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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

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ts1 is a neurovirulent spontaneous temperature-sensitive mutant of Moloney murine leukemia virus TB which causes hindlimb paralysis in mice. Previously, it had been shown that the temperature-sensitive defect resided in the *env* gene. At the restrictive temperature, the envelope precursor polyprotein, gPr80^{env}, is inefficiently processed intracellularly into two cleavage products, gp70 and Prp15E. This inefficient processing of gPr80^{env} is correlated with neurovirulence. In this study, it was shown that a single amino acid substitution, Val-25→Ile in gPr80^{env}, is responsible for the temperature sensitivity, inefficient processing of gPr80^{env} at the restrictive temperature, and neurovirulence of ts1. At the restrictive temperature, a steady-state level of nonprocessed, endoglycosidase H-sensitive gPr80^{env} remained in the endoplasmic reticulum of cells infected by ts1, but no endoglycosidase H-resistant gPr80^{env} and only trace amounts of gp70 were detected in the infected cells. Since the host cell-encoded processing protease resides in the *cis* cisternae of the Golgi apparatus, inefficient processing of gPr80^{env} at the restrictive temperature is most likely due to inefficient transport of gPr80^{env} from the endoplasmic reticulum to the *cis* cisternae of the Golgi apparatus rather than due to misfolded gPr80^{env} being a poor substrate for the processing protease at the restrictive temperature.

*ts*1 is a neurovirulent spontaneous temperature-sensitive mutant of Moloney murine leukemia virus TB (MoMuLV-TB) (22, 41, 43, 50). MoMuLV-TB causes lymphoma in mice after a long latency period of 179 days (48), whereas *ts*1 causes a progressive hindlimb paralytic disease in susceptible strains of mice after a much shorter latency period ranging from 24 to 80 days (22, 28, 42, 50). The hindlimb paralytic disease is characterized by a degenerative spongiform encephalomyelopathy (22, 41, 50), generalized body wasting (42, 50), severe thymic atrophy (22, 42), and drastic immunodeficiency (42). A similar hindlimb paralytic disease has been described for other strains of ecotropic MuLV isolated from wild mice (10) and experimentally generated in the laboratory (3, 18, 31, 43).

The neurovirulence of *ts*1 seems to be related to its temperature sensitivity (44). Only susceptible strains of adult mice that were infected with *ts*1 as neonates succumb to hindlimb paralysis (22, 44). Newborn mice have a lower body temperature (~34.0°C) than adult mice (~38.4°C) (5). The body temperature of newborn mice is permissive for the replication of *ts*1, and *ts*1 is able to replicate in target cells and spread to the central nervous system (CNS) (22, 41, 50). However, signs of hindlimb paralysis do not appear until the infected mice become young adults. In young adult mice, the body temperature is restrictive for the replication of *ts*1, and defective virus replication is correlated with the onset of signs of hindlimb paralysis. The first signs to appear are tremors in the hindlimbs, which rapidly progress to paraparesis, which is then followed by hindlimb paralysis. Histopathological studies indicate that spongiform changes occur in the brain stem and in both the grey and white matter of the spinal cord (28, 50).

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In previous studies, the close genetic relatedness between *ts*1 and MoMuLV-TB allowed us to explore the molecular basis for the neurovirulence of *ts*1 (38, 46, 47, 49). By constructing hybrid viruses between *ts*1 and both MoMuLV-TB and MoMuLV, it was shown that two regions in the *env* gene of *ts*1 are required to cause hindlimb paralysis (38, 46, 49). One region, in the *Xba*I-*Bam*HI restriction fragment (771 base pairs [bp], nucleotides [nts] 5767 to 6537), coded for the temperature sensitivity, inefficient processing of gPr80^{env} at the restrictive temperature, and neurovirulence of *ts*1 (38, 44, 46, 49). The other region, in the *Bam*HI-*Cla*I restriction fragment (1,138 bp, nts 6538 to 7675), coded for the enhanced ability of *ts*1 to replicate in the CNS relative to MoMuLV-TB (38, 49). A comparison of the amino acid sequences (deduced from the nucleotide sequences) encoded by the *env* genes of both *ts*1 and MoMuLV-TB showed that four amino acid substitutions in *ts*1, i.e., Val-25→Ile in gPr80^{env}, Ser-157→Ala in gPr80^{env}, Arg-430→Lys in gp70, and Ile-23→Val in p15E, were the results of genuine mutations in *ts*1 (38). Both the Ile-25 and the Ala-157 substitutions in gPr80^{env} were encoded by the *Xba*I-*Bam*HI region of *env*, whereas both the Lys-430 substitution in gp70 and the Val-23 substitution in p15E were encoded by the *Bam*HI-*Cla*I region of *env*.

In this study, fine-structure genetic mapping was done by exchanging restriction fragments containing one or more of the four amino acid substitutions in the envelope proteins of *ts*1 between *ts*1 and MoMuLV-TB to generate a new set of hybrid virus genomes. These hybrid viruses were assayed for the phenotypic characteristics of *ts*1. It was found that the Val-25→Ile substitution in gPr80^{env} was responsible for the temperature sensitivity, inefficient processing of gPr80^{env} at the restrictive temperature, and neurovirulence of *ts*1. Endoglycosidase H (endo H) sensitivity experiments

showed that a steady-state level of the nonprocessed gPr80^{env} remains in the endoplasmic reticulum (ER) of infected cells at the restrictive temperature. These results indicated that inefficient processing of gPr80^{env} at the restrictive temperature is most likely 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, rather than due to misfolded gPr80^{env} being a poor substrate for the processing protease at the restrictive temperature.

MATERIALS AND METHODS

Cells. Viruses were propagated in mouse TB cells, a thymus-bone marrow cell line derived from CFW/D mice (2), and titer was determined on 15F cells, a murine sarcoma-positive, leukemia-negative (S^+L^-) cell line (45). NIH 3T3 cells were used for transfection and immunoprecipitation experiments. All cell lines were maintained in Dulbecco modified Eagle medium supplemented with 6% fetal bovine serum and 4% bovine serum.

Viruses. The standard MoMuLV referred to in this report was described previously in Yuen et al. (46). The progenitor of MoMuLV-TB, a variant of MoMuLV, was isolated as described by Wong et al. (43) from the tissue extract of a sarcoma produced in a MoMuLV-murine sarcoma virus-infected BALB/c mouse provided by J. B. Moloney. Since its isolation, it has been propagated in TB cells and has been designated MoMuLV-TB. MoMuLV-TB has been molecularly cloned into Charon 21A, and clone *wt*-25 was used in these studies (47). *ts*1 has been molecularly cloned into Charon 21A, and clone *ts*1-19 was used in these studies (47).

