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Identification of Point Mutations in the Envelope Gene of Moloney Murine Leukemia Virus TB Temperature-Sensitive Paralytogenic Mutant *ts*1: Molecular Determinants for Neurovirulence

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***ts*1, a temperature-sensitive mutant of Moloney murine leukemia virus TB, induces hind-limb paralysis in mice. The DNA of both the *ts*1 and Moloney murine leukemia virus TB *env* genes has been sequenced, and the encoded amino acid sequences have been deduced from the DNA sequences. Four amino acids in the *ts*1 envelope protein have been identified which may be responsible for the *ts*1 phenotype, which includes temperature sensitivity, nonprocessing of Pr80^{env}, and neurovirulence.**

Murine leukemia viruses (MuLVs) induce neoplasias which are primarily of hematopoietic origin (10) and nonneoplastic disorders, which include deformed whiskers (16), age-related greying of coats (11), murine retrovirus-induced immunodeficiency disease (12), and spongiform encephalomyelopathy (for reviews, see references 4 and 8). The type of neoplasia or nonneoplastic disorder is dependent on the strain of MuLV and the strain of the infected mouse. Paralytogenic strains of MuLV have been isolated from wild mice (4, 5), and they have been experimentally generated in the laboratory from nonparalytogenic MuLVs (1, 6, 17, 21).

*ts*1 is a spontaneous temperature-sensitive mutant of Moloney MuLV TB (MoMuLV-TB) (21). Only susceptible strains of mice that are infected with *ts*1 as neonates succumb to hind-limb paralysis. Newborn mice have a lower body temperature (~34°C) than adult mice (~38.4°C) (3). In neonates, the body temperature is permissive, and *ts*1 is able to replicate in target cells and spread to the central nervous system (20). In young adult mice, the body temperature is nonpermissive, and the *env* precursor polyprotein, Pr80^{env}, is not processed intracellularly. As a result, Pr80^{env} accumulates in the infected cells (20, 22, 23, 25). Histopathological studies indicate that noninflammatory spongiform changes occur in both the grey and white matter of the brain stem and spinal cord (26). Compared with MoMuLV-TB, *ts*1 has an enhanced ability to replicate in the central nervous system (i.e., neurotropism) (20).

Both *ts*1 and MoMuLV-TB have been molecularly cloned (24). *ts*1 clone *ts*1-19 and MoMuLV-TB clone wt-25 were used in this study. Previously, subgenomic restriction fragments were exchanged between *ts*1, MoMuLV-TB, and MoMuLV (23, 25). Restriction maps of MoMuLV and of previously published hybrid constructs *ts*1wt-1, *ts*1wt-9, *ts*1wt-13, and *ts*1wt-14 (23, 25) are shown in Fig. 1. Molecular determinants for nonprocessing of Pr80^{env} and paralysis induction were localized in an *Xba*I-*Pst*I (nucleotides [nts] 5765 to 8264 and 1 to 567) restriction fragment which encodes for the *env* gene polyprotein, the long terminal repeat, and the 5′-noncoding region. The *Xba*I-*Pst*I fragment was subdivided into two domains, an *Xba*I-*Bam*HI (nts 5765 to 6537) restriction fragment and a *Bam*HI-*Pst*I (nts 6538 to 8264 and 1 to 567) restriction fragment (25). The determinant for temperature sensitivity and nonprocessing of Pr80^{env} resided in the *Xba*I-*Bam*HI fragment. The determinant for neurotropism resided in the *Bam*HI-*Pst*I fragment. Both domains were required for paralysis induction.

