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Role of a heterologous retroviral transport element in the development of genetic complementation assay for mouse mammary tumor virus (MMTV) replication

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ABSTRACT

The mouse mammary tumor virus (MMTV) is a type B retrovirus that is unique from other retroviruses in having multiple "tissue specific" and "hormone inducible" promoters. This unique feature has lead to the increasing interest in studying the biology of MMTV replication with the ultimate goal of developing MMTV based vectors for potentially targeted human gene therapy. In this report, we describe, for the first time, the establishment of an *in vivo* genetic complementation assay to study various aspects of MMTV replication. In the assay described here, the function of MMTV Rem/RmRE regulatory pathway has been successfully substituted by a heterologous retroviral constitutive transport element (CTE) from Mason Pfizer Monkey Virus (MPMV) for mature MMTV particle production. Our results revealed that in the absence of MPMV CTE or Rem/RmRE, RNA transcribed from MMTV Gag-Pol expression plasmids were efficiently transported to the cytoplasm. However, the presence of CTE was indispensable for Gag-Pol protein expression. In addition, we report the development of MMTV based vectors in which the packageable RNA was transcribed either from MMTV LTR or from a chimeric LTR, which could successfully be packaged and propagated by particles produced from MMTV Gag-Pol expression plasmids containing a heterologous transport element. The role of MPMV CTE in the transport of MMTV transfer vector RNA was not found to be significant. Development of such an assay should not only shed light on how MMTV regulates its gene expression, but also should provide additional molecular tools for delineating the packaging determinants for MMTV, which is imperative for the development of novel vectors for targeted and inducible gene therapy.

Introduction

The mouse mammary tumor virus (MMTV) is a type B retrovirus, which causes breast cancer and T cell lymphomas in mice (reviewed in Mustafa et al., 2000, 2003). Historically, MMTV has been classified as a simple retrovirus, a retrovirus that contains only the structural genes necessary for virus particle formation (*gag/env*) and those coding for enzymes required for virus replication (*pro/pol*) (Coffin, 1992). However, it has long been known that MMTV also encodes additional viral factors, *sag* (*superantigen*) (Acha-Orbea and Palmer, 1991; Marrack et al., 1991) and perhaps *Naf* (Salmons et al., 1990). Like other complex retroviruses such as human, simian, and feline immunodeficiency viruses (HIV, SIV, and FIV, respectively) in which Rev responsive element (RRE) interacts with Rev protein, MMTV Rem-Responsive Element (RmRE) present at the 3′ end of the genome interacts with Rem (Indik et al., 2005; Mertz et al., 2005; Müllner et al., 2008) as well as with HIV-1 Rev (Dangerfield et al., 2005). Because of the presence of these accessory genes and regulatory elements, it has recently been suggested that MMTV be classified as a complex murine retrovirus (Mertz et al., 2005).

In addition, MMTV is distinct from most other retroviruses in another aspect in that it maintains multiple promoters for the expression of its various genes (Arroyo et al., 1997; Günzburg et al., 1993; Miller et al., 1992); two promoters in the LTR and possibly two in the *env* reading frame (reviewed in Mustafa et al., 2000). Compared to that, a virus as complex as HIV, encoding at least ten genes, contains only one standard promoter in the LTR for its transcriptional needs. This makes MMTV more similar to the only other known retrovirus shown to contain an additional promoter in the *env* gene, the foamy virus (Meiering et al., 2001). MMTV promoters in the LTR are unique because they are inducible by steroid hormones due to the presence of hormone responsive elements (HREs) within the U3 region of the LTR, which contribute to these inducing effects (Ham et al., 1988). Because of these distinctive characteristics, MMTV LTR promoters have been widely used for transgene expression in the mouse model of human breast cancer (Ahmed et al., 2002; Andrechek et al., 2003; Ross and Solter, 1985; Stewart et al., 1984; Wagner et al., 1997), thus making them major candidate promoters to be used in human breast cancer gene transfer studies. The side effects associated with the conventional cancer

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chemotherapy have led to the development of alternative therapeutic approaches such as gene therapy or gene-directed enzyme prodrug therapy (GDEPT). In GDEPT, a drug-metabolizing transgene is selectively delivered into tumor cells where its "conditional" and "targeted" expression is under the influence of an inducible promoter, which can be controlled by providing the inducing agent. This aspect alone had prompted the use of MMTV promoters for "conditional" and "targeted" gene expression in gene therapy and GDEPT. In one such study, retroviral vectors containing MMTV LTR promoters have been used allowing the elevated transgene expression in both human and non-human cell lines that could be further improved by induction with dexamethasone (Klein et al., 2008). Therefore, the inducible and tissue-specific control of MMTV promoters make them valuable tools for the study of gene expression and the development of novel MMTV based vectors for targeted human gene therapy.

Before MMTV based vectors can be exploited for human gene therapy, it is important that the pertinent aspects of MMTV replication are understood. One of the hallmarks and very essential step of retroviral life cycle is the efficient and specific packaging of retroviral genomic RNA by the assembling virion particles. Very little is known about the packaging determinants of MMTV that allow the specific and preferential packaging of the genomic RNA into the virus particles over the cellular and other viral RNAs. Only one study exists and suggests that, like other retroviruses, the 5′end of the MMTV genome may contain some sequences responsible for RNA packaging (Salmons et al., 1989). In an attempt to study MMTV replication and RNA packaging, we report the establishment of a three plasmid *trans* complementation assay for MMTV similar to the assays that we and others have developed to study RNA packaging in other retroviruses such as HIV, SIV, FIV and MPMV (Naldini et al., 1996; Rizvi and Panganiban, 1993; Browning et al., 2001).

Briefly, in this assay, retroviral *cis* and *trans*-acting factors are separated into three individual plasmids. Two plasmids provide *gag/pol* and *env* structural and regulatory genes, whereas a third plasmid called transfer vector contains the minimal *cis*-acting sequences needed for RNA packaging, reverse transcription, and integration as well as a marker gene that allows monitoring the vector propagation into the target cells. Due to the presence of the packaging signal only in the transfer vector, its RNA serves as the only source for the packagable RNA into the virus particles produced by Gag-Pol expression plasmids. The three plasmids are co-transfected into the producer cells resulting in the generation of virus particles containing the packaged transfer vector RNA that is capable of transducing the gene of interest into the target cells, the replication of which is limited to a single round with no further chance of reinfection.

