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# 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

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**To better understand the molecular mechanism involved in retrovirus *ts*1-induced paralytic disease in mice, we constructed a panel of recombinant viruses between *ts*1 and the wild-type viruses Moloney murine leukemia virus (MoMuLV) and MoMuLV-TB, a strain of MoMuLV. These recombinant viruses were constructed in an attempt to identify the sequence(s) in the genome of *ts*1 which contains the critical mutation(s) responsible for the neurovirulence of *ts*1. Two functionally distinct sequences in the genome of *ts*1 were found to be responsible for its paralytogenic ability. One of these sequences, the 0.77-kilobase-pair *Xba*I-*Bam*HI (nucleotides 5765 to 6537) fragment which encodes the 5′ half of gp70 and 11 base pairs upstream of the *env* gene coding sequence, determines the inability of *ts*1 to process Pr80$^{env}$. The other sequence, the 2.30-kilobase-pair *Bam*HI-*Pst*I (nucleotides 538 to 8264 and 1 to 567) fragment, which comprises nearly two-thirds of the *env* gene, the long terminal repeat, and the 5′ noncoding sequence, determines the enhanced neurotropism of *ts*1. Replacement of any one of these two regions with the homologous region from either one of the two wild-type viruses resulted in recombinant viruses which either totally failed to induce paralysis or induced a greatly attenuated form of paresis in some of the infected mice.**

A group of temperature-sensitive (*ts*) mutants, *ts*1, *ts*7, and *ts*11, of a strain of Moloney murine leukemia virus (MoMuLV), MoMuLV-TB, induce hindlimb paralysis in 100% of susceptible strains of inbred mice inoculated shortly after birth (17). The latency period is about 21 to 42 days depending on the mutant used. The disease progresses rapidly and is invariably fatal. The characteristics of the pathology are vacuolated neurons and spongiform degeneration of the central nervous system (CNS) with absence of inflammation (15). The paralytogenic mutants also share a common defect in their inability at the restrictive temperature to process the *env* precursor protein, Pr80$^{env}$ (17).

To identify the mutation(s) in the genomes of the paralytogenic *ts* mutants which confers on them the inability to process Pr80$^{env}$ efficiently and the ability to cause hindlimb paralysis and to determine whether these two properties are due to mutation in one or more genes, we have constructed chimeric genomes of *ts*1, the prototype of the paralytogenic mutants, and MoMuLV or MoMuLV-TB (20). From these hybrid constructs, we have identified a 3.9-kilobase-pair (kbp) *Hin*dIII-*Pst*I (nucleotides [nt] 4895 to 8264 and 1 to 567) sequence of *ts*1 comprising the 3′ end of *pol* and all of *env*, the long terminal repeat (LTR), and the 5′ noncoding sequence to be responsible for the *ts* function, inefficiency in processing Pr80$^{env}$, and induction of paralysis (21). We have further extended these findings by demonstrating that the 1.6-kbp *Hin*dIII-*Bam*HI (nt 4895 to 6537) sequence within the 3.9-kbp fragment controls temperature sensitivity and processing of Pr80$^{env}$ but is not sufficient for induction of paralysis (20).

We have also established that *ts*1 replicates relatively better than wild-type (wt) virus in the CNS of infected mice (15). It was unclear whether this property results from an additional mutation within the *Hin*dIII-*Bam*HI fragment or a mutation in the *Bam*HI-*Pst*I fragment of the genome. Since the *Hin*dIII-*Bam*HI fragment consists of the 3′ end of *pol* and the 5′ end of gp70, to further delimit the sequence within this fragment of the *ts*1 genome which carries the mutation(s) that hindered efficient processing of Pr80$^{env}$, chimeric genomes were constructed of *ts*1 and MoMuLV-TB or MoMuLV with exchanges of subgenomic fragments within the *Hin*dIII-*Bam*HI fragment. The infectious recombinant virus obtained on transfection of each chimeric genome construct was tested in the appropriate assay system to determine its phenotype. The 0.77-kbp *Xba*I-*Bam*HI (nt 5765 to 6537) fragment, which contains the 5′ half of the gp70 domain together with 11 base pairs upstream of the *env* gene coding sequence, was found to be responsible for the inability of *ts*1 to process Pr80$^{env}$. To determine the DNA sequence responsible for the enhanced neurotropism, the replication efficiencies of MoMuLV-TB and *ts*1 in the spinal cords of mice sacrificed at different times postinoculation (p.i.) were compared with those of the recombinant viruses derived from different constructs. The 2.30-kbp *Bam*HI-*Pst*I (nt 6538 to 8264 and 1 to 567) fragment, which comprises nearly two-thirds of the *env* gene, the entire LTR, and the 5′ noncoding sequence, was found to be responsible for the enhanced neurotropism of *ts*1. The above investigations indicated that the neurovirulent determinants of *ts*1 virus reside in at least two functionally separate regions of the *ts*1 genome. The following is a report on these studies.

## MATERIALS AND METHODS

**Cells.** Viruses were propagated in mouse TB cells, a thymus bone marrow cell line derived from CFW/D mice (1), and assayed on 15F cells, a murine-sarcoma-positive, leukemia-negative ($S^+L^-$) cell line (22). NIH 3T3 cells were used for transfection experiments. All cell lines were maintained in Dulbecco modified Eagle minimal essential medium (DMEM) supplemented with 8% fetal calf serum.

