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VIRAL TRANSFER TECHNOLOGY **BRIEF COMMUNICATION**

Improved safety and titre of murine leukaemia virus (MLV)-based retroviral vectors

Y Zhao, W Low and MKL Collins

Department of Immunology, Windeyer Building, 46 Cleveland St, London WIP 6DB, UK

Many retroviral vectors based on murine leukaemia virus (MLV) contain the first 420 nucleotides of the gag gene, as this was reported to increase vector titre by increasing the efficiency of RNA packaging. In this study, deletion of this gag sequence from its original location did not decrease the titre of two retroviral vectors, pBabe puro and MFG-S⁻. The two vectors could be improved by replacing the gag sequence with a CTE from Mason–Pfizer monkey virus (MPMV). This substitution improved vector titre, while eliminating a region of homology between vector and packaging constructs. Gene Therapy (2000) **7,** 300–305.

Keywords: *retrovirus; retroviral vector; RNA packaging*

MLV-based retroviral vectors are the most widely used gene delivery vehicles in clinical gene therapy protocols.[1] The viral components of these vectors comprise *trans*-complementing genes (*gag*, *pol* and *env*) expressed in packaging cells and the *cis*-acting sequences (LTRs, primer binding sites and packaging signals) present within the vector. Inclusion of part of the *gag* gene sequence in vectors was shown to increase recombinant viral titre.[2,3] This *gag* sequence was reported to increase the amount of vector packaged into virions and was therefore thought to be an extension of the Ψ packaging signal.[3,4] However, retention of the *gag* sequence in vectors is not desirable as it could aid recombination in the packaging cells, leading to generation of replication-competent viruses.

Replication of retroviruses requires export of unspliced genomic RNA from nuclei to cytoplasm. The lentiviruses, such as HIV, encode a *trans*-acting regulatory protein (Rev), which binds to a *cis*-acting Rev responsive element (RRE) to facilitate the export of the unspliced genomic RNA.[5,6] Although the simple retroviruses do not encode a Rev-like protein, a *cis*-acting element, termed constitutive transport element (CTE), has been identified in MPMV, SRV and RSV.[7–10] These CTEs are believed to interact with cellular proteins to enhance export of genomic RNA.[11–13] It has been reported that such CTEs can substitute for RRE/Rev in HIV and SIV vectors.[7,10,14,15] In this study we demonstrate that the first 420 nucleotides of *gag* sequence in MLV can increase both cytoplasmic RNA level and packaging efficiency of a vector genome. Its substitution by the MPMV CTE results in MLV vectors with an increased titre (up to 10-fold higher).

To analyse the function of the 420 nucleotide *gag* sequence in MLV vectors, we deleted this from both pBabe puro and MFG-S⁻ as detailed in Figure 1. The titre of pBabe Δ*gag* ($1–2 \times 10^5$ IU/ml) was slightly increased in comparison with the parental vector (Figure 2a) and that of MFG Δ*gag* ($6–8 \times 10^5$ IU/ml) was the same as the parental vector. These titres were determined on human TE671 cells and are therefore lower than the reported titres for the parental vectors on 3T3 cells. These results do not agree with those of Bender *et al*[3] and Morgenstern and Land[16] who showed that inclusion of the *gag* sequence increased vector titre by five- to 10-fold. However, Kim *et al*[17] have previously reported that removal of the *gag* sequence from MFG does not decrease vector titre. To examine possible functions of the *gag* sequence further, we also moved it close to the 3′ LTRs of the vectors, resulting in pBabe C*gag* (Figure 1a) and MFG C*gag* (Figure 1b). The titres of these vectors were three- to four-fold higher than the parental vectors (Figure 2). Inclusion of the *gag* sequence in reverse orientation (MFG C*gag* R, Figure 1b) did not increase vector titre (Figure 2b).

