Effects of stoichiometry of retroviral components on virus production

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A study was conducted to investigate the effects of increasing the amount of each retroviral component on vector production. It was found that, while the components of both amphotropic and ecotropic vectors were expressed independently of each other in a transient transfection system, increasing the amount of the gag/gag-pol component resulted in a decrease in virus titres for the amphotropic particles but not ecotropic particles. Analyses of the virus stocks produced indicated that the negative effect on titres was closely linked to the availability of envelope proteins for virion incorporation. The negative effect was not observed for ecotropic particle production in 293T cells, where the ecotropic receptor was absent, but was manifested when production was conducted in 293/12 cells expressing the ecotropic receptor. This suggested that the premature interaction between envelope and receptor in producer cells could limit the amount of envelope available for virion incorporation. In designing optimal vector production systems it is essential, therefore, to balance the concentration of the vector components and to ensure that there is never an excess of Gag/Gag–Pol.

Introduction

The assembly of viruses is a complex, well-regulated process that is controlled by a variety of mechanisms including regulated gene expression, protein processing and specific morphogenic pathways (Swanstrom & Wills, 1997). In retroviruses, the stoichiometry of viral components is determined by splicing of the full-length transcript to produce a specific ratio of unspliced RNA to spliced RNA of about 2:1 (Katz et al., 1988). The former is either translated into the Gag/Gag–Pol proteins of the viral core or packaged, while the latter is translated into the envelope proteins. In addition, read-through or frameshift suppression of termination of translation of the full-length transcript ensures that the ratio of Gag to Gag–Pol proteins is maintained at about 20:1 (Hayman, 1978; Jamjoom et al., 1977; Oppermann et al., 1977). Finally, it has been suggested that fixed equilibria exist between full-length transcripts that are destined to be packaged and those that are translated (Sonstegard & Hackett, 1996). The importance of a fine balance of viral components has been illustrated by studies in which the ratios of components have been disrupted, resulting in reduced virus production (Felsenstein & Goff, 1988; Katz & Skalka, 1990). In this report, we have examined the effects of altering the stoichiometry of the components of retroviral vectors on the efficiency of gene transfer and vector particle production.

In retroviral vectors, the three components, genome, Gag/Gag–Pol and envelope, are generally dissociated in order to minimize the possibility of production of replication-competent virus. This dissociation has traditionally been achieved by integrating the separate cassettes into the genome of a cell line to construct a producer cell. This strategy inevitably disrupts the regulatory systems that have evolved, presumably, to deliver optimum ratios of components that are present in a replication-competent virus. This may lead to suboptimal levels of vector produced from producer cells, a situation that is relatively complex to correct in an integrated expression system. In recent years, however, transient methods of retrovirus vector production have been developed. In these systems, viral components are segregated into three different plasmids that are used in short-term transfections (Soneoka et al., 1995). Typically, vector particles are harvested 2 days post-transfection. This technology now makes it possible to study the effect of the amount of each component on virus
production simply by varying the amounts of the three plasmids used in the experiment.

Methods

- **Plasmid constructs**. pHIT60, pHIT111, pHIT123 and pHIT456 are plasmids that expressed murine leukaemia virus (MLV) gag/pol, lacZ-containing genome, ecotropic envelope and amphotropic envelope, respectively (Soneoka et al., 1995).

- **Cell culture**. 293T and 293 cells were maintained in DMEM containing 10% foetal calf serum and 1% penicillin, streptomycin and t-glutamine. NIH3T3 cells were maintained in DMEM containing 10% newborn calf serum and 1% penicillin, streptomycin and t-glutamine. 293/12 cells were derived from 293 cells and expressed the murine ecotropic MLV receptor (Ragheb et al., 1995). They were a kind gift from Richard Tun and Paula Cannon (University of Southern California School of Medicine, Los Angeles, USA).

- **Virus production and titration**. Viral vectors were produced by a transient transfection system that has been described previously (Soneoka et al., 1995). The virus stocks were harvested 48 h post-transfection and tittered on NIH3T3 cells in 6-well plates. The transduced cells were stained for β-galactosidase 48 h post-transduction as described previously (Soneoka et al., 1995).

