Molecular Cloning of a Feline Leukemia Virus That Induces Fatal Immunodeficiency Disease in Cats

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A replication-defective variant of feline leukemia virus was molecularly cloned directly from infected tissue and found to induce a rapid and fatal immunodeficiency syndrome in cats. Studies with cloned viruses also showed that subtle mutational changes would convert a minimally pathogenic virus into one that would induce an acute form of immunodeficiency. The data suggest that acutely pathogenic viruses may be selected against by current methods for isolation of the human and simian immunodeficiency viruses.

INFECTION WITH FELINE LEUKEMIA VIRUS (FeLV) is associated with a variety of fatal diseases in domestic cats, including proliferative syndromes such as lymphosarcoma and leukemia but, more commonly, antiproliferative syndromes such as aplastic anemia and fatal immunodeficiency disease (*1*). In fact, FeLV provided an important paradigm perceived by Gallo and colleagues in seeking a retroviral etiology for human AIDS (*2*). A major limitation of our understanding of the pathogenesis of human AIDS results from the lack of an adequate animal model for analysis of human immunodeficiency virus (HIV-1)–induced viremia and disease. We have recently identified a naturally occurring isolate of FeLV (designated FeLV-FAIDS) that consistently induces a fatal immunodeficiency syndrome in outbred specific-pathogen-free (SPF) cats that is similar to AIDS in humans (*3, 4*). Therefore, we are studying the molecular mechanisms of induction of FeLV-associated immunodeficiency in cats.

In both human AIDS and the simian immunodeficiency virus (SIV) model for AIDS, viral antigens and DNA are difficult to detect in fresh tissue, and for all analyses to date investigators have used virus passaged in vitro (*5*). A major advantage of the feline model is that one can readily detect viral antigens and DNA in vivo (*3, 4*). Thus direct molecular analysis of the virus is possible without initial propagation (and thereby biological selection) in vitro. Here we report the molecular cloning and nucleotide sequence of FeLV-FAIDS obtained directly from tissues of cats with immunodeficiency syndrome. A replication-competent virus derived from these cats does not induce immunodeficiency. However, we identify a type of virus that will induce fatal immunodeficiency disease; all examples of this virus are replication-defective and require the replication-competent virus as a helper for transmission.

Immunodeficiency induced by FeLV-FAIDS follows one of two fatal clinical courses depending on the age of the animal at the time of inoculation. Cats inoculated at 8 weeks of age or younger develop an acute disease within 6 months; older cats develop a more chronic disease course marked by an asymptomatic or prodromal period of one or more years, similar to that found in human AIDS. On the basis of variable sized endonuclease fragments detected with a hybridization probe specific for an exogenous FeLV long terminal repeat (LTR), two classes of FeLV were previously described in the tissues of cats with the immunodeficiency syndrome (*4*). "Common form" virus, characterized by a 3.4-kb Kpn I fragment derived from near its 3′ end, appeared at the onset of viremia and persisted throughout the lifetime of the infected cats. Just prior to the onset of disease, FeLV variant forms always appeared in bone marrow. The most frequent of these forms was variant A, which has a 2.1-kb Kpn I fragment derived from near its 3′ end; other variants were also observed, particularly in cats with more chronic forms of disease (*4*). The appearance of variants consistently predicted the onset of overt disease, and it was therefore suggested that these viral genomes are the pathogenic components of FeLV-FAIDS (*4*). However, the precise role of common form and different variant FeLVs in induction of immunodeficiency in cats was unclear.

We have cloned proviral and unintegrated forms of FeLV-FAIDS from the tissues of four cats with acute or chronic immunodeficiency disease. Six clones were isolated from bone marrow DNA of cats (Nos. 1082, 1220, and 1045) in which the variant forms of the virus predominated. In addition, proviral DNA was cloned from the small intestine of cat 1161 which had approximately equal numbers of copies of the common and variant A genomes. Proviruses were categorized as common form or variant on the basis of the 3′ fragment generated by digestion with Kpn I in a manner consistent with earlier studies (Fig. 1) (*4*). A 3.4-kb band corresponds to the common form genome, whereas a 2.1-kb band corresponds to the variant A genome.

