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Characterization of mouse lines transgenic with the human poliovirus receptor gene

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Two mouse lines transgenic with the human poliovirus receptor gene (PVR), TGM-PRG-1 and TGM-PRG-3, were characterized to determine whether transgene copy number and PVR expression levels influence susceptibility to poliovirus. The mouse lines have been bred for more than 10 generations and the transgene was stably transmitted to progeny as determined by Southern blot hybridization and restriction fragment length polymorphism. The transgene copy number is 10 in the TGM-PRG-3 mouse line and one in the TGM-PRG-1 mouse line. Abundance of PVR RNA is on average three-fold higher in TGM-PRG-3 relative to TGM-PRG-1 tissues, and the abundance of the receptor molecule is three-fold higher in TGM-PRG-3 central nervous system tissues compared to TGM-PRG-1 tissues as determined by Western blot analysis. When TGM-PRG-1 and TGM-PRG-3 mice were inoculated intracranially with a neurovirulent type III poliovirus strain, they developed clinical symptoms and CNS lesions characteristic of human poliomyelitis. These results indicate that the PVR gene is expressed as a functional receptor in the CNS of both mouse lines rendering the mice susceptible to poliovirus infection. Even though the two mouse lines have different copy numbers of the transgene and different levels of PVR RNA and protein, they are similar in their susceptibility to poliovirus.

Key words: Human poliovirus receptor, transgenic mouse lines, TGM-PRG-1 and TGM-PRG-3, poliovirus neurovirulence, poliovirus susceptibility, receptor dosage.

Introduction

Poliovirus is a small positive-stranded RNA virus of the Picornaviridae family. The natural host range of polioviruses, restricted to humans, has been attributed to the expression of the poliovirus receptor (pvr) [1, 2]. The pvr [3] is encoded by a single-copy gene located on human chromosome 19 [4]. The availability of human genomic clones encoding the PVR gene [4–6]

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enabled the construction of transgenic (TgPVR) mouse lines. Two mouse lines, TGM-PRG-1 and TGM-PRG-3 have now been characterized with respect to expression of the human poliovirus receptor, PVR gene copy number, and their susceptibility to wild-type III poliovirus.

The pvr is a member of the immunoglobulin superfamily [3] and is expressed as two secreted forms (β and γ) and two membrane-bound forms (α and δ) [4]. The membrane-bound receptor has three extracellular immunoglobulin-like domains [3, 7], a single transmembrane region, and a cytoplasmic tail. The secreted receptor lacks the transmembrane region. Unglycosylated forms of the receptor are 43 kd (δ) and 45 kd (α); a glycosylated intermediate form is 67 kd, and the fully glycosylated form is 80 kd in HeLa cells [8,9]. Polioviruses bind to the amino terminal and most distal domain of the receptor molecule, termed domain 1 [10–13]. The cellular function of pvr is not known, but is postulated to be involved in cell adhesion and/or cell-to-cell communication [14]. The α isoform of pvr is a serine phosphoprotein, potentially phosphorylated by calcium/calmodulin kinase II [15].

TgPVR mice are susceptible to types I, II and III polioviruses [5; Weeks-Levy *et al*., unpublished results]. Inoculation of neurovirulent polioviruses into the central nervous system (CNS) of TgPVR mice causes destruction of neurons leading to paralysis. Non-transgenic mice are unaffected by poliovirus inoculated into the CNS and lesions characteristic of poliovirus are not observed [5]. The target sites for poliovirus infection in transgenic mouse CNS are similar to those in humans, and the clinical signs are reminiscent of those in human poliomyelitis. The mouse homologue to pvr, designated mph, does not bind type I, type II or type III polioviruses [12, 16]; domain 1 of mph has only 52% amino acid identity with domain 1 of pvr. Since non-transgenic mice are susceptible to mouse-adapted poliovirus strains such as Lansing [17], these polioviruses presumably interact with a mouse receptor not yet identified [18, 19].

TgPVR mice have multiple uses. Because these mice express the human poliovirus receptor, poliovirus infections in the transgenic mice mimic human infection. These mice should be invaluable, not only in elucidating interactions of polioviruses with permissive cells, but also for the study of poliovirus pathogenesis [5, 20–22]. TgPVR mice are being used to study attenuation/neurovirulence contributed by specific nucleotides in type I, II and III poliovirus genomes [23; Weeks-Levy *et al*., unpublished results]. These mice are also being used as models to facilitate studies using poliovirus as a vector to deliver antigens of other viruses to sites responsible for mucosal infection and immunity [24–29]. This non-primate animal model is also being evaluated for use in neurovirulence testing of poliovirus vaccines [30–35; Deatly *et al*., unpublished results], and a disease protection test of inactivated poliovirus vaccine potency [36].

In this study, characterization of the TGM-PRG-1 and TGM-PRG-3 mouse lines has been performed to determine transgene copy number(s), transgene stability during multiple generations of breeding, and transgene RNA and receptor expression. Mice from both lines were also inoculated intra-cranially (IC) to compare their relative sensitivity to a neurovirulent type III poliovirus. Experimental evidence demonstrates that despite differences in transgene copy number and PVR RNA and protein expression levels, these two mouse lines share a similar susceptibility to poliovirus infection.

Results

Detection of the PVR gene in transgenic mouse offspring by PCR

To monitor the transmission of the PVR gene to transgenic mice progeny, a polymerase chain reaction (PCR) assay was developed. Specific PVR primers, SN1 and SN2, amplify 226 bp of a region encoding domain 1, the poliovirus binding site of the pvr. Primers for the mouse glyceraldehyde 3-phosphate dehydrogenase (G3PDH) gene were included in the reaction mix to ensure that DNA from both non-transgenic and transgenic mice were amplifiable in this assay (Fig. 1). The assay was optimized to detect one copy of the transgene per cell in 10^6 cells (data not shown). After PCR amplification, the products were visualized on a 1.5% agarose gel stained with ethidium bromide. Amplified PCR products specific for the transgene (226 bp) and the G3PDH (1 kb) control were produced from transgenic mouse DNA (1A, 1B1 and 1B2); whereas, only the 1 kb G3PDH PCR product was amplified from non-transgenic mice (NTGM) DNA. The 226 bp PCR product was purified

