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Site-Directed Mutagenesis of the Codon for Ile-25 in gPr80^{env} Alters the Neurovirulence of *ts*1, a Mutant of Moloney Murine Leukemia Virus TB

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ts1, a spontaneous temperature-sensitive mutant of Moloney murine leukemia virus TB, causes hind-limb paralysis in mice. A Val-25→Ile substitution in gPr80^{env} is responsible for temperature sensitivity, inefficient processing of gPr80^{env}, and neurovirulence. In this study, the Ile-25 in gPr80^{env} was replaced with Thr, Ala, Leu, Gly, and Glu by site-directed mutagenesis of the codon for Ile-25 to generate a new set of mutant viruses, i.e., *ts*1-T, -A, -L, -G, and -E, respectively. The phenotypic characteristics of these mutant viruses differed from those of *ts*1. For each mutant, the degree of temperature sensitivity was correlated with the degree of inefficient processing of gPr80^{env}, and the following rank order was observed for both parameters: *ts*1-E > *ts*1-G > *ts*1-L > *ts*1-A > *ts*1 > *ts*1-T. In FVB/N mice, mutant viruses of low and intermediate temperature sensitivity and inefficiency in processing of gPr80^{env} were neurovirulent and consistently caused mutant-specific disease profiles: *ts*1-T caused severe whole-body tremor, *ts*1-A generally caused hind-limb paralysis, and *ts*1-L generally caused a delayed-onset paraparesis. By 150 days postinfection, FVB/N mice that were infected with *ts*1-G and -E, mutants of high temperature sensitivity and inefficiency in processing of gPr80^{env}, had lymphoid leukemia instead of a neurological disease. These results suggest that the dynamics of gPr80^{env} processing are important in determining the neurovirulent phenotype in vivo.

A mutant of Moloney murine leukemia virus TB (MoMu LV-TB) (29), designated *ts*1, causes a progressive hind-limb paralytic disease in susceptible strains of mice (13, 19) that is characterized by a degenerative spongiform encephalomyelopathy (13, 19, 27, 38), generalized body wasting (28, 38), marked thymic atrophy (13, 28), and severe immunodeficiency (28). In addition to being temperature sensitive (29), *ts*1 inefficiently processes the envelope precursor polyprotein, gPr80^{env}, into gp70 and Prp15E at the restrictive temperature (30). Endoglycosidase H sensitivity experiments have indicated that at the restrictive temperature, an elevated steady-state level of nonprocessed gPr80^{env} remains in the endoplasmic reticulum (ER) of infected cells (21). Most likely the inefficient processing of gPr80^{env} is due to inefficient transport of gPr80^{env} from the ER to the *cis* cisternae of the Golgi apparatus, which contains the host cell-encoded processing protease (21). Inefficient processing of gPr80^{env} has been correlated with the neurovirulence of *ts*1 (30). The molecular determinant responsible for the temperature sensitivity, inefficient processing of gPr80^{env}, and neurovirulence of *ts*1 has been mapped to a very conservative amino acid substitution in gPr80^{env}, i.e., Val-25→Ile (21, 22).

In this study, to further examine the relationship between the inefficient processing of gPr80^{env} and the neurovirulence of *ts*1, a set of mutants of *ts*1 that were defective in processing of gPr80^{env} at the restrictive temperature to a greater or lesser degree than *ts*1 itself was made. To make the mutant viruses, the codon for Ile-25 in the gPr80^{env} of *ts*1 was mutagenized to code for both conservative and nonconservative changes in amino acids (Thr, Ala, Leu, Gly, and Glu) to yield *ts*1-T, -A, -L, -G, and -E, respectively. These mutant viruses were assayed for temperature sensitivity, processing of gPr80^{env}, and neurovirulence in FVB/N mice. The results of these assays showed that the degree of inefficient processing of gPr80^{env}, within a specific range, is correlated with the development of neurodegenerative disease. A low to intermediate degree of inefficient processing of gPr80^{env} is correlated with the emergence of the neurovirulent phenotype; however, if processing is too inefficient, there is no neurovirulence.

MATERIALS AND METHODS

Cells. Viruses were propagated in TB cells (3), an established tissue culture cell line that was derived from a mixed culture of fetal thymus and bone marrow from CFW/D mice (2). Titer of virus was determined by the modified direct focus assay (31) on 15F cells (3), a nontransformed, nonproducer, murine sarcoma-positive, leukemia-negative (S^+ L^-) cell line. NIH 3T3 cells were used for transfection experiments. All cell lines were maintained in Dulbecco modified Eagle medium supplemented with 6% fetal bovine serum and 4% bovine serum.

Viruses. MoMuLV-TB is a variant of MoMuLV (29, 36), and *ts*1 is a temperature-sensitive mutant of MoMuLV-TB (29). Both MoMuLV-TB and *ts*1 have been molecularly cloned, and clones *wt*-25 and *ts*1-19, respectively, were used in this study (35).

Plasmids. Plasmid p21 (34) consists of a pUC9 plasmid (25) that has the *Hin*dIII-*Bam*HI fragment of *ts*1 for an insert (1,643 base pairs [bp], nucleotides [nts] 4895 to 6537). This insert consists of the 3′ end of the *pol* gene and one-third of the *env* gene. Plasmid p22 (34) consists of a pUC9 plasmid that has the *Bam*HI-*Pst*I fragment of *ts*1 for an insert (2,294 bp, nts 6538 to 8264 and 1 to 567). This insert contains two-thirds of the *env* gene, the long terminal repeat, and the 5′ end of the *gag* gene. Plasmid p17 (34) consists of a pKC7 plasmid that has the *Sma*I-*Hin*dIII fragment of *ts*1 for an insert (4,864 bp, nts 31 to 4894). It contains part of the R

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region of the long terminal repeat, U5, the *gag* gene, and most of the *pol* gene. Nucleotides were numbered by the method of Shinnick et al. (20).

Oligonucleotides. Oligonucleotides were synthesized on an Applied Biosystems (Forester, Calif.) model 380B DNA synthesizer by the UTMDACC Macromolecular Synthesis Facility. The mutagenic oligonucleotide primers (21 nts) that were used to mutagenize the codon for Ile-25 in gPr80^{env} were (mismatched nucleotides are underlined) 5′-ATTGCCC ATAGCGTCTCCCGA-3′ (complementary to nts 5938 to 5958) for Ile-25→Leu, 5′-AATTGCCCATGTCGTCTCCC G-3′ (complementary to nts 5939 to 5959) for Ile-25→Thr, and 5′-AATTGCCCATNCCGTCTCCCG-3′ (complementary to nts 5939 to 5959), where N is a G for Ile-25→Ala, N is a C for Ile-25→Gly, and N is a T for Ile-25→Glu. The oligonucleotide primer (17 nts) that was used to verify the mutagenized nucleotides by nucleotide sequencing was 5′-GTCCACAGAGGGTGGTT-3′ (complementary to nts 5966 to 5982).

