Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line

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ABSTRACT

We report the development of an advanced system for transfer and expression of exogenous genes in mammalian cells based on Moloney murine leukemia virus (Mo MuLV). Extensive deletion/mutagenesis analysis to identify cis-acting signals involved in virus transmission has led to the design of a family of novel, highly efficient retroviral vectors and a partner helper-free packaging cell line. The pBabe retroviral vector constructs transmit inserted genes at high titres and express them from the Mo MuLV Long Terminal Repeat (LTR). Each of these vectors has been constructed with one of four different dominantly acting selectable markers, allowing the growth of infected mammalian cells in the presence of G418, hygromycin B, bleomycin/phleomycin or puromycin, respectively. The high titre ecotropic helper free packaging cell line, ΩE, was designed in conjunction with the pBabe vectors to reduce the risk of generation of wild type Mo MuLV via homologous recombination events. The ΩE cell line was generated with separate *gagpol* and ecotropic *env* expression constructs with minimal sequence overlap and decreased sequence homology achieved by 'codon wobbling'. Homologous *env* coding sequences were deleted from the pBabe vectors without diminishing recombinant vector titre. Together, the pBabe vectors and ΩE cell line should prove useful in experiments where highest frequencies of gene transfer, or concomitant expression of several different genes within a single cell are required with minimal risk of helper virus contamination.

INTRODUCTION

Helper free retroviral vector systems have been increasingly utilised in gene transfer experiments because they are designed for can cotransfer and expression of a gene plus a selectable marker (1, 2). Mo MuLV based retroviral vectors have been successfully applied to a number of studies including mutagenesis (3), promoter traps (4, 5), cell lineage analysis and oncogenesis within developing organs (6, 7) and are currently being used in the optimization of gene therapy regimes (8).

Existing retroviral systems however, still suffer from a number of limitations. First, some of the most widely employed retroviral vectors and packaging cell lines fail to produce recombinant titres as high as those of wild type Mo MuLV which are necessary for gene transfer into rare cell populations such as hematopoetic stem cells (9). Second, only a few retroviral vectors reproducibly express an inserted cloned gene after selection of the co-introduced selectable marker, and for the most part the selectable marker is limited to the *neo* gene from the Tn5 transposon which confers resistance to the aminoglycoside G418 in mammalian cells (10). Last, some vectors and packaging cell lines are more likely to interact in the generation of contaminating wild type Mo MuLV than others (11). Besides posing the hazard of viraemia in the host during gene therapy or whole animal experiments, such wild type virus leads to the mobilisation and spread of defective vector proviruses which can blur the precise interpretation of experiments usually afforded by the clonal nature of vector proviral integrations.

The goal of the work presented in this report was to produce a generally applicable Mo MuLV based retroviral vector system that would transmit and express inserted genes as efficiently as endogenous genes of Mo MuLV while minimising the risk of helper virus contamination.

MATERIALS AND METHODS

Construction of Retroviral Vectors

Subcloning was carried out via standard recombinant DNA techniques (12). Oligonucleotides were synthesised on an Applied Biosystems 380B and purified by denaturing acrylamide electrophoresis. Site directed mutagenesis was performed as described (13). Exact restriction maps and details of each of the constructs are available upon request.

Transfection, Retroviral Infection and Titration

Constructs were transferred into cells via calcium phospate mediated transfection employing a modified protocol (14) in

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which packaging cells were split onto 10 cm dishes at a density of 7.5×10^5 18 hour prior to transfection. 10 μg of construct DNA as a precipitate was applied to cells for 6 hours followed by 15% glycerol shock for 3 minutes with replacement of 1/2 normal volume of medium. Transiently produced virus was harvested 48 hours post glycerol shock.

Stable ecotropic packaging cell lines were generated by transfecting ecotropic packaging cell lines such as Ψ-2 (15) and ΩE with vector DNA, or infecting them with virus released from the transiently transfected amphotropic packaging cell line PA317 (16). Virus was obtained from stable producer cell lines by allowing the cells to reach confluence, removing their spent medium, replacing it with 1/2 volume of fresh medium and harvesting the conditioned medium 48 to 72 hours later.

Retroviral infections/titrations were performed by filtering producer cell culture supernatants through a 0.2 or 0.45 μm filter and adding polybrene to a final concentration of 8 μg/ml. Supernatants were added either neat or diluted into medium supplemented with 8 μg/ml polybrene before application in 2 ml volumes to a 10 cm dish that had been seeded with 7.5×10^5 cells 18−24 hour prior to infection. Infections proceeded for 2 hours, after which time the viral supernatant was removed and replaced with fresh medium. 24 hours later cells were split 1:20 into selective medium in 10 cm dishes, with medium changed every third day until drug resistant colonies were visible without the aid of microscopy. Drug selection levels used for vector infection of NIH 3T3, PA317, Ψ-2 and ΩE cells were as follows: G418: 1 mg/ml, hygromycin B: 50 or 150 (Ψ-2) μg/ml, phleomycin: 10−25 μg/ml, puromycin: 1.0−2.5 μg/ml.

Stable Expression Analysis: Northern Analysis, CAT Activity and v-Ha-*ras* Production

Total cellular RNA was prepared and subjected to Northern analysis according to standard protocols (12, 17). For quantitation of CAT activity, cells were grown just to the point of confluence on a 10 cm dish, then lysed in 300 μl of NP-40 buffer (0.65% v/v NP-40, 10 mM Tris-Cl pH 8.0, 1mM EDTA, 150 mM NaCl). Extracts were harvested and incubated at 68°C for 10 minutes before being assayed as described (18) and the results normalised for extract protein content (Protein Assay, BioRad).

Cells infected with a vector containing a v-Ha-*ras* gene were grown to 3/4 confluence, washed, starved in serum and methionine free medium for 30 minutes then metabolically labeled overnight with ^{35}S methionine in methionine free medium supplemented with 1% v/v FCS. Cells were lysed in phospholysis buffer (20 mM $NaPO_4$ buffer pH 7.5, 100 mM NaCl, 1% v/v Triton X-100, 0.5% w/v Na deoxycholate, and 0.1% w/v SDS) on ice for five minutes with periodic agitation. Cell lysates were sequentially incubated with rat monoclonal α v-Ha-*ras* antibody (Oncogene Science Inc.), rabbit α rat IgG (Cappel Labs), and protein A-Sepharose (Sigma). Bound material was denatured and electrophoresed through a 12.5% SDS polyacrylamide gel. The gel was fixed in10% v/v glacial acetic acid/40% v/v MeOH, incubated with 1M salycilic acid, washed in dH_2O, dried, and autoradiographed at −70°C with intensifying screen.