- **Western blot analysis**. The anti-p15, anti-gp70 and anti-β-galactosidase antibodies were purchased from Serotec, Quality Biotech and Sigma, respectively. Five hundred µg of protein from each plasmid was added to 10 µl of reporter lysis buffer from Promega. After boiling for 5 min in SDS loading buffer and loaded onto a 12% SDS–PAGE gel. Cells were lysed in reporter lysis buffer from Promega and centrifuged at 15000 r.p.m. for another 30 min. It was then dried. The paper was washed twice in 0.1% SDS–T containing 0.05% skimmed milk and the bands were detected by using the chemiluminescence detection kit from Amersham.

- **Reverse transcriptase (RT) assay**. The assay for RT was performed according to Goff et al. (1981). Briefly, 90 µl of virus supernatant was added to 10 µl 10 × RT buffer (500 mM Tris–HCl, pH 8.3; 0.5% NP-40; 50 µg/ml oligodeoxythymidylic acid; 100 µg/ml polyadenylic acid; 200 mM DTT; 6 mM MnCl2; 600 mM NaCl) containing 10 µCi [α-32P]dTTP (Amersham) and incubated at 37 °C for 2 h. Five µl of each reaction was then spotted onto DEAE paper (DE-81, Whatman) and air-dried. The paper was washed twice in 0.6 M NaCl, 0.06 M sodium citrate for 15 min each and then in 95% ethanol for 15 min. It was then air-dried and exposed overnight to an X-ray film.

Results

Establishing the conditions under which the components were not saturating during virus production

A preliminary study was carried out to determine the amount of DNA at which the components were not saturating during vector production. The plasmids used were those described by Soneoka et al. (1995): briefly, pHIT60, pHIT456 and pHIT111 respectively encode gag/pol, amphotropic envelope and lacZ-containing genome. Keeping the ratios of the three components equal, different amounts of DNA were used to transfect 293T cells. Transfections were performed in 6 cm dishes with FuGENE6 transfection reagent (Roche). The virus stocks were titrated on NIH3T3 cells and virus titres were measured by X-Gal staining as described previously (Soneoka et al., 1995). The results are shown in Fig. 1(a). It was found that the virus titre reached a maximum of 10^4 L.f.u./ml at 0.7 µg DNA used in the transfections. Using 0.1 µg of each plasmid produced a virus titre of about 10^3 L.f.u./ml. At this amount of DNA, it seemed likely that the components were not saturating, so this amount was used as a starting point for further analysis.

Expression of viral components occurred independently of each other in transected cells

In order to ascribe any variations in titre to quantitative differences in the amounts of the vector components, it was important to establish that variations in the amounts of DNA used in the transient transfections resulted in appropriate variations in the amounts of gene products. Western blot analysis was performed with lysates from the transected cells using anti-P15 (Gag), anti-gp70 (Env) and anti-LacZ antibodies (Fig. 2). As expected, the levels of gene products were proportional to the amounts of DNA used, with no obvious interdependence of the different components.
**Stoichiometry of retroviral components**

**Fig. 2.** Analysis of viral components in lysates of 293T cells transfected with different amounts of the three HIT plasmids. Thirty μg total protein was loaded in each lane. Antibodies used were anti-p15 (Gag) (a), anti-gp70 (Env) (b) and anti-β-galactosidase (LacZ) (c). Lanes: 1, 0.1 μg of all three plasmids; 2, 1 μg pHIT60, 0.1 μg pHIT111 and pHIT456; 3, 1 μg pHIT111, 0.1 μg pHIT60 and pHIT456; 4, 1 μg pHIT456, 0.1 μg pHIT60 and pHIT111; 5, 1 μg of all three plasmids; 6, 0.1 μg pHIT60, 1 μg pHIT111 and pHIT456.

**Fig. 3.** RT assay and Western blot analysis of virus stocks produced with different amounts of the three plasmids. (a) RT assay. Spots: 1, negative control; 2, 0.1 μg of all three plasmids; 3, 1 μg pHIT60, 0.1 μg pHIT111 and pHIT456; 4, 1 μg pHIT111, 0.1 μg pHIT60 and pHIT456; 5, 1 μg pHIT456, 0.1 μg pHIT60 and pHIT111; 6, 1 μg of all three plasmids; 7, 0.1 μg pHIT60, 1 μg pHIT111 and pHIT456. (b)–(c) Western blot analysis using anti-p15 antibody (b) and anti-gp70 antibody (c). Lanes: 1, negative control; 2, 0.1 μg of all three plasmids; 3, 1 μg pHIT60, 0.1 μg pHIT111 and pHIT456; 4, 1 μg pHIT111, 0.1 μg pHIT60 and pHIT456; 5, 1 μg pHIT456, 0.1 μg pHIT60 and pHIT111; 6, 1 μg of all three plasmids; 7, 0.1 μg pHIT60, 1 μg pHIT111 and pHIT456.