**Viruses.** The strain of Moloney murine leukemia virus

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(MoMuLV-TB) used in this study was isolated as described by Wong et al. (16) from the tissue extract of a sarcoma produced in an MoMuLV-infected BALB/c mouse provided by J. B. Moloney (7). Since its isolation, it has been propagated in TB cells. MoMuLV-TB has been single virus, single cell cloned on several occasions Clone LV30 was molecularly cloned (21). It has been shown by Shields and coworkers (10) and confirmed by us (unpublished data) that MoMuLV-TB can be distinguished from the standard MoMuLV by the electrophoretic mobility of the p30 protein. In this report, the two strains of MoMuLV will be referred to as MoMuLV and MoMuLV-TB.

*ts*1 is a spontaneous *ts* mutant of MoMuLV-TB isolated as described by Wong and co-workers (16).

**Virus assay.** The 15F assay has been described previously (14, 18).

**Virus DNAs.** The permuted genomes of *ts*1 and MoMuLV-TB were molecularly cloned into Charon 21A as described by Yuen et al. (21). The infectious recombinant viral DNAs *ts*1-19, *ts*1-20, and wt-25, each with one copy of the LTR, were used in the studies reported here. Plasmid 8.2, an infectious permuted MoMuLV genome with one copy of the LTR cloned into pBR322 at the *Hin*dIII site (11), is a gift from D. Baltimore. The viral DNA of p8.2 is referred to as wt-8.2.

**Isolation, purification, and analysis of viral DNAs.** Restriction fragments were separated by agarose gel electrophoresis. The desired fragments were electroeluted into dialysis bags. The DNA was filtered through glass fiber to remove agarose debris and then concentrated by ethanol precipitation. The DNA pellet was suspended in TE (10 mM Tris, 1 mM EDTA, pH 8.0) and then extracted with phenol-chloroform, chloroform, and ether.

Restriction enzymes were purchased from Bethesda Research Laboratories, Gaithersburg, Md., and the conditions for endonuclease digestion were those recommended by the supplier. Gel transfer and filter hybridization were carried out by the method of Southern (12).

**DNA transfection.** The calcium phosphate precipitation method of Graham and van der Eb (5) was used. Details of the procedures were as described by Yuen et al. (21).

**Recombinant plasmid and chimeric genome construction.** Restriction fragments were cloned into pUC9 (13), pUC18 (19), pKC7 (8), or pBR322. Transformations into *Escherichia coli* HB101 were carried out by standard procedures. Construction of the recombinant plasmids and chimeric genomes used was as described in the text.

**Viral infectivity assay of inoculated mice.** CFW/D mice less than 24 h old were inoculated intraperitoneally with 0.1 ml of virus suspension containing $10^6$ to $10^7$ infectious units per ml. Mice were sacrificed at 20, 25, and 30 days of age, and spleen as well as spinal cord extracts were prepared by removing the organs and homogenizing them in DMEM with a Potter-Elvehjem tissue grinder. The resulting suspensions were then centrifuged at 950 × *g*, the supernatants were decanted and filtered through 0.45-μm (pore size) sterile membranes (Millipore Corp., Bedford, Mass.), and appropriate dilutions were made in DMEM and assayed in 15F cells for infectious virus.

**Metabolic labeling of cells, immunoprecipitation, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of viral proteins.** Immunoprecipitation of intracellular virus-specific proteins and sodium dodecyl sulfate-polyacrylamide gel electrophoresis were performed as described previously (17, 18). Gels were fluorographed and exposed to X-ray film at −70°C.

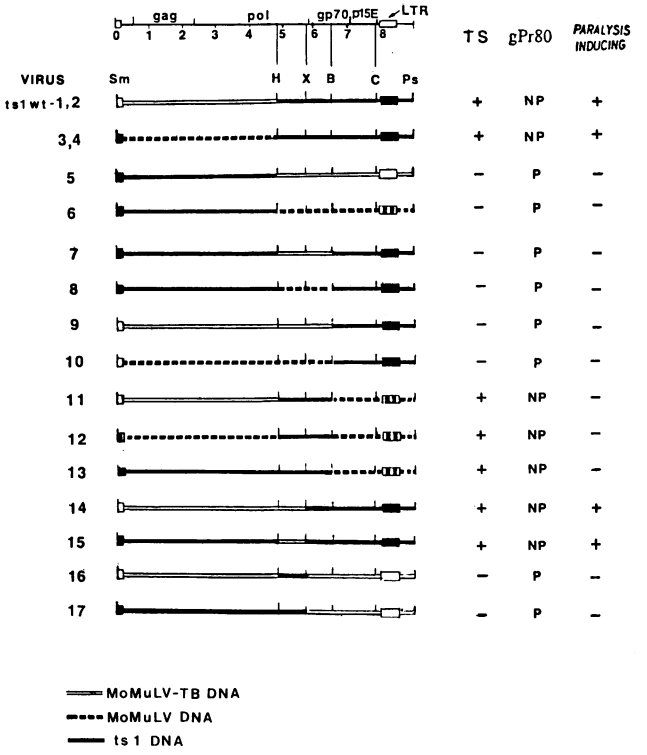


FIG. 1. Schematic representation of the genomes of hybrid viruses *ts*1wt-1 to *ts*1wt-17 and summary of their phenotypic characteristics. The data for *ts*1wt-1 to *ts*1wt-13 were previously published (21). B = *Bam*HI; C = *Cla*I; H = *Hin*dIII; Sm = *Sma*I; Ps = *Pst*I; X = *Xba*I; P = $Pr80^{env}$ processed to gp70 and p15E; NP = $Pr80^{env}$ not processed.