RESULTS

Effect of Splice Donor Mutations on Retroviral Vector Titre

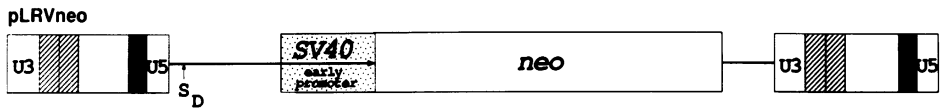
In the context of attempting to generate a retroviral vector that would most reliably express an inserted gene and a selectable marker it was decided to employ a DO (*D*irect *O*rientation) retroviral vector architecture (19). Here the promoter residing in the 5′ LTR is used to transcribe the inserted gene and an internal promoter is used for expression of a drug resistance marker (although this gene arrangement can be reversed), with both transcripts sharing a common termination/polyadenylation signal in the 3′ LTR. In such vectors it is advantageous to eliminate the unused splice donor in order to avoid artifacts, such as the activation of cryptic splice acceptors within inserted cDNAs (T. Claudio, personal communication).

Our initial attempts at assembling an efficient splice deficient DO vector (pLRV) led to the observation that sequence alterations in the splice donor region had adverse effects on the virus titre (Figure 1). pLRV was based on the unpublished pLJ construct whose design is essentially identical to the splice competent vector pDO-L (19) with the exception of an altered splice donor region (Figure 1) designed to inactivate the Mo MuLV splice donor. Unexpectedly, the recombinant titres of polyclonal Ψ-2 producer lines of both pLJ and pLRV were ten-fold lower (10^4 cfu/ml) than those of previous vectors such as pZipNeo SV(X) and pDO-L (10^5 cfu/ml). Since the only known variable introduced into the pLJ vector relative to pDO-L was the altered splice donor region, the possibility that this region might be responsible for the low titres associated with pLJ and its derivative pLRV was explored. The wild type Mo MuLV splice donor region was substituted for the mutant splice donor region of pLRV to generate pLRV S+, and the titres associated with polyclonal stable Ψ-2 producers of these vectors were quantitated. As the averaged results of two independent experiments in Figure 1 demonstrate, substitution of the wild type Mo MuLV splice donor region led to a six-fold increase in the titre of pLRV S+ over pLRV.

In order to generate a high titre DO type vector it was necessary to inactivate the Mo MuLV splice donor without adversely affecting vector titre. To this end, two point mutants of the Mo MuLV splice donor were made by site directed mutagenesis in M13 phage. The mutation designated (AG*G*T to AG*A*T) was designed to eliminate splicing by removal of the G residue which normally participates in lariat formation during splicing (20), while the 194 mutation (AGG*T* to AGG*C*) was chosen since it is present in the non-splicing acutely transforming defective retrovirus Harvey MuSV (21). Both mutations were introduced into the pLRV vector yielding pLRV 193 and pLRV 194. Titres of supernatants of polyclonal stable Ψ-2 producer cell lines of the vectors exceeded those of the producers of pLRV but not those of pLRV S+. Since the titres associated with pLRV 194 were the highest of the two vectors with point mutant splice donors, the 194 splice donor mutant was chosen for use in future DO retroviral constructs.

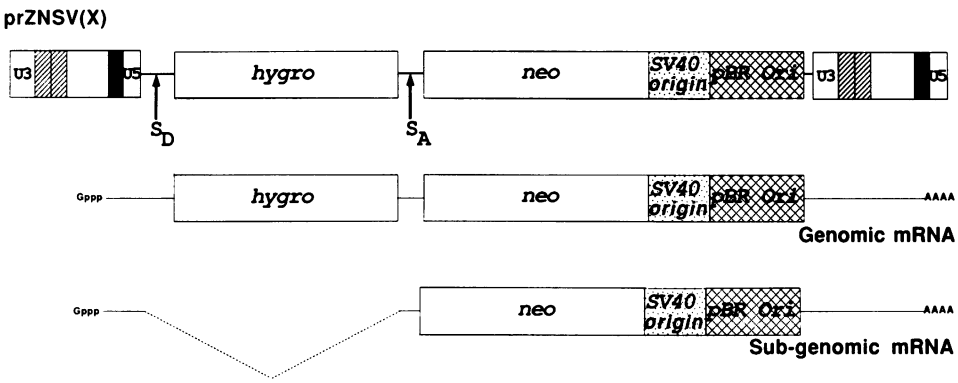
Functional Analysis of the 194 Splice Donor Point Mutation

To determine the degree to which the high titre 194 point mutation was effective in inhibiting splicing, it was introduced into the leader region of the splicing vector prZNSV(X) (a version of pZipNeoSV(X) with a simplified bacterial vector backbone with a limited number of restriction sites) to give prZNSV(X) 194. The *hph* gene which confers resistance to hygromycin B (22) was inserted into both prZNSV(X) and prZNSV(X) 194, and stable Ψ-2 producer cell lines established by selection in hygromycin B. Supernatants from both producer populations were used in titrations on NIH 3T3 cells using either G418 or hygromycin B selection. As shown in Figure 2, the $G418^r$ titre associated with the spliced mRNA was diminished by three orders of magnitude in prZNSV(X) 194 compared to prZNSV(X), while



	splice donor sequence	single provirus polyclonal G418 titre cfu/ml
wild type	agGTaagctggc	3.4 X 10^5
194	agGCaagctggc	2.3 X 10^5
193	agATaagctggc	1.4 X 10^5
pLJ	agATaag••••c	5.8 X 10^4

Figure 1. Effects of splice donor sequences on retroviral vector titre. The wild type Mo MuLV splice donor and two variants (created by site directed mutagenesis) were cloned into the vector pLRV. Each construct was transfected into PA317 cells and the transiently produced virus used to infect Ψ-2 cells, which were G418 selected to establish stable ecotropic producer lines. The supernatants of the four stable producer populations were used to infect NIH 3T3 cells. Subsequently the G418^r titres were quantitated. The results shown represent the average of two independent experiments.



