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Role of a Cytotoxic-T-Lymphocyte Epitope-Defined, Alternative *gag* Open Reading Frame in the Pathogenesis of a Murine Retrovirus-Induced Immunodeficiency Syndrome

Arti Gaur* and William R. Green

Department of Microbiology and Immunology and the Norris Cotton Cancer Center, Dartmouth Medical School, Lebanon, New Hampshire

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LP-BM5 murine leukemia virus-infected C57BL/6 mice develop profound immunodeficiency and B-cell lymphomas. The LP-BM5 complex contains a mixture of defective (BM5def) and replication-competent helper viruses among which BM5def is the primary causative agent of disease. The BM5def primary open reading frame (ORF1) encodes the single *gag* precursor protein (Pr60^{gag}). Our lab has recently demonstrated that a novel immunodominant cytotoxic-T-lymphocyte (CTL) epitope (SYNTGRFPPL) is expressed from a +1-nucleotide translational open reading frame of BM5def during the course of normal retrovirus expression. The SYNTGRFPPL CTL epitope may be generated from either of two initiation methionines present, ORF2a or ORF2b, located downstream of the ORF1 initiation site. This study investigates the role(s) of the alternative ORF2-derived *gag* protein(s) of BM5def in viral pathogenesis. We have examined the disease-inducing capabilities of mutant viruses in which the translational potential of either the initiating ORF2a or ORF2b AUG has been disrupted. Although these mutated viruses are capable of wild-type ORF1 expression, they are unable to induce disease. Our data strongly suggest the existence of a novel ORF2 product(s) that is required for LP-BM5-induced pathogenesis and have potentially broad implications for other retroviral diseases.

Genetically susceptible C57BL/6 (B6) mice infected with the LP-BM5 retroviral isolate (18) develop immunodeficiency similar to AIDS. This disease is called murine AIDS (MAIDS), stages of which are characterized by splenomegaly, lymphadenopathy, hypergammaglobulinemia, profound immunodeficiency, and B-cell lymphomas in the context of retroviral-induced immunodeficiency, generally similar to AIDS-related non-Hodgkin's lymphomas. Among the three classes of murine leukemia viruses (MuLV) in the LP-BM5 isolate, the BM5eco (and possibly recombinant mink cell focus-forming virus) MuLV function as helper viruses for the replication-defective BM5def, which is the primary etiological agent causing disease (1, 4, 13, 18). Two molecular clones of the defective genome, Du5H and DEF27, have been characterized from different isolates, with both revealing large common regions of *pol* and *env* that have been deleted (1, 5). The BM5def primary open reading frame (ORF1) encodes the single *gag* precursor protein (Pr60^{gag}). The BM5def *gag* sequences encoding the core proteins MA (p15), CA (p30), and NC (p10) have 80 to 90% homology to the analogous sequences in the Pr65^{gag} of other ecotropic MuLV. However, within a 25-amino acid (aa) segment at the carboxy-terminal end of p15 and another 25-aa region in p12, BM5def displays a distinctively high diversity from the nonpathogenic BM5eco and other typical replication-competent ecotropic MuLV (1, 5) (Fig. 1A).

All of the work done to understand LP-BM5-induced pathogenesis, including yeast two-hybrid screening for host proteins that bind BM5def *gag* protein, has focused conceptually on the BM5def ORF1-encoded Pr60^{gag} protein exclusively. The essential role of ORF1 in pathogenesis has been established by generating a myristylation-negative (Myr$^-$) Pr60^{gag} mutant of BM5def (Du5H clone) (12). Similar to other myristylated proteins, Pr60^{gag} on myristylation attaches to the inner leaflet of the plasma membrane. Following this, myristylated Pr60^{gag} may interact with other membrane-bound molecules leading to disease. The Myr$^-$ Pr60^{gag} mutant BM5def is unable to induce either the expansion of target B cells or the splenomegaly characteristic of MAIDS, demonstrating that an unaltered Pr60^{gag} ORF1 is necessary for viral pathogenesis (12). Other groups have also established the crucial roles of ORF1 coding regions of the Pr60^{gag} gene in pathogenesis. By examining the disease-inducing potential of MuLV-rescued BM5def carrying truncated *gag*, it has been proposed that the MA (p15)- and p12-encoding regions of BM5def *gag* are sufficient for pathogenicity (24). Also, substitution of the p15 and p12 sequences of BM5def virus with BM5eco-specific sequences eliminates its ability to induce MAIDS (17). Furthermore, by comparing chimeric viruses with mutated p12 regions, the unique nucleotide sequence in the BM5def p12^{gag} region, not homologous to BM5eco, appears crucial for MAIDS development (16, 17). Collectively, these observations (12, 16, 17, 24) have been taken as confirmation of the essential role of ORF1 in pathogenesis.

However, these studies have concentrated on modifying only nucleotide sequences and considering their resultant changes in the ORF1-derived Pr60^{gag} polypeptide. The potential that alternative open reading frames (and consequently proteins encoded by them) are embedded within the BM5def *gag* gene and may play an important role in pathogenesis of BM5def has not yet been studied. In addition to studying viral pathogenesis, our lab has focused on protective immune responses in

* Corresponding author. Mailing address: Dartmouth Medical School, Department of Microbiology and Immunology, 626W Borwell, One Medical Center Dr., Lebanon, NH 03756. Phone: (603) 650-5056. Fax: (603) 650-6223. E-mail: Arti.Gaur@dartmouth.edu.