## RESULTS

**Hybrid viruses *ts*1wt-11, *ts*1wt-12, and *ts*1wt-13.** In a previous study (20), recombinant viruses (*ts*1wt-1 to *ts*1wt-13) were successfully used in localizing the region of the *ts*1 genome which confers temperature sensitivity and inability to process $Pr80^{env}$ efficiently to the 1.6-kbp *Hin*dIII-*Bam*HI (nt 4895 to 6537) fragment. It was also shown that the *Hin*dIII-*Bam*HI along with the *Bam*HI-*Pst*I subgenomic fragments carry genetic determinants essential for the paralytogenicity of *ts*1 (Fig. 1). In the same study, it was reported that 4 of 22 mice injected with *ts*1wt-13 began to show early signs of paresis 39 to 95 days p.i. However, on further observation, symptoms of paresis in all of these mice regressed approximately 10 days later, and none of them developed total hindlimb paralysis as observed in *ts*1-injected mice. In the present study, an additional 14 mice were inoculated with *ts*1wt-13; at the same time, 14 mice were inoculated with *ts*1wt-11 to serve as controls. Consistent with the above finding, 8 of the 14 mice injected with *ts*1wt-13 also showed early signs of paresis 35 to 95 days p.i. The paresis regressed, and the mice survived for more than 6 months without further symptoms of CNS disease. In contrast, none of the 14 mice injected with *ts*1wt-11 showed any sign of paresis (Table 1). The reason for the highly attenuated form of paresis in some of the mice injected with *ts*1wt-13 is not known at present. However, since of the three recombinant viruses, *ts*1wt-11, *ts*1wt-12, and *ts*1wt-13 (Fig. 1), only the 5′ half of the *ts*1wt-13 genome is derived from *ts*1 in addition to the *Hin*dIII-*Bam*HI fragment that all

TABLE 1. Characterization of viruses produced by transfection of chimeric genomes in NIH 3T3 cells

| Virus | Titer ratio (34/39°C)[a] | Pr80$^{env}$ processing[b] | No. of mice injected | % of mice paralyzed[c] |
|---|---|---|---|---|
| wt-25[d] | 2.4 | P | >50 | 0 |
| *ts*1-19[d] | 100–1,000 | NP | >50 | 100 |
| *ts*1wt-7 | 2.1 | P | 12 | 0 |
| *ts*1wt-8 | 2.5 | P | 65 | 0 |
| *ts*1wt-9 | 1.8 | P | 38 | 0 |
| *ts*1wt-10 | 1.6 | P | 26 | 0 |
| *ts*1wt-11 | 100–1,000 | NP | 34 | 0 |
| *ts*1wt-12 | 100–1,000 | NP | 10 | 0 |
| *ts*1wt-13 | 100–1,000 | NP | 36 | 0[e] |
| *ts*1wt-14 | 100–1,000 | NP | 12 | 100[f] |
| *ts*1wt-15 | 100–1,000 | NP | 13 | 100 |
| *ts*1wt-16 | 4.0 | P | 5 | 0 |
| *ts*1wt-17 | 5.5 | P | 19 | 0 |

[a] Appropriately diluted virus was inoculated into two sets of plates seeded with 15F cells and allowed to adsorb for 40 min at 34°C. One set of plates was incubated at 34°C and the other at 39°C. The assay was read 4 to 5 days p.i.

[b] Infected cells grown at 39°C were pulsed for 30 min with [$^3$H]leucine and chased for 3 h. Cell extracts were immunoprecipitated with antiserum to MoMuLV gp70, analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, fluorographed, and exposed to Kodak X-ray film. P = Pr80$^{env}$ processed to gp70 and p15E; NP = Pr80$^{env}$ not processed.

[c] CFW/D or BALB/c mice within 48 h after birth were inoculated intraperitoneally with $10^5$ to $10^6$ infectious units of virus in 0.1 ml of growth medium. Mice were observed for up to 6 months p.i.

[d] Parental viruses.

[e] About 34% of these mice showed early signs of paresis, i.e., tremors and minor hindlimb ataxia, at 35 to 95 days p.i. However, these symptoms regressed by approximately 10 days after their onset without reaching total paralysis as seen in *ts*1-inoculated mice. These mice survived for at least 6 months p.i. without showing symptoms of neural involvement.

[f] All 12 mice inoculated with *ts*1wt-14 developed hindlimb paralysis similar to that induced by *ts*1, although the latency period was longer (30 to 100 days p.i). However, 2 of the 12 mice showed marked improvement about 50 days after the onset of paralytic symptoms.

three recombinant viruses have in common, it is possible that the 5′ half of the *ts*1 genome which includes part of the LTR may have in part contributed to induction of the highly attenuated form of paresis in some of the mice injected with *ts*1wt-13.