Splice Donor	Hygro Titre	G418 Titre
Wild type	3.6 X 10^5	1.5 X 10^5
194	1.9 X 10^5	1.4 X 10^2

Figure 2. Effect of splice donor point mutations on expression of a spliced gene. The *hph* gene (*hygro*) from pHygro Bgl II was inserted into the splicing vector prZNSV(X). Expression of *hygro* in prZNSV(X) hygro would be directed by non spliced mRNA while neo expression arrise from translation of spliced mRNA. To create prZNSV(X) 194 hygro the 194 splice donor mutation was cloned in place of the excised wild type splice donor of Mo MuLV (as a Kpn I to BamH I fragment) in prZNSV(X) hygro. Both constructs were transfected into PA317 cells and the transiently produced virus used to infect Ψ-2 cells, which were hygromycin B selected to establish stable ecotropic producer lines. The supernatants of the stable producer populations were used to infect NIH 3T3 cells and both hygro^r and G418^r titres were quantitated. The results shown represent the average of two independent experiments.

hygro^r titre derived from non-spliced mRNA was equivalent for both vectors.

Effect of *gag* Sequences on Vector Titre

With the use of the 194 splice donor mutant DO constructs with titres nearly as high as those of vectors with wild type splice donors such as pZipNeoSV(X) could be obtained. However, wild type Mo MuLV produces titres in excess of 10^6 pfu/ml in contrast to the aforementioned recombinant vector titres of 10^5 cfu/ml. On the other hand, the N2 vector, which was unique in its maintenance of *gag* coding sequences, possessed the capactiy to transmit a *neo*^{r} gene at titres tenfold higher than most other existing retroviral vectors (9).

As a direct test of their effect on vector titre, *gag* sequences were inserted into a conventional *gag*$^-$ DO vector, pneoSRV. Fusion of the *gag* sequences in frame with the *neo* gene produced the *gag*$^+$ construct pgagneoSRV. Two indepentent experiments measuring the respective virus titres revealed that indeed *gag* sequences can potentiate recombinant vector titre by an order of magnitude (see Figure 3A for averaged results).

To further explore the mechanism by which *gag* sequences exert their effect on vector titre, four additional vectors in which the *gagneo* fusion was separated into its constituent parts or expressed from the internal SV40 promoter were assembled, and two independent titrations performed. The results in Figure 3A can be summarised as follows: To attain increased recombinant vector titre, *gag* sequences must be inserted at their native position within wild type virus, adjacent to the Ψ-site, but not necessarily as a *gagneo* fusion. The high titres associated with *gag*$^+$ vectors were not due to helper virus since XC assays (23) of the Ψ-2 producers of the six vectors proved negative. Northern blot analysis of the vector derived mRNAs present in polyclonal Ψ-2

