Relationship between retroviral vector membrane and vector stability

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The present work studies the physico-chemical properties of retroviral vector membrane, in order to provide some explanation for the inactivation kinetics of these vectors and to devise new ways of improving 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 than 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 HCT116 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 with 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 retroviral vectors for therapeutic purposes.

INTRODUCTION

Recombinant retroviruses are one of the most commonly used vectors for gene therapy (Mountain, 2000; McTaggart & Al-Rubeai, 2002; http://www.wiley.co.uk/genmed/clinical). However, the low stability of retroviral 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 retroviral vectors is in the range of 2–8 h at 37 °C (Lee et al., 1998; Le Doux et al., 1999; Pizzato et al., 2001). The negative impact of 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 vectors’ 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 in their membrane. An earlier study by Aloia et al. (1988) showed that manipulation of membrane rigidity of Human immunodeficiency virus (HIV) could affect virus stability and infectivity. These observations constitute evidence for the complexity of the factors influencing the loss of infectivity of retroviral vectors. Thus, it is important to characterize retroviral vectors’ physico-chemical and biological properties in order to understand their low stability.

The present work aims to characterize the physico-chemical properties of the retroviral vector envelope produced in TE Fly A7 cells. For this characterization, vector envelope rigidity was assessed by electron paramagnetic resonance (EPR), the cholesterol and phospholipid composition of the
envelope was determined, and the protein thermal profiles and denaturation transitions were studied by differential scanning calorimetry (DSC). The envelope characteristics were instrumental in providing an understanding of the patterns in inactivation kinetics with storage temperature and in devising strategies for improving vector stability.

**METHODS**

**Cell culture.** Human-derived cell lines were used in this work – HT1080, HCT116 and TE Fly A7. TE Fly A7 is a TE 671 (human)-derived cell line producing retroviruses with amphotrophic envelopes. These retroviruses code for the reporter gene lacZ. The cell line HCT116 and the producer cell line TE Fly A7 were provided by Généthon (France), the cell line HT1080 was obtained from the ATCC. Cells were cultured in T-flasks (Nunc) in Dulbecco’s modified Eagle’s medium (DMEM) (4 g/L glucose, 2 g/L NaHCO3, 100 U penicillin ml–1, 100 μg streptomycin ml–1 and 5% fetal bovine serum (FBS; Life Technologies) supplemented with 100 U penicillin ml–1, 100 μg streptomycin ml–1 and 5% fetal bovine serum (FBS; Life Technologies) and incubated in a humidified incubator with 5% CO2 in the gas phase, at 37 °C. Cells were passaged 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).

**Virus production and purification.** For viral vector production, cells were grown nearly to confluency in DMEM (Life Technologies) supplemented with 5% FBS (Life Technologies) at 37 and 32 °C. Fresh medium was added to cells 24 h before the virus supernatant was harvested and then filtered with 0.45 μm filters (Whatman). Filtered viral supernatant was concentrated by ultracentrifugation using a Beckman L8-55M centrifuge (100 000 g for 1.5 h 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 with 137 mM NaCl, 15 mM Na2HPO4, 2.7 mM KCl, 0.5 mM MgCl2, pH 7.5. Spin label solution [1 mg 5-doxyl-stearic acid (Sigma) ml–1 plus 50 μg crystallized BSA (Sigma) ml–1 as carrier] was added to the purified virus (200 μl viral protein mg–1) and PBS was added up to 1 ml. The mixture was incubated for 2 h at room temperature, after which unbound spin label was removed from the mixture by centrifugation on a 20% (w/v) sucrose solution for 2 h at 200 000 g at 4 °C in a Beckman 90Ti rotor. The pellet virus was resuspended in PBS pH 7.2 and transferred to a quartz EPR flat-cell for aqueous samples.

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

**Membrane-lipid composition determination.** For cholesterol determination, samples of intact virus were adjusted to 0.2 M with NaCl and then extracted with 3 vols of chloroform/methanol (2:1, v/v). The resulting organic phase was lyophilised and analysed for cholesterol content by using an enzymic cholesterol kit from Sigma (ref. 401-25P) according to the manufacturer’s instructions. All samples and controls were performed in triplicate.

