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Neurodegenerative Disease Induced by the Wild Mouse Ecotropic Retrovirus Is Markedly Accelerated by Long Terminal Repeat and *gag-pol* Sequences from Nondefective Friend Murine Leukemia Virus

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The wild mouse ecotropic retrovirus (WM-E) induces a spongiform neurodegenerative disease in mice after a variable incubation period of 2 months to as long as 1 year. We isolated a molecular clone of WM-E (15-1) which was weakly neurovirulent (incidence, 8%) but was highly leukemogenic (incidence, 45%). Both lymphoid and granulocytic leukemias were observed, and these leukemias were often neuroinvasive. A chimeric virus was constructed containing the *env* and 3′ *pol* sequences of 15-1 and long terminal repeat (LTR), *gag*, and 5′ *pol* sequences from a clone of Friend murine leukemia virus (FB29). FB29 has been shown previously to replicate to high levels in the central nervous system (CNS) but is not itself neurovirulent. This finding was confirmed at the DNA level in the current study. Surprisingly, intraperitoneal inoculation of neonatal IRW mice with the chimeric virus ($FrCas^E$) caused an accelerated neurodegenerative disease with an incubation period of only 16 days and was uniformly fatal by 23 days postinoculation. Introduction of the LTR of 15-1 into the $FrCas^E$ genome yielded a virus ($FrCas^{EL}$) with a degree of neurovirulence intermediate between those of 15-1 and $FrCas^E$. No differences were found in the levels of viremia or the relative levels of viral DNA in the spleens of mice inoculated with 15-1, $FrCas^E$, or $FrCas^{EL}$. However, the levels of viral DNA in the CNS correlated with the relative degrees of neurovirulence of the respective viruses ($FrCas^E$ > $FrCas^{EL}$ > 15-1). Thus, the *env* and 3′ *pol* sequences of WM-E (15-1) were required for neurovirulence, but elements within the LTR and *gag-pol* regions of FB29 had a profound influence on the level of CNS infection and the rate of development of neurodegeneration.

The wild mouse ecotropic retrovirus (WM-E) originally isolated by Gardner and co-workers (15) causes a chronic neurodegenerative disease associated with spongiform degeneration of the grey matter and tremulous paralysis (1). In addition, this virus also causes leukemia of both lymphoid and granulocytic cell types (14, 16). The neurodegenerative disease is generally slow in onset, with incubation periods ranging from 2 to 12 months, and the incidence of disease can be unpredictable, even when molecularly cloned virus and inbred strains of mice are used. These features have made systematic study of the pathogenesis of the neurologic disease cumbersome.

In the original description of the neurologic disease, the overall incidence in wild mice over a 14-month observation period was only 15% (15). The heterogeneity in susceptibility appeared to be due in part to segregation of the *Akvr-1* (*Fv-4*) resistance gene (17). More subtle effects have been observed in inbred laboratory mice in which host resistance genes affect both the incidence and the tempo of the neurologic disease (20). These latter host genes have not been characterized.

In addition to host genetic resistance, the tempo of the disease is affected by stage of development of the mouse at which inoculation of the virus occurs. The most rapid disease has been observed in mice inoculated in utero during midgestation (35). In contrast, mice generally are completely resistant when the virus is inoculated after postnatal day 6 (22). It is thought that at least a component of this age dependence is mediated by the immune response (21).

The nature of the virus inoculum also influences the tempo of the disease. Brooks et al. (3) found that inoculation of concentrated virus shortened the incubation period and increased the severity of the disease. This could have been due to either the increased initial virus load per se or perhaps the existence of a minor population of highly virulent virus variants in the original virus stock. DesGroseillers et al. (10, 12) have used molecular approaches to define the viral sequences which influence neurovirulence. These studies revealed that the viral *env* gene of WM-E is an important determinant of neurovirulence (10, 28) but that viral long terminal repeat (LTR) sequences appeared to influence the tempo of the disease (12). They replaced the LTR of their infectious clone of WM-E (NE-8) with that of the thymotropic virus Moloney murine leukemia virus (Mo-MuLV). Interestingly, intrathymic inoculation of the chimeric virus induced accelerated neurologic disease with an incubation period of 40 to 100 days (compared with 100 to 200 days for NE-8).

Using similar approaches, we constructed a chimeric virus containing envelope sequences derived from an infectious molecular clone of WM-E and nonenvelope sequences derived from a strain of nondefective Friend murine leukemia virus (F-MuLV). This chimeric virus induced markedly accelerated neurodegenerative disease (incubation period, only 16 days) which affected 100% of the inoculated mice and was invariably fatal. Sequences within the viral LTR, as well as the *gag-pol* region of F-MuLV, were found to be

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responsible for this remarkable disease acceleration, and these sequences specifically enhanced the level of infection of the central nervous system (CNS).

MATERIALS AND METHODS

Mice, viruses, and inoculations. IRW mice are an inbred strain of the *Fv-1*$^{n/n}$ genotype derived, bred, and raised at the Rocky Mountain Laboratories. These mice are highly susceptible to leukemia induced by neonatal inoculation of both F-MuLV (36) and Mo-MuLV (M. Sitbon, H. Ellerbrok, F. Pozo, J. Nishio, S. F. Hayes, L. H. Evans, and B. Chesebro, J. Virol., in press) helper viruses and are also susceptible to CNS disease induced by neonatal inoculation of WM-E (26). Mice (24 to 48 h old) were inoculated intraperitoneally and, in one experiment, intracerebrally with 30 μl of virus stocks. Mice were weaned at 21 days, except for mice inoculated with the virus FrCas^{E}, which were not weaned. Virus stocks were prepared as described previously (32). The titers of virus stocks were as follows (in focus-forming units per milliliter): 15-1, 3×10^5; FrCas^{E}, 5.4×10^5; FrCas^{EL}, 3×10^5; CasFr^{E}, 1×10^6; FB29, 4×10^6. Clinical evaluations involved examination of the movements of mice in the cage and lifting each mouse by the tail to evaluate the normal reflex abduction of the hind limbs (1). Starting at 11 to 12 days postinoculation, mice were evaluated daily until 30 days of age. Mice were examined weekly thereafter for evidence of neurologic disease and after 4 months, when the first cases of leukemia were noted; the examinations included palpation for splenomegaly and lymphadenopathy under ether anesthesia.