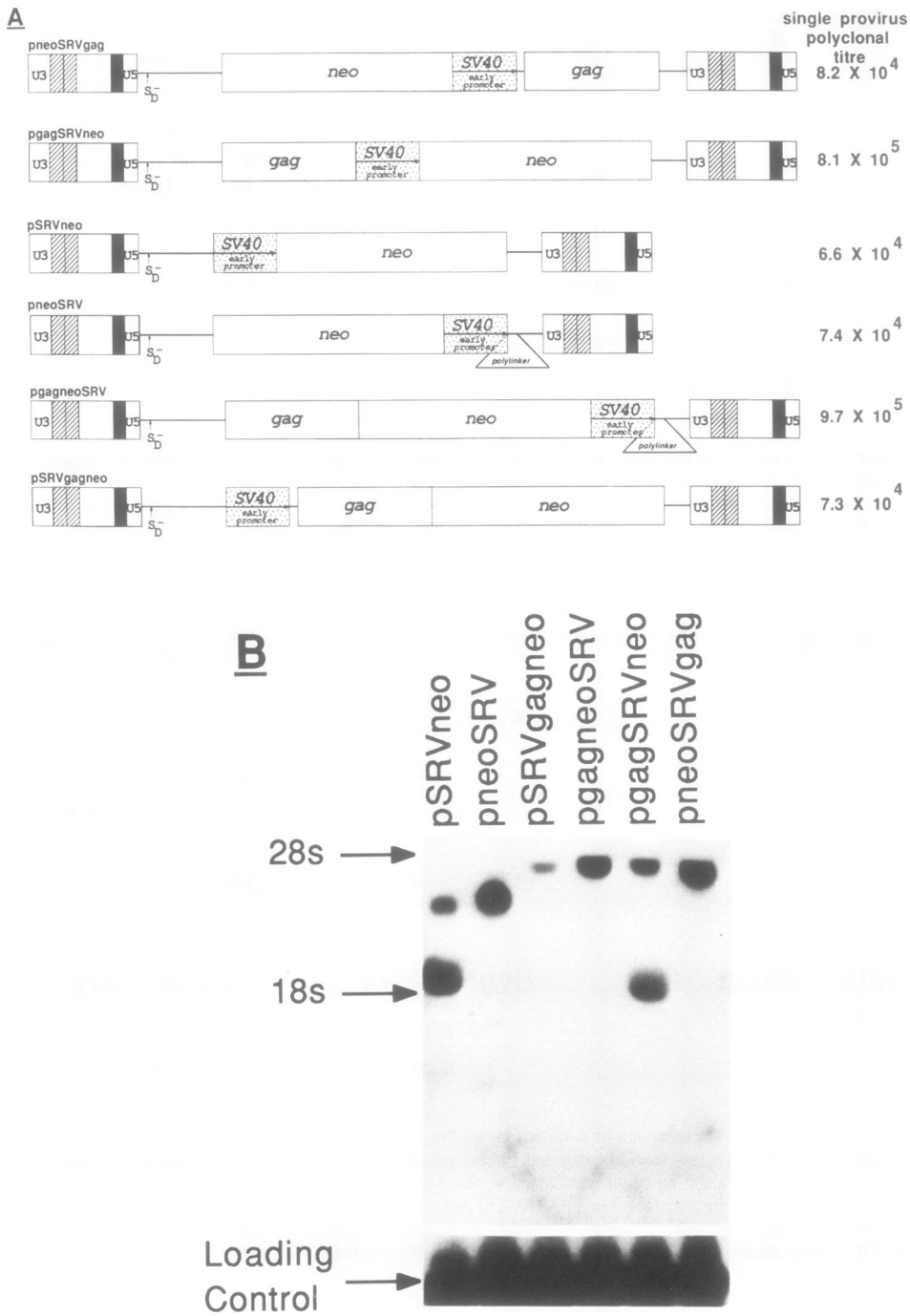


Figure 3. Effects of *gag* sequences on vector titre. A) Titration of pSRV derived constructs. Six constructs derived from the vector pSRV with *gag* sequences from a proviral clone of Ableson MuLV (44) in varying positions were assembled. Each construct was transfected into PA317 cells and the transiently produced virus used to infect Ψ-2 cells, which were G418 selected to establish stable ecotropic producer lines. The supernatants of the six stable producer populations were used to infect NIH 3T3 cells and the G418^r titres were quantitated. The results shown represent the average of two independent experiments. B) Northern Analysis of mRNA in stable Ψ-2 producer populations. Total RNA was prepared from each of the six Ψ-2 producer cell populations, and 10 μg of each sample electrophoresed through a formaldehyde/1% agarose gel. The gel was blotted onto a GeneScreen Plus membrane membrane and hybridised to ^{32}P-labelled probe made from a 1 kb Bgl II fragment containing the *neo* gene of Tn5. The blot was stripped by boiling and re-hybridised to a ^{32}P-labelled γ-actin probe to normalise for amounts of RNA loaded/transferred onto the blot.

producer cell lines used in the above titrations shown in Figure 3B demonstrates that *gag* sequences did not augment vector titre by increasing steady state levels of genomic vector RNA. Specifically, more genomic RNA was found in the pneoSRVgag producer population than the producer population harbouring the reciprocal construct pgagSRVneo, yet the titres associated with the latter producer cell line were ten-fold higher.

Effect of *env* Sequences on Vector Titre

Since *gag* coding sequences at the 5′ end of retroviral vectors had been shown to enhance vector titre, the contribution of

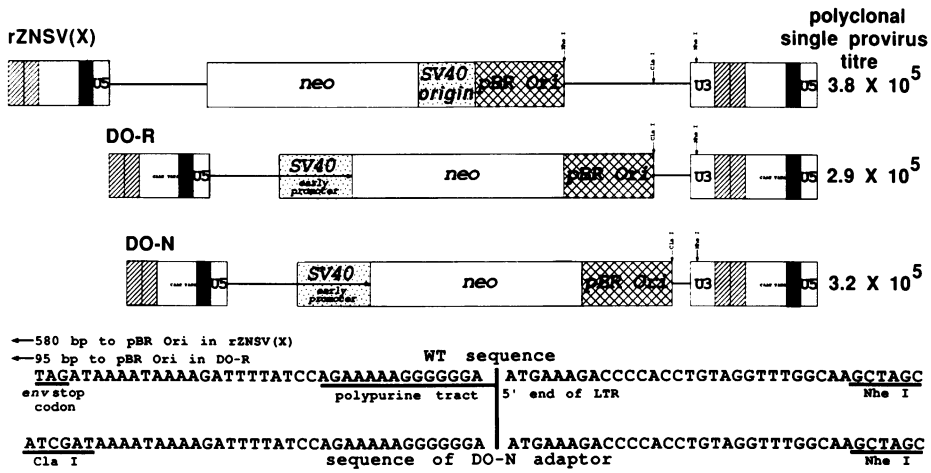


Figure 4. Effects of *env* sequences on vector titre. An adaptor composed of synthetic oligonucleotides was inserted in place of the excised Cla I to Nhe I fragment of pDO-R to delete its 95 bp of *env* coding sequences. The resultant construct, pDO-N, and two control vectors bearing different amounts of *env* coding sequences, prZNSV(X) and pDO-R, were transfected into PA317 cells and the transiently produced virus used to infect Ψ-2 cells, which were G418 selected to establish stable ecotropic producer lines. The supernatants of the stable producer populations were used to infect NIH 3T3 cells and the G418^r titres were quantitated. The results shown represent the average of two independent experiments.

sequences at the 3′ end towards vector titre was explored. It was of considerable interest to determine whether *env* coding sequences retained in all retroviral vectors were dispensable in a high titre retroviral vector. This point had become relevant in light of findings that vectors that retained the most wild type sequences, such as N2, were more likely to generate wild type virus as they were passaged in packaging cells, presumably via homologous recombination events (11). As a consequence, all *env* coding sequences 5′ of the *env* stop codon were deleted from the vector pDO-R yielding pDO-N. Supernatants of stable Ψ-2 producer populations of pDO-N, pDO-R and pZipNeo SV(X) (which contain 0 bp, 95 bp, and 580 bp of *env* coding sequences respectively) were titrated on NIH 3T3 cells. No decrease in titre was observed for the pDO-N vector relative to the *env*+ vectors as shown in Figure 4, indicating that *env* coding sequences are not required in *cis* during the Mo MuLV life cycle.

pBabe Vectors

Initial attempts to utilise pgagneoSRV (Figure 3A) as an expression vector invariably led to limited or non detectable expression of several genes inserted 3′ of the vector's internal SV40 early promoter. Substitution of the rat β actin or simian cytomegalovirus immeadiate early (CMV IE94) promoter for the SV40 early promoter in pgagneoSRV did not improve the vector's ability to express exogenous genes (data not shown). Because of these failures, an alternative approach that would utilise the strong promoter in the LTR for expression of an inserted gene while still maintaining *gag* sequences was sought.

In order to augment vector titre *gag* sequences must be located at the 5′ end of the vector next to the Ψ-site (see Figure 3A). However, at this position the initiator ATG of *gag* is the first ATG within a favorable context for translation initiation, precluding translation of genes inserted 3′ of these *gag* sequences (24). Therefore, a mutagenised *gag* cassette, lacking the normal initiator codon, from the vector pLNL 6 (25) was incorporated into two new DO vectors with expanded polylinkers but without functional splice donors and *env* coding sequences. The resultant high titre 'pBabe' vectors were designed to express inserted genes from the promoter within the LTR and an mRNA encoding drug resistance markers from the internal SV40 early region promoter (Figure 5). pBabe Neo and pBabe Hygro confer resistance to kanamycin/G418 or hygromycin B in procaryotes and eucaryotes, respectively, allowing quick recovery of integrated proviruses derived from these vectors as plasmids by 'shuttling' them into bacteria (1).

To quantitatively determine if the ATG$^-$ *gag* casette in the pBabe vectors would confer high titre characteristics to the vectors without inhibiting translation of inserted genes, the *cat* and v-Ha-*ras* genes were cloned into both vectors and their expression levels compared to those of these genes cloned into a conventional DO vector lacking *gag* sequences.

