, few of the particles in a preparation are infectious. The stability of the vectors also depends on a number of producer cell and production process related variables such as the producer cell line type, medium composition, production temperature and production system (Burns et al., 1993; Coroadinha et al., 2006b; Coroadinha et al., 2006d; Davis et al., 1997; McTaggart and Al-Rubeai, 2002). In fact, temperature has been one of the most studied culture conditions in terms of vector production. Nevertheless, only one study showed so far that the production of gammaretroviral vectors at different temperatures affects the stability of the vectors (Beer et al., 2003). Vectors produced at 32°C showed lower stability than vectors at 37°C; the authors related this lower stability to the level of cholesterol on the membrane. However, the mechanism underlying this lower stability is not fully understood.

2.5.2. Stability during purification

The vectors are more stable at low temperatures, with half-lives higher than 100 hours at 4°C (Beer et al., 2003; Cruz et al., 2006; Le Doux et al., 1999). For this reason whenever possible the downstream processes are performed
at 4°C to minimize the infectivity loss. Besides temperature, gammaretroviral vectors are also sensitive to acidic or basic pH having their optimal stability between pH 5.5 and 8.0 (Ye et al., 2003). High salt concentrations have also been reported to affect retroviral stability (Segura et al., 2005). Thus, prior to designing downstream processing strategies it is necessary to have a good knowledge of the stability of the vector and its susceptibility to different factors (i.e. temperature, pH, ionic strength, shear stress) so that in the end a higher amount of good quality vectors can be obtained.

2.5.3. Stability during storage
Downstream processing, final product formulation and storage temperature are determinants of the half-life of gammaretroviral and lentiviral vectors. These vectors can be stored as -85°C and in the lyophilized form at 4°C; however, their high sensitivity to adverse conditions make these processes highly prone to inactivation of the vectors. Although no mechanism has been identified as the cause of this inactivation, reports show that it is possible to improve the recovery of these processes by using compatible solutes such as hydroxyecctoin and firoin. It was possible to increase the recovery of these processes from 60% to 90% during freezing at -85°C and from 30% to 80% in lyophilisation. Also, it was possible to increase the stability at -85°C from 16 to 185 days and in the lyophilized form from 9 to 340 days at -20°C (Cruz et al., 2006). Still, improved storage solutions are necessary for vector production under GMP conditions, since the necessary safety and potency testing and the subsequent final product release for clinical application may take 6 to 12 months. Along with the time necessary to obtain the approval of the clinical protocol by the regulatory agencies, the
maintenance of the potency has to be assured for at least 1 to 2 years (Rodrigues et al., 2007a).

Finally, after production, purification and storage the vectors have to be applied to the cell culture (ex vivo) or patient (in vivo), another step taking place at 37°C where the vectors can lose infectivity. This step is even more delicate for gammaretroviral vectors, since they only can enter the cell nucleus during mitosis; thus, even in the host cell, loss of infectivity can occur (Andreadis et al., 1997).

2.5.4. Inactivation mechanisms

Work has been performed on the kinetics of gammaretroviral and lentiviral vector inactivation (Beer et al., 2003; Higashikawa and Chang, 2001). Nevertheless, none of these studies attempted to unravel the cause for the fast inactivation of these viruses. Looking at the composition of the retroviral particles (Figure 2) several components can be susceptible to degradation/inactivation, leading to infectivity loss in different steps of the infection process (described above). In Figure 4 possible mechanisms of inactivation and their consequences upon infection are represented. The envelope proteins can shed from the virus membrane, leading to the loss of the virus capacity to bind to the cell receptors and thus preventing viral entry (Figure 4b). Normally, envelope shedding is assumed as the main cause of gammaretroviral and lentiviral vector inactivation, but so far no convincing proof of this mechanism has been shown (Layne et al., 1992; Merten, 2004). The viral membrane can disrupt, leading to the loss of the viral proteins and viral genome (Figure 4c). The viral genome can suffer degradation with the consequent virus loss of capacity to synthesize viral DNA (Figure 4d). And, finally, the enzymes RT and IN can suffer
inactivation with the impediment of viral DNA synthesis and integration of
the gene of interest, respectively (Figures 4e and f).

\[ \text{Figure 4: Schematic representation of the possible mechanisms of inactivation of} \]
\[ \text{gammaretroviral and lentiviral vectors and their implications on the vector infection} \]
\[ \text{process. (a) No inactivation mechanism – normal infection process; (b) Envelope} \]
\[ \text{shedding – no entry of the vectors; (c) Membrane disruption – no entry of the} \]
\[ \text{vectors; (d) RNA degradation – no DNA synthesis; (e) RT inactivation – no DNA} \]
\[ \text{synthesis; and, (f) IN inactivation – no integration.} \]

Throughout this literature review it was possible to verify that
gammaretroviral and lentiviral vectors are very complex systems highly
prone to inactivation. Along the manufacture process several factors can
affect the stability of the vectors, through different effects on the particle. In
Table 2 are summarized the factors affecting stability of gammaretroviral
and lentiviral vectors along the manufacture process, together with the
effect on the particle, the mechanism responsible for this effect and possible
solutions to overcome such effects.
Table 2: Overview of the factors affecting vector stability during production together with the effect caused, the mechanism responsible for inactivation and a possible solution.

<table>
<thead>
<tr>
<th>Factors affecting stability</th>
<th>Effect</th>
<th>Inactivation mechanism</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>During production</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Producer cell</td>
<td>Changes in vector membrane composition</td>
<td>Unknown</td>
<td>Choose the best cell line (Beer et al., 2003)</td>
</tr>
<tr>
<td>Medium composition</td>
<td>- Changes in vector membrane composition</td>
<td>Unknown</td>
<td>Optimise culture medium (Coroadinha et al., 2006b; Coroadinha et al., 2006d)</td>
</tr>
<tr>
<td></td>
<td>- Protection of particle structure/components</td>
<td>Unknown</td>
<td></td>
</tr>
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<tr>
<td></td>
<td>- Direct effect on vectors</td>
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<td>No real solution</td>
</tr>
<tr>
<td><strong>During purification</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
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</tr>
<tr>
<td><strong>During storage</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Process steps</td>
<td>Particle destabilization due to osmotic stress</td>
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<td>Optimise conditions (Cruz et al., 2006)</td>
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<td>Temperature</td>
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</tr>
<tr>
<td><strong>During transduction</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature</td>
<td>Direct effect on vectors</td>
<td>Unknown</td>
<td>No solution</td>
</tr>
</tbody>
</table>
In most cases the infectivity loss is caused by vector disruption due to shear stress or physico-chemical factors (temperature, pH, ionic strength). Although there is no solution after disruption of the particle, the disruption can be avoided by using conditions that in most of the cases are already established. However, one of these factors affects vector stability by other means, and is correlated to vector inactivation in all stages of manufacture from production to cell transduction; this is temperature. Temperature has even a greater impact during the production stage because it affects the intrinsic stability of the particles produced and it has a direct effect on the particle in solution. Several studies have been performed on the inactivation kinetics of gammaretroviral vector and lentiviral vectors at different temperatures. Nevertheless, no mechanism of inactivation was identified so far to explain infectivity loss.

In this introduction it was possible to highlight that already some improvement on the stability of gammaretroviral vectors was obtained, especially during the storage process. Nevertheless, without understanding the real causes for vector loss of infectivity it is difficult to, substantially and specifically, improve the stability of these vectors from the production stage to gene transfer.

3. SCOPE OF THE THESIS

The main goal of this thesis is to identify the main causes of gammaretroviral and lentiviral vectors temperature inactivation during and after production in order to provide solutions to obtain more stable vectors. Throughout Chapter I it was possible to determine temperature as a major cause of these vectors instability in all manufacture stages. Temperature affects the
intrinsic stability of the vectors produced and has a direct effect on the particles in solution. Thus, this thesis was divided in two parts with complementary goals: (1) to understand the effect of culture temperature on gammaretroviral vector stability, with the purpose of choosing the best culture temperature; and, (2) to identify the inactivation mechanisms behind the thermo-sensitivity of gammaretroviral and lentiviral vectors. With the outcome of these two parts another objective arises that is the development of specific strategies to stabilize the vectors at different temperatures.

It is known that the production temperature affects the composition of the gammaretroviral vector membrane. In order to understand the effect of culture temperature on vector stability, a thorough study on the physicochemical properties of the vector membrane was performed with vectors produced at different temperatures. It was possible to correlate the different stabilities obtained with different vector membrane characteristics and to choose the best production temperature to obtain more stable vectors.

Gammaretroviral and lentiviral vectors have high thermo-sensitivity at 37°C, the production temperature that was shown to give higher stability to gammaretroviral vectors. At this temperature several viral components can suffer degradation/inactivation. Thus, a screening of the stability of several viral components was performed at 37°C for both gammaretroviral and lentiviral vectors in order to define the vectors inactivation mechanisms.

The knowledge of the main inactivation mechanism of both vectors opened the possibility to perform directed stabilization to improve the stability of purified gammaretroviral and lentiviral vectors and to verify that although both vectors have the same main inactivation mechanism, different stabilization strategies had to be implemented.
4. REFERENCES


expressing the human multidrug resistance 1 gene (MDR1). *Bone Marrow Transplant* **25 Suppl 2**, S114-117.


Introduction


Chapter 1


Introduction


Chapter 1


Online Databases:

Journal of Gene Medicine Clinical Trial Database:

http://www.wiley.co.uk/genetherapy/clinical
Chapter II

EFFECT OF CULTURE TEMPERATURE ON VECTOR STABILITY

Adapted from:

Effect of culture temperature on vector stability

ABSTRACT

The present work aims at studying the physico-chemical properties of gammaretroviral vector membrane, in order to provide some explanations for the inactivation kinetics of these vectors and to devise new ways to improve transduction efficiency. For this purpose, vectors with an amphotropic envelope produced by TE Fly A7 cells at two culture temperatures (37 and 32°C) were characterized by different techniques. Electron Paramagnetic Resonance (EPR) results showed that vectors produced at 32°C are more rigid then those produced at 37°C. Further characterization of vector membrane composition allowed us to conclude that the vector inactivation rate increases with elevated cholesterol to phospholipid ratio. Differential Scanning Calorimetry (DSC) showed that production temperature also affects the conformation of the membrane proteins. Transduction studies using HCT 116 cells and tri-dimensional organ cultures of mouse skin showed that vectors produced at 37°C have higher stability and thus higher transduction efficiency in gene therapy relevant cells as compared to vectors produced at 32°C. Overall, vectors produced at 37°C show an increased stability at temperatures below 4°C. Since vector membrane physico-chemical properties are affected in response to changes in culture temperature, such changes, along with alterations in medium composition, can be used prospectively to improve the stability and the transduction efficiency of gammaretroviral vectors for therapeutic purposes.
Chapter II

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1. INTRODUCTION

Recombinant gammaretroviruses are one of the most commonly used vectors for gene therapy (McTaggart and Al-Rubeai, 2002; 2004; Mountain, 2000). However, the low stability of gammaretroviral vectors, namely that of Murine Leukemia Virus (MLV)-derived vectors containing amphotropic envelope, is still hampering a wider clinical application of these viral vectors. It has been reported that the half-life of most gammaretroviral vectors is in the range of 2 to 8 hours at 37°C (Le Doux et al., 1999; Lee et al., 1998; Pizzato et al., 2001). The negative impact of a low stability is reflected not only in the dramatic reduction of the concentration of infectious particles but also in their efficacy in clinical applications. Several factors that affect vector stability have been studied including: production temperature, pH and additives. Nevertheless, it is not yet understood how all these factors affect the vector constitution with respect to their stability. Beer et al. (2003) have shown that the level of cholesterol in the viral membrane is correlated with virus stability and that vectors produced at different temperatures have different cholesterol levels at their membrane. An earlier study by Aloia et al. (1988) showed that manipulation of membrane rigidity of HIV could affect virus stability and infectivity. These observations constitute evidence of the complexity of the factors influencing the loss of infectivity of gammaretroviral vectors. Thus, it is important to characterize gammaretroviral vectors physico-chemical and biological properties in order to understand their low stability.

The present work aims at characterizing the physico-chemical properties of the gammaretroviral vector envelope produced in TE Fly A7 cells. For this characterization, vector membrane rigidity was assessed by EPR (Electron
Paramagnetic Resonance), the cholesterol and phospholipid composition of the membrane was determined and the envelope protein thermal profiles and denaturation transitions were studied by DSC (Differential Scanning Calorimetry). The membrane characteristics studied were instrumental to provide an understanding of the patterns in inactivation kinetics with storage temperature and to devise strategies for improving vector stability.

2. MATERIALS AND METHODS

2.1. Cell Culture

Human derived cell lines were used in this work – HT1080, HCT116 and TEFly A7. TEFly A7 is a TE 671 (human) derived cell line producing retroviruses with amphotropic envelope. These retroviruses code for the reporter gene lacZ. The cell line HCT 116 and the producer cell line TE Fly A7 were provided by Généthon (France), the cell line HT1080 is from ATCC. The cells were cultured in T-flasks (Nunc™, Roskilde, Denmark) in DMEM (4.5 g L⁻¹ glucose, Glutamax I) (Life Technologies, Paisley, UK) supplemented with penicillin/streptomycin (100 U mL⁻¹; 100 μg mL⁻¹) and 5% FBS (Life Technologies) and incubated in a humidified incubator with 5% CO₂ in the gas phase, at 37°C. The cells were passed 1/10 twice a week and were used up to 30 passages after thawing. The absence of mycoplasma in the cell cultures was checked regularly (PCR based mycoplasma test from ATCC, Rockville/MD, USA).

2.2. Virus Production and Purification

For viral vector production cells were grown nearly to confluence in DMEM (Life Technologies) with 5% FBS (Life Technologies) supplement at 37°C
and 32°C. Fresh medium was added to cells 24 hours before the virus supernatant was harvested and filtered with 0.45 μm filters (Whatman, Maidstone, UK). The filtered viral supernatant was concentrated by ultracentrifugation using a Beckman L8-55M centrifuge: 100 000 × g for 1.5 hours at 4°C in a Beckman 45Ti rotor. The pelleted virus was resuspended in PBS pH 7.2 and purified by centrifugation on a 20% (w/v) sucrose solution (Merck, Darmstadt, Germany) at 200 000 × g for 2 hours at 4°C in a Beckman 90Ti rotor.

2.3. Spin labelling

After the purification, the pelleted virus was suspended in 137 mM NaCl / 15 mM Na₂HPO₄ / 2.7 mM KCl / 0.5 mM MgCl₂, pH 7.5. Spin label working solution (1 mg mL⁻¹ 5-doxyl-stearic acid (Sigma, St. Louis, MO) plus 50 mg mL⁻¹ crystallized BSA (Sigma) as carrier) was added to the purified virus (200 μl per mg of viral protein) and PBS was added up to 1 ml. The mixture was incubated for 2 hours at room temperature, after which unbound spin label was removed from the mixture by centrifugation on a 20% (w/v) sucrose solution, for 2 hours at 200 000 × g and 4°C, in a Beckman 90Ti rotor. The pelleted virus was resuspended in PBS pH 7.2 and transferred to a quartz EPR flat cell for aqueous samples.

EPR spectra were measured in a Bruker ESR300 spectrometer, equipped with an Oxford Instruments variable temperature flow system. Spectra were obtained with a microwave power of 24 mW, from 5 to 42°C, after stabilization (typically 5 minutes) at each temperature. The hyperfine splitting constant 2Tᵥ was measured using the spectrometer software.
2.4. Membrane lipid composition determination

For cholesterol determination, samples of intact virus were adjusted to 0.2 M with NaCl and then extracted with 3 volumes of chloroform/methanol (2:1, v/v). The resulting organic phase was lyophilised and analysed for cholesterol content using an enzymatic cholesterol kit from Sigma (ref. 401-25P) (St Louis, USA) according to the manufacturers instructions. All samples and controls were performed in triplicate.

For phospholipid determination 0.1 mL sample of intact virus were mixed with 2.0 mL of chloroform and 2.0 mL of ferrithiocyanate reagent (27.03 g of ferric chloride hexahydrate (Merck, Darmstadt, Germany) and 30.4 g of ammonium thiocyanate (Merck) in 1 l of distilled water). The phospholipid concentration of the resulting organic phase was determined by reading the absorbance of each sample at 488 nm. All samples and controls were performed in triplicate.

2.5. Differential Scanning Calorimetry

Calorimetric experiments were performed on a MicroCal VP-DSC MicroCalorimeter (Northampton, USA) controlled by the VP-viewer program. Calibration of temperature and heat-flow were carried out according to MicroCal instructions. Solutions of gammaretroviral vectors produced at 37°C and 32°C were prepared in PBS buffer, pH 7.2. Sample and reference solutions were degassed for 8 minutes before the calorimetric data acquisitions. DSC scans were run at a constant heating rate of 2°C/min from 20 to 100°C and with an overpressure of about 30 psi to prevent bubble formation during heating. To assess the thermal unfolding reversibility, two sequential DSC scans with each protein solution were performed. No
endothermic peak was observed on reheating. The protein concentration for the virus produced at 37°C was 7.1 μM and that for the virus produced at 32°C was 11.2 μM.

Raw calorimetric data was converted to the excess heat capacity of unfolding by subtracting the instrumental baselines determined under identical conditions and dividing it by the scan rate and by the sample protein concentration. The melting temperature (T_m), the calorimetric (ΔH_{cal}) and the van’t Hoff (ΔH_{vH}) enthalpies were calculated using the software supplied with the instrument.

2.6. Virus stability assay

The first order decay constants were determined by titration of the infective virus on the purified sample. For that purpose, the target cells, HT1080, were infected with different dilutions of these samples and tested for expression of the lacZ gene (Carmo et al., 2004; Cruz et al., 2007). The titer of the purified samples at the beginning of the virus stability assay was 2.80×10^8 infectious particles per mL. The temperature inactivation profiles of the purified gammaretroviral vectors were determined by measuring virus titer versus time at several temperatures: -85, -20, 4, 17, 27 and 37°C. All of the samples were performed in triplicate.

The change in the concentration of infectious viruses with time can be described by the following equation:

\[
\frac{dX}{dt} = -kX
\]

which, upon integration, yields the concentration of active virus (X) at time t is given by:
\[ X = X_0 \exp(-kt) \]

where \( X_0 \) is the initial concentration of active virus and \( k \) is the virus decay rate constant. Best-fit values for virus decay rate constants were determined by using non-linear regression analysis to fit the data to equation.