Construction of hybrid virus genomes. Details of methods for the construction of hybrid virus genomes have been described previously (46, 49). Hybrid *ts*1wt-33 consists of a *ts*1 genome with a substituted *Bst*EII-*Bst*XI restriction fragment (116 bp, nts 5924 to 6039) of MoMuLV-TB. Hybrid *ts*1wt-32 is a reciprocal hybrid genome of *ts*1wt-33. Hybrid *ts*1wt-26 consists of a *ts*1 genome with a substituted *Bst*XI-*Bam*HI restriction fragment (498 bp, nts 6040 to 6537) of MoMuLV-TB. Hybrid *ts*1wt-31b consists of a *ts*1 genome with a substituted *Bam*HI-*Hpa*I restriction fragment (660 bp, nts 6538 to 7197) of MoMuLV-TB. Hybrid *ts*1wt-30 is a reciprocal hybrid genome of hybrid *ts*1wt-31b. Hybrid *ts*1wt-23 consists of a *ts*1 genome with a substituted *Hpa*I-*Cla*I restriction fragment (478 bp, nts 7198 to 7675) of MoMuLV-TB. Hybrid *ts*1wt-41 consists of a *ts*1 genome with two substituted restriction fragments of MoMuLV-TB, i.e., *Bst*XI-*Bam*HI and *Hpa*I-*Cla*I. Hybrid *ts*1wt-42 consists of an MoMuLV-TB genome with two substituted restriction fragments of *ts*1, i.e., *Bst*EII-*Bst*XI and *Bam*HI-*Hpa*I. The hybrid genomes were verified by mapping four restriction enzyme sites that were present in MoMuLV-TB but not in *ts*1. These four restriction site polymorphisms were identified by analysis of the nucleotide sequences of the *env* genes for both MoMuLV-TB and *ts*1 (38). In MoMuLV-TB, there is a *Sca*I site (nts 5870 to 5875) in the *Hin*dIII-*Bst*EII fragment (1,029 bp, nts 4895 to 5923), a *Sfi*I site (nts 6237 to 6249) in the *Bst*XI-*Bam*HI fragment (498 bp, nts 6040 to 6537), an *Apa*I site (nts 6992 to 6997) in the *Bam*HI-*Hpa*I fragment (660 bp, nts 6538 to 7197), and a *Hae*III site (nts 7204 to 7207) in the *Hpa*I-*Cla*I fragment (478 bp, nts 7198 to 7675). Nucleotides were numbered by the method of Shinnick et al. (36).

DNA transfection. NIH 3T3 cells were transfected with viral DNA by the calcium-phosphate precipitation method (13). Details of this procedure have been described previously in Yuen et al. (47).

Metabolic labeling, radioimmunoprecipitation, and SDS-PAGE. Immunoprecipitation of intracellular virus-specific proteins by using fixed *Staphylococcus aureus* (Pansorbin cells from Calbiochem Corp., La Jolla, Calif.) as an immunoabsorbent and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were performed as described previously (44, 45), except that the infected NIH 3T3 cells were double labeled with both L-[^{35}S]cysteine (50 μCi/ml) and L-[^{35}S]methionine (50 μCi/ml) and all immunoprecipitation steps were done at 4°C. Goat antiserum prepared against Rauscher MuLV gp69/71 (lot 79S000842) was obtained from Microbiological Associates, Inc., Bethesda, Md. Apparent molecular weights (M_r) of proteins were determined by plotting log M_r versus relative distance migrated (34). The ^{14}C-protein standards (Dupont, NEN Research Products, Boston, Mass.) had M_rs of 97,400 (phosphorylase B), 69,000 (bovine serum albumin), 46,000 (ovalbumin), 30,000 (carbonic anhydrase), and 12,300 (cytochrome *c*).

Endo H treatment. *Streptomyces plicatus* endo H (EC 3.2.1.96), purified from a recombinant *Escherichia coli* strain, was purchased from Boehringer GmbH, Mannheim, Federal Republic of Germany. The endo H digestions were optimized by the procedure described by Trimble and Maley (39). The washed Pansorbin cell pellets obtained from immunoprecipitation were washed two additional times with 10 mM Tris (pH 7.4)–1% (vol/vol) aprotinin–2 mM phenylmethylsulfonyl fluoride and one time with 1% (vol/vol) aprotinin–2 mM phenylmethylsulfonyl fluoride, and then they were suspended in 30 μl of antigen release buffer (10 mM Tris [pH 7.4], 0.2 M 2β-mercaptoethanol, 1% SDS). Antigen was released from the Pansorbin cell-antibody complex by boiling the mixture for 5 min, and the Pansorbin cells were removed by centrifugation. Reagents were added to the supernatant to give final concentrations of 50 mM sodium citrate (pH 5.3), 0.1 M 2β-mercaptoethanol, 0.02% SDS, 1 mM phenylmethylsulfonyl fluoride, 1% (vol/vol) aprotinin, 5 mM EDTA, 0.02% (wt/vol) acetylated bovine serum albumin, and 40.0 mU of endo H per ml in a volume of 50 μl. The samples were digested for 16 h at 37°C, and they were prepared for SDS-PAGE as described previously (45).

Mouse strain and inoculation procedure. The BALB/c mice (28, 42), the inbred CFW/D mice (2), and the procedure for intraperitoneal inoculations (47) have been described previously. The experiments involved with the CFW/D mice were conducted as a blind study.

RESULTS

Construction of hybrid viruses. The MoMuLV genome contains 8,332 nts (36) (Fig. 1). The *env* gene (1,998 bp, nts 5777 to 7774) codes for an envelope precursor polyprotein, gPr80^{env} (632 amino acids, encoded by nts 5876 to 7771) which is processed intracellularly by a host cell-encoded protease to yield two envelope proteins, gp70 (435 amino acids, encoded by nts 5876 to 7180) and Prp15E (196 amino acids, encoded by nts 7184 to 7771) (37). Prp15E is processed by a virus-encoded protease to yield p15E (180 amino acids, encoded by nts 7184 to 7723) and p2E (16 amino acids, encoded by nts 7724 to 7771) (37). In a previous study (38), four point mutations in the *env* gene of *ts*1 which resulted in amino acid substitutions that may be responsible for the phenotype of *ts*1 were identified. A G→A change at nt 5948 resulted in a Val-25→Ile substitution in gPr80^{env}, a T→G change at nt 6344 resulted in a Ser-157→Ala substitution in

