A Novel Truncated env Gene Isolated from a Feline Leukemia Virus-Induced Thymic Lymphosarcoma

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We PCR amplified the exogenous feline leukemia virus (FeLV)-related env gene species from lymphosarcomas induced by intradermally administered plasmid DNA of either the prototype FeLV, subgroup A molecular clone, F6A, or a new molecular clone, FeLV-A, Rickard strain (FRA). Of the nine tumors examined, six showed the presence of deleted env species of variable sizes in the tumor DNA. One env mutant, which was detected in a FRA-induced thymic lymphosarcoma, had a large internal deletion beginning from almost the N-terminal surface glycoprotein (SU) up to the middle region of the transmembrane (TM) protein of the env gene. The deduced polypeptide of this truncated env (tenv) retained the complete signal peptide and seven amino acids of the N-terminal mature SU of FRA env gene, followed by eight amino acids from the frameshift in the TM region. To study the biological function of tenv, we used a murine retrovirus vector to produce amphotropic virions. Infection of feline fibroblasts (H927), human fibrosarcoma cells (HT1080), or human B-lymphoma cells (Raji) led to pronounced cytotoxicity, while the tenv virus did not induce significant cytotoxicity to feline T-lymphoma cells (3201B) or human T-lymphoma cells (CEM). Together, these results convincingly demonstrated that the genetic events that led to truncation in the env gene occurred de novo in FeLV lymphogenesis and that such a product, tenv, could induce cytotoxicity to fibroblastic and B-lymphoid cells but not to T-lymphoid tumor cells. This type of selective toxicity might be potentially important in the development of the neoplastic disease.

Retroviruses are causative agents in the induction of lymphoid malignancies (leukemia-lymphoma complex) in mammals, including humans. In the domestic cat, an outbred species which has the highest incidence of lymphoid malignancies of any animal, the disease is naturally associated with chronic feline leukemia virus (FeLV) infection (8, 18). There is solid evidence to indicate that interactions between infectious FeLV and non-infectious inherited endogenous FeLV elements generate recombinant viral quasispecies which represent a variety of chimeric envelope glycoproteins depending on the extent of amino terminal portion replaced by the endogenous env sequences (9, 13, 24). The viral species with specific adaptive amino acid mutations and with certain sites of recombination are rapidly selected for replication efficiency and are overrepresented at later time points after infection (2, 3, 14). FeLVs with recombinant env genes are detected with high frequency in naturally as well as experimentally induced feline lymphosarcomas (2, 3, 10, 14, 23, 25). Evidence also exists to suggest that some defective env genes detected in FeLV-induced lymphosarcomas may be additional factors in the disease process (16, 23).

Although a previous study addressed the issue of in vivo derivation of defective env genes from an FeLV, subgroup A (FeLV-A) molecular clone (16), administration of an inoculum prepared by propagating the virus in feline cell cultures could not eliminate the possibility of introducing defective FeLV contaminants along with the replication-competent FeLV-A virus. In this report, we present data demonstrating in vivo generation of defective env genes which were detected in majority of lymphosarcomas induced by direct delivery of proviral DNA of molecular clones of FeLV-A by intradermal injection into specific-pathogen-free (SPF) cats. Detection of a spectrum of truncated env genes, all beginning from an inoculum of a single molecular species of FeLV and occurring in the lymphosarcomas induced, suggests that products of some of these defective env genes retained in tumor cells may have a role in the multistep process of FeLV pathogenesis. In this regard, we describe a highly truncated env gene product derived from one of these lymphosarcomas which displayed a pattern of selective cytotoxicity.

MATERIALS AND METHODS

Cell culture. H927 feline fibroblast and PA317 mouse amphotropic packaging cell lines were maintained in the Dulbecco modified Eagle medium high-glucose medium supplemented with 10% fetal bovine serum. The human HT1080 fibrosarcoma cell line was cultured in Eagle minimal essential medium with 10% fetal bovine serum. Raji and CEM cells and human B- and T-cell lines, respectively, were grown in RPMI medium supplemented with 10% fetal bovine serum. Feline 3201B T cells were maintained in 1:1 RPMI-Leibovitz’s L-15 medium supplemented with 20% fetal bovine serum. All media were purchased from Irvine Scientific Co.