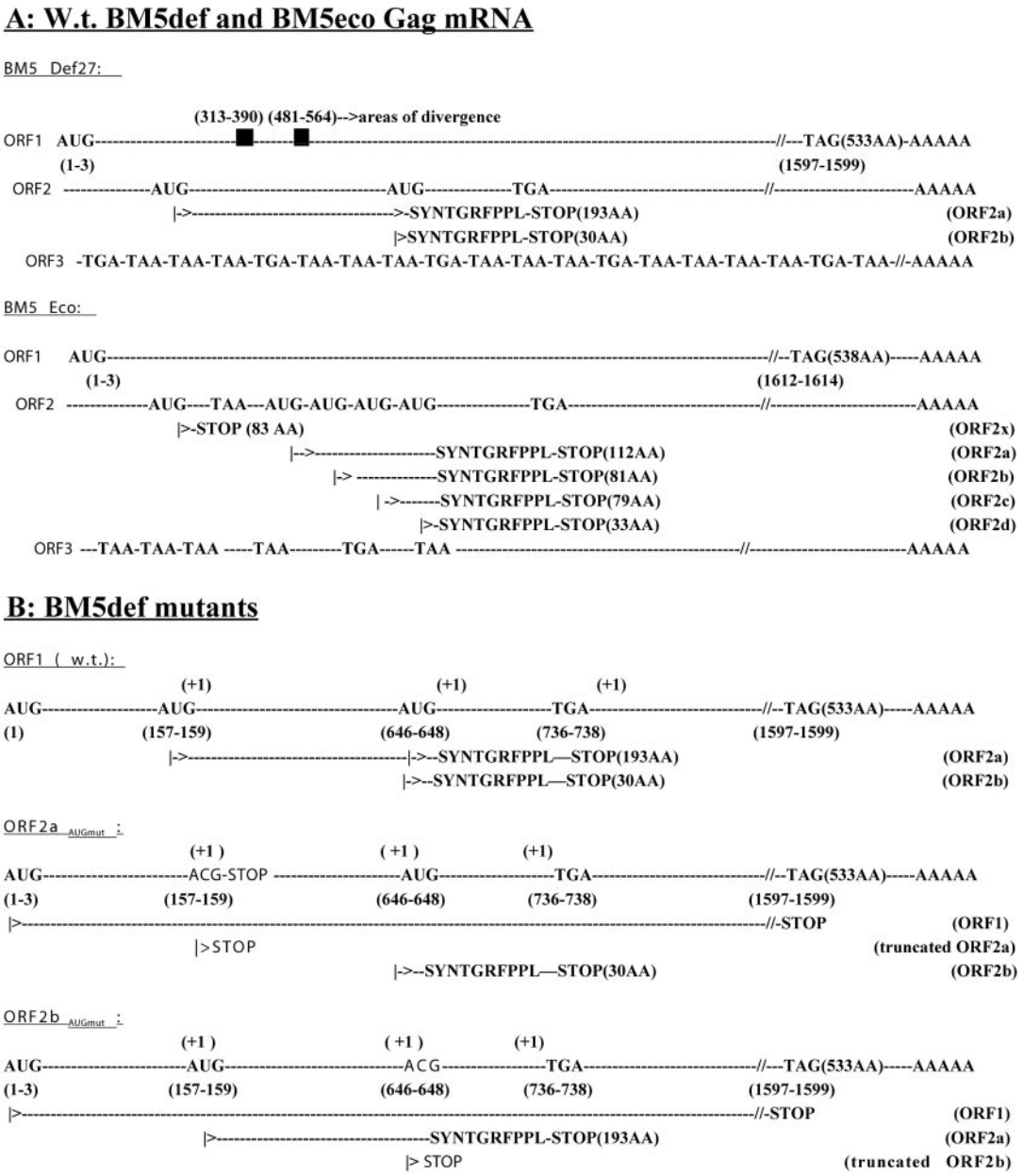


FIG. 1. (A) Differences in alternative ORF coding potentials of BM5eco and BM5def. (B) Generation of BM5defORF2a$_{AUGmut}$ and BM5defORF2b$_{AUGmut}$ viruses.

MAIDS-resistant BALB/c mice (20, 22, 26). We and others have shown that resistance in H-2^a and H-2^d mouse strains is primarily dependent on CD8$^+$ T cells (19, 20, 22, 23, 26, 27). Specifically, we have demonstrated that an H-2K^d-restricted immunodominant cytotoxic-T-lymphocyte (CTL) epitope is nontraditionally expressed from an alternative translational *gag* ORF during the course of normal in vivo and in vitro LP-BM5 retrovirus expression (22). Thus, we have shown that in MAIDS-resistant BALB/cByJ, C57BL/KsJ, and (BALB/c × C57BL/6)F$_1$ mice, CTLs raised against BM5eco or BM5def *gag* protein are directed against the conserved p30-encoding region, but in fact are specific for a novel epitope (SYNTG RFPPL) generated from a second, +1-nucleotide (nt) register defined as ORF2 (20, 22). Our lab also demonstrated that, in the context of the extended minigene constructs used for minimal epitope mapping, generation of SYNTGRFPPL depends on a naturally occurring ORF2 Met initiation codon (22). Hence, it seems likely that SYNTGRFPPL expression in the context of full-length BM5def *gag* is also the result of conventional AUG-directed translation. Thus, the SYNTGRFPPL epitope may be generated from either one of two potential initiation codons in BM5def *gag* ORF2, i.e., starting at 157 (ORF2a AUG) or 646 (ORF2b AUG) nt, respectively, downstream of the ORF1 AUG. According to the Kozak consensus sequence (15), ORF2a is in a strong, and ORF2b is in a very strong, initiation codon context (21, 22). This extended alter-

native *gag* ORF is unique for retroviral systems, especially when comparing BM5def to other ecotropic MuLV such as AKR623, Friend, and Moloney. Furthermore, given the usual frequent random occurrences of stop codons in the +1- and +2-nt registers, the extended BM5def ORF2 predicted maximum protein is unusually long at 193 aa (Fig. 1A).