Site-directed mutagenesis of codon for Ile-25. The *Hin*dIII-*Bam*HI insert of plasmid p21 (1,643 bp, nts 4895 to 6537) was subcloned into the replicative form of bacteriophage M13 mp18 (32). JM101 cells (32) were transfected with the resulting recombinant replicative form of M13mp18 to yield a recombinant clone that was designated M13-21.1. Single-stranded DNA was prepared from plaque-purified M13-21.1 bacteriophage by the minipreparation method (14). The mutagenic primers were hybridized to the M13-21.1 single-stranded DNA, and the Ile-25 codon in the *Hin*dIII-*Bam*HI insert was mutagenized with the oligonucleotide-directed in vitro mutagenesis system (Amersham Corp., Arlington Heights, Ill.), which is based on the phosphorothioate DNA method (15). TG1 cells (8) were transfected with replicative forms of M13-21.1 that contained mutagenized *Hin*dIII-*Bam*HI inserts, and bacteriophage were plaque purified (14). Single-stranded DNA was prepared from the bacteriophage by the minipreparation method. The mutagenized Ile-25 codon in full-length M13-21.1 single-stranded DNA was identified by dideoxy-chain termination nucleotide sequencing with an unmodified T7 DNA polymerase (Pharmacia LKB Biotechnology, Inc., Picastaway, N.J.) by a method that was based on that of Tabor and Richardson (23).

Construction of mutagenized virus genomes. JM101 cells were transfected with M13-21.1 single-stranded DNA that contained a mutagenized *Hin*dIII-*Bam*HI insert, and bacteriophage were plaque purified. The replicative form was prepared by the minipreparation method of Birnboim and Doly (4). The mutagenized *Hin*dIII-*Bam*HI insert was subcloned from the replicative form into plasmid pUC8 (25). Full-length virus genomes were constructed by ligating the *Hin*dIII-*Bam*HI insert of the pUC8 plasmid (1,643 bp, nts 4895 to 6537) to the *Bam*HI-*Pst*I insert of plasmid p22 (2,294 bp, nts 6538 to 8264 and 1 to 567) and the *Sma*I-*Hin*dIII insert of plasmid p17 (4,864 bp, nts 31 to 4894) by a method that was described in detail previously (34).

DNA transfection. NIH 3T3 cells were transfected with viral DNA by the calcium phosphate precipitation method (7). Details of this procedure have been described previously by Yuen et al. (35).

Metabolic labeling, radioimmunoprecipitation, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Immunoprecipitation of intracellular virus-specific proteins with fixed *Staphylococcus aureus* (Pansorbin cells from Calbiochem Corp., La Jolla, Calif.) as an immunoabsorbent and sodium dodecyl sulfate-polyacrylamide gel electrophoresis were performed as described previously (21, 30, 31). TB cells were infected with virus, and viral proteins were double labeled with both L-[^{35}S]cysteine (50 μCi/ml) and L-[^{35}S]methionine (50 μCi/ml). Goat antiserum prepared against Rauscher MuLV gp69/71 (lot 79S000842) was obtained from Microbiological Associates, Inc., Bethesda, Md.

Growth curves of viruses. TB cells (10^6) were seeded in 75-cm^2 T flasks, and the cells were incubated overnight at 34°C in medium that contained 2 μg of Polybrene per ml. The next day, the TB cells were infected with 1 ml of virus (10^6 infectious units [IU] per ml) in basal medium for a multiplicity of infection of 1. After an adsorption period of 30 min, 20 ml of complete medium (without Polybrene) was added to the flasks, and they were incubated at 34°C for 4 days. Samples of medium (1 ml) were taken at 24-h intervals and frozen at −80°C. The titers of the virus in the samples were determined with the modified 15F assay. The $\log_{10}$ IU/ml was plotted versus days postinfection (p.i.).

Mouse strain and inoculation procedure. Within 48 h after birth, FVB/N mice (Taconic, Germantown, N.Y.) were inoculated intrathymically with 10^5 to 10^6 IU of virus in 0.05 ml of growth medium by using a tuberculin syringe that was fitted with a 27-gauge needle. The housing and maintenance of the mice have been described previously (28).

Assay of infectious virus in tissues of inoculated mice. Mice that were inoculated with virus were sacrificed by intoxication with carbon dioxide. The amounts of infectious virus in homogenates (27) of the spleen and spinal cord were determined by the 15F assay.

RESULTS

Substitution of the codon for Ile-25 in gPr80^{env} of *ts*1 with those for Thr, Leu, Ala, Gly, and Glu. Previous studies have shown that a Val-25→Ile substitution in gPr80^{env} is responsible for the temperature sensitivity, inefficient processing of gPr80^{env}, and neurovirulence of *ts*1 (21, 22). To determine if the phenotype of *ts*1 would be changed by substituting the Ile-25 in gPr80^{env} with other amino acids, Ile-25 was substituted with five different amino acids, i.e., Thr, Ala, Leu, Gly, and Glu. The strategy was to substitute the hydrophobic side chain of Ile-25 with both conservative and nonconservative side chains (1, 17). The Leu side chain is the same size as that of Ile, but it has different branching. Ala and Gly have shorter side chains than that of Ile. The Thr side chain has a hydroxyl group that can form a hydrogen bond. The Glu side chain is hydrophilic, whereas the Ile side chain is hydrophobic. A set of mutants of *ts*1 that contained these five different Ile-25 substitutions in gPr80^{env} were constructed, i.e., *ts*1-T, -A, -L, -G, and -E (Fig. 1). The mutant *ts*1-T contains a Thr at amino acid position 25 in gPr80^{env}, *ts*1-L contains a Leu, *ts*1-A contains an Ala, *ts*1-G contains a Gly, and *ts*1-E contains a Glu. The phenotypic characteristics of the mutants are summarized in Table 1.

Temperature sensitivity of the viruses. *wt*-25, *ts*1, and *ts*1-T, -L, -A, -G, and -E were assayed for temperature sensitivity on 15F cells. In Table 1, the ratio of the titer of virus produced at the permissive temperature (34°C) to the titer of virus produced at the restrictive temperature (39°C) is shown for each virus. As found previously (21, 34, 35, 37), *wt*-25 was not temperature sensitive, and the titer ratio for *ts*1 was greater than 100. One mutant, *ts*1-T, was not as temperature sensitive as *ts*1, and the titer ratio of *ts*1-T was only ninefold greater than that of *wt*-25. The other four mutants of *ts*1 were more temperature sensitive than *ts*1. The rank order of the mutant viruses for temperature sensitivity was *ts*1-E > *ts*1-G > *ts*1-L > *ts*1-A > *ts*1 > *ts*1-T.