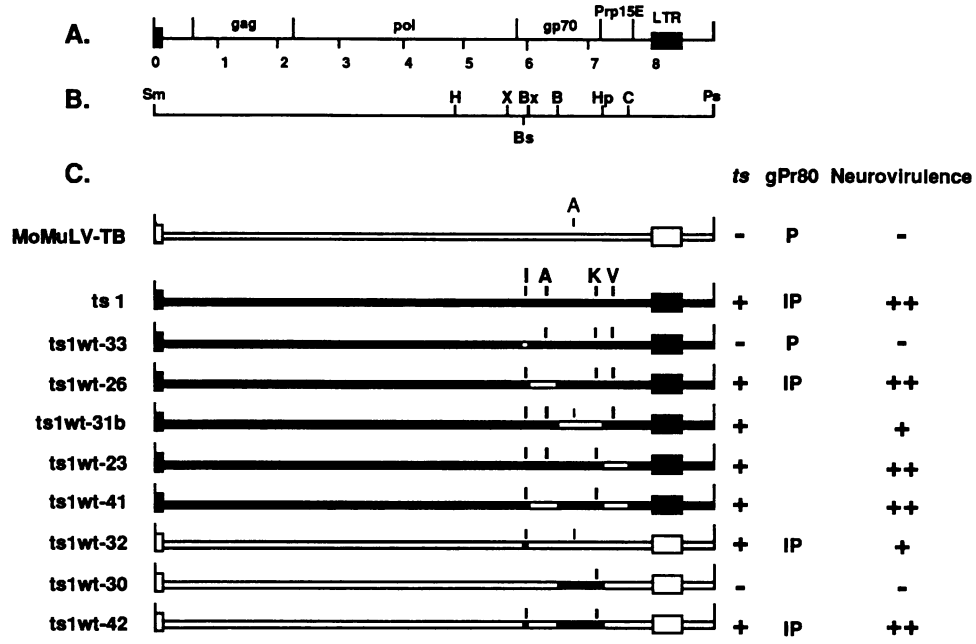

FIG. 1. Determination of the amino acid substitution responsible for the temperature sensitivity, inefficient processing of gPr80^{env}, and neurovirulence of *ts*1. (A) Genetic map of the MoMuLV genome. (B) Critical restriction sites used to construct hybrid virus genomes. Restriction site abbreviations: B, *Bam*HI; Bs, *Bst*EII; Bx, *Bst*XI; C, *Cla*I; H, *Hin*dIII; Hp, *Hpa*I; Ps, *Pst*I; Sm, *Sma*I; X, *Xba*I. (C) Genomes of hybrid viruses used to determine the phenotype of *ts*1. Hybrid genomes were constructed by exchanging homologous restriction fragments between *ts*1 (black) and MoMuLV-TB (white). The locations of the four amino acid substitutions (Val-25→Ile in gPr80^{env} [I], Ser-157→Ala in gPr80^{env} [A], Arg-430→Lys in gp70 [K], and Ile-23→Val in p15E [V]) encoded by the *env* gene of *ts*1 are shown above the *ts*1 genome. Restriction fragments containing amino acid substitutions of *ts*1 are *Bst*EII-*Bst*XI (I), *Bst*XI-*Bam*HI (A), *Bam*HI-*Hpa*I (K), and *Hpa*I-*Cla*I (V). The position of the Thr-347→Ala substitution in gp70 encoded by the MoMuLV-TB *env* gene is shown above the MoMuLV-TB genome (A). The Thr-347→Ala substitution in gp70 of MoMuLV-TB is encoded by the *Bam*HI-*Hpa*I restriction fragment. NIH 3T3 cells were transfected with the hybrid virus genomes to obtain the infectious hybrid viruses. The viruses were assayed for temperature sensitivity (*ts*), inefficient processing of gPr80^{env} (gPr80), and neurovirulence. For temperature sensitivity, + indicates that the ratio of foci produced on 15F cells (34.0°C/39.0°C) was greater than 100. For gPr80, P indicates that gPr80^{env} was efficiently processed to gp70 and Prp15E at 39°C, and IP indicates that gPr80^{env} was inefficiently processed at 39.0°C. For neurovirulence, ++ indicates hindlimb paralysis, + indicates paraparesis (a slight degree of paralysis which affects the hindlimbs), and − indicates no signs of paraparesis or hindlimb paralysis.

gPr80^{env}, a G→A change at nt 7164 resulted in an Arg-430→Lys substitution in gp70, and an A→G change at nt 7250 resulted in an Ile-23→Val substitution in p15E. Both the Ile-25 and the Ala-157 substitutions in gPr80^{env} are encoded by the *Xba*I-*Bam*HI region of *env* (771 bp, nts 5767 to 6537) which is responsible for the temperature sensitivity, the gPr80^{env} processing defect at the restrictive temperature, and the neurovirulence of *ts*1 (38, 44, 46, 49). Both the Lys-430 substitution in gp70 and the Val-23 substitution in p15E are encoded by the *Bam*HI-*Cla*I region of *env* (1,138 bp, nts 6538 to 7675) which is responsible for the enhanced ability of *ts*1 to replicate in the CNS relative to MoMuLV-TB (38, 49).

In this present study, individual amino acid substitutions in the envelope proteins of *ts*1 were assigned to specific phenotypic characteristics of *ts*1 by assaying a new set of hybrid viruses. A new set of hybrid genomes was constructed by exchanging restriction fragments between *ts*1 and MoMuLV-TB (Fig. 1). The codons for the four amino acid substitutions in the envelope proteins of *ts*1 are contained within unique restriction fragments (36, 38). The codon for the Ile-25 substitution in gPr80^{env} is in a *Bst*EII-*Bst*XI restriction fragment (116 bp, nts 5924 to 6039), the codon for the Ala-157 substitution in gPr80^{env} is in a *Bst*XI-*Bam*HI restriction fragment (498 bp, nts 6040 to 6537), the codon for the Lys-430 substitution in gp70 is in a *Bam*HI-*Hpa*I restriction fragment (660 bp, nts 6538 to 7197), and the codon for the Val-23 substitution in p15E is in a *Hpa*I-*Cla*I restriction fragment (478 bp, nts 7198 to 7675). In hybrids *ts*1wt-23, -26, -30, -31b, -32, and -33, single amino acid replacements were made which involved one of the amino acid substitutions encoded by the *env* gene of *ts*1 and the corresponding wild-type amino acid of MoMuLV-TB. In hybrids *ts*1wt-41 and -42, two amino acid replacements were made which involved two of the amino acid substitutions encoded by the *env* gene of *ts*1 and two of the corresponding wild-type amino acids of MoMuLV-TB. (Note that in MoMuLV-TB there is an Ala in position 347 of gp70 instead of a Thr, which is in both *ts*1 and MoMuLV [38] [Fig. 1].) The Thr-347→Ala substitution in gp70 probably arose during propagation of MoMuLV-TB virus after *ts*1 was selected from MoMuLV-TB but before the genome of MoMuLV-TB was molecularly cloned (i.e., clone *wt*-25) (38, 47). Both hybrids *ts*1wt-31b and *ts*1wt-32 contain the *Bam*HI-*Hpa*I restriction fragment (660 bp, nts 6538 to 7197) of MoMuLV-TB which codes for the Thr-347→Ala substitution in the gp70 of MoMuLV-TB.

All of the exchanged restriction fragments could be identified by restriction site polymorphisms between *ts*1 and MoMuLV-TB except for the *Bst*EII-*Bst*XI fragment. In MoMuLV-TB, the *Bst*XI-*Bam*HI fragment contains a *Sfi*I site (nts 6237 to 6249), the *Bam*HI-*Hpa*I fragment contains