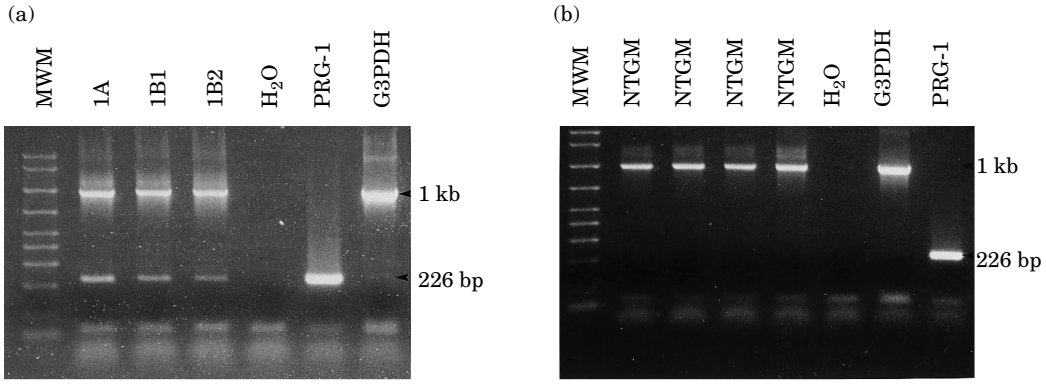

Figure 1. PCR assay to monitor transmission of the PVR gene to transgenic mouse offspring. 1A, 1B1 and 1B2 refer to blood samples from transgenic mice (a) and NTGM indicates blood obtained from non-transgenic mice (b). The molecular weight markers (MWM) (BioVentures) range in size from 2.0 to 0.05 kb. A control sample without DNA template is represented by H_2O. Also 0.5 pg of the genomic clone pWE-PRG-1 was amplified as a positive control for the transgene specific primers and 0.5 attamoles of G3PDH DNA (Clontech No. 5409-1) was amplified for a positive control for the G3PDH primers.

from an agarose gel and sequenced (data not shown) and identity to PVR sequences was confirmed [3].

Inheritance of the transgene is stable through generations of breeding

To determine the genetic stability of the 37 kb transgene during the breeding of transgenic mice, restriction fragment length polymorphism (RFLP) analysis was performed on progeny from multiple generations of transgenic mice. Differences in the RFLP maps of individual mice would indicate DNA rearrangements, deletions or insertions. These potential changes in the transgene could affect expression of a functional PVR gene product and, ultimately, susceptibility of that individual mouse (and subsequent progeny) to poliovirus infections.

Figure 2 is an example of an RFLP map of DNA from representatives of one generation of transgenic mice from different litters (lanes 1–3 and 6–8). Twenty micrograms of genomic DNA, purified from mouse tails and digested with *Bam*H1, were hybridized with a ^{32}P-H20A probe (see Materials and Methods). As illustrated, the RFLP maps of the different mice were identical to the RFLP maps of the respective PRG clones as expected, if no DNA rearrangements occurred. RFLP map analysis from four consecutive generations indicated no changes in transgene DNA from TGM-PRG-1 and TGM-PRG-3 mouse lines (data not shown). Since the RFLP patterns have not changed over ten generations, the PVR gene in these two mouse lines appears to be genetically stable.

Transgene copy number in TGM-PRG-1 and TGM-PRG-3 mice

Southern blot hybridization was used to compare relative copy numbers of the transgene in TGM-PRG-1 and TGM-PRG-3 mice. Twenty micrograms of *Bam*H1 digested genomic DNA were electrophoresed into an agarose gel, then transferred to a Zeta-Probe nylon membrane. The blot was hybridized simultaneously with: a ^{32}P-H20A probe, a ^{32}P-mouse macrophage colony stimulating factor gene (mCSF) probe, for detection of a single-copy gene [37] and used as an internal control for DNA equivalence; and a ^{32}P- 1 kb DNA marker probe. Hybridization to 10 kb, 3 kb and 1.2 kb fragments in TGM-PRG-1 mouse DNA, with additional hybridization to a 6 kb fragment in TGM-PRG-3 mouse DNA, represent PVR gene recognition by the H20A probe. Hybridization to the 4 and 3.5 kb DNA fragments represent the mCSF single copy mouse gene. Hybridization results (Fig. 3] indicate that mice representative of the TGM-PRG-3 line have a higher copy number than TGM-PRG-1 mice. In order to determine the actual transgene copy numbers in each mouse line, a Betascope (Betagen) was used to quantify [38] the amount of PVR specific probe and mCSF control probe hybridized to TGM-PRG-1 and

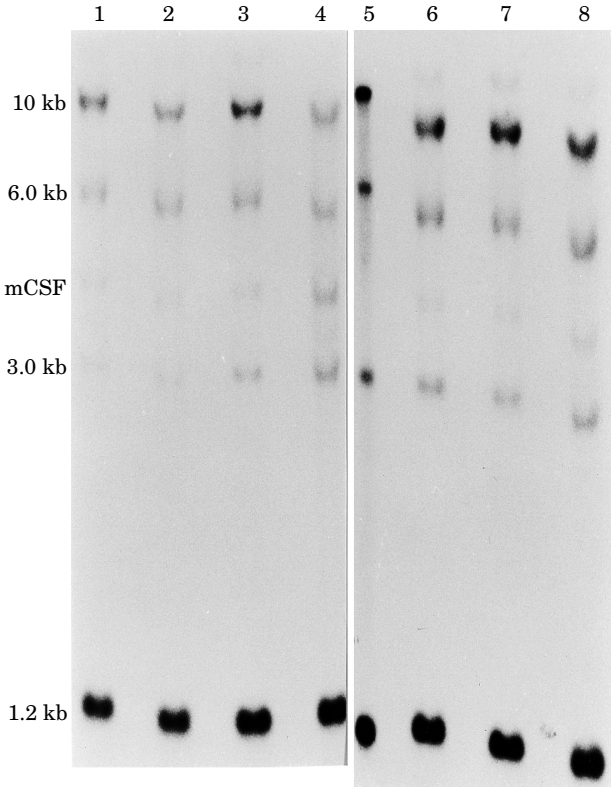

Figure 2. RFLP map of TGM-PRG-3 mice. Twenty micrograms of genomic DNA purified from tails of TGM-PRG-3 mice from multiple litters of the same generation were digested with *Bam*H1 (lanes 1–3 and 6–8). Thirty nanograms of *Bam*H1-digested pWE-PRG-3 were mixed with 20 μg of *Bam*H1-digested genomic DNA from non-transgenic mice (lane 4) and 30 ng of *BamH1*-digested pWE-PRG-3 (alone) is in lane 5. Thirty nanograms of pWE-PRG-3 represent 10 copies of the transgene in 20 μg of genomic DNA. The blot was hybridized with ^{32}P-H20A and ^{32}P-mCSF probes. The hybridized blot was exposed to XAR-5 film overnight.