In this study, we have eliminated the long terminal repeat and the 5′-noncoding region from the latter domain by constructing additional chimeric viruses *ts*1wt-18 and *ts*1wt-19 (Fig. 1). Details for the construction of hybrid virus genomes have been described (23, 25). Hybrid *ts*1wt-18 consists of a MoMuLV-TB genome with a substituted *ts*1 *Hin*dIII-*Cla*I restriction fragment. *ts*1wt-19 is a reciprocal

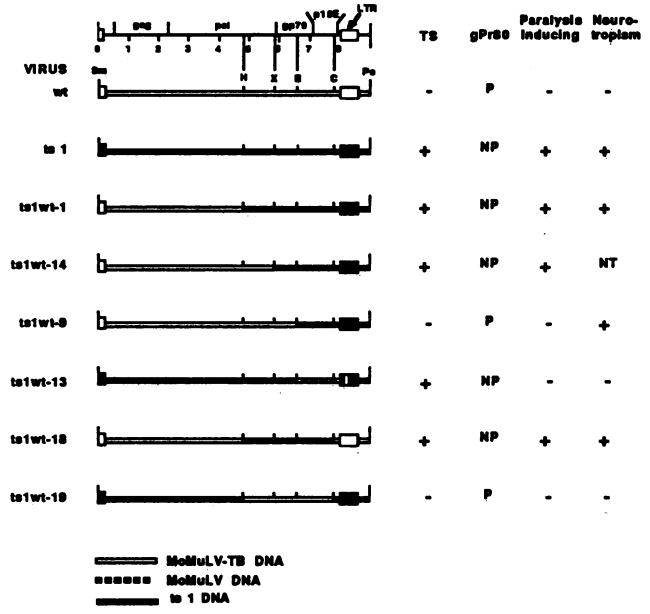

FIG. 1. Schematic representations of the genomes of *ts*1, MoMuLV-TB, and hybrid viruses *ts*1wt-1, *ts*1wt-14, *ts*1wt-9, *ts*1wt-13, *ts*1wt-18, and *ts*1wt-19. Hybrids *ts*1wt-1, *ts*1wt-9, *ts*1wt-13, and *ts*1wt-14 were constructed previously (23, 25). To the right of the genomes is a summary of the respective phenotypic characteristics. Critical restriction sites are indicated in a physical map of the MoMuLV genome (19). Neurotropism is defined as an enhanced ability to replicate in the central nervous system (brain and spinal cord) relative to MoMuLV-TB. Abbreviations: TS, temperature sensitivity; P, Pr80^{env} processed to gp70 and p15E; NP, Pr80^{env} not processed; NT, not tested. Restriction sites: B, *Bam*HI; C, *Cla*I; H, *Hin*dIII; Sm, *Sma*I; Ps, *Pst*I.

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TABLE 1. Comparison of nucleotide and amino acid changes in MoMuLV-TB and *ts*1 relative to MoMuLV in a region spanning the *env* gene from *Xba*I to *Cla*I

Nucleotide position[a]	Codon position[b]	Nucleotide base[c]			Amino acid[d]		
		Mo	Mo-TB	*ts*1	Mo	Mo-TB	*ts*1
5811	1	A	T	—	Asn	Ile	—
5845	3	A	—	G	Leu	—	—
5875	3	T	—	C	Thr	—	—
5948	1	G	—	A	Val	—	**Ile**
5958	2	C	T	T	Thr	Ile	Ile
6064	3	T	—	G	Pro	—	—
6188	1	C	T	—	Leu	—	—
6247	3	C	G	—	Arg	—	—
6292	3	T	—	C	Cys	—	—
6344	1	T	—	G	Ser	—	**Ala**
6367	3	C	T	T	Asn	—	—
6451	3	C	T	—	Ala	—	—
6499	3	T	—	G	Arg	—	—
6603	2	A	G	G	Asp	Gly	Gly
6640	3	G	T	T	Ser	—	—
6667	3	G	A	—	Gly	—	—
6721	3	A	G	G	Leu	—	—
6778	3	A	G	G	Gln	—	—
6796	3	A	—	T	Leu	—	—
6914	1	A	G	—	Thr	Ala	—
6994	3	G	—	A	Gly	—	—
6995	1	T	C	C	Ser	Pro	Pro
7038	2	G	A	A	Ser	Asn	Asn
7164	2	G	—	A	Arg	—	**Lys**
7207	3	C	—	T	Ala	—	—
7250	1	A	—	G	Ile	—	**Val**
7303	3	A	C	C	Gln	His	His
7312	3	A	G	G	Val	—	—
7327	3	G	A	—	Arg	—	—
7387	3	C	T	—	Val	—	—
7573	3	A	—	T	Gly	—	—

[a] Positions are numbered according to Shinnick et al. (19).