For phospholipid determination, 0.1 ml sample of intact virus was mixed with 2.0 ml chloroform and 2.0 ml ferrithiocyanate reagent [27-03 g ferric chloride hexahydrate (Merck) and 30-4 g ammonium thiocyanate (Merck) in 1 l 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.

**DSC.** Calorimetric experiments were performed on a MicroCal VP-DSC MicroCalorimeter controlled by the VP-viewer program. Calibration of temperature and heat-flow were carried out according to MicroCal instructions. Solutions of retroviral vectors produced at 37 and 32 °C were prepared in PBS buffer, pH 7.2. Sample and reference solutions were degassed for 8 min before the calorimetric data acquisitions. DSC scans were run at a constant heating rate of 2 °C min–1 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 were converted to the excess heat capacity of unfolding by subtracting the instrumental baseline determined under identical conditions and dividing it by the scan rate and by the sample protein concentration. The melting temperature (Tm), the calorimetric (ΔHcal) and the van’t Hoff (ΔHvH) enthalpies were calculated using the software supplied with the instrument.

**Virus stability assay.** The first-order decay constants were determined by titration of the infective virus on the purified sample. For that purpose, target cells, HT1080, were infected with different dilutions of these samples and tested for the expression of the lacZ gene (Cruz et al., 2000; Carmo et al., 2004). The titre of the purified samples at the beginning of the virus stability assay was 2.8×106 infectious particles ml–1. Temperature inactivation profiles of the purified retroviral vectors were determined by measuring virus titre against 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:

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

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 the equation.

**Transduction efficiency assays**

**Infection of HCT116 cells.** To determine the transduction efficiency of the virus produced at 37 and 32 °C a microplate assay was used (Le Doux et al., 1999). Briefly, HCT116 cells were seeded in a 96-well tissue plate (1.65×104 cells per well). After 24 h, medium was removed and dilutions of virus in culture medium with 8% polybrene (Sigma) ml–1 were added to each well. Two days after transduction, medium was removed and cells were washed once with 100 μl PBS with 1 mM MgCl2. After removal of the wash solution, 50 μl lysis buffer was added [PBS with 1 mM MgCl2 and 0.5% Nonidet P-40 (Roche Diagnostics)] to each well and the plate was incubated at 37 °C. After 30 min, 50 μl lysis buffer with 6 mM o-nitrophenyl β-D-galactoside (Sigma) warmed to 37 °C was added to each well and the plate was incubated at 37 °C for 1.5 h. The reaction was stopped by the addition of 20 μl 1 M Na2CO3. The optical
density was measured at 420 nm using an absorbance plate reader spectra MAX 340 (Molecular Devices); non-specific background at 650 nm was subtracted. Values for replicative wells without virus were subtracted as background. Values for each point are the means of at least triplicate wells.

Infection of primary human keratinocytes. Human keratinocytes were obtained from skin biopsies of healthy donors by enzymatic digestion following previously described methods (Rheinwald & Green, 1975). Human primary keratinocytes were seeded in 24-well tissue plates (1 x 10⁵ cells per well). After 48 h, cells were infected for 2 h with 10⁴ infectious particles per well of MLV vectors produced at 37 and 32°C in the presence of 7 µg polybrene ml⁻¹. Medium was replaced and 72 h later the cultures were stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) for Lac-Z transgene activity.

Infection of mouse skin micro-organs. Skin of 1-day-old mouse was cut into 0-3 mm slices using a TC-2 tissue sectioner (Sorvall). Slices were incubated in DMEM supplemented with 10% fetal calf serum (Hasson et al., 2005). After 24 h, slices were infected for 6 h with 10⁶ infectious particles of MLV vectors produced at 37 or 32°C in 200 µl in the presence of 7 µg polybrene ml⁻¹. Medium was replaced and 48 h later the skin was stained with X-Gal for Lac-Z transgene activity, and photos were taken under a binocular.