### 2.7. Transduction efficiency assays

#### 2.7.1. Infection of HCT116 cells

To determine the transduction efficiency of the virus produced at 37 and 32°C a microplate assay was used (Cruz et al., 2007; Le Doux et al., 1999). Briefly, HCT 116 cells were seeded in a 96-well tissue plate (1.65×10^4 cells well\(^{-1}\)). After 24 hours the medium was removed and dilutions of virus in culture medium with 8 \( \mu \)g mL\(^{-1}\) of polybrene (Sigma, Saint Louis, USA) were added to each well. Two days after transduction the medium was removed, and the cells were washed once with 100 \( \mu \)L of PBS with 1 mM MgCl\(_2\). After removal of the wash solution 50 \( \mu \)L of lyses buffer was added (PBS with 1 mM MgCl\(_2\) and 0.5% Nonidet P-40 (Roche Diagnostics, Mannheim, Germany)) to each well, and the plate was incubated at 37°C. After 30 minutes, 50 \( \mu \)L of lyses buffer with 6 mM ONPG (Sigma, Steinheim, Germany) warmed to 37°C was added to each well, and the plate was incubated at 37°C for 1h30. The reaction was stopped by the addition of 20 \( \mu \)L of 1 M Na\(_2\)CO\(_3\). The optical density was measured at 420 nm using an absorbance plate reader spectra MAX 340 (Molecular Devices, Sunnyvale, USA); nonspecific background at 650 nm was subtracted. Values for replicative wells without virus were subtracted as background. Values for each point are the averages of at least triplicate wells.
2.7.2. Infection of primary human keratinocytes

Human keratinocytes were obtained from skin biopsies of healthy donors by enzymatic digestion following previously described methods (Rheinwald and Green, 1975). Human primary keratinocytes were seeded in 24-well tissue plates (1×10⁴ cells well⁻¹). After 48 hours the cells were infected for 2 hours with 10⁴ infectious particles per well of MLV vectors produced at 37 and 32 °C in the presence of 7 μg mL⁻¹ polybrene. The medium was replaced and 72 hours later the cultures were stained with X-gal for Lac-Z transgene activity.

2.7.3. Infection of mouse skin micro-organs

Skin of one-day-old mouse was cut into 0.3 mm slices using TC-2 tissue sectioner (Sorvall, Newton, USA). The slices were incubated in DMEM supplemented with 10% FCS (Hasson et al., 2005). After 24 hours the slices were infected for 6 hours with 10⁶ infectious particles of MLV vectors produced at 37 or 32 °C in 200 μL⁻¹ in the presence of 7 μg mL⁻¹ polybrene. The medium was replaced and 48 hours later the skin was stained with X-gal for Lac-Z transgene activity and photos were taken under a binocular.

3. RESULTS

3.1. Gammaretroviral vector membrane rigidity and thermal transitions measured by EPR

Previous results reported by Beer et al. (2003) have shown that vectors produced at different temperatures are phenotypically different as a result of the changes in the cellular cholesterol levels. In addition, it has been known for more than two decades that the membrane properties of retroviruses change with temperature (Slosberg and Montelaro, 1982). Thus, to
characterize the gammaretroviral vectors used in this work, vector membrane rigidity was measured for each production temperatures (37 and 32°C). For this purpose, Electron Paramagnetic Resonance (EPR), a useful and reliable method to measure the membrane rigidity of retroviruses (Slosberg and Montelaro, 1982) was used. Figure 1 depicts the hyperfine splitting constant $2T_{II}$ for vectors produced at 37 and 32°C measured by EPR. As can be observed, the increase in temperature causes a general decrease in the value of the spectral parameter $2T_{II}$. Since $2T_{II}$ correlates with lipid bilayer rigidity, this decrease corresponds to a decrease in the membrane rigidity, i.e., the membrane becomes more fluid as temperature increases.

Figure 1: Plot of the hyperfine splitting constant $2T_{II}$ measured at different temperatures for purified retroviral vectors produced at 37°C (■) and 32°C (□). The p value is provided for each fitting. The arrows represent the transition point for each temperature.

The plots of $2T_{II}$ values versus temperature also allow the definition of the thermal transition temperatures, determined from the slope changes. Statistical analysis was performed in order to determine if the slopes presented in Figure 1 are statistically different. For this purpose, hypothesis
testing was performed and the results clearly indicated that the slopes at 37°C and at 32°C are different (p<0.00001 in both cases). These thermal break points have been attributed to a viral membrane phase transition (Slosberg and Montelaro, 1982).

The first conclusion taken from the results presented in Figure 1 is that production temperature has a marked effect upon the rigidity of the membrane of the gammaretroviral vectors. In fact, the gammaretroviral vectors produced at 32°C by TE Fly cells are more rigid (higher 2Tn) than those produced at 37°C. Consequently, for the gammaretroviral vectors produced at 32°C the membrane phase transition occurs at a temperature which is 10°C higher than for virus produced at 37°C.

3.2. Vector membrane composition

It has been shown that the cell membrane composition can be manipulated in several ways, from cholesterol sequestration or depletion to the inhibition of cholesterol biosynthesis (Beer et al., 2003; Simons and Toomre, 2000). Also, production temperature may affect membrane composition of the gammaretroviral vectors by changing cell metabolism and by affecting thermodynamically dependent processes such as vector budding.

To further study this phenomenon, the membranes of gammaretroviral vectors were evaluated in terms of the cholesterol to phospholipid molar ratio (C/P). This ratio has been widely used as a parameter for the study of the lipid composition of retrovirus membranes (Aloia et al., 1988; Aloia et al., 1993). The C/P molar ratio obtained for purified gammaretroviral vectors produced at 37°C was 0.50 ± 0.04 and for vectors produced at 32°C was 0.75 ± 0.08. Thus, the vectors show a 50% increase in the C/P ratio when the production temperature decreases from 37 to 32°C.
Since cholesterol is known for its stiffening and regulating effects upon membrane lipid phase behavior (Brown and London, 2000) it is not surprising that the rigidity of the vectors produced at 32°C is higher than that of the vectors produced at 37°C (Figure 1). In fact, it has been observed for MLV gammaretroviral vectors with amphotropic envelope that higher cellular cholesterol content leads to vectors with lower stability (Beer et al., 2003).

3.3. Differential scanning calorimetry

Calorimetric scans were performed with purified gammaretroviral vectors produced at 37 and 32°C and one transition was observed in both cases (Figure 2). The thermodynamic parameters obtained from the analysis of those endotherms are listed in Table 1.

![Differential Scanning Calorimetry heating curves of purified MLV vectors produced at 37°C (___) and 32°C (___).](image)

No difference was observed in the transition temperature between gammaretroviral vectors produced at 37°C and vectors produced at 32°C; nevertheless, a significant decrease in the calorimetric enthalpy was observed for the vector produced at 32°C. As a result, the van't Hoff to
calorimetric enthalpies ratio increased more than 4 fold for the retrovirus produced at 32°C, in comparison to the retrovirus produced at 37°C.

**Table 1**: Thermodynamic parameters for purified MLV vectors produced at 37°C and 32°C. $T_m$, transition temperature; $\Delta H_{\text{cal}}$, calorimetric enthalpy; $\Delta H_{\text{vH}}$, van’t Hoff enthalpy.

<table>
<thead>
<tr>
<th>Vectors produced at 37°C</th>
<th>$T_m$ (°C)</th>
<th>$\Delta H_{\text{cal}}$ (kcal mol$^{-1}$)</th>
<th>$\Delta H_{\text{vH}}$ (kcal mol$^{-1}$)</th>
<th>$\Delta H_{\text{vH}}/\Delta H_{\text{cal}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vectors produced at 32°C</td>
<td>71.5 ± 0.1</td>
<td>73 ± 8</td>
<td>192 ± 9</td>
<td>2.7 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>71.5 ± 0.1</td>
<td>16 ± 2</td>
<td>211 ± 7</td>
<td>13 ± 2</td>
</tr>
</tbody>
</table>

This ratio is related to the cooperative unfolding unit and thus the results suggest that the gammaretroviral surface protein produced at 37°C is folded in a dimer or trimer while the protein produced at 32°C is assembled in larger oligomers. It has been previously reported that the gammaretroviral Env molecule consists of a surface glycoprotein (SU) complexed with a trans-membrane protein (TM) and that these complexes are grouped into oligomers on the surfaces of the cell and of the virion (Rein et al., 1998). Moreover, it is not certain if membrane proteins form dimers, trimers or other oligomers given that it is possible that different conditions will affect molecular conformations. Although the enthalpies ratio increase is very significant, the hypothesis that the gammaretroviral proteins produced at different temperatures are folded in different manners must be further studied and evidences from other techniques are required to support these conclusions.
3.4. Vector inactivation rates

The results presented above show that the membrane properties of gammaretroviral vectors produced at 37 and 32°C are significantly different. To determine if these properties are related to vector stability, the thermal inactivation rates (k) were determined for each case, plotting vector titer along incubation time. Table 2 shows the first order inactivation rates, determined at -85, -20, 4, 17, 27 and 37°C, for the vectors and Figure 3 shows the corresponding half-life times. From these results it is possible to observe that the production temperature affects the inactivation rates at temperatures below 4°C, with the vectors produced at 37°C being more stable than those produced at 32°C, especially at standard storage temperatures.

Table 2: Vector first order inactivation rates (k, h⁻¹) measured at different temperatures for purified gammaretroviral vectors produced at 37 and 32°C; the standard error and the p-values are also shown.

<table>
<thead>
<tr>
<th>Incubation temperature</th>
<th>Inactivation rate (h⁻¹)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Production at 37°C</td>
<td>Production at 32°C</td>
</tr>
<tr>
<td>37°C</td>
<td>0.239 ± 0.005</td>
<td>0.25 ± 0.02</td>
</tr>
<tr>
<td>27°C</td>
<td>0.127 ± 0.005</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>17°C</td>
<td>0.07 ± 0.02</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>4°C</td>
<td>0.014 ± 0.005</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>-20°C</td>
<td>0.007 ± 0.001</td>
<td>0.0146 ± 0.0001</td>
</tr>
<tr>
<td>-85°C</td>
<td>0.0017 ± 0.0002</td>
<td>0.0027 ± 0.0004</td>
</tr>
</tbody>
</table>
Effect of culture temperature on vector stability

![Graph showing the relationship between incubation temperature (°C) and half-life (h)](image)

**Figure 3:** Vector half-life (h) measured at different temperatures for purified retroviral vectors produced at 37°C (■) and 32°C (□).

In fact, after incubation at 4°C for 24 hours, vectors produced at 37°C retained 70% of their infectivity against only 12% for the vectors produced at 32°C. This means that the physical changes observed in the vectors lead to substantially different transduction efficiency.

Vector inactivation kinetics were determined by plotting the inactivation rate and corresponding incubation temperature data according to a linearized form of the Eyring equation (Figure 4), an Arrhenius-like equation, given by:

\[
\ln\left(\frac{k}{T}\right) = -\frac{\Delta H^*}{R} \times \frac{1}{T} + \ln\left(\frac{k_B}{h}\right) + \frac{\Delta S^*}{R}
\]

Where \( k \) is the rate constant for the first order inactivation rate, \( T \) is the temperature in Kelvin, \( k_B \) is Boltzmann’s constant, \( h \) is Planck’s constant and \( R \) is the ideal gas constant. \( \Delta H^* \) and \( \Delta S^* \) are, respectively, the enthalpy and entropy of activation for the reaction.

Figure 4 shows that the vectors exhibit two different kinetic phases. The fact that different profiles were observed for vectors produced at different temperatures reflects the different characteristics of the vectors. Statistical
analysis was performed in order to determine if the slopes in Figure 4 are statistically different. For this purpose, hypothesis testing was performed and the results clearly indicated that the slopes are different, both at 37°C (p<0.00001) and at 32°C (p=0.00002). The most interesting observation is that the results presented in Figure 1 regarding the thermal break points are consistent with the kinetic phase transition temperatures shown in Figure 4, reinforcing the relevance of the vector membrane properties upon vector inactivation kinetics.

![Graph](image)

**Figure 4:** Temperature dependence of the first order inactivation rate of purified retroviral vectors produced at 37°C (A) and 32°C (B). The data are plotted according to the linearized form of the Eyring equation (Arrhenius-like), where T is the temperature (K) and k is the first order inactivation rate (h⁻¹). The p value is provided for each fitting.

**3.5. Vector transduction efficiency**

The results presented above show that membrane properties of the gammaretroviral vectors produced at 37 and 32°C are significantly different and that these differences can be correlated with the vector inactivation rates. To understand whether physical property changes caused by production of the vectors at the different temperatures are of relevance in
gene therapy applications, transduction of HCT116 cells and of two experimental systems of clinical relevance were tested: human keratinocytes that form in culture artificial skin for subsequent autologous transplantation (Del Rio et al., 2004), and micro-organ cultures of mouse skin, containing both the epidermal and dermal components of the tri-dimensional tissue (Gershonowitz et al., 2004; Hasson et al., 2005). To compare transduction efficiencies, these systems were infected with MLV vector preparations produced at 37 and 32°C, with and without incubation at 4°C for 24 hours.

In order to determine the infectivity of the vector preparations, before and after incubation at 4°C, the titers of all virus preparations were equalized by appropriate dilutions to $10^8$ infectious particles. The residual transduction efficiency, retained after incubation, for both the keratinocytes and the HCT116 cells are shown in Figure 5.

![Figure 5: Transduction efficiency of MLV vectors produced at 37 and 32°C, retained after incubation at 4°C for 24 hours. Purified retroviral vectors produced at 37°C (□) and 32°C (■), with and without incubation at 4°C for 24 hours, were used to infect HCT116 cells or human keratinocytes with equalized titer of infectious units.](image)

The data indicate that when equalized titers of vectors produced at different conditions infect human primary cells the number of infected keratinocytes is similar. The same occurs for HCT116 cells when infected with equalized
titers of infectious units. These results suggest that the instability of the vectors produced at 32°C, as shown by titration on HT1080 cells holds true also for primary human keratinocytes of therapeutic potential, as well as for HCT116 cells.

Using the mouse skin micro-organs cultures, the infection was also performed with equalized titers of infectious units of each viral preparation (Figures 6a to 6d).

![Figure 6](image)

**Figure 6:** Transduction efficiency of mouse skin micro-organs by MLV vector preparations. a) Infection with vectors produced at 37°C; b) infection with vectors produced at 37°C, after incubation at 4°C for 24 hours; c) infection with vectors produced at 32°C; d) infection with vectors produced at 32°C, after incubation at 4°C for 24 hours; e) mock infection (negative control skin).

The results indicated that both virus preparations, produced at 37 and 32°C, infected the skin, when equal amounts of infectious particles were added. Although this is a semi-quantitative method it seems that even with equalized titers, the vectors produced at 37°C are more efficient in transducing the tri-dimensional organ cultures. It should be noted that in order to infect the tri-dimensional organ culture the virus was absorbed to
the skin tissue for 6 hours at 37°C; during which time further inactivation of the virus produced at 32°C occurred. Histological analysis of the infected mouse skin micro-organs indicated that actively dividing epidermal keratinocytes were mostly infected by the MLV vector (Hasson et al., 2005). Taken together these results further indicate that vectors produced at 37°C would present higher transduction efficiency and be more stable in gene therapy applications compared with vectors produced at 32°C.

4. DISCUSSION

Due to the application of gammaretroviruses as vectors for gene therapy clinical indications, the generation of high titer stable vectors has received a great attention. Recently, two relevant papers were published regarding the stability of gammaretroviral vectors, one analyzed the inactivation kinetics of the vectors and have observed two different inactivation phases, depending on the storage temperature (Higashikawa and Chang, 2001), and the other studied the effect of the production temperature upon cell and virus membrane and the consequences to vector stability (Beer et al., 2003). The results presented herein provide a physico-chemical characterization of vectors produced by TE Fly A7 cells in order to understand why vectors produced at different temperatures present different transduction efficiency in relevant gene therapy applications.

The production temperature causes significant changes on the vector membrane composition. Our results show that there are two factors affecting the characteristics of viral vectors produced. First, the production temperature, by affecting thermodynamics of the cell, virus membrane and budding process, leads to rearrangements of lipids and proteins that
ultimately will dictate the viral membrane rigidity (Figure 1). Second, production temperature may change lipid metabolism leading to a selective availability of lipids in the cell membrane and consequently to vectors with different membrane compositions. Previous studies on HIV membrane lipids suggested that the cholesterol level in the viral membrane derives from specific interactions between the viral protein and the cellular lipids during the budding process (Aloia et al., 1988; Pessin and Glaser, 1980). Other studies have shown that cell membrane proteins, previously thought to be excluded from the budded virus (Gordon et al., 1988), are also incorporated into retroviruses (Hammarstedt et al., 2000). Furthermore, the DSC data suggests that the conformation of the envelope proteins is different for vectors produced at different temperatures. The protein’s ability to achieve multiple conformations requires an incompletely folded precursor state (O’Reilly and Roth, 2003) and production at lower temperatures is a condition known to change protein-folding pathway (Kjaer and Ibanez, 2003). As a result, when the precursor protein is produced at 32°C it can exist in a more folded state and hence not achieving the best conformation for membrane receptor binding. Also, different conformations may lead to a different sensitivity to the inactivation and thus to a higher or lower vector stability.

In addition, the correlation between the membrane phase transitions (Figure 1) and kinetic phase transitions (Figure 4), shows that the effect of incubation temperature upon the characteristics of the vectors has a direct impact upon the temperature dependence of the vector inactivation rate. One possible explanation for this may be the concomitant increase in the mobility of the envelope trans-membrane protein with temperature. The
 Effect of culture temperature on vector stability

effect of this increased mobility may have different consequences. On the one hand, higher mobility of the trans-membrane protein, TM, may have as a consequence an easier disruption of the bond between this protein and the surface glycoprotein SU thus reducing membrane receptor binding; on the other hand, a higher rigidity may hinder infection or vector internalization, meaning that there should be limits to the extent at which the virus properties can be manipulated in order to increase stability. In addition, it is possible that membrane phase transition observed as temperature increases, by leading to significant variations of trans-membrane protein mobility, alter the way in which that stability changes with temperature, thus giving rise to a new inactivation kinetic phase.

