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# Development of pathological lesions in the central nervous system of transgenic mice expressing the *env* gene of *ts*1 Moloney murine leukemia virus in the absence of the viral *gag* and *pol* genes and viral replication

Y Eugene Yu[1], Wonkyu Choe[1], Wenguang Zhang[1], George Stoica[2] and Paul KY Wong[1]

[1]*Science Park-Research Division, The University of Texas M.D. Anderson Cancer Center, Smithville, Texas 78957 and* [2]*Department of Veterinary Pathobiology, Texas A & M University, College Station, Texas 77843, USA*

**The *ts*1 Moloney murine leukemia virus causes a degenerative neurologic disease in mice characterized by the development of noninflammatory spongiform encephalomyelopathy. To determine whether *gag* and *pol* gene products and viral replication are necessary for the *ts*1-*env* gene product to cause neurodegeneration, we generated transgenic mice harboring only *ts*1-*env*. Neuropathological lesions were observed in mice expressing the transgene in the central nervous system. This implies that *gag* and *pol* gene products and viral replication are not necessary for *ts*1-*env* to cause a mild form of neurodegeneration in mice.**

**Keywords:** *ts*1-*env* transgenic mice; neuropathological lesions; *gag* and *pol* genes; viral replication

## Introduction

Like several other C-type murine retroviruses, *ts*1 Moloney murine leukemia virus (MoMuLV) can cause spongiform neurodegeneration when injected into susceptible strains of mice. We have been studying the *ts*1 model to gain insight into the mechanisms of spongiform degeneration induced by retroviruses (Wong 1990; Wong and Yuen 1992, 1994; Gonzales-Scarano *et al*, 1995).

Newborn mice infected with the *ts*1 virus develop mild generalized body tremors that progress to bilateral hind limb paralysis (McCarter *et al*, 1977; Wong *et al*, 1983). FVB/N, the mouse strain most susceptible to *ts*1 virus, develops disease 30–40 days postinfection (Wong *et al*., 1991). The disease syndrome is characterized by the development of noninflammatory spongiform encephalomyelopathy accompanied by neuronal loss, mild gliosis, and demyelination. These degenerative lesions predominately involve the lumbar spinal cord, brain stem, thalamus, and cerebellum (Stoica *et al*, 1993).

As a C-type murine retrovirus, the *ts*1 virus genome consists of three structural genes: *gag*, *pol*, and *env*. The *env* gene of *ts*1 virus has been shown to play an important role in virus-induced neurodegeneration (Yuen *et al*, 1986). To determine whether expression of the *gag* and *pol* genes and viral replication are necessary for *ts*1-*env* to cause neurodegeneration, we removed these two viral genes from the viral genome and generated transgenic mice harboring either one of two transgene constructs: *ts*1-*env*(F) (Yu *et al*, 1991) or *ts*1-*env*(G), in which long terminal repeats (LTRs) of MoMuLV or *ts*1 were used as the regulatory element, respectively (Figure 1). To construct the *ts*1-*env*(G) transgene (originally named pMDA6), the Nhe I-Sst I fragment in the 5′ LTR of p*ts*1-*env*(F) was replaced by a 384 bp Nhe I-Sst I fragment isolated from the 5′ LTR of the *ts*1 viral genome (Yu *et al*, 1991). This was done by multiple subcloning (Sambrook *et al*, 1989). The constructs were tested by transfection into NIH3T3 cells. The resulting expression of *ts*1 envelope proteins was confirmed by radioimmuno-

Correspondence: PKY Wong
Tel: 512-237-2403; fax: 512-237-2444.
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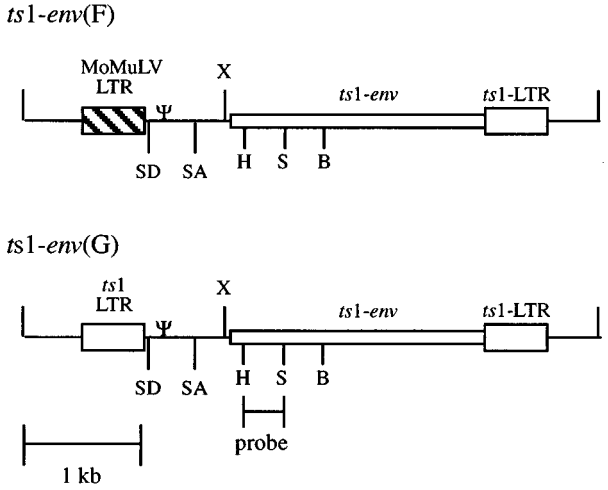


**Figure 1** Structures of the transgenes *ts*1-*env*(F) and *ts*1-*env*(G). SA, splice acceptor site; SD, splice donor site; $\psi$, packaging site; MoMuLV LTR, Moloney murine leukemia virus long terminal repeat; *ts*1-LTR, *ts*1 virus long terminal repeat; B, BamHI site; H, HpaI site; S, SmaI site; X, XbaI site.