Results and discussion

MPMV constitutive transport element (CTE) can substitute the function of Rem/RmRE nuclear export pathway

As a first step towards establishing MMTV *trans* complementation assay, we had to develop MMTV Gag-Pol expression plasmid capable of producing virus particles that can package and propagate MMTV transfer vector RNAs. It has recently been shown that the expression of MMTV Gag-Pol proteins from an unspliced genomic message is dependent on the presence of Rem/RmRE export pathway and that MMTV RNA can interact with the HIV-1 Rev protein to upregulate its gene expression (Dangerfield et al., 2005; Indik et al., 2005; Mertz et al., 2005; Müllner et al., 2008). Our goal was to develop a Gag-Pol expression vector in which Rem/RmRE sequences are eliminated to reduce the chances of recombination between the same elements

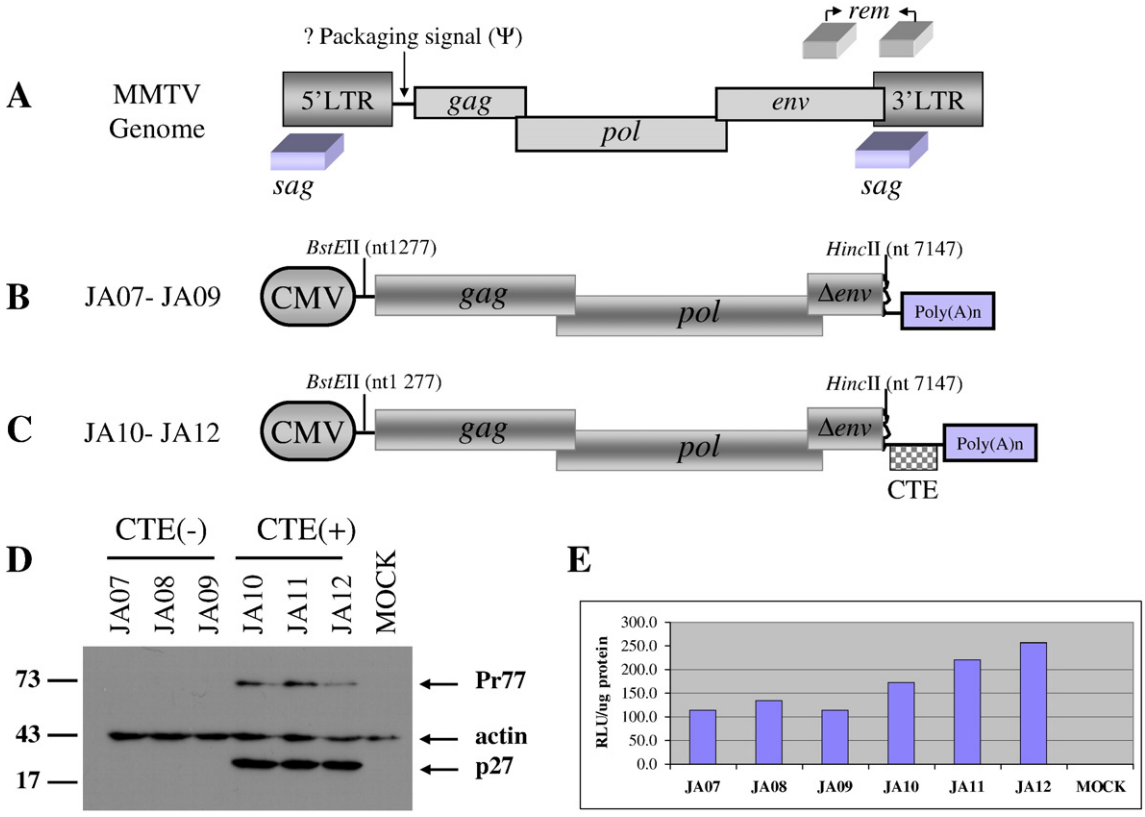

Fig. 1. MMTV *gag/pol* gene expression is mediated by MPMV CTE. (A) Schematic representation of a complete MMTV genome. (B and C) MMTV Gag-Pol expression plasmids with and without MPMV CTE. (D) Western blot analysis of transfected 293T cell lysates with MMTV Gag-Pol expression plasmids. Both processed and unprocessed forms of Gag protein are shown by arrows. As a control for protein loading, the same blot was stripped and reprobed with antibodies against β-actin. Cellular lysate from mock transfected 293T cells is shown as a control. (E) Transfection efficiencies were measured by luciferase activity from the co-transfected pGL3 control DNA using the Dual Luciferase Assay kit RLU, relative light units/microgram protein.

among the packaging constructs and the transfer vectors. Since it has already been shown that the MPMV constitutive transport element (CTE) can compensate for the Rev/RRE regulatory system in case of HIV and SIV (Bray et al., 1994; Rizvi et al., 1996), we tested whether the MPMV CTE could substitute for the function of Rem/RmRE export pathway in the case of MMTV. Therefore, a region encompassing MMTV *gag/pol* genes was cloned in either the absence or presence of MPMV CTE into expression plasmids (Figs. 1B and C). Designing MMTV Gag-Pol expression plasmids in such a fashion, which at the 5′end lacked region spanning R and most of U5 and at the 3′ end lacked all the sequences from nucleotide 7147 till the end of HYB MTV, removed most of the *cis*-acting sequences needed for packaging, reverse transcription, and integration. Therefore, the RNA expressed from these Gag-Pol expression plasmids can only express the viral structural proteins, however cannot be packaged and propagated by the assembling virus particles. Such an assumption was based on an earlier report of Salmons et al., in which replacing the 5′LTR of MMTV with that of Rous sarcoma virus (RSV) resulted in abolishing MMTV RNA packaging (Salmons et al., 1989). Such a design of the packaging construct, which removed most of the *cis*-acting elements, should diminish the possibility of recombination between the packaging construct and transfer vectors, which may result in generating replication competent virus.

Co-transfection of these Gag-Pol expression plasmids along with a control plasmid, pGL3 DNA, expressing firefly luciferase for transfection efficiencies (Ghazawi et al., 2006), demonstrated the successful expression of MMTV Gag-Pol proteins only in the presence of MPMV CTE (Fig. 1D). This was despite the fact that the transfection efficiencies for these expression plasmids (with and without CTE) were within two folds (Fig. 1E). Next, we wanted to investigate what may have contributed to the inability of the CTE (−) vectors to express Gag-Pol proteins. Towards this end, we fractionated the RNA obtained from the transfected 293T cells into cytoplasmic and nuclear fractions to assess whether the CTE (−) *gag/pol* RNAs were efficiently transported from the nucleus to the cytoplasm. As a first step, both nuclear and cytoplasmic fractions were DNase treated and PCR amplified using MMTV Gag specific primers (OTR567/OTR552) in order to ascertain that the RNA preparations were devoid of any contaminating plasmid DNA, which may compound the interpretation of our results. Fig. 2A.1 and A.2 show that both nuclear and cytoplasmic RNA preparations were free of any contaminating plasmid DNA. Having confirmed this, we conducted RT-PCR for 20, 25, and 30 cycles on the cDNAs prepared from both CTE (−) and CTE (+) cytoplasmic and nuclear RNA fractions using MMTV Gag primers (OTR567/OTR552) to evaluate the effect of the presence or absence of CTE on the transport of these RNAs. RT-PCR analysis revealed that both CTE (−) and CTE (+) MMTV *gag/pol* RNA were efficiently transported to the cytoplasm (Fig. 2B), however the proteins were expressed only by the CTE (+) RNAs (Fig. 1D). These results were rather surprising since the presence of CTE has largely been implicated in the efficient transport of unspliced messages of both cellular and viral genes (reviewed in Felber et al., 2007). Because of these unexpected results, we wanted to ensure that there was no artifact in our fractionation technique resulting in the damage of the nuclear membrane, which may have contributed to the leakage of RNAs transcribed from Gag-Pol expression plasmids from the nucleus to the cytoplasm. To rule out such a possibility, cytoplasmic RNA fractions were tested by RT-PCR for the presence of unspliced β-actin mRNA, which is found exclusively in the nuclear fraction unless the integrity of the nuclear membrane has been compromised during the fractionation process (Tan et al., 1995). Since we were looking for the lack of unspliced β-actin message, it was imperative for us to ensure that this lack of unspliced β-actin message in the cytoplasmic fraction was not due to the lack of amplifiable cDNAs in PCR reactions. Therefore, the RT-PCR was performed for 25 cycles as a multiplex in the presence of primers/competimers for 18S ribosomal RNA as an ancillary control. The results