PCR analysis of exogenously related env genes in cat tumor tissues. Genomic DNA was isolated from tumors of six pFRA-challenged cats, 5022, 5023, 5024, 5025, 5039, and 5041 (3); three pF6A-challenged cats, 5035, 5036, and 5051 (A. J. Phippis et al., unpublished data), and an SPF fetus tissue. PCR reactions were performed with Taq DNA polymerase (Gibco-BRL) to amplify env genes of FeLV-A and the recombinants between FeLV-A and the endogenous env elements from these DNA samples. The 5’ primer was made to the sequences conserved between FeLV-A and endogenous FeLV pol region (H18) (3), and the 3’ primer was complementary to the exonous 3’ long terminal repeat (LTR) sequence of molecular clone FeLV-A, Rickard strain (FRA), or F6A (H20) (3). The env sequence was also amplified by using the same strategy from the tumor of cat 4746-5, which was challenged with an FeLV-A Rickard plasma preparation and a mixture of in vitro-generated recombinant FeLVs (14, 24). Construction of mutant or chimeric env-expressing retroviral vector. PCR products of full-length env genes from tumors 4746-5, 5022, 5023, 5024, and 5025 and the 700-bp deleted env gene from tumor 5023 were cloned into the pCR2.1 vector (Invitrogen). To study recombinant env species harbored by these exper-
the encephalomyocarditis virus in front of the selection marker gene (NeoR) so

opWZLneo vector, a Moloney murine leukemia virus (MuLV)-based retroviral

quences) into the cloned env genes. The PCR products were then cloned into pW2L vector, a Moloney murine leukemia virus (MuLV)-based retroviral vector (6, 20). This vector contains an internal ribosome entry site (IRES) from the encephalomyocarditis virus in front of the selection marker gene (NeoR) so as to produce a bicistronic mRNA containing the gene of interest and Neo.

RNA isolation and RT-PCR. Total RNA was extracted from thymic tumor and normal splenic tissues of the cat 5023 with a RNasey Kit (Qiagen). The reverse transcriptase (RT) reaction was performed with 1 µg of RNA primed with oligo(dT). The sequences related to exogenous FeLV env species were amplified from the cDNA by using RB447 and RB448 primers. As a control, the GADPH sequence was amplified from the same cDNA preparations with the primer set RBS581 (CCACCATGGGAAATCTCGAG) and RBS582 (TTCTAGACGCAAGTCAGGTCCAC). To ensure the absence of genomic DNA contamination in the cDNA samples, experiments without RT enzyme were simultaneously run.

Sequence analysis. The cloned env genes in both pcR2.1 vector and pWZLneo vector were sequenced by using the M13 reverse and RB953 primers, respectively. The pWZLneo vector (AAAAGACGCAATTCGAGTGGTGG) was made to represent a complementary sequence to IRES of pWZLneo vector. Automated fluorescence-based cycle sequencing was conducted with the ABI Prism 377 DNA sequencer (Perkin-Elmer, Foster City, Calif.) and the ABI Prism Dye Terminator cycle-sequencing Kit (P/N 402808) as specified by the manufacturer.

Stable transfection of PA317 cells and conditioned medium preparation. To produce amphotropic viruses, the parental pWZLneo, the FeLV-B-like recombinant env and the env retroviral constructs were transfected into PA317 cells by use of Lipofectamine (Gibco-BRL) according to the manufacturer’s protocol. G418-containing medium was transferred to the plastic slides at different time points after infection. Then the cells were fixed with 10% paraformaldehyde and stored at

80°C. The TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling) assay was performed according to the manufacturer’s instructions (Boehringer Mannheim). ACE (Vector Labs) was used as color substrate.

RESULTS

Detection of env genes in experimentally induced tumors. To examine the env species in both pFRA- and pF6A-induced tumors (3; Phipps et al., unpublished data), exogenous-related env genes were PCR amplified with H18 and H20 primers. An SPF cat fetus DNA was included as a negative control. Lanes: 1, H2O; 2, SPF cat fetus; 3, tumor 5022; 4, tumor 5023; 5, tumor 5024; 6, tumor 5025; 7, tumor 5032; 8, tumor 5041; 9, tumor 5053; 10, tumor 5056; and 11, tumor 5051. Molecular sizes are indicated on the right.

![FIG. 1. Detection of deleted env species in lymphosarcomas in cats inoculated with FeLV-A proviral DNA. Genomic DNA was isolated from six pFRA-induced tumors and three pF6A-induced tumors. The exogenously related env genes were PCR amplified with H18 and H20 primers. An SPF cat fetus DNA was downloaded as a negative control. Lanes: 1, H2O; 2, SPF cat fetus; 3, tumor 5022; 4, tumor 5023; 5, tumor 5024; 6, tumor 5025; 7, tumor 5032; 8, tumor 5041; 9, tumor 5053; 10, tumor 5056; and 11, tumor 5051. Molecular sizes are indicated on the right.](http://jvi.asm.org/)

