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IDENTIFICATION OF A UBIQUITOUS FAMILY OF MEMBRANE PROTEINS AND THEIR EXPRESSION IN MOUSE BRAIN

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Summary

A family of genes encoding membrane proteins with a unique structure has been identified in DNA and cDNA clones of various eukaryotes ranging from yeast to human. The nucleotide sequences of three novel cDNAs from *Drosophila melanogaster* and mouse were determined. The amino acid sequences of the two mouse proteins have human homologs. The gene (*TMS1*) encoding the yeast member of this family was disrupted, and the resulting mutant showed no significant phenotype under several stress conditions. The expression of the mouse genes TMS-1 and TMS-2 was examined by *in situ* hybridization of sections from brain, liver, kidney, heart and testis of an adult mouse as well as in a 1-day-old whole mouse. While the expression of TMS-2 was found to be restricted to the central nervous system, TMS-1 was also expressed in kidney and testis. The expression of TMS-1 and TMS-2 in the brain overlapped and was localized to areas associated with glutamatergic excitatory neurons, such as the hippocampus and cerebral cortex. High-magnification analysis indicated that both mRNAs are expressed in neurons. Semiquantitative analysis of mRNA expression was performed in various parts of the brain. The conservation, unique structure and localization in the mammalian brain of this novel protein family suggest an important biological role.

Key words: membrane protein, mouse, yeast, brain, expression, cDNA.

Introduction

The yeast genome project identified approximately 6500 open reading frames (ORFs), some of which encode ubiquitous proteins that are present in all phyla (Nelissen et al., 1997). Several of the identified yeast proteins have unknown functions in mammals (Supek et al., 1996; Askwith and Kaplan, 1998). The function of many other ubiquitous proteins is still unknown, and disruption of their ORF in yeast results in no identifiable phenotype. However, the marked conservation of their sequences suggests a pivotal physiological function for most, if not all, of these proteins. An extensive classification of the genes encoding putative membrane proteins has been performed (Nelissen et al., 1997). However, for those potential membrane proteins that lack a known signature, computer analysis has failed to reveal their function. Biochemical analysis and cellular distribution may help to unravel the function of these proteins in the absence of a clear phenotype in their yeast null mutants. Another available source of information may be obtained by analysis of the source of expressed sequence tags (ESTs) that are published in various databases.

We have studied several conserved systems in eukaryotic cells from yeast to human that function in a similar fashion (Nelson, 1992, 1995; Kolarov et al., 1990; Supek et al., 1996). The information gained from the study of yeast cells may help to unravel the mechanism of action of the mammalian proteins and may also shed light on their involvement in hereditary and infectious diseases (Supek et al., 1997). Most of the hydrophobic proteins that contain several transmembrane helices function as receptors or transporters. The transporters are usually much more conserved than the receptors, and members of many of the transporter families are present in bacteria, yeast, *Caenorhabditis elegans*, *Drosophila* spp. and mammals. The family of neurotransmitter transporters is one example of such a phenomenon (Nelson, 1998). The neurotransmission cycle involves two key uptake systems operating in the plasma membrane and the synaptic vesicles (Nelson, 1998). cDNAs encoding most, but not all, of the neurotransmitter transporters have been cloned in the last few years. They include two families of vesicular transporter; one functions in the uptake of monoamines and the other of γ-aminobutyric acid (GABA) into the synaptic vesicles (Liu et al., 1992b; McIntire et al., 1997). There are other vesicular transporters whose cDNA has not yet been cloned. We decided to look for transporter function in two different ways. First, we examined the possibility that the orphan neurotransmitter transporter NTT4 is the vesicular glutamate transporter (Liu et

al., 1993; Luque et al., 1996; Masson et al., 1999), and then we searched for ESTs encoding hydrophobic proteins that are distributed in areas of the brain with an enriched glutamatergic innervation. We also assumed that the vesicular glutamate transporter may be part of a conserved gene family. In looking for yeast ORFs whose homologous proteins are expressed in mammalian brain, we came across novel ORFs that encode membrane proteins with quite unusual membrane topography. Two homologous cDNAs from mouse brain were obtained and sequenced, and their expression was studied by *in situ* hybridization.

Materials and methods

Bioinformatics

The database searches for homologous proteins or EST sequences were performed using the programs 'BLAST' or 'TBLASTN', respectively (GCG-Wisconsin Package Version 10.0, Genetics Computer Group, Madison WI, USA; NCBI, National Center of Biotechnology). We assessed the percentage identity by pairwise alignment using the program Bestfit. The multiple alignment was performed using the program Pileup (GCG) and the program Boxshade to visualize the results. Assembly of the *Drosophila* and mouse genes from their respective ESTs and partial sequencing were performed using the program DNAstar (Liu et al., 1992a). Hydropathy plots and the prediction of transmembrane segments were performed using the algorithm of Kyte and Doolittle (1982) distributed by Expasy on the Internet (http://expasy.hcuge.ch).

