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 genes 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 lymphomagenesis 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 over-represented 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 minority 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. Phipps 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 exogenous 3’ long terminal repeat (LTR) sequence of molecular clone FeLV-A, Rickard strain (FRA), or F6A (H20) (3). The env sequence was 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-
mmercially available Mo-μL-based retroviral env sequences) into the cloned 3
RNA isolation and RT-PCR. Total RNA was extracted from thymic tumor and normal splenic tissues of the cat 5023 with a RNeasy 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 RB581 (CCACCATGGGAAATTCATGGA) and RB582 (TCTAGACGG CAGGTCAGTCCAC). 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. RB953 was made to represent a complementary sequence to IRES of pWZLneo vector (AAAAGACGGCAATATGGTGG). Automated fluorescence-based cycle sequencing was conducted with the ABI Prism 377 DNA sequencer. Sequence analysis.

Transfection of PA317 cells and conditioned medium preparation. To produce amphotropic viruses, the parental pWZLneo, the FeLV-B-like recombinant env and the tvn retroviral constructs were transfected into PA317 cells by use of Lipofectamine (Gibco-BRL) according to the manufacturer’s protocol. After 48 h, the cells were split 1:10 and selected in G418-containing medium (400 μg/ml) for 10 days. As the selection of transfected cells was based on the expression of the G418-resistant gene from the same bicistronic proviral DNA carrying the env fragment, the use of WZLneo vector allowed virtually all selected resistant cells to express the env protein. The growth medium was changed every 3 days and G418-resistant colonies were isolated and expanded for further study.

When G418-resistant cells reached 80% confluence, the G418-containing growth medium was replaced with G418-free medium. The virus-containing cell supernatant fluids were harvested, passed through 0.45 μm (pore-size) filters (Gelman Sciences), and stored at −80°C for RT activity assay and infection of cell cultures.

RT activity assay. Titers of the viruses harvested were estimated by RT activity assay (5, 19). M-MuLV RT and GA-FeLV virus stock with known titres were included as standards. Each sample was tested in triplicate. Filtered conditioned medium (20 μl) was mixed with 25 μl of a cocktail containing 50 mM Tris (pH, 8.3), 10 mM dithiothreitol, 10 mM MgCl2, 60 mM NaCl, 0.05% NP-40, 2 μg of poly(dA·dT)12·18, and 3 μCi/ml α32P-dTTP. After 2 h at 37°C, 5 μl of the reaction mixture was transferred to 2 by-2-cm squares of DE81 chromatographic paper (Whatman International, Ltd.) and allowed to dry. The DE81 paper was washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.0015 M sodium citrate) for about 5 min each and then rinsed with ethanol. The dried filter paper squares were then transferred to scintillation vials for counting. The RT activity was used to obtain an estimate of the number of infectious particles by comparing it with that of a GA-FeLV-B preparation with a known virus titer.

Cell infection. B927 and HT1080 cells were seeded in six-well plates (2 × 105/well) the day before infection. On day 2, the cells were infected with 1 ml of individual virus preparations (approximately 5 × 106 infectious units per ml). After 12 h, the conditioned medium was replaced with G418-containing medium. The medium was changed every 3 days for up to 10 days. Aliquots (2 × 103) of Raji, CEM and 3201B cells were primed with 28 μl of Polybrene in 1 ml for 24 h prior to infection with conditioned medium containing approximately 105 infectious units per ml in the absence of Polybrene. At 12 h postinfection, the cells were resuspended with growth medium with or without G418.

MTT assay. Cytotoxic effect of the mutant env protein to Raji cells was measured by MTT assay. MTT is the yellow tetrazolium salt that can be converted to purple formazan dye by metabolically active cells. After treatment with Polybrene, Raji cells were mixed with conditioned medium as described above and transferred to a 96-well plate (2.5 × 103 cells/well for the 24-h postinfection measurement and 1.25 × 103 cells/well for the 48-h postinfection measurement). The MTT assay was conducted according to the manufacturer’s protocol (Boehringer Mannheim) after 24 and 48 h of infection.

TUNEL assay. An aliquot of Raji cell suspension (50 μl) treated with conditioned 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 deleted 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 5026; 8, tumor 5041; 9, tumor 5053; 10, tumor 5056; and 11, tumor 5051. Molecular sizes are indicated on the right.

Analysis of env for nucleotide and deduced amino acid sequence. The 700-bp env species from cat tumor 5023, namely, tenv, was cloned and sequenced. The tenv gene sequence was 100% homologous to CRA env (3), except for a large internal deletion of 1519 bp that shortened tenv. The deletion began from 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). 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). 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).
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 infection, 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{tenv} cytotoxicity to Raji cells. Along with morphological changes in \textit{tenv}-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{tenv}-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 μg 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{tenv} virus infection on Raji cells.

The same amount of Raji cells was infected with the same titer of \textit{tenv} 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{tenv} virus infection. Compared to vector-treated cells, there were 40% fewer \textit{tenv}-infected cells at 24 h postinfection. Furthermore, only 30% cells remained in \textit{tenv}-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{tenv} 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{tenv} 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{tenv} virus. To this end, to examine induction of apoptosis in \textit{tenv}-treated Raji cells, we performed a TUNEL assay to assess levels of apoptosis in vector virus- or \textit{tenv} virus-infected Raji cells. TUNEL assay is based on the detection of single- and double-stranded DNA breaks occurring in apoptosis. Because at 24 hour postinfection massive cell death (60% of vector-infected cells) was observed, Raji cells infected with \textit{tenv} 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{tenv} virus treatment resulted in approximately 45 ± 9% stained Raji cells with dark red nuclei, whereas only about 8 ± 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>
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<td>CEM</td>
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FIG. 4. Morphological changes in Raji cells induced by \textit{tenv} expression. Raji cells were treated with the vector-containing virus or \textit{tenv}-containing virus. At 24-hour postinfection, the cells treated with \textit{tenv} virus (panel B) displayed formation of large aggregates, whereas the cells treated with vector virus alone (panel A) remained primarily as single cells. Magnification, ×31.

FIG. 5. MTT assay of Raji cells infected with the \textit{tenv} virus. Cytotoxic effect of \textit{tenv} on Raji cells was measured by MTT assay at 450 nm 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{tenv}-treated cells. The standard deviations are also indicated.
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 tumored 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 of the factor, while alterations in the N-amino terminus do not appear to abolish gp55 activity (7).

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

**FIG. 6.** TUNEL assay of Raji cells infected with the tenv virus. Suspension of infected cells (50 μl) was transferred to plastic slides and left until dry. Apoptosis was detected with a red chromogenic substrate. Positively stained cells are the cells with dark red nuclei. (A) Vector-treated cells. (B) tenv-treated cells.
feline or human T-lymphoid tumor cells, namely, 3201B and CEM cells, did not manifest any significant cytopathicity. 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 cyto-

pathic 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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