In all cases, pBabe vector titres were greater than 10^6 cfu/ml, exceeding conventional *gag*$^-$ DO vector titres by at least an order of magnitude. No significant difference in stable *cat* activity was noted between the conventional *gag*$^-$ vector DO-R cat and pBabe Neo cat (Figure 6). Similarly, (cfu and ffu) titrations of stable producers of DO-R ras and pBabe Neo ras or pBabe Hygro ras on NIH 3T3 cells resulted in identical ratios of drug resistant colonies to morphologically transformed foci for both the *gag*$^-$ and ATG$^-$ *gag*$^+$ vectors (Figure 7A). Examination of the p21$^{v\text{-}Ha\text{-}ras}$ protein levels in drug resistant NIH 3T3 cells resulting from infection with the above vectors revealed no significant difference in the amount of p21$^{v\text{-}Ha\text{-}ras}$ produced by the *gag*$^-$ and ATG$^-$ *gag*$^+$ vectors (Figure 7B). Last, no aberrant electrophoretic mobility of the p21$^{v\text{-}Ha\text{-}ras}$ produced by the ATG$^-$ *gag*$^+$ pBabe vectors was observed, indicating the expressed p21$^{v\text{-}Ha\text{-}ras}$ was not a fusion protein resulting from initiation at an upstream *gag* ATG codon.

To expand the range of available drug selection schemes, two additional pBabe vectors with different drug selection markers were constructed. pBabe Bleo allows growth of infected cells in the presence of phleomycin, while pBabe Puro permits selection in medium containing puromycin (Figure 5). Both vectors maintain the high titre characteristic of the other pBabe vectors (J. P. Morgenstern, Ph. D. thesis), but are not shuttle vectors as they are not capable of conferring resistance in bacteria.

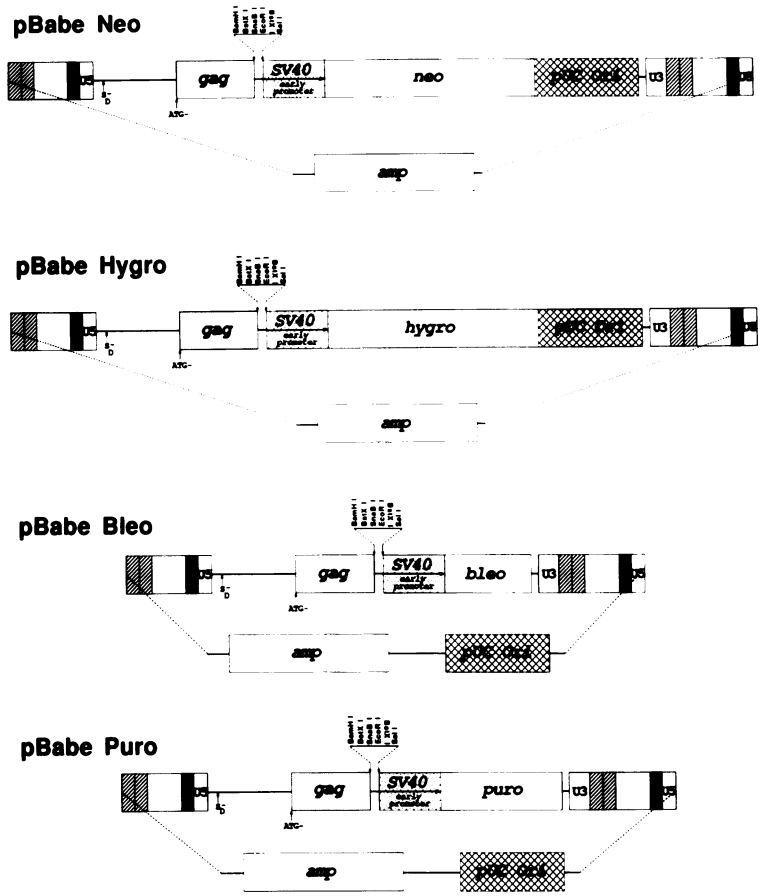
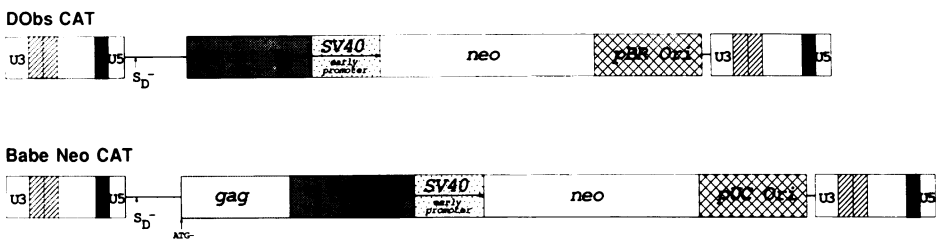


Figure 5. Schematic representation of pBabe vectors each containing a different selectable marker gene. Drug resistance genes were derived from the following plasmids: *neo*, pSV2 Neo (10), *hph*, pHygro Bgl II, *ble*, pUT 714 (45), and *pac*, pSV2 pac (46). The hatched boxes represent the 72 bp repeats of the enhancer and the blackened boxes the R region beteween the transcriptional start and the polyadenylation sites within the Mo MuLV LTRs.

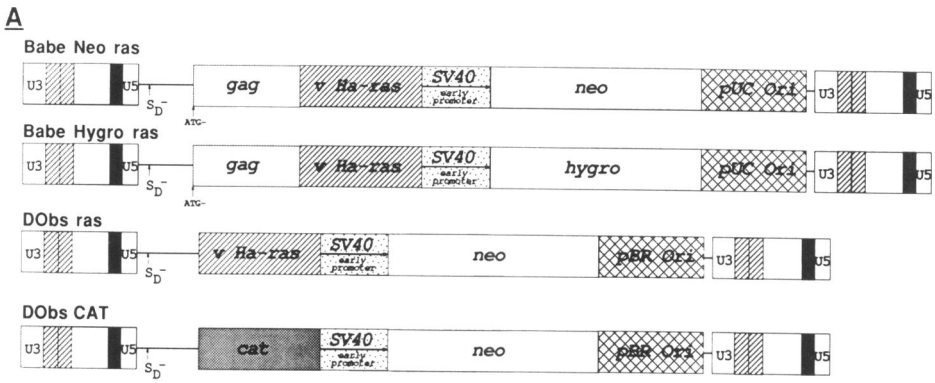


Construct	single provirus polyclonal (cfu/ml) G418^r titre	psi-2 single provirus polyclonal (cpm) cat activity	NIH 3T3 single provirus polyclonal (cpm) cat activity
DObsCAT	3.6×10^5	2.4×10^4	2.8×10^4
Babe Neo CAT	5.5×10^6	2.7×10^4	2.6×10^4

Figure 6. Comparison of titre and expression of a bacterial reporter gene by pBabe Neo and pDO-R. The *cat* gene from Tn9 was inserted into pBabe Neo and pDO-R. Both constructs were transfected into PA317 cells and the transiently produced virus used to infect Ψ-2 cells, which were G418 selected to establish stable ecotropic producer lines. The supernatants of the stable producer populations were used to infect NIH 3T3 cells and the G418^r titres were quantitated. The results shown represent the average of two independent experiments. The enzymatic CAT activity present in both the Ψ-2 stable producer populations and populations of NIH 3T3 cells pooled from the G418 titrations were assayed and normalised for protein content. The results shown are the average of two CAT assays on each cell population.