To investigate the mechanism of action of the *gag* sequence, we performed Northern hybridisation analysis on both cytoplasmic polyadenylated and packaged viral RNA, using a puro fragment as a probe. The deletion of the *gag* sequence in pBabe puro resulted in a reduction of unspliced genomic RNA accumulated in the cytoplasm (Figure 3a). However, an increased amount of genomic RNA was packaged (Figure 3b), in agreement with the increase in titre (Figure 2a). This suggests that deletion of *gag* sequence from its original position significantly increases RNA packaging efficiency. Insertion of the *gag* sequence adjacent to the 3′ LTR led to a higher level of genomic RNA accumulated in the cytoplasm in comparison with pBabe Δ*gag* (Figure 3a) and a small increase in cytoplasmic genomic RNA for MFG C *gag* (Figure 4a). Furthermore, an increased amount of genomic RNA was detected in virus particles (Figure 3b) in agreement with the further increase in titre (Figure 2a). These results demonstrate that transfer of the *gag* sequence adjacent to the 3′ LTR augmented vector titre, both by promoting

Correspondence: MKL Collins
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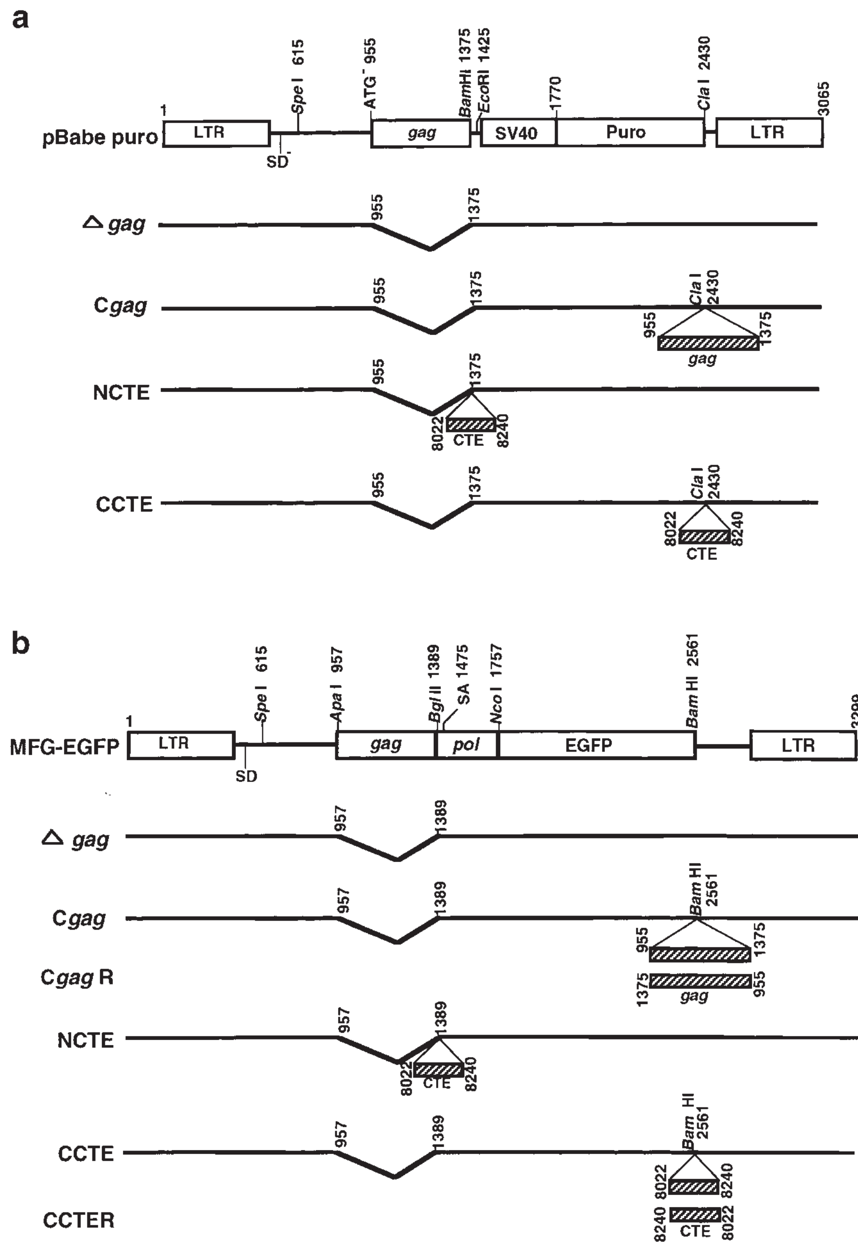