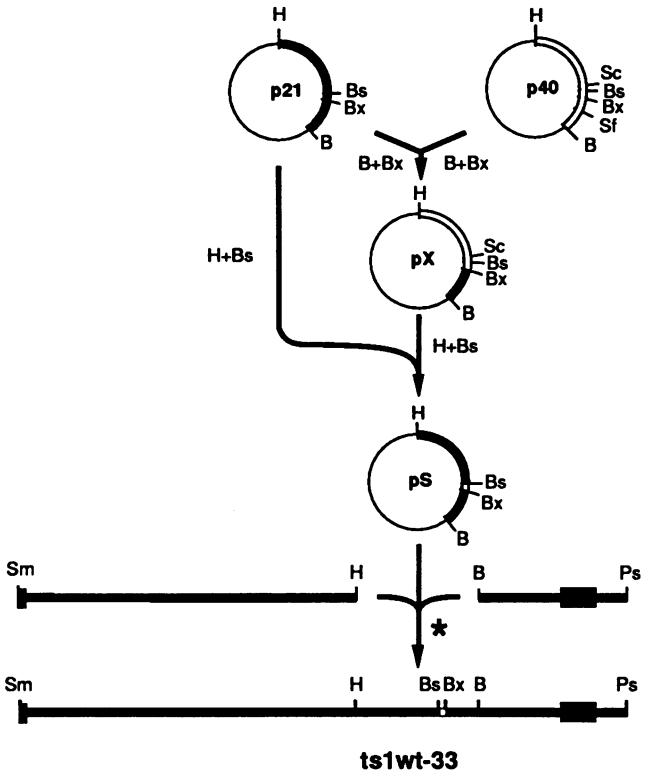

FIG. 2. Schematic diagram illustrating the construction of hybrid genome *ts*1wt-33. Plasmids p21 and p40 are pUC9 plasmids that contain the *Hin*dIII-*Bam*HI fragment (1,643 bp, nts 4895 to 6537) of *ts*1 (black) and MoMuLV-TB (white), respectively (46). Plasmid p40 contains two marker restriction sites, *Sca*I (nts 5870 to 5875) and *Sfi*I (nts 6237 to 6249), that are not present in p21. The *Bst*XI-*Bam*HI fragment (498 bp, nts 6040 to 6537) of p40 was replaced by the *Bst*XI-*Bam*HI fragment of p21 to yield plasmid pX. Plasmid pX contains the *Sca*I marker but not the *Sfi*I marker. The *Hin*dIII-*Bst*EII fragment (1,029 bp, nts 4895 to 5923) of pX was replaced by the *Hin*dIII-*Bst*EII fragment of p21 to yield plasmid pS. Plasmid pS does not contain the *Sca*I marker or the *Sfi*I marker. By a procedure described in detail previously (46), the *Hin*dIII-*Bam*HI fragment of pS was ligated to both the *Bam*HI-*Pst*I fragment (2,294 bp, nts 6538 to 8264 and 1 to 567) of plasmid p22 (46) and the *Sma*I-*Hin*dIII fragment (4,864 bp, nts 31 to 4894) of plasmid p17 (46) to yield hybrid *ts*1wt-33 (*). Restriction site abbreviations: B, *Bam*HI; Bs, *Bst*EII; Bx, *Bst*XI; H, *Hin*dIII; Ps, *Pst*I; Sc, *Sca*I; Sf, *Sfi*I, and Sm, *Sma*I.

an *Apa*I site (nts 6992 to 6997), and the *Hpa*I-*Cla*I fragment contains a *Hae*III site (nts 7204 to 7207). Since the *Bst*EII-*Bst*XI fragment is small (116 bp) and does not contain a restriction site polymorphism, exchanges involving the *Bst*EII-*Bst*XI fragment were accomplished by an indirect procedure as shown for the construction of hybrid *ts*1wt-33 in Fig. 2. Instead of exchanging the small *Bst*EII-*Bst*XI fragment of plasmid p40 with the corresponding fragment in plasmid p21 in one step, two larger fragments that contained restriction site polymorphisms (*Bst*XI-*Bam*HI [498 bp, nts 6040 to 6537] and *Hin*dIII-*Bst*EII [1,029 bp, nts 4895 to 5923]) were exchanged between p21 and p40 in two steps to yield plasmid pS. The final steps in the construction of *ts*1wt-33 from plasmid pS are shown in Fig. 2. After the hybrid genomes were constructed, they were transfected into NIH 3T3 cells to obtain infectious hybrid viruses.

Identification of the amino acid substitution in gPr80^{env} that is responsible for the temperature sensitivity of *ts*1. Previous studies showed that either the Ile-25 substitution or the Ala-157 substitution in gPr80^{env}, encoded by the *Xba*I-*Bam*HI region of *env*, was responsible for the temperature sensitivity of *ts*1 (38, 46, 49). In order to determine which of the two amino acid substitutions (Ile-25 or Ala-157) was responsible for temperature sensitivity, the panel of viruses in Fig. 1 was assayed for temperature sensitivity. A virus was considered to be temperature sensitive if the ratio of foci produced in 15F cells at the permissive temperature (34.0°C) to foci produced at the restrictive temperature (39.0°C) was greater than 100. In hybrid *ts*1wt-26, the Ala-157 in gPr80^{env} of *ts*1 was replaced by the wild-type Ser of MoMuLV-TB by exchanging the *Bst*XI-*Bam*HI restriction fragment (498 bp, nts 6040 to 6537) of *ts*1 with the corresponding *Bst*XI-*Bam*HI restriction fragment of MoMuLV-TB. Hybrid *ts*1wt-26 remained temperature sensitive even though the Ala-157 substitution in the gPr80^{env} of *ts*1 had been replaced by the wild-type Ser of MoMuLV-TB. This result indicated that the Ser-157→Ala substitution in gPr80^{env} was not responsible for the temperature sensitivity of *ts*1. In hybrid *ts*1wt-33, the Ile-25 in the gPr80^{env} of *ts*1 was replaced by the wild-type Val of MoMuLV-TB by exchanging the *Bst*EII-*Bst*XI restriction fragment (116 bp, nts 5924 to 6039) of *ts*1 with the corresponding *Bst*EII-*Bst*XI restriction fragment of MoMuLV-TB. However, hybrid *ts*1wt-33 was not temperature sensitive, and this result indicated that the Val-25→Ile substitution in gPr80^{env} was responsible for the temperature sensitivity of *ts*1. The result for hybrid *ts*1wt-32, a reciprocal of *ts*1wt-33, demonstrated that MoMuLV-TB could be converted into a temperature-sensitive virus by replacing the Val-25 in the gPr80^{env} of MoMuLV-TB with Ile. Furthermore, all of the viruses containing the Val-25→Ile substitution in gPr80^{env}, i.e., *ts*1 and *ts*1wt-23, -26, -31b, -32, -41, and -42, were temperature sensitive, whereas the viruses containing the wild-type Val-25 of MoMuLV-TB in gPr80^{env}, i.e., MoMuLV-TB and *ts*1wt-30 and -33, were not temperature sensitive. From these results, it can be concluded that the Val-25→Ile substitution in gPr80^{env}, not the Ser-157→Ala substitution in gPr80^{env} encoded by the *Xba*I-*Bam*HI region of *env*, is responsible for the temperature sensitivity of *ts*1.

Identification of the amino acid substitution that is responsible for inefficient processing of gPr80^{env} at the restrictive temperature. Previous studies showed that either the Ile-25 substitution or the Ala-157 substitution in gPr80^{env}, encoded by the *Xba*I-*Bam*HI region of *env*, was responsible for the inefficient processing of gPr80^{env} at the restrictive temperature (38, 44, 46, 49). In this study, it has been shown that a single amino acid substitution, Val-25→Ile in gPr80^{env}, is responsible for the temperature sensitivity of *ts*1. In order to determine whether the Val-25→Ile substitution in gPr80^{env} is also responsible for the inefficient processing of gPr80^{env} at the restrictive temperature, only the critical hybrid viruses in Fig. 1, i.e., *ts*1wt-26, -32, -33, and -42, were assayed for processing of gPr80^{env} at both the permissive (34.0°C [data not shown]) and the restrictive (39.0°C) temperatures. In these assays, infected NIH 3T3 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 [36, 38]). The pulse period was followed by a chase period of 0, 90, or 300 min. After the chase period, radiolabeled proteins were immunoprecipitated with anti-gp70 antisera, and they were separated by SDS-PAGE (Fig. 3 [data shown for only *ts*1wt-32 and -33 at the restrictive temperature]).