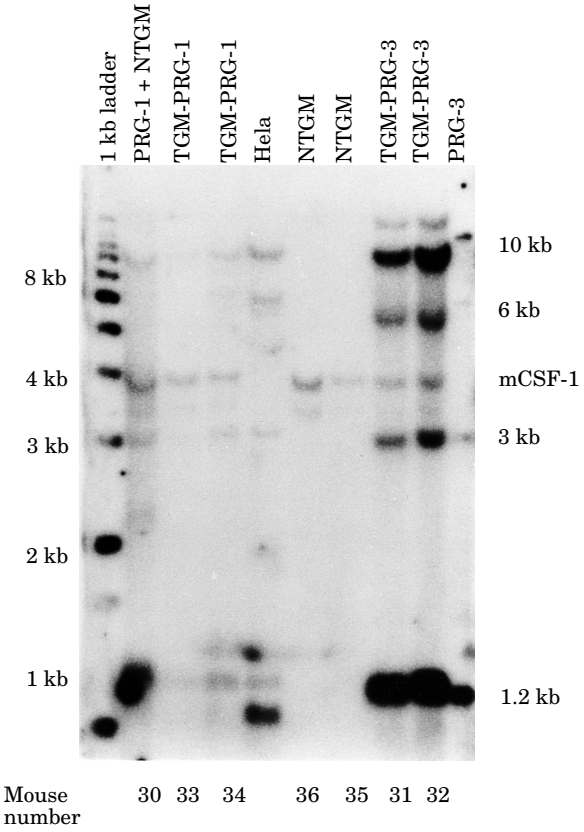

Figure 3. Determination of the transgene copy number in TGM-PRG-1 and TGM-PRG-3 mice. Twenty micrograms of mouse tail DNA and human genomic DNA (HeLa) were digested with *BamH1* and blotted onto a nylon membrane. The blot was hybridized with a ^{32}P-H20A probe, a ^{32}P-mCSF probe and a ^{32}P-1 Kb ladder probe. Three nanograms of the genomic clones PRG-3 (alone) and PRG-1 (in 20 μg of non-transgenic mouse DNA) were added as controls. NTGM represents non-transgenic mouse (DNA). The hybridized blot was exposed for 7 days. HeLa and the PRG-1 DNA mixed in NTGM DNA represent controls for single copy PVR genes.

TGM-PRG-3 DNA. Data from several experiments (not shown) indicated that the amount the PVR gene probe hybridizing to the *Bam*H1 fragments encoding PVR, determined by radiometric assays of TGM-PRG-1 mice No. 33 and No. 34, was similar to the cpm hybridizing to the single gene copy PVR *Bam*H1 fragments in HeLa cell DNA and the single gene copy mCSF DNA *Bam*H1 fragments in mouse genomic DNA. In TGM-PRG-3 mice No. 31 and No. 32, there was an average 10-fold increase in cpm hybridizing to DNA fragments encoding PVR relative to cpm hybridizing to mouse mCSF DNA fragments. The transgene copy numbers for each mouse line were determined by the ratio of the cpms that hybridized to PVR restriction endonuclease fragments divided by its probe size relative to the cpms hybridized to the 3 kb mCSF fragment divided by its probe size. By these relative comparisons, TGM-PRG-3 mice have ten copies and TGM-PRG-1 mice have one copy of the PVR gene. TGM-PRG-1 mice have a lower copy number than TgPVR1-17 mice (∼five copies), a homozygous mouse line bred at Columbia University [5], which was derived from the same founder mouse. After 10 generations of separate breeding, TGM-PRG-1 and TgPVR1-17 are considered to be different mouse lines [39].

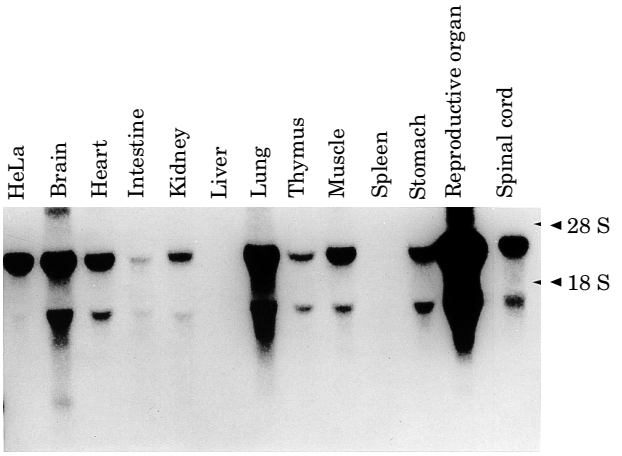

Figure 4. PVR RNA expression in transgenic mouse tissues. Fifteen micrograms of (denatured) total RNA samples purified from TGM-PRG-3 mouse tissues were electrophoresed on 1.2% agarose gels. The RNA was transferred to a nylon Zeta-Probe membrane and hybridized under stringent conditions with a ^{32}P-H20A probe. The hybridized blots were exposed for 4.5 days. The positions of the 28 S and 18 S rRNAs are also highlighted as determined by ethidium bromide staining of the RNA prior to transfer.