Figure 1 *pBabe (a) and MFG (b) based vectors, showing the location of gag and MPMV CTE sequences. To generate pBabe Δgag, the polymerase chain reaction (PCR) was used to produce an SpeI–BamHI fragment from pBabe puro[16] spanning the packaging signal and the mutated ATG at nucleotide 955 with a BamHI site introduced at the 3′ end. The SpeI–BamHI fragment was then used to replace the SpeI–BamHI fragment of pBabe puro, resulting in a deletion of 420 bp of gag sequence. Nucleotides 8022–8240 of Mason–Pfizer monkey virus (MPMV) with BamHI and EcoRI ends were amplified by PCR from plasmid pTMOwt, kindly provided by Eric Hunter.[26] This CTE fragment was cloned into pBabe Δgag, replacing the gag sequence to generate pBabe NCTE. The same CTE fragment, generated with ClaI ends by PCR, was inserted into the pBabe Δgag plasmid next to the vector 3′ LTR, resulting in pBabe CCTE. In a similar way, pBabe Cgag was constructed containing a 420 bp gag fragment, amplified by PCR with ClaI ends from pBabe puro. To construct MFG based vectors, a EGFP fragment, based on pEGFP-Cl (Clontech, Basingstoke, UK), was inserted into MFG-S⁻ (Somatix Therapy Corporation, Alameida, CA, USA) between the NcoI–BamHI sites, resulting in MFG-EGFP. MFG-EGFP was then digested with ApaI and BglII, the DNA was repaired with S1 nuclease and Klenow polymerase and ligated to generate MFG-EGFP Δgag. The CTE fragments were then introduced into the MFG-EGFP Δgag vector, either replacing gag or adjacent to the 3′ LTR, resulting in MFG-EGFP NCTE and MFG-EGFP CCTE, respectively. Insertion of the gag fragment adjacent to the 3′ LTR resulted in MFG-EGFP Cgag. The construction of all the plasmids was verified by sequence analysis using an ABI system.*

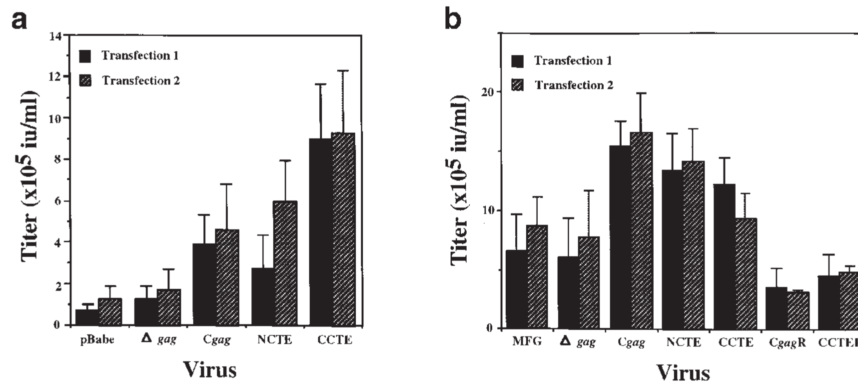

Figure 2 *Effects of gag and MPMV CTE sequences on vector titre. Vector DNA was transfected into the amphotropic packaging cell line, TEFLYA8[19] using Lipofectamine (Gibco, Paisley, UK), in the presence of 1/10 molar amount of pSV2neo (Clontech) in the case of the MFG derivatives. After 48 h, the producer cells were selected for 14 days by the addition of puromycin (1 μg/ml; Sigma, Poole, UK) or G418 (1 mg/ml; Gibco) for pBabe or MFG vectors, respectively. A minimum of 200 colonies was pooled, grown to confluence then incubated overnight in OptiMEM (Gibco). The virus-containing supernatant was filtered through 0.45 μm filters (Gelman Sciences, Ann Arbor, MI, USA). Human TE671 cells were infected for 5 h with serially diluted viruses in OptiMEM containing polybrene (8 μg/ml; Sigma). For each vector, virus titre was determined from triplicate infection with viruses harvested from two sets of transfections. For pBabe vectors, the infected cells were selected with puromycin (1 μg/ml) for 14 days and resistant colonies were counted (a). For MFG derived vectors, the infection efficiency was measured after 48 h infection on a FACScan (Becton Dickinson, Heidelberg, Germany). The percentage of cells expressing EGFP was measured and the viral titre and mean EGFP expression in infected cells was calculated (b).*

RNA accumulation in the cytoplasm and also by increasing packaging efficiency of RNA into virus particles.