[b] The position that the nucleotide occupies in the codon for the encoded amino acid.

[c] Dashes represent bases identical to those in MoMuLV. Abbreviations: Mo, MoMuLV; Mo-TB, MoMuLV-TB.

[d] Dashes represent amino acids identical to those in MoMuLV; boldface amino acids are unique to *ts*1. Abbreviations: Mo, MoMuLV; Mo-TB, MoMuLV-TB.

hybrid genome construct of *ts*1wt-18. The *Hin*dIII-*Cla*I restriction fragment (nts 4895 to 7675) contains the 3′ end of *pol* and most of *env*. NIH 3T3 cells were transfected with the DNA of the hybrid genomes to obtain infectious hybrid viruses. The hybrid viruses were assayed for temperature sensitivity, ability to process the $Pr80^{env}$ precursor polyprotein, and ability to induce paralysis after injection into neonatal mice. The data (Fig. 1) show that the *ts*1wt-18 construct has the same phenotypic properties as does *ts*1. Since *ts*1wt-18 contains the *ts*1 *env* gene but not the *ts*1 long terminal repeat, the neurovirulent determinants in *ts*1 reside entirely within the envelope gene in a restriction fragment bound by *Xba*I and *Cla*I (nts 5765 to 7675).

To pinpoint the mutations in the *ts*1 envelope gene which are responsible for its neurovirulence, the nucleotide sequence of the entire *env* gene was determined for both MoMuLV-TB and *ts*1. The inserts in MoMuLV-TB plasmids p27 and p40 (23) and *ts*1 plasmids p21 and p22 (23) were sequenced by the dideoxy chain termination method (18). There are 1,910 base pairs in the MoMuLV *Xba*I-*Cla*I restriction fragment. The MoMuLV-TB and *ts*1 *env* nucleotide sequences were compared to the standard MoMuLV sequence (19), and there were no deletions or insertions. The nucleotide differences between MoMuLV, MoMuLV-TB, and *ts*1 are listed for the *Xba*I-*Cla*I fragment in Table 1. Relative to MoMuLV, MoMuLV-TB had 18 differences, whereas *ts*1 had 23 differences. There are only 10 nts present in both MoMuLV-TB and *ts*1 which are not present in MoMuLV. *ts*1 inherited these 10 nts from MoMuLV-TB. The remaining eight changes in nucleotide in MoMuLV-TB probably arose during virus propagation after *ts*1 was isolated but before MoMuLV-TB wt-25 was molecularly cloned. Each of the remaining 13 changes in nucleotide in *ts*1 could have arisen during one of the following two time periods: the time period in which *ts*1 was being selected or the time period in which *ts*1 was propagated after selection but before *ts*1-19 was molecularly cloned.