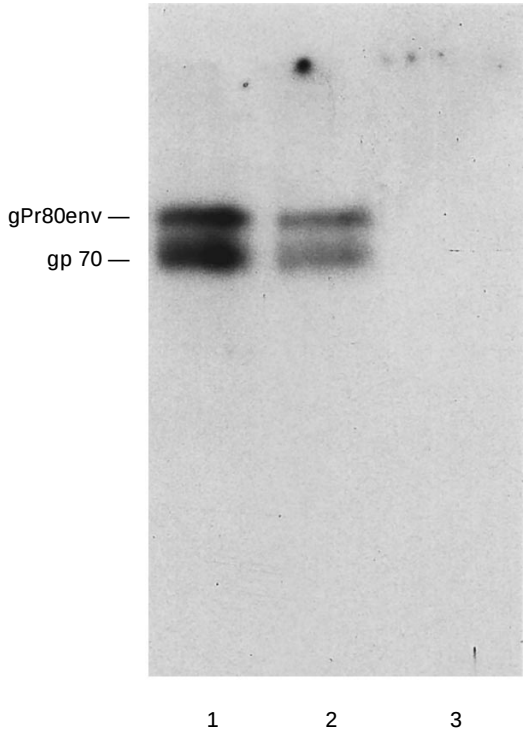


**Figure 2** Expression of the *env* gene of *ts*1 in NIH3T3 cells. Lane 1, cell lysate from *ts*1 virus-infected cells; lane 2, cell lysate from p*ts*1-*env*(F)-transfected and G418-resistant cells; lane 3, cell lysate from parental NIH3T3 cells. Cells used in the experiment were pulse-radiolabeled for 10 min with [$^{35}$S]methionine and [$^{35}$S]cysteine and chased for 120 min at 34°C. The expressed *env* proteins of *ts*1 (gPr80$^{env}$ and gp70) were identified by immunoprecipitation and SDS–PAGE using goat antiserum prepared against Rauscher MuLV gp69/71. The procedure has been described in detail elsewhere (Yu and Wong, 1992).

precipitation assay (see Figure 2 for *ts*1 envelope protein expressed in NIH3T3 cells harboring p*ts*1-*env*[F]; *ts*1 envelope protein was also detected in cells harboring p*ts*1-*env*[G] [Choe and Wong, unpublished result]).

p*ts*1-*env*(F) and p*ts*1-*env*(G) DNAs were then digested with EcoRI and HindIII to release the 5.2 kb microinjection fragment. DNA fragments purified using a QIAEX gel extraction kit (Qiagen Inc, Chatsworth, CA) were microinjected into the pronuclei of one-cell mouse embryos. Three different strains of mice – FVB/N, B6SJLF$_2$, and B6D2F$_2$ – were used for the transgenic studies. The B6SJLF$_2$ and B6D2F$_2$ founder mice were generated by DNX, Inc (Princeton, NJ) and by the Department of Molecular Genetics at MD Anderson Cancer Center, respectively; FVB/N founder mice were produced in our own transgenic mouse facility. Microinjection of DNA was conducted as described by Hogan *et al* (1986). Embryos surviving after microinjection were transferred into B6D2F$_2$ pseudopregnant foster mothers.

Twenty-one independent founder transgenic mice were produced by microinjection of the *ts*1-*env*(F) or *ts*1-*env*(G) transgene constructs into the pronuclei of embryos with FVB/N, B6SJLF$_2$, or B6D2F$_2$ genetic backgrounds. All transgenic founders were bred to FVB/N mice because this strain is most susceptible to the *ts*1 virus (Wong *et al*, 1991). To screen the transgenic mice, tail DNAs were isolated by the procedure of Laird *et al* (1991). Potential transgenic mice were routinely screened by PCR amplification. Using the primers 5′-ATGGCGCGTTCAACGCTCTCA-3′ and 5′-CTATGGCTCATACTCTATAGG-3′ (nucleotides [nt] 5777–5797 and 7753–7773, respectively, in the MoMuLV genome), a specific amplified product of the *ts*1-*env* gene of about 2 kb could be detected in PCR-amplified tail DNA from the transgenic mice. One advantage of using this pair of primers was that no 2 kb band could be detected in B6SJLF$_2$ or FVB/N nontransgenic mice, thus distinguishing the transgene with the possible endogenous retroviral sequences. Southern blot hybridization analyses (Yu *et al*, 1996) were used to establish individual transgenic lines by distinguishing the different integration sites. The 259 bp Hpa I-Sma I fragment of the *ts*1-*env* gene (nt 5819–6078) was used to generate the radiolabeled probe, which did not hybridize to any endogenous retroviral sequences. Southern blot analysis revealed that three founder mice had two integration sites. For example, in line EY1291 (Figure 3), only transgenic mice carrying integration site A expressed the detectable *ts*1-*env* gene in organs examined. Therefore, a total of 24 transgenic mouse lines were established at the beginning of the experiment. After every three

generations, Southern blot analyses were conducted on transgenic mouse lines chosen for further analysis to monitor the integrity of the inserted transgenes. So far, no rearrangement of transgenes has been observed.