Virus was quantified by focal immunofluorescence assay using type-specific anti-gp70 monoclonal antibodies 48, for viruses containing the F-MuLV *env* gene (8), and 667, for viruses containing the WM-E *env* gene (26). Virus titers are expressed as in vitro fluorescent focus-forming units per milliliter. *Fv-1* tropism was analyzed by "hitness" curves using NIH 3T3 and Balb 3T3 cells (29).

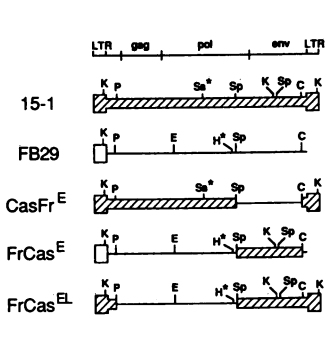
Pathologic studies. Clinically affected mice were killed by exsanguination after ether anesthesia and examined for gross pathologic changes. In some cases, tissues were fixed in 5% formaldehyde in phosphate-buffered saline (pH 7.4) for 24 h and paraffin-embedded blocks were sectioned and stained with hematoxylin and eosin. Diagnoses of leukemia types were made on the basis of the sites and character of organomegaly, as well as examination of imprints stained with Giemsa. Thymic lymphomas were characterized by gross thymic enlargement in addition to splenomegaly and lymphadenopathy. Nonthymic lymphomas were characterized by splenomegaly and lymphadenopathy but no thymic enlargement. Chloroleukemia was diagnosed when there was splenomegaly and lymphadenopathy, the lymph nodes having a characteristic green tinge. These latter leukemias were unaccompanied by thymic enlargement. Leukemic infiltration of the CNS was diagnosed either by imprint preparations of the meninges over the neocortex or by routine histologic examination of embedded material.

Molecular cloning of viral DNA and isolation of a WM-E-specific probe. NIH 3T3 cells were infected at a high multiplicity of infection with the Cas-Br-M strain of WM-E (originally obtained from Janet Hartley, National Institute of Allergy and Infectious Diseases), and to assure confluent infection, the cells were passed three times at a 1/10 dilution. These cells were used to infect new NIH 3T3 cells by cocultivation at a ratio of four uninfected cells to one infected cell. After 21 h, the DNA was extracted and subjected to high-salt precipitation as described by Hirt (19). The unintegrated closed circular viral DNA in the supernatant was digested with *Sal*I, which cuts once in the viral genome within the polymerase gene (7, 24), and cloned into plasmid pUC19, which was also cut with *Sal*I and subjected to alkaline phosphatase treatment. After transformation into *Escherichia coli* JM109, bacterial colonies were blotted onto nylon filters (Nytran; Schleicher & Schuell, Inc., Keene, N.H.). Of 2,500 recombinant plasmids, 12 hybridized to a WM-E-specific probe, WM^{xb} (described below), and 8 of these contained inserts of ≥8.2 kilobases (kb). Plasmid DNAs from these eight clones were partially purified by the alkaline lysis method (25) and cut with *Sal*I. DNA was transfected into NIH 3T3 cells as previously described (36), and virus was recovered from one of the clones (15-1) after four passages of the cells at a 1/5 dilution. This virus was used to infect Fisher rat embryo cells, from which virus stocks were prepared.

The 1504E strain of WM-E (4), obtained from M. Gardner (University of California, Davis, Dixon), was cloned by similar techniques with some modifications. In this case, the Hirt supernatant was subjected to further fractionation by ion-exchange chromatography to purify supercoiled DNA (18) and this DNA was digested with *Hin*dIII, which cuts the viral DNA 3′ of the *Sal*I site within the polymerase gene (7). After ligation into pUC19 and transformation, colonies were screened with a full-length viral DNA probe derived from FB29 (see below). We recovered 14 clones of ≥8.2 kb, one of which, clone 12-4, was used to produce a WM-E-specific probe essentially as described by Rassart et al. (33). As predicted on the basis of restriction endonuclease analysis of Hirt supernatant preparations of this virus (7), WM1504-E contains an *Xba*I site 263 bases 3′ of the *env* start codon and a *Bam*HI site 760 bases 3′ of the *env* start. This 0.5-kb *Xba-Bam* fragment was ligated into pUC19 cut with the same enzymes. The insert (WM^{xb}) was purified by electroelution, and its specificity was tested by Southern blot analysis using cloned viral DNAs from WM-E clones 15-1 and 12-4; ecotropic viruses Mo-MuLV, F-MuLV, and Akv; xenotropic virus NZB-IU6; and polytropic virus 13MCF (the latter two of which were kindly provided by A. Khan, National Institute of Allergy and Infectious Diseases). At high stringency (washing at 65°C in 0.1× SSPE [1× SSPE is 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA {pH 7.4}]–0.1% sodium dodecyl sulfate for 30 min), significant hybridization was detected only to the wild mouse viral DNAs (data not shown).

Construction of chimeric viral genomes. The two parental viruses used in construction of chimeric viruses were 15-1 (the infectious WM-E clone) in pUC19 and FB29, a clone of F-MuLV strain I^{-5} (36) cloned at the *Hin*dIII site in the 3′ end of the *pol* gene into a modified pUC19 plasmid from which the multiple cloning site between *Sph*I and *Eco*RI had been excised. The latter plasmid was kindly supplied by M. Sitbon (Laboratoire d'Immunologie et Virologie des Tumeurs, Hopital Cochin, Paris, France). DNA fragments were purified by electroelution from agarose gels (DNA Grade Ultrapure Agarose; Bio-Rad Laboratories, Richmond, Calif.). Enzymes were from New England BioLabs, and reactions were performed under conditions recommended by the manufacturer.