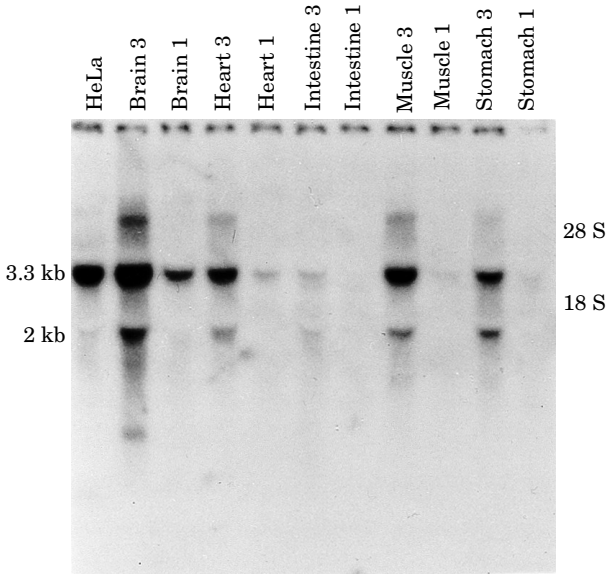

Figure 5. Comparison of PVR RNA expression in TGM-PRG-1 and TGM-PRG-3 mouse tissues. 15μg of total RNA was purified from selected tissues of TGM-PRG-1 (No. 33) and TGM-PRG-3 (No. 31) mice. These same two transgenic mice were used in determination of the transgene copy number (Fig. 3). After denaturation with glyoxal, the RNA was electrophoresed on a 1.2% agarose gel. The RNA was transferred to a Zeta-Probe nylon membrane and hybridized with a ^{32}P-H20A probe. Numbers 1 and 3 represent the transgenic mouse lines (TGM-PRG-1 and TGM-PRG-3, respectively) from which the tissues were derived. The hybridized membrane was exposed for 2 days.

Expression of the PVR RNA in transgenic mouse tissues

Since the expression of the PVR gene is necessary to confer susceptibility to polioviruses, it is important to determine which tissues are expressing PVR RNA in the transgenic mice. Northern blot analysis of total RNA from various organs demonstrated a major PVR RNA transcript of 3.3 kb and a minor 2 kb transcript. There are four PVR RNA isoforms as a result of different splicing events. All four PVR RNA isoforms, encoding the two secreted and two membrane-bound receptors, migrate at approximately 3.3 kb [4]. PVR RNAs are expressed in most tissues (Fig. 4), albeit at different abundances. High levels of PVR RNA were detected in the brain, spinal cord, heart, lung, muscle, stomach and reproductive organ(s), while levels in spleen or liver were very low or undetectable. Equivalent loading of the RNA samples was monitored by visualizing the 18 S and 28 S rRNAs by staining with ethidium bromide prior to the transfer of RNA to the membrane. In addition to the 3.3 and 2 kb RNA species, a 5.6 kb RNA species [3, 5] was also detectable in the brain and reproductive organ (Fig. 4), as well as in the heart, lung, muscle and stomach (as observed in longer film exposures; see Fig. 5). The 2 kb and 5.6 kb PVR RNA species are not always detectable in HeLa cells and TGM-PRG-1 tissues (Figs 4 and 5; and data not shown) presumably because of differences in their relative gene abundance.

The pattern of relative abundance and the tissue distribution of PVR RNA in the different tissues is very similar for TGM-PRG-1 and TGM-PRG-3; but higher levels of PVR RNA were detected in TGM-PRG-3 tissues compared to TGM-PRG-1 tissues. Since the 2 kb RNA is less abundant in TGM-PRG-1 as compared to the same TGM-PRG-3 tissues, it is probably not derived from the MPH gene [40, 41]. It is possible that the 2 kb RNA represents an alternative splice variant which is present in higher abundances in TGM-PRG-3 tissues than in TGM-PRG-1 tissues. The 2 kb RNA was also detected in total RNA purified from tissues of TgPVR1-7, TgPVR1-17 and TgPVR3-9 mice [5]. A 2 kb RNA

of low abundance was detected in the Northern blot hybridization analysis of poly A+ RNA from ICR-PVRTg1 mice and attributed to MPH RNA [40]. Our data described above suggest that this is not the case. The origin of the 5.6 kb RNA is not clear; however, it also was detected in human RNA samples (data not shown). The 5.6 kb transcript contains sequences other than those that hybridize to the PVR cDNA [3].

To compare the steady-state level of PVR RNA directly in select tissues from TGM-PRG-1 and TGM-PRG-3 mice, total RNA from brain, heart, muscle and intestine was analysed on the same membrane. As illustrated in Fig. 5, the 5.6, 3.3 and 2 kb PVR RNAs were present in greater abundance in tissues from the TGM-PRG-3 mouse (No. 33) relative to tissues from the TGM-PRG-1 mouse (No. 31). TGM-PRG-3 (No. 33) and TGM-PRG-1 (No. 31) were the same mice used to determine transgene copy numbers (Fig. 3). To confirm equivalent loading of RNA from the same tissue type, a ^{32}P-β-actin probe (Clontech) was also hybridized to this same membrane, after the PVR probe was removed (data not shown).

Betascope analysis of the cpm hybridized to the 3.3 kb PVR RNA in this experiment (Fig. 5) indicated an average three-fold higher level of PVR RNA in TGM-PRG-3 tissues relative to TGM-PRG-1 tissues (data not shown). Ethidium bromide staining of the RNAs prior to transferring to the membrane showed that equivalent amounts of 18 S and 28 S rRNAs were loaded in each lane and that the RNAs appeared intact. The higher levels of PVR RNA detected in TGM-PRG-3 mouse tissues correlate with the higher PVR gene copy number in the DNA of this mouse line relative to TGM-PRG-1 mice (see Fig. 3).