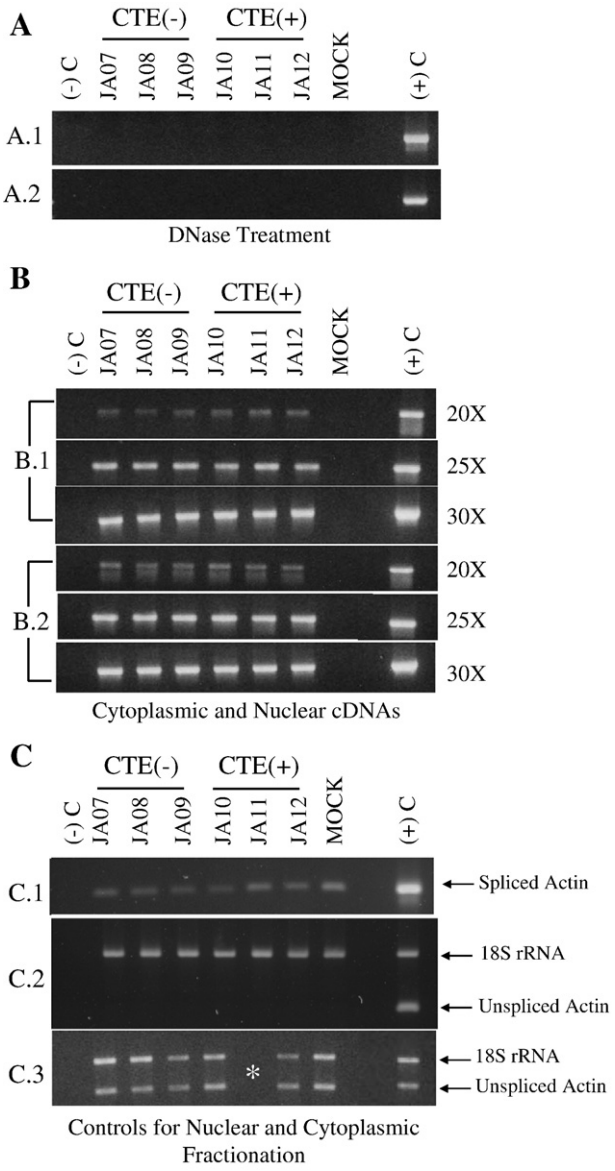

Fig. 2. Role of MPMV CTE in the transport of MMTV *gag/pol* RNA. (A) Cytoplasmic (A.1) and nuclear fractions (A.2) were isolated from the transfected 293T cells and DNase treated RNAs were amplified using MMTV Gag specific primers OTR567 and OTR552 to rule out the possibility of any contaminating plasmid DNAs from the transfected cultures (B) PCR of the cytoplasmic (B.1) and nuclear (B.2) cDNAs amplified using MMTV Gag specific primers described above, which will amplify 400 bp region of Gag. PCRs were conducted for 20, 25, and 30 cycles. (C) Control for nucleocytoplasmic fractionation technique. To demonstrate that the integrity of the nuclear membrane was maintained during nucleocytoplasmic fractionation, cytoplasmic RNA fractions were tested for the presence of unspliced β-actin mRNA, which is found exclusively in the nuclear fractions. C.1 shows the presence of spliced β-actin mRNA in the cytoplasmic fraction. C.2 shows the multiplex RT-PCR of cytoplasmic RNA fraction for unspliced β-actin mRNA that should be exclusively nuclear, thus could not be detected with 25 cycles of PCR. C.3 shows multiplex RT-PCR for unspliced β-actin mRNA in nuclear RNA fraction. (−) C and (+) C represents negative and positive controls, respectively; (*) denotes a sample that was lost.

of the RT-PCR show that spliced β-actin message was observed only in the cytoplasmic fraction (Fig. 2C.1). On the other hand, unspliced β-actin message was undetectable (Fig. 2C.2) even though it could be detected in the nuclear fractions (Fig. 2C.3). Amplification of 18S ribosomal RNA in multiplex PCR confirmed the presence of amplifiable cDNA. Taken together, these results suggest that no compromise was made on the integrity of the nuclear membrane during the fractionation process, thus it is reasonable to conclude that the MMTV *gag/pol* mRNAs were efficiently transported even in the absence of CTE. Therefore, the efficient MMTV *gag/pol* gene expression by the CTE

(+) RNA observed cannot be attributed to the RNA transport only and suggest that perhaps there are other underlying mechanisms, which regulates gene expression following RNA transport.

Two recent reports have shown that MMTV unspliced *gag/pol* genomic message is dependent on Rem/RmRE pathway and any perturbations in this pathway result in the inefficient transport of *gag/pol* message, consequently resulting in the lack of *gag* gene expression. One of these studies was conducted in the wild type virus by mutating the Rem protein (Mertz et al., 2005), while the other one was conducted in the presence of the entire viral sequences (expressed via cytomegalovirus (CMV) promoter and polyadenylated by bovine growth hormone (BGH) poly A) by introducing deletions in the RmRE (Müllner et al., 2008). On the other hand, the sub-genomic MMTV Gag-Pol expression plasmids described in this study contained limited viral sequences, apart from the *gag/pol* sequences, and therefore the lack of other viral sequences in these expression plasmids may have contributed in part in their efficient transport even in the absence of CTE. Consistent with this possibility, in contrast to other retroviruses, the RNA transport of MMTV sub-genomic expression plasmids containing only envelope cDNA was not found to be influenced by Rem/RmRE pathway (Müllner et al., 2008). It is therefore reasonable to hypothesize that the additional sequences present on the unspliced RNA in the two recently published studies (Mertz et al., 2005; Müllner et al., 2008) may have synergistically contributed along with the *gag/pol* sequences in entrapping *gag/pol* message in the nucleus. It would therefore be interesting to investigate how much sequences, other than the *gag/pol* in the sub-genomic context, would be needed to disrupt the RNA transport in the absence of CTE, if at all.