Yeast strains

Saccharomyces cerevisiae strains W303-1b were used in this study. The haploid strain was *MATa*, *leu2 his3 ade2 trp1 ura3*. Δ*VMA10* was (*MATa leu2 his3 ade2 trp1 VMA10::URA3*) and Δ*TMS1* was (*MATa leu2 his3 ade2 trp1 TMS1::URA3*). The cells were grown on YPD medium (1 % yeast extract, 2 % bactopeptone and 2 % dextrose with 50 mmol l^{-1} Mes or Mops as buffers), and the medium was titrated to the required pH with NaOH. Plates were supplemented with 2 % agar. Minimal medium was prepared with 0.67 % yeast nitrogen base and 2 % dextrose added with the appropriate auxotrophic substances. Yeast transformation was performed as described previously (Ito et al., 1983; Elble, 1992). Yeast cells (5 ml culture volume) were grown overnight, and 0.5 ml of the cell suspension was centrifuged for 10 s at 18 000 ***g***. We added 10 µl of salmon sperm DNA (10 mg ml^{-1}) and 1 µg of plasmid DNA to the pellet. Then 500 µl of PLATE (10 mmol l^{-1} Tris-HCl, pH 7.5, 1 mmol l^{-1} EDTA, 40 % PEG 4000 and 0.1 mol l^{-1} lithium acetate) was added to the mixture. The cells were incubated with PLATE overnight and were seeded on selective plates.

Disruption of the TMS1 *gene in yeast cells*

Knockout of the *TMS1* gene was performed as follows. The full gene of *TMS1* flanked by approximately 400 base pairs (bp) from each side of the reading frame was obtained by amplification by polymerase chain reaction (PCR) of yeast genomic DNA. The gene was cloned to a commercial T/A vector pGEM-T easy (Promega, Madison, WI, USA). The gene was then cloned into a *NotI* site of the shuttle vector pYES. Disruption of the gene was conducted using the HANNAH method (Supekova et al., 1995). DNA fragments from the 5′ and the 3′ flanking regions were connected by PCR to the two ends of the *URA3* marker, and the DNA construct was used directly for transformation of the W303 wild-type strain. Yeast strains were grown on minimal medium in the absence of uracil. Colonies that grew on the selective medium were selected, checked by PCR for homologous recombination and analyzed for their phenotype. For DNA preparation, yeast cells were grown in selective medium or in YPD to the stationary phase, and the cells were centrifuged for 2 min at 2000 ***g***. STET (100 µl; 50 mmol l^{-1} EDTA, 5 % Triton X-100, 8 % sucrose and 50 mmol l^{-1} Tris-HCl, pH 8) and 0.2 g of glass beads were added to the cell pellet, and the mixture was vortexed for 20 min. Additional STET (100 µl) was added, and the mixture was boiled for 3 min, cooled for 1 min on ice and centrifuged for 10 min at 18 000 ***g*** in an Eppendorf centrifuge. A fraction (100 µl) of the supernatant was removed and placed in an Eppendorf tube containing 50 µl of 7.5 mol l^{-1} ammonium acetate. The mixture was incubated for 1 h at –20 °C and centrifuged for 10 min at 18 000 ***g***. A fraction (100 µl) of the supernatant was removed to a new tube, and 200 µl of 100 % ethanol at –20 °C was added. The mixture was centrifuged for 30 min at 18 000 ***g***, the pellet was washed with 70 % ethanol and the DNA was dissolved in 20 µl of Tris/EDTA (TE) buffer (pH 8).

Construction of HA, Flag and myc *epitope-tagged TMS proteins*

To detect the expression of mouse TMS cDNAs in tissue-cultured cells, the proteins were epitope-tagged at their C termini. The N terminus may span the membrane, and tagging this end might therefore alter the assembly of the protein in the membrane. The HA epitope tag (YPYDVPDYA) was added to the C terminus of TMS-1 by PCR, and the correct insertion of the DNA fragment was verified by DNA sequencing. For insertion of the Flag epitope tag, the TMS cDNAs were cloned in-frame by PCR into *Bam*HI and *Eco*RI sites of the pcDNA3 (Invitrogen, Carlsbad) vector that contained the Flag tag (DYKDDDDK). The same primers were also used to clone the TMS cDNA into pSecTag vector (Invitrogen, Carlsbad) containing the *myc* epitope tag (EQKLISEEDLN).