**Construction of hybrid viruses *ts*1wt-14, *ts*1wt-15, *ts*1wt-16, and *ts*1wt-17.** The 1.6-kbp *Hin*dIII-*Bam*HI fragment of *ts*1, which consists of the 3′ end of the *pol* gene as well as the 5′ half of gp70, was shown to confer on *ts*1 its temperature sensitivity and inefficiency in processing Pr80$^{env}$ (20). To further delimit the DNA sequence within this subgenomic fragment which is responsible for the above phenotypic expressions, recombinant plasmids isolated in a previous study (20) were used to construct an additional set of hybrid genomes *ts*1wt-14, *ts*1wt-15, *ts*1wt-16, and *ts*1wt-17.

Endonuclease restriction mapping showed that both the *ts*1 and MoMuLV-TB genomes differ from that of MoMuLV in the loss of the *Xba*I site at nt 5325 but retain the *Xba*I site at nt 5765, which is 11 base pairs upstream from the coding sequence of the *env* gene (21). This *Xba*I site is also located midway between the *Hin*dIII and *Bam*HI sites of the 1.6-kbp fragment. Hybrid genomes of *ts*1 and MoMuLV-TB were constructed by exchange of homologous *Hin*dIII-*Xba*I fragments between the two genomes.

To construct a viral genome that contains the *ts*1 subgenomic fragment from *Hin*dIII-*Xba*I and the MoMuLV-TB fragment from *Xba*I-*Pst*I (Fig. 2), we began with p1, a pUC9 recombinant plasmid containing the *ts*1 *Hin*dIII-*Pst*I fragment, and p14, a pUC9 recombinant plasmid containing the MoMuLV-TB *Hin*dIII-*Pst*I fragment (20). Since the *Xba*I site found in the LTR of MoMuLV-TB is missing in *ts*1, the intact *Xba*I-*Pst*I (nt 5765 to 8264 and 1 to 567) fragment could be isolated from p1. The *Xba*I-*Xba*I sequence was deleted from p14 to produce p42, which was further treated to remove the shorter *Xba*I-*Pst*I fragment to obtain the viral *Hin*dIII-*Xba*I MoMuLV-TB fragment still attached to pUC9. Ligation of the *ts*1 *Xba*I-*Pst*I fragment from p1 to the *Hin*dIII-*Xba*I fragment of MoMuLV-TB to produce p47 restored the viral *Hin*dIII-*Pst*I fragment. To obtain the complete genome, this newly constructed chimeric *Hin*dIII-*Pst*I fragment was isolated from p47 and ligated to both p17 and p18, recombinant pKC7 plasmids containing the *Sma*I-*Hin*dIII fragment from nt 31 to 4895 of *ts*1 and MoMuLV-TB, respectively (20), to produce constructs 14 and 15. The recombinant viruses obtained upon transfection in NIH 3T3 cells were designated *ts*1wt-14 and *ts*1wt-15.

The reciprocal hybrid was constructed with p28, a pUC9 recombinant plasmid containing the *Hin*dIII-*Bam*HI fragment of *ts*1 and the *Bam*HI-*Pst*I fragment of MoMuLV-TB, and p14 (Fig. 3). The *Xba*I-*Xba*I viral fragment was isolated from p14 and inserted into the *Xba*I site of p41, the recombinant plasmid obtained by deleting the *Xba*I-*Xba*I fragment from p28, to generate the recombinant plasmid p45. p45 contains the chimeric *Hin*dIII-*Pst*I fragment that is *ts*1 from

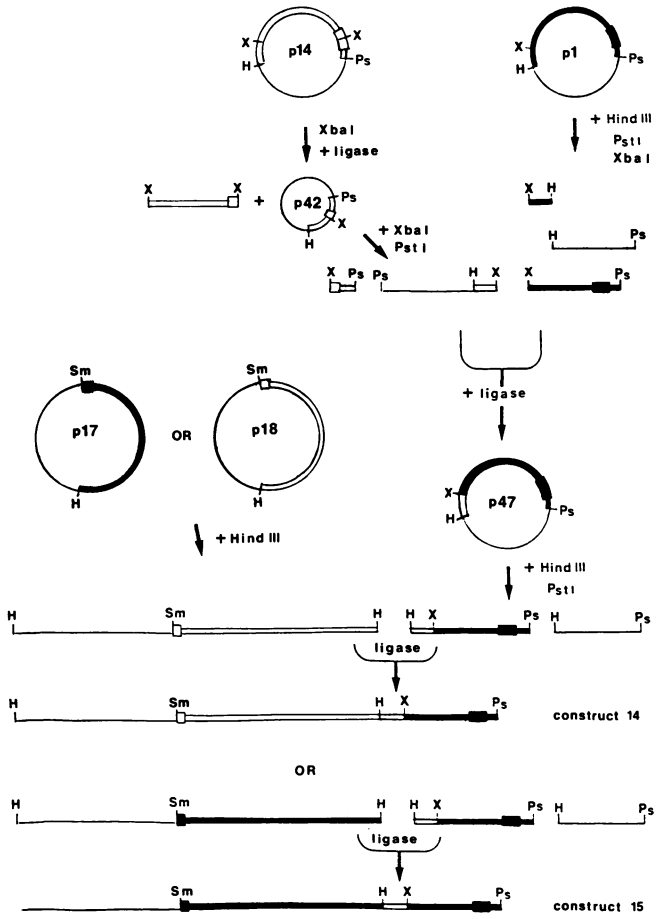


FIG. 2. Schematic representation of the construction of a chimeric *Hin*dIII-*Pst*I viral sequence consisting of an MoMuLV-TB *Hin*dIII-*Xba*I fragment and a *ts*1 *Xba*I-*Pst*I fragment and construction of the chimeric genome constructs 14 and 15. Details are described in the text. =, pMuLV-TB DNA; ■, *ts*1 DNA; —, plasmid DNA. Abbreviations are as defined in the legend to Fig. 1.