With a 0-min chase in both *ts*1wt-32- and -33-infected cells, most of the radiolabel in the immunoprecipitates was

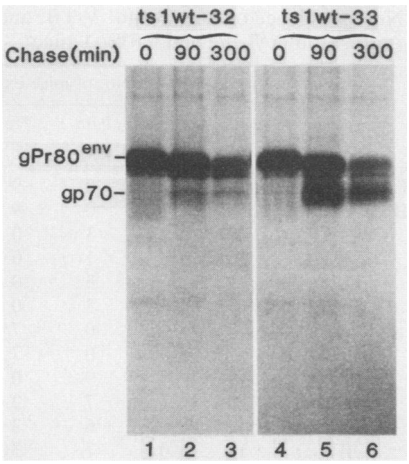

FIG. 3. Pulse-chase analysis of gPr80^{env} processing by *ts*1wt-32 and -33 at the restrictive temperature. NIH 3T3 cells were infected with *ts*1wt-32 and -33 and incubated at 39.0°C. The infected cells were pulse-radiolabeled for 10 min with both L-[^{35}S]cysteine and L-[^{35}S]methionine and chased for 0, 90, or 300 min. The radiolabeled cells were lysed, and samples of the lysate containing 2×10^5 cpm were treated with anti-gp70 antisera. Immunoprecipitated proteins were separated by SDS-PAGE on a 9.0% polyacrylamide gel.

incorporated into gPr80^{env}, and radiolabeled gp70 was not detected (Fig. 3, lanes 1 and 4). With a 90-min chase in the *ts*1wt-33-infected cells, processing of radiolabeled gPr80^{env} into the cleavage products of gp70 and Prp15E was evident (lane 5), and processing continued throughout the 300-min chase period (lane 6). At 300 min, very little radiolabeled gPr80^{env} remained in the *ts*1wt-33-infected cells. The radiolabeled gPr80^{env} had been processed into the cleavage products of gp70 and Prp15E, and these cleavage products were then transported to the plasma membrane, incorporated into virions, and exported from the cell. However, in the *ts*1wt-32-infected cells, the processing of radiolabeled gPr80^{env} was very inefficient at 90 and 300 min (lanes 2 and 3). Only a faint gp70 band was present at both 90 and 300 min (Fig. 3, lanes 2 and 3) instead of the intense, prominent gp70 band that was present for *ts*1wt-33 (lanes 5 and 6). In *ts*1wt-32-infected cells there was some decrease in intensity of the gPr80^{env} band at 300 min (lane 3), and this may be due to protein degradation. In studies of a Rauscher MuLV temperature-sensitive mutant (*ts*26) which is also defective in processing the envelope precursor polyprotein at the restrictive temperature, the nonprocessed envelope precursor polyprotein has been shown to be degraded (32).

Hybrid *ts*1wt-33, which consists of a *ts*1 genome except for a single nucleotide change which resulted in the replacement of Ile-25 by Val in gpPr80^{env}, processed gPr80^{env} into gp70 and Prp15E at the restrictive temperature (Fig. 3, lanes 5 and 6), whereas the reciprocal hybrid, *ts*1wt-32, inefficiently processed gPr80^{env} at the restrictive temperature (lanes 2 and 3). These results indicate that the Val-25→Ile substitution in gPr80^{env} is responsible for the inefficient processing of gPr80^{env} at the restrictive temperature. The results of the assays for both hybrids *ts*1wt-26 and -42 confirm the results for hybrids *ts*1wt-32 and -33. In hybrid *ts*1wt-26, the Ala-157 in the gPr80^{env} of *ts*1was replaced by the wild-type Ser of MoMuLV-TB by exchanging the *Bst*XI-*Bam*HI restriction fragment (498 bp, nts 6040 to 6537) of *ts* 1with the corresponding fragment of MoMuLV-TB. Hybrid *ts*1wt-26 inefficiently processed gPr80^{env} at the restrictive temperature even though the Ala-157 substitution in gPr80^{env} had been replaced by the wild-type Ser of MoMuLV-TB. This result indicated that the Ser-157→Ala substitution in gPr80^{env} was not responsible for the inefficient processing of gPr80^{env} at the restrictive temperature. On the basis of these gPr80^{env} processing results and the temperature sensitivity results, it can be concluded that the Val-25→Ile substitution in gPr80^{env}, not the Ser-157→Ala substitution in gPr80^{env}, is responsible for both the temperature sensitivity and the inefficiency in processing gPr80^{env} at the restrictive temperature of *ts*1.

Intracellular location of the nonprocessed gPr80^{env} at the restrictive temperature. The MoMuLV gPr80^{env} precursor polyprotein is vectorially transported into the lumen of the ER during synthesis (37). In the ER, gPr80^{env} is cotranslationally modified with endo H-sensitive mannose-rich asparagine-linked oligosaccharides (27, 37, 40), and monomers of gPr80^{env} are assembled into oligomers (25). The oligomers are transported to the *cis* cisternae of the Golgi apparatus, where subunits of gPr80^{env} in the oligomers are rapidly cleaved by a host cell-encoded protease to yield subunits consisting of heterodimers of gp70 and Prp15E (9). The processed oligomers are then transported to the medial cisternae of the Golgi apparatus, where the endo H-sensitive mannose-rich asparagine-linked oligosaccharides are converted into endo H-resistant complex-type asparagine-linked oligosaccharides (37, 40). In subsequent steps, the endo H-resistant oligomers, consisting of subunits of gp70-Prp15E heterodimers, are transported to the plasma membrane (37). However, there are cases in which the nonprocessed envelope polyprotein of some dual-tropic MuLVs (8) and of a mutant Rauscher MuLV with a small deletion in the *env* gene (21) are transported to the cell surface. Since *ts*1 inefficiently processes gPr80^{env} at the restrictive temperature, it was of interest to determine the intracellular location of the nonprocessed gPr80^{env}.