To examine expression of the transgene, total cellular RNAs were isolated and analyzed using standard procedures (Sambrook *et al*, 1989). Selected transgenic mice and negative controls were scarified, and tissues were collected from cerebral cortex, brain stem, spinal cord, thymus, and spleen. RNAs were extracted from homogenized tissues with guanidine thiocyanate and then purified by phenol extraction and ethanol precipitation. Expression of transgene mRNA was examined by RNase protection assay. The radiolabeled probe was obtained by *in vitro* transcription of plasmid p72-E3, which has the 259 bp Hpa I-SmaI fragment of *ts*1-*env* gene cloned into the SmaI site of pSP72. After digestion with BamHI, transcription with T7 polymerase produced an RNA transcript of 307 nt. As an internal control, the ubiquitously expressed mouse cyclophilin message RNA was used. The 192 bp antisense radiolabeled probe was synthesized using a linearized pTRI-cyclophilin-mouse plasmid purchased from Ambion, Inc (Austin, Texas), which contains a 103 bp cDNA insert of a highly conserved region of the mouse cyclophilin gene spanning exons 1 and 2 (Hasel and Sutcliffe, 1990).

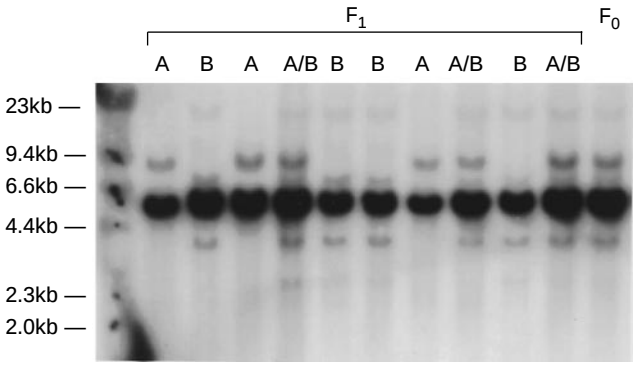


**Figure 3** Southern blot analysis of EY1291 transgenic mice. The analysis was conducted as described elsewhere (Sambrook *et al*, 1989). DNA (10 $\mu$g) isolated from PCR-positive transgenic mice was digested with BamHI. The 259 bp HpaI-SmaI fragment of the *ts*1-*env* gene (nt 5819–6078) was used to generate the radiolabeled probe, which did not hybridize to any endogenous retroviral sequences. $F_0$, DNA isolated from the founder mouse; $F_1$, DNAs isolated from the $F_1$ mice in EY1291 line. Lane A, transgenic mice with integration site A; lane B, transgenic mice with integration site B; lane A/B, transgenic mice with integration sites A and B.

The results of RNase protection assays showed that mice from nine transgenic lines expressed *ts*1-*env* RNA (Table 1). Among them, the founder mice of two lines had a B6SJL$F_2$ genetic background, while the rest had an FVB/N genetic background. Two of the nine lines harbored the *ts*1-*env*(G) transgene construct. Transgene expression was about 30 to 200 times lower than the viral expression observed in the same organs of *ts*1-infected mice. The levels of transgene expression in cerebral cortex, brain stem, and spinal cord were similar among all nine lines (see Figure 4). Seven independent lines shared a similar pattern of expression in all organs examined, in the following rank order: thymus > spleen > cerebral cortex=brain stem=spinal cord (Figure 4 and Table 1). In any specific organ, however, the absolute levels of expression could differ among these lines. For example, all five organs from line EY338 mice apparently displayed levels of transgene expression 20 times lower than those from line EY1698 mice. Similar patterns of expression of the transgene suggested that the LTR controlled the expression of the transgene in these lines and that transgene expression was independent of the influence of the regulatory elements surrounding the transgene in the chromosome. Two other lines did not follow the general pattern. In line EY388, expression of the *ts*1-

**Table 1** Organ-specific expression of *ts*1-*env* gene[a]

| *Transgenic lines* | *Transgene* | *Strain background of founder* | *Brain stem* | *Cerebral cortex* | *Spinal cord* | *Spleen* | *Thymus* |
|---|---|---|---|---|---|---|---|
| EY338 | *ts*1-*env*(F) | FVB | − | − | − | + | ++ |
| EY388 | *ts*1-*env*(F) | FVB | − | − | − | ± | − |
| EY554 | *ts*1-*env*(G) | FVB | ±[b] | ±[b] | ±[b] | ++ | +++ |
| EY972 | *ts*1-*env*(G) | FVB | ±[b] | ±[b] | ±[b] | ++ | +++ |
| EY994 | *ts*1-*env*(F) | FVB | ±[b] | ±[b] | ±[b] | ++ | +++ |
| EY1291 | *ts*1-*env*(F) | FVB | + | + | + | ± | ± |
| EY1605 | *ts*1-*env*(F) | FVB | ± | ± | ± | +++ | ++++ |
| EY1695 | *ts*1-*env*(F) | B6SJL | ±[b] | ±[b] | ±[b] | ++ | +++ |
| EY1698 | *ts*1-*env*(F) | B6SJL | ± | ± | ± | +++ | ++++ |

[a]−, No expression detected; ±, lowest level of expression; +, relatively low level of expression; ++, moderate level of expression; +++, high level of expression; ++++, highest level of expression. [b]Detected only about 50% of the time