Expression in HEK 293 cells and immunofluorescence

HEK 293 cells were grown under 5 % CO_2 in glucose-rich Dulbecco's modified Eagle medium supplemented with 10 % foetal calf serum (Sigma, St Louis, MI, USA), 2 mmol l^{-1} glutamine, 25 µg ml^{-1} penicillin and 25 µg ml^{-1} streptomycin. Cells (10^5 cells per 3.5 cm plate containing coverslips coated with 0.1 % gelatin) were transfected by using a calcium phosphate kit (Sigma) with the plasmids pcDNA3 (Invitrogen) as a control, HA-tagged TMS1 in pcDNA3 and Flag-tagged

TMS2 in pcDNA3. The cells were cotransfected with green fluorescence protein pEGFP plasmid to assess the transfection efficiency (Clontech, Palo Alto, CA, USA). Two days after transfection, the cells were washed with phosphate-buffered saline (PBS) and fixed with 4 % paraformaldehyde for 30 min. After fixation, the cells were washed with TBS (50 mmol l^{-1} Tris, 150 mmol l^{-1} NaCl, 1 mmol l^{-1} $CaCl_2$, pH 7.4) and permeabilized with 0.2 % Triton X-100 for 10 min. Blocking of non-specific epitopes was achieved by incubation with 200 μg ml^{-1} normal goat anti-mouse immunoglobulin (NGG Jackson Immuno Research, West Grove, CA, USA) for 30 min. The cells were incubated with 10 μg of the antibody against HA (Babco) or Flag (Kodak) in 100 μl of TBS (placed carefully on the coverslip) for 1 h and then washed five times with 2 ml of TBS. Second antibody of *cy*3-conjugated anti-mouse IgM (0.2 μg in 0.1 ml) was added (Jackson Immuno Research, West Grove) and, after incubation for 30 min, the cells were washed three times with 2 ml of TBS. Mounting of the coverslip was performed with 29 mmol l^{-1} *n*-propyl gallate (Sigma) in Mowiol (Hoechst). Cell staining was visualized by fluorescence microscopy with the barrier filters BA515IF (green) or BA590 (red), and the cells were photographed using a digital camera (CCD SenSys photometrics). The pictures were processed using Adobe Photoshop.

Hybridization histochemistry

Adult mouse brain and spinal cord (frontal and sagittal sections), adult mouse peripheral organs (testis, kidney, heart and liver; frontal or sagittal sections) and the whole body of a 1-day-old mouse (sagittal sections) were used for *in situ* hybridization using ^{35}S-labelled oligonucleotide probes. A CO_2-anaesthetized C5BL/6 adult mouse was decapitated, and the brain was removed and immediately frozen and stored at −80 °C until use. Peripheral tissue was also collected in this way. A CO_2-anaesthetized 1-day-old mouse was also directly frozen on dry ice. Cryostat sections of the brain and organs (12 μm) were mounted on slides previously coated with 2 % 3-aminopropyltriethoxysilane in acetone (EGA Steinheim, Germany), and fixed in 4 % paraformaldehyde (in PBS, pH 7.4) for 20 min followed by three washes of 5 min each in PBS.

Four oligonucleotide probes were designed for TMS-1 and three probes for TMS-2 in regions of the cDNA that exhibit no significant homology (Genosys Biotechnology Inc., TX, USA). For analysis of TMS-1 expression, oligonucleotides corresponding to base sequences 254–313, 950–1010, 966–1025 and 1157–1216 were synthesized. For TMS-2, oligonucleotides corresponding to base sequences 328–387, 1006–1065 and 1182–1242 were synthesized. Sense oligonucleotides corresponding to the sequences at position 254–313 for TMS-1 and 328–387 for TMS-2 were also synthesized. The oligonucleotide probes were labelled at the 3′ end with [^{35}S]dATP (New England Biolabs, USA). Corresponding sense probes were also synthesized. The procedures for oligonucleotide tailing and *in situ* hybridization have been described previously (Luque et al., 1995). To analyze the signal at the cellular level, hybridized sections were exposed to film (Hyperfilm-βmax, Amersham) at 4 °C for 3 weeks, after which they were dipped in photographic emulsion. A semiquantitative analysis of the distribution of mRNA signals was performed with the aid of an image analyzer (MCID, Imaging Research, St Catherine's, Ontario, Canada). The Nissl-counterstained sections were examined with brightfield and darkfield optics using a Leica Leitz DMRB microscope and image analyzer. Data are expressed as arbitrary units derived from optical density measurements of autoradiographic films.