Limited restriction site variability was noted and it was generally confined to the extracellular glycoprotein (gp70) gene (Fig. 1). Only two differences, Kpn I and Sac II sites in variant gp70, were observed between the prototype common form clone

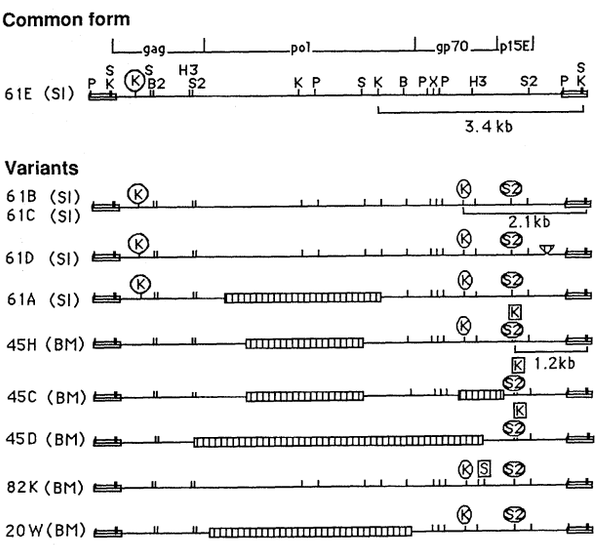

Fig. 1. Restriction site maps of FeLV variants. Each clone was named with a letter after the last two digits of the number of the cat from which they were isolated, for example, clone 61E was obtained from cat 1161 (*19*). Letters in parentheses show the tissue DNA from which the clone was isolated: SI, small intestine, BM, bone marrow. The boundaries of deleted regions of the genome (shown as striped boxes) are estimated as midway between the nearest restriction sites. The Kpn I site (in oval symbols) would generate a 3′ Kpn I digestion fragment consistent with a variant A genome (*4*); the additional Kpn I site in the isolates from cat 1045 (in a square) would generate a 1.2-kb terminal Kpn I fragment that was defined as variant B in this cat in a previous study (*4*). Restriction sites indicated correspond to: Bgl II (B2), Hind III (H3), Kpn I (K), Pst I (P), Sma I (S), Sst II (S2), and Xho I (X). Only divergent sites are indicated in the maps of variant clones.

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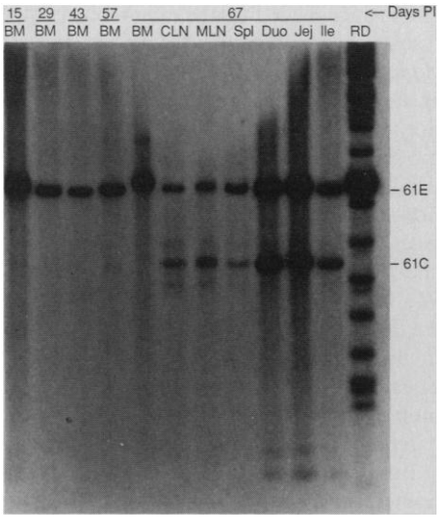

Fig. 2. Analysis of viral DNA present in sequential bone marrow samples and in various tissues at necropsy in cats inoculated with 61C/61E. Cat 1541 was inoculated with 10^5 focus-forming units of a viral mixture of 61C plus 61E, and BM DNA was analyzed at the intervals indicated by PI (days post-infection) by blot hybridization (*19*) until death at 67 days PI. MLN and CLN refer to mesenteric and colonic lymph node, respectively; Spl refers to spleen; and Ile, Jej, and Duo designate intestinal ileum, jejunum, and duodenum, respectively. RD refers to DNA from FeLV-infected cells used as hybridization standard (*19*). The higher molecular weight band (4.5 kb) in the 67-day BM sample is due to incomplete digestion.

61E (from cat 1161) and two of the variant genomes (61B and 61C) cloned from the same cat. The Sac II site was present in all of the variant clones analyzed, and the Kpn I site (that would generate a 2.1-kb Kpn I fragment consistent with a variant A virus) was found in all but two clones (45C and 45D) each of which had deletions in that region of gp70. The only restriction site polymorphism outside gp70 was a Kpn I site in the *gag* gene found in all clones derived from animal 1161.

Internal deletions were frequent in variant proviruses. Six of ten clones contained deletions centered around the *pol* gene, one contained a deletion in gp70 in addition to a deletion in *pol* (clone 45C), and one had a small deletion in the transmembrane protein coding sequence (clone 61D). Many deletions probably arose de novo in the infected cats since all three clones derived from cat 1045 contained the same restriction site polymorphism in gp70, but had different deletions.