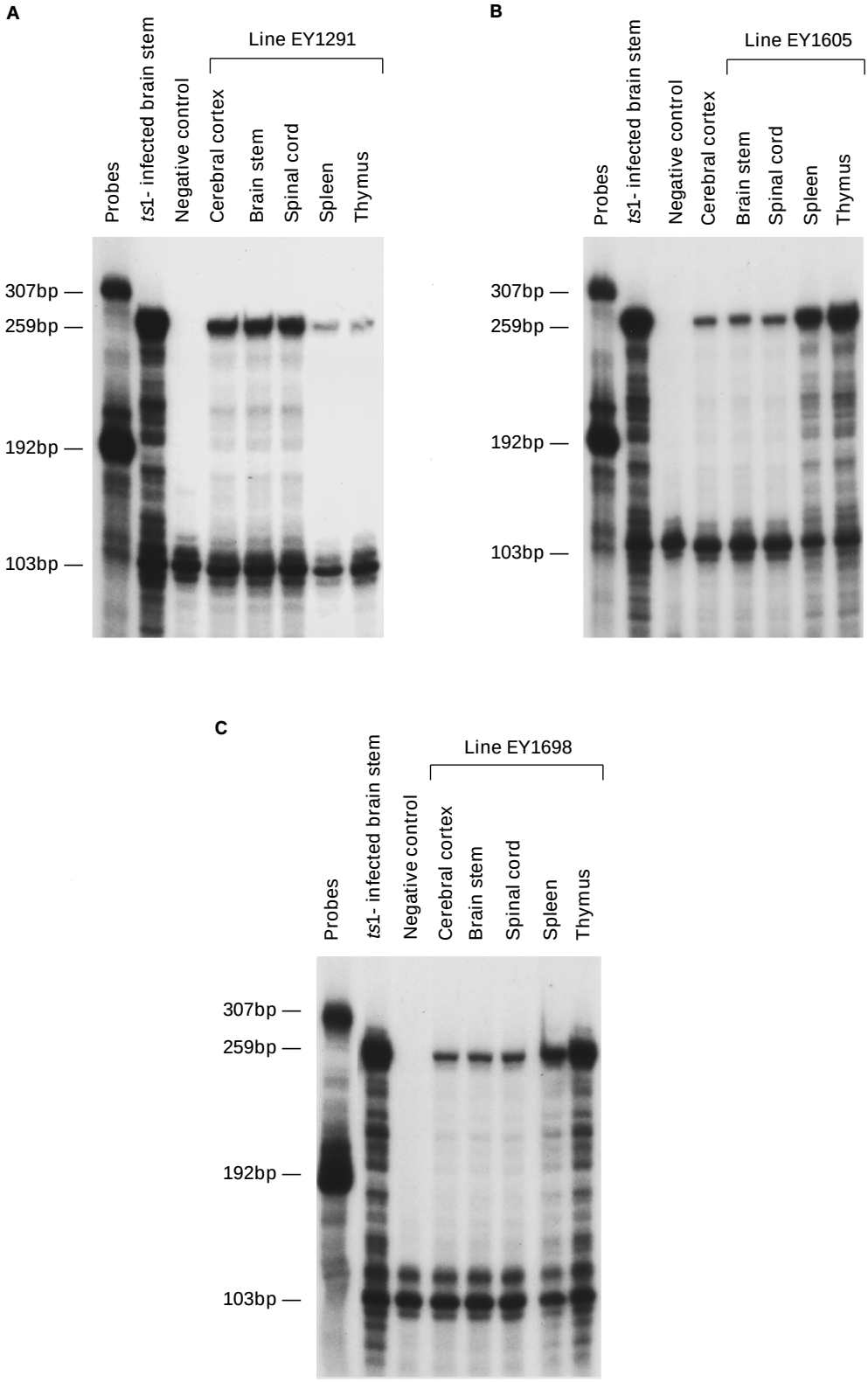


**Figure 4** Detection of *ts*1-*env* transcripts in organs of *ts*1-*env* transgenic mice by RNase protection assay. Lane 1, radiolabeled probe RNAs; lane 2, 1.0 $\mu$g of total RNA from the brain stem of *ts*1 virus-infected mice; lane 3, 10 $\mu$g of total RNA from the brain stem of a nontransgenic littermate; lane 4–8, 10 $\mu$g of total RNA from cerebral cortex, brain stem, spinal cord, spleen, and thymus of transgenic mice, respectively. The RNAs used in lanes 3–8 were isolated from mice in line EY1291 (A), mice in line EY1605 (B), and mice in line EY1698 (C). A 259 bp stretch of the *ts*1-*env* probe and a 103 bp stretch of mouse cyclophilin probe were protected by transgene RNA and mouse cyclophilin RNA, respectively.

*env* gene could only be detected in spleen (data not shown); in line EY1291, the highest level of expression was in cerebral cortex, brain stem, and spinal cord (Figure 4A). It may be that, in these two lines, one or more host regulatory elements influenced expression of the transgene.

The organs of transgenic mice were analyzed for the presence of *ts*1 envelope proteins. These proteins were undetectable by radioimmunoprecipitation assay, Western blotting, or immunohistochemical staining (data not shown). The same results were observed in transgenic mice expressing either the HIV gp120 envelope gene (Toggas *et al*, 1994) or the *env* gene of Cas-Br-E murine retrovirus (Kay *et al*, 1993). Our methods may have failed to detect protein products of the retroviral *env* genes in transgenic mice because (a) the expressed protein levels were under the threshold of detection or (b) the antibodies used were not sufficiently sensitive. It has been shown that retroviral proteins are, in general, weakly immunogenic and that it is difficult to generate sensitive immunohistochemical probes (Wiley and Gardner, 1993).