Regarding vector membrane composition, higher inactivation rates were observed for vectors with higher C/P molar ratios as previously reported (Beer et al., 2003). However, a correlation between stability and C/P ratio may not always be observed. The type of lipids incorporated at different temperatures may be different resulting in different membrane behaviours (Figure 1) and ultimately in different sensitivities of the inactivation rate to incubation temperature. Additionally, the fact that the cellular metabolism is different at various temperatures, may lead to a modified C/P ratio associated with a modified phospholipid composition (e.g., more saturated fatty acids would lead to a higher rigidity). It was also observed that some changes that occur in the vectors membrane may have an effect upon the entry of the vector into the cell leading to different transduction efficiencies (Figures 5 and 6). This may be due to the change of the conformation of the envelope proteins or to the change of the membrane rigidity.
Several papers focusing on the function of lipid micro-environments in the cell surface, known as lipid rafts, have shown that these domains are able to selectively include or exclude proteins and lipids, thus being privileged sites for virus budding (Nguyen and Hildreth, 2000; Simons and Ikonen, 1997). This results in a specific, rather than random, accumulation of certain cellular lipids, such as cholesterol, sphingomyelin, and the ganglioside GM1, within the viral envelope. The fact that retroviruses exhibit a rigidity and a cholesterol content higher than those of the plasma membrane of the cells where they are produced (Slosberg and Montelaro, 1982) strengthens the likelihood of virus budding in these high rigidity sphingolipid/cholesterol-based structures, which was suggested for HIV-1 (Aloia et al., 1993; Nguyen and Hildreth, 2000). The fact that rafts are dynamic structures that can be altered in terms of their size and lipid composition in response to extracellular triggers (Simons and Toomre, 2000) opens a window for improving vector stability by manipulating vector characteristics through specific changes in the production temperature and medium composition (Gény-Fiamma et al., 2004).

Gammaretroviral vectors have the drawback of low stability, and several studies were performed to understand the factors that affect their stability, such as production temperature, envelope protein, pH and additives. Only few studies are directed at understanding how these factors affect vector constitution and its impact upon stability. This work aimed at assessing physico-chemical properties of the vectors membrane and relating them to vector stability in order to evaluate the possibility to increase vector stability for gene therapy clinical applications. The results obtained indicate that the culture temperature affects vector membrane properties both in terms of
lipid composition and in terms of protein conformation, leading to changes in vector stability and inactivation kinetics. This demonstrates that the effect of culture temperature upon vector stability can be exerted through changes in the physico-chemical properties of the vector. Thus, since the concentration or ratios of individual lipid components can affect the stability of gammaretroviral vectors, more attention has to be paid as to how quantitative changes in individual lipids and cholesterol caused by cultivation conditions (e.g. medium composition) or by the host cell type affects vector stability.

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5. REFERENCES


Chapter II


Chapter II


**Online Databases:**

Journal of Gene Medicine Clinical Trial Database:
http://www.wiley.co.uk/genetherapy/clinical
Chapter III

INACTIVATION MECHANISMS OF GAMMARETROVIRAL VECTORS

Adapted from:

ABSTRACT

The loss of gene transfer capacity in gammaretroviral vectors constitutes a major disadvantage in the development of these vectors for gene therapy applications. In the present work the loss of vectors capacity to perform reverse transcription was studied as a possible explanation for the low stability of gammaretroviral vectors from the production stage to the target cell gene transfer event.

Inactivation studies were performed with MLV vectors at 37°C and several residual activities were tested, including viral infectivity, reverse transcription capacity, RT activities and viral RNA stability.

The results indicate a high correlation between loss of infectivity and the capacity of the virus to perform the initial steps of reverse transcription. To further understand the thermo-sensitivity of the reverse transcription process, the two enzyme activities of reverse transcriptase (RT) were investigated. The results indicate that while the inactivation rate of the DNA polymerase is faster than that of RNase H, the decline of these two enzyme activities is significantly slower than that of reverse transcription. Also, viral RNA stability is not implicated in the loss of the virus capacity to perform reverse transcription as the rate of viral RNA degradation was very slow. Furthermore, it was observed that the amount of viral RNA that entered the cells decreased slowly due to viral inactivation at 37°C.

The reverse transcription process is thermo-labile and this sensitivity determines the rate of gammaretroviral inactivation. Strategies targeting stabilization of the reverse transcription complex should be pursued to improve the applicability of gammaretroviral vectors in gene therapy studies.
Chapter III

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1. INTRODUCTION

Gammaretroviral vectors, especially the murine leukemia virus (MLV)-based vectors, have been widely used as gene delivery vehicles in clinical gene therapy protocols (McTaggart and Al-Rubeai, 2002; Pages and Bru, 2004; Thomas et al., 2003). The high potential of gammaretroviruses as vectors is due to the broad range of susceptible host cells, to stable integration of the transferred gene into the host chromosomes and to their relatively low immunogenicity (Andreadis et al., 1999; McTaggart and Al-Rubeai, 2002). Despite these advantages, there are still problems hampering a wider application of these vectors, including non-specific integration of the viral DNA, the requirement for division of the target cell, the difficulty in obtaining high titers and the instability of the virus, leading to a rapid loss of gene transfer capacity (Andreadis et al., 1999; McTaggart and Al-Rubeai, 2002; Pages and Bru, 2004). The low stability of these vectors is a critical issue with respect to production, storage and quality of the viral preparations, since infectivity of the virus declines fast at 37°C (Andreadis et al., 1999; McTaggart and Al-Rubeai, 2002), the standard temperature for production and transduction. Extensive work has been done in order to stabilize viral vector infectivity; however, since the mechanisms of inactivation are not known, it has been difficult to achieve substantial viral stabilization. For example, different reagents have been added to viral preparations in an effort to increase stability; although it has been possible to increase the storage half-life of the vectors during freezing, an increase in the stability at 37°C was not achieved (Cruz et al., 2006). Some viral components may be susceptible to degradation/inactivation affecting gene transfer, i.e. the genomic viral RNA, capsid proteins, envelope proteins and
the enzymes reverse transcriptase (RT) and integrase, yet there are no published studies on the inactivation/degradation of any of these viral components.

The process of reverse transcription is complex, comprising several steps: (1) the viral RNA is first reverse transcribed into the minus DNA strand by the RNA-dependent DNA polymerase function of RT; (2) viral RNA is subsequently hydrolyzed by the RNase H activity of the RT; and (3) the minus strand DNA serves as a template for synthesis of the second DNA strand by the DNA-dependent DNA polymerase function of the RT. During synthesis of the double stranded DNA, RT performs two template switches, strand-transfers, to generate the long terminal repeats (LTRs) (Telesnitsky and Goff, 1997; Whitcomb and Hughes, 1992). Successful completion of the reverse transcription process is obligatory for all subsequent steps of the infection (Whiting and Champoux, 1998). Thus, it is logical to consider the thermo-sensitivity of the reverse transcription process as a possible mechanism of gammaretroviral vector inactivation. The aim of this work is to evaluate the thermo-sensitivity of the steps involved in the formation of proviral DNA and its correlation to the rapid inactivation of gammaretroviral infectivity at physiological temperatures.

2. MATERIALS AND METHODS

2.1. Gammaretroviral vector production and purification

MLV vectors pseudotyped with amphotropic envelope were obtained from supernatant of human TE FLY A7 packaging cell line, derived from TE 671 cells (ECACC no. 89071904) transformed with the plasmid pMFGSnlsLacZ (Pizzato et al., 2001) (kindly provided by Dr. Otto Merten from Généthon,
France). Cells were cultured in DMEM medium (Gibco, Paisley, UK) supplemented with 4.5 g L\(^{-1}\) of glucose (Merck, Darmstadt, Germany), 6 mM of glutamine (Gibco) and 5\% (v/v) FBS (Gibco). After three days of culture the medium was replaced, MLV vectors were produced during the following 24 hours and the supernatant was filtered with 0.45 \(\mu\)m filters (Cruz et al., 2007). The viral supernatant was concentrated by ultracentrifugation in a Beckman Optima XL-100 centrifuge (Beckman Coulter Inc., Fullerton, CA, USA): 100 000 \(\times\) g for 1.5 hours at 4 °C using a Beckman 45Ti rotor (Burns et al., 1993). The pelleted virus was resuspended in storage buffer (10 mM Tris pH 7.2, 2 mM MgCl\(_2\) and 0.01\% Tween 80) and purified by centrifugation on a 20\% (w/v) sucrose solution at 200 000 \(\times\) g for 2 hours at 4 °C using a Beckman 90Ti rotor (Carmo et al., 2006; Landazuri et al., 2006). The final virus pellet was resuspended in storage buffer. The total yield of the concentration and purification steps was 10\% and the titer of the final viral preparation was 4.5\(\times\)10\(^7\) infectious particles mL\(^{-1}\).

2.2. Inactivation studies

After resuspension of the viral pellet in storage buffer, this viral preparation was incubated in a CO\(_2\) incubator at 37 °C and samples were removed, before incubation (zero time) and 1, 2, 4, 6, 8, 10, 12, 24 and 48 hours post-incubation. These samples were aliquoted for each test and stored at -85 °C. Pre-incubated samples were thawed and tested for infectivity, endogenous and intracellular reverse transcription activity, reverse transcriptase activity and RNA degradation. The same set of viral samples was used to perform all tests. Activities in viral samples, without pre-incubation (zero time) were taken as 100\%.


2.3. Quantitation of infectious MLV

To determine retroviral infectivity, HCT 116 (ATCC CCL-247) target cells were seeded in 96-well, flat-bottomed plates (Starstedt, Newton, USA) at a density of 1.65×10⁴ cells per well and incubated for 24 hours. Infections were carried out by replacing the medium with 20 μL of dilutions (10⁻¹ to 10⁻⁴) of viral supernatants in DMEM medium containing 8 μg mL⁻¹ polybrene (Sigma, Steinheim, Germany), followed by incubation at 37°C for 4 hours (Cruz et al., 2007). After adsorption, 180 μL of fresh medium was added and cells were incubated for 2 days. The medium was aspirated and the cells were washed with 100 μL of PBS; afterwards, fixation solution (100 μL) containing 0.75% (v/v) formaldehyde 37% (v/v) (Merck) and 5.1% (v/v) of glutaraldehyde (Sigma) in PBS was added for 2 minutes and the cells were further washed with 100 μL of PBS. X-Gal dye (100 μL) consisting of 5 mM K₃Fe(CN)₆ (Merck), 5 mM K₄Fe(CN)₆ (Merck), 1 mM MgCl₂ (Merck) and 200 mg mL⁻¹ X-gal (Stratagene, La Jolla, CA, USA) in dimethylformamide (Riedel deHaën, Seelze, Germany) was added, and LacZ-positive (blue) cells were counted after 24 hours of incubation at 37°C. Three dilution sets were performed for each sample tested.

2.4. Endogenous reverse transcription

Endogenous DNA synthesis reactions were performed as described elsewhere (Fassati and Goff, 1999; Haseltine et al., 1979). The reaction mixture consisted of 50 mM Tris-HCl pH 8.3, 6 mM MgCl₂, 50 mM NaCl, 1 mM DTT, 1 mM dNTP, 0.01% (v/v) Nonidet P40 (Roche Diagnostics, Mannheim, Germany) and 50 μL of a purified viral sample (1 mg mL⁻¹ protein), in a final volume of 100 μL. Three reactions were performed for
each sample. The endogenous reactions were incubated at 37°C for 2 hours and then warmed to 75°C for 10 minutes. Viral DNA products were quantified by real-time PCR using a method described elsewhere (Carmo et al., 2004). The U5-R sequence of the MLV genome (Figure 1b, primers 1) was amplified with the following primers: forward primer - 5’ ATT GAC TGA GTC GCC CGG 3’; reverse primer - 5’ AGC GAG ACC ACA AGT CGG AT 3’. The R-U3 sequence of the genome (Figure 1c, primers 2) was amplified using primers: forward primer - 5’ TGA AAT GAC CCT GTG CC 3’; reverse primer - 5’ AGT CAA TCG GAG GAC TG 3’. The amount of DNA produced was linearly proportional to the quantity of virus added in the endogenous reaction and the amount of viral RNA (template) present in the samples was not limiting.

2.5. Intracellular reverse transcription

Target cells HCT116 were seeded in 6-well plates (Nunc, Roskilde, Denmark) at a density of 5×10⁶ cells per well and incubated for 24 hours. Infections were carried out by replacing the medium with 500 µL of the viral samples that were previously incubated at 37°C for several periods of time, for each time point 3 wells were infected. Polybrene 8 µg mL⁻¹ was added to the viral samples during the infection. The virus was allowed to adsorb to the cells for 4 hours, then the cells were washed 3 times with PBS, and fresh medium was added (Julias et al., 2001). Twenty hours after infection, the total DNA was isolated using the high pure PCR template preparation kit (Roche Applied Science) and eluted with 200 µL of elution buffer. The DNA products were quantified using the real-time PCR methods described above, to quantify the U5-R and the R-U3 DNA.
2.6. Reverse transcriptase activity

To determine DNA polymerase activity the RetroSys™ C-Type RT activity kit (Innovagen, Lund, Sweden) was used. RT was released from the virions using Triton X-100 (0.5%). The primer/template polyrA/(polydT) was used and incorporation of bromodeoxyuridine followed. The product was quantified colourimetrically following incubation with the supplied phosphatase-conjugated antibody against bromodeoxyuridine. All reactions were linear with respect to incubation time and amount of virus added and three dilution sets were performed for each sample.

2.7. Reverse transcriptase RNase H activity

To determine the activity of the RNase H an established fluorescence assay (Parniak et al., 2003) was appropriately modified. Briefly, the hybrid RNA/DNA substrate had to be changed and thus the following RNA sequence was used: 5’ AAAUGAAAGACCCCGCUGA - fluorescein 3’ hybridized with the DNA sequence: 5’ Dabcyl – TCAGCGGGGTTCTTTATTT 3’. The design of the hybrid substrate took into consideration the following factors: 1. the melting temperature (Tm) for this hybrid was 60°C to ensure stability over a range of temperatures for the enzyme assay; and, 2. the RNA sequence of the hybrid corresponds to the 3’ end of PBS-R section.

Reaction mixtures (50 µL) containing 0.5 µM RNA/DNA hybrid (Sigma-Proligo, Paris, France), 50 mM Tris, pH 8.0, 60 mM KCl were added to wells of WhiteSorp Fluoronunc microplates (Nunc) (Parniak et al., 2003). Viral samples were pre-treated with Triton X-100 1% (Sigma). Reactions were started by adding 50 µL of viral samples in 50 mM Tris, pH 8.0, 60 mM KCl,
10 mM MnCl₂ and incubation was performed at 37°C for 3 hours. Three reactions were performed for each sample. Reactions were quenched with 50 μL of 0.5 M EDTA, pH 8.0. Fluorescence arising from RNA hydrolysis and release of fluorescein molecule was measured with a Cary Eclipse microplate fluorescence spectrophotometer (Varian), using an excitation wavelength of 490 nm and an emission wavelength of 520 nm.

2.8. Viral genomic RNA degradation

To evaluate viral genomic RNA degradation an already established RT real-time PCR method was modified (Carmo et al., 2004). Briefly, viral samples were incubated at 75°C for 10 minutes to release RNA and treated with DNase I to destroy DNA derived from lysed cells. The viral RNA was transcribed into cDNA using the first strand cDNA synthesis kit (Roche Diagnostics) and a specific primer for the U3 sequence of the viral RNA (5’ CTA ACC AAT CAG TTC GCT TCT CGC 3’, See Figure 1a, primer 3). The viral cDNA was quantified by real-time PCR using the following primers for the R sequence of the viral genome: forward primer - 5’ ATT GAC TGA GTC GCC CGG 3’; reverse primer - 5’ AGC GAG ACC ACA AGT CGG AT 3’ (see Figure 1b, primers 1).

To determine the amount of intact parental viral RNA inside the infected cell the protocol described above to observe intracellular reverse transcription was used. Four hours after infection RNA was isolated from infected cells using the High Pure Viral Nucleic Acid kit (Roche Applied Science) and eluted with 50 μL of elution buffer.
2.9. Statistical measurements

Three complete sets of experiments were performed and a representative experiment is presented. For all the tests, data points presented are average of at least three replicates of viral samples; the standard deviation is indicated in all figures. In Figures 2, 3 and 6 the standard deviation is almost always below 5%, making it difficult to be visualized in the graphics. Half-lives (t½) of the different RT activities, of viral RNA and of infectivity were calculated by using linear regression analysis to fit the data at the linear range. Standard deviations were calculated from the regression analysis.

3. RESULTS

3.1. Thermo-lability of the reverse transcription process

Synthesis of proviral DNA following virus entry entails a complex set of interactions involving the RT, the genomic RNA template, as well as additional viral factors (Figure 1). The process of reverse transcription was analyzed in this work using both the endogenous viral DNA synthesis reaction and the synthesis of proviral DNA in the infected cell. In the endogenous reaction, purified virus is permeabilized with a non-ionic detergent (NP40) and incubated with deoxyribonucleotides triphosphate, thus enabling all the steps of reverse transcription that culminate in the production of a functional proviral DNA, including the two LTRs (Schultz et al., 1995; Telesnitsky and Goff, 1997). In the first step of the reverse transcription process, tRNAPro primes DNA synthesis to the 5’ end of the RNA (U5-R, see Figure 1a). To specifically measure the thermo-lability of this step, endogenous RT reactions were performed with purified MLV
samples that have been pre-incubated at 37°C for different time periods. The initial DNA produced in the virions was quantified using a real-time PCR to amplify the U5-R region (Figure 1b, primers 1).

**Figure 1**: Initial steps of the reverse transcription process. (a) Minus strand DNA synthesis initiates from the tRNA^\text{5′}^\text{U5} primer, annealed to the PBS (primer binding site) region of the plus sense RNA genome, and proceeds to the 5′ end of the RNA. (b) After RNase H degradation of the R and U5 sequences of the resulting RNA-DNA hybrid, 1st strand transfer occurs (c) with the annealing of the 3′ DNA R sequence to the complementary 3′RNA R sequence. Minus strand DNA synthesis R-U3 sequence resumes, accompanied by further RNase H digestion of the template RNA. The grey boxes represent viral RNA and the white boxes represent the cDNA. The black arrows represent the primers employed in the real-time PCR methods (Materials and methods section) used to quantify the viral DNA products: 1 – Reverse transcribed before 1st strand transfer; 2 – Reverse transcribed after 1st strand transfer. Arrow 3 represents the primer used in the method to quantify full length RNA.

It was noticed that viral samples before the endogenous reaction already contained small amount of viral DNA. This fact was noticed previously by other investigators (Chen et al., 2001; Trono, 1992; Zhu and Cunningham,
1993). To ensure that this background DNA (approximately 3% of total synthesis) did not affect the results, a control endogenous reaction without detergent was carried out. Under these conditions dNTP did not penetrate the viral envelope and the amount of background DNA remained constant. In addition, the amount of background DNA in virions, before the endogenous reaction, was subtracted from the amount obtained after the reaction (Figure 2). It is clear from these results that the capacity of virions to initiate the process of reverse transcription declines rapidly with a t½ of 2.3 ± 0.5 h. In a parallel experiment virus infectivity was tested and the results indicated a rapid decline in viral infectivity following pre-incubation at 37°C, with a t½ of 1.7 ± 0.2 h. It is worth noting that with the exception of the 1 hour time point, the virus capacity to initiate reverse transcription was similar to the reduction in gammaretroviral infectivity (Figure 2); thus, analysing the next step of the reverse transcription process, i.e. the 1st strand transfer, was essential.