A 240 nucleotide sequence from MPMV has been shown to promote the nuclear export of unspliced RNA.[7,8,11] We therefore examined whether the 240 nucleotide MPMV CTE could enhance MLV titre (Figure 1). This sequence shares no homology with the MLV packaging constructs, or human endogenous or infectious retroviruses. It is within the non-coding region of MPMV and its longest potential open reading frame is 24 amino acids. In pBabe puro, the MPMV CTE enhanced vector titre three- to six-fold when substituted in the original *gag* position (pBabe NCTE, Figure 2a) or 10-fold when inserted adjacent to the 3′ LTR (pBabe CCTE, Figure 2a). In MFG a two- to three-fold enhancement of titre was observed when the MPMV CTE was inserted in either position, and insertion in the reverse orientation did not enhance titre (Figure 2b, MFG NCTE, MFG CCTE and MFG CCTER).

The MPMV CTE has been identified as a *cis*-acting nuclear export element in MPMV or HIV.[17,18] We therefore examined whether it increased MLV cytoplasmic RNA. Northern hybridisation analysis showed that insertion of the MPMV CTE, either replacing the *gag* sequence or adjacent to the 3′ LTR, promoted the accumulation of unspliced genomic RNA in the cytoplasm. The origin of the RNA species intermediate in size between the genomic and puro RNAs in the CTE constructs is not clear (Figure 3a); it is not included in genomic RNA quantification and was not detected in packaged RNA (Figure 3b). A six-fold increase of RNA was accumulated in the cytoplasm in comparison with pBabe puro, which corresponded to a 15-fold increase in comparison with the parental pBabe Δ*gag* (Figure 3a). Again a modest increase in cytoplasmic genomic RNA was observed when the MPMV CTE was inserted in MFG (Figure 4a). Because the CTE promoted accumulation of unspliced genomic RNA in the cytoplasm, it also increased the amount of RNA packaged in virions (Figure 3b, pBabe NCTE) in agreement with the observed increase in titre (Figure 2). EGFP expression in cells infected with the MFG-based viruses is shown in Figure 4b. Improved expression compared with parental MFG was detected with the Δ*gag*, NCTE and CCTE vectors. The combination of improved titre and EGFP expression makes the MFG-NCTE the most attractive for future use.

In this study, deletion of the MLV *gag* sequence from the commonly used pBabe and MFG-S^{-} vectors did not decrease viral titres. This finding appears to contradict previous studies in which viral titres or RNA packaging were compared in vectors with or without the *gag* sequence.[2,3,4,16] However, there is another recent report showing that deletion of the *gag* sequence from MFG did not affect vector titre.[17] The reason for the discrepancies between results remains unclear. It is possible that use of the newer packaging cell lines[19–21] which express higher levels of viral proteins, eliminates effects of the *gag* sequence.

The *gag* sequence has a modest (2.5-fold) effect on accumulation of vector RNA in the cytoplasm, when included in the pBABE vector. This may partly explain its observed effect on RNA packaging in a previous study, where total cellular RNA and packaged RNA were measured.[3] Significantly, this MLV *gag* sequence has also been reported to allow rex-independent expression from HTLV-1-based vectors, by enhancing nuclear export.[22] However, deletion of the *gag* sequence does not eliminate RNA export or viral titre, as is the case for previously characterised retroviral CTEs.[9,18] If MLV contains a classic CTE, it must lie within sequences which cannot easily be deleted because they contain LTR, primer binding or packaging functions. In our study, the *gag* sequence can