The MoMuLV *Xba*I-*Cla*I fragment encodes for 637 amino acids of the envelope polyprotein. The amino acids encoded by the *env* gene for both *ts*1 and MoMuLV-TB have been deduced from their respective nucleotide sequences (Fig. 2 and Table 1). Together MoMuLV-TB and *ts*1 have 11 amino acid differences relative to MoMuLV. Of these 11 amino acid differences, 5 (Ile, Gly, Pro, Asn, and His) are present in both MoMuLV-TB and *ts*1. Two amino acids, Ile and Ala, are present in MoMuLV-TB but not in *ts*1. In the *ts*1 *Xba*I-*Bam*HI domain, there are two unique amino acids, Ile and Ala. There are also two unique amino acids in the *ts*1 *Bam*HI-*Cla*I domain, Lys and Val. Only one of these unique *ts*1 amino acids, Val, occurs in a known functional domain of the envelope polyprotein (Fig. 2). These results indicate that the unique Ile or Ala at the amino-terminal end of the envelope polyprotein is responsible for *ts*1 temperature sensitivity and nonprocessing of $Pr80^{env}$. The unique Lys at the carboxy end of gp70 or the unique Val at the amino-terminal end of p15E is responsible for *ts*1 neurotropism.

There is significant amino acid sequence similarity between the *ts*1 envelope polyprotein and the envelope polyproteins of three other MuLVs including Cas-Br-E, Akv, and Friend (14). Cas-Br-E is a wild mouse paralytogenic MuLV (14), whereas Akv is a nonparalytogenic MuLV (9). The wild-type strain of Friend MuLV is nonparalytogenic (7), but paralytogenic strains have been isolated by passaging the wild-type strain in rats (6). The amino acid sequences of the envelope polyproteins of MoMuLV, MoMuLV-TB, *ts*1, Cas-Br-E, Akv, and wild-type Friend MuLV were aligned according to Rassart et al. (14). The amino acid sequence similarities of the envelope polyproteins of MoMuLV-TB, *ts*1, Cas-Br-E, Akv, and wild-type Friend MuLV to MoMuLV were calculated to be 99, 99, 75, 83, and 85%, respectively.

TABLE 2. Unique amino acids in the *ts*1 envelope polyprotein[a] compared with those of MoMuLV, Mo-MuLV-TB, Cas-Br-E, Akv, and wild-type Friend MuLV at the same positions

Nucleotide position[b]	Amino acid[c]					
	Mo	Mo-TB	*ts*1	Cas	Akv	Fr
5948	Val	—	**Ile**	—	—	—
6344	Ser	—	**Ala**	Thr	—	—
7164	Arg	—	**Lys**	Pro	—	Lys
7250	Ile	—	**Val**	Val	Val	Val

[a] These amino acids are present in the *ts*1 envelope polyprotein (*Xba*I-*Cla*I) but not in MoMuLV-TB.

[b] The nucleotide position in the *ts*1 *env* gene which alters the MoMuLV-encoded amino acid is numbered according to Shinnick et al. (19). Abbreviations: Mo, MoMuLV; Mo-TB, MoMuLV-TB; Cas, Cas-Br-E.

[c] Dashes represent amino acids identical to those in MoMuLV, and boldface amino acids are unique to *ts*1.