Analysis of env for nucleotide and deduced amino acid sequence. The 700-bp env species from cat tumor 5023, namely, env, was cloned and sequenced. The env gene sequence was 100% homologous to FRA env (3), except for a large internal deletion of 1519 bp that shortened env. The deletion began near the N-terminal surface glycoprotein (SU; 6099 of FRA) up to the mid-transmembrane (TM) region (7617 of FRA). Both ends of deletion were flanked by a direct repeat of six nucleotides (Fig. 2). In addition, this deletion also resulted in a frameshift that gave rise to a premature stop codon (7645 of FRA). Comparison of the deduced polypeptide from this sequence to that of FRA indicated that it retained the complete signal peptide and seven amino acids of the N-terminal mature SU of FRA env protein, followed by a sequence of
eight altered amino acids resulting from the frameshift in the TM region (Fig. 2).

Induction of cell morphological changes and cell death by 
tenv expression. To study the biological function of 
tenv, we used a vector (pWZLneo) to express the 
tenv protein (Fig. 3). The WZLneo vector is a Moloney MuLV-based retroviral vector 
that contains an IRES sequence upstream of the amino-glycoside phosphotransferase (Neo') gene. Existence of IRES 
allows selected G418-resistant clones to express theoretically 
both 
tenv and Neo' genes from the same RNA. To confirm the 
expression of 
tenv, RT-PCR was performed on 
tenv-stably 
transfected PA317 clones. The right size mRNA of 
tenv was 
readily detected in the 
tenv-transduced cells but not vector-transfected cells (data not shown). Cell free viral supernatant 
fluids from the stably transfected clones of amphotropic PA317 
packaging cell line were used to infect fibroblastic and lymphoid cell lines.

The feline H927 fibroblasts and the human HT1080 fibrosarcoma cells were infected with the 
tenv virus harvested and 
pooled from two virus-producing PA317 clones. After 12 h of 
infecction, cells were placed in G418-containing medium and 
cultured for at least 6 days. Unexpectedly, we failed to obtain 
any G418-resistant clones either from H927 or HT1080 cells 
infected with the 
tenv virus. These experiments were repeated 
twice, and the results were the same. Microscopic examination 
of the cells revealed hallmarks of apoptosis such as nuclear 
condensation and surface blebbing.

The 
tenv virus was also used to infect human Raji B cells. In 
five independent experiments with virus preparations from two 
stably transfected clones, we consistently observed formation 
of large cellular aggregates which occurred as early as 4 h 
postinfection. The aggregates could be disrupted mechanically 
but reformed quickly even when the infected cells were 
cultured in the medium without viruses. As illustrated in Fig. 4, 
the Raji cells treated with the 
tenv virus displayed cell aggregations of 15 to 50 cells each, whereas the Raji cells infected 
with vector or full-length env virus (data not shown) under 
identical virus and cell concentrations for infection remained 
primarily as single cells.

In contrast to the B cells, parallel studies with other lym-

FIG. 2. Nucleotide and deduced amino acid sequence of 
tenv. Starting from FRA env ATG (positions 5968 to 5970), the deduced amino acids of 
tenv and comparison to that of FRA env are depicted. The sequence in the dashed box represents the signal peptide. The sequence in the solid box represents the N-terminal 
portion of pFRA mature SU which is retained in 
tenv. The sequence underlined indicates a relevant portion of FRA mid-TM region which is altered in 
tenv. The deletion junction is highlighted in gray. Asterisks indicate the same amino acids between 
tenv and FRA env. The diamond denotes the premature stop codon.

FIG. 3. Schematic representation of Moloney MuLV-based WZLneo vector. The dashed boxes indicate the Moloney MuLV LTRs. The packaging signal sequences 
(Ψ+), ATG minus deleted gag (Δgag), IRES, and the Neo' gene are marked. The 
tenv gene was cloned into EcoRI site shown as E. The vertical arrows indicate the 
relative positions of ATG and the premature stop codon (+).
phoid cells such as CEM and 3201B cells and human and feline T-cell lines, respectively, revealed no significant morphological changes or cytotoxic effects. These results with different cell lines are summarized in Table 1.

Quantification of \textit{tnv} cytotoxicity to Raji cells. Along with morphological changes in \textit{tnv}-infected Raji cells, we also repeatedly observed a decrease in viable cell numbers relative to those treated with vector or full-length recombinant \textit{env} viruses. Thus, we wanted to further examine cell death in \textit{tnv}-transduced Raji cell clones after G418 selection as described above for H927 and HT1080 cells. However, Raji cells were quite resistant to G418 treatment. For example, in experiments with up to 1,000 \( \mu \)g of G418 per ml, uninfected Raji cells remained viable even after treatment. For that reason, we decided to use MTT assay to quantify cytotoxic effects of \textit{tnv} virus infection on Raji cells.