Expression of pvr in brain tissues of TGM-PRG-1 and TGM-PRG-3 mice

The brains of 4–6-week-old male TGM-PRG-1 and TGM-PRG-3 mice were removed following their perfusion. Membrane fractions were prepared from the brain homogenates. Aliquots containing 10.24, 12.8, 16 and 20 μg of the membrane fractions were subjected to 8% PAGE. The electrophoresed proteins were transferred to PVDF membranes and hybridized with 5H5, a monoclonal antibody to the human poliovirus receptor [12], as well as a monoclonal antibody to actin as a standard reference. Actin and pvr proteins were detected by chemiluminescence. The pvr was only detected in transgenic mouse preparations (data not shown); and a higher abundance was present in the brains of TGM-PRG-3 mice relative to TGM-PRG-1 mice. The levels of pvr and actin were determined by densitometry, then graphed (Fig. 6). Since the levels of actin were similar for TGM-PRG-1 and TGM-PRG-3 protein extracts, the levels of pvr from each mouse line were compared directly. The average ratio of pvr from TGM-PRG-3 to TGM-PRG-1 brains is 2.77. This result correlates with the average three-fold difference in abundance of the PVR RNA in TGM-PRG-3 and TGM-PRG-1 tissues.

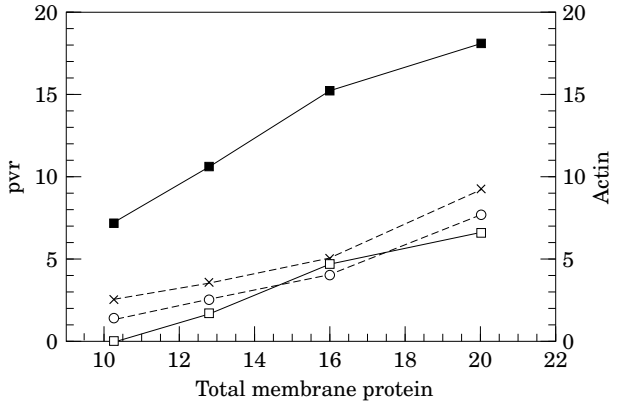

Figure 6. Comparison of pvr abundance in TGM-PRG-1 and TGM-PRG-3 mouse brains by Western immunoblots. Membrane fraction proteins of brain homogenates from male TGM-PRG-3 and TGM-PRG-1 were loaded onto 8% PAGE gels (10.24, 12.8, 16 and 20 μg). The electrophoresed proteins were transferred to PVDF filters and incubated for 1 h with monoclonal antibodies to pvr and actin. The abundances of pvr (63–67 kD) and actin (47 kD) were visualized following chemiluminescence and a 30 min exposure to Bio Max ML film. Prestained molecular weight markers (MWM, Novex) were electrophoresed to monitor the size of the bands. The amounts of pvr and actin in each lane were quantified using a densitometer, then graphed with respect to the amount of protein loaded in each lane. –■–, pvr: PRG-3; ---X---, pvr: PRG-1; –□– actin: PRG-3; ---O---, actin: PRG-1.

Susceptibility of TGM-PRG-1 and TGM-PRG-3 mice to neurovirulent type III poliovirus

Mice transgenic with the PVR gene are susceptible to infection with wild-type polioviruses

Table 1. Comparison of the susceptibility of TGM-PRG-1 and TGM-PRG-3 mouse lines to wild-type III poliovirus

Dose/mouse*	Mouse line	No. of mice affected†/No. inoculated
7.2 logs	TGM-PRG-1	10/10
6.2 logs	TGM-PRG-1	9/10
5.2 logs	TGM-PRG-1	5/10
4.2 logs	TGM-PRG-1	1/10
7.2 logs	TGM-PRG-3	10/10
6.2 logs	TGM-PRG-3	9/10
5.2 logs	TGM-PRG-3	4/10
4.2 logs	TGM-PRG-3	1/10

* Doses are in $TCID_{50}$/ml.
† Affected = clinical signs of paralysis or death; of the mice affected, 94% first developed paralysis, then died; 6% survived paralysis.
Brains of selected mice were examined for lesions characteristic of poliovirus.
PD_{50} for TGM-PRG-1 mice = 5.20 log $TCID_{50} \pm 0.73$.
PD_{50} for TGM-PRG-3 mice = 5.30 log $TCID_{50} \pm 0.73$.

when inoculated IC; while non-transgenic mice are not susceptible (5, 42; C. Weeks-Levy *et al.*, unpublished results). To compare poliovirus susceptibility of the TGM-PRG-1 and TGM-PRG-3 mouse lines, separate groups of mice were inoculated IC with four different doses of type III poliovirus wild-type Leon strain. Inoculated mice were observed daily, for up to 21 days, for clinical signs of poliomyelitis. The total number of mice affected per dose is shown in Table 1. As illustrated, TGM-PRG-1 and TGM-PRG-3 mice were sensitive to Leon virus inoculated in the CNS in a dose-dependent manner. The incidence of clinical signs (paralysis and death) increased with increasing doses of virus. Because Leon is a wild-type III poliovirus, the number of mice surviving paralysis was extremely low (6%). The proportion of mice affected at each dose was similar for the two mouse lines. Neuropathological evaluation of brain sections from the inoculated mice demonstrated the presence of degenerating neurons, or lesions characteristic of human poliomyelitis (Fig. 7). The frequency and severity of these lesions correlated positively with the respective clinical signs, i.e. the higher the dose of poliovirus, the more severe and widespread the neuropathology.

The paralytic dose (PD_{50}), or the dose at which 50% of the mice were paralysed, was calculated for the two mouse lines. The PD_{50} of TGM-PRG-1 mice was 5.20 log $TCID_{50} \pm 0.73$ and 5.30 log $TCID_{50} \pm 0.73$ for TGM-PRG-3 mice (Spearman-Karber 50% endpoints $\pm$95% confidence intervals were determined as described in [34]). TGM-PRG-1 and TGM-PRG-3 mice were compared by clinical score. No significant difference between the mouse lines was observed (>0.25), indicating that they have similar susceptibilities to wild-type III poliovirus inoculated IC.