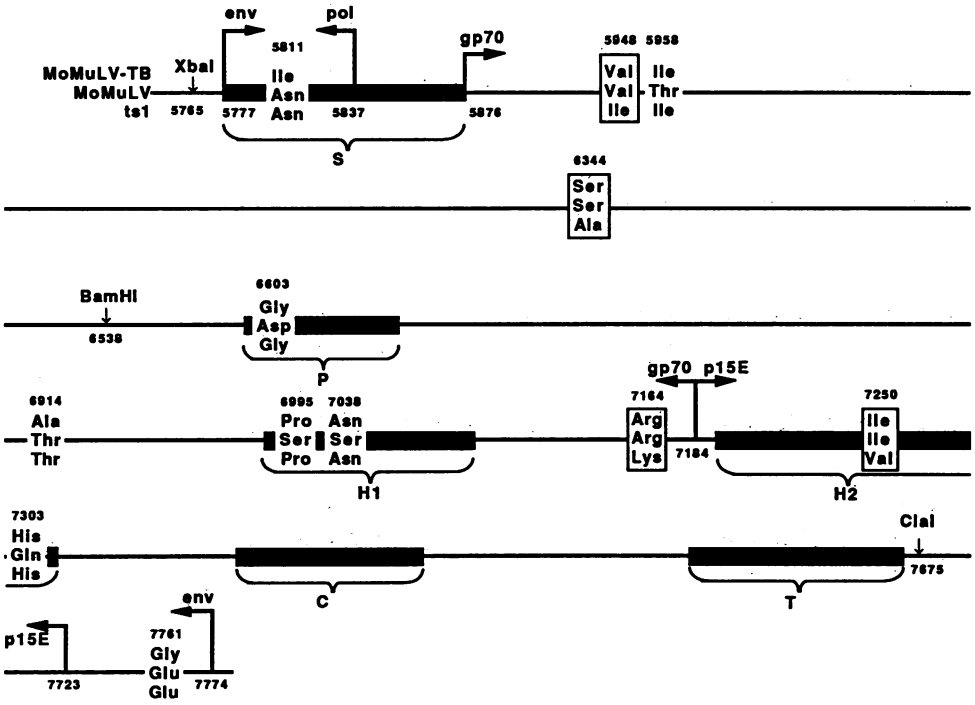

FIG. 2. Comparison of the amino acid sequences of polyproteins encoded by the MoMuLV-TB and *ts*1 *env* genes to that of MoMuLV. The amino acid sequence of the MoMuLV envelope polyprotein was deduced from the nucleotide sequence by Shinnick et al. (19). The MoMuLV envelope polyprotein is represented by a thin solid line, except at positions in which amino acid changes occur in MoMuLV-TB or *ts*1. The amino acid sequences of MoMuLV-TB and *ts*1 were deduced from the respective nucleotide sequences. MoMuLV-TB amino acid changes are written above the line, and *ts*1 changes are written below. The position of the altered nucleotide is written above the amino acid. If the altered amino acid is unique to *ts*1, it is boxed. Domains within the envelope polyprotein are represented as heavy solid lines. Abbreviations: S, signal sequence (nts 5777 to 5876) (19); P, proline-rich domain (nts 6559 to 6697) (7); H1, gp70 hydrophobic domain (nts 6992 to 7075) (9); H2, p15E hydrophobic domain (nts 7187 to 7312) (9); C, highly conserved C-terminal *env* domain in p15E (nts 7388 to 7468) (2); T, p15E transmembrane domain (nts 7583 to 7675) (9, 13).

The unique amino acids encoded by the *ts*1 *env* gene are compared with amino acids of five other MuLVs at the same positions in Table 2. In the *Xba*I-*Bam*HI domain, there are two unique *ts*1 amino acids, Ile and Ala. Val is conserved in all of the MuLVs except *ts*1, which has an Ile. Ser is conserved in four of the MuLVs, but the paralytogenic viruses have amino acid substitutions as follows. *ts*1 has an Ala, and Cas-Br-E has a Thr. In the *Bam*HI-*Cla*I domain, there are also two unique *ts*1 amino acids, Lys and Val. At the Lys position, Friend MuLV also has a Lys, but Cas-Br-E has a Pro, whereas the other three MuLVs have an Arg. The final unique *ts*1 amino acid site resides in the p15E hydrophobic domain (9, 13) (Fig. 2). All MuLV strains have a Val at this site except for the nonparalytogenic MoMuLV strains, which have an Ile.

The significance of amino acid substitutions in the *ts*1 envelope polyprotein in relation to the structure and function of the protein on a molecular level is not known. MuLV-induced spongiform encephalomyelopathy may serve as an animal model for neurological diseases induced by human retroviruses. Tropical spastic paraparesis is a human T-cell lymphotropic virus type I-induced paralytic disease, and like *ts*1, its primary clinical feature is the development of paralysis in the lower legs and body (15). An increased understanding of retroviral neural pathogenesis at the genetic and molecular level, based on an animal model, should provide important new approaches for the prevention and treatment of the diverse human diseases caused by this unique group of viruses.

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