Based on these observations, the present study examines the role(s), if any, of the alternative ORF2-derived *gag* protein(s) of BM5def in viral pathogenesis. Significantly, as the pathogenic BM5def viral component is replication incompetent, the putative ORF2 protein(s) it encodes cannot be crucial to replication. Rather, we hypothesize that in addition to the primary ORF1-encoded $Pr60^{gag}$, BM5def ORF2 translation proceeding from the ORF2a and/or ORF2b AUG(s) gives rise to protein(s) of 193 or 30 aa in length (maximally), and one or both of these ORF2 products are required for LP-BM5 viral pathogenesis per se.

MATERIALS AND METHODS

Viruses. LP-BM5 was prepared in our laboratory as previously described (11). The molecular clone for the wild-type (wt) BM5def, p127/A1, generously provided by Sisir Chattopadhyay, was used to generate the BM5defORF2a_{AUGmut} or BM5defORF2b_{AUGmut} mutant viruses. A QuickChange kit from Stratagene was used for site-directed mutagenesis. The primers used to generate the BM5defORF2a_{AUGmut} mutant were 5′ CCACTAGACGGTACTTTTAATTT AGAC and 3′ AATGTCTAAATTAAAAGTACCGTCTAG. To generate the BM5defORF2b_{AUGmut} mutant, the primers used were 5′ GTATTGTAACTGA CCGTTACCCCCCAAACG and 3′ CGTTTGGGGGGTAACGGTCAGTTAC AATAC.

Mice. Seven-week-old male C57BL/6NCr mice were purchased from the National Cancer Institute, housed at the Dartmouth Medical School animal facility, and infected intraperitoneally with 2.5×10^4 PFU of LP-BM5 or BM5eco-rescued wt BM5def or BM5defORF2a_{AUGmut} or BM5defORF2b_{AUGmut} viruses at 8 to 10 weeks of age.

Activational and immunosuppressive parameters of MAIDS pathogenesis. Enzyme-linked immunosorbent assay determinations of serum immunoglobulin (Ig), splenomegaly measurements, *allo*-CTL and ^{51}Cr release assays, and proliferation assays in response to mitogen were carried out as previously described (11).

BM5def *gag* GFP-tagged fusion proteins. We used the enhanced green fluorescent protein (EGFP) to generate the wt or mutant $ORF1_{AUG}$EGFP or $ORF2_{AUG}$EGFP fusion proteins. The GFP fragment was subcloned out of the pEGFP-N1 plasmid (BD Biosciences, Clontech) and in frame with the AUGs of the BM5def ORF1, BM5def ORF2a, or BM5def ORF2b.

Viral load. By using a real-time SYBR-Green/iCycler iQ-based quantitative assay that our lab has recently developed (7), quantitative reverse transcription-PCR (qRT-PCR) assays were used to quantify BM5def and BM5eco *gag* RNA levels. Primers used in this qRT-PCR assay were designed to specifically distinguish between BM5def *gag* and BM5eco *gag* and have been described previously (7). It is important to note that primers for BM5eco *gag* were generated by using unique BM5eco sequences found at base pairs 1188 and 1370 relative to the BM5eco *gag* initiating ATG and these defining nucleotides specific to BM5eco (and not to BM5def) were positioned at the 3′ end.

RESULTS AND DISCUSSION

Presence of alternative reading frames in BM5def and generation of BM5defORF2a_{AUGmut} and BM5defORF2b_{AUGmut} mutant viruses. By using site-directed mutagenesis, we introduced a series of nucleotide changes in the BM5def molecular clone, Def27, to disrupt the translational potential of either the ORF2a or ORF2b AUG-defined reading frames. These mutants carrying disrupted ORF2a or ORF2b AUGs are referred to as either BM5defORF2a_{AUGmut} or BM5defORF2b_{AUGmut} viruses, respectively. The mutations in ORF2a or ORF2b were carefully designed so as to alter neither the sequence integrity nor the preferred mammalian codon usage (8) of ORF1. The BM5defORF2a_{AUGmut} virus was generated by mutating the most-5′ ORF2 start AUG (nt 157 to 159) to ACG and additionally introducing a stop codon at position 169 to 171 (Fig. 1A and B). The BM5defORF2b_{AUGmut} virus was generated by mutating the more-3′ ORF2 AUG (nt 646 to 648) to ACG, as it was impossible to engineer an adjacent stop codon without altering the amino acid coding sequence in ORF1 (Fig. 1B). Viral stocks were produced by generating stable transfectants of the wt BM5def (as a transfection control) or the mutated viruses in SC-1^{eco} cells that are chronically infected with the helper BM5eco virus. BM5eco-rescued viral stocks, prior to injection into B6 mice, were confirmed for the presence of BM5eco and BM5def (wt or mutants) by RT-PCR and real-time qRT-PCR assays (7) (data not shown). In addition, the mutations were verified by sequencing. Viral stocks were adjusted for delivery of an equivalent infectious titer of 2.5×10^4 PFU, as determined by the XC-plaque assay (25) (data not shown). Additionally, viral genomic RNA was isolated from the stocks and converted to cDNA, and viral RNA copy numbers were determined by qRT-PCR by using standard curves. We determined that our standard infectious viral titer of 2.5×10^4 PFU of LP-BM5 averaged approximately 1.3 million (±0.2 million) copies of BM5eco RNA and 1.93 million (±0.05 million) copies of BM5def RNA. Similarly, the number of viral genomes per 2.5×10^4 PFU for the BM5eco-rescued viral preparations were as follows: (i) wt BM5def (transfection control), 1.14 million (±0.14 million) copies of BM5eco RNA and 1.97 million (±0.04 million) copies of BM5def RNA; (ii) BM5defORF2a_{AUGmut}, 1.12 million (±0.2 million) copies of BM5eco RNA and 1.82 million (±0.04 million) copies of BM5def RNA; (iii) BM5defORF2b_{AUGmut}, 1.32 million (±0.2 million) copies of BM5eco RNA and 1.9 million (±0.03 million) copies of BM5def RNA.

