

The major homology region of bovine leukaemia virus p24^{gag} is required for virus infectivity *in vivo*

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In order to gain insight into the role of the major homology region (MHR) in the infectious potential of bovine leukaemia virus (BLV), mutations were introduced into the capsid gene of an infectious molecular clone. A provirus that was designed to contain only a slightly modified version of the MHR (substitution of phenylalanine 147 with a tyrosine) was still infectious *in vivo*. Furthermore, the provirus loads were not significantly different from those obtained with a wild-type virus. A second mutant was designed to analyse a mild modification of the MHR at the level of arginine 150. The substitution of this residue with a lysine completely destroyed the infectious potential of the recombinant virus. Finally, a third mutant that was deleted in the MHR region was unable to infect the host. Thus it appears that the integrity of the MHR domain is essential for BLV infectivity *in vivo*.

The retrovirus *gag* genes encode three main structural proteins: matrix (MA), capsid (CA) and nucleocapsid (NC) (Leis *et al.*, 1988). These proteins appear to exert similar functions in the replicative cycle of all retroviruses. However, there is little amino acid conservation among different retroviruses except for a stretch of 20 amino acids within the capsid gene that has been called the major homology region (MHR) (Wills & Craven, 1991). Despite this high conservation during evolution, mutations in the MHR region have remarkably different effects. Indeed, most deletions or insertion mutations in the CA domain of murine leukaemia virus blocked the assembly of the mutant proteins into virions (Hansen *et al.*, 1990). By contrast, most of the CA domain (including the MHR) of Rous sarcoma virus (RSV) was found to be

dispensable for particle assembly and release (Craven *et al.*, 1995). Deletions in the MHR domain of human immunodeficiency virus (HIV) blocked replication in cell culture and reduced the ability to form virus particles (Dorfman *et al.*, 1994). Within the MHR domain, three residues, Gln-138, Glu-142 and Arg-150, are always conserved among different retroviruses (Wills & Craven, 1991). A fourth residue (either Tyr or Phe) at position 147 appears to be invariably aromatic. These sequences also overlap a T cell epitope that is recognized in bovine leukaemia virus (BLV)-infected animals (Majer *et al.*, 1994). In fact, purified p24 capsid protein stimulates the incorporation of [³H]methyl-thymidine into CD4⁺- and CD8⁺-enriched T cell populations. By peptide scanning of the p24 sequence, two major epitopes were identified within the p24 protein at positions 31 to 55 (PGSQVWIQTLRLAILQAD-PTPADLE) and 141 to 165 (AESYVEFVNRLQISLADNLPD-GVPK). This last epitope thus coincides with the MHR located between amino acids Ile-136 and Leu-155.

In order to evaluate the importance of this region for the infectious potential of BLV, recombinant proviruses were constructed and their behaviour was analysed *in vivo* (Willems *et al.*, 1994). The provirus 344, which was cloned into the plasmid pBLVHind, was previously shown to be infectious *in vivo* and to induce tumours in sheep. We therefore used this provirus to evaluate the importance of the MHR region. We first determined the nucleotide sequence of its p24 gene. It appeared that the p24^{gag} nucleotide sequence of the 344 provirus was highly related to that of the T15 variant (Rice *et al.*, 1987). Only 10 point substitutions differed between the two (data not shown). In terms of protein sequence, a single amino acid was changed: the Val at position 209 was replaced by an Ile.

In order to gain insight into the role of the MHR region on the infectious potential of BLV, three mutants were considered. A first construct called pBLVgag147 was designed to contain only a slightly modified version of MHR. The residue at position 147 appears to be invariably aromatic, being either a Phe in the BLV–human T-lymphotropic virus (HTLV) subfamily or a Tyr in the HIV–simian immunodeficiency virus (SIV) group. In mutant pBLVgag147, the Phe at position 147 was replaced by a Tyr generating a highly related MHR domain

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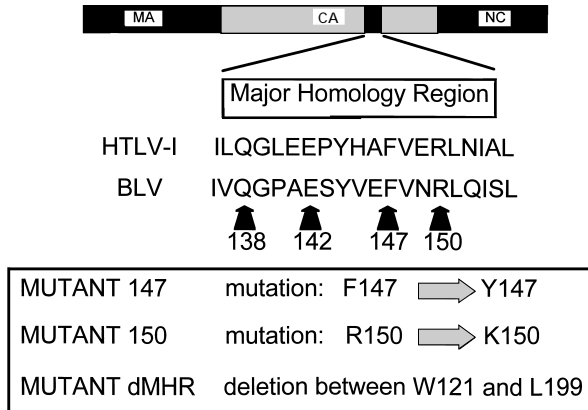


Fig. 1. Schematic representation of the Gag polyprotein and description of the mutants. The MHR is located in the BLV and HTLV-I capsid genes. The four highly conserved residues Gln-138 (Q), Glu-142 (E), Phe-147 (F) and Arg-150 (R) are indicated. To gain insight into the role of the MHR region in the infectious potential of BLV, two substitutions were introduced at amino acids Phe-147 and Arg-150. The first mutation (Phe-147 → Tyr) was designed to give a highly similar version of the MHR. The second substitution was performed to analyse a mild modification of the MHR (Arg-150 → Lys). Finally, a third mutant was deleted of the entire MHR region between residues Trp-121 and Leu-199.