*Hin*dIII to *Xba*I and MoMuLV-TB from *Xba*I to *Pst*I. Complete genomes were restored by ligating the *Hin*dIII-*Pst*I viral fragment from p45 to both p17 and p18 at the *Hin*dIII site to produce constructs 16 and 17. The recombinant viruses obtained on transfection were designated *ts*1wt-16 and *ts*1wt-17.

**Characterization of the infectious recombinant viruses *ts*1wt-14, *ts*1wt-15, *ts*1wt-16, and *ts*1wt-17.** The infectious viruses *ts*1wt-14, *ts*1wt-15, *ts*1wt-16, and *ts*1wt-17 were tested with respect to temperature sensitivity (Table 1), efficiency in processing $Pr65^{gag}$ (Fig. 4, odd-numbered lanes) and $Pr80^{env}$ (Fig. 4, even-numbered lanes) at the nonpermissive temperature, and ability to induce hindlimb paralysis (Table 1). *ts*1wt-14 and *ts*1wt-15 were temperature sensitive. The intracellular viral $Pr80^{env}$ protein of *ts*1wt-14- or *ts*1wt-15-infected cells (Fig. 4, lanes 8 and 10, respectively) was not processed to gp70 and p15E, whereas $Pr65^{gag}$ was processed to p30, p15, p12, and p10. Since the viral proteins were immunoprecipitated with anti-p30, the other *gag* gene products, p15, p12, and p10, were not precipitated (lanes 7 and 9). *ts*1wt-14 and *ts*1wt-15 also retained their ability to induce paralysis in mice. In contrast, *ts*1wt-16 and *ts*1wt-17 were

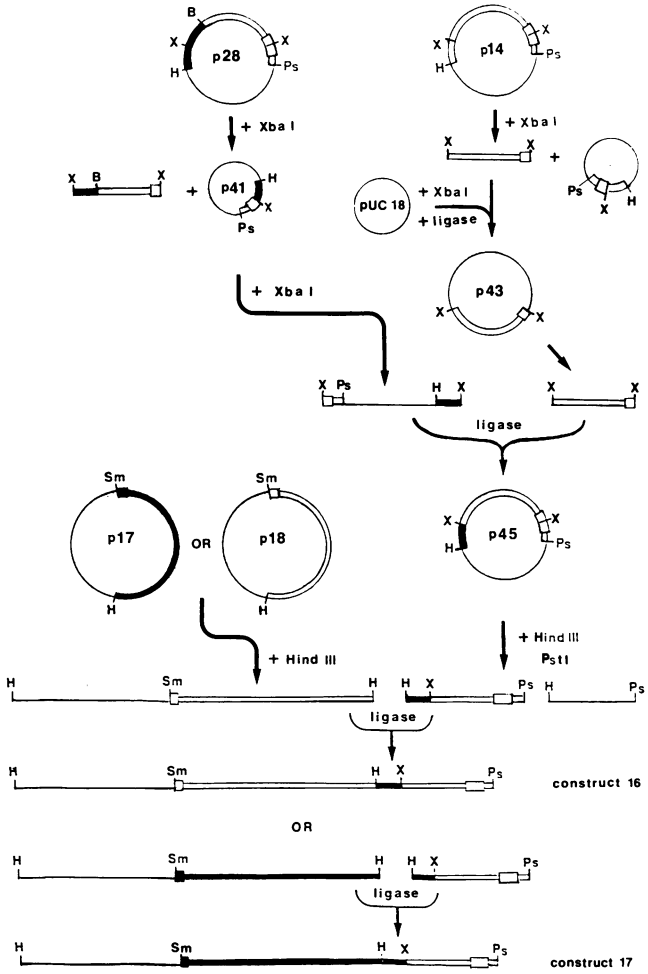


FIG. 3. Schematic representation of the construction of a chimeric *Hin*dIII-*Pst*I viral sequence consisting of a *ts*1 *Hin*dIII-*Xba*I fragment and an MoMuLV-TB *Xba*I-*Pst*I fragment and construction of the chimeric genome constructs 16 and 17. Details are described in the text. =, pMuLV-TB DNA; ■, *ts*1 DNA; —, plasmid DNA. Abbreviations are as defined in the legend to Fig. 1.