To define the pathogenicity of common form virus, and determine if variants readily arise from it de novo, we performed the following experiments. A feline embryo fibroblast cell line (AH927) was transfected with cloned common form (61E) DNA; reverse transcriptase (RT) was detected in these cultures by 12 days, as was proviral DNA with the expected 3.4-kb 3′ terminal Kpn I fragment. Sixteen 8-week-old SPF cats were then inoculated with 10^5 focus-forming units of 61E virus [titered by the clone 81 assay (*6*)] derived from transfected cells. Each inoculated cat became viremic within 2 to 4 weeks and each has remained viremic since then. None has developed any signs of immunodeficiency disease after 10 months to more than 2 years. One cat developed a T cell lymphosarcoma after 21 months but the others remain healthy, indicating that FeLV-61E is minimally pathogenic and, like other chronic retroviruses, is capable of inducing lymphosarcoma with a long latency. In periodic bone marrow samples taken from two of these cats and in other tissues examined at necropsy, we have detected 61E virus (that originally appeared coincident with viremia) with no evidence of the 2.1-kb Kpn I fragment observed in symptomatic cats inoculated with FeLV-FAIDS. By comparison, the average survival time of 8-week-old SPF cats inoculated with the original FeLV-FAIDS strain was 3 months and disease was always preceded by the appearance in bone marrow of high copy numbers of variant genomes (*4*). The results from cats inoculated with 61E indicate that common form virus present in the original FeLV-FAIDS isolate does not induce immunodeficiency disease and that variants associated with immunodeficiency do not arise from common form virus at high frequency in vivo.

We then transfected all of the apparently full-length variant clones (61B, 61C, and 82K) into AH927 cells. Measurable RT activity was not detected in any of the cultures for a 6-week period; by comparison, clone 61E produced measurable RT activity within 2 weeks. A feline T cell line was also transfected with these clones and failed to produce measurable RT (but these cells did support growth of 61E). To evaluate the possibility that these variant genomes have a limited host cell range that does not include fibroblasts and T cells, we cotransfected the DNA from two apparently full-length clones, 61B and 61C, into AH927 cells with a selectable marker [pxfMoAneo (*7*)] containing the neomycin resistance gene. Cells that had taken up the marker DNA were identified by their ability to grow in the presence of G418. Cell cultures containing proviral DNA were obtained from single cell clones; none of these were producing measurable RT activity. Levy *et al.* (*8*) showed that RT can be detected in cell lines transfected with a replication-competent clone of HIV-1, even if the particular cell line was not receptive to infection by the virus. By analogy, our results suggested that all the variant genomes under study were replication-defective. Low levels of intracellular viral messenger RNA (mRNA) and extracellular viral p27 antigen were produced in each of two cell cultures containing 61B and one of two cell cultures containing 61C, indicating that each genome was transcriptionally competent and perhaps was packaged as noninfectious viral particles.

We then attempted to rescue the defective variants as transmissible virus from viral mRNA-positive cultures by infection with 61E virus. Two weeks after infection, supernatant from these cultures was transferred onto fresh AH927 cells. The inoculated cells developed approximately equal copy numbers of 61C and 61E DNA, as judged by blot hybridization, indicating successful transmission of the variant genome. When four 8-week-old SPF cats were inoculated with the 61C/61E virus mixture, each developed viremia and a fatal immunodeficiency disease within 4 months that was typical of that observed in cats inoculated with the original FeLV-FAIDS isolate (*3*).

In cats inoculated with 61C/61E, 61E was detected in the bone marrow 2 to 3 weeks after inoculation, and in all tissues examined at death (Fig. 2). This result is consistent with results obtained from cats inoculated with 61E alone, and with previous studies of cats inoculated with FeLV-FAIDS that indicated no discernible tissue specificity of common form virus for replication in vivo (*4*). However, in contrast to the results of previous studies in which FeLV-FAIDS variants were consistently found in bone marrow just prior to disease (*4*), three of four cats inoculated with 61C/61E did not have detectable levels of 61C virus in the bone marrow preceding onset of disease or at postmortem (for example, Fig. 2). However, 61C was detected at high copy number in intestinal DNA, the tissue from which both 61C and 61E clones were originally isolated, with lower amounts in lymph node and spleen (Fig. 2). The cats inoculated with 61C/61E had enteritis and histological evidence of damage to intestinal epithelium.

Identification of a replication-competent, minimally pathogenic clone and a replication-defective pathogenic clone allows the possibility of constructing a replication-competent virus that will induce immunodeficiency disease. We used an Xho I site near to the carboxyl terminus of the polymerase gene to construct chimeric viruses with exchanged regions of the 61E and 61C genomes (Fig. 3A). A construct containing the 5′LTR-*gag-pol* of 61C and the *env*-3′LTR of 61E (CCEE) was incapable of producing infectious virus; this localized the replication defect (or defects) in clone 61C to the 5′ region. The reciprocal construct, containing

the *env*-3′LTR of 61C (EECC), was replication-competent and, when inoculated into seven 8-week-old cats, induced fatal immunodeficiency in all seven within 3 to 8 weeks—the shortest disease course that we have observed with any inoculum (*3, 4*). Like the 61C variant in cats inoculated with 61C/61E, the number of copies of the EECC viral genome was highest in intestinal tissue in four of four cats examined although, unlike 61C, the EECC genome was also present in the bone marrow of each cat (Fig. 3B). The clinical pattern of disease was also similar (*3*). Furthermore, viral antigen was detected in damaged intestinal epithelia suggesting that the virus may play a direct role in cytopathic depletion in that tissue (Fig. 4). Intestinal cytopathicity is also observed in the intractable diarrhea and wasting syndromes that characterize human AIDS (*9*) and in the acute lethal immunodeficiency of macaques inoculated with SIV isolate PBj-14 (*10*).