The same amount of Raji cells was infected with the same titer of \textit{tnv} or vector viruses. MTT converting activity was then measured at different time points after infection. As shown in Fig. 5, there was clearly a decrease in the number of metabolically active cells when the cells were monitored for up to 48 h after \textit{tnv} virus infection. Compared to vector-treated cells, there were 40\% fewer \textit{tnv}-infected cells at 24 h postinfection. Furthermore, only 30\% cells remained in \textit{tnv}-treated Raji cells compared to that of vector control at 48 h postinfection (Fig. 5). Although the observed cytotoxic effect was most likely related to the kinetics of \textit{tnv} protein expression, this issue could not be properly evaluated because of the lack of appropriate antiserum against this highly truncated \textit{env} product with altered TM terminal sequences.

Evidence for induction of apoptosis to Raji cells by \textit{tnv} expression. Formation of cell aggregates was previously reported for FeLV-C-treated 3201B feline T-lymphoid cells. Such morphological changes were followed by induction of apoptosis (15, 17). Similarly, we observed that clumping of Raji cells was induced following infection with the \textit{tnv} virus. To this end, to examine induction of apoptosis in \textit{tnv}-treated Raji cells, we performed a TUNEL assay to assess levels of apoptosis in vector virus- or \textit{tnv} virus-infected Raji cells. TUNEL assay is based on the detection of single- and double-stranded DNA breaks occurring in apoptosis. Because at 24 h postinfection massive cell death (60\% of vector-infected cells) was observed, Raji cells infected with \textit{tnv} or vector viruses were collected at 4, 8, and 18 h after infection for apoptosis assessment. For each slide, three fields were counted for the total number of cells and the cells that were stained positive. At 18 h postinfection, \textit{tnv} virus treatment resulted in approximately 45 \( \pm \) 9\% stained Raji cells with dark red nuclei, whereas only about 8 \( \pm \) 3\% of the cells treated identically with vector alone

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Description of cell lines</th>
<th>Observed cytotoxicity</th>
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<tbody>
<tr>
<td>Raji</td>
<td>Human B-cell lymphoma</td>
<td>Yes</td>
</tr>
<tr>
<td>HT1080</td>
<td>Human fibrosarcoma</td>
<td>Yes</td>
</tr>
<tr>
<td>H927</td>
<td>Feline embryofibroblast</td>
<td>Yes</td>
</tr>
<tr>
<td>3201B</td>
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<td>No</td>
</tr>
<tr>
<td>CEM</td>
<td>Human T-cell lymphoma</td>
<td>No</td>
</tr>
</tbody>
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![FIG. 4. Morphological changes in Raji cells induced by \textit{tnv} expression. Raji cells were treated with the vector-containing virus or \textit{tnv}-containing virus. At 24-hour postinfection, the cells treated with \textit{tnv} virus (panel B) displayed formation of large aggregates, whereas the cells treated with vector virus alone (panel A) remained primarily as single cells. Magnification, \( \times 31 \).](http://jvi.asm.org/)

![FIG. 5. MTT assay of Raji cells infected with the \textit{tnv} virus. Cytotoxic effect of \textit{tnv} on Raji cells was measured by MTT assay at \( A_{595} \) at 24 h (A) and 48 h (B) postinfection. The open bars represent the vector virus-treated cells, and the gray bars represent the \textit{tnv}-treated cells. The standard deviations are also indicated.](http://jvi.asm.org/)
virus displayed this characteristic of apoptosis. Representative results of this assay are shown in Fig. 6.

**Analysis of tenv expression in the tumor tissue.** To determine whether the deleted *env* gene was indeed expressed in the tumor cells from which *tenv* sequences were isolated, we conducted RT-PCR of the total RNA obtained from the tumor tissue and a normal tissue (spleen) from the 5023 tumor cat. As shown in Fig. 7, a PCR product was readily detected in the tumor tissue but not in the spleen. The size of the product corresponded well to the expected 453 bp from the *tenv* gene sequence. The quality of the RNA derived form the tissue was verified by the detection of the *GADPH* gene product.

**DISCUSSION**

In analogy to murine retrovirus-induced disease processes, we predicted that certain FeLV *env* recombinant or truncated *env* glycoproteins would be involved in signal transduction processes that regulate cell proliferation, cell death, or phenotypic expression. The basis for this prediction was the presence of numerous reports on functions of chimeric or truncated *env* glycoproteins of MuLVs different from the primary role of cell receptor recognition for entry into host cells. For example, infection of an interleukin-2 (IL-2)-dependent T-lymphoma cell line, specifically with the recombinant mink cell focus-forming (MCF) virus, confers factor-independent growth (26).