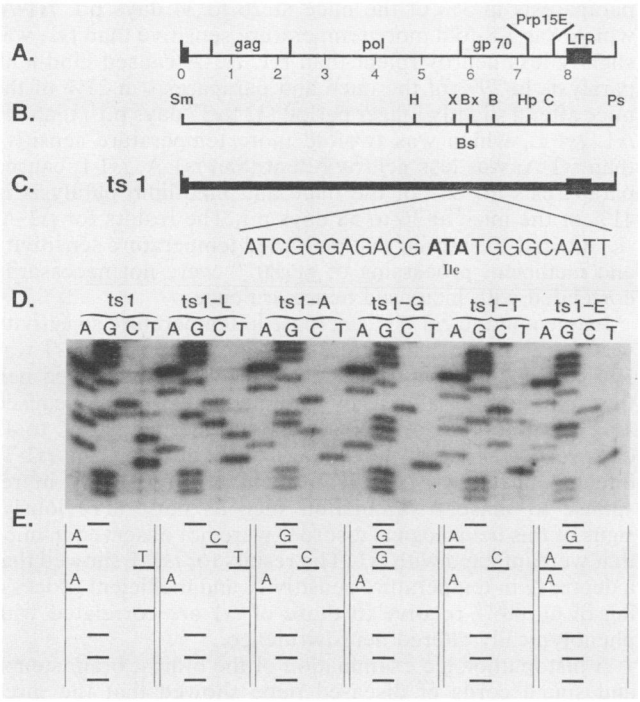

FIG. 1. Molecular characterization of mutant viruses. (A) Genetic map of MoMuLV genome (20). LTR, Long terminal repeat. (B) Restriction map of MoMuLV genome (20, 21, 34). Restriction site abbreviations: B, *Bam*HI; Bs, *Bst*EII; Bx, *Bst*XI; C, *Cla*I; H, *Hin*dIII; Hp, *Hpa*I; Ps, *Pst*I; Sm, *Sma*I; and X, *Xba*I. (C) Genome of *ts*1 (21). The codon for Ile-25 in gPr80^{env} (nts 5948 to 5950) is located in a *Bst*EII-*Bst*XI restriction fragment (116 bp, nts 5924 to 6039). (D) Nucleotide sequence of the codon (nts 5948 to 5950) in the *Hin*dIII-*Bam*HI insert of plasmid p21 (*ts*1) and the mutagenized *Hin*dIII-*Bam*HI fragments that were used to construct *ts*1-T, -L, -A, -G, and -E. The terminating dideoxynucleotide (A, G, C, or T) is written above the four sequencing lanes of each clone. (E) Schematic diagram of the bands in the autoradiograph that are located in the region of the mutagenized codons. Symbols for nucleotides which are inside the codon (nts 5948 to 5950) are written above the bands. ATA codes for Ile, CTA codes for Leu, GCA codes for Ala, GGA codes for Gly, ACA codes for Thr, and GAA codes for Glu.

Another feature besides titer ratio which distinguished some of these mutant viruses from *ts*1 at both the permissive and restrictive temperatures was the size of the foci formed on 15F cells. A focus is formed as a result of the rescue of an integrated sarcoma virus in a 15F cell by an infecting helper MuLV which is followed by the spread of progeny virus to neighboring 15F cells (3, 26). These infected 15F cells become transformed, round up, and detach from the surface of the tissue culture dish (12). The resulting plaquelike "hole" surrounded by piled, rounded cells is a focus. Therefore the size of a focus may indicate the replication efficiency of a MuLV. Since the size of a focus is dependent on experimental conditions such as the incubation period following the infection of the 15F cells, all of the viruses in this set of experiments were assayed at the same time and under the same experimental conditions. The size of the foci formed by *wt*-25 was used as a standard. For each virus, the foci produced at 39°C were smaller than those produced at 34°C. At the permissive temperature, *wt*-25, *ts*1, and *ts*1-T, -A, and -L formed large foci, whereas the two extremely temperature-sensitive viruses, *ts*1-G and -E, formed small foci. At the restrictive temperature, *wt*-25 and the least

TABLE 1. Phenotypic characteristics of *wt*-25, *ts*1, and *ts*1-T, -A, -L, -G, and -E

Virus	Titer ratio[a] (34°C/39°C)	Processing of gPr80^{env}[b]		Neurovirulence[c]
		39°C	34°C	
wt-25	1.8	P	P	No signs
*ts*1	3.0×10^2	IP	P	Hindlimb paralysis
*ts*1-T	16.0	P*	P	Severe whole-body tremor
*ts*1-A	7.5×10^2	IP	P*	Hindlimb paralysis
*ts*1-L	1.5×10^3	IP	P*	Paraparesis
*ts*1-G	4.2×10^3	IP	P*	No signs
*ts*1-E	2.2×10^4	IP	IP	No signs

[a] Two sets of 60-mm dishes were seeded with 15F cells for the modified 15F assay. Stocks of virus containing between 2×10^6 and 2×10^7 IU/ml were diluted in basal medium. Samples of diluted virus (0.5 ml) were added to both sets of 15F cells, and adsorption proceeded for 40 min at 34°C. After adsorption, one set of dishes was incubated at 34°C and the other set was incubated at 39°C. Foci were counted 5 days p.i. for the 34°C assays and 4 days p.i. for the 39°C assays. The titer ratio (34°C/39°C) is the average of two experiments.

[b] Summary of the results shown in Fig. 3. P, gPr80^{env} efficiently processed to gp70 and Prp15E; P*, gPr80^{env} partially processed; IP, gPr80^{env} inefficiently processed.

[c] Summary of the assays of neurovirulence of the viruses in FVB/N mice from Table 2.

temperature sensitive of the mutant viruses, *ts*1-T, formed medium-sized foci, *ts*1 and *ts*1-A and -L formed small foci, and the two extremely temperature-sensitive mutant viruses, *ts*1-G and -E, formed very small foci. Thus the size of the foci produced at the restrictive temperature decreased as the temperature sensitivity of the viruses increased. The small foci produced by *ts*1-G and -E at 34°C suggested that the replication efficiencies of these two mutant viruses were low even at 34°C.

Growth curves of the viruses in TB cells at permissive temperature. To determine whether mutants *ts*1-G and -E replicated inefficiently at 34°C, growth curves of *ts*1-G and -E in TB cells were compared with those of *ts*1 and *ts*1-T, -A, and -L (Fig. 2). (The TB cell line is similar to the 15F cell line except that 15F cells contain an integrated replication-negative, transformation-negative murine sarcoma virus [3]). At 4 days p.i., the values of the titers for *ts*1 and *ts*1-T, -A, and -L, were clustered within 0.3 $\log_{10}$ unit in an interval between 7.5 and 7.8 $\log_{10}$ IU/ml, but the titers of *ts*1-G and -E (6.7 and 5.1 $\log_{10}$ IU/ml, respectively) were much lower. These results show that the two extremely temperature-sensitive mutants, *ts*1-G and -E, replicated less efficiently in vitro than *ts*1 and *ts*1-T, -A, and -L at the permissive temperature.

Processing of gPr80^{env} of the viruses at both permissive and restrictive temperatures. *wt*-25, *ts*1, and *ts*1-T, -L, -A, -G, and -E were assayed for processing of gPr80^{env} at both 34 and 39°C (Fig. 3). Anti-gp70 antiserum was used to immunoprecipitate radiolabeled proteins from lysed infected TB cells after a chase period of 120 min. At the permissive temperature (Fig. 3A), processing of radiolabeled gPr80^{env} was efficient for *wt*-25, *ts*1, and *ts*1-T (lanes 1, 2, and 3); processing was partial for *ts*1-L, -A, and -G (lanes 4, 5, and 6); and processing was inefficient for *ts*1-E (lane 7). At the restrictive temperature (Fig. 3B), processing of radiolabeled gPr80^{env} was efficient for *wt*-25 (lane 1), partial for *ts*1-T (lane 3), and inefficient for *ts*1 and *ts*1-L, -A, -G, and -E (lane 2, 4, 5, 6, and 7). *ts*1-E was the only mutant that inefficiently processed gPr80^{env} at both the permissive and restrictive temperatures. These results show that the degree of ineffi-