Several earlier and recent studies, including ours, have used sub-genomic HIV, SIV, and FIV Gag-Pol expression plasmids, however, majority of these expression plasmids have included sequences such as RRE, MPMV CTE, or cellular CTE, and at times contained β-globin intron (Bray et al., 1994; Rizvi et al., 1996; Browning et al., 2001; Bor et al., 2006; Swartz et al., 2007). When post-transcriptional regulatory elements replaced Rev/RRE function in these expression plasmids, protein expression was measured and was found to be efficiently expressed. It is therefore plausible to speculate that the *gag/pol* RNA from these sub-genomic expression plasmids may have been efficiently transported even in the absence of post-transcriptional regulatory elements if they contained only limited viral sequences other than the *gag/pol* sequences. Along the same line, Butsch et al. have analyzed the nucleocytoplasmic transport of unspliced HIV-1 *gag/pol* RNA and Gag protein production from sub-genomic expression plasmids with or without a post-transcriptional regulatory element from spleen necrosis virus (SNV) (Butsch et al., 1999). In the absence of any export element they still observed the transport of HIV-1 *gag/pol* unspliced RNA, however, the inclusion of SNV transcriptional regulatory element only modestly increased (2 folds) the cytoplasmic accumulation of unspliced RNA in sharp contrast to a significant increase in Gag protein expression (81,000 pico grams compared to no detectable Gag). The modest increase in the RNA transport in their observations cannot be sufficient to account for such a significant increase in Gag production in the presence of SNV post-transcriptional regulatory element. Thus, it is reasonable to suggest that the inclusion of MPMV CTE in MMTV Gag-Pol expression plasmids augmented MMTV Gag-Pol protein expression by enhancing the translation of MMTV *gag/pol* RNAs as has recently been proposed for the translation of unspliced mRNA containing MPMV CTE (Bor et al., 2006; Jin et al., 2003; Swartz et al., 2007).

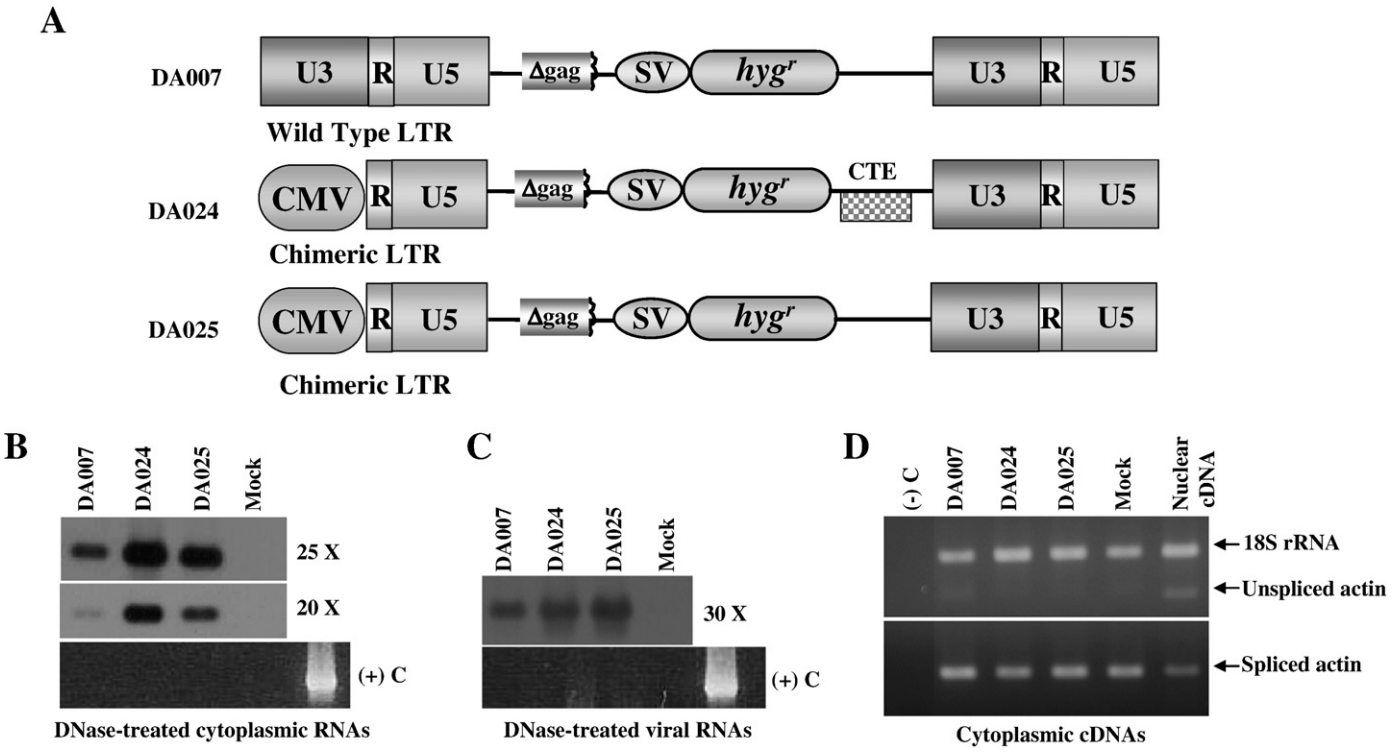

Fig. 3. Role of MPMV CTE in MMTV transfer vector RNA transport and packaging. (A) Schematic representation of MMTV transfer vectors. (B) PCR of the cytoplasmic cDNAs amplified for 20 and 25 cycles using MMTV specific primers (OTR 463 and OTR 676), which would amplify 560 bp region of 3′end of transfer vector RNA and hybridized using a probe from MMTV U3 region (Upper panel). The lower panel represents DNase-treated cytoplasmic RNAs, which were amplified using MMTV specific primers to eliminate the possibility of any contaminating plasmid DNA from the transfected cells; + C, plasmid as a positive control. (C) PCR of the viral cDNAs amplified using the same viral specific primers used for amplification of cytoplasmic cDNAs and hybridized using a probe from MMTV U3 region. PCR was performed for 30 cycles (Upper panel). As mentioned earlier for cytoplasmic RNAs, viral RNAs were also treated with DNase and amplified using MMTV specific primers to confirm the absence of any contaminating plasmid DNA (Lower panel); + C, plasmid as a positive control. (D) Control for nucleocytoplasmic fractionation technique as described in Fig. 2C and Materials and methods. The upper panel shows the multiplex RT-PCR for unspliced β-actin mRNA that should be exclusively nuclear. The spliced β-actin mRNA was present in the cytoplasmic fraction (bottom panel), while the unspliced β-actin message could not be detected with 25 cycles of PCR (upper panel); however it could be detected in the nuclear fraction used as a control (Upper panel, last lane).