Another example is release of a lymphoid cell line from the IL-2 requirement when MCF SU is coexpressed in these cells along with either erythropoietin receptor (EpoR) or the structurally related molecule, IL-2 receptor β (12). A chimeric *env* glycoprotein, gp55 of polycythemia-inducing Friend spleen focus-forming virus (SFFV), which is a product of MCF-derived extracellular SU domain fused to an ecotropic-derived TM segment, is an abnormally processed defective protein. This protein is needed to bind to EpoR to transform erythropoietic cells in the virus-induced disease (11, 22).

To pursue this prediction in FeLV pathogenesis, we describe here experiments that provided direct evidence for the first time for in vivo generation of not only viruses with recombinant *env* glycoprotein (3; Phipps et al., unpublished data) but also a spectrum of truncated glycoprotein genes, all beginning from an inoculum of a single molecular species of FeLV and occurring in the lymphosarcomas induced. Since the proviral plasmid DNA was inoculated intradermally into the cats, the composition of the inoculum could not be questioned. Initially, we selected a single truncated species, *tenv*, to examine its structure and function in cell cultures. The deduced polypeptide of the cloned *tenv* gene indicates that besides the complete signal peptide and the first seven amino acids of the mature SU of FRA, *tenv* does not contain any other SU sequence. The C-terminal portion of eight amino acids is unique because of the large internal deletion (1,519 bp) and frameshift in the TM region. The nucleotide sequence of the C-terminal region, however, corresponds fully to the FRA TM sequence. Thus, it appears that *tenv* is a direct derivative of the FRA parental virus rather than of any recombinant generated in vivo. In some regards, however, *tenv* displays structural similarity to SFFV gp55. Besides the internal deletion of 585 bp, one striking feature in gp55 nucleotide sequence is a single-base-pair insertion that changes the reading frame (1, 4, 27). Interestingly, although each SFFV isolate may differ in the type or position of the base pair inserted, the resulting SFFV *env* proteins all end with the same unique five to six C-terminal amino acid sequence (21). There is evidence that EpoR activation by gp55 is indeed dependent on sequences at the C-terminal position of the base pair inserted, the resulting SFFV *env* proteins all end with the same unique five to six C-terminal amino acid sequence.

For functional studies of *tenv*, we used a WZLneo retrovirus vector to transduce this gene into a few different cell types. The *tenv* virions were produced in amphotropic PA317 packaging cell line and used to infect a number of fibroblastic and lymphoid cell lines. The findings were striking. While feline fibroblasts (H927), human fibrosarcoma (HT1080), and human Raji B cells exhibited cytotoxic response to *tenv* virus infection, the
feline or human T-lymphoid tumor cells, namely, 3201B and CEM cells, did not manifest any significant cytotoxicity. The differential cytotoxicity is interesting since all of the lymphoid cell lines were infected with the same virus titer for the same amount of cells. The fibroblasts, H927 and HT1080 cells, even received less amount of virus. Still the findings will be more convincing once reagents are available to determine the levels of env protein expression in the various cell lines tested. Additionally, it will be necessary to extend the study to other target cell lines and natural feline cell populations to increase the significance of this cytotoxicity. It should, however, be noted that in contrast to the observed effect of env on the fibroblasts and B cells, infection of these cells with a full-length recombinant env-containing murine retroviruses or the vector viruses did not induce any morphological changes or cytopathic effects. Thus, the changes observed were specific for env expression and not due to nonspecific murine retrovirus infection. Furthermore, there appears to be a target cell specificity for env as well. In the limited study, the morphological changes induced in H927, HT1080, and Raji cells by env appeared to be similar to some of the hallmarks of apoptosis. The results implied, but certainly did not prove, that the de novo-generated env could be critical in lymphomagenesis. Conceivably, while the T-tumor cells may be resistant to cell death by expression of this novel protein, other cell types like B lymphoid cells and stromal (fibroblasts) cells may be targets of selective killing by this product. This type of selective cytotoxic effect may potentiate compensatory proliferation of the resistant cells in the tumorigenic process.

In conclusion, we have documented conclusive proof for the in vivo generation of truncated env genes in FeLV-induced neoplasia. Since the majority of the experimental tumors display one or more discrete species of variably truncated FeLV env genes, it is quite likely that at least some of them will have functional consequences. In this regard, the truncated version tested here illustrates a cytotoxic property which is specific for cell types.

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