(Fig. 1). A second mutant was designed to analyse a mild modification of the MHR. Indeed, among the four highly conserved residues within the MHR, three of them, Gln-138, Glu-142 and Arg-150, are always conserved among the different retroviruses (Fig. 1). In mutant pBLVgag150, the Arg-150 was replaced by a Lys. This substitution maintains the basic hydrophilic character of the amino acid but modifies the length of the side chain. Finally, a third mutant was designed to evaluate the importance of the entire MHR region in the infectious potential of the virus. Therefore, an in-frame deletion of the sequences coding for the MHR was performed to construct provirus pBLVdMHR (Fig. 1).

To construct the three recombinant proviruses, site-directed mutagenesis was performed by PCR of the pSGgag template plasmid using synthetic oligonucleotides that contain the mutations. The pSGgag construct was obtained by the insertion of the 1.7 kb *SacI*–*Bam*HI insert of pBLVHind, which contains an infectious BLV provirus, into the *SacI*–*Bgl*II sites of the pSG5 vector. The resulting mutagenized plasmids harbouring the Phe-147 → Tyr-147 and Arg-150 → Lys-150 mutations were called pSGgag147 and pSGgag150, respectively.

To construct the pSGgagdMHR plasmid, a deletion with the *Stu*I and *Pst*I restriction enzymes was performed on unmethylated pSGgag DNA. The joining of both ends was performed by insertion of two oligonucleotides, 5' CCTG-GAAGCTTCTGCA 3' and 5' GAAGCTTCCAGG 3'. The resulting plasmid pSGgagdMHR thus contained a deletion of the gag coding sequence between the *Stu*I (position 1102 following the numerotation of Rice *et al.*, 1987) and *Pst*I (at position 1343) restriction sites.

Three recombinant proviruses were constructed with the modified gag genes from plasmids pSGgag147, pSGgag150 and pSGgagdMHR. Therefore, the *Nco*I (at position 577) to *Clal* (at position 1464) inserts from these vectors were exchanged with the corresponding fragment of the pBLVHind plasmid yielding pBLVgag147, pBLVgag150 and pBLVdMHR constructs. All the constructs were completely sequenced in order to check for the presence of the desired mutation and for the lack of modification due to *Taq* DNA polymerase mistakes. The integrity of the recombinant proviruses was checked by restriction analysis and sequencing. Furthermore, the expression of the Tax and p24^{gag} proteins was measured in transient transfection assays. No significant difference was observed between the wild-type and mutated viruses (data not shown).

The behaviour of the different proviruses was directly analysed *in vivo*. As a positive control, two sheep (animals 297 and 298) were injected intradermally with the wild-type provirus construct (cloned in pBLVIX; Willems *et al.*, 1995). These animals were maintained under controlled conditions at the National Institute for Veterinary Research (Uccle, Belgium). Seroconversion, as revealed by the presence of antibodies directed towards the envelope gp51 protein, occurred 21 and 33 days post-injection, respectively. As a control, the animals were kept together with an uninfected sheep (animals 288). Under these conditions, we did not observe natural transmission of the virus (reviewed by Kettmann *et al.*, 1994). When the pBLVgag147 recombinant was injected into sheep 213 and 299, the animals quickly seroconverted after 28 and 25 days respectively. These seroconversion times did not significantly differ from those of the wild-type virus. In contrast, the pBLVgag150 and pBLVdMHR recombinants were unable to infect their host. It thus appears that the deletion of the MHR domain destroyed the infectious potential of the virus. Even a mild mutation at the level of Arg-150 was deleterious to the virus.

From these data, we can conclude that the MHR domain is essential to the infectious potential of BLV *in vivo*. Since even a mild mutation at the level of Arg-150 kills the virus, one could have expected that the substitution of Phe-147 for Tyr also interferes with virus replication *in vivo*. Therefore, the provirus loads were measured 6 months after infection by a semi-quantitative PCR amplification. Peripheral blood circulating leukocytes from the different sheep were lysed and their DNA was amplified by 22 cycles of PCR using primers specific for the *tax* gene. The amplified fragments were then separated on an agarose gel and analysed by Southern blot hybridization using a virus probe. Under these conditions, a 1 kb fragment was amplified in samples from sheep infected with the wild-type virus (animals 298 and 299, Fig. 2). As a control for quantification, 10-fold serial dilutions of the pBLVIX plasmid DNA were amplified in parallel (Fig. 2). As a negative control for PCR contamination, no fragments were observed when the amplification was performed with PBMCs from the uninfected

