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Adaptive Evolution of a Tagged Chimeric Gammaretrovirus: Identification of Novel *cis*-Acting Elements that Modulate Splicing

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Retroviruses are well known for their ability to incorporate envelope (Env) proteins from other retroviral strains and genera, and even from other virus families. This characteristic has been widely exploited for the generation of replication-defective retroviral vectors, including those derived from murine leukemia virus (MLV), bearing heterologous Env proteins. We investigated the possibility of "genetically pseudotyping" replication-competent MLV by replacing the native *env* gene in a full-length viral genome with that of another gammaretrovirus. Earlier, we developed replication-competent versions of MLV that stably transmit and express transgenes inserted into the 3′ untranslated region of the viral genome. In one such tagged MLV expressing green fluorescent protein, we replaced the native *env* sequence with that of gibbon ape leukemia virus (GALV). Although the GALV Env protein is commonly used to make high-titer pseudotypes of MLV vectors, we found that the *env* replacement greatly attenuated viral replication. However, extended cultivation of cells exposed to the chimeric virus resulted in selection of mutants exhibiting rapid replication kinetics and different variants arose in different infections. Two of these variants had acquired mutations at or adjacent to the splice acceptor site, and three others had acquired dual mutations within the long terminal repeat. Analysis of the levels of unspliced and spliced viral RNA produced by the parental and adapted viruses showed that the mutations gained by each of these variants functioned to reverse an imbalance in splicing caused by the *env* gene substitution. Our results reveal the presence of previously unknown *cis*-acting sequences in MLV that modulate splicing of the viral transcript and demonstrate that tagging of the retroviral genome with an easily assayed transgene can be combined with *in vitro* evolution as an approach to efficiently generating and screening for replicating mutants of replication-impaired recombinant viruses.

Keywords: retrovirus; murine leukemia virus; envelope; evolution; splicing

Introduction

The genomic RNA of simple retroviruses such as murine leukemia virus (MLV) encodes three genes, *gag*, *pol*, and *env,* which are each indispensable for replication. The Gag and Pol proteins are translated from the full-length, unspliced transcript, which also serves as the packaged and transmitted genome. A fraction of the transcript undergoes a splicing event that places the *env* open reading frame near the 5′ cap, permitting translation of the

Abbreviations used: MLV, murine leukemia virus; Env, envelope; IRES, internal ribosome entry site; GFP, green fluorescent protein; GALV, gibbon ape leukemia virus; MFI, mean fluorescence intensity; MOI, multiplicity of infection; RT, reverse transcriptase.

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surface and transmembrane subunits of the envelope (Env) protein, which mediates the binding and entry of the virus into the host cell through interaction with cognate receptors on the cell surface. Retroviruses have evolved Env proteins that utilize a variety of cellular receptors,[1] and retrovirus particles can functionally incorporate Env proteins from other retroviral strains and genera, and even from other virus families, when expressed in the same cell. This attribute has been exploited extensively to generate pseudotyped replication-defective retroviral vectors that exhibit redirected or broadened tropism and greater biophysical stability.[2] Furthermore, "genetically pseudotyped" infectious retroviruses can arise during infection by natural recombination events that result in replacement of *env* sequences with those of a co-infecting or endogenous virus,[3,4] and such *env* gene alterations can play an important role in virulence or pathogenicity and cross-species transmissibility.

The construction of chimeras between different strains, species or genera of retroviruses represents a useful strategy for investigating retrovirus biology, and a large number of such chimeras have been reported.[5–10] However, production of replication-competent chimeric viruses requires not only that the proteins encoded by the heterologous sequence functionally substitute for any replaced proteins, but also that the modification of the viral genome itself not impair replication. In fact, artificially constructed chimeric retroviruses are very often partially or completely replication-impaired.[7,8,10–13] The availability of fully replication-competent forms of such viruses would permit more thorough characterization and provide new tools for probing retroviral biology, and the identification of mutations that impart or improve replication competence may itself provide novel insights into retroviral replication.

One elegant approach to circumventing a block to replication in an impaired virus, and which does not require knowledge of the mechanism responsible for the block, is to employ adaptive evolution. The methodology used in this study combines adaptive evolution with genetic tagging of the virus to simplify identification of efficiently replicating mutants. We previously developed replication-competent variants of MLV containing an internal ribosome entry site (IRES)-transgene insert in the virus's 3′ untranslated region (UTR)[14–16] These viruses replicate to high titer and can efficiently transmit and express exogenous genes in mammalian cells. Additionally, the use of a fluorescent reporter transgene, such as green fluorescent protein (GFP), in these viruses allows spread to be easily tracked and quantified by flow cytometry or by fluorescence microscopy. The IRES-GFP cassette is very well tolerated in the MLV genome, is retained over a large number of cell-free passages in culture, and its presence is compatible with replication kinetics comparable to wild-type virus.

Here, we employed this tagged retrovirus system to monitor the consequences of experimentally introduced genetic alterations to the viral genome. We describe a GFP-tagged chimeric virus in which the *env* gene in an MLV genome was replaced with the *env* of another gammaretrovirus, gibbon ape leukemia virus (GALV). While the amphotropic MLV Env protein utilizes the Pit-2 phosphate transporter for viral entry, the GALV Env protein utilizes the Pit-1 phosphate transporter.[1] Surprisingly, although the GALV Env protein is commonly used to make high-titer pseudotypes of standard MLV vectors,[17–19] the chimeric virus exhibited greatly reduced replication kinetics.

However, retroviruses display a high inherent rate of mutation, thought to be in large part a consequence of the error-prone nature of reverse transcriptase,[20] they can evolve rapidly to overcome obstacles to their propagation. This property has proven experimentally useful for the generation of recombinant retroviruses with desirable qualities and in the analysis of retrovirus proteins.[21–23] In this study, we took advantage of this property to select for variants of the MLV-GALV chimera that had acquired replication-enhancing mutations. We found that prolonged cultivation of cells exposed to the chimeric virus allowed the natural selection of rapidly replicating variants and that repeated attempts to generate such variants resulted in the selection of viruses with mutations in different locations. Our results reveal previously unidentified elements in MLV that control the extent to which the viral transcript undergoes splicing, and illustrate a novel approach to generating and identifying efficiently replicating variants of replication-impaired recombinant retroviruses.

Results

Construction and adaptive evolution of a tagged MLV-GALV chimera

To evaluate the possibility of altering the receptor specificity of MLV by incorporation of a heterologous *env* gene, we replaced the *env* gene of a GFP-tagged, replication-competent proviral clone of MLV[15] with that of GALV. In the resulting construct, designated GZAP-GFP (Figure 1(a)), a short portion of the signal peptide-coding sequence of the MLV *env* was retained in order to avoid alteration of the MLV *pol* sequence that overlaps with *env*. The retroviral Env signal peptide serves to target the Env protein for translation in the endoplasmic reticulum. The Env of GZAP-GFP thus contains a hybrid signal peptide, but is expected to be of fully GALV origin when mature, according to analysis by the SignalP program.[24] Whereas the Env signal peptide of MLV is 33 amino acid residues,[25] that of GZAP-GFP and wild-type GALV were predicted by SignalP to be 35 and 42 amino acid residues, respectively. Because the length of the hybrid signal