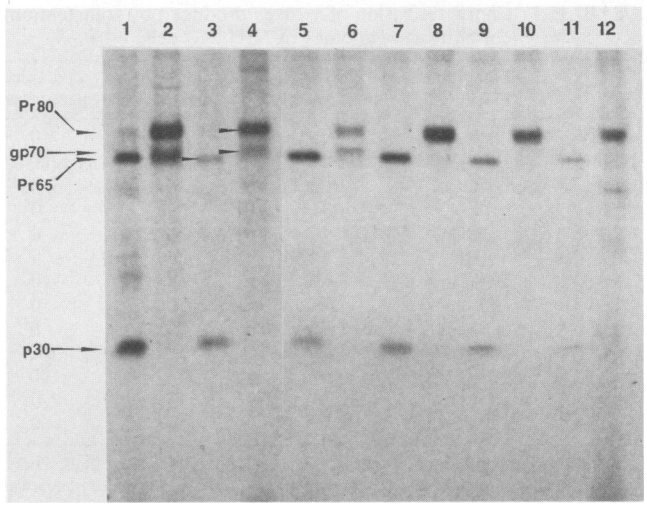


FIG. 4. Metabolic labeling and immunoprecipitation with anti-p30 and anti-gp70 of intracellular viral proteins of cells infected with MoMuLV-TB (lanes 1 and 2), *ts*1wt-16 (lanes 3 and 4), *ts*1wt-17 (lanes 5 and 6), *ts*1wt-14 (lanes 7 and 8), *ts*1wt-15 (lanes 9 and 10), or *ts*1 (lanes 11 and 12). Odd-numbered lanes = viral proteins immunoprecipitated with anti-p30. Even-numbered lanes = viral proteins immunoprecipitated with anti-gp70.

not temperature sensitive and were able to process both $Pr65^{gag}$ (Fig. 4, lanes 3 and 5) and $Pr80^{env}$ (Fig. 4, lanes 4 and 6) intracellularly at the nonpermissive temperature. They also failed to induce paralysis in mice. We have previously shown, using constructs 7 to 13 (Fig. 1), that a mutation(s) in the 1.6-kbp *Hin*dIII-*Bam*HI sequence from nt 4895 to 6537 of *ts*1 is responsible for its temperature sensitivity and inability to process $Pr80^{env}$ efficiently (21). Taken together, these results indicate that the 0.77-kbp *Xba*I-*Bam*HI *ts*1 fragment from nt 5765 to 6537, which lies within the 5′ half of the gp70 domain, contains the mutation(s) which not only prevents processing of $Pr80^{env}$ but also causes the temperature sensitivity of *ts*1. These results further show that mutations within the *Xba*I-*Pst*I fragment from nt 5765 to 8264 and 1 to 567 determine the paralytogenic phenotype of *ts*1. This finding is consistent with previous observations (20) that substitution of the *Hin*dIII-*Bam*HI fragment from nt 4895 to 6537 of wt virus with the homologous *ts*1 fragment failed to generate virus capable of inducing paralysis and that substitution of the *Bam*HI-*Pst*I fragment from nt 6537 to 8264 and 1 to 567 of the wt genome with the homologous *ts*1 fragment does not produce virus capable of inducing paralysis. Thus, from the two sets of constructs, 7 to 13 and 14 to 17, we conclude that although a mutation(s) in the *Xba*I-*Bam*HI fragment of *ts*1 prevents the processing of $Pr80^{env}$, causes temperature sensitivity, and plays a role in induction of paralysis, some other factor encoded by the *Bam*HI-*Pst*I fragment is also necessary for induction of paralysis. To identify the other factor(s) encoded by the *Bam*HI-*Pst*I fragment which plays a role in induction of paralysis, the following experiments were conducted.

**Identification of the region in the *ts*1 genome responsible for its ability to invade and replicate in the CNS.** We have previously shown that *ts*1, when compared with the wt, has an enhanced ability to infect and replicate in the CNS (15). We therefore attempted to determine the region in the *ts*1 genome which is responsible for the observed enhanced neurotropism. Since the *Xba*I-*Bam*HI subgenomic fragment, which is responsible for inefficient processing of $Pr80^{env}$,

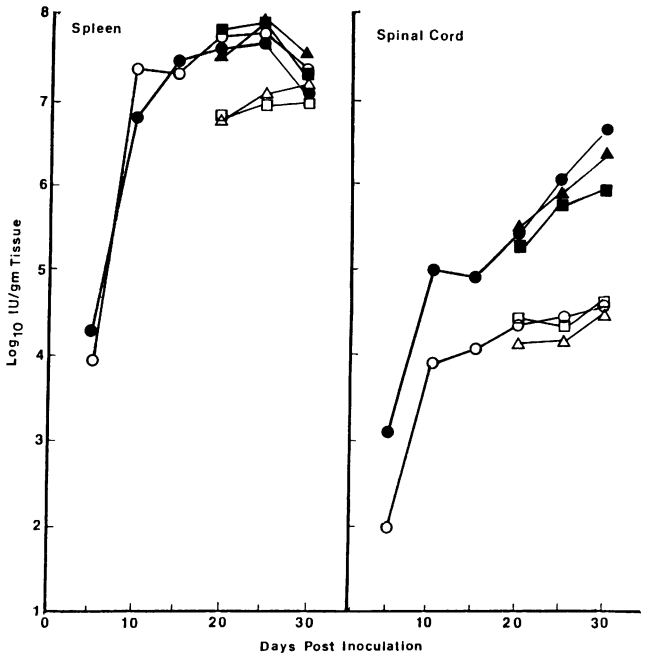


FIG. 5. Virus titers in spleens and spinal cords of MoMuLV-TB-, *ts*1-, *ts*1wt-9-, *ts*1wt-10-, *ts*1wt-11-, or *ts*1wt-13-infected BALB/c mice. Newborn mice were inoculated intraperitoneally with 0.1 ml of virus suspension containing $10^5$ to $10^6$ infectious units per ml. Virus-infected mice were sacrificed at 20, 25, or 30 days of age, and spleen and spinal cord extracts were assayed for viral infectivity. MoMuLV-TB and *ts*1 controls were from previously published results (15), which also included virus titers from 5, 10, and 15 days of age. Each point represents the average titer obtained from 3 mice. Symbols: ●, *ts*1 titer; ○, MoMuLV-TB titer; ▲, *ts*1wt-9 titer; ■, *ts*1wt-10 titer; △, *ts*1wt-11 titer; □, *ts*1wt-13 titer.