**Titre variations with different ratios of components**

Table 1 shows the different combinations that were used and the virus titres obtained. Fig. 3 shows the corresponding

Table 1. Effect of each component on virus titre

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Amount of plasmid used in transfection (μg)</th>
<th>Titre (l.f.u./ml)</th>
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<td></td>
<td>pHIT60</td>
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* pHIT456 was used to provide the amphotropic envelope while pHIT123 was used to provide the ecotropic envelope.
when 0·1 µg of each was used. This is as expected and shows the maxima and minima that might be expected in this study. The first noteworthy conclusion from the other data is that, at the 0·1 µg level, the Gag components saturated the system, as an increase in pHIT60 to 1 µg yielded no increase in transducing titre (Table 1, expt 2). The data in Fig. 3 (lanes 2 and 3) suggest that expression levels of Gag and Env proteins and the RT levels were as would be expected from the DNA input. There was no aberrant expression of any of the components. This suggests that there was sufficient Gag in the system to package all of the RNA genomes produced from 0·1 µg pHIT111 and to pick up all of the envelope produced from 0·1 µg pHIT456. In contrast, increases in levels of the genome (pHIT111) or the amphotropic envelope (pHIT456) did yield significant increases in titre (Table 1, expts 3 and 4), suggesting that they were both limiting. Increasing the genome had about a 2-fold greater effect than the amphotropic envelope, which supports the notion that the most limiting component in this particular production system is the genome. Again, expression of the protein components was as expected from the DNA input (Fig. 3, lanes 4 and 5). A prediction from these observations would be that, if the levels of both the genome and amphotropic envelope components were raised while maintaining Gag at the low 0·1 µg level, a more substantial increase in titre would be seen compared with that observed when either genome or amphotropic envelope was increased. This is exactly what was seen (Table 1, expt 6). In fact, titres were only 2-fold lower than when all three plasmids were used at 1·0 µg. The first simple message from this study is therefore that it is pointless to use relatively large amounts of the Gag/Gag–Pol expression plasmid in a transient production system. However, the observation shown in Table 1 (expt 2) leads one to go beyond that rather bland statement to say that, in fact, relatively high levels of the gag/gag–pol plasmid are inhibitory. Transducing titres dropped 4-fold when the amount of gag/gag–pol plasmid was raised to 1·0 µg while keeping the genome and envelope plasmids at 0·1 µg. Presumably, this is because an excess of Gag/Gag–Pol components would lead to the production of relatively high levels of defective particles in the cells. These particles could interfere with transduction, either by competing for the other components, genome and envelope, in the producer cell or by competing for access to the target cells.

The envelope and genome components were found to be limiting, since the virus titre could be increased by increasing the amounts of these components. In order to determine the amount of plasmid at which each limiting component reached saturation in the system, two plasmids (pHIT60 and either pHIT111 or pHIT456) were kept at 0·1 µg while the amount of the third was increased in step-wise increments of 0·2 µg. The corresponding virus titre of each transfection was measured and a graph of virus titre versus amount of plasmid was plotted (Fig. 1b). It was found that the virus titre reached a maximum at 0·8 µg pHIT456 and 1 µg pHIT111. Hence, the optimal ratio of the amounts of the three plasmids that was required for the most efficient vector production was 1:8:10 (pHIT60:pHIT456:pHIT111).

The negative effect of gag/gag–pol on virus titre was not due to infection interference by defective particles

In order to test the hypothesis that the decrease in virus titre observed during increased Gag/Gag–Pol expression was due to empty enveloped particles, which might have saturated the target-cell receptors, or to ‘bald’ particles, which bound non-specifically to the target cells and obstructed binding of transducing particles, empty particles containing amphotropic and ecotropic envelopes were produced by transfecting 293T cells with 1 µg pHIT60 and 0·1 µg pHIT456 (amphotropic envelope) or pHIT123 (ecotropic envelope). ‘Bald’ particles were also produced by transfecting 293T cells with 1 µg pHIT60. Five hundred µl of the defective virus stock was mixed with 500 µl of the virus stock produced by using 0·1 µg of all three plasmids (Table 2). The resulting virus mixture was titrated on NIH3T3 cells. The results showed that there was no decrease in titre when either bald, amphotropic or ecotropic empty particles were present in the virus stocks. This suggested that the decrease in titre was not due to obstruction of receptors by defective particles. Given this observation, it seemed likely that the reduced titre was due to events that occurred during virus production in the transfected cells and which affected the quality of the virus particles.