Ability of BM5def ORF2a and ORF2b AUGs to initiate translation of GFP fusion proteins. In order to determine the translational potential of the alternative ORF2a and ORF2b AUGs, we first generated BM5defORF1$_{AUG}$EGFP (as a positive control) and BM5defORF2a_{AUG}EGFP and BM5def ORF2b_{AUG}EGFP tagged viruses. These constructs allow for the expression of the ORF1 or ORF2a or ORF2b AUG-defined reading frames as fusions to the N terminus of EGFP (Fig. 2A). Thus, the EGFP gene, including its endogenous stop codon, was cloned at the C terminus of, and in the same reading frame as, either the ORF1, ORF2a, or ORF2b AUGs, with no intervening in-frame stop codons. For the BM5def ORF2a_{AUG}EGFP construct, EGFP was cloned 5′ of the ORF2b initiation AUG to ensure that all EGFP expression would necessarily emanate only from the ORF2a initiation AUG. Stable transfectants of the EGFP-tagged BM5def viruses were generated in SC-1^{eco} cells and analyzed for EGFP fusion protein expression by fluorescence-activated cell sorting (FACS). GFP expression was detected in cells carrying BM5defORF1$_{AUG}$EGFP as expected and also by BM5def ORF2a_{AUG}EGFP- or BM5defORF2b_{AUG}EGFP-transfected cells (Fig. 2A). Importantly, BM5defORF2a/2b AUG-directed EGFP expression was detected at somewhat lower (24% lower total mean fluorescence intensity) but still very substantial levels compared to the conventional BM5def ORF1-directed levels. Additionally, to ascertain how much EGFP expression in

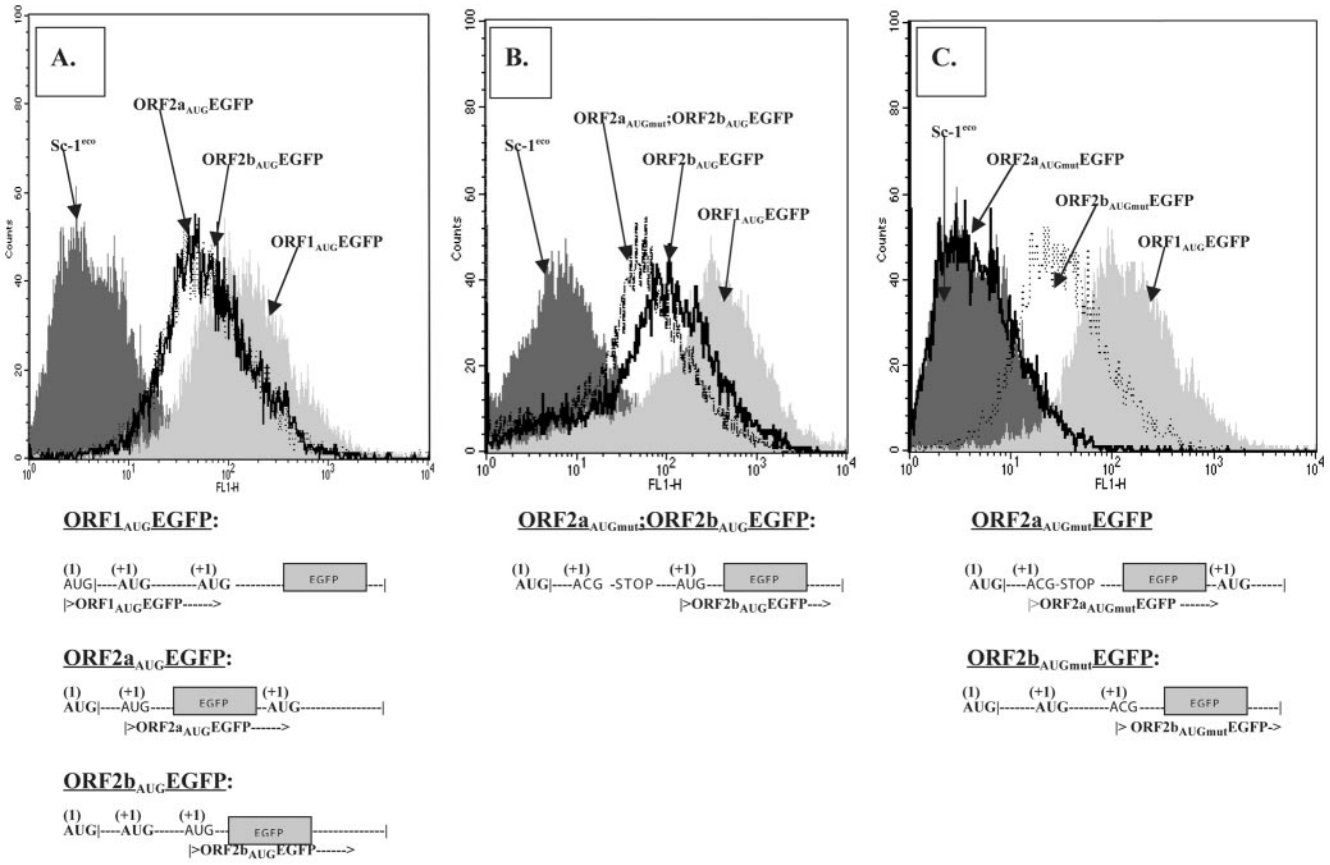
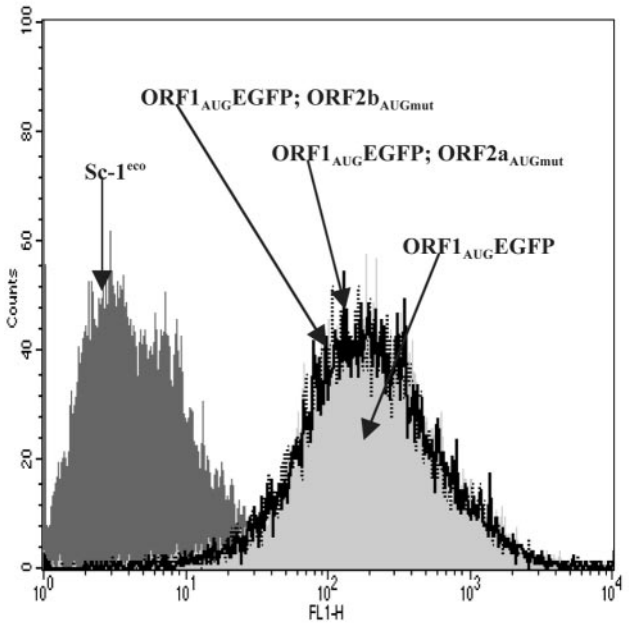