For neuropathological assessments, transgenic and control mice were perfused (under anesthesia) with 2% paraformaldehyde or 10% neutral buffered formalin via the left heart ventricle. Tissues from all body organs were then routinely examined histologically. From the central nervous system (CNS), multiple coronal sections were cut and processed. Processing of tissues for paraffin embedding, routine hematoxylin and eosin (H&E) staining, and immunohistochemistry for astrocyte detection were performed as previously described elsewhere (Stoica *et al*, 1993). On H&E-stained sections, evidence of neurodegeneration including spongiform changes was observed in 63% of transgenic mice expressing the transgene in the CNS (Table 2). These neurodegenerative changes were characterized by small aggregates of membrane-bound vacuoles within neuropil or perikaryons of the motor neurons (Figure 5A–D). The lesions observed were mild to moderate and comparable to stage I (10–15 days post inoculation, d.p.i.) spongiform lesions seen in *ts*1-infected mice (Stoica *et al*, 1993). Although pathological lesions were observed occasionally in the cerebral cortex and cerebellum, the spongiform degeneration observed in transgenic mice was mainly seen in the ventral horn of the lumbar spinal cord (Figure 5C, D) and brain stem (Figure 5A, B), the same areas where intact *ts*1 virus induced the most severe neurodegeneration (Figure 6). This regional specificity may suggest that motor neurons in these anatomic sites are more susceptible or vulnerable to the effect of the *ts*1 envelope proteins. In addition, vacuolized glial cells (primarily astrocytes) were occasionally observed. Gliosis was observed in only four transgenic mice and was classified as mild. Such areas were composed primarily of reactive astrocytes. Typically, astrocytes positive for glial fibrillary acidic protein appeared as aggregates of a few extensively branched, reactive astrocytes (Figure 7). The above-mentioned neuropathological lesions were not seen in nontransgenic littermates or in transgenic mice expressing no detectable *ts*1-*env* RNA in the CNS (e.g. Figure 5E, F and Figure 7B). Histopathological examinations were also conducted on thymus, heart, muscle, lung, liver, pancreas, spleen, intestine, stomach, kidney, uterus, and testis isolated from transgenic mice and nontransgenic littermates, and no abnormality including spongiform change was detectable in any of the samples.

**Table 2** Correlation between expression of *ts*1-*env* in the CNS and neuropathological lesions observed in transgenic mice[a]

| *Transgenic lines* | *Strain background of founder* | *Level of* ts*1*-env *expressed in CNS*[b] | *Spongiform or neuronal degeneration*[c] |
|---|---|---|---|
| EY388 | FVB | − | 0/9 |
| EY1291 | FVB | + | 12/18 |
| EY1605 | FVB | ± | 5/8 |
| EY1698[d] | B6SJL | ± | 3/6 |
| Nontransgenic littermates | FVB | − | 0/8 |

[a]Animals used in the experiment were between 8 and 12 months of age. [b]See footnote a in Table 1 for key. [c]No. mice with lesion/total no. mice. [d]Transgenic mice used in experiment have been backcrossed to FVB mice for at least 6 generations

No sign of neurological defect was detected in any transgenic mice except for five of 91 transgenic mice from line EY1291 between 8 and 12 months of age that exhibited adduction reflex of the hind limbs when held by their tails. This reflex is an early clinical sign of neurologic disorder in mice infected with *ts*1 virus (Wong, 1990) and other neurovirulent murine retroviruses (Stansly, 1965; Kay *et al*, 1993). No hind limb adduction has been observed in age-matched transgenic mice from EY1605 (49 mice examined) and EY1698 lines (35 mice examined) or in nontransgenic littermates (104 mice examined).

The aforementioned result is consistent with previous transgenic studies of retroviral envelope genes. Like *ts*1 virus, HIV and Cas-Br-E virus can induce neuropathological lesions in infected hosts. In the absence of *gal*, *pol*, or other viral gene expression, transgenic mice expressing either the gp120 *env* gene of HIV (Toggas *et al*, 1994) or the *env* gene of Cas-Br-E virus (Kay *et al*, 1993) in the CNS have been shown to develop neurodegeneration. However, when compared with transgenic mice harboring the *env* gene from the Cas-Br-E virus, *ts*1-*env* transgenic mice develop neuropathological lesions at younger ages (8–12 months *versus* 15–24 months in Cas-Br-E *env* transgenic mice). This difference is interesting because the latency of neurodegeneration in *ts*1-infected mice is generally