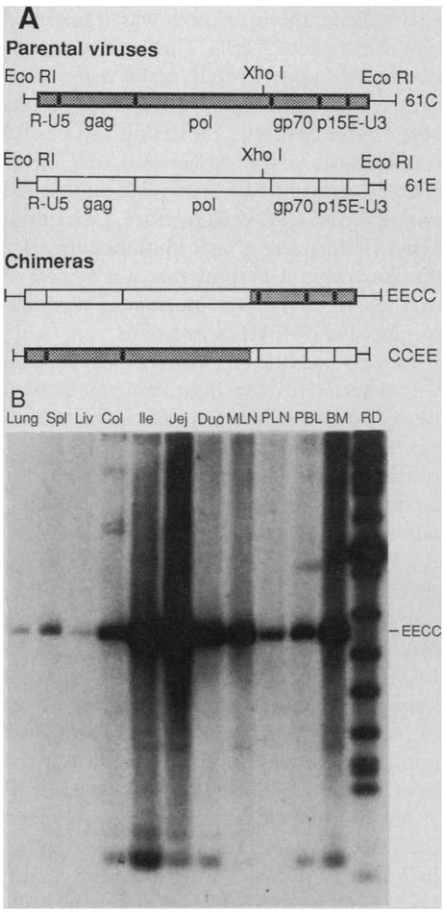

Fig. 3. Generation and testing of chimeric genomes of 61E and 61C. (**A**) Construction of reciprocal 61E and 61C chimeric genomes (*20*). The growth and pathogenicity of each clone after transfection was as follows. Parental viruses: 61C, replication-defective, causes immunodeficiency disease when rescued with 61E; 61E, replication-competent, minimally pathogenic. Chimeras: EECC, replication-competent, causes feline immunodeficiency disease; CCEE, replication-defective. (**B**) Analysis of the viral DNA in the tissues of a cat (1807) inoculated with EECC. Cats were inoculated and monitored as described in Fig. 2; cat 1807 survived 29 days after inoculation. The relative copy number of FeLV genomes in the tissues of cat 1807 was typical of the three cats inoculated with EECC that were analyzed in detail. Abbreviations: Spl, spleen; Liv, liver; Col, colon; Ile, ileum; Jej, jejunum; Duo, duodenum; MLN, mesenteric lymph node; PLN, pharyngeal lymph node; PBL, peripheral blood lymphocytes; BM, bone marrow. No viral DNA was detected in the brain or salivary gland of cat 1807.

We determined the nucleotide sequence of the 61C component of the pathogenic EECC virus chimera and compared its nucleotide and deduced amino acid sequences with the minimally pathogenic 61E virus sequence (*11*). This region contains the 3′ terminal 219 nucleotides of the *pol* open reading frame, the complete 1953-nucleotide *env* gene predicted to encode a 421–amino acid extracellular glycoprotein, and a 198–amino acid transmembrane protein (p15E), as well as the complete 482-nucleotide 3′ LTR (Fig. 5). No amino acid differences were observed between 61C and 61E in the region encoding p15E. Two coding differences in 61C were observed near the carboxyl terminus of the *pol* open reading frame; however, these changes were not found in another variant, 82K, which also induced fatal immunodeficiency in cats when inoculated along with 61E (*12*). These findings suggest that the substitutions in the *pol* region of 61C may not contribute to its pathogenicity. The LTRs differ by three nucleotides, two changes within the U3 region, and a one-nucleotide deletion at the boundary between U3 and R. The most pronounced differences were observed within the gp70 gene, where the 61C sequence differs from that of 61E by 11 scattered amino acid changes (17 single nucleotide changes), plus a six–amino acid (18 bp) insertion and a six–amino acid deletion. The additional Kpn I site found in variant gp70 genes is due to a single base change near the deletion. Both of these changes occur in a region of gp70 that is highly variable in other isolates of FeLV (*13*); the additional Sac II site found in all variant genomes examined to date is within the insertion (that corresponds to an imperfect direct repeat, underlined in Fig. 5), and similarly is in a region of gp70 that is also highly variable among FeLV isolates (*13*). In summary, studies in vivo implicate the *env*-3′LTR of 61C as the region encoding the lymphocytopathic and enterocytopathic determinants of the virus, and nucleotide sequence comparisons indicate that relatively subtle changes in gp70 or the LTR probably correspond to the critical viral determinants.