Taken together, our results indicate that the lack of MMTV Gag-Pol protein expression from the CTE (−) RNAs reflects their inability to be translated efficiently and suggest that there are further links between the efficiently transported MMTV *gag/pol* RNA and its translation in the presence of MPMV CTE indicating complexities in post-transcriptional gene regulation. Thus, it is conceivable to suggest that the MMTV Gag-Pol protein expression by CTE containing RNAs may have been facilitated by enhancing polyribosome association of these RNAs as has recently been shown for HIV-1 *gag/pol* CTE containing RNA using splicing regulatory SR protein, 9G8 using 293T cells (Swartz et al., 2007). The results presented here successfully demonstrate that MMTV Rem/RmRE regulatory pathway for the expression of *gag/pol* genes in a sub-genomic context could be substituted with MPMV CTE, which interacts with cellular factors thereby obviating the need of any virally encoded factors to be provided in *cis* or *trans*. In addition, the use of heterologous transport element such as MPMV CTE should also minimize the chances of recombination with similar sequences present on the transfer vectors, thus making the *trans* complementation assay safer.

Role of MPMV CTE in the transport and packaging of MMTV transfer vector RNAs

To determine whether the particles produced by MMTV Gag-Pol expression plasmids containing the MPMV CTE are biologically relevant and capable of encapsidating and propagating MMTV transfer vector RNA, a series of MMTV transfer vectors were generated (Fig. 3A). DA007 is a transfer vector based on HYB MTV molecular clone (Shackleford and Varmus, 1988) and contains the sequences at the 5′end up to 400 bp of Gag, a region, which has been broadly implicated in augmenting RNA packaging in a number of retroviruses (reviewed in D'Souza and Summers, 2005; Darlix, 1986). At the 3′ end, it includes sequences from nucleotides 7484 until the end of the 3′LTR and therefore does not contain the newly identified Rem protein (Fig. 3A). In addition, it contains *hygromycin B phosphotransferase* gene expressed from the simian virus 40 (SV 40) promoter (SV-hyg^r) as a marker to monitor vector RNA propagation in the target cells.

We used the three plasmids *trans* complementation assay to determine MMTV transfer vector RNA packaging and propagation potentials. Towards this end, the MMTV transfer vector (DA007) and the MMTV Gag-Pol expression plasmid containing MPMV CTE (JA10) were co-transfected along with a vesicular stomatitis virus envelope protein G (VSV-G) expression construct, MD.G (Naldini et al., 1996) into 293T producer cells and the transfected cultures were dexamethasone induced as described previously (Salmons et al., 1989). Supernatants containing virus particles were harvested 72 h post-transfection and used without concentration to infect human HeLa CD4+ cells and to isolate virion RNA. Transfected 293T cells were fractionated into nuclear and cytoplasmic RNA fractions to determine the successful transport of the transfer vector RNA, while whole cell protein extracts were isolated to determine transfection efficiencies. Infected cultures were selected with medium containing hygromycin B to identify successfully transduced cells by the appearance of hygromycin resistance (Hyg^r) colonies.

The expression of transfer vector (DA007) RNA, its transport from the nucleus to the cytoplasm, and its ability to be packaged into virus particles were analyzed by RT-PCR. These analyses revealed that DA007 RNA was either poorly expressed or inefficiently transported to the cytoplasm (Fig. 3B) and consequently was packaged at a reduced level into the virus particles (Fig. 3C). Consistent with the RNA packaging results, the propagation of DA007 RNA was also poor (Table 1). These results could be explained on the basis that DA007 did not contain either MPMV CTE or an intact Rem/RmRE regulatory system to facilitate the nuclear export of the transfer vector RNA. Therefore, we generated another transfer vector (SS008), which is similar to DA007 except that *hygromycin* gene has been replaced with *enhanced green fluorescence protein (EGFP)* gene and MPMV CTE was cloned downstream of the SV-EGFP cassette (Fig. 4A). Vector propagation in case of SS008 was determined by monitoring EGFP expression by Fluorescent Activating Cell Sorting (FACS) analysis. Cloning of MPMV CTE in SS008 did not significantly enhance RNA transport (Fig. 4B) despite high transfection efficiency (data not shown) and therefore continued to show low packaging and transduction efficiencies (Figs. 4C, E, and Table 1). Since the presence of MPMV CTE in SS008 (when compared to DA007, Figs. 3B and 4B) did not significantly improve RNA transport suggesting that either its presence is not important in the subgenomic context when only limited viral sequences are present or the transfer vector RNAs were not efficiently expressed. It is therefore plausible to propose that the poor expression and consequently poor packaging and propagation in DA007 and SS008 were due to the poor transcription of these vectors containing MMTV LTR promoters in human cells.

In order to overcome the potential block of MMTV promoter and to enhance the expression of the transfer vectors in human cells, the U3 region of the 5′LTR containing the TATAA box was replaced with the human cytomegalovirus (hCMV) promoter to create a chimeric 5′LTR in DA007 and SS008, generating DA025 and SS014, respectively. In DA025 and SS014, the 3′LTR was maintained without any modifications (Figs. 3A and 4A). To further assess the role of MPMV CTE in efficiently expressed MMTV subgenomic RNAs, we cloned the MPMV CTE downstream of the SV-hyg^r cassette in DA025 or SV-EGFP cassette in SS014, generating DA024 and SS013, respectively (Figs. 3A and 4A). Following *trans* complementation, the cytoplasmic RNA fraction from the transfected cells was isolated and analyzed to monitor vector RNA expression and transport.

Test of these transfer vector RNAs (DA025, DA024, SS014, and SS013) revealed that the MMTV transcripts containing the chimeric 5′ LTR were efficiently expressed (Figs. 3B and 4B) when compared to DA007 or SS008, which contained the wild type 5′LTR, suggesting that the chimeric 5′LTR promoter can overcome the potential block of MMTV promoter in human cells. Figs. 3B and 4B show that the presence of CTE in the transfer vector (DA024 and SS013) did not have pronounced effect on the transport of these RNAs when compared to the vectors that were lacking the MPMV CTE (DA025 and SS014). This is despite the fact that at least in the case of SS014, which showed lower transfection efficiency when compared to SS013 (data not shown), transfer vector RNA was still properly transported (Fig. 4B). Since these results were unexpected, we made sure that no compromise was made on the integrity of the nuclear membrane, which could have leaked the

Table 1
Propagation of MMTV transfer vector RNAs in the presence or absence of MPMV CTE