![Figure 2: Correlation of viral DNA synthesis in the endogenous reverse transcription reactions with viral infectivity. Initiation of viral DNA synthesis (U5-R DNA), 1st strand transfer (R-U3 DNA) and infectivity profile (IP) of viral samples pre-incubated at 37°C were measured as described in the Materials and methods section. Viral infectivity and DNA synthesized in the viral preparations, prior to incubation at 37°C, were taken as 100%. The results obtained in this study gave standard deviations lower than 5%, thus it is difficult to visualize the error bars in the graphic.](image-url)
During synthesis of minus-strand DNA, the RNase H activity of RT degrades the RNA moiety in the RNA:DNA hybrid. This facilitates the annealing of the minus-strand DNA R sequence to the complementary R at the 3’end of the viral RNA and, consequently, synthesis of the minus-strand DNA R-U3 takes place (Ben-Artzi et al., 1993). To investigate whether the 1st strand transfer is a thermo-labile step, specific primers were applied to amplify the R-U3 DNA sequence (Figure 1c, primers 2). The R-U3 DNA synthesized, in viral preparations that were pre-incubated at 37°C, was determined by subtracting the background DNA present before the reaction from that obtained after the endogenous reaction. The results presented in Figure 2 show that the amount of DNA synthesized after the 1st strand transfer decreases rapidly with a t½ of 1.9 ± 0.1 h. This profile strongly resembles that of viral infectivity decline, with a t½ of 1.7 ± 0.2 h.

To further analyse the relationship between the thermo-lability of the first template switch and the decline in viral infectivity, the initial steps of reverse transcription in the cell cytoplasm following infection were measured. Viral samples that were pre-incubated at 37°C for various intervals were used to infect cell cultures, total cell DNA was extracted and real-time PCR analysis with specific viral primers was carried out (Figures 1b and 1c). The results presented in Figure 3 show that the 1st strand transfer (R-U3) rapidly decrease with profiles of inactivation similar to that of infectivity decline (t½ of R-U3 2.3 ± 0.1 h versus t½ of 1.7 ± 0.2 h for infectivity). In repeated experiments the first template switch decline was faster than that of the initiation of RT reaction.
Figure 3: Correlation of viral DNA synthesis inside the cell with viral infectivity. Initiation of viral DNA synthesis (U5-R DNA), 1st strand transfer (R-U3 DNA) and infectivity profile (IP) of viral samples pre-incubated at 37°C were measured as described in the Materials and methods section. Viral infectivity and DNA synthesized in the cell, prior to pre-incubation at 37°C, were taken as 100%. The results obtained in this study gave standard deviations lower than 5%, thus it is difficult to visualize the error bars in the graphic.

3.2. Thermo-lability of the RT enzymatic activities

During the reverse transcription process, the RT performs 2 different enzyme activities for the complete synthesis of double stranded proviral DNA, i.e., RNA and DNA dependent DNA polymerase and ribonuclease H. The thermo-lability of the DNA polymerase activity was tested both in viral samples and with purified RT enzyme, pre-incubated at 37°C, using an exogenous template: primer, polyA:oligoT (Figure 4). The decline rates of virion associated DNA polymerase activity and of the purified RT pre-incubated at 37°C were similar, with half-lives of 5 ± 1 h and 4.5 ± 0.1 h, respectively.

To confirm that the residual enzyme activity measured in this experiment came from RT molecules present inside the virus during the pre-incubation step at 37°C, the amount of RT activity outside of the virus, i.e., RT activity
in a control virion sample not treated with detergent was assessed. The results indicated that less than 10% of total DNA polymerase activity was present outside the virions during the pre-incubation step (data not shown).

![Graph](image)

**Figure 4:** Reverse transcriptase inactivation profile during incubation at 37°C of purified viral samples and of purified RT enzyme. The initial RT activity, prior to incubation at 37°C, was taken as 100%.

During the reverse transcription process, RNase H activity is required for the 1st template switch (Figure 1c). One possible explanation for the high thermo-sensitivity of this step (Figure 2) is that RNase H inactivation is fast, therefore determining the rate of the first template switch. To directly determine the thermo-stability of RT RNase H, an established enzyme assay was applied (Parniak et al., 2003). In this assay, the substrate is a 3' end-fluorescein labelled RNA (20-nucleotides) annealed to a 5' end-Dabcyl-modified DNA. RNase H cleaves the RNA substrate to release a fluorescein-labelled product generating a fluorescence signal. Given that the published methods to quantify the RT RNase H activity have used purified enzymes, while in this work enzymes in the viral particles were evaluated, it was necessary to modify the standard method. Furthermore, since the referred method has been developed to quantify HIV RT RNase H activity, additional
adaptation for MLV RNase H was required (see Materials and methods section). In this context, the sequence of the RNA substrate used here corresponded to that of MLV PBS-U5 regions.

Optimization of the RNase H reaction conditions included the selection of non-ionic detergent concentration, duration of the reaction and the amount of virus. Under optimal conditions a linear correlation between viral concentration and RNase H activity was obtained, with a narrow variability of replicates (Figure 5a).

The kinetics presented in Figure 5b indicate that the inactivation profile of RT RNase H activity in samples pre-incubated at 37°C ($t^{1/2}$ of 10.3 ± 1.5 h) is slower than that of DNA polymerase activity and does not appear to correlate with the decline rate of the 1st strand transfer step ($t^{1/2}$ of 2.3 ± 0.1 h, Figures 2 and 3).

![Figure 5](image_url)

**Figure 5**: (a) RNase H activity as a function of virus concentration. Data points represent fluorescence intensity produced during 3 h incubation. The means ± SD for triplicate reactions are presented. (b) Profiles of RT RNase H activity of viral samples, pre-incubated at 37°C. The initial RNase H activity, prior to incubation at 37°C, was taken as 100%. 

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3.3. Kinetics of viral genome degradation

The reverse transcription process to produce a complete proviral DNA is rather slow, 8 to 12 hours in HIV (Kim et al., 1989), and requires an intact viral RNA template. It is possible that during pre-incubation at 37°C the viral RNA is rapidly degraded thus affecting the synthesis of proviral DNA and consequently viral infectivity. To directly follow the rate of viral RNA degradation, a method was devised to specifically measure intact full size viral genomic RNA. A primer with homology to the U3 region of the viral RNA was used for the RT step (Figure 1a, primer 3), such that the cDNA produced will have only one R sequence, derived from the 5’ end of the viral RNA template (Figure 1). After cDNA synthesis a real-time PCR method was used to specifically amplify the cDNA R sequence at the 5’end of the viral genome (Carmo et al., 2004). Using this procedure, any internal nick within the genomic RNA template would result in the abortion of cDNA synthesis prior to the R region. Consequently, PCR amplification of the R sequence will occur only from full size cDNA derived from intact genomic RNA.

The results presented in Figure 6a show that the kinetics of genomic RNA degradation within the virion pre-incubated at 37°C (t½ of 21 ± 2 h) is much slower than the decline of the 1st template switch (2.3 ± 0.1 h, Figure 3). As the rate of viral RNA degradation in intact virions may not necessarily reflect the situation in the cytoplasm following infection, we next tested the integrity of the viral genomic RNA in the cell cytoplasm following infection. This analysis can also show the efficiency of viral entry to the cell. To this end, cells were infected with viral samples pre-incubated at 37°C; total RNA was isolated from cells 4 hours after infection and subjected to RT-PCR
analysis, to specifically measure levels of intact viral RNA, as described above (Figure 6b).

**Figure 6**: Rate of viral RNA degradation during pre-incubation at 37°C. (a) RNA in purified viral samples; (b) Viral RNA in the cytoplasm of cells infected with viral samples pre-incubated at 37°C. The initial intact RNA concentration, prior to incubation at 37°C, was taken as 100%. The calculated ratio of intact viral RNA in the cell versus the amount of DNA synthesized (RNA/DNA U5-R) at each time point is also presented in Figure 6b. The results obtained in this study gave standard deviations lower than 5%, thus it is difficult to visualize the error bars in the graphic.

Surprisingly, the results indicated that the quantity of viral genomic RNA that enters the cells declined very slowly due to pre-incubation of the virus at 37°C. As compared with a fresh virus (100%), virus pre-incubation for 8 hours at 37°C reduced by only 47% the amount of intact intracellular viral RNA. Furthermore, from this data it was possible to calculate the ratio of intact viral RNA versus the amount of DNA (U5-R) that is synthesized within the cell for each pre-incubated viral sample. As seen in Figure 6b, the ratio of template RNA to product DNA following infection, increased with time by 2 fold at 6 hours pre-incubation.
4. DISCUSSION

Gammaretroviral vectors have been shown to be relatively unstable at physiological temperatures (37°C) (Carmo et al., 2006; Higashikawa and Chang, 2001), presenting a major drawback for their applications in gene therapy. Gammaretroviral vector inactivation has been studied under different conditions, especially at different temperatures, providing some information about their inactivation kinetics (Carmo et al., 2006; Cruz et al., 2006). Nevertheless, the mechanism responsible for viral inactivation is still not known as several viral components may suffer degradation/inactivation. The reverse transcription constitutes a complex process, performed in the cytoplasm, which requires functionality of several viral constituents, like reverse transcriptase, viral RNA, nucleocapsid protein, among others. Therefore, it is reasonable to hypothesize that viral capacity to perform reverse transcription is diminished by incubation at 37°C and this can affect viral infectivity.

The results show that the decrease of gammaretroviral infectivity presented a high correlation to the loss of virus capacity to perform reverse transcription, both in the endogenous reaction and following infection in the cell (Figures 2 and 3). Specifically, early steps of the reverse transcription process, initiation (U5-R synthesis) and 1st strand transfer (R-U3 synthesis), were affected by temperature, with the 1st strand transfer step presenting the highest sensitivity. This thermo-sensitivity is somehow expected since the 1st strand transfer is one of the most complex steps in reverse transcription, requiring native conformation of the components present in the reverse transcription complex. Furthermore, several studies have suggested that the 1st strand transfer is a highly inefficient step even under optimal conditions.
(Haseltine et al., 1979; Kulpa et al., 1997). To ensure that in the endogenous reaction the reduction of reverse transcription efficiency was not caused by the use of Mg$^{2+}$ instead of Mn$^{2+}$, the preferred ion for MLV RT in exogenous reactions (Malmsten et al., 1998; Roth et al., 1985), these reactions were also performed with MnCl$_2$. The results have shown that the loss of the virus capacity to perform reverse transcription was the similar in both conditions (data not shown).

The results from testing the thermo-sensitivity of the RT enzymatic activities clearly indicate that RNase H activity is more thermo-stable than the DNA polymerase; furthermore, the rate of decrease in infectivity is much faster than the rate of inactivation of the enzymatic activities. Taken together, these observations suggest that an initial step of infection, either prior to or during initiation of reverse transcription, is highly thermo-sensitive and determines the rate of viral inactivation. To study early steps of infection, the entry of the parental viral genomic RNA into the cell was analyzed as well as the stability of viral RNA within the virion. To this end, a strategy to specifically measure intact viral RNA in the cell was devised, using a quantitative RT-PCR. Surprisingly, levels of intact parental viral RNA in the cell, following infection, declined very slowly (Figure 6b). Correspondingly, intact genomic RNA within purified virions was also shown to be relatively stable to incubation at 37°C (Figure 6a). As the amount of viral RNA that entered the cells declined at a slow rate as compared to the viral infectivity and the initiation of reverse transcription, it follows that entry of viral RNA into the cell is not a rate limiting factor. This conclusion was further supported by the increased ratio of intact viral RNA to the amount of viral DNA (U5-R) produced in the cell following
infection (Figure 6b). Thus, the amount of intracellular intact viral RNA is not restraining the synthesis of viral DNA. This observation is a strong confirmation that the vector loses its capacity to insert the gene of interest by not being able to transcribe its RNA into DNA.

In Figure 7 a summary of the tests performed in this work is presented as well as the calculated half-lives ($t_{1/2}$) for each step. It is clear that pre-incubation of virus at 37°C affects the infection process after the entry step, most probably at the 1st template switch of the reverse transcription process. RT inactivation and RNA degradation appear to play a minor role, if any, in the loss of viral infectivity. Taken together, this analysis provides a solid explanation for the low stability of gammaretroviral vectors from the production stage to gene transfer into the target cell.

![Diagram](image)

**Figure 7**: Summary of the tests performed in the analysis of gammaretroviral thermo-sensitivity and half-lives obtained in each test. Viral samples were pre-incubated at 37°C for several periods of time and tested for: (a) endogenous reverse transcription, U5R DNA - initiation of DNA synthesis and RU3 DNA - 1st strand transfer; (b) viral RNA degradation; (c) RT DNA polymerase and RNase H activities; (d) intact viral RNA entry into host cells; (e) intracellular reverse transcription, U5R DNA - initiation of DNA synthesis and RU3 DNA - 1st strand transfer; and, (f) viral infectivity.
Several studies have shown that integrity of the reverse transcription complex is essential for completion of the reverse transcription reaction (Fassati and Goff, 1999). This complex is comprised of viral RNA, RT, integrase and nucleocapsid proteins (Fassati and Goff, 1999; Gotte et al., 1999; Zhang et al., 2002). The nucleocapsid proteins have several roles during reverse transcription i.e. unwinding the viral RNA, stimulating reverse transcription, facilitating strand transfer and increasing the processivity of DNA synthesis (Cameron et al., 1997; Gotte et al., 1999; Nisole and Saib, 2004; Zhang et al., 2002). Therefore, changes in the viral nucleoprotein conformation, during pre-incubation at 37°C, may alter the capacity of the reverse transcription complex to perform the initial steps of reverse transcription. Since very little is known of this viral complex once in the cytoplasm, further studies of its thermo-sensitivity and the correlation to viral infectivity must await the development of new assays. Another important constituent of the reverse transcription complex is the primer tRNA, its proper interaction with the reverse transcriptase enzyme and the template RNA influence the initiation of the reverse transcription process (Gotte et al., 1999). Thus, it would be interesting to study the stability of this primer molecule at 37°C as well as the stability of its binding with RT and template RNA.

The findings reported herein have practical relevance, since stabilization of the reverse transcription complex should now be pursued in order to improve the stability of gammaretroviral vectors. This could have important implications in the production and storage of gammaretroviral vectors for gene therapy applications.
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5. REFERENCES


Chapter III


Inactivation mechanisms of gammaretroviral vectors


Chapter III


Chapter IV

INACTIVATION MECHANISMS OF LENTIVIRAL VECTORS

Adapted from:

Inactivation mechanisms of lentiviral vectors

ABSTRACT

Lentiviral vectors are an important tool for gene transfer research and gene therapy purposes. However, the low stability of these vectors affects their production, storage and efficacy in pre-clinical and clinical settings. In the present work the mechanism underlying the thermo-sensitivity of the lentiviral vector was evaluated.

For lentiviral vectors, pseudotyped by amphotropic and RDpro envelopes, the capacity to perform reverse transcription is lost rapidly at 37°C with high correlation to the infectivity loss. The vector with RDpro envelope presented a higher stability than that with amphotropic envelope for both the reverse transcription process and virus infectivity. Reverse transcriptase enzyme inactivation and viral template RNA degradation were not implicated in the lost of the virus capacity to perform reverse transcription. Furthermore, early steps in the infection process do not determine the rate of viral inactivation as the amount of viral RNA and p24 protein entering the cells decreased slowly for both vectors.

Taken together, it can be concluded that the reverse transcription process is thermo-labile and thus determines the rate of lentiviral inactivation. Strategies to stabilize the reverse transcription process should be pursued to improve the applicability of lentiviral vectors in gene therapy.


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1. INTRODUCTION

Lentiviral vectors are in the forefront of gene delivery vehicles for research and application in clinical settings (Cockrell and Kafri, 2007). These vectors have the advantages of MLV-derived gammaretroviral vectors and, most importantly, the capacity to stably integrate the gene of choice, even in non-dividing cells (Cockrell and Kafri, 2007; Klages et al., 2000; Zufferey et al., 1998). Lentiviral vectors have already been used successfully to transduce cell types such as retinal cells, pancreatic islets, cells of the central nervous system, and progenitor and differentiated haematopoietic cells, both in vitro and in vivo (Verhoeyen and Cossut, 2004).

One of the major challenges of these vectors is their production, since the development of stable producer cell lines has been a major hurdle, mainly due to the intrinsic cytotoxicity of constitutive expression of some lentiviral proteins (Breckpot et al., 2007). Although producer cell lines have already been developed (Broussau et al., 2008; Ikeda et al., 2003; Klages et al., 2000), lentiviral vectors are still mostly produced transiently by transfection of 293T cells with 3 or 4 coding plasmids (Farley et al., 2007; Zhang et al., 2001).

The production of lentiviral vectors is affected both by low titers and by the low stability of vector infectivity. The low stability of these vectors at 37°C is a critical issue with respect to production, storage and the efficacy of viral preparations in clinical studies; nevertheless, viral stability issue has been poorly addressed (Higashikawa and Chang, 2001; Strang et al., 2004). Several viral components may be susceptible to degradation/inactivation affecting viral infectivity namely, genomic viral RNA, capsid proteins, envelope proteins and the enzymes reverse transcriptase (RT) and integrase. Recently,
our laboratory showed that a key inactivation mechanism of MLV-based gammaretroviral vectors at 37°C is the loss of virus capacity to perform reverse transcription (Carmo et al., 2008), raising the possibility of a similar inactivation mechanism affecting lentiviral vectors. The capacity to produce lentiviral vectors pseudotyped with different envelope proteins is critical to enable gene targeting to specific tissues and cells in clinical applications (Cronin et al., 2005; Verhoeyen and Cosset, 2004). VSV-G envelope (VSV-G Env) is often used to pseudotype lentiviral vectors. However, to ensure reproducible vector quality among productions, a stable producer cell line was chosen in this work; given its high cytotoxicity the VSV-G pseudotype can not be used in stable producer cell lines (Breckpot et al., 2007; Cronin et al., 2005). Furthermore, VSV-G pseudotypes are inactivated by human serum complement, another drawback of this envelope protein (DePolo et al., 2000; Strang et al., 2004). Hence, two different proteins, the amphotropic and the RDpro envelopes (ampho Env and RDpro Env) were selected as pseudotypes in this work; both are resistant to human serum complement, infect clinical relevant cells and can be expressed in stable producer cell lines (Cronin et al., 2005; Sandrin et al., 2002; Stitz et al., 2000; Strang et al., 2004). Additionally, it is known that vectors pseudotyped with the RD114 envelope are more thermo-stable than vectors pseudotyped with amphotropic envelope (Cosset et al., 1995).