The chimeric viral genomes are illustrated in nonpermuted form in Fig. 1. For construction of FrCas^{E}, the FB29 plasmid was cut with *Sph*I and *Cla*I and the 8.6-kb fragment containing LTR, *gag*, and 5′ *pol* sequences, along with plasmid pUC19, was ligated to a 2.6-kb *Sph*I-*Cla*I fragment contain-



	FV-1 Tropism	∝gp70 Reactivity 48	667	Viremia
15-1	N	-	+	4.0 X 10^4 (9)
FB29	NB	+	-	6.8 X 10^5 (9)
CasFr^E	n.t.	+	-	2.6 X 10^4 (6)
FrCas^E	NB	-	+	5.6 X 10^4 (10)
FrCas^{EL}	n.t.	-	+	1.6 X 10^4 (6)

FIG. 1. Schematic representation of the DNA genomes of parental viruses 15-1 and FB29 and chimeric viruses CasFr^E, FrCas^E, and FrCas^{EL}. The gene boundaries are depicted above. The restriction sites used in the constructions and for confirmation of the respective structures were *Cla*I (C), *Eco*RI (E), *Hin*dIII (H), *Kpn*I (K), *Pvu*I (P), *Sal*I (Sa), and *Sph*I (Sp). Asterisks indicate the sites at which the viral DNA was cloned into the vector. Note that the FB29 and FrCas^E plasmids contain one LTR, whereas the 15-1, CasFr^E, and FrCas^{EL} plasmids contain 2 LTRs. After transfection and subsequent infection, the proviruses contain both 5′ and 3′ LTRs. *Fv-1* tropism as a marker of the p30 gene was determined by hitness analysis on NIH 3T3 and Balb 3T3 cells. Anti-gp70 monoclonal antibody reactivity was analyzed on live NIH 3T3 cells infected with the respective viruses. The viremia titers represent the levels of viremia of IRW mice infected as neonates and bled at 21 to 26 days postinoculation. Titers are represented as mean fluorescent focus-forming units per milliliter of serum. The numbers in parentheses represent the numbers of mice analyzed. n.t., Not tested.

ing the *env* and 3′ *pol* sequences from 15-1. This fragment from 15-1 contains an internal *Sph*I site, necessitating partial digestion with this enzyme. For construction of the CasFr^E genome, the 8.6-kb *Cla*I-*Sph*I fragment of 15-1 containing the two 15-1 LTRs, the *gag* and 5′ *pol* genes, and plasmid pUC19 was isolated. Since this vector contained an internal *Sph*I site, this fragment was the product of partial *Sph*I digestion. This fragment was ligated to the 2.6-kb *Sph*I-*Cla*I fragment from FB29 containing the *env* and 3′ *pol* sequences. The chimeric genome FrCas^{EL} was assembled in two steps. A fragment of FrCas^E from the *Pvu*I site 5′ of the *gag* gene to the *Hin*dIII site, including the entire *gag* and 5′ *pol* gene, was ligated to a *Cla*I-*Pvu*I fragment from 15-1, including both 15-1 LTRs, and cloned into pBR322 cut with *Cla*I and *Hin*dIII. This insert was then ligated to the 2.6-kb *Hin*dIII-*Cla*I fragment of FrCas^E containing at its 5′ end 80 bases between the *Hin*dIII-*Sph*I site in the 3′ *pol* gene derived from FB-29 and the *Sph*I-*Cla*I region containing the *env* gene derived from 15-1. This ligation product was cloned into pUC19 cut with *Hin*dIII and treated with alkaline phosphatase. Constructions were validated by restriction site analysis of the viral DNAs, *Fv-1* tropism of the respective viruses as a marker of the *gag* gene, and monoclonal antibody reactivity of the respective viruses as a marker of the *env* gene. The source of the LTR in the chimeric genome FrCas^{EL} was determined by *Kpn*I digestion. The FrCas^E plasmid contained only one LTR, whereas the 15-1 plasmid contained two tandemly duplicated LTRs. Digestion of FrCas^{EL} with *Kpn*I, which cuts within the R region of the LTR, yielded a 0.5-kb fragment, confirming the existence of the duplicated LTR of 15-1 in this construction. To minimize the chances that altered virulence of a chimeric virus was due to a cloning artifact, viral DNAs from three independent clones were transfected into NIH 3T3 cells and the resulting viruses were used to infect neonatal IRW mice. For each chimeric viral genome, all three virus isolates behaved in the same manner.

Southern blot analysis. For Southern blot analysis of genomic DNA, tissue was homogenized in 10 mM Tris–1 mM EDTA with a Dounce homogenizer. Sodium dodecyl sulfate and proteinase K were added to final concentrations of 1% and 0.36 mg/ml, respectively, and the homogenate was incubated for 4 h at 50°C. After phenol-chloroform extraction, samples were treated with RNase (70 μg/ml) at 37°C for 60 min. After phenol-chloroform extraction, the DNA was ethanol precipitated in 2 M ammonium acetate and washed twice with 95% ethanol and the pellet was dissolved in 10 mM Tris–1 mM EDTA (pH 7.4). This technique consistently yielded high-quality DNA with 260/280 ratios of 1.7 to 1.8.

For Southern blot analysis, 10 μg of tissue DNA was cut with *Bam*HI, yielding an internal 3.4-kb fragment from 15-1 and the chimeric viruses which hybridized to probe WM^{xb}. *Bam*HI was also used to cut DNAs from tissues of FB29-inoculated mice, yielding a 0.8-kb internal fragment which hybridized to F-MuLV-specific probe E57BS (27), kindly provided by Serge Fischelson (Laboratoire d'Immunologie et Virologie des Tumeurs). The DNA was fractionated in 1% agarose gels and blotted onto nitrocellulose paper. Preincubation was in 0.05 mg of sheared salmon sperm DNA per ml–5× Denhardt solution–3× SSPE at 63°C for 4 h (25). The probes were labeled with [^{32}P]dCTP using the random-primer technique (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) to a specific activity of 1×10^8 to 2×10^8 dpm/μg. Hybridizations were performed with 200 to 300 ng of labeled probe in a 5-ml volume at 63°C for approximately 24 h. Blots were washed for 30 min at the same temperature with 2× SSPE–0.1% sodium dodecyl sulfate and subsequently in 0.1% SSPE–0.1% sodium dodecyl sulfate at 63°C. The blots were exposed to Kodak X-Omat AR film with intensifying screens at −70°C, and the autoradiograms were scanned with a laser densitometer (LKB Ultrascan XL; LKB Produkter AB, Bromma, Sweden).