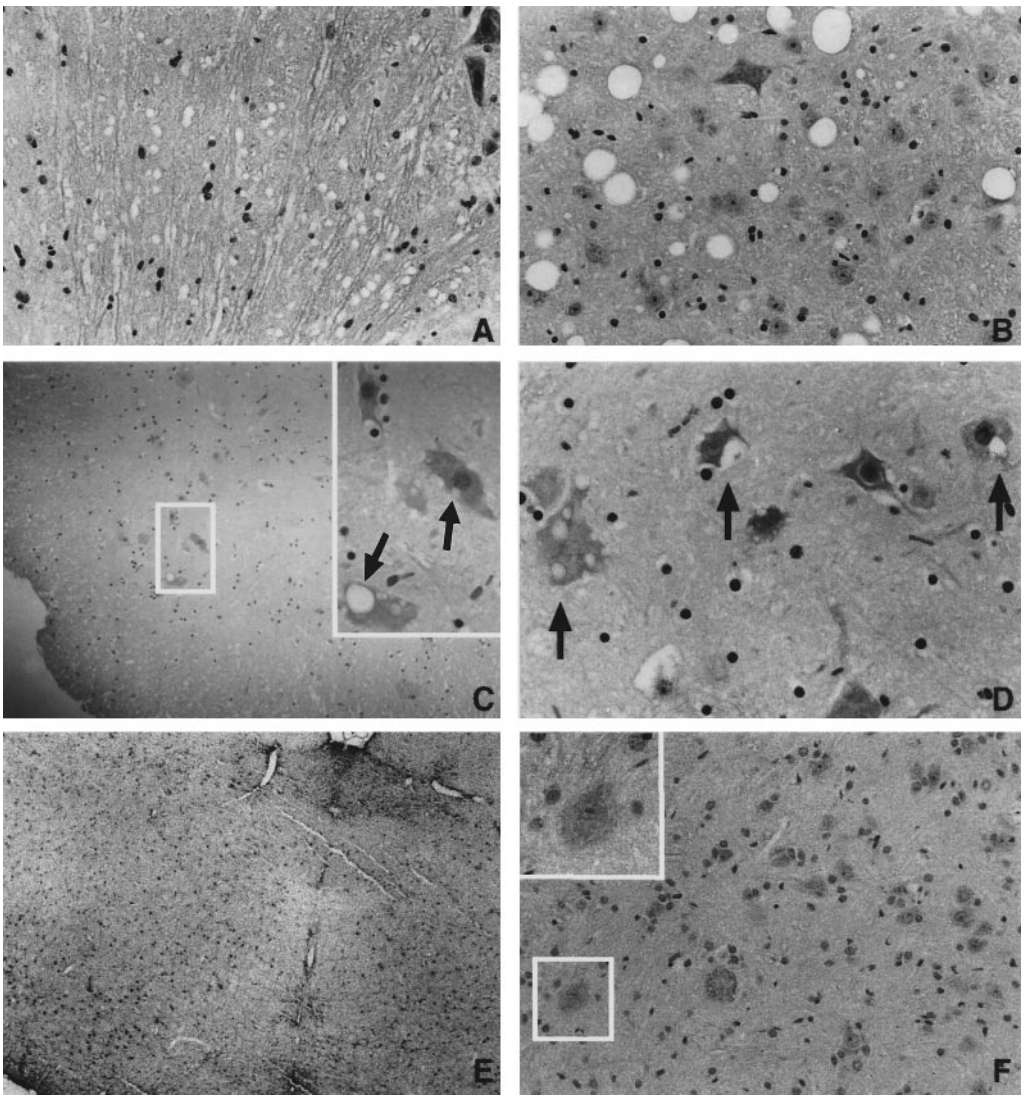


**Figure 5** Spongiform or neuronal degeneration observed in transgenic mice. Neuropil spongiform change was observed in the brain stems of (A) 11-month-old transgenic mouse no. 1978 from line EY1698 (H&E, X120) and (B) 9-month-old transgenic mouse no. 1237 from line EY1291 (H&E, X120). Vacuolar degenerative change in motor neurons (arrows) was observed in the ventral horns of the lumbar spinal cords of (C) 9-month-old transgenic mouse no. 1154 from line EY1605 (H&E, X50; inset X220) and (D) 8-month-old transgenic mouse no. 1240 from line EY1291 (H&E, X240). No degenerative change was observed in (E) the brain stem of 12-month-old nontransgenic littermate no. 1613 from line EY1291 (H&E, X22) and (F) the ventral horn of the lumbar spinal cord of a 8-month-old nontransgenic littermate no. 1246, from line EY1291 (H&E, X120; inset X240).

shorter than in Cas-Br-E-infected mice (Wong, 1990). Thus, it is possible that the envelope proteins of *ts*1 are more potent than those of the Cas-Br-E virus.

Like the transgenic mice carrying the Cas-Br-E *env* gene, the transgenic mice expressing *ts*1-*env* also showed incomplete penetrance of the neuropathological phenotype (Table 2; 50–75% penetrance in the *ts*1-*env* transgenic mice *versus* 33–52% penetrance in the Cas-Br-E *env* transgenic mice (Kay *et al*, 1993). Indeed, incomplete penetrance of the transgene-associated phenotype has been reported in many transgenic animal lines. Some investigators have attributed this to the genetic heterogenicity of transgenic mice (Schulz *et al*, 1992; Qiao *et al*, 1994), while others have provided evidence that absence of the phenotype in some of transgenic mice correlated with the low level of transgene transcript (Propst *et al*, 1990; Overbeek, 1994; Propst *et al*, 1994) or with instability of the transgene mRNA (Stewart *et al*, 1993). Still others have considered it an inherent feature in expression of some specific transgenes (Pereira *et al*, 1994). To examine the correlation between the level of transgene expression and the occurrence of detectable pathological lesions in the CNS of our transgenic mice with an FVB inbred