NIH 3T3 cells were infected with *ts*1wt-32 and -33 at the restrictive temperature (39.0°C), pulse-radiolabeled with both L-[^{35}S]cysteine and L-[^{35}S]methionine for 10 min, and chased for 0, 90, or 300 min. After the chase period, the radiolabeled cells were lysed, and gp70 and gPr80^{env} were immunoprecipitated with anti-gp70 antisera. The radiolabeled proteins, treated or not treated with endo H, were separated by SDS-PAGE (Fig. 4). Endo H hydrolyzes mannose-rich asparagine-linked oligosaccharides from the polypeptide backbone of glycoproteins to yield apoproteins with increased electrophoretic mobilities (39). In all chase periods for both *ts*1wt-32 and -33, the glycosylated gPr80^{env} (Fig. 4, odd-numbered lanes) was hydrolyzed by endo H to an unglycosylated core polypeptide (Fig. 4, even-numbered lanes) with an increased electrophoretic mobility and an M_r of 63,000 (7), and endo H-resistant gPr80^{env} was not detected (Fig. 4, even-numbered lanes). These results indicated that at the restrictive temperature gPr80^{env} is not transported beyond the *cis* cisternae of the Golgi apparatus in both *ts*1wt-32 and -33-infected cells (19). However, the intensity of the gp70 bands in lanes 9 and 11 indicated that the endo H-sensitive gPr80^{env} of *ts*1wt-33 is processed by the host cell-encoded processing protease in the *cis* cisternae of the Golgi apparatus, whereas there is very little processing of the endo H-sensitive gPr80^{env} of *ts*1wt-32 (lanes 3 and 5).

Analysis of the amino acid substitution in gPr80^{env} that is responsible for the neurovirulence of *ts*1. In this report, neurovirulence is defined as the ability of a virus to cause either paraparesis or hindlimb paralysis in mice. Paraparesis is defined as a partial or slight degree of paralysis of the

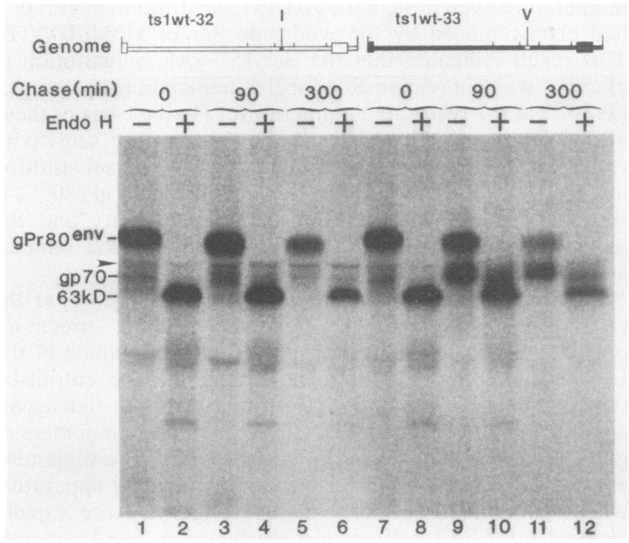

FIG. 4. Endo H sensitivity of gPr80^{env} at the restrictive temperature in *ts*1wt-32 and -33-infected cells. NIH 3T3 cells were infected with *ts*1wt-32 and -33 and incubated at 39.0°C. The infected cells were pulse-radiolabeled for 10 min with both L-[^{35}S]cysteine and L-[^{35}S]methionine and chased for 0, 90, or 300 min. The radiolabeled cells were lysed, and samples of the lysate containing 2×10^5 cpm were treated with anti-gp 70 antisera. Radiolabeled proteins were released from the immunoprecipitates. The released proteins were treated with endo H (+) or untreated (−), and then they were separated by SDS-PAGE on a 9.0% polyacrylamide gel. The arrowhead designates an unidentified protein (lanes 3, 4, 5, and 6) which coprecipitated with gp70 and gPr80^{env}; it has an M_r of 77,000, which is the same as that of the BiP protein (11).

hindlimbs. A useful diagnostic sign of paraparesis is the retraction of the hindlimbs when the mice are suspended by their tails. In addition to retraction and weakness of the hindlimbs, frequent tremors of the hindlimbs are also observed. Hindlimb paralysis is defined as partial or complete loss of function and voluntary movement of the hindlimbs. *ts*1 is a neurovirulent retrovirus, and it causes hindlimb paralysis in mice (22, 41, 50). In previous studies, it was shown by constructing hybrid viruses between *ts*1 and both MoMuLV-TB and MoMuLV that two regions of the *env* gene of *ts*1, i.e., *Xba*I-*Bam*HI (771 bp, nts 5767 to 6537) and *Bam*HI-*Cla*I (1,138 bp, nts 6538 to 7675), are required to cause hindlimb paralysis. It was also found that the *Bam*HI-*Cla*I restriction fragment only enhanced the ability of *ts*1 to replicate in the CNS relative to MoMuLV-TB and that the *Bam*HI-*Cla*I restriction fragment did not code for neurovirulence, as did the *Xba*I-*Bam*HI restriction fragment (38, 49).

The *Xba*I-*Bam*HI restriction fragment of *ts*1 encodes for two amino acid substitutions in gPr80^{env}, i.e., Val-25→Ile and Ser-157→Ala (38). In this study, in order to determine which one of the two amino acid substitutions (Ile-25 or Ala-157) was responsible for the neurovirulence of *ts*1, mice were intraperitoneally inoculated with the panel of viruses shown in Fig. 1. The infected mice were observed for signs of hindlimb paralysis, and the results are shown in Table 1 and Fig. 1. During a postinoculation period of 5 months, mice that were infected with MoMuLV-TB and hybrids *ts*1wt-30 and -33 showed no signs of hindlimb paralysis (−), mice that were infected with hybrids *ts*1wt-31b and -32 had paraparesis (+), and mice that were infected with *ts*1 and hybrids *ts*1wt-23, -26, -41, and -42 had hindlimb paralysis (++). Only viruses which contained the Val-25→Ile substitution in gPr80^{env} were neurovirulent (+ or ++) (*ts*1 and hybrids *ts*1wt-23, -26, -31b, -32, -41, and -42), whereas the viruses which contained the Val-25 in gPr80^{env} were not neurovirulent (−) (MoMuLV-TB and hybrids *ts*1wt-30 and -33). These results indicate that the Val-25→Ile substitution in gPr80^{env} is responsible for the neurovirulence of *ts*1, and this is the same amino acid substitution that is responsible for the temperature sensitivity and inefficient processing of gPr80^{env} at the restrictive temperature of *ts*1.

TABLE 1. Neurovirulence of *ts*1, MoMuLV-TB, and the hybrid viruses in BALB/c and CFW/D mice[a]

Virus	Mouse strain	No. of mice inoculated	No. of mice exhibiting			
			Paralysis	Paraparesis	No signs	Neurovirulence[b]
MoMuLV-TB	BALB/c	9	0	0	9	−
MoMuLV-TB	CFW/D	9	0	0	9	−
*ts*1	BALB/c	9	6	3	0	++
*ts*1	CFW/D	10	9	1	0	++
*ts*1wt-23	BALB/c	9	6	3	0	++
*ts*1wt-26	BALB/c	9	6	3	0	++
*ts*1wt-30	BALB/c	7	0	0	7	−
*ts*1wt-30	CFW/D	7	0	0	7	−
*ts*1wt-31b	BALB/c	9	0	9	0	+
*ts*1wt-31b	CFW/D	10	1	7	2	+
*ts*1wt-32	BALB/c	9	0	6	3	+
*ts*1wt-32	CFW/D	10	0	7	3	+
*ts*1wt-33	BALB/c	9	0	0	9	−
*ts*1wt-41	BALB/c	7	5	2	0	++
*ts*1wt-41	CFW/D	12	7	5	0	++
*ts*1wt-42	BALB/c	9	6	3	0	++
*ts*1wt-42	CFW/D	11	7	4	0	++

[a] Within 48 h after birth, mice were inoculated intraperitoneally with 10^5 to 10^6 infectious units of virus in 0.1 ml of growth medium and observed for signs of hindlimb paralysis for 5 months. The CFW/D mice were treated as a blind study.