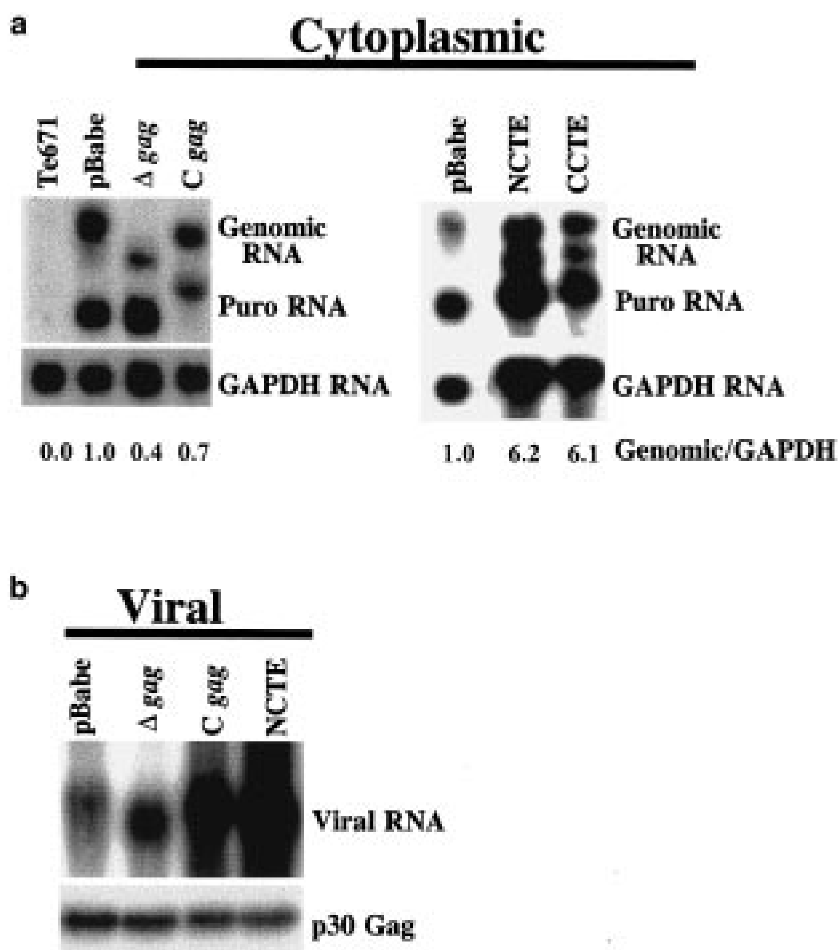

Figure 3 *Effects of gag and MPMV CTE sequences on cytoplasmic RNA level and packaging of pBabe puro vectors. (a) Cytoplasmic RNA was isolated by the guanidine isothiocyanate/caesium chloride method, then mRNA was isolated using a poly(A) Quick kit (Stratagene Europe, Amsterdam, The Netherlands). A 500 bp puro fragment was labelled with ^{32}P dCTP using the Prime-a-Gene system (Promega, Southampton, UK) and used to hybridise to the RNA samples resolved on a denaturing agarose gel, then transferred to nitrocellulose. A GAPDH fragment was used as a loading control. The relative amount of virus genomic RNA to GAPDH was determined by densitometry of autoradiographs; this ratio was set to 1.0 for pBabe puro and those of the others were normalised to this. (b) To prepare viral RNA, supernatant was harvested from packaging cells, then viral particles were pelleted by centrifugation at 1500 g for 3 h and were lysed in 4 M guanidium isothiocyanate solution. DNA and proteins were extracted with phenol/chloroform in the presence of 0.2 M sodium acetate (pH 4.0). Viral RNA was then concentrated by ethanol precipitation with yeast tRNA (50 μg) as a carrier. RNA was analysed by Northern hybridisation with a puromycin probe (upper panel). 1/20 volume of the same supernatant was analysed by SDS-PAGE and Western blot with an anti-Gag antibody (lower panel).*

also enhance packaging of cytoplasmic RNA, but only when it is present at the 3′ end of the vector genome. This demonstrates that it does not have to be part of a continuous packaging signal extending from ψ and that it therefore may not be involved in genome dimerisation and nucleocapsid binding.[23]