ΩE: a High Titre Third Generation Ecotropic Helper-Free Packaging Cell Line

As previously noted, wild type virus is much more likely to arise when passaging the *gag*$^+$ vector N2 in helper-free packaging cell lines relative to *gag*$^-$ vectors (11). The provirus-like packaging construct lacking Ψ (packaging) sites present in 'first generation' packaging cell lines, such as Ψ-2 cells, could be restored to a wild type provirus by a single recombination event,



Construct	G418^r cfu/ml	hygro^r cfu/ml	ffu/ml
Babe Neo ras	4.4 X 10^5	0	4.1 X 10^5
Babe Hygro ras	0	2.5 X 10^5	2.7 X 10^5
DO-R ras	2.5 X 10^4	0	2.3 X 10^4
DO-R cat	2.6X 10^5	0	0

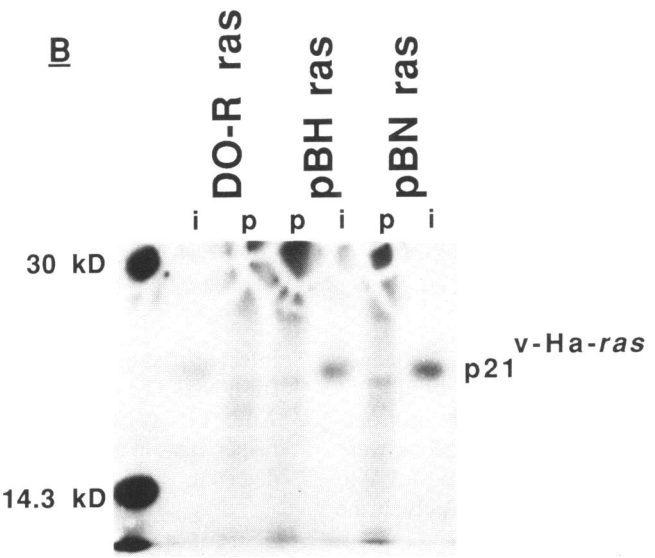


Figure 7. Comparison of titre and expression of a viral oncogene by pBabe Neo/pBabe Hygro and pDO-R. A) The v-Ha-*ras* gene of Ha MuSV was BamH I linkered and inserted into pBabe Neo, pBabe Hygro and pDO-R. All three constructs transfected into Ψ-2 cells and the resultant G418^r or hygromycin B^r colonies pooled as stable producer populations. The supernatants of the stable producer populations were used to infect NIH 3T3 cells and the G418^r, hygromycin B^r and morphological focus forming titres were quantitated. Supernatants of Ψ-2 producers of pDO-R cat were included as a negative control for focus forming ability. B) The amount of p21$^{v\text{-}Ha\text{-}ras}$ present in pooled G418^r or hygromycin B^r NIH 3T3 cell populations infected with pBN ras, pBH ras or pDO-R ras was examined by immunoprecipitation/SDS PAGE analysis. p: pre-immune rat serum as the primary antibody; i: rat monoclonal α p21$^{v\text{-}Ha\text{-}ras}$ as the primary antibody.

apparently enhanced by the addition of homologous *gag* sequences in N2. This led investigators to generate the 'second generation' helper free packaging cell line PA317 whose packaging construct lacks both a Ψ-site and a 3′ LTR, necessitating two recombination events with a vector to restore a wild type proviral structure (16). Although for the most part helper virus is rarely observed in PA317 cells, it still has been detected (26). By placing the the *gagpol* and *env* reading frames on separate (Ψ site and 3′ LTR minus) constructs 'third generation' cell lines (27−29) restrict the generation of wild type virus to three separate recombination events.

To further reduce the probability of the aformentioned three recombination events occurring during the passaging of the *gag*$^+$pBabe vectors, an improved third generation ecotropic packaging cell line was constructed (Figure 8A). First, the region of overlap between the Ψ-site/3′ LTR minus *gagpol* and *env* expression constructs was reduced to the minimum of 61 bp (found in different reading frames of the *pol* and *env* genes of Mo MuLV) in an effort to decrease the possibility that they would recombine to form a contiguous *gag/pol/env* product (Figure 8B). Transversion mutations were introduced into at least the third base of each of the final 20 codons of the *pol* gene in the construct pJ4Ω 5′ WT gagpol. This 'codon wobbling' maintains the primary protein sequence of the integrase region of the *pol* open reading