RESULTS

Neurovirulence and leukemogenesis of WM-E molecular clone 15-1. The virus derived by transfection of clone 15-1 was inoculated intraperitoneally or intracerebrally into 74 neonatal IRW mice (9×10^3 FFU per mouse), and the mice were observed for evidence of clinical disease (Fig. 2). Mice which exhibited clinical signs of disease were killed and autopsied. By 43 weeks of observation, 97% of the mice had either been killed because of clinical disease (62%) or died between the weekly observation periods without pathologic diagnosis (35%). An unexpectedly high incidence of leukemia was observed (45% of mice inoculated). Most of the leukemias were lymphocytic, but a significant number of granulocytic leukemias (chloroleukemias) were also observed (Table 1).

The incidence of neurological disease was relatively low and could be divided clinically into two types. Tremulous hindlimb paralysis associated with muscle atrophy, characteristic of WM-E disease, was seen in only six (8%) of the mice and had a relatively long incubation period of more than 25 weeks. The autopsies of these mice were essentially unremarkable, with no evidence of leukemia or gross CNS abnormalities. These mice, unfortunately, were not examined histologically. A second type of neurologic disease seen in 11 mice (15%) consisted of acute flaccid paralysis of the hindlimbs without tremor. These are signs suggestive of cord transection, perhaps due to an acutely developing space-

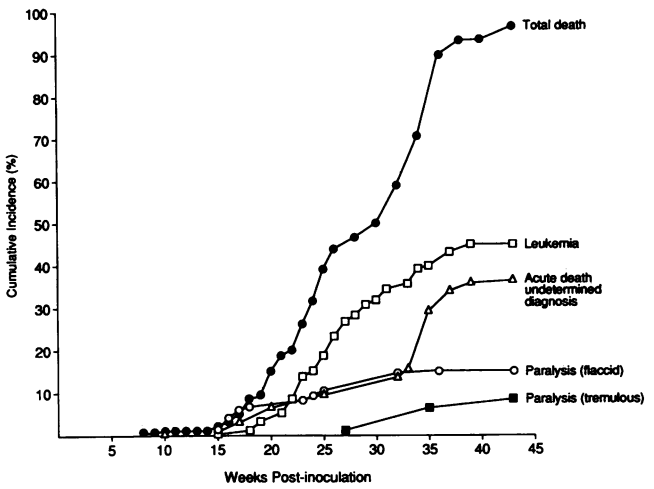


FIG. 2. Leukemogenicity and weak neurovirulence of WM-E molecular clone 15-1. Seventy-four IRW mice were inoculated with 15-1 and monitored for 44 weeks. The curves represent the cumulative incidence of disease as a function of time postinoculation.

occupying lesion. Autopsies of these mice revealed evidence of leukemia in 6 of 11 mice, with leukemic infiltrates in the meninges (Fig. 3A). Of the remaining five mice with flaccid paralysis, three had no evidence of leukemia, but gross and microscopic examination of the CNS revealed massive intraparenchymal hemorrhages involving the hindbrain and rostral spinal cord (Fig. 3B). The symmetry of the lesions suggested that the vascular defect occurred in one of the larger feeder arteries. Of the 11 mice with flaccid paralysis, 2 were not autopsied. This form of paralysis was rapidly fatal, and mice rarely survived for more than 24 h after the clinical diagnosis was made. As mentioned above, in addition to leukemia and neurologic disease, a significant proportion of the inoculated mice (35% overall) died between the weekly examinations. These mice exhibited neither signs of chronic neurologic disease (tremor or reflex abnormalities) nor evidence of significant splenomegaly or lymphadenopathy by palpation. Although the cause of death is not certain, it is possible that these mice developed acute flaccid paralysis due to either hemorrhage or undetected leukemia. Cumulatively, the results of this study indicated that molecularly cloned virus 15-1 was highly leukemogenic but only weakly neurovirulent. Furthermore, among the 17 mice which ultimately developed neurologic disease, only 6 exhibited clinical evidence of the chronic degenerative disease caused by WM-E.

Rationale for construction of chimeric viruses. Previous studies using an infectious center assay indicated that a strain of F-MuLV (FB29) replicated to high levels in the CNS but did not cause neurologic disease (31). A comparison of the levels of viral DNA in the CNS relative to those in the spleens of mice inoculated as neonates with either 15-1 or FB29 revealed a striking difference (Fig. 4). Whereas 15-1 and FB29 viral DNAs were detected in the spleens of mice 14 days postinfection, detectable levels of viral DNA were observed in the CNS only in mice inoculated with FB29. These results suggested that the weak neurovirulence of 15-1 was due in part to its limited ability to replicate in the CNS, a function which might be complemented by sequences from FB29. We therefore constructed chimeric viruses with 15-1 and FB29 (Fig. 1).

TABLE 1. Types of leukemia induced by 15-1

Leukemia type[a]	No. of mice	%
Thymic lymphoma	9	27
Nonthymic lymphoma	10	30
Chloroleukemia	12	37
Thymic lymphoma and chloroleukemia	2	6

[a] Diagnoses were based on gross examination at autopsy and, in some cases, imprint preparations and histopathologic examinations. The criteria used are elaborated in Materials and Methods.