This study evaluates the thermo-sensitivity of the reverse transcription process in lentiviral vectors pseudotyped with different envelope proteins and its correlation to the rapid vector inactivation at physiological temperatures. The data presented herein indicate that the envelope protein
affect the thermo-stability of the reverse transcription and thus vector infectivity.

2. MATERIALS AND METHODS

2.1. Lentiviral vectors production and purification

eGFP encoding lentiviral vectors pseudotyped with amphotropic envelope and lentiviral vectors pseudotyped with RDpro envelope were obtained from supernatant of STAR-A cell line (ECACC no. 04072118, Salisbury, UK) and of STAR RDpro cell line (ECACC no. 04072117), respectively, cell lines developed at the University College London (Ikeda et al., 2003). Both cells lines were co-transfected with pSELECT-puro-mcs (InvivoGen, Toulouse, France) and pSIN-CSGW (kindly provided by Dr. Adrian J. Thrasher from UCL, UK) at a ratio of 1:20 and selected with 3 µg mL⁻¹ puromycin. Cells were cultured in DMEM medium (Sigma, Steinheim, Germany) supplemented with 4.5 g L⁻¹ of glucose (Merck, Darmstadt, Germany), 2 mM of glutamine (Gibco, Paisley, UK) and 10% (v/v) FBS (Gibco). After three days of culture the medium was replaced, lentiviral vectors were produced during the following 24 hours and the supernatant was filtered with 0.45 µm filters. The viral supernatant was concentrated by ultracentrifugation in a Beckman Optima XL-100 centrifuge (Beckman Coulter Inc., Fullerton, CA, USA): 70 000 × g for 1.5 hours at 4°C using a Beckman 45Ti rotor (Hanawa et al., 2002; Reiser, 2000). The pelleted virus was resuspended in storage buffer (10mM Tris (Calbiochem, Darmstadt, Germany) pH 7.2, 2 mM MgCl₂ (Merck) and 0.01% Tween 80 (Merck)) and purified by centrifugation on a 20% (w/v) sucrose (Fluka, Steinheim, Germany) solution at 100 000 × g for 2 hours at 4°C using a Beckman 90Ti rotor (Mok et al., 2007). The final virus
pellet was resuspended in storage buffer. The total yield of the concentration and purification steps was 15% for vectors pseudotyped with amphotropic Env and 20% for vectors pseudotyped with RDpro Env. The titer of the final viral preparation was \(7 \times 10^5\) infectious particles (IP) mL\(^{-1}\) for vectors with amphotropic Env and \(4 \times 10^6\) ip mL\(^{-1}\) for vectors with RDpro Env.

### 2.2. Inactivation studies

After resuspension of the viral pellet in storage buffer, the viral preparations were incubated at 37°C and samples removed, before incubation (zero time) and 0.5, 1, 1.5, 2, 4, 6, 8 and 10 hours post-incubation. These samples were aliquoted for each test and stored at -85°C. Pre-incubated samples were thawed and tested for residual infectivity, endogenous and intracellular reverse transcription activity, reverse transcriptase activity, RNA degradation, RNA entry and p24 entry. The same set of pre-incubated viral samples was used to perform all tests. Activities in viral samples, without pre-incubation (zero time) were taken as 100%.

### 2.3. Quantitation of infectious lentiviral vectors

To determine lentiviral infectivity, 293T target cells were seeded in 24-well, flat-bottomed plates (Nunc, Roskilde, Denmark) at a density of \(5 \times 10^4\) cells per well and incubated for 24 hours. Infections were carried out by replacing the medium with 150 μL of dilutions of viral samples in DMEM medium containing 8 μg mL\(^{-1}\) polybrene (Sigma, Steinheim, Germany), followed by incubation at 37°C for 4 hours. After adsorption, viral solutions were removed and 500 μL of fresh medium were added. At 48 hours post infection the cells were trypsinized, resuspended and the percentage of cells expressing GFP was determined by flow cytometry using a CYFlow Space
flow cytometer (Partec, Münster, Germany). Three dilution sets were performed for each sample tested.

2.4. Endogenous reverse transcription

Endogenous DNA synthesis reactions were performed as described elsewhere (Fassati and Goff, 2001). The reaction mixture consisted of 100 mM Tris-HCl (Sigma) pH 8.3, 6 mM MgCl₂ (Merck), 15 mM NaCl (Merck), 1 mM dithiothreitol (Merck), 2 mM dNTP (Roche Diagnostics, Mainheim, Germany), 0.01% (v/v) Nonidet P40 (Roche Diagnostics) and 50 µL of a purified viral sample (1 mg mL⁻¹ protein), in a final volume of 100 µL. Three reactions were performed for each sample. The endogenous reactions were incubated at 37°C for 3 hours and then warmed to 75°C for 10 min. Viral DNA products were quantified by real-time PCR using the FastStart DNA Master SYBR Green I kit (Roche Diagnostics) on a LightCycler® instrument (Roche Diagnostics). The U5-R sequence of the HIV genome was amplified with the following primers: forward primer - 5’-GCT AAC TAG GGA ACC CAC -3’; reverse primer - 5’-GCT AGA GAT TTT CCA CAC TGA -3’. The R-U3 sequence of the genome was amplified using primers: forward primer - 5’-GAC CAA TGA CTT ACA AGG C-3’; reverse primer - 5’-AGC AGT GGG TTC CCT A-3’. Using the R-U3 primers the results will only show the decrease of the virus capacity to perform 1st strand transfer, since all the samples have been previously affected by the loss of capacity to perform the initiation step. Furthermore, the amount of DNA produced was linearly proportional to the quantity of virus added in the endogenous reaction and the amount of viral RNA (template) present in the samples was not limiting.
2.5. Intracellular reverse transcription

Target cells 293T were seeded in 6-wells plates (Nunc) at a density of $1 \times 10^6$ cells per well and incubated for 24 hours. Infections were carried out by replacing the medium with 500 µL of the viral samples pre-incubated at 37°C for several periods of time, for each time point 3 wells were infected. Polybrene 8 µg mL$^{-1}$ was added to the viral samples during the infection. The virus was allowed to adsorb to the cells for 4 hours, then the cells were washed 3 times with PBS, and fresh medium was added (Julias et al., 2001). Twenty hours after infection, the total intracellular viral DNA was isolated using the high pure PCR template preparation kit (Roche Diagnostics) and eluted with 200 µL of elution buffer. The DNA products were quantified using the real-time PCR methods described above, to quantify the U5-R and the R-U3 DNA sequences.

2.6. Reverse transcriptase activity

To determine DNA polymerase activity the RetroSys™ RT activity kit (Innovagen, Lund, Sweden) was used. RT was released from the virions using Triton X-100 (0.5% v/v, Sigma). The primer/template polyrA/(polydT) was used and incorporation of bromodeoxyuridine followed. The product was quantified colourimetrically following incubation with the supplied phosphatase-conjugated antibody against bromodeoxyuridine. All reactions were linear with respect to incubation time and amount of virus added and three dilution sets were performed for each sample.

2.7. Viral genomic RNA degradation

To evaluate viral genomic RNA degradation an already established real-time RT-PCR method used for MLV vectors was modified (Carmo et al., 2008;
Carmo et al., 2004). Briefly, viral samples were incubated at 75°C for 10 minutes to release RNA and treated with DNase I (Sigma) to digest DNA derived from lysed cells. The viral RNA was transcribed into cDNA using the first strand cDNA synthesis kit (Roche Diagnostics) and a specific primer for the R-U3 sequence of the viral RNA (5’-AGC AGT GGG TTC CCT A-3’). The viral cDNA was quantified by real-time PCR using the following primers for the U5-R sequence of the viral genome: forward primer - 5’-GCT AAC TAG GGA ACC CAC -3’; reverse primer - 5’-GCT AGA GAT TTT CCA CAC TGA -3’.

To determine the amount of intact parental viral RNA inside the infected cell the protocol described above to observe intracellular reverse transcription was used. Four hours after infection RNA was isolated from infected cells using the High Pure Viral Nucleic Acid kit (Roche Diagnostics) and eluted with 50 μL of elution buffer.

2.8. Entry of p24 protein

To determine the amount of p24 viral capsid protein inside the infected cell the protocol described above to observe intracellular reverse transcription was used. In this method, cells were washed three times with PBS 4 hours after infection, trypsinized and washed again with PBS. HIV p24 was liberated from infected cells by lyses with 1% (v/v) Nonidet P40. The concentration of viral p24 protein was determined using an enzyme immunoassay (Innotest®, Innogenetics®, Ghent, Belgium) as recommended by the manufacturer.
2.9. Statistical analysis

Three complete sets of experiments were performed and a representative experiment is presented. For all the tests, data points presented are average of at least three replicates of viral samples; the standard deviation is indicated in all figures. Half-lives ($t_{1/2}$) of the endogenous and intracellular reverse transcription processes, of RT activity, of viral RNA and of infectivity were calculated by using linear regression analysis to fit the data at the linear range. Standard deviations were calculated from the regression analysis.

3. RESULTS

3.1. Thermo-lability of the reverse transcription process

The process of reverse transcription depends on specific interactions between the RT enzyme, the genomic RNA template/tRNA primer, as well as additional viral proteins. Synthesis of linear double strand proviral DNA requires several distinct steps: (i) initiation of (-)DNA synthesis; (ii) 1st strand transfer; (iii) initiation of (+)DNA synthesis; (iv) 2nd strand transfer; and, (v) completion of a linear double stranded DNA (Abbink and Berkhout, 2008; Telesnitsky and Goff, 1997). Recently, we have shown that initial steps of the reverse transcription reaction, notably initiation of DNA synthesis and 1st strand transfer of MLV-derived gammaretroviral vectors are thermo-labile, and that this sensitivity determines the rate of retroviral infectivity decline (Carmo et al., 2008). We, therefore, tested the thermo-sensitivity of the reverse transcription reaction of lentiviral vectors. The thermo-
sensitivity of two reverse transcription reactions, endogenous and intracellular, was compared (Figure 1).

In the endogenous reaction, purified vectors that were pre-incubated at 37°C were permeabilized by a non-ionic detergent (Nonidet P40) and incubated with deoxyribonucleotides triphosphate, thus enabling all steps of reverse transcription that culminate in the production of a functional proviral DNA, including the two LTRs (Telesnitsky and Goff, 1997). As purified viral samples, before the endogenous reaction, already contained small amount of DNA, as reported previously (Chen et al., 2001; Trono, 1992), a control endogenous reaction without detergent was carried out. Under these conditions, dNTP did not penetrate the viral envelope and the amount of DNA remained constant. The background DNA in virions, before endogenous reaction, was subtracted from the amount of DNA obtained after the reaction.

In the intracellular reverse transcription reaction, viral samples that were pre-incubated at 37°C for various intervals were used to infect host cells and total DNA was extracted after 16 hours. By performing these two reactions in parallel it was possible to validate the data of reverse transcription thermo-sensitivity.

DNA products of the two initial steps of the reverse transcription process, i.e. initiation of DNA synthesis, using primers for the U5-R sequence, and the 1st strand transfer, using primers for the R-U3 sequence, were quantified in both reactions. For the lentiviral vectors with amphotropic envelope, the results obtained in the endogenous reactions are shown in Figure 1a, and the results obtained for the intracellular reactions are depicted in Figure 1b. It is clear from these two assays that the capacity of the virus to initiate the
process of reverse transcription and to perform 1st strand transfer declines rapidly due to pre-incubation at 37°C. The half-life time (t½) obtained from the endogenous reaction is 1.1 ± 0.1 h for the initiation and 1.0 ± 0.2 h for the 1st strand transfer, and from the intracellular reaction t½ was 1.3 ± 0.1 h for the initiation and 1.0 ± 0.1 h for the 1st strand transfer. In a parallel experiment, viral infectivity was tested and the results indicated a rapid decline in infectivity following pre-incubation at 37°C, with a t½ of 0.75 ± 0.03 h, and a very similar profile to that obtained for the two reverse transcription reactions (Figures 1a and 1b).

![Graphs showing viral DNA synthesis and infectivity](image)

**Figure 1:** Correlation of viral DNA synthesis in the endogenous and the intracellular reverse transcription reactions with viral infectivity, of lentiviral vectors with amphotropic Env (a and b, respectively) and of lentiviral vectors with RDpro Env (c and d, respectively). Initiation of DNA synthesis (U5-R DNA), first strand transfer (R-U3 DNA) and infectivity profile (IP) of viral samples pre-incubated at 37°C were measured as described in the Material and Methods section. Viral infectivity and DNA synthesized in the viral preparations, prior to incubation at 37°C, were taken
The tests described above were also performed with lentiviral vectors pseudotyped with RDpro envelope. In Figure 1c it is possible to observe the products of initiation and the 1st strand transfer of the endogenous reverse transcription and in Figure 1d the products of initiation and 1st strand transfer of the intracellular reverse transcription, together with the infectivity profile. The results confirm that the reverse transcription reaction is thermo-sensitive with profiles of inactivation very similar to that of infectivity loss. The t½ were 2.4 ± 0.5 h and 2.7 ± 0.4 h for the initiation and the 1st strand transfer of the endogenous reaction, respectively, and 2.0 ± 0.2 h and 2.4 ± 0.2 h for the initiation and the 1st strand transfer of the intracellular reaction, respectively. The decline in infectivity showed a t½ of 1.3 ± 0.1 h. Lentiviral vectors pseudotyped with amphi Env showed higher thermo-sensitivity in infectivity as compared with the RDpro vector. Interestingly, higher thermo-sensitivity of amphi Env vector was also observed in the reverse transcription process.

3.2. Thermo-lability of the RT DNA polymerase activity and of the viral RNA genome

For the synthesis of viral DNA several viral components need to be functional, including RT and the template viral RNA. Thus, the stability of these two viral components was analysed (Figure 2).

Loss of the DNA polymerase enzyme activity was observed after pre-incubation of the vectors at 37°C, using an exogenous template: primer, polyA:oligoT (Figure 2a). The decline rates of virion associated DNA polymerase activity for lentiviral vectors pseudotyped with amphotropic envelope and with RDpro envelope were similar, with a t½ higher than 10 h for both vectors.
Chapter IV

Figure 2: Reverse transcriptase inactivation profile (a) and viral RNA degradation profile (b) during incubation at 37°C of lentiviral vectors with amphotropic Env and of lentiviral vectors with RDpro Env. The initial RT activities and RNA concentrations, prior to incubation at 37°C, were taken as 100%.

To evaluate the rate of viral RNA degradation in the virion, a method that specifically measures full length viral genomic RNA, previously established for MLV-based gammaretroviral vectors, was adapted for lentiviral vectors (Carmo et al., 2008). Briefly, a primer with homology to the U3 region of lentivirus viral RNA was used for the RT step, such that the cDNA produced have only one R sequence, derived from the 5’ end of the viral RNA template. After cDNA synthesis a real-time PCR method was used to specifically amplify the cDNA R sequence at the 5’ end of the viral genome. Any internal nick within the genomic RNA template would result in the abortion of cDNA synthesis prior to the R sequence and thus to a low signal in the real-time PCR.

The results presented in Figure 2b show that the kinetics of genomic RNA degradation within virions pre-incubated at 37°C has a t½ higher than 10 h for both lentiviral pseudotypes.
3.3. Thermo-sensitivity of viral entry to the host cell

As shown above (Figure 2b), viral RNA in the virion remains almost intact for more than 10 hours at 37°C, yet, vectors pre-incubated at 37°C lost the capacity to perform synthesis of proviral DNA from the viral RNA. Thus, one needs to check if the vectors lost their capacity to enter cells during incubation at 37°C. To this end, cells were infected with lentiviral vectors with either amphi Env or RDpro Env pre-incubated at 37°C; viral RNA was isolated from the cells 4 hours after infection and subjected to real-time RT-PCR analysis, to specifically measure levels of intact viral RNA in cells. To validate this method, two experiments were performed. First, cells non permissive to lentiviral vectors with RDpro (NIH 3T3 cells (Rasko et al., 1999)) were infected with these vectors in parallel to infection of 293T cells and the protocol to quantify the viral RNA inside infected cells was followed. The results showed that only 10% of the vectors that entered 293T cells were detected in NIH 3T3 cells. These results demonstrate that the amount of viral RNA attached to cells (10%) does not affect the quantification of intracellular RNA in the permissive cells. Second, lentiviral vectors with or without envelope were used to infect 293T cells and the protocol to quantify viral RNA inside infected cells was followed. The results have shown that less than 1% of the vectors without envelope entered the cells. We concluded therefore, that the amount of viral RNA adsorbed to the cell surface following infection is low and can be controlled.

Intracellular viral RNA quantification following infection (Figures 3a and 3b) indicated a very slow decline due to the pre-incubation at 37°C. For both lentiviral vector pseudotypes, more than 50% of the intact viral RNA was still able to enter the host cells after 6 hours of pre-incubation.
From these data, the ratio of intact viral RNA entering the cell versus the amount of DNA (U5-R) synthesized within the cell was calculated for each pre-incubated viral sample, this ratio indicates the efficiency of the reverse transcriptase machinery to synthesize DNA from viral RNA. Around 10 copies of viral RNA give rise to 1 copy of viral DNA.

As shown in Figures 3a and 3b the ratio of template RNA to product DNA, following infection, increased with time for both lentiviral vectors pseudotypes, this shows that an increase of RNA that is not reverse transcribed into DNA occurred along incubation time. Nevertheless, for lentiviral vectors with ampho Env after 2 hours of pre-incubation a 2-fold increase was observed in the ratio RNA/U5-R DNA, and for vectors with RDpro Env, only after 4 hours a 2-fold increase was verified.

To validate that the amount of intact viral RNA in the host cell could be used as a measure of viral entry, the amount of p24 viral capsid protein of
the pre-incubated viral samples that enters the host cells was quantified. Cells were infected with viral samples pre-incubated at 37°C for several periods of time (the same samples used for verifying the amount of intact RNA entering the cells), 4 hours after infection cells were extensively washed, trypsinized, lysed and the amount of p24 protein was quantified (Figure 4).

![Graph](image_url)

**Figure 4:** Viral p24 proteins in the cytoplasm of cells infected with lentiviral vectors with amphotropic Env and with lentiviral vectors with RDpro Env, pre-incubated at 37°C. The initial p24 concentrations, in cells infected with viral samples prior to incubation at 37°C, were taken as 100%.