RESULTS

Retroviral 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 & Montelaro, 1982). Thus, to characterize the retroviral vectors used in this work, vector membrane rigidity was measured for each production temperature (37 and 32°C). For this purpose, EPR, a useful and reliable method for measuring the membrane rigidity of retroviruses (Slosberg & Montelaro, 1982), was used. Fig. 1 depicts the hyperfine splitting constant 2TII 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 2TII. Since 2TII 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. The plots of 2TII values against 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 Fig. 1 are statistically different. For this purpose, hypothesis testing was performed and the results clearly indicate that the slopes at 37 and 32°C are different (P<0.00001 in both cases). These thermal break points have been attributed to a viral membrane phase-transition (Slosberg & Montelaro, 1982).

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

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 (Simons & Toomre, 2000; Beer et al., 2003). Also, production temperature may affect membrane composition of the retroviral vectors by changing cell metabolism and by affecting thermodynamically dependent processes such as vector budding.

To study further this phenomenon, the membranes of retroviral vectors were evaluated in terms of cholesterol to phospholipid molar ratio (C/P). This ratio has been widely used as a parameter for the study of lipid composition of retrovirus membranes (Aloia et al., 1988, 1993). The C/P molar ratio obtained for purified retroviral 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 behaviour (Brown & 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 (Fig. 1). In fact, it has been observed for MLV retroviral vectors with amphiprotic envelope that higher cellular cholesterol content leads to vectors with lower stability (Beer et al., 2003).
**DSC**

Calorimetric scans were performed with purified retroviral vectors produced at 37 and 32 °C and one transition was observed in both cases (Fig. 2). The thermodynamic parameters obtained from the analysis of those endotherms are listed in Table 1. No difference was observed in the transition temperature between retroviral vectors produced at 37 or 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 fourfold for the retrovirus produced at 32 °C, in comparison to the retrovirus produced at 37 °C. This ratio is related to the cooperative unfolding unit and thus the results suggest that the retroviral 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 reported previously that the retroviral env molecule consists of a surface glycoprotein (SU) complexed with a transmembrane protein (TM) and that these complexes are grouped into oligomers on the surfaces of the cell and of the virion (Zhao et al., 1997; 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 retroviral proteins produced at different temperatures are folded in different manners must be studied further and evidence from other techniques is required to support these conclusions.

**Vector inactivation rates**

The results presented above show that the membrane properties of retroviral 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 by plotting vector titre against incubation time. Table 2 shows the first-order inactivation rates, determined at −85, −20, 4, 17, 27 and 37 °C, for the vectors and Fig. 3 shows the corresponding half-lives. 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. In fact, after incubation at 4 °C for 24 h, vectors produced at 37 °C retained 70% of their

**Table 1.** Thermodynamic parameters for purified MLV vectors produced at 37 and 32 °C

<table>
<thead>
<tr>
<th></th>
<th>Tm (°C)</th>
<th>ΔHcal (kcal/mol)</th>
<th>ΔHvH (kcal/mol)</th>
<th>ΔHvH/ΔHcal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vectors produced at 37 °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>Vectors produced at 32 °C</td>
<td>71.5 ± 0.1</td>
<td>16 ± 2</td>
<td>211 ± 7</td>
<td>13 ± 2</td>
</tr>
</tbody>
</table>

**Table 2.** Vector first-order inactivation rates (k, h⁻¹) measured at different temperatures for purified retroviral vectors produced at 37 and 32 °C

Standard error and the P values are also shown.

<table>
<thead>
<tr>
<th>Incubation temperature (°C)</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</td>
<td>0.239 ± 0.005</td>
<td>0.25 ± 0.02</td>
</tr>
<tr>
<td>27</td>
<td>0.127 ± 0.005</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>17</td>
<td>0.07 ± 0.02</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>4</td>
<td>0.014 ± 0.005</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>−20</td>
<td>0.007 ± 0.001</td>
<td>0.0146 ± 0.0001</td>
</tr>
<tr>
<td>−85</td>
<td>0.0017 ± 0.0002</td>
<td>0.0027 ± 0.0004</td>
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infectivity compared with only 12% for 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 (Fig. 4), an Arrhenius-like equation, given by:

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

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. \( \Delta H^\circ \) and \( \Delta S^\circ \) are the enthalpy and entropy of activation for the reaction, respectively.