We have replaced the *gag* sequence with the MPMV CTE to produce improved versions of the pBabe puro and MFG-S⁻ vectors. The increase in titre obtained by this inclusion of the MPMV CTE suggests that nuclear export of parental vector RNA is suboptimal. Even the modest increase in titre of these new vectors, three-fold in the case of MFG-S⁻ with the MPMV CTE, will extend the potential applications of retroviral vectors, which are often limited by low titre. In the case of pBabe puro, removal of the *gag* sequence also allows alignment of the start codon of the transgene with that of *gag*, which should enhance transgene expression as an internal ribosome entry site facilitates *gag* translation.[24] The new vectors will also allow elimination of any sequence homology between vectors and packaging constructs, eliminating the risk of recombination in packaging cells. This is particularly valuable for packaging systems which generate retroviral vectors by transient transfection,[21,25] as multiple copies of vector and packaging constructs are present within a single cell which increases the risk of recombination. It will also be crucial for the preparation of high titre viruses for clinical use.

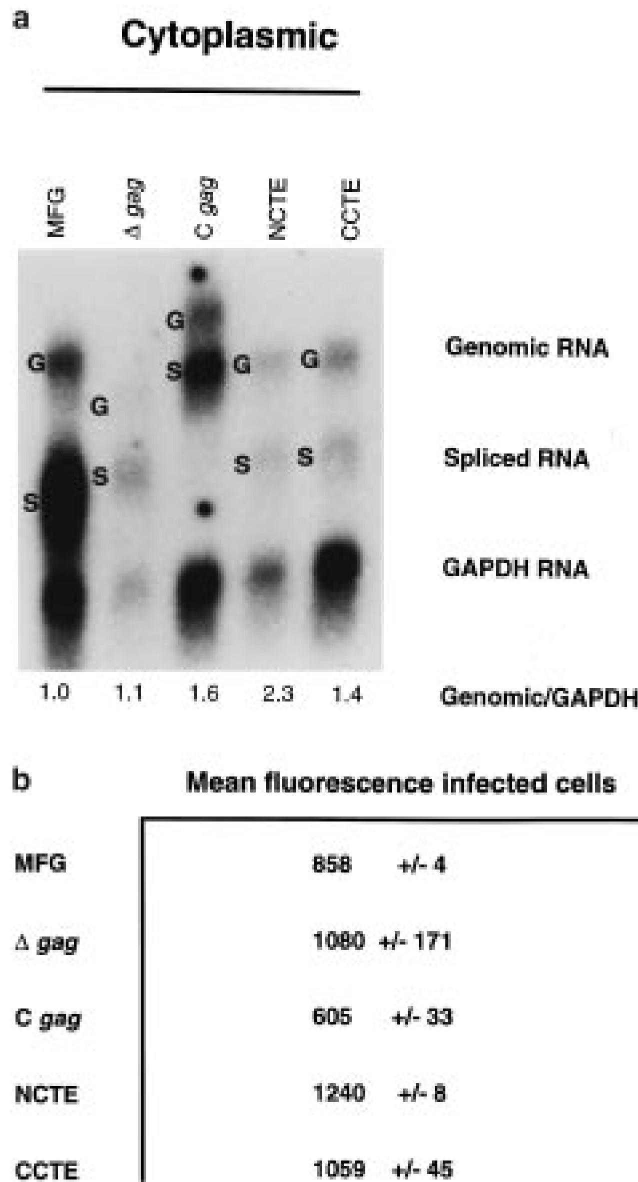

Figure 4 *Effects of gag and MPMV CTE sequences on MFG vectors. (a) poly(A)+ RNA was isolated from bulk populations of vector-transfected packaging cells. RNA was analysed by Northern hybridisation with an 800 bp EGFP and a GAPDH probe. The relative amount of virus genomic RNA to GAPDH was determined by excision of appropriate areas of membrane and scintillation counting; this ratio was set to 1.0 for MFG and those of the others were normalised to this. (b) Supernatants from bulk populations of vector-transfected packaging cells were used to infect human TE671 cells. Infected cells were defined by FACScan as those within a region that included only 0.02% of uninfected cells, which comprised between 20 and 70% of the infected cell populations. EGFP expression in two independently infected populations was determined, the mean fluorescence ± s.e. from the two infections is shown.*

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