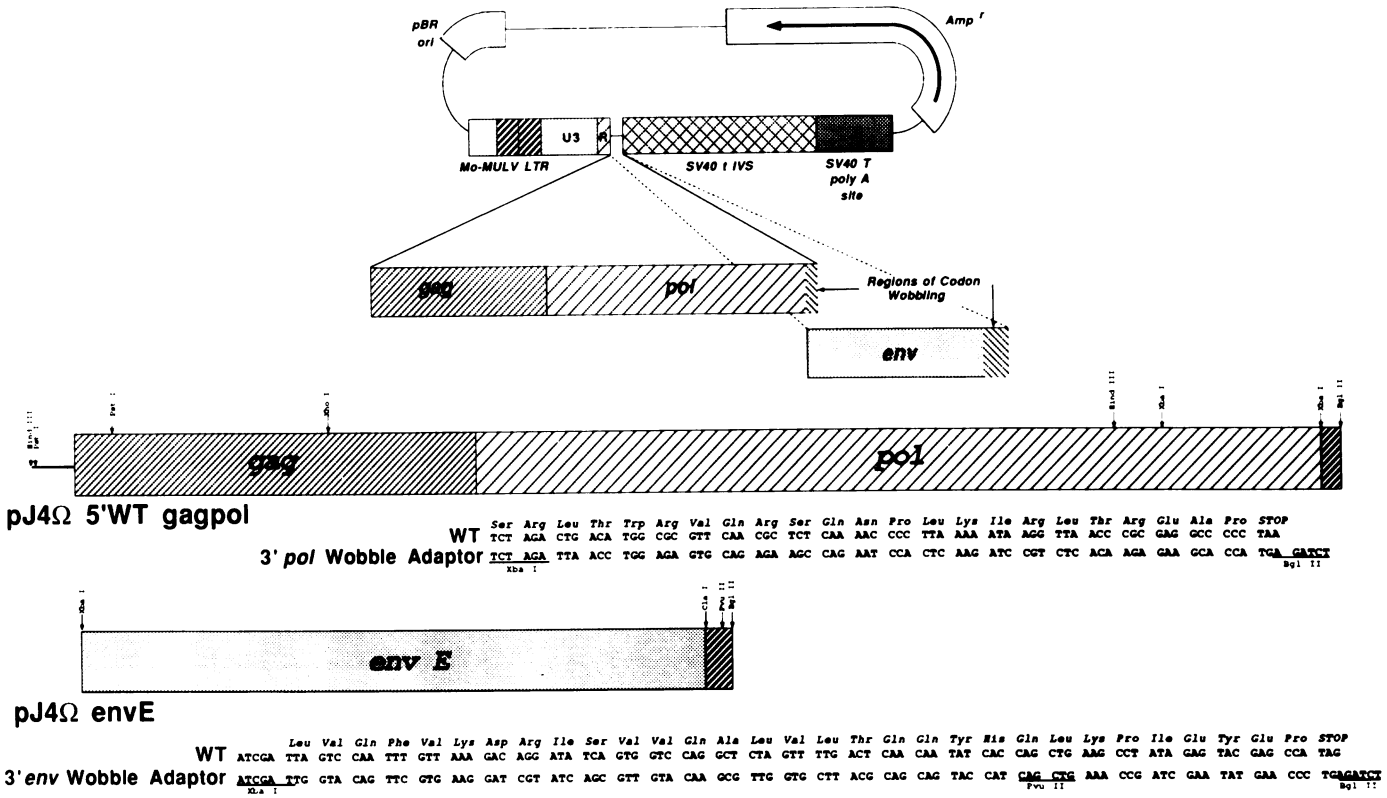


Figure 8. Schematic representation of ΩE third generation packaging *gagpol* and *env* constructs in pJ4Ω (47). The exact derivation of pJ4Ω gagpol and pJ4Ω envE, including a comparison of wild type Mo MuLV coding sequence and codon 'wobble' adaptors used to decrease nucleotide sequence homology between the constructs is indicated. The wobble adaptors were subcloned into pJ4Ω, after which the *gagpol* orf was inserted as a fragment from a Pst I to Xba I partial digest of the NB-tropic proviral clone pMov 9-2 (48) (positions 739 to 5325 of Mo MuLV) and the ecotropic *env* coding region was inserted as an Xba I to Cla fragment (positions 5766 to 7674 of pMo Mu LV).

frame (orf), but decreases the primary nucleotide sequence homology over the 61 bp region of *pol/env* overlap in pJ4Ω 5′ WT gagpol and pJ4Ω envE to 55%. At the 3′ end of the ecotropic *env* expression construct pJ4Ω envE the final 31 codons were wobbled (decreasing homology with the ecotropic Mo MuLV to 62%), and all sequences 3′ of the *env* stop codon (polypurine tract and 3′ LTR) were deleted (Figure 8B).

Both constructs were co-transfected with the pSV2 gpt marker (30) into NIH 3T3 cells, and 14 days later 192 mycophenolic acid resistant colonies were picked and assayed for reverse transcriptase activity in their supernatants (31). Twelve clones with the highest reverse transcriptase levels (as well as Ψ-2 cells as a positive control) were infected with an amphotropic stock of pBN cat. Subsequently, G418 resistant colonies were pooled, and their supernatants used in titrations on NIH 3T3 cells. The above procedure was repeated for the three packaging clones yielding the highest titres in the intial experiment. Clone 2E1, which gave titres 50% as high as Ψ-2 (15) or the 'third generation' GP+E (27) packaging cells, was designated as the ΩE packaging cell line.

As a preliminary test of the ΩE cell line's propensity for generating wild type virus, a polyclonal population of ΩE cells with pBN cat proviruses from the above titrations were passaged for four weeks and their undiluted supernatant used to infect 7.5×10^5 NIH 3T3 cells. In order to promote any potential helper virus to spread, the infected NIH 3T3 cells were not selected in G418 and were passaged for two weeks in the presence of 2 μg/ml of polybrene. Undiluted supernatant from the infected/passaged NIH 3T3 cells was used to infect 7.5×10^5 fresh NIH 3T3 cells, all of which were split and selected in G418. After 14 days no G418^r colonies, indicative of contaminating helper virus in the initial ΩE pBN cat producer supernatants, were detected.

DISCUSSION

By exploring the role of coding sequences of the Mo MuLV genome as *cis* acting signals in the retroviral life cycle, we have been able to develop a highly efficient retroviral based gene transfer system for stable expression within mammalian cells. While a number of laboratories have reported either high titre retroviral vectors or safe helper free packaging cell lines, we have extended their initial observations and designed a series of highly efficient vectors in conjunction with a packaging cell line.

Initial work with DO retroviral vectors revealed that alterations in the splice donor region of Mo MuLV can have deleterious effects on vector titre. A similar effect was noted by another group, however they failed to observe a significant effect of *gag* sequences on vector titre (32). In order to avoid splicing artifacts, a splice donor point mutant which did not adversely affect vector titre was made and demonstrated to inhibit splicing (as infered by a 10^3-fold decrease in G418^r titre) when introduced into the vector pZipNeo SV(X). A similar inhibition of splicing was obtained when the identical splice donor mutation from Ha MuSV was introduced into the splicing vector N2 (33).

Work presented in this paper is also in agreement with results showing that *gag* sequences enhance retroviral vector titre (25, 34). These findings indicate that *gag* sequences achieve their effect by directly increasing the efficiency of packaging of RNA into budding virions, in *cis*, and without the need for their translation. In addition, our work shows that unlike the core region of the Ψ-packaging site (35), the *gag* sequence portion of the Ψ-site cannot be moved from its native position within the Mo MuLV genome and still maintain its activity.