The results show that the amount of p24 protein that entered cells declined very slowly due to pre-incubation at 37°C; at 6 hours of pre-incubation, 50% of the p24 proteins still entered the cells, for both lentiviral vectors, confirming the results of viral RNA entry.

### 4. DISCUSSION

Lentiviral vectors are a powerful tool for gene transfer and gene therapy applications. However, several hurdles exist in the large scale production and efficient application of lentiviral vectors. Low thermo-stability of the virus affects the final yield of vectors produced, as well as the quality/efficacy of the preparations to be used in clinical trials. Hence, a
better knowledge of how lentiviral vectors lose infectivity is necessary in order to devise strategies for vector stabilization. Recently, our laboratory described that the mechanism of gammaretroviral vectors (based on MLV) infectivity loss was related to the thermo-sensitivity of reverse transcription process (Carmo et al., 2008). Due to similarity of gammaretrovirus and lentivirus, the sensitivity of the reverse transcription process at 37°C was studied as a possible mechanism of lentiviral vector inactivation; vectors pseudotyped with two different Env proteins (amphotropic and RDpro) were used.

The results indicated that for both lentiviral vectors pseudotypes the capacity to perform reverse transcription at 37°C decreases rapidly and with high correlation to the vector infectivity loss (Figure 1). Similar thermo-sensitivity was obtained for the endogenous and the intracellular reverse transcription reactions. Two distinct steps of the reverse transcription process were found to be thermo-labile, initiation of (-) DNA synthesis and the 1st strand transfer. The observations with lentiviral vectors are comparable to the ones obtained for gammaretroviral vectors, in terms of the inactivation mechanism (Carmo et al., 2008). It was also observed that neither RT enzyme inactivation nor viral RNA degradation have a role in the loss of the virus capacity to perform reverse transcription, since these two viral components are rather stable at 37°C (Figure 2). It should be noted that, RT and RNA from lentiviral vectors showed higher stability as compared with gammaretroviral vectors. It is known that RT from gammaretroviral and lentiviral vectors have different structures, lentivirus RT is a dimer of two different subunits and both contain DNA polymerase activity, while gammaretrovirus RT is a monomer (Telesnitsky and Goff,
1997), which may be related to their different thermo-stabilities. The effect of pre-incubation at 37°C on viral entry into the host cell was studied by measuring the amount of intact viral RNA and of p24 capsid protein that entered the cells (Figures 3 and 4). As the amount of these two viral components following cell entry declined at a slower rate, when compared to viral infectivity, it follows that entry of viral RNA into the cell is not a rate-limiting factor for infectivity decline. Since the ratio of intact viral RNA to the amount of viral DNA produced after infection increased along pre-incubation time, it is possible to infer that vectors lose their capacity to transcribe RNA into DNA and that the amount of RNA inside the cell does not restrict reverse transcription. Thus, the first conclusion drawn from this work is that loss of the virus capacity to perform reverse transcription is a mechanism of lentiviral vectors inactivation.

An overview of the tests performed in the present study is presented in Figure 5, in addition to the thermo-sensitivity (t½) of each step. It is clear that pre-incubation of virus at 37°C affects the infection process after the entry step for both lentiviral pseudotypes. Vectors pseudotyped with RDpro Env are more stable than amphi Env pseudotype with respect to viral infectivity and interestingly this higher stability is also observed in the reverse transcription process. In vectors with RDpro Env the limiting thermo-labile step is the initiation of DNA synthesis and not the 1st strand transfer, as observed for vectors pseudotyped with amphotropic Env and as previously shown for gammaretroviral MLV vectors (Carmo et al., 2008).
Figure 5. Overview of the tests performed in the analysis of lentiviral thermosensitivity and half-lives obtained in each test for vectors with amphotropic Env and for vectors with RDpro Env. Viral samples were pre-incubated at 37°C for several periods of time and tested for: (a) endogenous reverse transcription, U5-R DNA – initiation of DNA synthesis and R-U3 DNA – first strand transfer; (b) viral RNA degradation; (c) RT inactivation; (d) intact viral RNA entry into host cells; (e) viral p24 protein entry into host cells; (f) intracellular reverse transcription, U5-R DNA – initiation of DNA synthesis and R-U3 DNA – first strand transfer; and, (g) viral infectivity.

It is worth noting that the higher stability of vectors with RDpro Env was not reflected by different rates of RT enzyme inactivation nor in the rate of RNA degradation. Also, the amount of viral RNA and of viral p24 protein that entered the host cells declined with similar rates for both pseudotyped vectors. Nevertheless, the ratio of intact viral RNA to the amount of viral DNA produced after infection increased at different rates for the two pseudotyped vectors, with Ampho Env showing a faster increase. Thus, the second conclusion that is drawn from this work is that lentiviral vector pseudotyped with RDpro Env is more stable than vector with ampho Env and that higher virus stability is due to a slower loss of the capacity to perform reverse transcription.
Interestingly, it was possible to observe that the efficiency to start reverse transcription was lower in lentiviral vectors than for gammaretroviral vectors. This efficiency is given by the RNA/U5-DNA ratio at zero time of pre-incubation. In lentiviral vectors 1 copy of U5-DNA is synthesized per 10 copies of viral RNA that enter the cell while in gammaretroviral vectors the value is 1 per 5 copies (Carmo et al., 2008).

The reverse transcription process occurs in the cytoplasm in a complex which includes viral RNA, tRNA, reverse transcriptase, nucleocapsid proteins and integrase. The integrity of this complex is of utmost importance for the completion of proviral DNA synthesis (Fassati and Goff, 2001; Gotte et al., 1999; Nermut and Fassati, 2003). In this work it was observed that neither viral RNA nor RT suffer inactivation at 37°C, and thus other components should be thermo-sensitive. The nucleocapsid protein (NC) plays an essential role in several steps of the reverse transcription process. It unwinds viral RNA, facilitates the strand transfers and improves the processivity of the DNA polymerase (Gotte et al., 1999; Nisole and Saib, 2004; Thomas and Gorelick, 2008). It is possible that at 37°C the native NC loses the conformation required for a functional reverse transcription complex, leading to inactivation of reverse transcription. Another important constituent of the reverse transcription complex is the primer tRNA, its proper interaction with the reverse transcriptase enzyme and the template RNA influence the initiation of the reverse transcription process (Gotte et al., 1999). Further studies on the stability of these two components at 37°C, as well as the stability of their interactions with RT and template RNA should be pursued to assess the cause for the fast inactivation of the reverse transcription complex.
Two different envelopes were used in this work for pseudotyping lentivirus, amphotropic and RDpro, both derive from gammaretroviruses. The amphi Env (derived from Murine Leukemia Virus) efficiently pseudotype lentiviral vectors while the RD114 Env (derived from Feline Endogenous Virus) has a very low capacity to pseudotype lentiviral vectors (Sandrin et al., 2002). Consequently, RD114 Env was engineered to be used in lentiviral vectors, by substitution of the cytoplasmic tail with that of MLV Env (Sandrin et al., 2004). Furthermore, the R peptide cleavage site sequence was replaced with that of a matrix-capsid cleavage site in HIV-Gag to create RDpro (Strang et al., 2004). A small change in Env can lead to a change in the overall vector stability, by providing higher protection to the reverse transcription complex.

The findings reported in the present study have profound practical relevance as stabilization of the reverse transcription process should be pursued to improve the stability of lentiviral vectors, one possible solution for stabilization being the modification of the Env protein. This has important implications for the production and storage of lentiviral vectors and consequently in their use as gene therapy vectors.

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5. REFERENCES


Chapter IV


Chapter V

STABILIZATION OF GAMMARETROVIRAL AND LENTIVIRAL VECTORS

Adapted from:

ABSTRACT

The low stability of gammaretroviral and lentiviral vectors affects their production making high quality clinical preparations a difficult goal to achieve. Recently, our laboratory has shown that the main inactivation mechanism for both these vectors is the loss of their capacity to perform reverse transcription. The aim of the present work was to increase the stability of gammaretroviral and lentiviral vectors through stabilization of the reverse transcription process, at 37°C and at 4°C.

The results have shown that it is possible to increase the stability of reverse transcription and, consequently, the infectivity of purified gammaretroviral vectors by adding recombinant human albumin (rHSA) to the storage buffer, both at 37°C and at 4°C. For lentiviral vectors it was observed that further protection was needed. This was achieved by adding lipids to the storage buffer, using a mixture of lipoproteins and rHSA. The difference of stabilization between gammaretroviral and lentiviral vectors was validated by performing stabilization tests with vectors possessing different envelope proteins and produced by different cell lines.

The results presented in this work show that it is possible to increase the half-life of purified gammaretroviral and lentiviral vectors by increasing the stability of the reverse transcription process at 37°C and at 4°C, but the two vectors have different stabilization requirements: for gammaretroviral vectors addition of rHSA is enough and for lentiviral vectors it is necessary to add both lipoproteins and rHSA.
Chapter V

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1. INTRODUCTION

Vectors derived from virus of the Retroviridae family, especially gammaretroviral and lentiviral vectors, have been widely used in gene transfer protocols and gene therapy clinical trials (Cockrell and Kafri, 2007; McTaggart and Al-Rubeai, 2002). These vectors present the main advantage of being able to insert the gene of interest into the host-cell genome, with lentiviral vectors being able to integrate the gene in both dividing and non-dividing cells whereas gammaretroviral vectors require dividing cells to integrate (Cockrell and Kafri, 2007; Klages et al., 2000; McTaggart and Al-Rubeai, 2002). Nevertheless, major production hurdles still exist given the difficulty in obtaining the necessary number of infectious particles required for a clinical trial (Breckpot et al., 2007; Merten, 2004); this difficulty is not only due to the low productivity of producer cells but also due to the high instability of these vectors. Both gammaretroviral and lentiviral vectors are known to have a short half-life at 37°C (Carmo et al., Submitted; Carmo et al., 2008; Higashikawa and Chang, 2001), a critical temperature for almost all the manufacturing steps from production to gene transfer. Several groups studied the inactivation kinetics of these vectors (Beer et al., 2003; Carmo et al., 2006; Higashikawa and Chang, 2001) and also work has been performed regarding the stabilization of the vectors at storage temperatures (Carmo et al., 2006; Cruz et al., 2006). Recently, the main mechanism responsible for these vectors drop in infectivity at 37°C was identified as being the loss of the virus capacity to perform reverse transcription (Carmo et al., Submitted; Carmo et al., 2008). This mechanism is thought to be caused by a conformational change of the reverse transcription complex at this biological temperature. Thus, it becomes possible to devise and test novel strategies to
stabilize the cause of the fast inactivation of gammaretroviral and lentiviral vectors.

Several compounds have been commonly used to stabilize viral vaccines: sugars such as sucrose, sorbitol and lactose; Fetal Bovine Serum (FBS); or, proteins like albumin and gelatine (Brandau et al., 2003). Sugars are known to stabilize gammaretroviral vectors (Cruz et al., 2006), but since they have the ability to increase the solvent surface tension as well as weaken interactions with macromolecular surfaces, their stabilizing effect is more pronounced when a change of the physical state of the water is involved, like freezing and lyophilisation (Brandau et al., 2003; Coroadinha et al., 2006a; Howell and Miller, 1983; Lee and Timasheff, 1981). Proteins on the other hand stabilize by performing non-specific interactions with container surfaces, e. g. the viral membrane, preventing disruption or loss of conformation (Brandau et al., 2003). Albumin is also known to enhance enzyme activity, and is therefore a good candidate to stabilize gammaretroviral and lentiviral vectors.

The aim of this work was to increase the stability of gammaretroviral and lentiviral vectors through stabilization of the reverse transcription process, at temperatures relevant for production and utilization, 37°C and 4°C. This was achieved by reducing the loss of the virus capacity to perform reverse transcription, using different formulations according with the specific needs of gammaretroviral and lentiviral vectors.
2. MATERIALS AND METHODS

2.1. Gammaretroviral vector production and purification

Murine Leukemia Virus (MLV) - derived vectors pseudotyped with amphotropic envelope were obtained from supernatant of human TE FLY A7 packaging cell line (kindly provided by Dr. Otto Merten from Généthon, France) and MLV-derived vectors pseudotyped with GALV envelope were obtained from supernatant of 293FLEX packaging cell line (Coroadinha et al., 2006b). TE FLY A7 cells were cultured in DMEM medium (Gibco, Paisley, UK) supplemented with 4.5 g L⁻¹ of glucose (Merck, Darmstadt, Germany), 6 mM of glutamine (Gibco) and 5% (v/v) FBS (Gibco) and 293Flex cells were cultured in DMEM medium (Gibco) supplemented with 4.5 g L⁻¹ of glucose (Merck), 4 mM of glutamine (Gibco) and 10% (v/v) FBS (Gibco). TE FLY A7 cells were inoculated at 2×10⁴ cells cm⁻² and 293FLEX cells at 4×10⁴ cells cm⁻². After three days of culture the medium was replaced, MLV vectors were produced during the following 24 hours and the supernatant was filtered at 0.45 µm (Cruz et al., 2007). The viral supernatant was concentrated by ultracentrifugation in a Beckman Optima XL-100 centrifuge (Beckman Coulter Inc., Fullerton, CA, USA): 100 000 × g for 1.5 hours at 4°C using a Beckman 45Ti rotor (Burns et al., 1993; Carmo et al., 2008). The pelleted virus was resuspended in storage buffer (Tris (Calbiochem, Darmstadt, Germany) pH 7.2, 10 mM MgCl₂ (Merck) and 0.01% Tween 80 (Merck)) and purified by centrifugation on a 20% (w/v) sucrose (Fluka, Steinheim, Germany) solution at 200 000 × g for 2 hours at 4°C using a Beckman 90Ti rotor (Carmo et al., 2008; Landazuri et al., 2006).
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The final virus pellet was resuspended in storage buffer or in storage buffer with a stabilizing agent.

2.2. Lentiviral vector production and purification

Lentiviral vectors pseudotyped with amphotropic envelope and lentiviral vectors pseudotyped with RDpro envelope were obtained from supernatant of STAR-A cell line (ECACC no. 04072118) and of STAR-RDpro cell line (ECACC no. 04072117), respectively, cell lines developed at the University College London (Ikeda et al., 2003). Both cells lines were co-transfected with pSELECT-puro-mcs (InvivoGen, Toulouse, France) and pSIN-CSGW (kindly provided by Dr. Adrian J. Thrasher from UCL, UK) at a ratio of 1:20 and selected with 3 µg mL⁻¹ puromycin. Cells were cultured in DMEM medium (Sigma) supplemented with 4.5 g L⁻¹ of glucose (Merck), 2 mM of glutamine (Gibco) and 10% (v/v) FBS (Gibco). Cells were inoculated at 6×10⁴ cells cm⁻²; after three days of culture the medium was replaced, lentiviral vectors were produced during the following 24 hours and the supernatant was filtered at 0.45 µm. The viral supernatant was concentrated by ultracentrifugation in a Beckman Optima XL-100 centrifuge: 70 000 × g for 1.5 hours at 4°C using a Beckman 45Ti rotor (Carmo et al., Submitted; Reiser, 2000). The pelleted virus was resuspended in storage buffer (10 mM Tris pH 7.2, 2 mM MgCl₂ and 0.01% Tween 80) and purified by centrifugation on a 20% (w/v) sucrose solution at 100 000 × g for 2 hours at 4°C using a Beckman 90Ti rotor (Carmo et al., Submitted; Mok et al., 2007). The final virus pellet was resuspended in storage buffer or in storage buffer with a stabilizing agent.
2.3. Inactivation studies

The following compounds were tested as stabilizers: bovine serum albumin fraction V (BSA, Merck cat no 112018, Darmstadt, Germany), at 0.4 and at 0.6 mg mL⁻¹; human serum albumin (HSA, Sigma cat no A1653, Steinheim, Germany), at 0.4 and at 0.6 mg mL⁻¹; recombinant human albumin (rHSA, Sigma cat no A7223), at 0.05 to 1.0 mg mL⁻¹; chemically defined lipid concentrate (GBCO cat no 11905-03), at 1% (v/v); cholesterol lipid concentrate (GIBCO cat no 0010025DG), at 0.2% (v/v); cholesterol (Sigma cat no C3045), at 0.03 mg mL⁻¹; and, CelPro-LPS (Hyclone, cat no SV30094.01, Logan, USA), at 0.05% to 1% (v/v).

After resuspension of the viral pellet, preparations were incubated at 37°C and samples removed, immediately before incubation (zero time) and at 0.5, 1, 1.5, 2, 4, 6, 8, 10, 12, 16 and 20 hours post-incubation. For the inactivation studies at 4°C, viral preparations were incubated at 4°C, and samples removed, before incubation (zero time) and at 2, 4, 6, 8, 12, 24, 48, 72, 96, 120, 144, 168, 192 and 216 hours post-incubation. All samples were aliquoted for each test and stored at −85°C. Pre-incubated samples were thawed and tested for residual infectivity and endogenous reverse transcription activity. The same set of pre-incubated viral samples was used to perform all tests. Activities in viral samples, without pre-incubation (zero time) were considered as 100%.

2.4. Serum delipidation

FBS lipid content was removed by a delipidation method described elsewhere (Cham and Knowles, 1976). Two additional steps of i) vacuum
evaporation followed by ii) dialysis against PBS in 100 volumes with three buffer exchanges at 4°C were performed.

Total lipid extraction was performed to FBS and delipidated FBS as described by Bligh and Dyer (Bligh and Dyer, 1959), followed quantification of phospholipids to assess the percentage of lipids removal. Concentration of total phospholipids was determined by a colorimetric assay based on complex formation between ammonium ferrothiocyanate and the phospholipids (Stewart, 1980). The removal of phospholipids from the FBS was higher than 95% (Rodrigues et al., Submitted).

2.5. Quantitation of infectious gammaretroviral vectors

To determine gammaretroviral infectivity of vectors with amphotropic envelope and with GALV envelope, HCT 116 (ATCC CCL-247) and TE 671 target cells were used, respectively. Target cells were seeded in 96-well, flat-bottomed plates (Sarstedt, Newton, USA) at a density of 1.65×10⁴ cells per well and incubated for 24 hours (Cruz et al., 2007). Infections were carried out by replacing the medium with 20 μL of dilutions (10⁻¹ to 10⁻⁹) of viral supernatants in DMEM medium containing 8 μg mL⁻¹ polybrene (Sigma), followed by incubation at 37°C for 4 hours. After adsorption, 180 μL of fresh medium was added and cells were incubated for 2 days. The medium was aspirated and the cells were washed with 100 μL of PBS; afterwards, fixation solution (100 μL) containing 0.75% (v/v) formaldehyde 37% (v/v) (Merck) and 5.1% (v/v) of glutaraldehyde (Sigma) in PBS was added for 2 minutes and the cells were further washed with 100 μL of PBS. X-Gal dye (100 μL) consisting of 5 mM K₃Fe(CN)₆ (Merck), 5 mM K₄Fe(CN)₆ (Merck), 1 mM MgCl₂ (Merck) and 200 mg mL⁻¹ X-gal (Stratagene, La Jolla, CA, USA) in dimethylformamide (Riedel deHaën, Seelze, Germany) was added, and
LacZ-positive (blue) cells were counted after 24 hours of incubation at 37°C. Three dilution sets were performed for each sample tested.