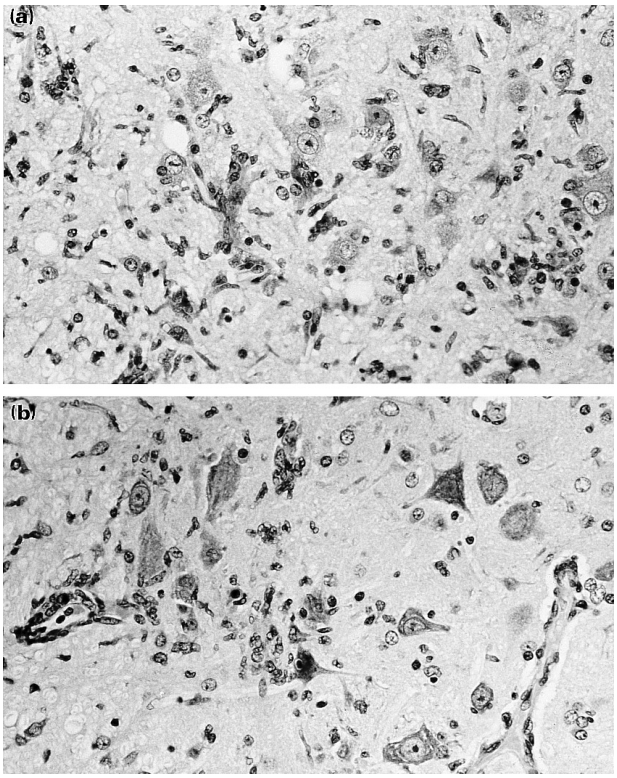

Figure 7. Neuropathology in the brains of TGM-PRG-1 and TGM-PRG-3 inoculated IC with Leon type III poliovirus. Pictured are light microscopic photomicrographs of the brainstem nuclei from TGM-PRG-3 mouse T3950209 inoculated with 7.2 logs of Leon (a) and TGM-PRG-1 mouse T10950015 inoculated with 6.2 logs of Leon (b). Both mice succumbed to disease on day 6 post-inoculation. (a) Many preserved neurons are present among damaged neurons undergoing infrequent neuronal satellitosis and neuronophagia with leukocyte/glial response in the neuropil. (b) Neuronal degeneration and loss accompanying leukocyte/glial response and vascular endothelial hyperplasia in the neuropil. Magnification of both photographs is 160 ×.

Discussion and Conclusions

The stability and expression of the PVR transgene has been studied in two TgPVR mouse

lines, TGM-PRG-1 and TGM-PRG-3. The two mouse lines differ in the transgene copy number integrated into their genomes. Ten copies are present in TGM-PRG-3 genomes and one copy is present in TGM-PRG-1 genomes. TGM-PRG-1 and TGM-PRG-3 mouse lines exhibit stable transmission of the transgene from generation to generation as determined by PCR and RFLP map analysis. PVR gene copy number differences correlate with the relative abundances of PVR RNA and protein in tissues from each mouse line. On average, the 3.3 kb PVR RNA is three-fold more abundant in TGM-PRG-3 tissues compared with TGM-PRG-1 tissues. The pvr is about three-fold more abundant in the brains of TGM-PRG-3 mice than TGM-PRG-1 mice. In addition to gene copy number, the expression levels of PVR may be affected by the transcriptional activity of the mouse genes surrounding the transgene integration site(s) in each of these transgenic mouse lines.

Most transgenic mouse tissues express PVR RNA, including those tissues in which poliovirus does not replicate [43, 44]. Since the 3.3 kb PVR RNA tissue distribution does not correlate directly with poliovirus tissue tropism, other factors must play a role in poliovirus susceptibility [45]. A productive poliovirus infection might require a particular form of the receptor. Potential differences in pvr molecules may be related to the membrane-bound and secreted isoform variations [4], degree of glycosylation [9], or other post-translational modifications. The form of the receptor which interacts with poliovirus may be specifically multimeric, heteromeric or monomeric. None-the-less, the form of the pvr molecule which facilitates a poliovirus infection is (at least) present on cells of the brain of PVR transgenic mice. In addition, pvr may not be the only host-encoded determinant of poliovirus tissue tropism [3, 21, 44]. Expression differences of host factors required for poliovirus translation and replication may exist among host cells in different tissue types. In this case, the block to poliovirus susceptibility must occur after virus binding and cell entry [46].

Our results suggest that both the TGM-PRG-1 and TGM-PRG-3 mouse lines express ample levels of functional receptor molecules necessary for efficient and effective poliovirus binding and cell entry. Therefore, all the regulatory elements required for transcription and translation of the PVR gene are present in the DNA of these transgenic mice. The data obtained by IC inoculation of poliovirus also indicate that the appearance of clinical signs of poliomyelitis in the mouse is dependent on the infecting dose of poliovirus.

Susceptibility to poliovirus inoculated IC correlates directly with PVR gene copy numbers integrated into the mouse genomes in three lines of transgenic mice previously described: ICR-PVRTg1 (10 copies), ICR-PVRTg21 (three copies) and ICR-PVRTg5 (two copies) [6]. However, when our transgenic mouse lines were inoculated with wild-type III poliovirus, TGM-PRG-3 and TGM-PRG-1 mice shared a similar susceptibility to poliovirus, despite a 10-fold higher PVR gene copy number in TGM-PRG-3 mice. Differences in genetic background between the ICR-PVRTg21, ICR-PVRTg1 and ICR-PVRTg5 mouse lines and TGM-PRG-1 and TGM-PRG-3 mouse lines may help determine susceptibility to poliovirus. This hypothesis is supported by the data obtained after inoculation of mouse-adapted polioviruses into 10 different strains of mice which showed a wide spectrum of sensitivities to poliovirus [47]. Since none of the mouse lines tested encoded the PVR gene, the difference in susceptibility to infection with poliovirus must have been due to other factors, required for replication or translation of poliovirus. Limiting levels of those host factors may exist in either C57BL6/J-derived or ICR-derived TgPVR mice, accounting for the difference in the dependence of susceptibility on receptor abundance. However, ICR-PVRTg21, TGM-PRG-3 and TGM-PRG-1 mice demonstrated nearly identical susceptibilities when challenged intraspinally with two less neurovirulent polioviruses, vaccine 3B and WHO/III reference virus (Deatly & Taffs, unpublished results).