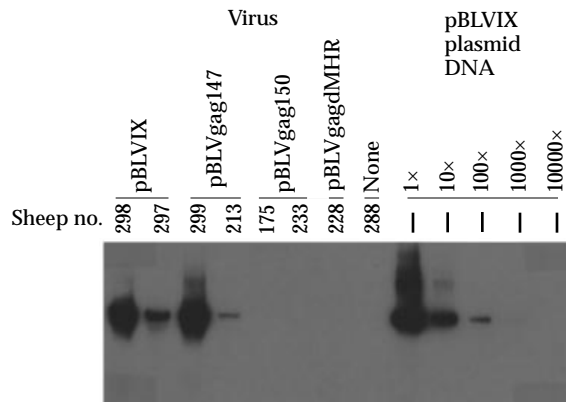


Fig. 2. Analysis of provirus loads *in vivo*. Blood samples from the sheep were cleared and washed three times in lysis buffer. The pellets were then resuspended in PCR buffer, digested with proteinase K and boiled. Ten μ l aliquots (out of 500) were amplified by 22 cycles of PCR with two oligonucleotides, A (position 6989 following the numerotation of Rice *et al.*, 1987; 5' CTCTTCGGGATCCATTACCTGA 3') and B (position 8000; 5' CCTGCATGATCTTTCATACAAAT 3'). After PCR, the samples were analysed by Southern blot hybridization using a BLV probe (SacI insert from plasmid pBLV344). Serial dilutions of the wild-type provirus cloned in plasmid pBLVIX were amplified in parallel under the same conditions. These experiments were performed at least in triplicate.

sheep 288. The PCR amplifications were also negative with PBMCs from sheep 175, 233 (injected with the pBLVgag150 recombinant) and 228 (injected with pBLVgagdMHR). The PCR data thus perfectly correlated with the presence of anti-gp51 antibodies as measured by the ELISA procedure. The provirus loads measured in sheep 213 and 299 infected with pBLVgag147 were similar to those detected in animals 297 and 298 infected with the wild-type virus (Fig. 2). It thus appears that the recombinant virus pBLVgag147 was able to propagate efficiently within the host and that the conservative substitution of Phe-147 with a tyrosine had no influence on the provirus loads.

In conclusion, we used a series of three mutants that were mutated in the MHR domain to varying degrees. A first provirus was designed to contain only a slight modified version of the MHR. In this mutant, the substitution with a tyrosine did not destroy the infectious potential of the virus. This observation should be compared with data from other systems. The substitution of Phe-147 in the HIV-1 MHR had only a moderate effect in cell culture (Mammano *et al.*, 1994). In contrast, the replacement of this phenylalanine by a tyrosine strongly reduced virus infectivity of RSV (Craven *et al.*, 1995). However, the recombinant RSV had still detectable infectivity. In the BLV system, it appears that the virus mutated at residue 147 is still infectious *in vivo* but also propagates efficiently within the animal. The provirus loads were indeed similar to those obtained with wild-type virus as measured 6 months after infection. For these three viruses, it thus appears that the phenylalanine may be switched with another aromatic residue without loss of infectious potential. This suggests that the

mutation does not significantly modify the structure of the MHR domain. Similarly, the evolutionary process appears to have selected for either aromatic residue at position 147. A tyrosine is encountered in HIV-1, HIV-2 and SIV whereas a phenylalanine appears the most frequent amino acid in the other replication-competent retroviruses (feline immunodeficiency virus, bovine immunodeficiency virus, visna/maedi virus, murine leukaemia virus, HTLV, equine infectious anaemia virus, RSV and BLV). The evolutionary conservation of the aromatic character of this residue strongly suggests that it has an important function in virus replication. Indeed, both in HIV and RSV, the removal of the extended side chain by substitution with alanine or serine prevented virus replication and affected virion morphogenesis (Mammano *et al.*, 1994; Craven *et al.*, 1995).

The second mutant that we constructed was designed to analyse a mild modification of the MHR at the level of Arg-150. The mutation of this highly conserved residue into a lysine maintains the basic hydrophilic character of the amino acid but modifies the length of the side chain. However, this substitution completely destroyed the infectious potential of the recombinant BLV. In contrast, a conservative substitution at this position was shown to have only a moderate effect on virus replication in cell culture of the HIV virus (Mammano *et al.*, 1994). In RSV, the replacement of the arginine by a glutamine decreased virus infectivity, whereas substitution by a leucine completely killed the virus (Craven *et al.*, 1995). Altogether, these data indicate that the mutation at the level of Arg-150 had remarkably different effects depending on the virus. Despite its high evolutionary conservation, this residue has some functional plasticity that still allows virus infectivity.

Finally, a third mutant was designed to evaluate the importance of the entire MHR region in virus infectivity. This drastic deletion did not allow the recombinant virus to infect sheep. This result was expected since the mutation of Arg-150 only also destroyed infectivity. However, one should recall here that most of the capsid domain of RSV, including the MHR, was found to be dispensable for particle assembly and release (Wills & Craven, 1991). Since mutations at the level of the arginine affected infectivity it thus appears that the infectious potential requires additional steps that cannot always be analysed in cell culture.

In summary, our data highlight the biological relevance of the MHR region of BLV *in vivo* and demonstrate that the integrity of the MHR domain is essential for its infectious potential.

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