A reasonable hypothesis would be that the overall transducing titre is a function of the probability that sufficient envelope and genome molecules become associated with the same particles. If either genome is absent or envelope is insufficient in a given particle, that particle will not be a transducing particle. Instead, it takes either the genome or envelope out of the pool from which transducing particles are formed, acting essentially as a competitive inhibitor. The higher the Gag/Gag–Pol concentration, for any given genome and envelope concentrations, the greater is the probability that genome and sufficient envelope molecules will not be present on the same particle and so the lower will be the transducing titre. This hypothesis predicts that anything that increases the chances of genome and sufficient envelope being co- incor- porated into a particle will increase titre. In particular, it may be possible to ameliorate the inhibitory effect of Gag/Gag–Pol by driving incorporation of either genome or envelope at high efficiencies. This can be tested.

The negative effect of the gag/gag–pol component on virus titre was only manifested at low levels of genome and envelope

The data in Table 1 (expts 3, 4, 7 and 8) show that increasing the level of the gag/gag–pol plasmid from 0·1 µg to 1·0 µg did not lead to a reduction in titre when either the amphotropic envelope or genome component was present at
Table 2. Effect of defective particles on virus titre
Culture supernatants were obtained from 293T cells transfected with 0·1 µg pHIT60, pHIT111 and pHIT456 (amphotropic transducing particles), 1 µg pHIT60 and 0·1 µg pHIT456 (empty amphotropic particles), 1 µg pHIT60 and 0·1 µg pHIT123 (empty ecotropic particles) or 1 µg pHIT60 (empty bald particles) or from untransfected 293T cells (mock).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Amphotropic transducing particles</th>
<th>Empty amphotropic particles</th>
<th>Empty ecotropic particles</th>
<th>Empty bald particles</th>
<th>Mock</th>
<th>Virus titre (l.f.u./ml)</th>
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<td>3.05 ± 1.5 × 10^3</td>
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The negative effect of $gag$/$gag$–$pol$ on virus titre was dependent on the type of envelope used

The amphotropic envelope was found to be limiting during virus production. One explanation for this observation was that a significant proportion of envelope protein was taken out of the pool that was available for incorporation due to premature interaction with their receptors in the producer cells. This could be tested by using an envelope protein the receptor of which was not expressed in the producer cells. If the hypothesis were true, this envelope would not be limiting during virus production.

The ecotropic envelope only mediates productive infection of murine cells. Its receptor has been identified and has been shown to be absent in human cells (Albritton et al., 1989). The absence of receptor for the ecotropic envelope in 293T cells was confirmed by transducing the cells with either amphotropic or ecotropic particles containing the pHIT111 genome. While the amphotropic particles yielded titres of $4.3 ± 0.4 × 10^6$, the cells were not transduced by the ecotropic particles, indicating the absence of the ecotropic receptor.

The transduction experiments described above were repeated with pHIT123 (ecotropic envelope expression plasmid) in place of pHIT456. The results are presented in Table 1. As with the amphotropic envelope, increasing the amount of genome plasmid from 0·1 to 1·0 µg resulted in a 24-fold increase in titre, indicating that the genome component was limiting. In addition, increasing the amount of genome plasmid brought the titre close to that achieved with 1 µg of all three plasmids, suggesting that genome was the only limiting component. This was confirmed by the fact that increasing the amount of envelope plasmid from 0·1 to 1·0 µg did not bring about a significant increase in titre. Hence, unlike the amphotropic envelope, the ecotropic envelope was not limiting during virus production, supporting the notion that there was less amphotropic envelope available for incorporation. These data are compatible with the idea that the presence of cognate receptor limits envelope availability.

Based on the observations made in the previous section, these results also predict that an increase in the $gag$/$gag$–$pol$ component would not result in a decrease in titre. This was indeed the case, as increasing the amount of pHIT60 from 0·1 to 1·0 µg did not reduce the titre at all. It was very likely that the absence of a reduction in titre was due to the ecotropic envelope being saturating. Hence, the probability of finding sufficient envelope on the particles was still high, even when particle formation was increased with increased Gag/Gag–Pol expression.