FIG. 2. (A) BM5def *gag* $ORF1_{AUG}$- versus $ORF2_{AUG}$- directed EGFP expression. (B) BM5def $ORF2b_{AUG}$EGFP versus BM5def$ORF2a_{AUGmut}$;$ORF2b_{AUG}$EGFP expression. (C) GFP expression in viruses carrying either the $ORF2a_{AUGmut}$ or $ORF2b_{AUGmut}$ mutations. In all three panels, the dark gray filled-in histograms are the negative controls of untransfected Sc-1^{eco} cells, while the light gray filled-in histograms represent Sc-1^{eco} cells transfected with BM5def$ORF1_{AUG}$EGFP. The black solid line and dashed line represent the following: (A) Sc-1^{eco} cells transfected with BM5def$ORF2a_{AUG}$EGFP or BM5def$ORF2b_{AUG}$EGFP viruses, respectively; (B) Sc-1^{eco} cells transfected with BM5def$ORF2b_{AUG}$EGFP or BM5def$ORF2a_{AUGmut}$;$ORF2b_{AUG}$EGFP viruses, respectively; (C) Sc-1^{eco} cells transfected with BM5def $ORF2a_{AUGmut}$EGFP or BM5def$ORF2b_{AUGmut}$EGFP viruses, respectively. These data are representative of three independent experiments. FACS analyses for cells in panels A and C and in Fig. 3 were carried out on the same day while that for panel B was done independently. Each histogram depicts a stable transfectant population which is representative of at least two independently transfected populations that were tested for GFP expression by FACS.

the BM5def$ORF2b_{AUG}$EGFP construct originated from the ORF2b AUG specifically (and not as a read-through from the upstream intact ORF2a AUG), we also generated a construct wherein the ORF2a AUG was mutated and the EGFP gene was cloned 5′ of and in the same reading frame as the ORF2b (BM5def$ORF2a_{AUGmut}$;$ORF2b_{AUG}$EGFP, Fig. 2B). SC-1^{eco} cells transfected with this BM5def$ORF2a_{AUGmut}$; $ORF2b_{AUG}$EGFP virus expressed considerable GFP (only 37% lower total mean fluorescence intensity than the BM5def$ORF2b_{AUG}$ carrying the unmutated ORF2a AUG), confirming that the ORF2b initiation AUG was independently capable of translating a fusion protein without any read-through from the upstream intact ORF2a AUG (Fig. 2B).

To verify that the GFP was being translated from the initiation AUGs of ORF2a or ORF2b and that the introduced mutations were effective in disrupting ORF2a or ORF2b AUG-initiated translation, EGFP was similarly engineered into the BM5def$ORF2a_{AUGmut}$ and BM5def$ORF2b_{AUGmut}$ viruses (Fig. 2C). To generate the BM5def$ORF2a_{AUGmut}$EGFP virus, EGFP was cloned 5′ of the ORF2b initiation AUG. Compared to the BM5def$ORF2a_{AUG}$EGFP, the total mean fluorescence intensity of BM5def$ORF2a_{AUGmut}$EGFP was consistently lowered in three independent experiments by >99%; essentially this mutant did not appear to express detectable GFP (Fig. 2C). These data clearly show that the ORF2a AUG is capable of initiating translation of a fusion protein, and the introduced mutations inhibit ORF2a AUG-initiated translation. In contrast, mutation of the ORF2b initiation codon did not completely inhibit the EGFP signal (Fig. 2C), as predicted from the results in Fig. 2B. Rather, the mutated BM5def$ORF2b_{AUGmut}$EGFP expressed substantial GFP (only 30% lower total mean fluorescence intensity than BM5def$ORF2b_{AUG}$), most probably due to the read-through from the intact ORF2a AUG (also see below).