2.6. Quantitation of infectious lentiviral vectors

To determine lentiviral infectivity, 293T target cells were seeded in 24-well, flat-bottomed plates (Nunc, Roskilde, Denmark) at a density of 5×10^4 cells per well and incubated for 24 hours. Infections were carried out by replacing the medium with 150 μL of dilutions of viral samples in DMEM medium containing 8 μg mL⁻¹ polybrene (Sigma), followed by incubation at 37°C for 4 hours. After adsorption, viral solutions were removed and 500 μL of fresh medium was added. At 48 hours post infection the cells were trypsinized, resuspended and the percentage of cells expressing GFP was determined by flow cytometry using a CYFlow Space flow cytometer (Partec, Münster, Germany). Three dilution sets were performed for each sample tested.

2.7. Endogenous reverse transcription

Endogenous DNA synthesis reactions were performed as described elsewhere (Carmo et al., 2008; Fassati and Goff, 2001). The reaction mixture consisted of 50 mM Tris-HCl (Sigma) pH 8.3, 6 mM MgCl₂ (Merck), 50 mM NaCl (Merck), 1 mM DTT (Merck), 1 mM dNTP (Roche Diagnostics, Mainheim, Germany), 0.01% (v/v) Nonidet P40 (Roche Diagnostics) and 50 μL of a purified gammaretroviral vectors sample and of 100 mM Tris-HCl pH 8.3, 6 mM MgCl₂, 15 mM NaCl, 1 mM DTT, 2 mM dNTP, 0.01% (v/v) Nonidet P40 and 50 μL of a purified lentiviral vectors sample, in a final volume of 100 μL. Three reactions were performed for each sample. The endogenous reactions were incubated at 37°C for 3 hours and then warmed to 75°C for 10 minutes. The endogenous reverse transcription reaction can
only be performed with concentrated and purified vector samples, since the medium constituents interfere with the reaction; to obtain a quantifiable DNA product the vectors have to be at high concentration. Viral DNA products were quantified by real time PCR using the FastStart DNA Master SYBR Green I kit (Roche Diagnostics) on a LightCycler® instrument (Roche Diagnostics). The R-U3 sequence of the MLV genome was amplified using primers: forward primer - 5' TGA AAT GAC CCT GTG CC 3'; reverse primer - 5' AGT CAA TCG GAG GAC TG 3' (Carmo et al., 2008) and the R-U3 sequence of the HIV genome was amplified using primers: forward primer - 5'-GAC CAA TGA CTT ACA AGG C-3'; reverse primer - 5'-AGC AGT GGG TTC CCT A-3' (Carmo et al., Submitted). The amount of DNA produced was linearly proportional to the quantity of virus added in the endogenous reaction and the amount of viral RNA (template) present in the samples was not limiting.

2.8. Statistical measurements

Three sets of experiments were performed and a representative experiment is presented. For all the tests, data points presented are averages of at least three replicates of viral samples; the standard deviation is indicated in all figures. Half-lives (t½) of the endogenous reverse transcription processes and of infectivity were calculated by using linear regression analysis to fit the data within the linear range. Standard deviations were calculated from the regression analysis.
3. RESULTS

3.1. Stabilization of gammaretroviral vectors

In the first stabilization test at 37°C, several types of albumin were used: bovine serum albumin (BSA), human serum albumin (HSA) and recombinant human albumin (rHSA). Gammaretroviral vectors with amphotropic envelope were produced using TE FLYA7 cells, purified and resuspended in storage buffer (Tris 10 mM pH 7.2, 2 mM MgCl₂ and 0.01% Tween 80) and in storage buffer with 0.4 mg mL⁻¹ of the different albumins. Purified viral preparations and a sample from the supernatant of producer cells containing viral vectors were incubated at 37°C, and samples were removed at different time points (Figure 1a).

Figure 1: Inactivation profiles of gammaretroviral vectors with amphotropic Env: infectivity (a) and reverse transcription process (b) at 37°C. Vectors were purified, resuspended in storage buffer (SB), SB with 0.4 mg mL⁻¹ BSA, HSA and rHSA, and incubated at 37°C for several periods of time. The inactivation profile of vectors in the cells supernatant at 37°C is also shown in terms of infectivity (a). Viral infectivity and DNA synthesized in the viral preparations, prior to incubation at 37°C, were considered as 100%.

It is possible to observe that the stability of the purified samples increased by adding albumin as a stabilizing agent. The half-lives (t½) obtained were of
1.9 ± 0.1 h for the viral preparation in storage buffer (SB), 5.1 ± 0.5 h for the viral preparation with BSA, 5.5 ± 0.8 h for the viral preparation with HSA and 4.5 ± 0.5 h for the viral preparation with rHSA. It is also possible to observe that by adding albumin to the viral preparations the viral stability of the vectors is nearly restored to the stability of the vectors in the supernatant (t½ of 6 ± 1 h). Due to the fact that recombinant human albumin is considered a safer component than albumin derived from animal sources, the rest of the work performed on the stabilization of gammaretroviral vectors was performed with rHSA. This increase in vector stability after adding rHSA was due to an increase in the stability of the reverse transcription process (Figure 1b). By adding rHSA to the purified vectors an increase of the reverse transcription process stability occurred with the t½ being 2.5 ± 0.1 h in SB and 11 ± 4 h in SB with rHSA. To establish the best rHSA concentration for stabilizing gammaretroviral vectors a screening was performed from 0.05 mg mL⁻¹ to 1 mg mL⁻¹; the results show that the best stabilizing concentration is 0.6 mg mL⁻¹ with a t½ of 5 ± 2 h for infectivity; higher concentrations of rHSA do not further increase stability (data not shown). From the results shown it is possible to infer that a way was devised to stabilize the main mechanism of gammaretroviral vectors inactivation at 37°C. Although this temperature is rather important for production and gene transfer of the vectors, during the production stage other temperatures are also relevant, such as 4°C, important during purification and storage of the viral preparations. So the next step was to verify the effect of rHSA on the stability of the vectors infectivity and reverse transcription process at 4°C (Figure 2).
Figure 2: Inactivation profiles of gammaretroviral vectors with amphotropic Env: infectivity (a) and reverse transcription process (b) at 4°C. Vectors were purified, resuspended in storage buffer (SB) or in SB with 0.6 mg mL⁻¹ rHSA, and incubated at 4°C for several periods of time. Viral infectivity and DNA synthesized in the viral preparations, prior to incubation at 37°C, were considered as 100%.

Adding rHSA to the SB increased the stability, in terms of infectivity, of the vectors from a t½ of 85 ± 15 h to a t½ of 191 ± 35 h (Figure 2a) with a corresponding increase on the stability of the reverse transcription process (Figure 2b) from a t½ of 113 ± 33 h to a t½ of 232 ± 67 h.

3.2. Stabilization of lentiviral vectors

Similar tests to those performed with gammaretroviral vectors - use of BSA, HSA, rHSA and supernatant - were performed with lentiviral vectors with amphotropic envelope, using the best concentration to stabilize gammaretroviral vectors obtained above, 0.6mg mL⁻¹ (Figure 3a). The results show that albumin has only a small stabilizing effect in lentiviral vectors. The half-lives obtained with lentiviral vectors were of 0.8 ± 0.1 h in SB, 1.5 ± 0.2 h in SB with BSA, 2.0 ± 0.1 h in SB with HSA and with rHSA, while the t½ in the supernatant was of 6.7 ± 0.4 h.
Figure 3: Inactivation profiles of lentiviral vectors with amphotropic Env: infectivity at 37°C. Vectors were purified, resuspended in storage buffer (SB), SB with 0.6 mg mL⁻¹ BSA, HSA and rHSA (a) or SB with 10% FBS or 10% delipidated FBS (b), and incubated at 37°C for several periods of time. The inactivation profile of vectors from the cells supernatant at 37°C is also shown in terms of infectivity (a). Infectivity in the viral preparations, prior to incubation at 37°C, was considered as 100%.

In this case albumin did not restore the stability of the vectors in the supernatant; this fact was also observed in the reverse transcription process with half-lives of 1.1 ± 0.1 h being observed in the SB and of 3.2 ± 0.5 h in SB with rHSA. A test was performed to understand if complete FBS, at the same concentration as in the culture medium (10%), would have a stabilizing effect similar to the cells supernatant on the vectors (Figure 3b). By adding 10% of FBS to the storage buffer the vectors half-life increased from 0.8 ± 0.1 h to 8 ± 1 h, restoring the stability of the vectors obtained in the supernatant. This increase was correspondent to an increased stability of the reverse transcription process from a t½ of 1.1 ± 0.1 h to a t½ of 12 ± 1 h. This clearly indicates that other component of the FBS apart from BSA should be stabilizing the vectors. FBS is a complex mixture of compounds from proteins to sugars, salts and lipids. Several papers describe the
importance of the lipids on the stability of lentivirus (Campbell et al., 2002; Graham et al., 2003), making them good candidates for stabilizing these vectors. One way to confirm this is to remove the lipids from FBS, using established methods (Cham and Knowles, 1976; Slater and Robertson, 1979) to generate a delipidated FBS and to observe its stabilizing effect on the vectors (Figure 3b). In Figure 3b the infectivity profiles of vectors in SB, in SB with rHSA and in SB with 10% FBS, are also shown as a term of comparison. When the lipids are removed from the FBS, an effect similar to that of rHSA is observed, with vectors having a t½ of 1.8 ± 0.1 h. The lipid composition of FBS is rather complex, including cholesterol, triglycerides, phospholipids, non sterified fatty acids, among others (Spector et al., 1980). Since lipids are non-soluble in aqueous systems, it was decided to evaluate lipid mixtures that are normally used as complement of serum-free culture media as possible stabilizers. Four different liquid mixtures were tested: a cyclodextrin based lipid concentrate containing cholesterol; a chemically defined lipid concentrate containing saturated and non-saturated fatty acids and cholesterol; a solution of cholesterol; and a purified lipoprotein solution derived from bovine serum (CelPro-LPS) containing lipoproteins and cholesterol. The experiments were performed with concentrations given by the manufacturers as corresponding to 10% FBS in the media. All these components were tested with and without 0.6 mg mL⁻¹ rHSA. The results have shown that only the lipoproteins solution (LPS) together with rHSA is able to increase the stability of lentiviral vectors above the stability of the vectors with rHSA with a t½ of 6.9 ± 0.4 h for infectivity and a t½ of 11 ± 2 h for the reverse transcription process. Thus, the mixture of LPS with rHSA was able to restore the stability of the vectors in the supernatant.
A screening of the best concentration of rHSA and of LPS to be used to stabilize the vectors was performed from 0.05 to 1 mg mL\(^{-1}\) of rHSA and from 0.05\% to 1\% of LPS, but the best concentrations were the ones already used, 0.6 mg mL\(^{-1}\) of rHSA and 0.3\% LPS. In fact, this concentration of LPS corresponds to 0.3 mg mL\(^{-1}\) of cholesterol, the concentration of cholesterol in medium with 10\% FBS. Above these concentrations no increase in the stability was observed over the one established with 0.6 mg mL\(^{-1}\) of rHSA and 0.3\% LPS (data not shown).

The stabilizing effect of SB with rHSA and LPS was also observed in lentiviral vectors incubated at 4\°C (Figure 4). The results show that SB with rHSA and LPS have a high stabilizing effect in terms of infectivity (Figure 4a) and in terms of the reverse transcription process (Figure 4b).

![Graphs showing the percentage of initial infectious particles and DNA amount over time for different conditions](image)

**Figure 4**: Inactivation profiles of lentiviral vectors with amphotropic Env: infectivity (a) and reverse transcription process (b) at 4\°C. Vectors were purified, resuspended in storage buffer (SB), in SB with 0.6 mg mL\(^{-1}\) rHSA and in SB with 0.6 mg mL\(^{-1}\) rHSA and 0.3\% lipoproteins solution (LPS), and incubated at 4\°C for several periods of time. Viral infectivity and DNA synthesized in the viral preparations, prior to incubation at 37\°C, were considered as 100\%.
3.3. Stabilization of gammaretroviral and lentiviral vectors with different envelope proteins

To verify if the different results obtained, in terms of stabilization, between gammaretroviral and lentiviral vectors could be generalized to other gammaretroviral and lentiviral vectors, a similar set of stabilization experiments were performed also to gammaretroviral vectors with GALV Env derived from 293FLEX cells and to lentiviral vectors with RDpro Env derived from STAR-RDpro cells. In this case the experiments were performed at 37°C and the best formulations for stabilization were used for both vectors: 0.6 mg mL⁻¹ rHSA; 10% FBS; and, 0.6 mg mL⁻¹ rHSA with 0.3% LPS. Both the infectivity and the reverse transcription process were observed. The results are shown in Figure 5, for gammaretroviral vectors with GALV Env, and in Figure 6, for lentiviral vectors with RDpro Env.

![Figure 5](image_url)

**Figure 5**: Inactivation profiles of gammaretroviral vectors with GALV Env: infectivity (a) and reverse transcription process (b) at 37°C. Vectors were purified, resuspended in storage buffer (SB), in SB with 0.6 mg mL⁻¹ rHSA, in SB with 0.6 mg mL⁻¹ rHSA and 0.3% lipoproteins solution (LPS) and in SB with 10% FBS, and incubated at 37°C for several periods of time. The inactivation profile of vectors from the cells supernatant at 37°C is also shown in terms of infectivity (a). Viral infectivity and DNA synthesized in the viral preparations, prior to incubation at 37°C, were considered as 100%.
Figure 6: Inactivation profiles of lentiviral vectors with RDpro Env: infectivity (a) and reverse transcription process (b) at 37°C. Vectors were purified, resuspended in storage buffer (SB), in SB with 0.6 mg mL⁻¹ rHSA, in SB with 0.6 mg mL⁻¹ rHSA and 0.3% lipoproteins solution (LPS) and in SB with 10% FBS, and incubated at 37°C for several periods of time. The inactivation profile of vectors from the cells supernatant at 37°C is also shown in terms of infectivity (a). Viral infectivity and DNA synthesized in the viral preparations, prior to incubation at 37°C, were considered as 100%.

rHSA stabilizes gammaretroviral vectors with GALV Env both in terms of infectivity and in terms of the reverse transcription process similarly to FBS. Further addition of LPS to the storage buffer with rHSA had no effect on the stability of gammaretroviral vectors with GALV Env. rHSA does not stabilize RDpro Env lentiviral vectors, it is necessary to add LPS to obtain the same stabilization obtained with FBS. These results confirm the results shown before, i.e., that gammaretroviral vectors are stabilized with rHSA alone and that lentiviral vectors need lipoprotein supplement addition to the rHSA to obtain a similar type of stabilization.
4. DISCUSSION

The production of gammaretroviral and lentiviral vectors present several problems, one of the most critical being the low stability of these vectors. The manufacture of gene therapy vectors comprises several steps: production, purification, storage, and, application of the vectors for gene transfer. Through all these steps vectors suffer inactivation and this affects the final quality and efficacy of the vectors preparations. In this work, several compounds and formulations were used to stabilize gammaretroviral and lentiviral vectors, through stabilization of the reverse transcription process, at two important temperatures for production and use, 37°C and 4°C.

Several compounds are normally used to stabilize viruses in commercial vaccines (Brandau et al., 2003); in this study we started by evaluating stabilizers for the reverse transcription process, and thus the vectors, at 37°C. Albumin, a known enzyme activity stabilizer, was able to increase the stability of purified gammaretroviral vectors, as measured by their half-lives by several hours, (Figure 1a) by increasing the stability of the reverse transcription process (Figure 1b). Furthermore, the results showed that by adding albumin to purified vectors the stability of the vectors approaches the stability of the vectors in cells supernatant, showing that purification does not damage the vectors, instead it removes stabilizing compounds, such as BSA, from the solution.

Since several types of albumin tested, BSA, HSA and rHSA, provided a similar stabilization, rHSA was chosen to be used in the rest of the work with gammaretroviral vectors as it is produced by genetically modified yeast;
Chapter V

thus, as it is not from an animal source like the other albumins it presents higher safety (Chuang et al., 2002; Kobayashi, 2006).

During purification and storage gammaretroviral vectors are usually kept at 4°C (Rodrigues et al., 2007), the stabilizing effect of rHSA at this temperature was also tested. It was possible to verify that rHSA protects the reverse transcription process not only at 37°C but also at 4°C increasing the stability of the vectors by 2-fold (Figure 2).