Our results indicate that variants of FeLV that induce feline immunodeficiency are not readily generated in vivo from a molecularly cloned common form virus. The types of change observed (point mutations, deletions, and duplications) are not typical of the variation found among other FeLVs, in which diversity is generated by recombination between a horizontally transmissible virus, of which 61E is a prototype, and endogenous FeLV-related sequences (*14*). We infer from the sequence homology in *env*-LTR and the identical restriction maps in the *gag* and *pol* regions that either the disease-inducing variants arose from common form or both arose from the same parental FeLV.

In studies with FeLV-FAIDS, variant genomes usually occurred at higher copy number than common form in the tissue DNA of cats with clinical immunodeficiency (*4*). Here we report that this population of variant viruses is predominantly replication-defective, although we are unable to say if other naturally occurring immunosuppressive variants of FeLV consist primarily of defective genomes. The ability of a defective virus to outgrow a replication-competent helper has also been observed with defective interfering particles that modulate replication in several other viral systems (*15*). Our results suggest that the LTR of the defective variants may have a stronger promoter than the helper virus or that the variant may code for a protein product that directly or indirectly stimulates its own replication. The variant and helper viruses may differ in the time it takes for each to establish superinfection interference, as has been proposed for cytopathic avian retroviruses (*16*). Many of the variants contain large internal deletions, corresponding to a region within the *env* gene intron, that may change the ratio of spliced and unspliced mRNA and result in an even higher level of *env* protein expression (*17*).

The diversity in the pathogenic potential of feline retroviruses suggests that similar diversities may occur in HIV-infected humans and SIV-infected simians. Since each of the variants we isolated were taken directly from tissue DNA and were not subjected to the requirement of replication competence or selection by growth in vitro, we suggest that an important subset of pathogenic variants of HIV may not be readily detected by the current techniques for isolation, all of which involve cocultivation of peripheral blood lymphocytes with immortalized T cell lines or normal peripheral blood cells (*5*). These results may explain some recent observations with SIV, where attenuation of virus pathogenicity occurs on passage in vitro (*18*), perhaps due to loss of a minor, acutely cytopathic and possibly

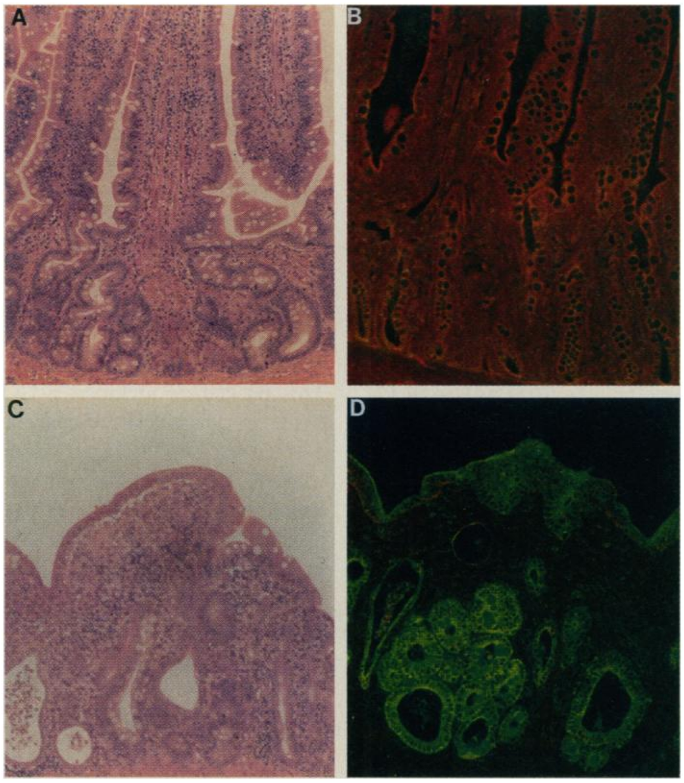

Fig. 4. Cytopathic damage and replication in small intestinal germinal (crypt) epithelium of a cat inoculated with EECC. (A and B) Paraffin sections of normal control cat intestine with intact intestinal villi. (**A**) Hematoxylin and eosin (H&E) staining. (**B**) Immunofluorescence staining of p27 viral antigen (*3*); no viral replication evident in crypt cells. (C and D) Intestine of a cat with immunodeficiency disease. Severe damage to crypt epithelium and atrophy of intestinal villi have occurred. (**C**) H&E stain. (**D**) Immunofluorescence staining showing extensive viral replication (p27 antigen) in damaged crypt cells.