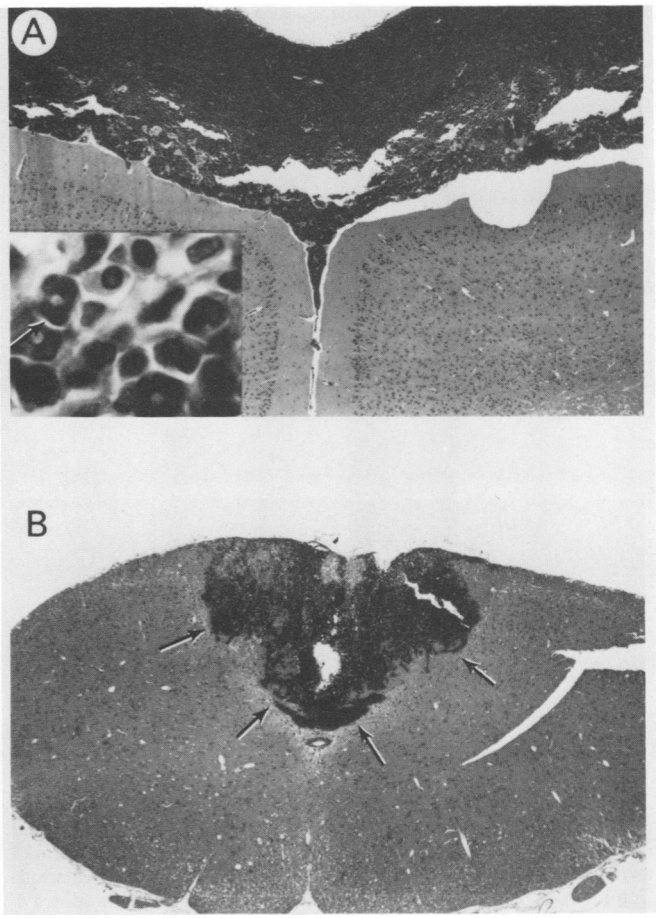


FIG. 3. Sections illustrating two causes of acute flaccid paralysis associated with long-term infection with virus 15-1. (A) Coronal section through the frontal lobes of the cerebral cortex from a mouse with chloroleukemia (magnification, ×40 before enlargement; hematoxylin and eosin stain). A massive meningeal infiltrate of leukemia cells is evident. At higher magnification (inset), donut-shaped nuclei (arrow) characteristic of murine granulocytes can be seen (magnification, ×1,000 before enlargement). (B) Coronal section through the middle of the medulla oblongata showing an intraparenchymal hemorrhage (margins of which are delimited by arrows) involving the dorsal aspect (magnification, ×40 before enlargement; hematoxylin and eosin stain).

Pathogenicity of recombinant viruses. Since the neurovirulence of WM-E has previously been mapped to the *env* gene (10, 28), we introduced the *env* and 3′ *pol* sequences of 15-1 into FB29 (FrCas^E; Fig. 1). Neonatal IRW mice inoculated with this chimeric virus exhibited a severe neurologic disease characterized by tremor and paralysis of both hind- and forelimbs beginning on day 16 postinoculation. This was

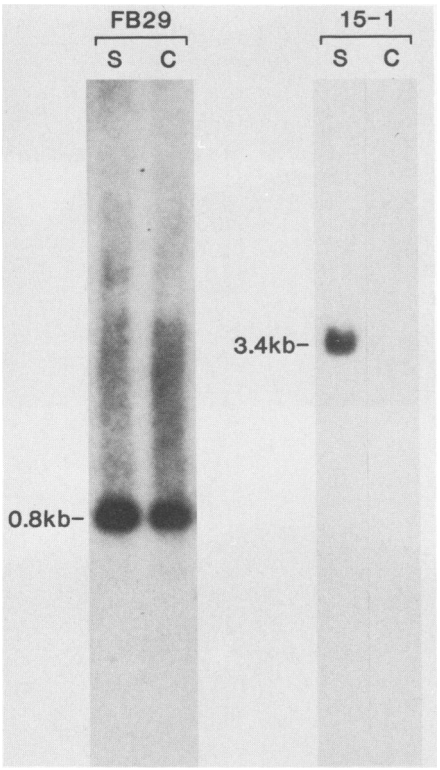


FIG. 4. Difference between FB29 and 15-1 in the capacity to infect the CNS early. Southern blots are shown of spleen (S) and spinal cord-cerebellum (C) tissues taken from IRW mice inoculated as neonates with FB29 or 15-1 and killed at 14 days postinoculation. Ten micrograms of DNA was cut with *Bam*HI and separated in 1% agarose gels. The FB29 blot was hybridized with an F-MuLV-specific probe, and the 15-1 blot was hybridized with a WM-E-specific probe. Exposure times for the autoradiographs were 4 days for FB29 and 3 days for 15-1.

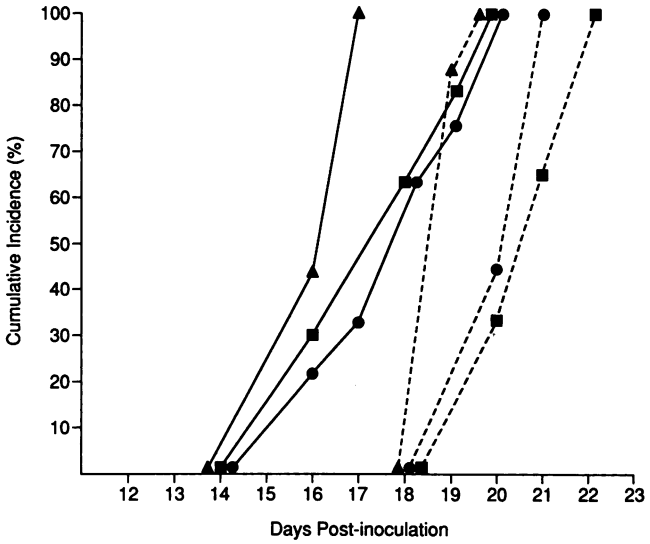


FIG. 5. Induction of a highly reproducible disease by FrCas^E. Three litters of IRW mice represented by different symbols (8 to 10 mice per litter) were inoculated as neonates with 1.6×10^4 focus-forming units of FrCas^E intraperitoneally and monitored daily for evidence of neurologic disease. Solid lines represent the cumulative incidence of clinical disease manifested by tremor, hind- and fore-limb paralysis, and wasting. Dotted lines show the tempo of death for each litter.

followed by immobility of the mice in the cage, lack of weight gain, and finally death by 20 to 25 days. The disease induced by FrCas^E was highly reproducible (Fig. 5). Pathologic studies revealed spongiform degeneration without inflammatory infiltrates involving primarily the caudal segments of the CNS from the midbrain through the lumbar spinal cord (Fig. 6). The vacuolation was localized to the grey matter and involved both the neuropil and neuron cell bodies. These findings are consonant with the pathologic changes induced by field isolates of WM-E (1), suggesting that the disease induced by FrCas^E simply represented the result of more severe and rapidly progressive CNS pathologic changes.