[b] Degree of neurovirulence: ++, hindlimb paralysis; +, paraparesis (a slight degree of paralysis which affects the hindlimbs); −, no signs of paraparesis or hindlimb paralysis.

Analysis of the amino acid substitutions encoded by the *env* gene of *ts*1 that are responsible for hindlimb paralysis. There were two classes of neurovirulent viruses in Fig. 1 and Table 1. The viruses in one class (++) caused hindlimb paralysis in mice, and they were more neurovirulent than the viruses in the other class (+), which caused paraparesis. Only viruses which contained both the Val-25→Ile substitution in gPr80^{env} and the *Bam*HI-*Hpa*I restriction fragment (660 bp, nts 6538 to 7197) of *ts*1 caused hindlimb paralysis (*ts*1 and hybrids *ts*1wt-23, -26, -41, and -42), whereas viruses which contained both the Val-25→Ile substitution in gPr80^{env} and the *Bam*HI-*Hpa*I restriction fragment of MoMuLV-TB caused paraparesis (hybrids *ts*1wt-31b and -32). This result confirms our earlier finding that only one region of the *env* gene of *ts*1, i.e., *Xba*I-*Bam*HI, is required for neurovirulence, whereas two regions of the *env* gene of *ts*1 are required for hindlimb paralysis, i.e., *Xba*I-*Bam*HI and *Bam*HI-*Cla*I (38, 46, 49).

In this study, the size of the latter region has been reduced from a 1,138-bp *Bam*HI-*Cla*I restriction fragment to a 660-bp *Bam*HI-*Hpa*I restriction fragment. The *Bam*HI-*Cla*I restriction fragment of *ts*1 coded for two amino acid substitutions, i.e., Arg-430→Lys in gp70 and Ile-23→Val in p15E, whereas the *Bam*HI-*Hpa*I restriction fragment coded for only the Arg-430→Lys substitution in gp70. As a result, the Ile-23→Val substitution in p15E was eliminated from having a role in causing hindlimb paralysis. In hybrid *ts*1wt-30, the

*Bam*HI-*Hpa*I restriction fragment of *ts*1was introduced into the genome of MoMuLV-TB, but this hybrid virus was not neurovirulent. However, when both the *Bam*HI-*Hpa*I restriction fragment of *ts*1 and the *Bst*EII-*Bst*XI restriction fragment of *ts*1were introduced into the genome of MoMuLV-TB, the resulting hybrid virus, *ts*1wt-42, caused hindlimb paralysis. Since the *Bst*EII-*Bst*XI restriction fragment coded for the Val-25→Ile substitution in the gPr80^{env} of *ts*1 and the *Bam*HI-*Hpa*I restriction fragment coded for the Arg-430→Lys substitution in gp70, this result suggested that both the Val-25→Ile substitution in the gPr80^{env} of *ts*1 and the Arg-430→Lys substitution in the gp70 of *ts*1 are required for hindlimb paralysis.

However, there is an additional amino acid difference between *ts*1 and MoMuLV-TB in gp70 which must be considered when analyzing these hybrid viruses. In MoMuLV-TB there is an Ala in position 347 of gp70 instead of the Thr which is in both *ts*1 and MoMuLV-TB (Fig. 1). The Thr-347→Ala substitution in the gp70 of MoMuLV-TB probably arose during propagation of MoMuLV-TB after *ts*1was selected from MoMuLV-TB but before the genome of MoMuLV-TB was molecularly cloned (i.e., clone wt-25) (38, 47). At this time it is not known whether the Ala-347 in the gp70 of hybrids *ts*1wt-31b and -32 has an effect on their neurovirulence. Nevertheless, it can be concluded that the Val-25→Ile substitution in gPr80^{env} is responsible for the neurovirulence of *ts*1 and that both the Vzl-25→Ile substitution in gPr80^{env} and the region of gp70 encoded by the *Bam*HI-*Hpa*I restriction fragment are required for hindlimb paralysis.

DISCUSSION

*ts*1 is a mutant retrovirus which causes hindlimb paralysis in susceptible strains of mice (22, 41, 43, 50). In order to pinpoint individual *ts*1 amino acid substitutions encoded by the *env* gene to specific phenotypic characteristics of *ts*1, a new set of hybrid viruses was constructed, and they were assayed for phenotypic characteristics of *ts*1 (Fig. 1). The assays of the new hybrid viruses showed that the Val-25→Ile substitution in gPr80^{env} is responsible for the temperature sensitivity, inefficient processing of gPr80^{env} at the restrictive temperature, and neurovirulence of *ts*1. However, hybrid viruses *ts*1wt-31b and -32 showed that the Val-25→Ile substitution in gPr80^{env} alone was not sufficient to cause hindlimb paralysis, but only paraparesis. An additional amino acid substitution, most likely the Arg-430→Lys substitution in gp70 encoded by the *Bam*HI-*Hpa*I restriction fragment (660 bp, nts 6538 to 7197), in combination with the Val-25→Ile substitution in gPr80^{env} was required for hindlimb paralysis. These results are consistent with our previous observation that two functionally distinct regions in the *env* gene of *ts*1 are required to induce hindlimb paralysis (49).

The results presented in this report for hybrid viruses *ts*1wt-31b and -32 demonstrate that the Val-25→Ile substitution in gPr80^{env} is responsible for the neurovirulence of *ts*1. This finding appears to contradict the results from a previous report (49) that hybrid viruses *ts*1wt-11, -12, and -13, which also contain the Val-25→Ile substitution in gPr80^{env}, were not neurovirulent (although hybrid *ts*1wt-13 did cause a temporary paraparesis in 34% of the infected mice). This seeming contradiction may be due to nucleotide sequence polymorphisms in the *Bam*HI-*Pst*I fragments (2,294 bp, nts 6538 to 8264 and 1 to 567) that were used to construct hybrids *ts*1wt-11, -12, -13, and -32. The *Bam*HI-*Pst*I fragment of hybrid *ts*1wt-32 was derived from MoMuLV-TB, whereas the *Bam*HI-*Pst*I fragments of hybrids *ts*1wt-11, -12, and -13 were derived from MoMuLV. Nucleotide sequence analysis of a 1,872-nt region (nts 6538 to 8264 and 1 to 145, which encodes for the 62% of the *env* gene and the entire long terminal repeat) in the *Bam*HI-*Pst*I fragments of both MoMuLV-TB and MoMuLV has identified 35 differences in nucleotide sequence (38, 48). It has been shown that nucleotide differences in the U3 region of the long terminal repeats of MoMuLV and MoMuLV-TB have resulted in differences in the disease profiles induced by MoMuLV and MoMuLV-TB (48). Possibly the *Bam*HI-*Pst*I fragment of MoMuLV in hybrids *ts*1wt-11, -12, and -13 attenuates the potential neurovirulence of these hybrid viruses, which is determined by the Val-25→Ile substitution in gPr80^{env}.