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              10        20        30        40        50        60        70        80        90       100       110       120
               |         |         |         |         |         |         |         |         |         |         |         |
     .....     Leu            Lys|leader >
61C  .....       T               A
61E  5958.CCCAGCTCAGACGATCCATCGAGATGGAAAGTCCAACGCACCCAAAACCCTCTAAAGATAAGACTCTCTCGTGGAACTTAGTGTTTCTGGTGGGATCTTATTCACAATAGACAT
env >.....                         M  E  S  P  T  H  P  K  P  S  K  D  K  T  L  S  W  N  L  V  F  L  V  G  I  L  F  T  I  D  I
pol >..... P  S Ser D  D  P  S Arg W  K  V  Q  R  T  Q  N  P  L  K  I  R  L  S  R  G  T

                         Pro   Met
61C            |gp70 >     C     G                                                   C
61E  AGGAATGGCCAATCCTAGTCCACACCAAATATATAATGTAACTTGGGTAATAACCAATGTACAAACTAACACCCAAGCTAATGCCACCTCTATGTTAGGAACCTTAACCGATGTCTACCC
      G  M  A  N  P  S  PHis QIle Y  N  V  T  W  V  I  T  N  V  Q  T  N  T  Q  A  N  A  T  S  M  L  G  T  L  T  D  V  Y  P

                                                     Met                - - - - - - Gly      Pro
61C                                                   G                 -----------------G G      C
61E  TACCCTACATGTTGACTTATGTGACCTAGTGGGAGACACCTGGGAACCTATAGTCCTAAGCCCAACCAATGTAAAACACGGGGCACGTTACCCTTCCTCAAAATATGGATGTAAAACTAC
      T  L  H  V  D  L  C  D  L  V  G  D  T  W  E  PIle V  L  S  P  TAsnValLysHisGlyAlaArg Y  PSer S  K  Y  G  C  K  T  T

                .......//.......
61C          G .......//.......C                                             Ile         Pro
61E  AGATAGAA.6320..//..6965.TTAGGTAACTACAGCAACCAAACAAACCCTCCCCCATCCTGCCTATCTATTCCGCAACACAAGCTGACCATATCTGAAGTATCAGGGCAAGGACTG
      D  R .......//....... L  G  N  Y  S  N  Q  T  N  P  P  P  S  CLeu S  I  PGln H  K  L  T  I  S  E  V  S  G  Q  G  L

                                                    His                AspTyrLeuThrAlaProArg
61C                                                  C                 G  TATCTAACCGCCCCGCGG
61E  TGCATAGGGACTGTTCCTAAGACCCACCAGGCTTTGTGCAATAAGACGCAACAGGGACATACAGGGGCGCAC------------------TATCTAGCCGCCCCCAATGGCACCTATTGG
      C  I  G  T  V  P  K  T  H  Q  A  L  C  N  K  TGln Q  G  H  T  G  AHis-  -  -  -  -  -  Y  L  A  A  P  N  G  T  Y  W

                                                    Leu
61C                                                  T
61E  GCCTGTAACACTGGACTCACCCCATGCATTTCCATGGCGGTGCTCAATTGGACCTCTGATTTTTGTGTCTTAATCGAATTATGGCCCAGAGTGACTTACCATCAACCCGAATATGTGTAC
      A  C  N  T  G  L  T  P  C  I  S  M  A  V  L  NTrp T  S  D  F  C  V  L  I  E  L  W  P  R  V  T  Y  H  Q  P  E  Y  V  Y

                    Gly               |p15E >  ......./                                                               •
61C                  G                   G     .......//.......   |LTR  U3 >
61E  ACACATTTTGCCAAAGCTGTCAGGTTCCGAAGAGAACCAATA.7324..//..7956.GAATGAAAGACCCCCTACCCAAAATTTAGCCAGCTACTGCAGTGGTGCCATTTCACAAGGCA
      T  H  F  A  K  AVal R  F  R  R  E  P  I ......./

61C                                                  G
61E  TGGAAAATTACTCAAGTATGTTCCCATGAGATACAAGGAAGTTAGAGGCTAAAACAGGATATCTGTGGTTAAGCACCTGGGCCCCGGCTTGAGGCCAAGAACAGTTAAACCCCGGATATA

61C  A
61E  GCTGAAACAGCAGAAGTTTCAAGGCCGCTGCCAGCAGTCTCCAGGCTCCCCAGTTGACCAGAGTTCGACCTTCCGCCTCATTTAAACTAACCAATCCCCATGCCTCTCGCTTCTGTACGC

61C                                        -|R >                                                               |U5 >
61E  GCGCTTTCTGCTATAAAACGAGCCATCAGCCCCCAACGGGCGCGCAAGTCTTTGCTGAGACTTGACCGCCCCGGGTACCCGTGTACGAATAAACCTCTTGCCGATTGCATC../..8440
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Fig. 5. Nucleotide sequence analysis of the *env*-3′LTR of 61C and comparison to the corresponding region of 61E virus. Portions of the nucleotide sequence of 61E (*11*) are shown along with the single-letter translation of the *pol* and *env* proteins and nucleotide coordinates along the 8440-nucleotide genome. The corresponding sequence of clone 61C was determined (*21*) and only those nucleotide and amino acids that differ are shown here. Two stretches of complete nucleotide identity are not shown and are indicated by ..//.. Where differences result in a change in the deduced amino acid sequence, the three-letter amino acid code is shown. Dashes indicate gaps.

defective virus. Thus, our results suggest a need for direct isolation of HIV and SIV genomes from tissue DNA.