Although the neurovirulence of WM-E has been mapped to the viral *env* gene, the studies described here show that sequences outside the *env* gene can accelerate the tempo of the disease. Since the LTR embodies sequences which regulate transcription, it appeared likely that the LTR of FB29 is responsible for the accelerated neurovirulence of FrCas^E. We therefore introduced into the DNA of FrCas^E the LTRs of 15-1 (FrCas^{EL}; Fig. 1). The genome of FrCas^{EL} was distinguished from 15-1 only in that it contained *gag* and 5′ *pol* sequences from FB29. Of 17 mice inoculated with FrCas^{EL} and monitored for 60 days, evidence of neurologic disease was observed in 60% (Fig. 7). Affected mice exhibited tremor and abnormalities in the abduction reflex, but complete paralysis was not observed. Clinically affected mice were first noted at 27 days postinoculation, and the disease incidence increased relatively slowly thereafter. Thus, the incidence, tempo, and severity of the neurologic disease induced by FrCas^{EL} was intermediate between those of FrCas^E and 15-1. These results indicated that although the FB29 LTR in FrCas^E contributed to its markedly accelerated neurovirulence, this effect could also be influenced by elements within the FB29 *gag-pol* region. Whether the accelerated neurovirulence of FrCas^E was a consequence of additive effects of these two regions of the viral genome is not known.

Both LTR and *gag-pol* sequences from FB29 specifically increase the level of CNS infection. Differences in the tempo of the neurodegenerative diseases induced by FrCas^E, FrCas^{EL}, and 15-1 could be due to differences in the initial doses of virus inocula or possibly to differences in the relative levels of virus replication in the mice. However, the titers of the initial inocula of the viruses were comparable (3×10^5 to 5×10^5/ml; see Materials and Methods). In addition, the levels of viremia measured at 21 to 26 days postinoculation were also comparable for these viruses (Fig. 1, viremia). To examine the levels of virus replication specifically within the CNS, the relative levels of viral DNAs in spleens and CNS tissue were examined (Fig. 8). Whereas the levels of viral DNAs in the spleens of mice inoculated with 15-1, FrCas^E, and FrCas^{EL} were comparable, the levels of viral DNAs in the CNS were different (FrCas^E > FrCas^{EL} > 15-1). These data are summarized in Table 2, which also shows that although viral DNA was not detected in the CNS of 15-1-inoculated mice 14 days postinoculation, low but detectable levels were seen in two of three mice at 60 days postinoculation. Taken together, the results indicate that both LTR and *gag-pol* sequences from FB29 had a profound influence on the level of CNS infection. However, the CNS/spleen ratio of viral DNA in FrCas^E-inoculated mice was similar to that of mice inoculated with FB29 (Table 2),

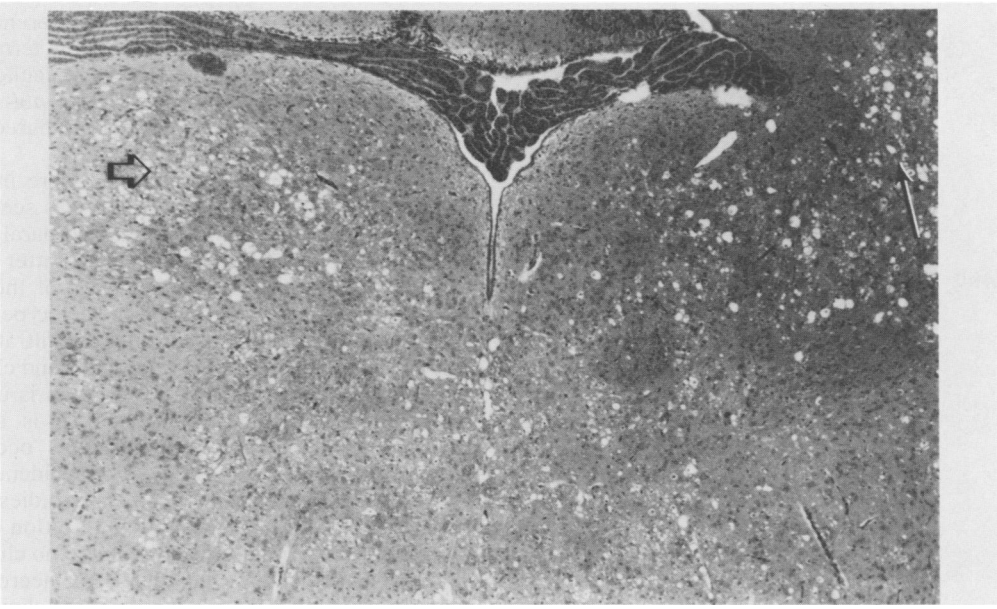


FIG. 6. Slightly oblique coronal section of the pons taken from an IRW mouse at 16 days postinoculation with FrCas^E. The mouse exhibited clinical signs of neurological disease. Spongiform degeneration is evident in the grey matter and involves the vestibular nuclei (narrow arrow) and the substantia grisea centralis (thick arrow) (magnification, 40× before enlargement; hematoxylin and eosin stain).

indicating that the level of CNS infection correlated with relative neurovirulence only for those viruses containing 15-1 *pol-env* sequences.

LTR *gag-pol* sequences of 15-1 contain the determinants of leukemogenesis. Although the *pol-env* sequences of 15-1 contain determinants of neurovirulence, this phenotype was only weakly expressed in combination with 15-1 LTR and *gag-pol* sequences. 15-1 itself was highly leukemogenic (Fig. 2). Chimeric virus CasFr^E, which consisted of FB29 *pol-env* sequences on a 15-1 background (Fig. 1) was also leukemogenic and induced leukemias of the same cell types and tempo as those induced by the parent virus, 15-1. Of 39 IRW neonates inoculated intraperitoneally with this chimeric virus, 13 (33%) had developed leukemia during 31 weeks of observation. This incidence is comparable to that induced by the donor of the non-*env* sequences (15-1) at this time after inoculation (34%) (Fig. 2). In addition, the incubation period of the leukemias induced by CasFr^E was similar to that of those induced by 15-1 (18 weeks) (Fig. 2). Of the 13 leukemias induced by CasFr^E during this period, there were 7 thymic lymphomas, 2 nonthymic lymphomas, and 4 chloroleukemias. This mixture of leukemia types is reminiscent of the leukemias induced by 15-1 (Table 1). Unlike the virus from which the *pol-env* sequences were derived (FB29), no erythroleukemias were seen. This result was expected, since previous studies by DesGroseillers and Jolicoeur (11) and Chatis et al. (6) indicated that strong determinants of leukemogenesis are harbored by the viral LTR. Interestingly, many of the leukemias included by CasFr^E were neuroinvasive, like those induced by 15-1. Meningeal infiltrates were observed in four of seven thymomas, two of two nonthymic lymphomas, and three of four chloroleukemias. Only two of these mice, however, exhibited signs of neurological disease (flaccid paralysis of the hindlimbs). We have never detected CNS hemorrhage in CasFr^E-inoculated mice. These results indicated that introduction of FB29 *pol-env* sequences into the 15-1 genome had little influence on the leukemogenesis determined by the 15-1 LTR *gag-pol* sequences.