Fig. 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 Fig. 4 are statistically different. For this purpose, hypothesis testing was performed and the results clearly indicated that the slopes are different, both at 37 \( (P<0.00001) \) and 32 °C \( (P=0.00002) \). The most interesting observation is that the results presented in Fig. 1, regarding the thermal break points, are consistent with the kinetic phase-transition temperatures shown in Fig. 4, reinforcing the relevance of the vector membrane properties upon vector inactivation kinetics.

**Vector transduction efficiency**

The results presented above show that membrane properties of retroviral 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 artificial skin in culture 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 h.

In order to determine the infectivity of vector preparations before and after incubation at 4 °C, the titres of all virus preparations were equalized by appropriate dilutions to \( 10^4 \) infectious particles. The residual transduction efficiency, retained after incubation, is shown in Fig. 5 for both the keratinocytes and the HCT116 cells. The data indicate that when equalized titres 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 titres 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-organ cultures, the infection was also performed with equalized titres of infectious units of each viral preparation (Fig. 6a–d). 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 titres, 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 onto the skin tissue for 6 h 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.

**DISCUSSION**

Due to the application of retroviruses as vectors for gene therapy clinical applications, the generation of high titre stable vectors has received great attention. Recently, two relevant papers were published regarding the stability of retroviral vectors: one analysed the inactivation kinetics of the vectors and observed two different inactivation phases, depending on the storage temperature (Higashikawa & Chang, 2001); the other studied the effect of the production temperature upon cell and virus membrane and the consequences for vector stability (Beer et al., 2003).

The results presented herein provide a physico-chemical characterization of vectors produced by TE Flp A7 cells in order to understand why vectors produced at different temperatures present different transduction efficiencies in relevant gene therapy applications.

The production temperature causes significant changes in 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 (Fig. 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 (Pessin & Glaser, 1980; Aloia et al., 1988). 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 suggest 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 & Roth, 2003) and production at lower temperatures is a condition known to change the protein-folding pathway (Kjaer & 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 achieve the best conformation for membrane receptor binding. Also, different

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**Fig. 5.** Transduction efficiency of MLV vectors produced at 37 and 32°C, retained after incubation at 4°C for 24 h. Purified retroviral vectors produced at 37 (grey) and 32°C (white), with and without incubation at 4°C for 24 h, were used to infect HCT116 cells or human keratinocytes with equalized titre of infectious units (as titrated on HT1080 cells). The number of X-Gal stained cells after infection with the 37 and 32°C virus preparations, prior to incubation at 4°C, is taken as 100%.

**Fig. 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 h. (c) Infection with vectors produced at 32°C. (d) Infection with vectors produced at 32°C, after incubation at 4°C for 24 h. (e) Mock infection (negative control skin).
conformations may lead to a different sensitivity to the inactivation and thus to higher or lower vector stability.

In addition, the correlation between the membrane phase-transitions (Fig. 1) and kinetic phase-transitions (Fig. 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 TM protein with temperature. The effect of this increased mobility may have different consequences. On the one hand, higher mobility of the TM protein 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 to 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 TM protein mobility, alter the way in which 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 reported previously (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 behaviors (Fig. 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 higher rigidity). It was also observed that some changes that occur in the vector membrane may have an effect upon the entry of the vector into the cell, leading to different transduction efficiencies (Figs 5 and 6). This may be due to the change of conformation of the envelope proteins or to the change of 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 (Simons & Ikonen, 1997; Nguyen & Hildreth, 2000). 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 & 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 & 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 & 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).

Retroviral vectors have the drawback of low stability, and several studies have been performed to understand the factors that affect their stability, such as production temperature, envelope protein, pH and additives. Only few studies have been directed at understanding how these factors affect vector constitution and its impact upon stability. This work aimed to assess physico-chemical properties of the vector membrane and relate them to vector stability in order to evaluate the possibility of increasing 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 retroviral vectors, more attention has to be paid to how quantitative changes in individual lipids and cholesterol caused by cultivation conditions (e.g. medium composition) or by the host-cell type affect vector stability.

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