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18. For example, (i) macaques inoculated with molecular clone–derived, replication competent SIV-macaque isolate BK28 [previously referred to as HTLV-4; H. Kornfeld, N. Riedel, G. A. Viglianti, V. Hirsch, J. I. Mullins, *Nature (London)* **326**, 610 (1987)] have become persistently infected but show no signs of lymphocyte subset deficits or immunodeficiency disease within 12 months after inoculation (M. Murphey-Corb, L. Martin, H. Kornfeld, J. I. Mullins, unpublished observations); (ii) macaques inoculated with undiluted, tissue-derived SIV (isolate PBj-14) develop acute onset lethal disease, whereas virus obtained from this pool biologically cloned by two sequential terminal dilutions and passaged in human lymphocytes caused infection but no acute disease (P. N. Fultz, personal communication); (iii) similarly, SIV (isolate $Delta_{915}$) extensively passaged in tissue culture has attenuated pathogenicity in vivo relative to virus that has undergone two passages in vitro (G. Baskin *et al.*, in preparation).
19. DNA from cat 1082 BM was fractionated by the method of Hirt [B. Hirt, *J. Mol. Biol.* **26**, 365 (1967)] to enrich for unintegrated viral DNA, whereas clones were obtained from total cellular DNA of cats 1161, 1045, and 1220. A bacteriophage vector prepared by Bam HI digestion [λL47.1; W. A. M. Loenen and W. J. Brammar, *Gene* **10**, 249 (1980)] was chosen for DNA from

cats 1082 BM and 1220 BM to clone unintegrated circular viral DNA (which Bam HI will cut once and therefore linearize). To obtain clones with intact proviruses, DNA from cats 1045 BM and 1161 SI was first cut with Eco RI (which does not cleave within the FeLV-FAIDS genome), and fractionated on a sucrose gradient. Fractions containing DNA of sufficient length to potentially contain full-length proviruses, but within the capacity of the bacteriophage vector [λgtWESλB; P. Leder, D. Tiemeier, L. Enquist, *Science* **196**, 175 (1977)] were pooled. The libraries were prepared and screened with the exU3 probe. Proviruses were subcloned into the plasmid vector pUC18 for subsequent analysis. These procedures, the origin of the RD(FeLV)-2 cell line used as hybridization standard (lane marked RD), and the generation of restriction maps and blot hybridization were generated as previously described [J. I. Mullins, J. W. Casey, M. O. Nicolson, N. Davidson, *Nucleic Acids Res.* **8**, 3287 (1980); J. I. Mullins, J. W. Casey, M. O. Nicolson, K. B. Burck, N. Davidson, *J. Virol.* **38**, 688 (1981)]. The most intense band in the RD lane is 3.6 kb in length and corresponds to approximately 22 copy per cell intensity, whereas the remaining bands correspond to single copy per cell intensity.

20. Plasmids containing the genome of 61E and 61C were digested with Eco RI and Bam HI. A fragment representing the 3′ portion of each virus was gel-purified in low melting agarose, treated with the Klenow fragment of DNA polymerase I to fill in recessed ends, and subcloned into Sma I–digested pUC19. These subclones were subsequently digested with Eco RI and Xho I and ligated with a gel-purified Eco RI–Xho I fragment representing the 5′ portion of either 61E or 61C. The structure of the plasmids containing full-length chimeric viruses was verified by restriction enzyme digestion.
21. Deletion subclones were generated by digestion of the Bam HI–Eco RI subclone of 61C (Fig. 4) with exonuclease Bal 31 [M. Poncz, M. Solowiejczyk, E. Schwartz, S. Surrey, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4298 (1982)], ligated into M13mp18, and sequenced by the Sanger dideoxy method as described [F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977)].
22. We thank V. Stallard for technical assistance. Supported by grants from the American Foundation for AIDS Research (J.O.), by NIH CA43216 (E.A.H. and J.I.M.), and by the Massachusetts AIDS Research Council and NIH CA01058 (J.I.M.). J.O. was supported in part by a fellowship from the Interdisciplinary Programs in Health under EPA assistance agreement CR812699, and P.R.D. by a postdoctoral fellowship from NIH (CA07966). DNA sequence analysis was performed using Intelligenetics program made available through BioNet computer resource.