Lentiviral vectors are very similar to gammaretroviral vectors in terms of production and stability. Thus, on the first test performed with lentiviral vectors the concentration of albumin optimized for gammaretroviral vectors, 0.6 mg mL⁻¹, was used. Interestingly, the stabilization of lentiviral vectors with albumin was very discrete (Figure 3a) compared to the one obtained with gammaretroviral vectors. By performing a test to purified vectors with 10% FBS in storage buffer it was observed that the stability of lentiviral vectors obtained in the supernatant was restored, meaning that other FBS compound beside BSA stabilize the vectors. Looking at the composition of FBS, the lipids arise as good candidates for stabilizing agents. Cholesterol has been described as being critical for lentivirus structure and infectivity (Campbell et al., 2002), and has been appointed also to affect the stability of gammaretroviral vectors (Beer et al., 2003; Carmo et al., 2006). Other lipids have also been related to a higher production of lentiviral vectors (Mitta et al., 2005). Accordingly, to check the importance of the lipids composition of FBS on the stability of lentiviral vectors, we used delipidated serum. Through delipidation, cholesterol, triglycerides, phospholipids and non-sterified fatty-acids are removed from FBS, without removal of proteins (Cham and Knowles, 1976; Slater and Robertson, 1979). Delipidated FBS
 resulted in the same stabilization than the one obtained with rHSA in lentiviral vectors (Fig. 3b), confirming that the lipids of FBS are necessary to stabilize lentiviral vectors. Several lipid mixtures were used to stabilize lentiviral vectors but only with a mixture of lipoproteins and rHSA it was possible to obtain a stability similar to the one obtained with 10% FBS. This mixture was shown to stabilize the reverse transcription process of lentiviral vectors at 37°C and at 4°C, as well as infectivity (Figures 3b, 4a and 4b). Lipoproteins are complex structures composed of several lipids, including cholesterol and phospholipids, and proteins (Olson, 1998). They act as lipid transporters, being responsible for the distribution of lipids through the blood (Olson, 1998). Albumin is also responsible for the transport of lipids through the blood, specifically fatty acids (Trigatti and Gerber, 1995). It is possible that these structures together with rHSA form a protection arrangement around the membrane of lentiviral vectors. As albumin is also known to associate tightly with cell surfaces (Dziarski, 1994), this lipoproteins/rHSA complexes can associate with the vector membrane protecting the structure and preventing conformational changes more efficiently than rHSA alone does; specifically, the conformation of the proteins that form the reverse transcription complex. Since these proteins are though to suffer conformational change at 37°C causing the loss of the virus capacity to perform reverse transcription (Carmo et al., Submitted). The gammaretroviral and lentiviral vectors used in this work have different stabilization needs, although they have the same main inactivation mechanism. To validate that these results could be applied to other gammaretroviral and lentiviral vectors, tests were performed with gammaretroviral vectors with GALV Env produced by 293FLEX cells and
with lentiviral vectors with RDpro Env produced by STAR RDpro cells (a 293 derived cell line). The results obtained confirmed the previous conclusions (Figure 5 and 6). To stabilize gammaretroviral vectors rHSA alone can be used and to stabilize lentiviral vectors LPS has to be used together with rHSA. Hence, stabilization is neither cell line dependent nor envelope protein dependent, gammaretroviral and lentiviral vectors produced with cell lines derived from HEK293 have different stabilizing needs even when the envelope protein is the same (amphotropic). Instead, the difference in stabilization seems to be vector dependent. Lentiviral vectors seem to be more sensitive than gammaretroviral vectors for two reasons: the half-lives of purified lentiviral vectors are lower than the ones of gammaretroviral vectors; and lentiviral vectors seem more sensitive to serum component removal from the solution, showing a higher drop of stability after purification than gammaretroviral vectors. Although gammaretrovirus and lentivirus come from the same virus family, there are some structural differences between them, one of them being the fact that the mature lentivirus has a different core shape than gammaretrovirus (Auerbach et al., 2007; Ganser-Pornillos et al., 2008). In addition, in the synthesis of viral DNA, the reverse transcription complex formed is composed of different proteins (Fassati and Goff, 1999, 2001). Thus, it is possible that lentiviral vectors need higher structural protection to have higher stability at 37°C and 4°C.

The results presented herein are of high practical relevance for the production of gammaretroviral and lentiviral vectors. First, strategies were devised to increase the stability of purified gammaretroviral and lentiviral vectors at 37°C and at 4°C; secondly it was shown that although
gammaretroviral and lentiviral vectors have the same main inactivation mechanism, lentiviral vectors need higher protection with respect to stabilization. Further work should be performed in order to understand how it is possible to increase the protection of the vectors from conformational changes.

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5. REFERENCES


Chapter V


Stabilization of gammaretroviral and lentiviral vectors


Chapter VI

DISCUSSION AND CONCLUSIONS
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1. DISCUSSION

Gene therapy is a powerful tool with the potential to correct both inherited and acquired diseases. As reviewed in Chapter I, gammaretroviral vectors are amongst the most studied vectors for gene therapy to date, since they are able to stably insert the gene of interest allowing to definitively cure a disease caused by inexistence or deficiency of such a gene. Recently, lentiviral vectors have emerged as the possible solution for some problems of gammaretroviral vectors, given that they are able to infect non-dividing cells, which is not the case of gammaretroviral vectors. These two vector systems present the most desirable characteristics to cure monogenic recessive diseases.

However, the similarities among these vectors also exist with respect to manufacturing difficulties as in both cases it is complicated to produce high titer preparations. One of the reasons for this difficulty is their low stability which affects the final yield of vectors produced, as well as the quality/efficacy of the preparations to be used in pre-clinical and clinical trials. The fast inactivation of these vectors is present in all the steps of the manufacturing process, since it can be caused by several conditions inherent to the process, and after manufacture, during gene transfer. Temperature is one of the production conditions that have higher impact on vector stability and infectivity loss, since it affects the intrinsic stability of the produced particles and causes loss of infectivity. This work aimed at understanding the effect of temperature on vector stability at these two levels: first, by studying the effect of production temperature on gammaretroviral vector stability, with the final purpose of determining the best temperature for manufacturing (Chapter II); and second, by unravelling the inactivation
mechanisms related with the thermo-sensitivity of gammaretroviral and lentiviral vectors (Chapter III and IV). The final goal of these studies was to use the knowledge obtained to perform direct and specific stabilization (Chapter V).

1.1. The effect of culture temperature on vector stability

When initiating the work described in this thesis it was already known that the production temperature affected the composition of gammaretroviral vector membrane (Beer et al., 2003). However, only the membrane cholesterol composition had been studied and there was no direct correlation between membrane properties and vector stability. Thus, in order to understand what changes on vector membrane, caused by production at different temperatures, could be related to the vector stability, a thorough study on the physico-chemical properties of the vector membrane was performed (Chapter II). This study was performed with gammaretroviral vectors produced at 32 and 37°C. Viral membranes were characterized in terms of rigidity, cholesterol and phospholipids composition; the protein thermal profiles and denaturation transition were also studied. It was observed that production temperature causes significant changes in the vector membrane composition and on the conformation of envelope proteins. Consequently, vectors produced at 32°C presented lower stability at temperatures below 4°C and lower transduction efficiencies in clinically relevant models.

The culture temperature can affect vector membrane composition in two ways: (1) it can affect the thermodynamics of the cell, virus membrane and budding process, leading to rearrangements of lipids and proteins that will dictate the viral membrane rigidity; and (2) it may change lipid metabolism,
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leading to a selective availability of lipids in the cell membrane and consequently to vectors with different membrane composition. Both these hypotheses can lead to lower stability of the vectors since both can affect the cholesterol/phospholipids ratio. This ratio and specifically the concentration of cholesterol are of the utmost importance to maintain vector stability; several reports show that both must be kept within a certain interval to obtain infectious retroviral particles (Beer et al., 2003; Campbell et al., 2002; Graham et al., 2003).

Overall, it was shown that culture temperature has a direct effect on vector stability by causing changes on membrane properties. Production at 37°C was shown to be the best solution for obtaining more stable vectors. This knowledge was used throughout the thesis. From this point on, all vectors were produced at 37°C, so that in all studies vectors with higher stability could be used. Lentiviral vectors were also produced at 37°C, the similarity between gammaretroviral and lentiviral vectors in terms of constitution, structure and production led to the assumption that culture temperature would affect lentiviral vectors similarly.

1.2. Inactivation mechanisms of gammaretroviral and lentiviral vectors

In Chapter III the inactivation mechanisms of gammaretroviral vectors were studied. During incubation at 37°C gammaretroviral vectors show a fast loss of their capacity to perform reverse transcription with a similar profile of infectivity loss. This fact was noticed both inside the virus (endogenous reaction) and intracellularly. To understand if the viral entry was also affecting viral infectivity, the entry of viral RNA of vectors pre-incubated at 37°C was studied. It was possible to exclude the loss of the virus capacity to enter the host cell as the responsible mechanism for the fast inactivation of
the vectors. These latter results further showed that there is an increase on
the amount of RNA untranscribed inside the cell along the incubation time,
implicating the loss of the virus capacity to synthesize DNA. With these
observations it was concluded that the main mechanism of gammaretroviral
vectors inactivation at 37°C is the loss of the virus capacity to perform
reverse transcription.

The same results were obtained in the study of the inactivation mechanisms
of lentiviral vectors (Chapter IV). Lentiviral vectors pseudotyped with two
different envelope proteins, amphotropic and RDpro, lose their infectivity at
37°C by losing their capacity to perform reverse transcription. Again,
evidence that the entry of the vectors has only a minor role in this
inactivation was experimentally obtained.

Lentiviral vectors with amphotropic and RDpro Env showed different
stabilities in terms of infectivity; as already reported in the literature, vectors
with RDpro Env are more stable. It was shown that this higher stability is a
result of a more stable reverse transcription process. The viral entry of both
vectors presented a similar profile, meaning that the envelope protein does
not affect viral entry (its function on infectivity) but rather RDpro Env gives
higher stability to the conformation of the viral particle.

With results obtained in Chapter V, it was also possible to conclude that the
loss of the virus capacity to perform reverse transcription is the main
inactivation mechanism of gammaretroviral and lentiviral vectors not only
at 37°C, but also at 4°C.

The reverse transcription process involves several viral constituents - RT,
viral RNA, NC, IN and tRNA - constituting the reverse transcription
complex, where DNA synthesis occurs. It was shown that both RT and viral
RNA are rather stable molecules at 37°C and cannot be correlated with the virus loss of capacity to perform reverse transcription, both for gammaretroviral and lentiviral vectors. Interestingly, some differences were observed between the stability of gammaretroviral and lentiviral vectors RT and RNA. Both lentiviral RT and RNA are more stable than gammaretroviral RT and RNA. There are some distinctions described in the literature between MLV RT and HIV RT that can give advantage for HIV RT in terms of stability, one of them being that RT from gammaretroviral and lentiviral vectors have different structures and conformations. The lentivirus RT is a dimmer of two different subunits and both contain DNA polymerase activity, while the gammaretrovirus RT is a monomer (Telesnitsky and Goff, 1997). However, no reason for higher RNA stability was encountered. Gammaretroviral and lentiviral vectors also showed differences in terms of the efficiency to start reverse transcription, with lentiviral vectors showing lower efficiency to initiate reverse transcription than gammaretroviral vectors.

With evidences that RT and RNA have no role on the loss of reverse transcription capacity, it was hypothesized that other viral components important for reverse transcription, such as nucleocapsid proteins or tRNA, may suffer a structural change at 37°C reducing DNA synthesis.

The indication that the main inactivation mechanism of gammaretroviral and lentiviral vectors at 37°C and at 4°C is the loss of the virus capacity to perform reverse transcription has important implications in the production and storage of these vectors. Stabilization of the reverse transcription process was pursued in Chapter V to improve the stability and consequently the final quality of pre-clinical and clinical preparations.
1.3. Stabilization of gammaretroviral and lentiviral vectors

Based on the knowledge that: (1) gammaretroviral and lentiviral vectors are more stable in the cell culture supernatant than after purification; and (2) BSA, the major constituent of FBS, used to supplement culture media, is a stabilizer of viruses by preventing loss of conformation, BSA became the obvious candidate to be included in a formulation for stabilizing these vectors. Experiments confirmed that albumin stabilizes purified gammaretroviral vectors by stabilizing the reverse transcription process, at both 37°C and 4°C, with the recovery of the stability obtained in the supernatant (Chapter V). Several types of albumin were used in this work, but recombinant human serum albumin (rHSA) was chosen due to its higher safety (it is not from animal origin). Nevertheless, albumin only moderately increased the stability of purified lentiviral vectors. Lipids are other constituents of FBS, and are also known to affect the stability of retroviral vectors. In fact, by adding rHSA and lipoproteins to purified lentiviral vectors the stability obtained from the supernatant was restored at both 37°C and 4°C.

It was also shown in Chapter V that the decrease in vector stability during the purification process is not due to damage of the viral particles; instead, removal of stabilizing compounds, such as BSA and lipids, occurs resulting in loss of vector stability.

To validate the stabilization difference between gammaretroviral and lentiviral vectors, vectors were produced in different cell lines and pseudotyped with different envelope proteins. The difference in stabilization was shown to be vector derived and neither cell line nor envelope protein dependent. Although gammaretrovirus and lentivirus come from the same
virus family, there are some structural differences between them, one of them being the fact that the mature lentivirus has a core shape different from that of the retrovirus (Auerbach et al., 2007; Ganser-Pornillos et al., 2008). In addition, in the synthesis of viral DNA, the reverse transcription complex formed is composed of different proteins (Fassati and Goff, 1999, 2001). Thus, it is possible that lentiviral vectors need higher structural protection to increase stability at 37°C and 4°C.

The knowledge of which serum components can stabilize gammaretroviral and lentiviral vectors permits protecting these vectors, from the production to the storage stages, with the addition of albumin and lipoproteins.

2. FUTURE RESEARCH DIRECTIONS INTO STABILIZATION OF GAMMARETROVIRAL AND LENTIVIRAL VECTORS

2.1. Inactivation mechanisms of gammaretroviral and lentiviral vectors

From the results obtained in Chapter III and IV it was possible to hypothesize that a change of conformation of NC and tRNA could be responsible for the loss of the vector capacity to perform reverse transcription. Further studies should be performed on the stability of these two viral components.

tRNA molecule is indispensable for the reverse transcription process, being the primer for the RT to start the reaction (Telesnitsky and Goff, 1997). In order for the synthesis of minus-strand DNA to occur, the tRNA primer must be positioned onto the PBS sequence of the viral RNA template (Abbink and Berkhout, 2008). However, both the viral RNA template and the tRNA are highly structured and, in the absence of appropriate conformation, the annealing between tRNA and the viral template might be
hindered (Gotte et al., 1999). If the tRNA would lose conformation at 37°C there would be no annealing between these two molecules, preventing the initiation of reverse transcription. Verification of this annealing step along incubation at 37°C should be performed to understand if this is the cause for the fast loss of the virus capacity to perform reverse transcription. Nevertheless, the annealing of tRNA to RNA can only be used to explain the loss of the virus capacity to initiate reverse transcription. It was observed in Chapters III and IV that reverse transcription is also affected by incubation at 37°C at the 1st strand transfer. Thus, other viral component must be affected during incubation at 37°C.

The nucleocapsid (NC) protein is considered to be a nucleic acid chaperone that catalyses structural rearrangement in RNA or DNA to form thermodynamically stable conformations. During reverse transcription it is essential for unwinding of tRNA, promoting the annealing between tRNA and RNA, stimulating the strand transfers and mediating alterations in the structure of the template to increase the processivity of DNA synthesis. Two zinc finger motifs are responsible for all these functions of NC (Gotte et al., 1999; Thomas and Gorelick, 2008). Thus, it would be important to understand if NC loses capacity to help reverse transcription by verifying the zinc finger motifs’ activities during incubation at 37°C. Certainly, each activity per se would not determine the real intracellular activity in the reverse transcription complex. Thus, it would also be necessary to verify the zinc finger activities in an environment that mimics the interactions between tRNA, RNA, RT and NC (Anthony and Destefano, 2007).
In Table 1 is represented the future work that could be performed to improve the knowledge on the inactivation mechanisms of gammaretroviral and lentiviral vectors.

**Table 1** – Future research directions to improve the knowledge on the inactivation mechanisms of gammaretroviral and lentiviral vectors.

<table>
<thead>
<tr>
<th>Future work</th>
<th>Goal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verify tRNA conformation loss at 37˚C</td>
<td>Determine if tRNA loss of conformation is responsible for the virus loss of capacity to perform reverse transcription</td>
</tr>
<tr>
<td>Verify tRNA and RNA annealing after incubation at 37˚C</td>
<td></td>
</tr>
<tr>
<td>Verify NC conformation loss at 37˚C</td>
<td>Determine if NC loss of conformation or zinc finger motifs activities are responsible for the virus loss of capacity to perform reverse transcription</td>
</tr>
<tr>
<td>Verify NC zinc finger motifs activities after incubation at 37˚C</td>
<td></td>
</tr>
<tr>
<td>Verify NC zinc finger motifs interaction with RNA, tRNA and RT after incubation of the vectors at 37˚C</td>
<td></td>
</tr>
</tbody>
</table>

### 2.2. Stabilization of gammaretroviral and lentiviral vectors

From the study of the culture temperature effect on vector stability (Chapter II) it became clear that temperature has a high impact on the lipidic composition of the vector membrane. This lipidic composition is critical for the vector stability. In fact, recent studies have shown that the addition of a different carbon source in the culture media increases vector stability through changes in the vector membrane lipidic composition (Coroadinha et al., 2006a; Coroadinha et al., 2006b). Furthermore, it was also observed that addition of lipidic complements to the production culture media improves the stability of gammaretroviral vectors (Rodrigues et al., Submitted). Thus, further studies should be performed regarding different culture conditions, in order to obtain vectors with the membrane properties that provide the best stability.
Discussion and Conclusions

Having identified the main inactivation mechanism of gammaretroviral and lentiviral vectors made possible to increase the half-life of purified vectors. This data can be used to further improve vector stability. Genetic modifications can be performed to reduce the thermo-sensitivity of the reverse transcription complex, more specifically to tRNA or NC. Given the results obtained with two differently pseudotyped lentiviral vectors, the use of different envelope proteins to pseudotype vectors that could protect even more the reverse transcription complex is another possibility.

In Table 2 is represented the future work that could be performed to improve the stability of gammaretroviral and lentiviral vectors, based on the work reported here.

Table 2 – Future research directions to improve the stability of gammaretroviral and lentiviral vectors.

<table>
<thead>
<tr>
<th>Future work</th>
<th>Goal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study the effect of other culture conditions (medium composition and additives) on vector stability</td>
<td>Determine the best combination of culture conditions to obtain more stable vectors</td>
</tr>
<tr>
<td>Genetic modification of the viral constituents involved in reverse transcription</td>
<td>Stabilize the reverse transcription process to obtain more stable vectors</td>
</tr>
<tr>
<td>Test the stability of vectors pseudotyped with different envelope proteins</td>
<td></td>
</tr>
</tbody>
</table>

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3. CONCLUSIONS

During the processes of manufacture and clinical application of gammaretroviral and lentiviral vectors, temperature was recognized as the factor that has higher impact on vector stability (Chapter I). Temperature affects the intrinsic stability of the vectors and causes the inactivation of the vectors in solution. However, there was no explanation on how these effects are actually exerted. In this work, the mechanisms by which temperature affects the stability of gammaretroviral and lentiviral vectors were recognized. First, by studying the effect of culture temperature on gammaretroviral vector stability it was observed that different culture temperatures cause the production of vectors with different membrane composition, rigidity and Env proteins conformation. This led to differences in the stability of the vectors, with vectors produced at 37°C presenting higher stability. Second, the loss of the virus capacity to perform reverse transcription was identified as the main mechanism responsible for the thermo-sensitivity of gammaretroviral and lentiviral vectors. The knowledge obtained from these two parts, was further used to increase vector stability. Storage formulations were developed for gammaretroviral and lentiviral vectors which greatly improved the stability of purified vectors at both 37°C and 4°C by conferring protection to the reverse transcription process. Overall, this thesis increased the knowledge and allowed solutions to be obtained for the stability problem of gammaretroviral and lentiviral vectors and raised future perspectives on how this stability can still be further improved.
4. REFERENCES


Chapter VI