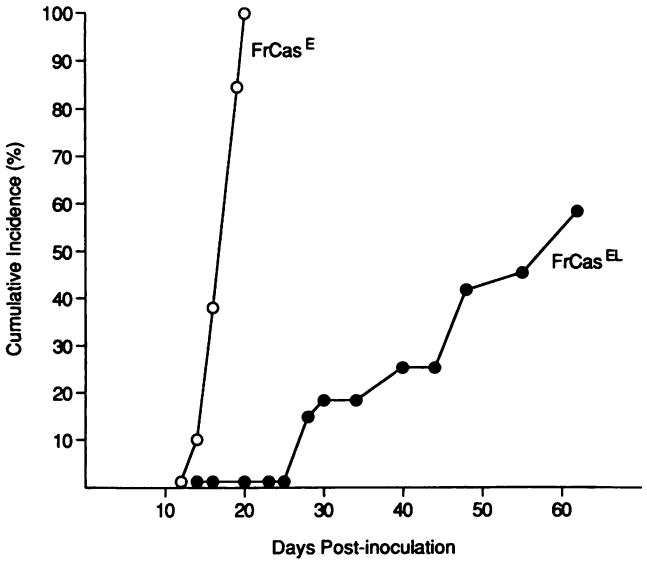


FIG. 7. FrCas^{EL} intermediate between 15-1 (compare with Fig. 2, closed squares) and FrCas^E in neurovirulence. Nineteen neonatal IRW mice were inoculated intraperitoneally with 1.6×10^4 focus-forming units of FrCas^E, and 26 neonatal IRW mice were inoculated with 1×10^4 focus-forming units of FrCas^{EL}. The mice were monitored daily for evidence of neurologic disease. The disease induced by FrCas^{EL} was of a slower tempo than that induced by FrCas^E. In addition, the disease induced by FrCas^{EL} consisted of tremor and abduction reflex abnormalities, but complete paralysis was not seen during the observation period.

DISCUSSION

An infectious molecular clone of WM-E (15-1) was isolated which, after neonatal inoculation, was found to be leukemogenic but exhibited only weak neurovirulence. IRW mice inoculated with this virus developed leukemias of both

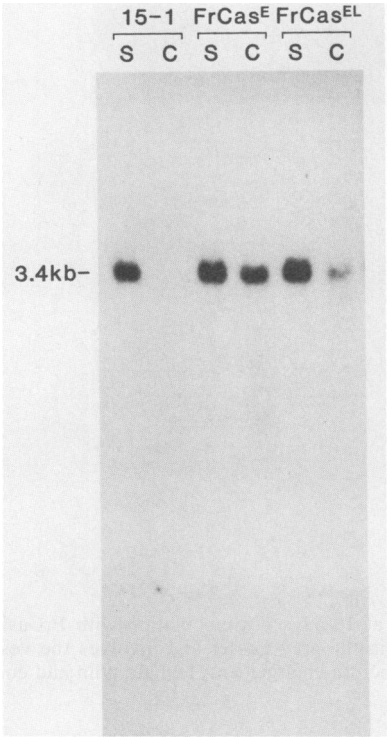


FIG. 8. Southern blot of DNAs extracted from spleen (S) and spinal cord-cerebellum (C) tissues 14 days postinoculation of neonatal mice with 15-1, FrCas^E, or FrCas^{EL}. Samples (10 μg) of DNA were treated as described in the legend to Fig. 4. The relative levels of viral DNA were comparable for spleens but differed significantly for CNS tissue, in which they correlated with the relative neurovirulence of the respective virus.

lymphoid and granulocytic types after approximately an 18-week incubation period. This variety of cell types was similar in terms of both frequency and tempo to those induced by biologically cloned Cas-Br-M (14), from which 15-1 was molecularly cloned. Chimeric viruses constructed between WM-E and amphotropic viruses (23) indicated that determinants of leukemogenicity exist within the LTR, *gag-pol*, and *pol-env* regions of the WM-E genome. In the current study, we observed no measurable differences in leukemogenicity between 15-1 and CasFr^E, in terms of either the incubation period or the spectrum of leukemia cell types. This suggests that the proposed WM-E *pol-env* determinant of leukemogenicity (23) is probably shared by FB29 and 15-1.

TABLE 2. Early infection of the CNS determined by FB29 LTR and *gag-pol* sequences

Virus	Days post-inoculation	Mean (range) densitometric units[a]		CNS/spleen ratio
		CNS	Spleen	
FB29	14	6 .0 (5.8–6.3)	7.5 (6.8–8.8)	0.8
15-1	14	<0 .1[b]	7.5 (7.3–7.7)	<0.01
15-1	60	≤0 .2[c](<0.1–0.25)	6.2 (4.7–8.0)	≤0.04
FrCas^E	14	7 .7 (6.9–9.1)	8.7 (8.0–9.0)	0.9
FrCas^{EL}	14	2 .2 (1.0–2.9)	8.4 (7.2–9.6)	0.3

[a] DNA samples (10 μg) from three mice per group were digested with *Bam*HI, and Southern blots were probed with the F-MuLV-specific probe for FB29-inoculated mice and the WM-E-specific probe for mice inoculated with 15-1 and the chimeric viruses. Densitometry was performed after 4 days of exposure of the autoradiogram for FB29 and 12 days of exposure for 15-1 and the chimeric viruses.

[b] No signal was detected for 15-1-inoculated mice at 14 days postinoculation.

[c] A weak but detectable signal was seen in two of three mice analyzed at 60 days postinoculation with 15-1.