Changes in envelope incorporation upon increasing the amount of envelope component

In order to attribute the lack of ability to increase the titre to the physical saturation of envelope proteins on the particles, the amounts of virion-associated amphotropic and ecotropic envelopes were investigated. Virus stocks were produced by
transfecting 293T cells with 0.1 or 1.0 µg pHIT123 or pHIT456. Aliquots of 0.1 µg pHIT60 and pHIT111, respectively, were used to supply the gag/gag–pol and genome components for all the samples. From the results described in the previous section, the Gag/Gag–Pol component was expressed independently from the other components and there would be similar numbers of particles in all the samples.

Western blot analyses were performed on the lysates and culture supernatants of the transfected cells. As expected, a 3-fold increase in envelope expression was detected in the cell lysate for both ecotropic and amphotropic envelopes when more of the corresponding plasmid was used for transfection (Fig. 4, lanes 2 and 4). There was also a 2-fold increase in envelope detected in the supernatant when pHIT456 was increased in the transfection (Fig. 4, lane 7), indicating that the amphotropic envelope was limiting at 0.1 µg pHIT456 and that the envelope found on the particles increased when the amount of pHIT456 was increased to 1.0 µg. This could account for the increase in titre brought about by increasing the amount of pHIT456 during virus production. In contrast, there did not seem to be a significant increase in the amount of ecotropic envelope detected in the virus supernatant when the amount of pHIT123 was increased from 0.1 to 1.0 µg, indicating that there was no significant change in the amount of virion-associated envelope (Fig. 4, lane 9). This suggested that, at 0.1 µg pHIT123, the envelope was saturating on the particles. No more envelope proteins could be accommodated on the particles, despite increasing expression in the cells with 1 µg pHIT123. This would explain why there was no increase in titre upon increasing the amount of pHIT123 from 0.1 to 1.0 µg.

The fact that the ecotropic but not amphotropic envelope was saturating at 0.1 µg suggested that there was more ecotropic envelope available for virion incorporation. This was in agreement with the notion that a proportion of the amphotropic envelope was sequestered through premature interaction with its receptors in the 293T cells. The hypothesis could be tested further by expressing the ecotropic receptor in cells that were used for the production of ecotropic particles.

### Production of ecotropic particles in 293 cells expressing the ecotropic receptor

In order to investigate the effect of premature envelope–receptor interaction on virus production, a study on the production of ecotropic particles was performed in 293/12 cells. These cells expressed the ecotropic receptor and could be transfected with high efficiencies (Ragheb et al., 1995). As a control, the same set of experiments was also conducted in 293 cells, which did not express the ecotropic receptor. The results are presented in Table 3. In general, the titres were lower compared with the virus stocks that were produced in 293T cells. This was expected, since there was a lack of plasmid amplification due to the absence of the large T antigen. The profile of the titres produced in the control 293 cells mirrored that of 293T cells: there was an increase in titre only when the genome component was increased, and no decrease in titre was observed upon increasing the amount of gag/gag–pol component, indicating that only the genome, and not the envelope component, was limiting. In the 293/12 cells, however, the profile was more similar to the production of amphotropic particles in 293T cells: apart from increasing the amount of

### Table 3. Effect of receptor expression on virus production

Different amounts of plasmids were used to transfect cells in 6 cm dishes with FuGENE6 transfection reagent (Roche). Virus titres were measured as l.f.u./ml as observed by X-Gal staining.

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<thead>
<tr>
<th>Experiment</th>
<th>Amount of plasmid used in transfection (µg)</th>
<th>Virus titre (l.f.u./ml)</th>
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envelope detected in the supernatant (Fig. 5) by its receptor in the 293 cells. This result was in complete agreement with the hypothesis that the ecotropic envelope was being sequestered, indicating that the ecotropic envelope was no longer saturating. This result was in complete agreement with the notion that this interaction might limit the pool of envelope available for incorporation. The reduction of available envelope resulted in a decrease in the number of virus particles that contained sufficient envelope protein for incorporation into virions in 293/12 cells. An obvious conclusion from these observations was that the presence of the ecotropic receptor resulted in the envelope component becoming limiting during virus production. This was probably due to the sequestration of envelope proteins by the receptors, thereby reducing the pool of envelope available for incorporation. The reduction of available envelope resulted in a decrease in the number of virus particles that contained sufficient envelopes, leading to a reduction in the number of transducing particles, which was subsequently manifested as a decrease in virus titre.