Transfer vector	Description of the transfer vector	Marker gene	Titers (CFU/ml±SD)[a,b]	% EGFP positive cells[c]
DA007	Wild type MMTV LTR+400 bp Gag	*Hygromycin*	47±4	–
DA024	Chimeric MMTV LTR+400 bp Gag+CTE	*Hygromycin*	1530±41	–
DA025	Chimeric MMTV LTR +400 bp Gag	*Hygromycin*	714±8	–
SS008	Wild type MMTV LTR+400 bp Gag +CTE	*EGFP*	–	0.2
SS013	Chimeric MMTV LTR+400 bp Gag+CTE	*EGFP*	–	18.5
SS014	Chimeric MMTV LTR+400 bp Gag	*EGFP*	–	8.9
Mock	No DNA (control)	–	≤1	≤1

[a] CFU/ml, colony forming units per ml of non-concentrated supernatant from the transfected cultures. SD, standard deviation.
[b] Each value represents a mean of three experiments performed in duplicates.
[c] EGFP positive cells represents the phenotypic analysis of the transduced target cells. Single cell suspension of the infected HeLa CD4+ cells were prepared and 10,000 cells per group were counted and analyzed using Becton Dickinson FACS.

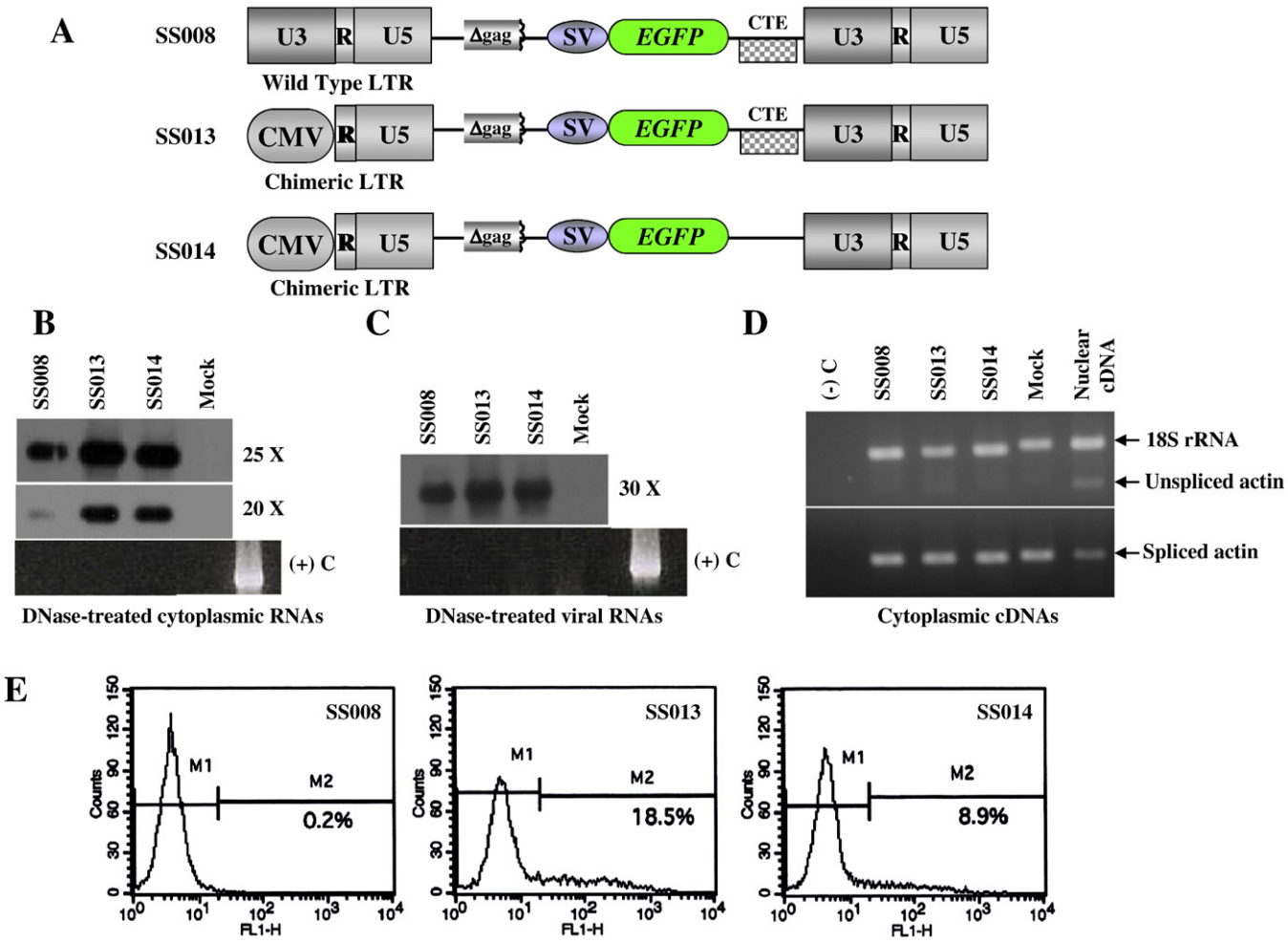

Fig. 4. Role of MPMV CTE in packaging and propagation of MMTV transfer vector RNAs. (A) Schematic representation of MMTV transfer vectors. SS008, SS013, and SS014 are transfer vectors similar to DA007, DA024 and DA025, respectively, containing *EGFP* as a marker gene instead of *hygromycin*. In addition, SS008 contains MPMV CTE when compared to DA007. (B) PCR of the cytoplasmic cDNAs amplified using MMTV specific primers for 20 and 25 cycles as described in Fig. 3B. (C) PCR of the viral cDNAs amplified using the same viral specific primers and probed as described in Fig. 3C. (D) Control for nucleocytoplasmic fractionation technique as described in Fig. 3D. (E) Flow cytometric analysis of the transduced target cells by FACS. Forty-eight hours post infection, cells were trypsinized, washed with PBS, and ten thousand cells were analyzed for the expression of *EGFP* from cells that may have been transduced by MMTV transfer vector RNA containing *EGFP* gene.

transfer vector RNA from the nucleus to the cytoplasm during the fractionation process. To ensure this, cytoplasmic RNA fractions were tested via RT-PCR by detecting the unspliced and spliced β-actin mRNA. The absence of unspliced β-actin message in the cytoplasmic RNA fraction (in either the presence or absence of CTE) assured that our fractionation technique was intact (Figs. 3D and 4D). Therefore, the transport of the transfer vector RNA we observed was bonafide and not compounded by any technical artifacts.