Two types of neurologic disease were induced by 15-1, chronic tremulous paralysis, which was seen in only 8% of the inoculated mice, and acute flaccid paralysis, which was observed in 15% of the mice. The latter syndrome was caused by either leukemic infiltration of the CNS or acute intraparenchymal hemorrhage. The cell type of the leukemia did not appear to affect its capacity to infiltrate the meninges, since this occurred in both lymphomas and chloroleukemias. It is not clear whether this observation is unique to leukemias induced by 15-1 or whether it is a more general phenomenon. Unless neurologic signs occur, one is not inclined to look at the CNS for evidence of leukemic infiltrates. It was apparent from the studies of mice inoculated with CasFr^E that leukemic infiltration of the meninges often occurred in mice which exhibited no clinical neurologic signs, and further damage caused by necrosis and hemorrhage is probably required to precipitate clinical disease. The acute CNS hemorrhage seen in 4% of the inoculated mice was curious but unexplained. WM-E is thought to replicate in endothelial cells (30), and it is thus conceivable that infection of these cells could lead to vascular fragility. Although we are not aware of studies with WM-E, infection with F-MuLV (9) and Rauscher leukemia virus (2) is associated with thrombocytopenia which could secondarily lead to CNS hemorrhage. On the basis of these observations, it is suggested that care should be taken in evaluating the etiology of paralysis that appears late in the course of infection with leukemogenic retroviruses.

Despite the rather unimpressive primary neurovirulence of 15-1, a chimeric virus (FrCas^E) containing the *env* gene and 3′ *pol* sequences from 15-1 and LTR, *gag*, and 5′ *pol* sequences from F-MuLV strain FB29 was highly neurovirulent. FrCas^E induced paralytic disease with an incubation period of only 16 days and an incidence of 100% and was fatal by 23 days postinoculation. Examination of the CNS revealed typical spongiform degeneration of the grey matter without evidence of inflammatory cells. Thus, this virus appeared to recapitulate the disease induced by WM-E (1) but with a remarkably accelerated tempo and enhanced severity. This result was unexpected, since FB29 itself induces erythroleukemia in IRW mice (36) but has no known potential for neurovirulence. It is unlikely that the accelerated neurovirulence of FrCas^E was due to a cloning artifact, since three independently derived clones yielded viruses with the same phenotype.

Although FB29 does not induce neurologic disease, both infectious-center assays (31) and Southern blot analysis (Fig. 4; Table 2) indicated that FB29 replicated to high levels in the CNS. In contrast, 15-1 replicated in the spleen but viral DNA was not detected in the CNS until late postinoculation (Table 2). Introduction of *pol-env* sequences from 15-1 into the genome of FB29 resulted in a virus which replicated to high levels in the CNS and also caused neurologic disease. These results confirmed the importance of the *env* gene in the neurovirulence of WM-E (10, 28) but also indicated that non-*env* sequences strongly influenced the level of virus replication in the CNS and consequently the tempo and severity of the neurologic disease. We showed through construction of chimeric virus FrCas^{EL} that both the LTR and *gag-pol* regions of FB29 increased the level of virus

infection of the CNS and contributed to the acceleration of disease.

The influence of the LTR on CNS replication was not unexpected. Retroviral LTRs contain transcriptional enhancer elements which bind *trans*-acting cellular proteins (37) and can function in a cell type-specific fashion (13, 38). DesGroseillers et al. (12) have previously shown that introduction of the Mo-MuLV LTR into WM-E resulted in a chimeric virus which induced accelerated neurologic disease after intrathymic but not intraperitoneal infection. Since the Mo-MuLV LTR appears to contain thymotropic enhancer elements, it was suggested that high-level virus replication in the thymus might secondarily increase the level of CNS infection. In the current study, however, the level of splenic infection (Fig. 4; Table 2) and the level of viremia were independent of the source of the viral LTR. Thus, the CNS should have been exposed to similar levels of $FrCas^{EL}$ and $FrCas^{E}$. However, $FrCas^{E}$ infected the CNS at higher levels than did $FrCas^{EL}$. This suggests that the enhanced CNS replication exhibited by $FrCas^{E}$ was CNS specific.

The influence of *gag-pol* sequences from FB29 on the level of CNS infection was unexpected. This effect also appeared to be CNS specific, since 15-1 and $FrCas^{EL}$ infected spleens at comparable levels (Fig. 4; Table 2) and produced similar levels of viremia (Fig. 1). Robinson and co-workers (34) have found that the capacity of avian leukosis viruses to induce osteopetrosis mapped to a region including the 5′ half of the *gag* gene and immediately adjoining 5′ noncoding sequences. Interestingly, a sequence has been identified in Rous sarcoma virus within the 5′ half of the *gag* gene which functions as a transcriptional enhancer in vitro (5). Whether this element is the determinant for the osteopetrotic potential of avian leukosis virus is not clear. Such an enhancer has not been identified in murine retroviruses. Finer-mapping studies of the FB29 *gag-pol* sequence will be required to identify the sequence responsible for increasing CNS replication.

For viruses 15-1, $FrCas^{EL}$, and $FrCas^{E}$, there was a direct correlation between the level of CNS infection and both the tempo and severity of the neurologic disease. However, CNS infection, although necessary, was not sufficient for induction of neurologic disease. The levels of viral DNA in the CNS relative to the spleens of mice inoculated with FB29 and $FrCas^{E}$ were comparable (Table 2). However, only the latter virus was neurovirulent. Two possible mechanisms appear to be consistent with these observations. FB29 and $FrCas^{E}$ might infect distinct populations of cells in the CNS, and this tropism is determined by the respective envelope glycoproteins. This hypothesis implies that unique receptors exist for the WM-E glycoprotein in the CNS. Alternatively, FB29 and $FrCas^{E}$ could infect the same cells in the CNS, but the presence of WM-E *pol-env* sequences in the CNS is cytotoxic. Immunohistochemical studies (to be reported elsewhere) tend to support the former hypothesis, since FB29 has been detected exclusively within CNS vascular cells (endothelial cells and pericytes), whereas $FrCas^{E}$ was found both within vascular and extravascular elements of the CNS.

In the current study, we found that major determinants of CNS tropism map within the viral LTR as well as the *gag-pol* region but that induction of neuropathologic changes depends on the *pol-env* region of the viral genome. Combining the LTR *gag-pol* region of the FB29 strain of F-MuLV with the *pol-env* sequences of WM-E clone 15-1 yielded a chimeric virus with markedly enhanced neurovirulence compared with any other isolate of WM-E so far described. By virtue of the highly reproducible disease induced by $FrCas^{E}$, this virus should provide a useful model for more systematic study of the pathogenesis of retrovirus-induced neurodegenerative disease.

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