Further experimentation is in progress to evaluate susceptibility of TGM-PRG-1 and TGM-PRG-3 mice to CNS inoculation with type III poliovirus strains of varying neurovirulence (and by other routes of inoculation) to determine if the relative susceptibilities of these two mouse lines to polioviruses can be differentiated. Preliminary data suggest that TGM-PRG-1 and TGM-PRG-3 mouse lines share similar susceptibilities to polioviruses of lower neurovirulent potential as well (Deatly & Taffs, unpublished data). In addition to 50% lethal and paralytic dose comparisons, other parameters of clinical disease and neurovirulence are under evaluation using statistical analysis to assess further the susceptibility of these transgenic mouse lines to type III polioviruses. Additional experimentation is also planned to study pvr

expression in poliovirus susceptible and resistant tissues to elucidate tissue tropism of poliovirus infections in these transgenic mouse lines.

Materials and methods

Generation of mouse lines transgenic with PVR

DNAs of PVR genomic clones, PRG1 and PRG3, were microinjected into (C57BL6/J × CBA/J) zygotes to produce founder mice [5]. The progeny from founder mice were used to establish transgenic mouse lines TgPVR1-17, TgPVR3-9, TgPVR1-7 (bred at Columbia University), and TGM-PRG-1 and TGM-PRG-3 bred at Lederle-Praxis Biologicals (now Wyeth-Lederle Vaccines and Pediatrics) or Charles River Laboratories (Wilmington, MA, U.S.A.). The TgPVR1-17 and TGM-PRG-1 mouse lines were derived from the same founder mice transgenic with the PRG1 genomic clone (pWE-PRG-1). TGM-PRG-1 mice were barrier-derived and bred under viral antigen free conditions at Charles River Laboratories. Homozygous males were bred to C57BL6/J (non-transgenic) females to produce hemizygous offspring. Hemizygous TgPVR1-17 males and females were bred together to produce a line homozygous for the transgene. The TgPVR1-7 mouse line was derived from a different founder mouse also transgenic with the PRG1 genomic clone. TgPVR3-9 and TGM-PRG-3 mouse lines were derived from the same founder mouse transgenic with the PRG3 genomic clone (pWE-PRG-3) [5]. TGM-PRG-3 mice were barrier derived and bred under viral antigen free conditions at Charles River Laboratories. Homozygous males were bred to C57BL6/J (non-transgenic) females to produce hemizygous offspring.

PRG1 and PRG3 genomic clones are each 37 kb and share 26 kb of sequence. Unique 11 kb sequences are present at the 5′ end of PRG-3 and 3′ end of PRG-1 [5]. Restriction fragment length polymorphism (RFLP) can be used to distinguish DNA from TGM-PRG-1 and TGM-PRG-3 mouse lines because of sequence differences at the 5′ and 3′ ends of their transgenes.

PCR detection of PVR in transgenic mouse genomic DNA

A rapid and sensitive PCR assay was developed to detect the PVR gene in transgenic mouse progeny. One microlitre of mouse blood was mixed with 20 μl of GeneReleaser (BioVentures, Tennessee) and the cells were lysed through a series of temperature changes in a thermocycler (65°C for 30 s, 8°C for 30 s, 65°C for 90 s, 97°C for 3 min, 8°C for 1 min, 65°C for 3 min, 97°C for 1 min, 65°C for 3 min, and 80°C soak). A two-step PCR was subsequently performed with the released DNA utilizing a pair of 22-mer primers specific to domain 1 of the PVR gene (5′ CCA CCC AGG TGC CCG GCT TCT T 3′ position 101–122; and 5′ CTC AGC GAG GCA TTC CGC AGC T 3′ position 326-305; [3] along with a pair of control primers homologous to a region of the mouse glyceraldehyde 3-phosphate dehydrogenase (G3PDH) gene (5′ TGA AGG TCG GTG TGA ACG GAT TTG GC 3′ and 5′ CAT GTA GGC CAT GAG GTC CAC CAC 3′) (Clontech, cat No. 5409-1, No. 5409-3). The PCR reaction mix contained 10 mM Tris pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM dNTP mix, 50 pmol of each primer and 2.5 units of Amplitaq (Perkin Elmer, Wilton. CT, U.S.A.). After 40 cycles of denaturation (94°C, 1 min) and annealing/elongation (72°C, 3 min), the amplified products were electrophoresed on an 1.5% agarose gel containing 50 μg/ml ethidium bromide and visualized under UV light.

Genomic DNA purification and Southern blot hybridization

Mouse genomic DNA was purified using the Genome DNA Isolation Kit (BIO 101) and then digested with *Bam*H1 overnight at 37°C. Equivalent amounts of digested genomic DNA were loaded onto 0.8% agarose gels to separate the different restriction endonuclease fragments by size. After the gel was immersed in 1.5 M NaCl, 0.4 M NaOH for at least 30 min, the DNA fragments were transferred overnight to a Zeta-Probe nylon membrane (Bio-Rad) in the presence of denaturation buffer (0.4 M NaOH, 1 M NaCl). A 1.2 kb *Xho*I DNA fragment of pSVL-H20A (representing 67% of a full length PVR cDNA; [3]) was nick-translated with α-^{32}P-dATP. After prehybridization for 2 h, membrane-bound DNA was hybridized with $2.5–6 \times 10^7$ cpm ^{32}P-H20A overnight at 65°C. If applicable, $5–6 \times 10^7$ cpm of ^{32}P-*Eco*R1/*Hind*III (1076 bp) fragment of mouse macrophage colony stimulating factor (mCSF; ATCC No. 63057) probe, and $1 \times 10^5–1 \times 10^6$ cpm of ^{32}P-1 kb ladder) were also added to the hybridization mix containing

6 × SSC, 6 × Denhardt's solution, 500 μg/ml denatured and sheared salmon sperm DNA, and 0.5% SDS. The hybridized blots were washed in 0.5% SDS, 0.3 M NaCl, 20 mM Na_2PO_4, and 0.2 mM EDTA twice at room temperature for 1 min each and twice at 45°C for 30 min each, followed by washes in 0.5% SDS, 0.015 M NaCl, 1 mM Na_2PO_4, and 0.1 mM EDTA, once at 45°C and once at 65°C for 30–60 min each.