BM5def ORF1-encoded *gag* is expressed in the BM5def$ORF2a_{AUGmut}$ or BM5def$ORF2b_{AUGmut}$ mutants. To confirm that the mutations introduced in BM5def ORF2a or ORF2b AUGs are in no way detrimental to the translation of



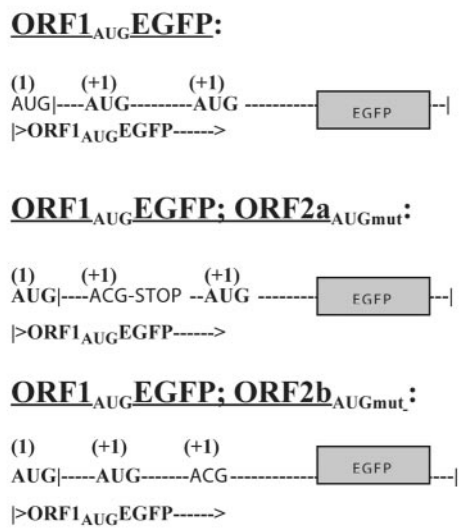


FIG. 3. ORF1$_{AUG}$EGFP expression in viruses carrying either the ORF2a$_{AUGmut}$ or ORF2b$_{AUGmut}$ mutations. The dark gray filled-in histogram is the negative controls of untransfected Sc-1^{eco} cells while the light gray filled-in histogram represents Sc-1^{eco} cells transfected with BM5defORF1$_{AUG}$EGFP. The black solid line and dashed line represent Sc-1^{eco} cells transfected with BM5defORF1$_{AUG}$EGFP cloned into BM5defORF2a$_{AUGmut}$ or BM5defORF2b$_{AUGmut}$ viruses, respectively. These data are representative of three independent experiments.

ORF1, which is required for viral pathogenesis, we also generated viruses with the ORF1$_{AUG}$EGFP fusion construct in both the BM5defORF2a$_{AUGmut}$ and BM5defORF2b$_{AUGmut}$ viruses. The EGFP fragment was cloned in frame with the initiating AUG, and at the C terminus, of ORF1 in both mutants (Fig. 3). The wt BM5def carrying the ORF1$_{AUG}$EGFP fusion protein defined above was used as a positive control in analogous FACS experiments. ORF1$_{AUG}$EGFP expression was detected in the BM5defORF2a$_{AUGmut}$ or BM5def ORF2b$_{AUGmut}$ viruses at levels indistinguishable from this positive control (Fig. 3). These data confirm that ORF1 AUG-initiated translation is not disrupted in the BM5def ORF2a$_{AUGmut}$ or BM5defORF2b$_{AUGmut}$ mutants, thus allowing us to address the potential need for ORF2 translation for pathogenesis under conditions of normal levels of required ORF1 product.

Activational and immunosuppressive parameters of MAIDS pathogenesis are not induced by the BM5defORF2a$_{AUGmut}$ or BM5defORF2b$_{AUGmut}$ mutants. To assess the pathogenicity of BM5def viruses mutated to inhibit translation from their alternative ORF2 AUGs, B6 mice were infected with rescued preparations carrying BM5eco virus and either the BM5def ORF2a$_{AUGmut}$ or BM5defORF2b$_{AUGmut}$ mutant virus or with positive (LP-BM5 and wt BM5def rescued with BM5eco) or negative (phosphate-buffered saline) controls. Splenomegaly, as a standard activational parameter characteristic of MAIDS (10, 11), was determined 11 weeks postinfection (wpi) by spleen weight (Fig. 4A). At 5, 9, and 11 wpi, serum IgG2a and IgM levels were determined (11 wpi; Fig. 4B), as a measure of the generalized hyper-Ig of MAIDS. Compared to the LP-BM5 or wt BM5def rescued control-infected mice, B6 mice infected with either the BM5defORF2a$_{AUGmut}$ ($P < 0.001$) or BM5defORF2b$_{AUGmut}$ ($P < 0.001$) virus did not induce measurable splenomegaly or hypergammaglobulinemia. This pattern was replicated in three additional independent experiments.

In addition, a standard panel of immunosuppressive parameters was measured at termination 11 wpi: responses to mitogens by T (ConA) versus B (lipopolysaccharide) cells, as well as the generation of alloantigen-specific (anti-H-2^{d}) CTL activity. Again, in contrast to LP-BM5 or wt BM5def rescued control-infected mice, B6 mice infected with the BM5eco-rescued BM5defORF2a$_{AUGmut}$ or BM5defORF2b$_{AUGmut}$ mutants did not develop disease, i.e., they had normal mitogen responses (Fig. 4C; data representative of four independent experiments, $P < 0.001$) and generation of CTL activity (Fig. 4D; data representative of three independent experiments). These results were further confirmed by flow cytometric analyses for the characteristic spleen cell phenotypic changes that are observed in uninfected versus LP-BM5-infected B6 mice (9, 14). LP-BM5 or wt BM5def rescued control-infected mice showed the expected expanded populations of CD19^{+} CD23^{-} and CD4^{+} Thy1.2^{-} spleen cells, but these changes were not observed in mice infected with the BM5defORF2a$_{AUGmut}$ or BM5defORF2b$_{AUGmut}$ mutant (data not shown).