alone is not enough for induction of paralysis (although it is necessary), it is likely that the *Bam*HI-*Pst*I fragment, which is also required for induction of paralysis, may confer on *ts*1 enhanced neurotropism. If this is the case, genomes of the recombinant viruses *ts*1wt-11, *ts*1wt-12, and *ts*1wt-13, in which the *Bam*HI-*Pst*I sequence is derived from the wt, should behave like wt MoMuLV in its relative inability to replicate in the CNS, whereas genomes of the recombinant viruses *ts*1wt-7, *ts*1wt-8, *ts*1wt-9, and *ts*1wt-10, in which the *Bam*HI-*Pst*I sequence is derived from *ts*1, should retain the enhanced ability of *ts*1 to replicate in the CNS. To test this hypothesis, newborn CFW/D or BALB/c mice were injected with representatives from both groups of recombinant viruses, *ts*1wt-9 and *ts*1wt-10 representing the latter and *ts*1wt-11 and *ts*1wt-13 representing the former group of recombinant viruses. Mice were sacrificed at 20, 25, and 30 days postinjection, and spleen and spinal cord extracts were assayed for infectious virus as described previously (15). Data on MoMuLV-TB and *ts*1 controls were from previously published results (15).

Virus titers in the spleens of *ts*1-, wt-, *ts*1wt-9-, or *ts*1wt-10-inoculated mice were at similar levels between 20 and 30 days p.i. (Fig. 5). Although the virus titers in the spleens of *ts*1wt-11- and *ts*1wt-13-inoculated mice were about fivefold lower than the titers of wt- or *ts*1-injected mice at 20 and 25 days p.i., by 30 days p.i. the virus titers were almost similar to those of *ts*1- or wt-injected mice. In contrast, virus titers from spinal cords were significantly higher in *ts*1-, *ts*1wt-9-, or *ts*1wt-10-inoculated mice than in mice inoculated with wt, *ts*1wt-11, or *ts*1wt-13. By day 30 p.i., virus titers in the spinal cords of *ts*1-, *ts*1wt-9-, or *ts*1wt-10-inoculated mice were about 50- to 100-fold higher than those found in the spinal cords of wt-, *ts*1wt-11-, or *ts*1wt-13-inoculated mice.

Our results indicate that the recombinant viruses *ts*1wt-9 and *ts*1wt-10, like *ts*1, retained their enhanced ability to replicate in the CNS, whereas the recombinant viruses *ts*1wt-11 and *ts*1wt-13, like wt MoMuLV-TB, replicated less well in the CNS.

## DISCUSSION

Our previous studies (17) demonstrated that the paralytogenic mutants of MoMuLV-TB are defective in processing the *env* precursor protein ($Pr80^{env}$). The nonparalytogenicity of another *ts* mutant, *ts*3, and the parental wt virus, which process $Pr80^{env}$ normally, suggests that an alteration(s) of the viral genome of the paralytogenic mutants may be the cause of the paralytic phenotype and that accumulation of $Pr80^{env}$ may play a role in induction of paralysis.

We had further shown (20) that a mutation(s) in the *Hin*dIII-*Pst*I fragment of the *ts*1 genome, which comprises the 5′ end of *pol*, the *env* gene, the LTR, and the 5′ noncoding sequence, was responsible for the temperature sensitivity, inefficiency in processing $Pr80^{env}$, and paralytogenicity. Construction of additional chimeric genomes with exchanges of homologous subgenomic fragments within the *Hin*dIII-*Pst*I fragment between *ts*1 and wt (20) indicated that, whereas replacement of the 1.6-kbp *Hin*dIII-*Bam*HI fragment of the wt genome with the homologous fragment from the *ts*1 genome was sufficient to prevent processing of $Pr80^{env}$ and induce temperature sensitivity, it was not enough to convert an otherwise wt virus into one capable of inducing paralysis. However, in one case, *ts*1wt-13 (Table 1), about 34% of the mice injected showed early clinical signs of paresis, i.e., tremors and minor hindlimb ataxia, at 35 to 95 days p.i. These symptoms, however, regressed by approximately 10 days after the onset of the symptoms without totally paralyzing the hindlimbs. No other disease symptoms indicating neuronal involvement were observed thereafter. The reason for this highly attenuated form of paresis induced by *ts*1wt-13 in some of the injected mice is not known at present. Since of the three recombinant viruses *ts*1wt-11, *ts*1wt-12, and *ts*1wt-13 (Fig. 1) only the 5′ half of the *ts*1wt-13 genome is derived from *ts*1 in addition to the *Hin*dIII-*Bam*HI fragment that all three recombinant viruses have in common, it is possible that the 5′ half of the *ts*1 genome, which includes part of the LTR, may in part contribute to induction of this highly attenuated form of paresis. Using chimeric genome constructs 14 to 17 together with previous findings on the recombinant viruses derived from constructs 7 to 13, the present study showed that a mutation(s) in the *Xba*I-*Bam*HI portion of the *ts*1 genome prevents the processing of $Pr80^{env}$ intracellularly and induces the temperature sensitivity of *ts*1.

