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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

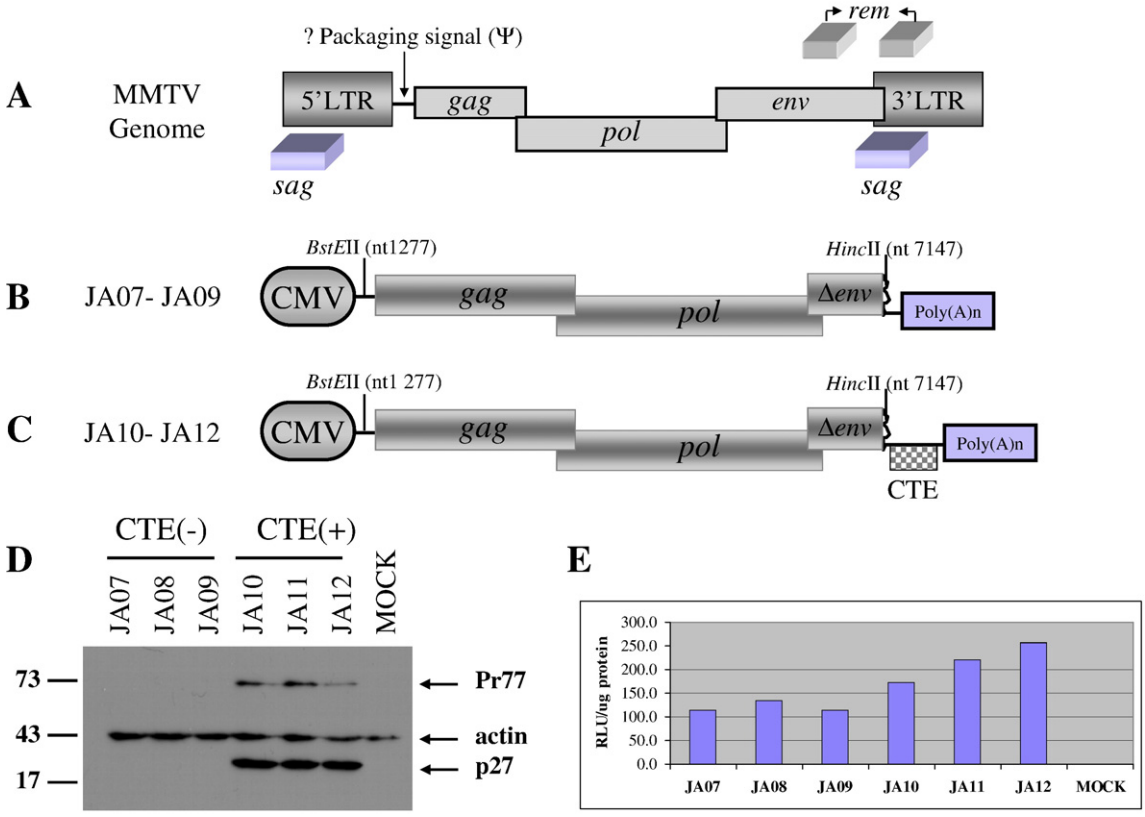

Fig. 1. MMTV *gag/pol* gene expression is mediated by MPMV CTE. (A) Schematic representation of a complete MMTV genome. (B and C) MMTV Gag-Pol expression plasmids with and without MPMV CTE. (D) Western blot analysis of transfected 293T cell lysates with MMTV Gag-Pol expression plasmids. Both processed 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.