Determination of BM5def and BM5eco *gag* RNA levels in infected mice. On termination of each experiment, we verified the presence of the appropriate control and experimental viruses in infected mice and quantified their expression by qRT-PCR assays for BM5eco and BM5def (7). Although there was no evidence of MAIDS induction by the BM5def ORF2a$_{AUGmut}$ or BM5defORF2b$_{AUGmut}$ preps, mice infected with these mutants expressed substantial levels of both BM5eco and BM5def (Fig. 5). BM5def *gag* RNA was more abundant than BM5eco *gag* RNA in spleen cells from both control and viral mutant-infected mice. These data are similar to what our lab has observed previously (7) as well as to BM5def and BM5eco *gag* RNA levels determined in the viral stocks that were generated in vitro and used for infecting mice

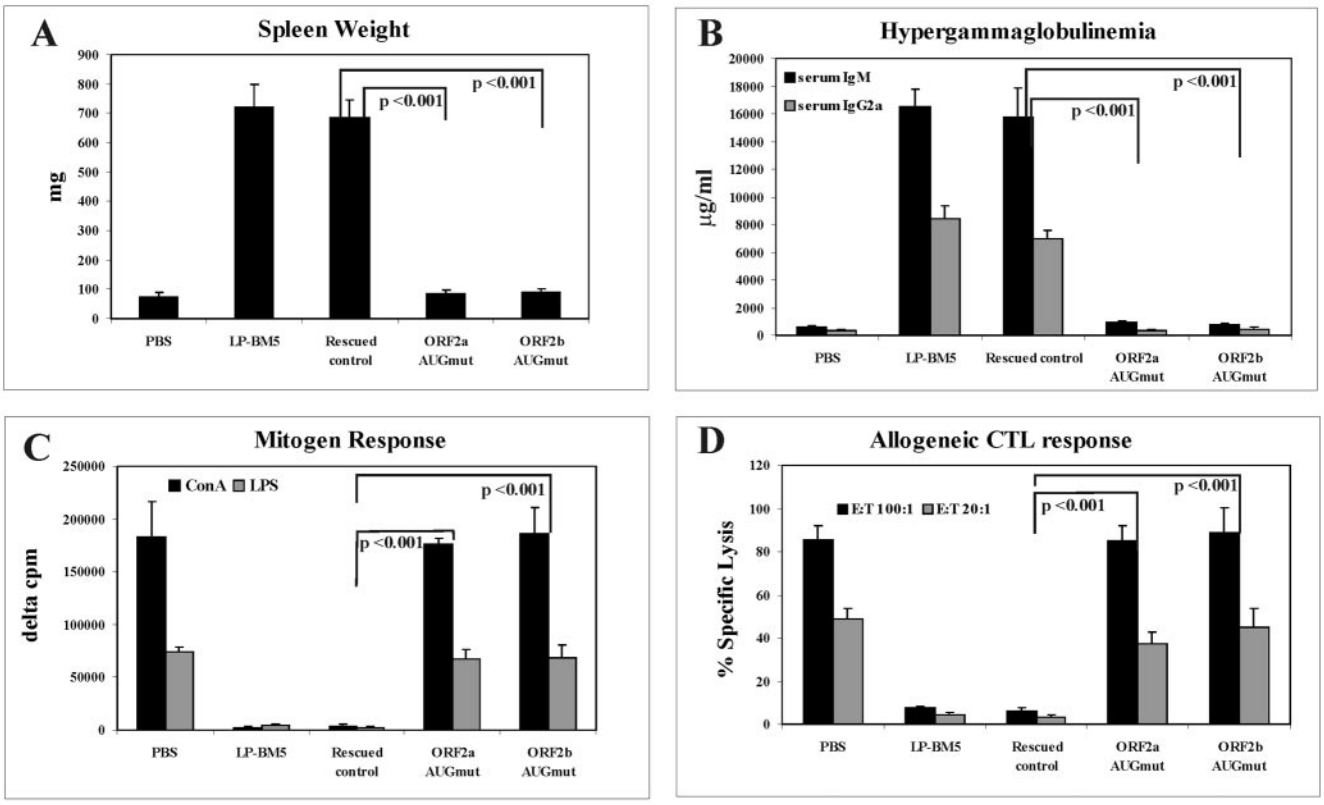


FIG. 4. Susceptibility of BM5eco-rescued wt or mutant BM5def-infected B6 mice to MAIDS. (A) Spleen size (mg). (B) Serum IgM and IgG2a response. (C) Spleen cell proliferation in response to T-cell mitogen (ConA, 2 μg/ml) and B-cell mitogen (lipopolysaccharide, 10 μg/ml). (D) Allogeneic cytotoxic-T-lymphocyte responses of spleen cells at 11 wpi. E:T, effector-to-target ratio. Means and standard deviations represent three mice per group and the data are representative of four independent experiments for panels A, B, and C and three independent experiments for panel D. Statistical analyses were carried out on LP-BM5-infected mice and B6 mice infected with either BM5defORF2a$_{AUGmut}$ or BM5defORF2b$_{AUGmut}$ ($P < 0.001$ by Student t test). PBS, phosphate-buffered saline.

in this study (data not shown). BM5eco *gag* and BM5def *gag* RNA levels from mice infected with the BM5def ORF2a$_{AUGmut}$ or BM5defORF2b$_{AUGmut}$ mutant viruses were only about two- to threefold lower than the viral loads for wt rescued control and LP-BM5-infected mice (Fig. 5). It seems very unlikely that this degree of difference in viral load would be sufficient to account for the absence of disease induction after infection by the mutated BM5def preparations. The higher viral loads in the positive controls, in this case infection with nonmutated viral preps, seem to simply reflect the fact that in full-blown MAIDS the characteristic extensive lymphoproliferation is a major contributor to the spread of the virus, in addition to multiple rounds of infection by free virions. This concept that B6 background mouse strains resistant to MAIDS pathogenesis still exhibit a substantial viral load indicative of normal initial infection and early viral spread has been further supported by both viral titration experiments and the study of several MAIDS-resistant versus -susceptible B6 strains, with either of the genes knocked out (CD154, CD40, CD80/86) or as chimeric CD40 transgenics (9, 10, 11). As we have previously discussed in some detail (9), these comparisons across mouse strains have shown that distinctly measurable disease can be observed under conditions that result in a few-fold lower BM5def viral load at termination of the experiment. In addition, on termination of each experiment, by using RT-PCR we isolated the BM5def viruses from spleen cells and sequenced them. Sequencing data confirmed the presence of only the wt BM5def in the mice infected with the positive control viral preps, versus only the appropriate mutant sequence in the mice infected with the experimental viruses. An