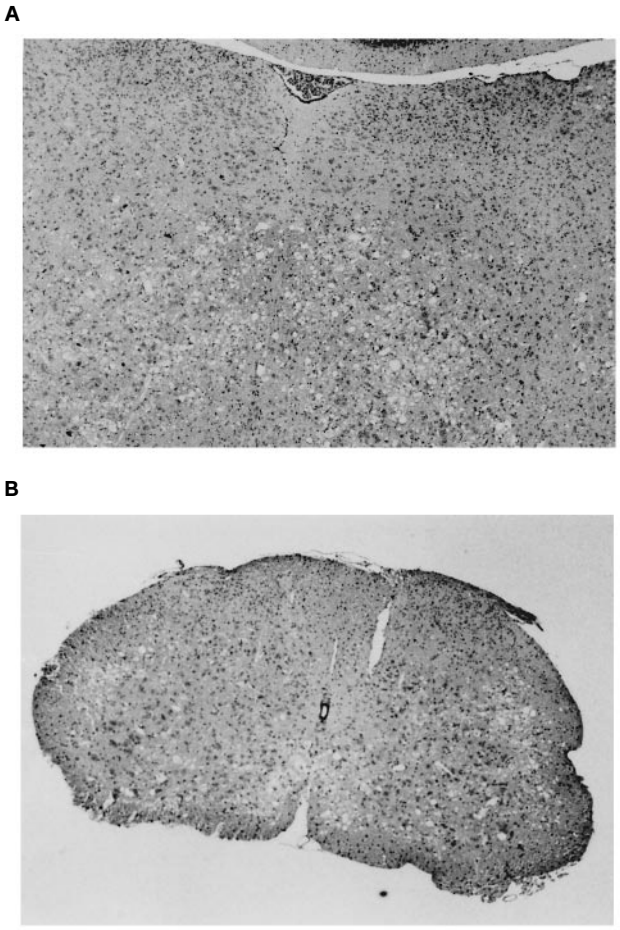


**Figure 6** Spongiform degeneration observed in *ts*1 virus-infected mice. Extensive spongiform changes were observed in the brain stem (A) and spinal cord (B) of a *ts*1 virus-infected FVB mouse killed at 35 dpi (H&E, X22).

genetic background, we compared *ts*1-*env* expression in four transgenic mice from line EY1291. As shown in Figure 8, neuropathological lesions were only observed in mice expressing the higher level of the transgene in the CNS (mouse no. 1380 and no. 1781). Therefore, like certain mouse lines carrying other transgenes (Propst *et al*, 1990, 1994; Overbeek, 1994), incomplete penetrance of the phenotype observed in the *ts*1-*env* transgenic mice may be due to the variability in the levels of transgene expression. However, in the EY1698 line, the genetic heterogenicity may also contribute to the incomplete penetrance of the phenotype because the strain background of the founder mouse is B6SJL.

How *ts*1 virus induces spongiform encephalomyelopathy is not yet fully understood (Gonzales-Scarano *et al*, 1995). It is possible that pathogenic mechanisms differ between *ts*1 virus-infected mice and transgenic mice carrying the *ts*1-*env* gene since every cell in transgenic mice harbors the *ts*1-*env*