The above observation may provide some insight into the nature of the proteolytic cleavage involved in the processing of $Pr80^{env}$ in MuLV. It has been proposed (6) that the primary translational product of the *env* gene is synthesized with a leader peptide (LP) (codons 1 through 33 of the *env* gene), which is then cleaved at the Thr-Ala peptide bond in the rough endoplasmic reticulum accompanying glycosylation of the *env* precursor. The *env* precursor is subsequently cleaved in the rough endoplasmic reticulum to the glycoprotein gp70 and Pr15E (i.e., p15E-p2E). This cleavage is

probably accomplished by cellular trypsinlike enzymes acting on the Arg-Glu peptide bond between the glycoprotein and the N-terminal residue of p15E. Since the cleavage of the LP precedes the cleavage of $Pr80^{env}$ into gp70 and p15E, one may postulate that, if the cleavage of the LP is blocked, it may prevent processing of $Pr80^{env}$. Our present study implicated a mutation(s) in the *Xba*I-*Bam*HI portion of the *env* gene as responsible for the inability of *ts*1 to process $Pr80^{env}$. The *Xba*I-*Bam*HI fragment includes the sequence which codes for the LP. Two hypotheses may be made. (i) A change in the 5′ end of the *env* gene product alters the configuration of the polypeptide, rendering it not recognizable by the cleavage enzyme. (ii) A mutation in the C-terminal end of the LP or the N-terminal end of $Pr80^{env}$ may prevent cleavage of the LP from the *env* gene precursor product, therefore blocking cleavage of $Pr80^{env}$ to gp70 and p15E. We are currently sequencing the *ts*1 and parental wt MoMuLV-TB subgenomic fragments in this region to determine whether such a mutation has indeed occurred.

Since inefficient processing of $Pr80^{env}$ alone does not result in paralysis, the crucial question is whether the paralytogenic mutants have other mutations, besides their inability to process its *env* precursor protein, which contribute to paralytogenesis. Since the CNS is affected and the *Bam*HI-*Pst*I fragment of *ts*1 is necessary for induction of paralysis, it seems likely that a mutation(s) in the *Bam*HI-*Pst*I fragment confers on *ts*1 the ability to directly invade and replicate in nerve cells.

The present study (Fig. 5) demonstrated that the recombinant viruses *ts*1wt-9 and *ts*1wt-10, whose genomes consist of the *ts*1 sequence from the *Bam*HI-*Pst*I site, retain their enhanced ability to replicate in the CNS. In contrast, the reciprocal recombinant viruses *ts*1wt-11 and *ts*1wt-13, whose genomes consist of the wt sequence from the *Bam*HI-*Pst*I site, do not possess enhanced ability to replicate in the CNS. Neurotropism, therefore, appears to be a unique property of the paralytogenic mutant, and the genetic determinant(s) for this phenotype is located in the *Bam*HI-*Pst*I fragment, which comprises the 3′ half of gp70, p15E, the LTR, and the 5′ noncoding sequence. The enhanced neurotropism of *ts*1 may be determined by a mutation(s) in the *env* gene, the LTR, or both. Alterations to the *env* gene may increase the affinity of the envelope glycoprotein of the paralytogenic virus for the surface receptor of neurons or it may induce a special interaction with the cell membrane so that the virus is more rapidly internalized into the nerve cell. A comparison of the oligonucleotide maps of a neurotropic and a nonneurotropic MuLV isolated from wild mice suggests that difference of a single oligonucleotide, which probably maps in the *env* region of the genome, may be responsible for the difference in pathogenicity (9). It is also possible that the ability of *ts*1 to infect neurons is attributable to a mutation(s) in the LTR, which enhances its expression in nerve cells. That the LTR determines the thymotropism of the standard MoMuLV (4) and the erythroleukemogenicity of Friend MuLV has been previously demonstrated (2, 3). Additional hybrid genomes of *ts*1 and wt are being constructed to determine whether both the *env* and LTR are involved in the enhanced neurotropism of *ts*1.

Histopathologic studies have shown the progressive development of lesions in the CNS which parallels the progression of clinical symptoms (J. F. Zachary, C. Knupp, and P. K. Y. Wong, Am. J. Pathol., in press). In particular, vacuolar degeneration of the neurons of the brain stem and spinal cord associated with motor function have been demonstrated. Ultrastructurally, dilatation of the endoplasmic reticulum and some budding virus particles have been observed in neurons associated with motor function. Budding virus and cytopathic changes associated with viral infection have also been observed in other cell types of the CNS. However, it does not appear that involvement of these cell types plays a critical role in induction of hindlimb paralysis.

Although a substantial amount of $Pr80^{env}$ was detected in the spinal cords of *ts*1-inoculated mice (15), it remains to be seen whether accumulation of $Pr80^{env}$ actually occurs in neurons. Nevertheless, it is tempting to suggest that intraneuronal accumulation of envelope precursor protein is the basis of the cell damage underlying this retroviral neurological disorder. Accumulation of viral protein on the cell membrane could impair the normal semipermeability and transport functions of cells and lead to leakage, degeneration, and the characteristic spongiform changes observed in the CNS. It could also block neuronal muscular transmission, resulting in paresis. Studies to delineate these possibilities are now in progress in our laboratory.

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