The packaging of DA024, DA025, SS013, and SS014 into the viral particles was determined by RT-PCR on the viral RNAs, which suggested that the presence of CTE did not significantly influence the packaging of these RNAs (Figs. 3C and 4C) and was in agreement with the vector RNA transport data (Figs. 3B and 4B). Finally, propagation of these transfer vector RNAs was determined by infecting the target cells with virions produced from the transfected 293T cells. Vector propagation was monitored either by selecting the target cells with media containing hygromycin B in the case of DA025 and DA024 or by monitoring EGFP expression by FACS analysis in the case of SS014 and SS013. Consistent with the packaging data, these analyses revealed that the transfer vector RNAs, with or without CTE, were propagated efficiently and were within a range of two folds (Table 1 and Fig. 4E).

Taken together, these results suggest that while MPMV CTE is required for the expression of MMTV *gag/pol* genes, its role in the transport, expression, and packaging of MMTV transfer vector RNA was not found to be important. This differential role of MPMV CTE could possibly be explained on the basis that MPMV CTE is required when *gag/pol* sequences are present in totality to overcome the effect of the putative negative regulatory elements (NREs) within the *gag/pol* region. Similar situation has been observed in the case of HIV and SIV requiring Rev/RRE regulatory system for *gag/pol* expression, the function of which could be compensated by MPMV CTE (Bray et al., 1994; Rizvi et al., 1996). Since our transfer vectors contained only 400 bp of Gag, the putative NREs may not be present in this short region. As for the LTR sequences in our transfer vectors, it has recently been shown that MMTV LTR region does not contain *cis*-acting repressor or inhibitory sequences requiring a regulatory pathway such as Rev/RRE for their transport from the nucleus to the cytoplasm (Dangerfield et al., 2005).

The three plasmids *trans* complementation assay described here for MMTV is the first demonstration in which MMTV genome has been separated into two separate plasmids and pseudotyped by an unrelated viral envelope glycoprotein. So far, successful development of MMTV Gag-Pol expression plasmids has evaded most investigators since cloning of MMTV *gag/pol* sequences have been greatly hampered due to the presence of inhibitory sequences in the *gag* gene (Shackleford and Varmus, 1988) and much was not known about how MMTV regulates its gene expression. Because of these caveats, to

our knowledge, no study so far has been successful in developing MMTV Gag-Pol expression plasmids capable of producing MMTV virus particles efficiently. Expression plasmids containing MPMV CTE (JA10- JA12) not only express *gag/pol* genes, but also the virus particles produced by them are capable of packaging and propagating the MMTV RNA in the biologically relevant assay described here. This *trans* complementation assay, in which we have separated the ability to express *gag/pol* genes from the source of the packageable RNA should also allow us to mutate the putative MMTV packaging sequences in the subgenomic viral context, since the packaging sequences have been shown (for a number of retroviruses) to extend into *gag* gene (D'Souza and Summers, 2005; Lever, 2007).

As discussed earlier, the inducible and tissue-specific control of MMTV promoters has brought to the limelight the idea of developing MMTV based vectors for "targeted" and "conditional" gene expression especially of those genes whose constitutive expression could be toxic to cells. The present study was undertaken to test the proof of principle towards developing an *in vivo* packaging and transduction assay for MMTV. Some of the vectors described here, for this purpose, contained the chimeric LTR harboring hCMV promoter to increase the vector RNA expression in the producer cells and in addition needed an internal promoter to express the marker genes in target cells. However, following infection, during the course of reverse transcription of the transfer vector RNA, the U3 region of the 3′LTR will be duplicated and one copy will be transferred to the 5′LTR resulting in the restoration of the wild type LTR in the integrated provirus. Such a translocation of the U3 region containing promoter sequences could bring the expression of the therapeutic gene under the influence of the LTR promoter, facilitating its inducible expression, thereby obviating the need of using an internal promoter for the expression of the gene of interest. Alternatively, MMTV promoters could replace SV 40 promoter, used for expressing *hygromycin* or *EGFP* genes in our transfer vectors, for inducible gene expression as has recently been shown (Klein et al., 2008). The results presented in this study strongly suggest that MMTV based vectors can transduce genes efficiently and such vectors can be further modified to be used for *in vivo* gene delivery studies. The *trans* complementation assay described here should also open new avenues of research towards delineating the packaging determinants for MMTV in a quantitative fashion, which is imperative for the development of safe and efficient MMTV based vectors for potentially "inducible" and "targeted" gene delivery in the fight against a number of human ailments.

Materials and methods

Plasmid construction

All plasmids described were constructed using the HYB MTV molecular clone (Shackleford and Varmus, 1988). In order to generate MMTV Gag-Pol expression constructs, sequences from HYB MTV containing *gag/pol* region between BstEII (nt 1277) and HincII (nt 7147) sites were ligated into the *Eco*RV site of an expression plasmid containing the pBR322 ori, hCMV promoter, and bovine growth hormone (BGH) poly A signal. The ligated DNA mixture was transformed into a number of bacterial strains (JM109, DH5α, STBL-1, and HB101) to stabilize the clones because most of the transformants either did not survive or had deletions in the sequences cloned in the expression plasmid. Out of the several hundred clones screened, only three clones were successfully identified from HB101 bacterial strain, which did not have any deletions in the *gag/pol* genes and were named JA07-JA09 (Fig. 1B). Next, a 234 bp DNA fragment with flanking EcoRV site containing MPMV CTE was cloned into the blunted NotI site of JA07-JA09, and the resulting clones containing the CTE in the sense orientation were named JA10-JA12, respectively (Fig. 1C).

MMTV transfer vectors (Figs. 3A and 4A) were created through several stages of cloning. Briefly, the 5′ sequences of MMTV genome up to 400 bp of Gag was amplified using OTR551 (sense, 5′ gcatcgatAATGCCGCGCCTGCAGCAGA 3′; nucleotides 1–20 of HYB MTV 5′ U3 region, sequences in lowercase artificially create a ClaI site) and OTR552 (antisense, 5′ cgactagtgatatcGTTCCCCTGGTCCCA-TAAG 3′; nucleotides 1885–1867 of HYB MTV Gag, sequences in lowercase artificially create SpeI and EcoRV sites) and HYB MTV as the template. Using the same template, the MMTV 3′ region was PCR amplified using OTR553 (sense, 5′ gcactagtgctagcCCTA-CATGGTTCTGGGAAAATTCT 3′, nucleotides 7485–7509 of HYB MTV envelope, sequences in lowercase artificially create SpeI and NheI sites) and OTR554 (antisense, 5′ cagggtaccGCTGCCGCAGTCGGCCGACC 3′; nucleotides 9877–9857 of HYB MTV, sequences in lowercase artificially create a KpnI site). The resulting 5′end and 3′end PCR products were digested separately with ClaI/SpeI and SpeI/KpnI, respectively and ligated to the ClaI and KpnI sites of a pUC derived pIC (Marsh et al., 1984) based cloning vector, TR277, in a three-way ligation creating DA001. This was followed by insertion of the SV-hyg^r cassette at the artificially created NheI site of DA001 creating the final clone DA007. To replace the MMTV U3 region containing the promoter sequences, the R/U5 region up to 400 bp of Gag was amplified using OTR617 (sense, 5′ cgcaagcttgagctcGAGTAAACTTGCAACAGTCC 3′; nucleotides 1163–1182 of HYB MTV 5′end sequences in lowercase artificially create HindIII and SacI sites) and OTR552 as the anti-sense primer. The resulting PCR product was digested with SacI and SpeI and was ligated along with the SpeI-KpnI fragment from DA001 (containing the 3′ region of MMTV) to the SacI-KpnI sites of a pcDNA3 based vector (PP071) in a three way ligation generating an intermediate clone DA018. Next, SV-hyg^r-MPMV CTE cassette was inserted at the artificially created NheI site of DA018 creating the final clone DA024. DA025 is a transfer vector similar to DA024 and was created by the insertion of the SV-hyg^r cassette without CTE at the artificially created NheI site of DA018 as described above. Another set of transfer vectors namely SS008, SS013, and SS014 were generated, which are similar to DA007, DA024, and DA025, respectively, however contain *EGFP* as a marker gene instead of *hygromycin*. In addition, SS008 differs from DA007 in another aspect in that it contains MPMV CTE in the presence of the wild type 5′LTR.