Taken together, the effects of both the splice donor region and *gag* sequences on retroviral vector titre strongly argue that the packaging region of Mo MuLV extends both 5′ and 3′ of the previously arbitrarily defined Ψ-site (15). Hence the core sequences of the Ψ-site are necessary and sufficient for conferring the packaging function in *cis* to an RNA transcript (35, 36), while the flanking sequences modulate the efficiency of the packaging function. Indeed one investigation has even implicated sequences as far 5′ as the U5 region as being involved in Mo MuLV genome packaging (37). When simultaneously incorporated into the pBabe vectors, the 194 splice donor point mutation (2.3×10^5 cfu/ml for pLRV 194) and *gag* sequences (8.1×10^5 cfu/ml for pgagSRVneo) interact multiplicatively to yield stable vector titres in excess of 2×10^6.

Converse to the findings for *gag* sequences, deletion of all *env* coding sequences had no effect on Mo MuLV based retroviral vector titre, demonstrating the lack of any *cis* acting replication signals within the *env* gene. Exclusion of non essential *env* sequences from retroviral vectors should eliminate homologous recombination between such vectors and second and third generation packaging constructs (see discussion of packaging constructs below).

By incorporating the above findings into a single construct, the pBabe vectors basically fulfil the potential of the DO design for retroviral vectors, transmitting and expressing exogenous genes, subsequent to selection, at high levels. These vectors rely on the Mo MuLV LTR's promoter for transcription of inserted genes, which has proven to be the more efficient than most internal promoters in a number of cell types (38, 39). As yet there is no evidence that the ATG$^-$ *gag* sequences responsible for the high titre nature of the pBabe vectors will compromise expression of an inserted gene at the level of translation. Currently more than eight genes have been successfully expressed within the pBabe vectors or those of similar design (33, L. Penn, M. Brooks and H.L., unpublished). Therefore, it is likely that this lack of inhibition of translation by ATG$^-$ *gag* sequences is a general phenomenon and therefore an inherent characteristic of these vectors. No aberrant splicing interactions with inserted genes have been observed in our experience with any vector containing the 194 splice donor mutation incorporated in the pBabe vectors.

None of the resistance activities encoded in the *neo*, *hph*, *ble*, or *pac* genes of the pBabe vectors overlap, making it possible to introduce four different genes into a single cell. This capability could be of great use in the study of any number of biological phenomena involving the interaction of multiple genes. In this context it is also worth mentioning that the selection for *pac* expression with puromycin is the most powerful selection procedure. Moreover, because pBabe Neo and pBabe Hygro are shuttle vectors with inverted BstX I restriction sites for efficient cDNA cloning (40), they are candidate vectors for use in the construction of cDNA expression libraries for use in complementation assays in mammalian culture systems (J. P. Morgenstern, Ph. D. thesis, University College, London).

The pBabe vectors harbour limited potential to yield wild type virus via homologous recombination with defective proviral 'packaging' constructs in helper free packaging cell lines (11). In contrast to many N2 based *gag*$^+$ vectors that maintain intact 5′ proviral structures (41, 42), the Ψ-site in the pBabe vectors is flanked by both an attenuated splice donor and ATG$^-$ *gag* sequences. At the pBabe vectors' 3′ end, all *env* sequences have been deleted, confining recombination to sequences within the polypurine tract and 3′ LTR, neither of which are present in the packaging constructs of the PA317 or ΩE packaging cell lines. All other vectors, except those whose 3′ ends are derived from pBabe constructs (33), contain somewhere between 100 to 550 bp of p15E coding sequences.

The high titre ΩE cell line represents a third generation helper free packaging cell line designed specifically to prevent recombination between the packaging constructs themselves, and the pBabe retroviral vectors (see previous paragraph). The successful use of codon wobbling to alter nucleotide sequence but maintain coding information in the *gagpol* and *env* packaging constructs of the ΩE cell line indicates that this may be a generally applicable method for reducing the probability of recombination between two known homologous coding sequences. To enable introduction of the pBabe vectors into a wide variety of mammalian cells, an amphotropic analog the ΩE cell line is currently under construction.

During the construction of the ΩE packaging cell line, two other groups have published Mo MuLV based third generation packaging cell lines (27−29). Both ΨCRE/ΨCRIP and GP+E/GP + envAm12 use separate *gagpol* and *env* packaging constructs lacking Ψ-sites and LTRs at their 3′ ends. Sequential transfection with different selectable markers was used to transfer the two Ψ-CRE/CRIP and GP+envAm12 packaging constructs into cells, whereas the GP+E and ΩE constructs were cotransfected together with a single marker. Here there may be an advantage to sequential introduction given the high propensity of DNA molecules to recombine during calcium phosphate transfection (43), although no helper virus has yet been detected in the ΩE cells.

The Ψ$^-$ CRE/CRIP *gagpol* and *env* packaging constructs contain reciprocal linker insertion mutations that destroy the *gag* and *env* open reading frames, rather than the reciprocal deletions found in the GP+E/GP + envAm12 and ΩE packaging constructs. It is likely that the large (>4 kb) region of overlap in the *gagpol* region CRE/CRIP packaging constructs will be much more prone to participate in recombination than the relatively small overlap (500 bp) in the GP+E/GP + envAm12 constructs. Here, greater care has been taken to prevent recombination in the ΩE constructs, with only a 66 bp region of overlap, whose sequence homology has been reduced to 55% by codon wobbling.

At the 3′ end of each of the *env* packaging constructs of the three cell lines, only the pJ4Ω envE construct has been designed to eliminate all sequences 3′ to the *env* stop codon. In the two other *env* packaging constructs the polypurine tract, inverted repeat at the LTR boundary, and portions of the 3′ LTR are retained. The significance of overlap of these sequences with those found in retroviral vectors (other than *env*$^-$ vectors such as pBabe) became apparent when helper virus was detected during shuttling of recombinant virus between Ψ-CRE and PA317 cell lines. Recombination in this case appears to have occurred between the sequences 3′ to *env* in one of the Ψ$^-$CRE

packaging constructs and the retroviral vector (29). Although not formally proven, one may expect that the reduction of sequence overlap and homology in the Ω packaging constructs will probably aid in the reduction of the frequency of helper formation in the ΩE cell line relative to the other Mo MuLV based third generation packaging cell lines.

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