The notion that the ecotropic envelope was limiting in 293/12 cells would be confirmed if an increase in envelope expression led to a corresponding increase in the envelope associated with the particles. Western blot analyses performed on ecotropic virus stocks produced in 293/12 cells revealed that this was indeed the case (Fig. 5). An increase in the amount of pHIT123 from 0·1 to 1·0 µg resulted in a 3-fold increase in envelope detected in the supernatant (Fig. 5b, lanes 1 and 4), indicating that the ecotropic envelope was no longer saturating. This result was in complete agreement with the hypothesis that the ecotropic envelope was being sequestered by its receptor in the 293/12 cells.

Discussion

We have shown in this study that overexpression of the Gag/Gag–Pol component had a negative effect on virus titre when the amphotropic envelope was used in the production of MLV-based vectors. This effect seemed to be intimately linked to the envelope being limiting during virus production. The fact that envelope was limiting implied that there was not sufficient envelope protein for incorporation into virions in order to produce particles of good transduction efficiency. Hence, it was conceivable that an increase of particle formation, brought about by an increase in Gag/Gag–Pol expression, would reduce even further the probability of finding sufficient envelope on the particles, due to the distribution of the envelope over a larger number of particles. This would result in a decrease in the number of transducing particles, leading to a reduction in virus titre.

The limitation by envelope was shown to be due to the presence of its receptor in the producer cell, presumably through premature envelope–receptor interaction. Premature interaction between virus envelope and the cognate receptor in the infected cell has been reported previously (Delwart & Panganiban, 1989; Matano et al., 1993). Our results on the production of ecotropic particles in 293/12 cells were consistent with the notion that this interaction might limit the pool of envelope available for incorporation. In addition, the incorporation of heterologous proteins into virus particles was shown to be reduced when their cognate ligands were co-expressed in the producer cells (Henriksson & Bosch, 1998; Henriksson et al., 1999). It was shown subsequently that interaction between those proteins and their receptors precluded their incorporation into virions. In human immunodeficiency virus, the envelope–receptor interaction in infected cells was shown to be prevented by the action of the vpu gene product, which down-regulated the CD4 receptor (Willey et al., 1992).

The strategies used by other retroviruses have not been reported (Swanstrom & Wills, 1997). In simple retroviruses such as MLV, which lack any accessory genes, it is conceivable that this could be achieved by expressing more envelope than is required for incorporation during virus production. The importance of production of sufficient envelope has been noted before, when it was observed that there was a threshold number of provirus copies required for efficient virus production (Odawara et al., 1998). Interestingly, there seemed to be a correlation between the numbers of provirus copies required for virus production and to achieve interference. In the light of our results on the envelope–receptor interaction, this could reflect the need for production of sufficient envelope to down-regulate the receptors. Efficient virus production was achieved when there were enough provirus copies to down-regulate receptor expression. This also led to virus interference through the reduction of available receptors for the entry of viruses with similar receptor usage. The importance for all viral genes to be present in the right proportions was also noted by Odawara et al. (1998). This is the first report of a systematic study describing the effects of each component on virus production. Our results suggest that it is essential to ensure that there is never an excess of gag/gag–pol when designing an...
optimal vector production system. Although the study was performed by using a transient transfection system, it would be reasonable to extrapolate the result to include vector production from stable packaging cell lines. In the latter system, the ratio of the number of copies of the gag/gag-pol gene to the env gene has never been taken into account during the selection of clones when constructing packaging cell lines. The results of this study indicate that this might be important for obtaining clones that produce virus stocks with higher titres.

Finally, the results of this study have revealed that a large number of empty particles lacking genome were present when all three plasmids were used in equal amounts. These particles could be filled by increasing the amount of the genome component, resulting in a virus stock with a high infectivity to particle ratio. One possible explanation is that there is a lack of proximity between the newly synthesized Gag protein and the genome transcript that is to be packaged. This proximity could be achieved in the wild-type virus because the gag gene products are translated from the full-length transcript, which can also be packaged. Alternatively, the wild-type packaging signal could be suboptimal. A packaging signal with the maximum affinity for the Gag proteins might not have evolved, as the strength of interaction would exclude the binding of ribosomes and hence down-regulate Gag expression. A study of these possibilities would shed light on the relationship between translation and packaging and might provide useful information that could be used to produce better-quality virus stocks with higher infectivity to particle ratios.

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