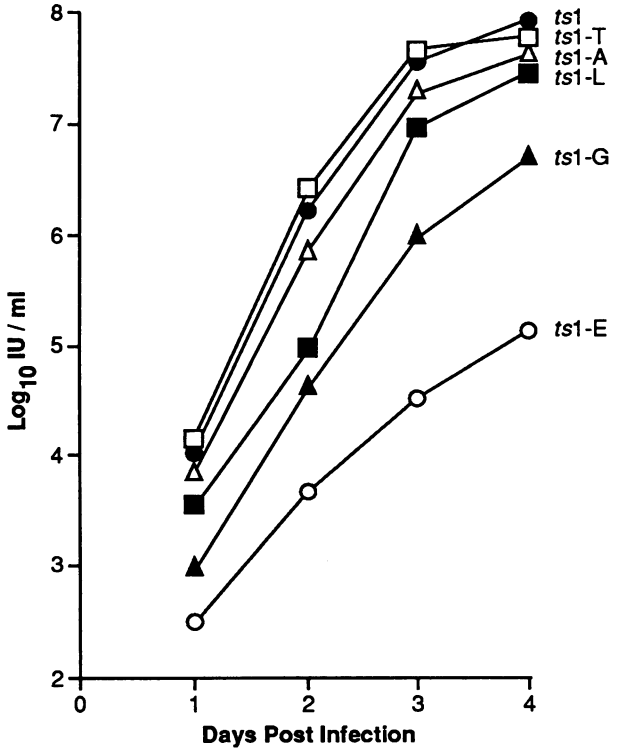

FIG. 2. Growth curves of the viruses in TB cells. The titers of infectious *ts*1 and *ts*1-T, -A, -L, -G, and -E that were released into the medium by infected TB cells (34°C) were assayed at 24-h intervals.

cient processing of gPr80^{env} for each mutant is correlated with the degree of temperature sensitivity of the mutant.

Neurovirulence of the viruses in FVB/N mice. Two amino acid substitutions in the gPr80^{env} of *ts*1 are required for *ts*1 to cause hind-limb paralysis in BALB/c and CFW/D mice, i.e., Val-25→Ile and most likely Arg-430→Lys (21, 22, 37). In a previous study (21), viruses that were hybrids of *ts*1 and *wt*-25 (*ts*1wt-31b and -32) and contained the Val-25→Ile substitution in gPr80^{env} but not the Arg-430→Lys substitution were constructed. Both *ts*1wt-31b and -32 generally caused paraparesis instead of hind-limb paralysis in BALB/c and CFW/D mice. However, in more recent studies we have found that, after a shorter and more uniform latency period, FVB/N mice are more susceptible to the neurological disease that is induced by *ts*1 than are the BALB/c and CFW/D mice (P. Wong, unpublished data). In FVB/N mice, hybrids *ts*1wt-31b and -32 cause hind-limb paralysis instead of paraparesis. In this study viruses were assayed for neurovirulence in FVB/N mice.

Within 48 h after birth, newborn FVB/N mice were inoculated intrathymically with *wt*-25, *ts*1, or *ts*1-T, -L, -A, -G, or -E. For 150 days, the infected mice were checked daily for signs of neurological disease (21, 38). Diseased mice were sacrificed when they became moribund, and the spleens and spinal cords were homogenized for assays of infectious virus. Table 2 shows the disease profiles of the infected mice, and Fig. 4 shows the cumulative incidence of the neurological disease. Mutants *ts*1-T, -A, and -L caused neurological disease after longer times p.i. than did *ts*1, but by 150 days p.i., no signs of neurological disease were evident in mice that were infected with mutant *ts*1-G or -E. *ts*1 caused hind-limb paralysis in 97% of the mice and paraparesis in 3% of the mice at 26 to 34 days p.i. *ts*1-A, which was 3.8-fold more temperature sensitive than *ts*1, was slightly less neurovirulent than *ts*1. *ts*1-A caused hind-limb paralysis in 79% of the mice and paraparesis in 21% of the mice after a slightly longer period (32 to 37 days p.i.) than did *ts*1. *ts*1-L, which was twofold more temperature sensitive than *ts*1-A, was less neurovirulent than *ts*1-A. *ts*1-L caused paraparesis in 59% of the mice and hind-limb paralysis in 41% of the mice at 36 to 58 days p.i. The results for *ts*1-A, -L, -G, and -E showed that increased temperature sensitivity and inefficient processing of gPr80^{env} were not necessarily correlated with increased neurovirulence.

In one mutant, *ts*1-T, a decrease in temperature sensitivity was correlated with a decrease in neurovirulence. *ts*1-T was 19-fold-less temperature sensitive than *ts*1 and caused neither hind-limb paralysis nor paraparesis but instead caused severe whole-body tremor in 100% of the mice at 32 to 45 days p.i. Near the terminal stage of the disease, the *ts*1-T-infected mice were severely tremulous, especially in response to sensory stimulation such as noise. Previously, signs of this neurological disorder were not observed in mice that were infected with *ts*1. The results for *ts*1-T showed that a decrease in temperature sensitivity and inefficient processing of gPr80^{env} relative to those of *ts*1 are correlated with phenotypically altered neurovirulence.

A histopathologic examination of the brains, brain stems, and spinal cords of diseased mice showed that the three neurovirulent mutants of *ts*1 (*ts*1-T, -A, and -L) produced a noninflammatory spongiform degeneration that was similar to that produced by *ts*1 (data not shown). However, the altered profiles of neurological disease in mice that were infected with *ts*1-A, *ts*1-L, and especially *ts*1-T probably reflect a topographical distribution and severity of spongiform degeneration within the central nervous system (CNS) different from that caused by *ts*1. Verification of these putative differences in spongiform degeneration will require a detailed histopathologic and morphometric analysis of the CNS of diseased mice.

Mutants *ts*1-G and -E were much more temperature sensitive than *ts*1 and *ts*1-T, -A, and -L, but *ts*1-G and -E were not neurovirulent. By 150 days p.i., most of the mice that were infected with *ts*1-G and -E had lymphoid leukemia. The livers and spleens of these leukemic mice were very enlarged. The livers weighed about 5 times more than those of the uninfected control mice, and the spleens weighed about 10 times more than those of the controls. FVB/N mice that were infected with *wt*-25 also developed lymphoid leukemia that was histopathologically similar to that caused by *ts*1-G and -E (data not shown). Previous studies have shown that *wt*-25 causes T-cell lymphoma in BALB/c mice (36). It is not known why *wt*-25 causes lymphoid leukemia in FVB/N mice. The neoplastic disease caused by *wt*-25 and *ts*1-G and -E in FVB/N mice appears, morphologically, to be a more fulminant form of the disease observed in BALB/c mice. Histologic evaluation of the liver showed marked accumulation of neoplastic lymphocytes within sinusoids and other vessels as well as effacement of periportal zones by neoplastic infiltrates. Normal splenic architecture was destroyed by massive infiltrations of cytomorphically similar lymphocytes. These results showed that *ts*1 retains the oncogenic potential of its progenitor, MoMuLV-TB, but the short latency period of the hind-limb paralytic disease that is induced by *ts*1 precludes the development of lymphoid leukemia, which has a longer latency period.