20 November 1987; accepted 15 January 1988

Site-Directed Mutagenesis of Two Trans-Regulatory Genes (*tat*-III, *trs*) of HIV-1

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Point mutations were introduced into the overlapping trans-regulatory genes (*tat*-III and *trs*) of human immunodeficiency virus type 1 (HIV-1), and the mutants were evaluated for virus expression. The results showed that *tat*-III has a positive trans-acting role and is required for transcriptional activation. A chain terminating mutation early in the *trs* gene resulted in an increase in transcription of viral messenger RNA as measured by nuclear transcription experiments, but only one major species of viral messenger RNA (1.8 kilobases) was detected, and little or no viral structural proteins were made. Thus, the *trs* gene product is essential for expression of virus structural proteins but, at the same time, may have a negative trans-regulatory role in transcription. Cotransfection of the point mutant proviruses defective in *tat* or *trs* with each other or with a complementary DNA clone containing *tat* and *trs* sequences restored the normal transcription pattern and subsequent virus production.

The prototype human immunodeficiency virus (HIV-1) contains, in addition to the structural genes *gag*, *pol*, and *env*, at least five accessory genes termed *sor*, *R*, *tat*-III, *trs*, and 3′-*orf* (*1*). The *tat* and *trs* genes are of particular interest since both are essential for virus expression (*2–5*). However, the precise functions of these genes in the virus life cycle and pathogenesis are not well understood. The sequences responsive to *tat* are located downstream from the initiation site for transcription, and several studies based on the use of deletion mutants suggest that *tat* increases the efficiency of messenger RNA (mRNA) utilization (post-transcriptional activation) (*5, 6*) or the level of steady-state mRNA either by direct transcriptional activation or mRNA stabilization (*7*). Parallel studies showed that nuclear extracts from HIV-infected cells contain transcriptional activator or activators specific for the HIV-1 long terminal repeat (LTR) (*8*). The *trs* gene (also referred to as *art*) may also act at more than one level, as an antirepressor at the translational level (*4*), and influence the relative abundance of viral mRNAs of large and small molecular weight (*5*). Because the *tat* and *trs* genes overlap, large deletions usually affect both genes and may further affect potential target sequences or secondary structures of the transcribed mRNA (*2–5*). We therefore used oligonucleotide-directed mutagenesis to introduce independently single translational stop codons in the 5′ portion of each gene. Analysis of these mutants indicates that both *tat* and *trs* regulate virus production at transcriptional and post-transcriptional levels.

Using a biologically active HIV-1 clone, HXB2 (*2*), we constructed two mutants with independent changes in the *tat* and *trs* genes (see Fig. 1) that we isolated by means of the double-primer extension method (*9*). This procedure included a GC primer (15 bases in length) previously designed for pGC1 vector (*10*) and a mutated oligonucleotide (21 bases). One mutant contained a termination codon (TGA) in place of the methionine initiator codon (ATG) in the *tat* gene [mutant 80:*tat*(−)]. The other contained a stop codon (TGA) in place of the glycine residue 6 (GGA) in the *trs* gene and simultaneously a leucine (CTG) codon in place of arginine (CGG) at residue 52 in the *tat* reading frame [mutant 107:*trs*(−)]. We then transfected the mutants into COS-1 cells (an African Green monkey kidney-derived fibroblastic cell line), which are highly permissive for virus production but are refractory to subsequent reinfection (*5*). The efficiency of transfection of mutant and wild-type clones appeared comparable as monitored by Southern hybridization of cellular DNA harvested 48 hours after transfection. The use of COS-1 cells allowed us to compare, qualitatively and quantitatively, the mutants for RNA and protein production. Mutant viruses derived from these cells can also be assayed for their infectivity in permissive lymphoid cell lines by cocultivation or by cell-free transmission.

Initially, we screened the mutants for *tat* activity using a transient chloramphenicol acetyltransferase (CAT) assay in which the bacterial acetyltransferase gene was used as an indicator (*11*). Cotransfection of the mutant genomes and an LTR-CAT plasmid into COS-1 cells was mediated by calcium phosphate precipitation. The activity of *tat* was measured as percentage conversion of ^{14}C-labeled chloramphenicol to its acetylated metabolites (Fig. 2). The plasmid HXB2 was used as control for wild-type activity, and the complementary DNA construct C15-CAT, which contains the HIV-1 3′ LTR upstream of CAT (*12*), served as a negative control. In mutant 80 the CAT activity was completely abrogated. However, in mutant 107, the level of CAT activity was only mildly reduced relative to that of the wild type. No significant difference was detected between the LTR-CAT alone and cotransfected LTR-CAT and the *tat*(−) clone over the linear range of the time

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