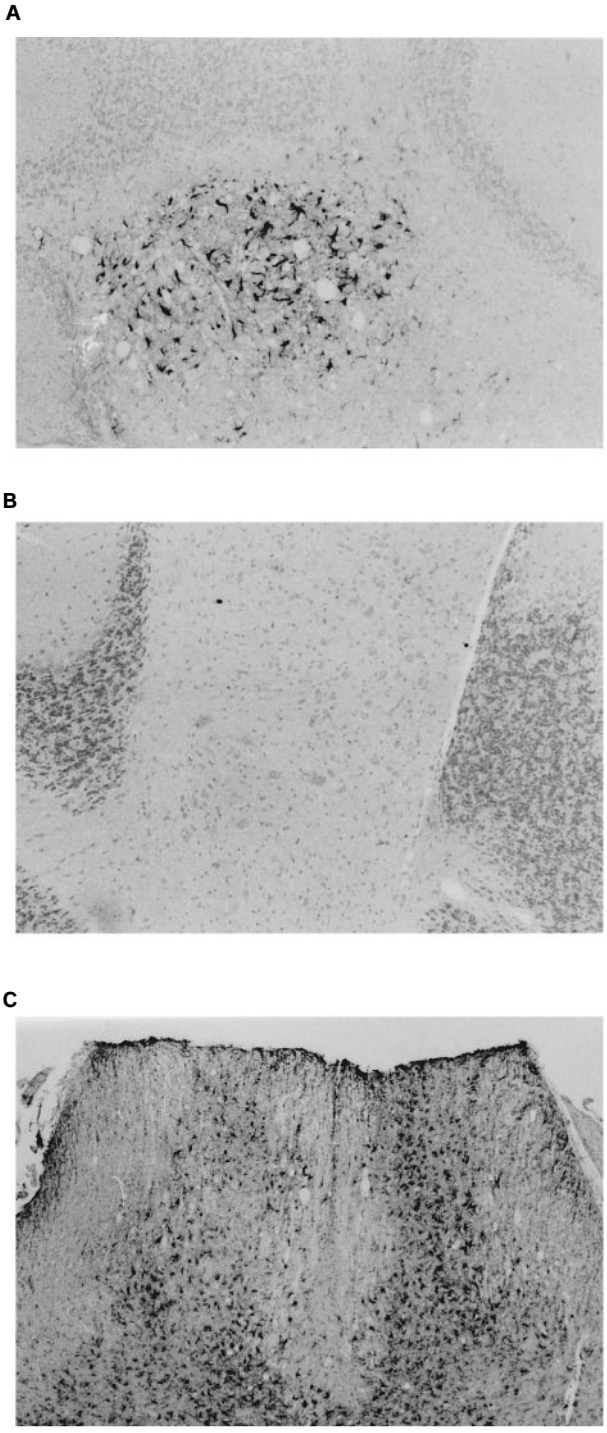


**Figure 7** Astrocytosis observed in transgenic mice and *ts*1 virus-infected mice. Using an immunocytochemical approach (in which an anti-glial fibrillary acidic protein antibody was used as described in detail in Stoica *et al*, 1993), astrocytosis was observed within an area of spongiform change in the cerebellum of a transgenic mouse (no. 2018, line EY1291, H&E, X50) (A). No astrocytosis was detected in a similar area of an age-matched nontransgenic littermate (no. 2014, H&E, X50) (B). Much more extensive astrocytosis was observed in the CNS of *ts*1 virus-infected mice, e.g. astrocytosis in the brain stem of a *ts*1 virus-infected FVB mouse killed at 35 dpi (H&E, X22) (C).

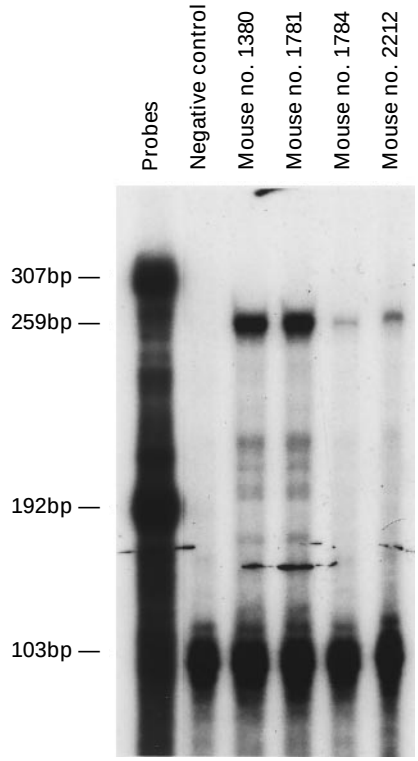


**Figure 8** Correlation between *ts*1-*env* RNA levels in the brain stem and pathological lesions observed in the spinal cord in individual mice from line EY1291. Total RNAs (10 $\mu$g) isolated from the brain stems were used in the RNase protection assay. The radiolabeled probes were synthesized using linearized plasmids p72-E3 and pTRI-cyclophilin-mouse. The spongiform or neuronal vacuolization was only observed in the spinal cords of mouse no. 1380 and mouse no. 1781.

gene, whereas vacuolized neurons in *ts*1-infected mice appear not to be infected with the virus. It is also possible that the neuropathological lesions observed in transgenic mice are induced by the *env* transcript instead of the envelope proteins since we could detect no *ts*1 envelope proteins in transgenic mice. Nevertheless, even if the neuropathological lesions were induced by the *ts*1 envelope proteins, it would still be interesting to complement the present study by examining the role of the *gag* and *pol* gene products in *ts*1 virus-induced neurodegeneration by establishing transgenic mice carrying the *gag* or *pol* gene; this would also help us paint a more complete picture of the *in vivo* effect of the individual viral gene product of *ts*1 virus.

The transgenic study reported here has also yielded some interesting information about transgene expression under the control of the MoMuLV LTR. C-type murine retrovirus LTRs have been used by many investigators to drive expression of their transgenes (Khillan *et al*, 1987; Aizawa *et al*, 1990; Sutrave *et al*, 1990; Sasaki *et al*, 1993; Conti, 1995). In this study, the 5′ LTRs in constructs *ts*1-*env*(F) (Yu *et al*, 1991) and *ts*1-*env*(G) contained one direct repeat (an enhancer sequence) and two direct repeats, respectively. However, our EY1605 and EY1698 transgenic mice (harboring *ts*1-*env*(F)) expressed higher levels of the transgene than did EY554 and EY994 transgenic mice (harboring *ts*1-*env*(G)) in all organs examined. This suggests that the site of transgene integration may be more important than the number of direct repeats in the LTR. Another interesting result is that, in most of our transgenic mice, the pattern of *env* gene expression (thymus > spleen > cerebral cortex=-brain stem=spinal cord) was similar to that seen in *ts*1-infected mice (i.e. higher expression in the organs of the immune system than in the organs of the CNS). In all the transgenic lines we examined, there was no difference in the levels of *ts*1-*env* expression among cerebral cortex, brain stem, and spinal cord. This suggest that the quality and quantity of *trans* elements that can interact with the regulatory elements in transgenes might be similar among cerebral cortex, brain stem, and spinal cord in all transgenic mice, no matter where the transgene is integrated.

In summary, the results reported here confirm that the *gag* and *pol* gene products and viral replication are not necessary for *ts*1-*env* to induce a mild form of neurodegeneration in transgenic mice.

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