Replication of the viruses in spleens and spinal cords of FVB/N mice. A number of factors may be important in

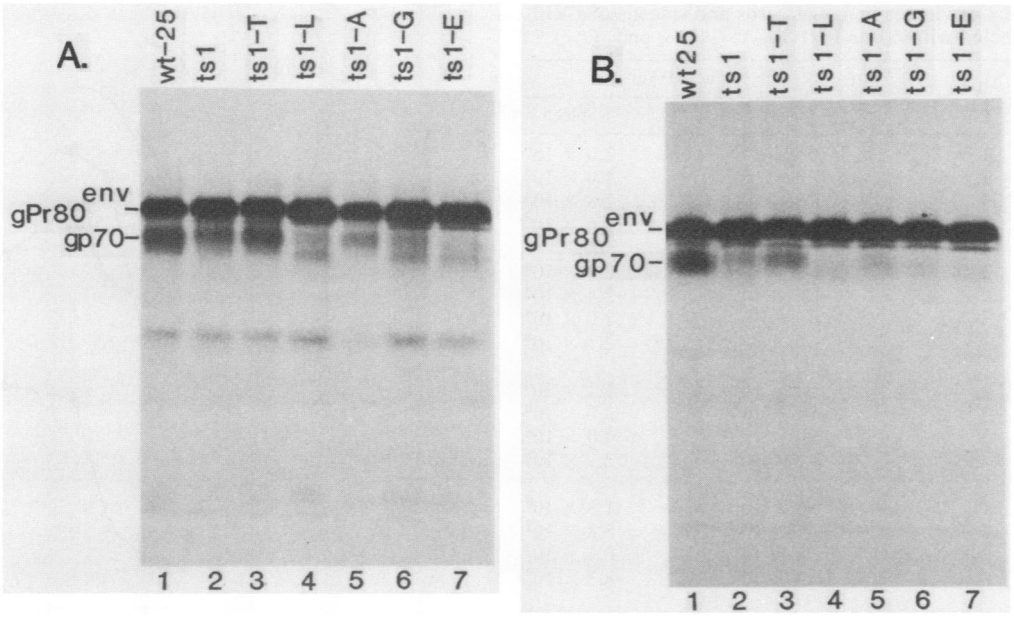

FIG. 3. Pulse-chase analysis of the processing of $gPr80^{env}$ at permissive and restrictive temperatures. TB cells were infected with *wt*-25, *ts*1, and *ts*1-T, -L, -A, -G, and -E and incubated at 34 or 39°C. The infected cells were pulse-radiolabeled for 10 min with both L-[^{35}S]cysteine and L-[^{35}S]methionine (there are 24 cysteines and 7 methionines in $gPr80^{env}$ and 20 cysteines and 2 methionines in gp70 [20, 21]). The pulse period was followed by a chase period of 120 min. The radiolabeled cells were lysed, and samples of the lysate containing 2×10^5 cpm were treated with anti-gp70 antiserum. Immunoprecipitated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 9.0% polyacrylamide gel. (A) Permissive temperature; (B) restrictive temperature.

determining the neurovirulence of a virus, i.e., efficiency of replication in peripheral tissues, efficiency in crossing the blood-brain barrier, and efficiency of replication in the CNS (10). In this study, it was shown that the in vitro replication efficiencies of mutants *ts*1-G and -E were less than those of *ts*1 and *ts*1-T, -A, and -L at 34°C. To determine if there was a relationship between replication efficiency in vivo and neurovirulence, the titer of virus was determined in the spleens (a peripheral tissue that is an important site of primary viral replication [19, 27, 37]) and the spinal cords of diseased moribund FVB/N mice (Table 3). The results show that the titers of the neurovirulent viruses (*ts*1 and *ts*1-T, -A, and -L) in both the spinal cord and the spleen were higher than those of the nonneurovirulent viruses (about 10-fold higher in the spinal cord and about 50-fold higher in the spleen than that of *ts*1-G and about 40-fold higher in the spinal cord and about 100-fold higher in the spleen than that of *ts*1-E).

To determine whether the titers of *ts*1 and the mutant

TABLE 2. Neurovirulence of *wt*-25, *ts*1, and *ts*1-T, -A, -L, -G, and -E in FVB/N mice[a]

Virus	No. of mice inoculated	No. of mice exhibiting:			
		Hind limb paralysis[b]	Paraparesis[c]	Severe whole-body tremor[d]	No signs[e]
*ts*1	30	29	1	0	0
*ts*1-T	18	0	0	18	0
*ts*1-A	14	11	3	0	0
*ts*1-L	17	7	10	0	0
*ts*1-G	9	0	0	0	9[f]
*ts*1-E	18	0	0	0	18[f]
wt-25	8	0	0	0	8[f]

[a] Within 48 h after birth, FVB/N mice were inoculated intrathymically with 10^5 to 10^6 IU of virus in 0.05 ml of growth medium. The mice were observed for signs of neurological disease for 150 days.
[b] Total loss of voluntary movement in the hindlimbs.
[c] Weakness and partial loss of voluntary movement in the hindlimbs (21).
[d] Severely tremulous, especially in response to sensory stimulation such as noise.
[e] By 150 days p.i.
[f] By 150 days p.i., these mice developed lymphoid leukemia: *ts*1-G infected, 9 of 9; *ts*1-E infected, 17 of 18; and *wt*-25 infected, 8 of 8.

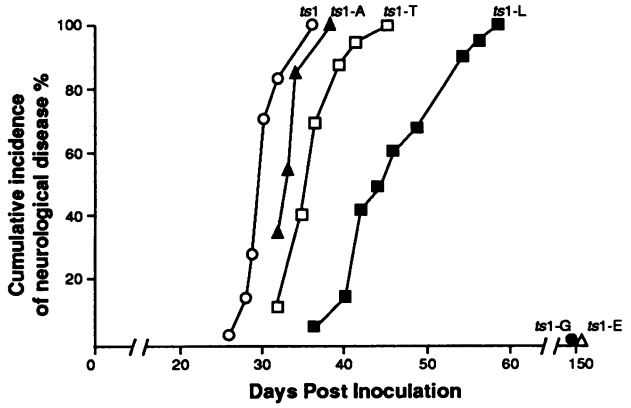

FIG. 4. Neurovirulence of *ts*1 and *ts*1-T, -A, -L, -G, and -E in FVB/N mice. Within 48 h after birth, FVB/N mice were inoculated intrathymically with 10^5 to 10^6 IU of virus in 0.05 ml of growth medium. The numbers of mice that were inoculated with each virus were 30 with *ts*1, 18 with *ts*1-T, 14 with *ts*1-A, 17 with *ts*1-L, 9 with *ts*1-G, and 18 with *ts*1-E. The mice were observed for signs of neurological disease for 150 days p.i.