Purification of RNA and Northern blot hybridization

Various organs from mice were removed and homogenized with a polytron homogenizer (Brinkmann Instruments, Westbury, NY, U.S.A.) in 4 M guanidine thiocyanate, 25 mM sodium citrate (pH 7), and 0.1 M β-mercaptoethanol. After homogenization, sodium lauryl sarkosyl was added to a final concentration of 2% and the samples were layered onto 1.25 ml 5.7 M CsCl cushions and centrifuged at 36 000 rpm in a SW50.1 rotor for 20 h at 20°C to pellet the RNA. The RNA pellet was resuspended in DEPC-H_2O, extracted once with equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol only, then ethanol precipitated in the presence of 0.3 M sodium acetate (pH 5.6). The precipitated RNA was centrifuged at 14 000 rpm in a microfuge at 4°C. 15 μg of total RNA was denatured with 1 M glyoxal, 50% DMSO and 5 mM NaH_2PO_4 (pH 7) for 1 h at 50°C. The denatured RNA samples were loaded onto 1.2% agarose gels containing 10 mM NaH_2PO_4 (pH 7) and electrophoresed to separate the RNA by size. The agarose gels were lightly stained with 50 ml of 10 mg/ml ethidium bromide in 500 ml 10 mM NaH_2PO_4 (pH 7) for 15–30 min to confirm the integrity of the 28S and 18 S ribosomal RNA in each RNA sample under UV light illumination. The gels were soaked in 10 mM NaOH, 0.025 M NaCl for 15 min and then the RNA was transferred to a Zeta-Probe nylon membrane (Bio-Rad, Hercules, CA, U.S.A.) overnight in the presence of 10 mM NaOH, 0.025 M NaCl. The RNA blot was rinsed in 2 × SSC for 15 min and prehybridized in 10% PEG, 5 × SSPE (0.75 M NaCl, 5 mM NaH_2PO_4 and 5 mM EDTA), and 250 μg/ml denatured and sheared salmon sperm DNA for 1–2 h, before overnight hybridization at 65°C with 2×10^7 cpm ^{32}P-H20A (1.2 kb *Xho I* DNA fragment of pSVL-H20A). The hybridized blot was washed twice in 2 × SSC, 0.1% SDS at room temperature (15–30 min), followed by two washes in 0.1 × SSC, 0.1% SDS at 50°C, 30 min each.

Detection of pvr in membrane fractions of Tg mouse brains

TGM-PRG-1 and TGM-PRG-3 male mice were first anaesthetized and perfused with PBS (Gibco-BRL, Grand Island, NY, U.S.A.) to remove the blood. The brains were homogenized in homogenizing buffer [0.025M sucrose, 1 mM EDTA pH 8, 20 mM Tris-HCl, pH 7.2 and protease inhibitor cocktail tablets (Boehringer Mannheim, Indianapolis, IN, U.S.A.)] with a polytron homogenizer (Brinkmann Instruments) to prepare a 10% emulsion (w/v). The homogenates were centrifuged at 400 × g, for 5 min (4°C), then the supernatants were centrifuged at 14 000 *g*, for 30 min at 4°C. A total of 250 μl of homogenizing buffer, plus 1% NP40, was added to the pellets, incubated on ice for 1 h, then centrifuged at 14 000 g, for 30 min (4°C). Protein concentrations of the membrane fractions (supernatants) were determined using a micro-BCA assay (Pierce, Rockford, Ill.). Various concentrations of proteins were heated at 85°C in sample buffer containing 25mM DTT for 5 min, and then loaded onto 8% acrylamide gels (Novex, San Diego, CA) for PAGE analysis. After the proteins were transferred to PVDF filters (Novex), the filters were blocked overnight in the presence of commercially prepared 1% blocking reagent (Boehringer Mannheim, Indianapolis, IN) plus 5% sheep serum (Sigma, St Louis, MO). The membrane proteins from mice of each of the two lines were incubated for 3–4h with monoclonal antibodies to pvr (1:10 hybridoma supernatant of 5H5) and actin (1:100 of mouse ascites fluid, clone AC-40; Sigma A4700) in blocking reagent with gentle shaking at room temperature. The filters were washed twice blocking with TBS containing 0.1% Tween 20, followed by 0.5% blocking reagent for 10 min each at room temperature, and then the filters were incubated with 40 mU/ml anti-mouse/anti-rabbit IgG horseradish peroxidase (HRP) (Boehringer Mannheim), for 30 min at room temperature. The filters were washed four times with TBS containing 0.1% Tween 20 for 15 min each at room temperature. The relative abundances of pvr and actin were visualized following detection using a Boehringer Mannheim Chemiluminescence Western Blotting Kit (cat No. 1520709) and a 30 min exposure to Bio-Max ML

film (Kodak, Rochester, NY, U.S.A.). The relative amounts of actin and pvr proteins were quantified using a GS-700 densitometer (Bio-Rad).

Intracranial inoculation of TGM-PRG-1 and TGM-PRG-3 mice

An animal protocol was approved by the WLVP Animal Care and Use Committee. After anaesthetization with 2.5% Avertin (Tribromoethyl Alcohol and Tertiary Amyl Alcohol; Sigma, St Louis, MO, U.S.A.) (at 0.01 ml/gram of body weight), 4–6 week old TGM-PRG-1 and TGM-PRG-3 mice were inoculated IC with 30 µl of four different doses (7.2 , 6.2, 5.2 and 4.2 log $TCID_{50}$) of Leon type III poliovirus using a 27 gauge 3/16" needle. Inoculated mice were observed daily for 21 days for lethargy, weakness, paralysis or death. The mice were euthanized when clinical signs of poliomyelitis developed or on the 21st day post-inoculation. The degree of neurovirulence was evaluated by clinical score. Mice surviving 21 days were assigned a '0'. Mice developing paresis or paralysis were assigned a '1'. A score of '2' was assigned for mice that died or were paralysed and euthanized. Clinical scores were evaluated by contingency table analysis using maximum likelihood fitting. At the time of sacrifice, CNS tissues were removed and fixed in 10% neutral-buffered formalin and processed for paraffin embedment. Sections of 6 µm were cut with a microtome and stained with Gallocyanin.

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