Although at present the molecular mechanism responsible for the neurovirulence of *ts*1 is not known, it is clear from the studies reported here that it correlates with the temperature sensitivity and the defect in processing gPr80^{env} at the restrictive temperature of *ts*1 (38, 44, 46, 49). In newborn mice, the body temperature is permissive for the replication of *ts*1, and *ts*1 is able to replicate in target cells and spread to the CNS (5, 41, 50). In adult mice, the body temperature is restrictive for the replication of *ts*1, and *ts*1 induces the degeneration of neural cells in the CNS. Possibly the gPr80^{env} processing defect of *ts*1 is responsible for the degeneration of neural cells in the CNS. In order to investigate the mechanism of induced neural cell degeneration by *ts*1, we sought to determine the intracellular location of the nonprocessed gPr80^{env} at the restrictive temperature. The gPr80^{env} of hybrid *ts*1wt-33 was processed at the restrictive temperature, whereas the gPr80^{env} of *ts*1wt-32 was inefficiently processed (Fig. 3). Unlike the gPr80^{env} of *ts*1wt-33, the gPr80^{env} of *ts*1wt-32 is temperature-sensitive, and at the restrictive temperature the gPr80^{env} of *ts*1wt-32 is probably misfolded (12). There are two possible reasons for the inefficient processing of the misfolded gPr80^{env} of *ts*1wt-32. The first possibility is that the misfolded gPr80^{env} is not transported from the ER to the *cis* cisternae of the Golgi apparatus (4, 6, 11, 20, 30), which contains the host cell-encoded processing protease (9), and as a result a pool of nonprocessed gPr80^{env} remains in the ER. The second possibility is that the misfolded gPr80^{env} is efficiently transported to the *cis* cisternae of the Golgi apparatus, but the misfolded polyprotein is a poor substrate for the host cell-encoded processing protease, and as a result a pool of nonprocessed gPr80^{env} remains in the *cis* cisternae of the Golgi apparatus. Experiments have not been done to distinguish between these two possibilities, but most likely the pool of misfolded gPr80^{env} remains in the ER and not in the *cis* cisternae of the Golgi apparatus, because endo H-resistant gPr80^{env} is not detected (8, 19, 21, 30).

If inefficient processing of gPr80^{env} induces the degeneration of neural cells, it must be taken into consideration that there are different molecular forms of gPr80^{env} in the infected cells, and possibly a threshold steady-state level of a specific molecular form of gPr80^{env} induces the degeneration of neural cells in the CNS. At the permissive temperature, there are two major molecular forms of gPr80^{env} in the ER. There are both newly synthesized monomers of endo H-sensitive gPr80^{env} and oligomers, consisting of subunits of endo H-sensitive gPr80^{env}, which were assembled from the monomers. The steady-state pool of gPr80^{env} monomers is maintained by the influx of newly synthesized monomers into the pool of gPr80^{env} monomers and the efflux of monomers from the pool of gPr80^{env} monomers to form subunits

of oligomers. The steady-state pool of oligomers is maintained by the influx of the oligomer subunits into the pool of oligomers and the efflux of oligomers from the ER to the *cis* cisternae of the Golgi apparatus, where processing of the gPr80^{env} subunits occurs. As a result, at the permissive temperature there is a constant ratio between steady-state levels of endo H-sensitive gPr80^{env} in monomers and endo H-sensitive gPr80^{env} in subunits of oligomers in the ERs of infected cells.

However, Fig. 4 shows that in cells that were infected at the restrictive temperature by hybrid *ts*1wt-32 (which contains the Val-25→Ile substitution in gPr80^{env}), a steady-state level of endo H-sensitive gPr80^{env} is maintained in the ER, probably as a result of inefficient transport of gPr80^{env} to the *cis* cisternae of the Golgi apparatus. Since the gPr80^{env} of *ts*1 is a temperature-sensitive protein, at the restrictive temperature the protein is probably misfolded (12). In studies of the vesicular stomatitis virus G protein (6, 20) and the influenza hemagglutinin protein (4, 11), it has been shown that proper folding of the monomer forms of both the G protein and the hemagglutinin protein in the ER is required for oligomerization and transport from the ER to the Golgi apparatus. If this finding also applies to *ts*1, then at the restrictive temperature the steady-state level of misfolded gPr80^{env} in monomers in the ER is probably much higher than that of gPr80^{env} in subunits of oligomers in the ER. Possibly a high steady-state level of misfolded monomers of gPr80^{env} in the ER induces degeneration of neural cells, and this is the reason for the degenerative effect of *ts*1 on neural cells in the CNS.

Another aspect of the replication of *ts*1 is its enhanced ability to replicate in the CNS relative to MoMuLV-TB (41); previously we have shown that the *Bam*HI-*Cla*I restriction fragment (1,138 bp, nts 6538 to 7675) in the *env* gene of *ts*1 is responsible for this ability (38, 49). In this report, it was shown (Table 1 and Fig. 1) that the Arg-430→Lys substitution in gp70 which is encoded by the *Bam*HI-*Hpa*I restriction fragment (660 bp, nts 6538 to 7197) was most likely responsible for this property of *ts*1. The Arg-430→Lys substitution is located in the carboxy-terminal portion of gp70 (36, 38). The putative ecotropic receptor binding region for MuLVs has been localized in the carboxy-terminal portion of gp70 (16, 26). Possibly, the Arg-430→Lys substitution in gp70 alters the affinity of the gp70 surface protein of *ts*1 for the ecotropic receptor protein (1), thereby increasing the infectivity of *ts*1 in neural cells of the CNS. Alternatively, the alteration in gp70 caused by the Arg-430→Lys substitution may enable *ts*1 to bind to a receptor molecule on neural cells that is different from the ecotropic receptor protein. It has been proposed that the neurovirulent wild mouse ecotropic MuLVs WM1504-E and Cas-Br-E infect neural cells by using a receptor protein that is not the same as the ecotropic receptor protein (23, 24). The ecotropic WM1504-E MuLV has a greater in vitro binding affinity for neural cells of the spinal cord than the lymphomagenic amphotropic WM1504-A virus (23). Furthermore, it has also been reported that human immunodeficiency virus type 1 is able to enter neural cells other than through the CD4 receptor, which is the receptor for human immunodeficiency virus type 1 in lymphoid cells (15).

It is not clear why *ts*1 is neurovirulent whereas other ecotropic MuLVs with phenotypic properties similar to those of *ts*1 are not neurovirulent. A temperature-sensitive mutant of Rauscher MuLV has been isolated (*ts*26), and like *ts*1 it is inefficient in processing gPr80^{env} at the restrictive temperature (32), yet *ts*26 does not cause hindlimb paralysis in mice (14). The mere replication of MuLVs in the CNS is not sufficient for neurovirulence either. MoMuLV-TB replicates in neural cells of the CNS to some extent and causes slight histological lesions (41), but it is not neurovirulent (50). Furthermore, it has been reported that mice which express large amounts of MoMuLV in the CNS do not develop neurological disease (35). An understanding of the molecular mechanism of the neurovirulence of *ts*1 may be important for studying the growing list of human neurological diseases which have been implicated as being caused by retroviruses, which include human immunodeficiency virus type 1-associated neurological diseases (17), multiple sclerosis (29), and tropical spastic paraparesis (33).

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