TABLE 3. Titers of viruses in spinal cords and spleens of FVB/N mice infected with *ts*1 and *ts*1-T, -A, -L, -G, and -E[a]

Virus	Mouse no.	Titer (IU/g)[b]	
		Spinal cord	Spleen
*ts*1	1	3.6×10^7	2.0×10^8
	2	5.0×10^7	1.0×10^8
	3	5.8×10^7	1.8×10^8
	Avg	4.8×10^7	1.6×10^8
*ts*1-T	1	2.0×10^7	2.0×10^8
	2	4.0×10^7	2.4×10^8
	3	4.5×10^7	2.0×10^8
	Avg	3.5×10^7	2.1×10^8
*ts*1-A	1	5.6×10^7	1.1×10^8
	2	1.8×10^7	3.0×10^8
	3	6.0×10^6	4.0×10^8
	Avg	2.7×10^7	2.7×10^8
*ts*1-L	1	5.8×10^7	1.3×10^8
	2	6.0×10^7	5.8×10^7
	3	1.3×10^8	6.1×10^7
	Avg	8.3×10^7	8.3×10^8
*ts*1-G	1	3.2×10^6	1.6×10^6
	2	3.0×10^6	4.7×10^6
	3	3.6×10^6	4.2×10^6
	Avg	3.3×10^6	3.5×10^6
*ts*1-E	1	4.0×10^5	1.6×10^6
	2	8.0×10^5	4.8×10^6
	3	7.5×10^5	2.6×10^6
	Avg	6.5×10^5	3.0×10^6

[a] Mice infected with *ts*1 or *ts*1-T, -A, or -L were sacrificed when they showed severe signs of neurological disease. Mice infected with *ts*1-G and -E were sacrificed when they showed signs of lymphoid leukemia.

[b] Titers of virus in homogenates of the spinal cord and spleen were determined by the modified 15F assay. Titers are the average IUs per gram of spinal cord or spleen for three mice.

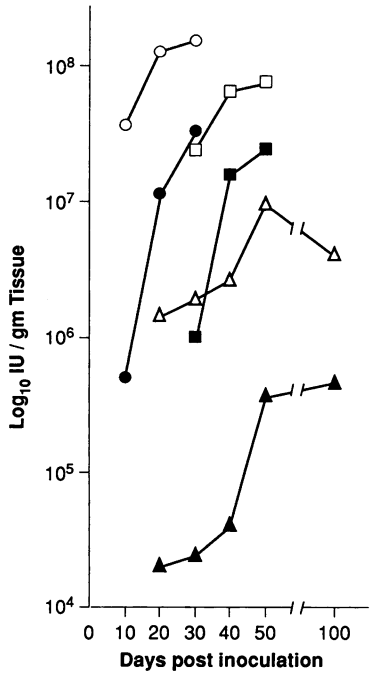

FIG. 5. Time course study of the titers of *ts*1 and *ts*1-L and -E in the spinal cords and spleens of infected mice. At various intervals, mice were sacrificed and the spleens and spinal cords were homogenized. The titers of virus in the two organs were plotted versus days p.i. Symbols: open, spleen; closed, spinal cord; ○ and ●, *ts*1; □ and ■, *ts*1-L; △ and ▲, *ts*1-E.

viruses in the spleens and spinal cords of diseased moribund mice were representative of the replication efficiencies of these viruses in these two organs in earlier stages of disease, the titers of *ts*1 and two representative mutants (*ts*1-L and -E) were determined in a time course study. In both organs throughout the course of disease, the titer of *ts*1-E, a mutant of extreme temperature sensitivity, was at least 10-fold lower than those of *ts*1 and *ts*1-L, mutants of intermediate temperature sensitivity (Fig. 5). These results are in agreement with the results for the diseased moribund mice (Table 3). Therefore it can be concluded that the nonneurovirulent viruses (*ts*1-G and -E) replicated less efficiently in vivo than the neurovirulent viruses (*ts*1 and *ts*1-T, -A, and -L). These results indicate that in addition to inefficient processing of gPr80^{env}, a high replication efficiency is correlated with neurovirulence.

DISCUSSION

The gPr80^{env} of *ts*1 is a temperature-sensitive protein (30). One method that has been used to study temperature-sensitive proteins is to substitute the temperature-sensitive amino acid with other amino acids (1, 17). This approach was used to study the temperature sensitivity, inefficient processing of gPr80^{env}, and neurovirulence of *ts*1. The Ile-25 in the gPr80^{env} of *ts*1 was substituted with five different amino acids (Thr, Ala, Leu, Gly, and Glu) to yield the mutant viruses *ts*1-T, -A, -L, -G, and -E, respectively. Each amino acid substitution for Ile-25 resulted in a mutant with a phenotype that was different from that of *ts*1. The rank order of the mutant viruses for temperature sensitivity was *ts*1-E > *ts*1-G > *ts*1-L > *ts*1-A > *ts*1 > *ts*1-T (Table 1). The degree of temperature sensitivity of each mutant correlated with the degree of inefficient processing of gPr80^{env} at the restrictive temperature (Fig. 3). The mutant with the least conservative substitution for Ile-25, *ts*1-E (Ile-25→Glu), was also the most temperature sensitive and processed gPr80^{env} with the least efficiency. These results suggest that the degree of misfolding of gPr80^{env} increases as the Ile-25 substitution becomes less conservative.

In previous studies, it was shown that inefficient processing of gPr80^{env} was correlated with the neurovirulence of *ts*1 (21, 27, 30). In this study, it was shown that low and intermediate degrees of inefficient processing of gPr80^{env} were correlated with neurovirulence in mutants *ts*1-T, -A, and -L but that extremely inefficient processing of gPr80^{env} was not correlated with neurovirulence in mutants *ts*1-G and -E. It is possible that the gPr80^{env} polyprotein has more than one functional role during neuropathogenesis. During the early stages of neuropathogenesis, when the body temperature (~34.0°C) of the newborn mouse is permissive for the replication of *ts*1 (5), efficient processing of gPr80^{env} to gp70 and Prp15E may be necessary for *ts*1 to infect target cells and spread in peripheral tissues (13, 27, 38). A threshold titer of *ts*1 in the peripheral tissues may be a prerequisite for invasion of the CNS (19). In later stages of the disease, when the body temperature (~38.4°C) of the young adult mouse is restrictive for the replication of *ts*1 (5), misfolding of gPr80^{env} in the ER may have a role in inducing the degener-

ation of neural cells (21, 27). Therefore replication efficiency may be important during early stages of neuropathogenesis, and misfolding of gPr80^{env} may be important during late stages.

Mutants *ts*1-G and -E did not replicate efficiently either in vitro in TB cells at 34°C (Fig. 2) or in vivo in the spleens and spinal cords of infected mice (Table 3, Fig. 5). These results suggest that a low replication efficiency of *ts*1-G and -E either in the peripheral tissues or in the spinal cord was responsible for the nonneurovirulence of *ts*1-G and -E. On the one hand, *ts*1-G and -E may have replicated inefficiently in the peripheral tissues and thus mounted an ineffective invasion of the CNS, with no neurological disease induced. On the other hand, the replication efficiencies of *ts*1-G and -E in the peripheral tissues may have been sufficient for effective invasion of the CNS, but the replication efficiencies of these two mutants in the CNS might have been too low to induce neurological disease. The data presented in this report do not distinguish between these two possibilities. However, the finding that *ts*1-G and -E caused lymphoid leukemia in FVB/N mice suggests that the replication efficiencies of *ts*1-G and -E were high enough for the two mutants to be oncogenic but not neurovirulent.