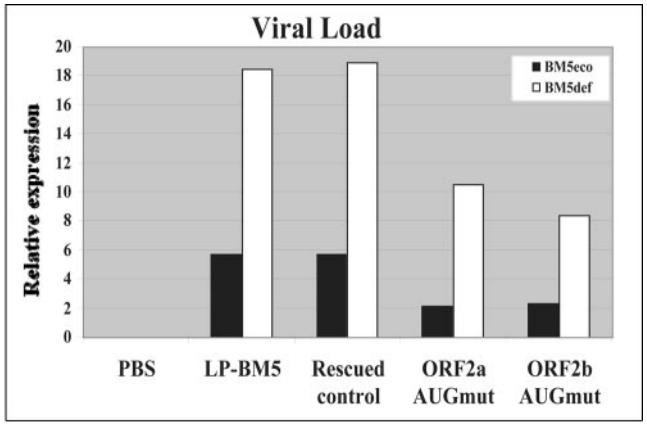


FIG. 5. Viral load for BM5eco *gag* and BM5def *gag* RNA quantified by qRT-PCR from spleen cells of infected mice at 11 wpi. These qRT-PCR data are representative of RNA isolated from infected spleens in four independent experiments.

inherent advantage of this system is that any reversion of the mutated to the wt sequence would have resulted in a defective virus that would have caused disease. These data clearly suggest that a virus different from what was initially injected into the mice had not arisen to detectable levels as a result of reversion during the 11-week course of infection.

In summary, based on (i) both activational and immunodeficiency parameters of MAIDS (Fig. 4), (ii) viral load data (Fig. 5), and (iii) sequence analysis of viruses isolated from mice 11 wpi, and ORF1/ORF2 expression data (Fig. 2 and 3), it is evident that the viral mixes consisting of either wt BM5eco-rescued ORF2a$_{AUGmut}$ or ORF2b$_{AUGmut}$ BM5def virus, each with intact wt ORF1 expression capability, are essentially unable to induce disease. These data strongly suggest the existence of a novel ORF2 product(s) that is required for LP-BM5-induced pathogenesis. The ORF2 product(s) may be initiated from either the ORF2a or ORF2b AUG (Fig. 2), as disruption of either one of their translational potentials leads to inhibition of disease (Fig. 4). One interpretation of these findings is that there is a requirement for both protein products of 193 and 30 aa, produced as separate entities from the ORF2a and ORF2b AUGs, respectively. These proteins may be independently essential in targeting different specific host cellular proteins. Alternatively or in addition, the two alternative ORF2 translational products may function together to cause disease by independently interacting with the same host cell protein target or combining to form a single ORF2a/ORF2b heterodimer.

As an option to two separate ORF2 protein products, it is also possible that only a single protein initiating from the ORF2a AUG is required. Thus, the data from the BM5defORF2b$_{AUGmut}$EGFP mutant virus (Fig. 2C) show that, despite disrupting the initiation AUG of ORF2b, GFP is still expressed (albeit at a reduced level) as a fusion protein, apparently from the unaltered ORF2a AUG which is in the same +1-nt reading frame as ORF2b (Fig. 2B). Importantly, however, the BM5defORF2b$_{AUGmut}$ virus is unable to cause disease. This finding could be explained based not on the ORF2b AUG mutation as a block to translation initiation at this site but rather on the basis of the introduction of a missense mutation at the ORF2b site in the ORF2a AUG-initiated maximal ORF2 protein. Thus, the presence of threonine instead of methionine may be deleterious to the structure of the extended protein produced from the ORF2a AUG.

The results presented in this paper support the existence of an alternative translation initiation mechanism in BM5def for the expression of an ORF2-encoded *gag* product(s) and its requirement in LP-BM5-induced pathogenesis. These data have obvious implications for pursuing the molecular mechanisms by which BM5def causes disease and studies are currently under way to identify the nature and function of the necessary ORF2 product(s). Our data are interesting to compare to the results of other labs that have also studied atypically encoded CTL epitopes, including in human immunodeficiency virus (2, 3, 6, 28). In particular, and comparable to our novel BM5def ORF2a and/or ORF2b AUG(s)-encoded 193 or 30 aa protein(s), a previously unobserved, conserved 87-residue protein encoded by influenza A virus has recently been identified by first defining a CTL epitope encoded in an alternative translational ORF (6). This immunogenic influenza virus protein, PB1-F2, recognized by CD8$^+$ T cells, is derived from a +1 alternative reading frame and may play a critical role in pathogenesis by inducing apoptosis in host immune cells that respond to influenza virus infection (6). Our identification of an immunodominant CTL epitope novelly expressed from an alternative translational *gag* ORF (20, 22), combined with our present data that the ORF2-encoded extended *gag* product(s) have a functional role in disease induction, raise the possibility that alternative reading frame-derived proteins may also be crucial in other disease models.

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