Transfections and infections of cells

The producer 293T cells were used for transfection and the HeLa CD4$^+$ cells were used for infection and both were maintained at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 7% calf serum, respectively. Approximately 16 h before the transfection, 293T cells were seeded in six-well plates at a density of 0.4×10^6 per well. Approximately, 2–4 h before transfection, media was changed and the cells were transfected by calcium phosphate method as described earlier (Mustafa et al., 2005). Briefly, a total of 10 microgram (μg) of DNA (3.3 μg of each plasmid) was used to prepare the DNA cocktail. As a control for the transfection efficiency, 250 ng of the firefly luciferase expression vector pGL3 control (Promega, Madison, WI) was also introduced in the DNA cocktail. Transfection efficiencies in the cultures were determined by testing a portion of the cells using the Dual-Luciferase Reporter Assay System (Promega). The luciferase values obtained were normalized to the protein concentration in the lysates as determined by the protein assay reagent.

Cells that were transfected with MMTV transfer vectors containing 5′wild type LTR (DA007 and SS008) were induced by 10^{-6} M dexamethasone as described previously (Salmons et al., 1989). Virus released in the supernatants was harvested 72 h post transfection, cleared of any cell debris, and used to infect HeLa CD4$^+$ cells, which were plated a day prior to infection at a density of 0.4×10^6 per 60-millimeter plate in the presence of DEAE-dextran sulfate as described previously (Browning et al., 2001). Forty-eight hours post infection, cells were selected with media containing hygromycin B or trypsinized, washed with PBS, and analyzed on a Becton Dickinson Fluorescent

Activating Cell Sorting (FACS) using the CellQuest software. Hyg^r colonies were selected for 10–12 days with media containing hygromycin B, stained with crystal violet dye, and counted as described previously (Browning et al., 2001).

Ultracentrifugation of virus particles

In order to remove cellular debris, supernatants from tranfected cultures were centrifuged at 4000 rpm for 10 min. Following which, viral particles were filtered using 0.2-micron syringe filters and pelleted by ultracentrifugation using SW28 rotor at 24,000 rpm for 2 h at 4 °C. TNE buffer (50 mM Tris–Cl, pH 7.4, 100 mM NaCl, and 1 mM EDTA, pH 8.0) was used to resuspend the viral pellets to isolate viral RNA using the Trizol LS reagent.

Nucleocytoplasmic fractionation of transfected cells and RNA isolation

To fractionate RNA into nuclear and cytoplasmic fractions, transfected cells were taken off from the plates and resuspended in cold diethylpyrocarbonate (DEPC)-treated RLN buffer (50 mM Tris pH 8, 140 mM NaCl, 1.5 mM $MgCl_2$) supplemented with 0.5 % NP40 and incubated on ice for 2 min to gently lyse the cells without disrupting the nuclei. The intact nuclei were spun down at 300 ×*g* for 2 min and 70% of the cytoplasmic fractions were carefully moved to fresh eppendorf tubes containing 1 ml Trizol reagent for subsequent RNA isolation. Nuclear RNA was also isolated from the nuclear pellets by Trizol method. Pelleted virions in TNE buffer were lysed in 500 μl of Trizol LS reagent containing 8 μl of polyacryl (used as a carrier) prior to RNA isolation. Purified RNA was resuspended in nuclease-free water and its concentration was determined by spectrophotometry.

Reverse transcriptase polymerase chain reaction (RT-PCR)

To ensure that our RNA preparations were free of any DNA contamination, 2.5 μg of cytoplasmic and nuclear RNA fractions as well as one fifth of viral RNA were DNase treated and reverse transcribed as described previously (Mustafa et al., 2005). The PCRs for unspliced β-actin were performed as a multiplex in the presence of primers/competimers for 18S ribosomal RNA as a control for cDNA addition in PCR reactions (18S Quantum competimer control, Ambion, Austin, TX). PCR reactions were performed for varying cycles at various annealing temperatures depending upon the melting temperature of the primer set used. To monitor the transport of CTE (−) and CTE (+) *gag/pol* RNA, both DNased cytoplasmic and nuclear fractions were amplified using MMTV gag specific primers OTR567 (sense, 5′ ccactagtATGGGGGTCT CGGGCTC 3′; nucleotides 1485–1501 of HYB MTV Gag, sequences in lowercase represents two dummies bp followed by SpeI) and OTR552, which would amplify 400 bp region of Gag. To study vector RNA transport and packaging, both cellular and viral DNased RNAs as well as cDNAs were amplified using MMTV specific OTR 643 (sense, 5′ cctctagaTGCGAAGAGCCTTGACCAAG 3′; sequences in lowercase represents 2 dummies bp followed by XbaI site followed by 8482–8501 nt of MMTV 3′ LTR) and OTR 676 (antisense; 5′ GAGGTTGAGCGTCTCTTTCTATT 3′; nucleotides 9067–9048 of U3 region of MMTV 3′ LTR), which would amplify 560 bp region of the transfer vector RNA. Products of PCR were analyzed on 2% agarose gels in the presence of ethidium bromide. Some of the gels were further processed for Southern blot analysis. Probes for hybridization were generated by PCR amplification using OTR643 and OTR676 and hybridization was performed by using the AlkPhos Direct Labeling Kit (Amersham, Arlington Heights, IL).

Western blot analysis

For western blot analysis, cellular lysates were prepared from the 293T cells transfected with MMTV Gag-Pol expression plasmids 72 h post transfection. 200 μg of cellular lysates were used for the detection of viral protein expression using antibodies specific for MMTV Gag protein as described earlier (Mustafa et al., 2000). As a control for protein loading, the same blot was stripped and reprobed with antibodies against cellular house keeping gene, β-actin.

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