In addition to replication efficiency, the degree of misfolding of gPr80^{env} at the restrictive temperature has also been correlated with neuropathogenesis. The neurovirulence of *ts*1 and *ts*1-T, -A, and -L differed in both disease profile (Table 2) and time of development of severe signs of neurological disease (Fig. 5). The reason for the different degrees of neurovirulence of these mutant viruses is not known. However, we previously proposed that neural-cell degeneration in the CNS of *ts*1-infected mice is induced by an elevated steady-state level of misfolded gPr80^{env} in the ER of neural cells (21). Possibly Val-25 has an important role in the normal-folding pathway of gPr80^{env} (21) and substitution of Val-25 with other amino acids results in different degrees of misfolding of gPr80^{env} (1, 17). The degree of misfolding of gPr80^{env} could possibly determine both the steady-state level of misfolded gPr80^{env} in the ER and the reduced amount of properly folded gPr80^{env} that is transported from the ER to the *cis* cisternae of the Golgi apparatus. Therefore, the Ile, Thr, Ala, and Leu substitutions which occupy amino acid position 25 in the gPr80^{env} of *ts*1 and *ts*1-T, -A, and -L, respectively, may affect the folding and transport of gPr80^{env} in neural cells to different degrees, which in turn results in different degrees of neural-cell degeneration for each of these viruses.

The neural-cell degeneration induced by *ts*1 and *ts*1-T, -A, and -L could be explained by either a direct or an indirect mechanism. In the direct mechanism, neural-cell damage could result from the binding of misfolded gPr80^{env} to a host cell protein in the ER of a neural cell. There is experimental evidence that proteins of other viruses can bind to host cell proteins in the ER. The Friend spleen focus-forming virus gp55 glycoprotein binds to the erythropoietin receptor in the ER, which results in the proliferation of erythroid cells (9, 33). Another viral protein, the E19 glycoprotein of adenovirus, binds to class I antigens of the human histocompatibility complex in the ER and prevents the class I antigens from being transported from the ER to the plasma membrane (16). Elevated levels of misfolded gPr80^{env} may induce the degeneration of neural cells by interacting with a specific host cell regulatory protein in the ER or by retaining a specific host cell protein in the ER. The different degrees of neurovirulence exhibited by *ts*1 and *ts*1-T, -A, and -L could have resulted from the interaction of different levels of misfolded gPr80^{env} with a specific host cell protein in the ER of neural cells.

An indirect mechanism could involve a superinfection mechanism of cell killing by retroviruses. This mechanism has been reviewed by Temin (24), and it has been proposed that host cells are killed by an accumulation of unintegrated viral DNA in the superinfected cells, by an overexpression of viral proteins in the superinfected cells, or simply by the toxicity of the massive superinfection. During the late stages of neuropathogenesis, when the body temperature (~38.4°C) of the young adult mouse is at the restrictive temperature, preinfected neural cells may be susceptible to superinfection because they are deficient in gp70 as a result of inefficient processing of gPr80^{env} to gp70 and Prp15E (27, 30). However, the variability in the neurovirulence of *ts*1 and *ts*1-T, -A, and -L could be due to differences in the ability of each of these viruses to superinfect preinfected neural cells at the restrictive temperature. The ability of a virus to superinfect a preinfected cell depends on both the susceptibility of the preinfected cell to superinfection (26) and the infectivity of the superinfecting virus (11). At the restrictive temperature, the susceptibility of a preinfected neural cell to superinfection should increase with increased misfolding of gPr80^{env} in the ER (i.e., decreased levels of cellular gp70), but the infectivity of the superinfecting virus should decrease with increased misfolding of gPr80^{env} in the ER (i.e., decreased amounts of gp70 on the surface of the superinfecting virus particle). Although it is possible that there are differences in the superinfectivity of *ts*1 and *ts*1-T, -A, and -L, it is not possible to predict the rank order of *ts*1 and *ts*1-T, -A, and -L for this putative superinfectivity, since the susceptibility of preinfected neural cells to superinfection should increase for the same reason that infectivity of the superinfecting virus should decrease.

It is intriguing that mutant *ts*1-T caused severe whole-body tremor instead of hind-limb paralysis. A similar neurological disorder that is characterized by excessive tremulousness, spasticity, and immobility has been reported in mice that were infected with the chimeric MuLV pNEMO-1 (6). pNEMO-1 MuLV consists of a Cas-Br-E MuLV genome in which the long terminal repeat is substituted with that of MoMuLV. Mice that are infected with pNEMO-1 MuLV have a different topographical distribution of spongiform degeneration in the brain than mice that are infected with Cas-Br-E, a MuLV that causes hind-limb paralysis. It is interesting that the signs of the neurological disorder that are induced by *ts*1-T are quite similar to those that are induced by pNEMO-1 MuLV. DesGroseillers et al. (6) have suggested that the neurological disorder induced by pNEMO-1 MuLV is determined by the long terminal repeat of the virus. However, the neurological disorder that is induced by *ts*1-T results from an Ile-25→Thr substitution in the gPr80^{env} of *ts*1, not from sequences in the long terminal repeat, which suggests that the topographical distribution of spongiform degeneration in the CNS can be determined by different regions of the viral genome. Possibly the decreased temperature sensitivity and the increased efficiency in processing of the gPr80^{env} of *ts*1-T relative to those of *ts*1 are responsible for a new pattern of viral spread within the CNS. In our laboratory, studies to determine if the topographical distribution of spongiform degeneration in the brains, brain stems, and spinal cords of mice that are infected with *ts*1-T is different from that in mice infected by *ts*1 (38) are now in progress.

In this report it has been shown that amino acid substitutions for Ile-25 in gPr80^{env} alter the neurovirulence of *ts*1 in

FVB/N mice. The neurovirulence of *ts*1 and *ts*1-T, -A, and -L is correlated with the ability of these viruses to invade and replicate to a high titer in the CNS and with the misfolding of gPr80^{env} in the ER of neural cells. The neurovirulence of the Cas-Br-E MuLV has also been mapped to the *env* gene (18), but the neurovirulence of the Cas-Br-E MuLV has been attributed to the binding of gp70 to a specific cellular receptor, not to misfolding of gPr80^{env} in the ER of neural cells. Further studies in our laboratory are directed at a more detailed molecular characterization of the role of misfolded gPr80^{env} in the neurovirulence of *ts*1 and *ts*1-T, -A, and -L.

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LITERATURE CITED

1. **Alber, T., and B. W. Matthews.** 1987. Structure and thermal stability of phage T4 lysozyme. Methods Enzymol. **154**:511–533.
2. **Ball, J. K., T. Y. Huh, and J. A. McCarter.** 1964. On the statistical distribution of epidermal papillomata in mice. Br. J. Cancer **18**:120–123.
3. **Ball, J. K., J. A. McCarter, and S. M. Sunderland.** 1973. Evidence for helper independent murine sarcoma virus. I. Segregation of replication-defective and transformation-defective viruses. Virology **56**:268–284.
4. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
5. **Crispens, C. G., Jr.** 1978. Handbook of the laboratory mouse, p. 139. Charles C Thomas, Publisher, Springfield, Ill.
6. **DesGroseillers, L., E. Rassart, Y. Robitaille, and P. Jolicoeur.** 1985. Retrovirus-induced spongiform encephalopathy: the 3′-end long terminal repeat-containing viral sequences influence the incidence of the disease and the specificity of the neurological syndrome. Proc. Natl. Acad. Sci. USA **82**:8818–8822.
7. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
8. **Jones, D. S., F. Nemoto, Y. Kuchino, M. Masuda, H. Yoshikura, and S. Nishimura.** 1989. The effect of specific mutations at and around the *gag-pol* gene junction of Moloney murine leukaemia virus. Nucleic Acids Res. **17**:5933–5945.
9. **Li, J., A. D. D'Andrea, H. F. Lodish, and D. Baltimore.** 1990. Activation of cell growth by binding of Friend spleen focus-forming virus gp55 glycoprotein to the erythropoietin receptor. Nature (London) **343**:762–764.
10. **Lustig, S., A. C. Jackson, C. S. Hahn, D. E. Griffin, E. G. Strauss, and J. H. Strauss.** 1988. Molecular basis of Sindbis virus neurovirulence in mice. J. Virol. **62**:2329–2336.
11. **Marsh, M., and A. Helenius.** 1989. Virus entry into animal cells. Adv. Virus Res. **36**:107–151.
12. **McCarter, J. A.** 1977. Genetic studies of the ploidy of Moloney murine leukemia virus. J. Virol. **22**:9–15.
13. **McCarter, J. A., J. K. Ball, and J. V. Frei.** 1977. Lower limb paralysis induced in mice by a temperature-sensitive mutant of Moloney leukemia virus. J. Natl. Cancer Inst. **59**:179–183.
14. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101**:20–78.
15. **Nakamaye, K. L., and F. Eckstein.** 1986. Inhibition of restriction endonuclease Nci I cleavage by phosphorothioate groups and its application to oligonucleotide-directed mutagenesis. Nucleic Acids Res. **14**:9679–9698.
16. **Paabo, S., B. M. Bhat, W. S. M. Wold, and P. A. Peterson.** 1987. A short sequence in the COOH-terminus makes an adenovirus membrane glycoprotein a resident of the endoplasmic reticulum. Cell **50**:311–317.
17. **Pakula, A. A., and R. T. Sauer.** 1989. Genetic analysis of protein stability and function. Annu. Rev. Genet. **23**:289–310.
18. **Paquette, Y., Z. Hanna, P. Savard, R. Brousseau, Y. Robitaille, and P. Jolicoeur.** 1989. Retrovirus-induced murine motor neuron disease: mapping the determinant of spongiform degeneration within the envelope gene. Proc. Natl. Acad. Sci. USA **86**:3896–3900.
19. **Prasad, G., G. Stoica, and P. K. Y. Wong.** 1989. The role of the thymus in the pathogenesis of hind-limb paralysis induced by ts1, a mutant of Moloney murine leukemia virus-TB. Virology **169**:332–340.
20. **Shinnick, T. M., R. A. Lerner, and J. G. Sutcliffe.** 1981. Nucleotide sequence of Moloney murine leukaemia virus. Nature (London) **293**:543–548.
21. **Szurek, P. F., P. H. Yuen, J. K. Ball, and P. K. Y. Wong.** 1990. A Val-25-to-Ile substitution in the envelope precursor polyprotein, gPr80^{env}, is responsible for the temperature sensitivity, inefficient processing of gPr80^{env}, and neurovirulence of *ts*1, a mutant of Moloney murine leukemia virus TB. J. Virol. **64**:467–475.
22. **Szurek, P. F., P. H. Yuen, R. Jerzy, and P. K. Y. Wong.** 1988. Identification of point mutations in the envelope gene of Moloney murine leukemia virus TB temperature-sensitive paralytogenic mutant *ts*1: molecular determinants for neurovirulence. J. Virol. **62**:357–360.
23. **Tabor, S., and C. C. Richardson.** 1987. DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. Proc. Natl. Acad. Sci. USA **84**:4767–4771.
24. **Temin, H. M.** 1988. Mechanisms of cell killing/cytopathic effects by nonhuman retroviruses. Rev. Infect. Dis. **10**:399–405.
25. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
26. **Weiss, R. A.** 1984. Experimental biology and assay of RNA tumor viruses, p. 209–260. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Wong, P. K. Y., C. Knupp, P. H. Yuen, M. M. Soong, J. F. Zachary, and W. A. F. Tompkins.** 1985. *ts*1, a paralytogenic mutant of Moloney murine leukemia virus TB, has an enhanced ability to replicate in the central nervous system and primary nerve cell culture. J. Virol. **55**:760–767.
28. **Wong, P. K. Y., G. Prasad, J. Hansen, and P. H. Yuen.** 1989. *ts*1, a mutant of Moloney murine leukemia virus-TB, causes both immunodeficiency and neurologic disorders in BALB/c mice. Virology **170**:450–459.
29. **Wong, P. K. Y., L. J. Russ, and J. A. McCarter.** 1973. Rapid, selective procedure for isolation of spontaneous temperature-sensitive mutants of Moloney leukemia virus. Virology **51**:424–431.
30. **Wong, P. K. Y., M. M. Soong, R. M. MacLeod, G. E. Gallick, and P. H. Yuen.** 1983. A group of temperature-sensitive mutants of Moloney leukemia virus which is defective in cleavage of *env* precursor polypeptide in infected cells also induces hind-limb paralysis in newborn CFW/D mice. Virology **125**:513–518.
31. **Wong, P. K. Y., M. M. Soong, and P. H. Yuen.** 1981. Replication of murine leukemia virus in heterologous cells: interaction between ecotropic and xenotropic viruses. Virology **109**:366–378.
32. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.
33. **Yoshimura, A., A. D. D'Andrea, and H. F. Lodish.** 1990. Friend spleen focus-forming virus glycoprotein gp55 interacts with the erythropoietin receptor in the endoplasmic reticulum and affects receptor metabolism. Proc. Natl. Acad. Sci. USA **87**:4139–4143.
34. **Yuen, P. H., D. Malehorn, C. Knupp, and P. K. Y. Wong.** 1985. A 1.6-kilobase-pair fragment in the genome of the *ts*1 mutant of Moloney murine leukemia virus TB that is associated with temperature sensitivity, nonprocessing of Pr80^{env}, and paralytogenesis. J. Virol. **54**:364–373.
35. **Yuen, P. H., D. Malehorn, C. Nau, M. M. Soong, and P. K. Y. Wong.** 1985. Molecular cloning of two paralytogenic, temperature-sensitive mutants, *ts*1 and *ts*7, and the parental wild-type Moloney murine leukemia virus. J. Virol. **54**:178–185.

36. **Yuen, P. H., and P. F. Szurek.** 1989. The reduced virulence of the thymotropic Moloney murine leukemia virus derivative MoMuLV-TB is mapped to 11 mutations within the U3 region of the long terminal repeat. J. Virol. **63**:471–480.
37. **Yuen, P. H., E. Tzeng, C. Knupp, and P. K. Y. Wong.** 1986. The neurovirulent determinants of *ts*1, a paralytogenic mutant of Moloney murine leukemia virus TB, are localized in at least two functionally distinct regions of the genome. J. Virol. **59**:59–65.
38. **Zachary, J. F., C. J. Knupp, and P. K. Y. Wong.** 1986. Non-inflammatory spongiform polioencephalomyelopathy caused by a neurotropic temperature-sensitive mutant of Moloney murine leukemia virus TB. Am. J. Pathol. **124**:457–468.