Results

Cloning and interruption of the yeast TMS1 *gene*

The *TMS1* gene was identified as ORF YDR105C in the yeast genome project and assigned as a potential membrane protein without known function (Nelissen et al., 1997). We synthesized two oligonucleotides flanking the ORF by 400 and 380 bp at the 5′ and the 3′ ends of the gene containing the restriction sites *Eco*RI and *Kpn*I, respectively. The gene was cloned to the same restriction sites of YPN2 plasmid (Noumi et al., 1991). The *URA3* gene was used as a selectable marker for the disruption of *TMS1*, which was performed by a method that utilizes exclusively PCR as described previously (Supekova et al., 1995). Two pieces of the DNA fragment were generated by PCR and introduced into the two sides of the *URA3* gene, as described in Materials and methods. The yeast cells were transformed with the PCR-generated DNA fragments, and the transformed cells were selected on uracil-free minimal plates. Three independent colonies were picked, grown in the appropriate minimal medium and subjected to analysis for gene interruption by PCR. The three colonies of Δ*TMS1* were shown to be lacking the part of the reading frame encoding 430 amino acid residues from the ORF starting at residue 24. The disrupted mutant Δ*TMS1* was viable and did not exhibit any major growth defect. The effects of several stress conditions on the growth of mutant Δ*TMS1* were analyzed. These included metal ions such as Co^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Ni^{2+}, Li^{+} and Cd^{2+}, temperature and osmotic stresses, and the effects of several chelating agents. We also tested several toxic chemicals, such as antimycin A and valinomycin, and uncouplers such as NH_4Cl and carbonyl cyanide *p*-(trifluoromethoxy)phenyl-hydrazone (FCCP). The effects of different carbon and nitrogen sources and oxidative stress induced by H_2O_2 were also tested. So far, we have identified no phenotype in the *TMS1* disruptant mutants.

Construction and sequencing of Drosophila *TMS1d and mouse TMS-2 from ESTs*

A search in the EST database revealed several *Drosophila* and mouse ESTs that presumably belong to new cDNAs in the respective organisms. By performing a search with each of them, it became apparent that they constitute two new cDNAs that encode proteins homologous to the yeast *TMS1*. They were denoted as mouse TMS-2 and *Drosophila* TMS1d.

Construction of the TMS-2 and TMS1d cDNAs was performed by multiple alignment of all published EST sequences. A consensus sequence of all ESTs of the respective cDNAs was deduced. Alignment of the assembled sequences suggested that there are ESTs that encode full-size cDNAs of the respective genes. To verify the newly assembled sequences, we obtained the EST clones containing the largest cDNA of each gene. The cDNA was sequenced and showed complete identity to the assembled sequences. These cDNAs were used for the cell biology studies. The sequences of TMS1d and TMS-2 were deposited in GenBank with the accession numbers AF181686 and AF181685, respectively.

The search also revealed another presumably full-size cDNA from the mouse library that was given the accession number L29441 in GenBank. Alignments of this sequence with available ESTs indicated that the published sequence was missing 270 bp from its 5′ end. The full-size EST was obtained from GenBank, sequenced and termed TMS-1. The DNA sequence of TMS-1 downstream from nucleotide 270 was identical to L29441. The sequence of TMS-1 was deposited in GenBank with the accession number AF181684.

A similar approach was used to construct the *Drosophila melanogaster* cDNA encoding TMS1d. The largest EST was ordered, and its sequence was verified by DNA sequencing. The sequencing confirmed that the EST-assembled sequence is identical to the full-size EST that was obtained. A search in GenBank failed to identify the *Drosophila* gene encoding the ESTs, but a gene encoding a homologous protein in *Drosophila virilis* was cloned, sequenced and deposited in January 1999 in GenBank with the accession number AF096709 (Hill et al., 1995). The TMS homologue is situated near the tyrosyl-tRNA gene and the failed axon connection protein (*fax*) gene.

TMS1 *is a member of a ubiquitous gene family*

We performed a homology search for yeast *TMS1* in GenBank and found three homologues, the mouse L26647, the rat TPO1 (Krueger et al., 1997) and the human Diff33 genes. Another search for homologues in the EST databases led to the identification of many ESTs with high homology, which were quite abundant in fungi, plants, *Drosophila*, *Caenorhabditis elegans* and mammals. All the ESTs belonged to genes encoding hypothetical proteins whose function is still unknown. Pairing of the homologous sequences showed a marked similarity. The results suggested that they are all members of the same gene family and that they share 30–80 % identity with mouse TMS-1 (Table 1). Human MUSTETU and rat TPO1 are the only cDNAs from this family that have been studied previously (Lebel and Mes-Masson, 1994; Krueger et al., 1997). The TPO1 protein exhibits approximately 37 % identity with both mouse TMS-1 and TMS-2, and it was identified as a member of a novel gene family (Krueger et al., 1997). The relatively low percentage identity among these rodent membrane proteins suggests that they represent the expression of three different genes of the same family. Therefore, we expect a third mouse gene highly homologous to rat TPO1 to be discovered. This idea is supported by the observed percentage identity among TMS proteins from vertebrates and invertebrates. Mouse TMS-1 showed 42 and 43 % identity with the TMS proteins from *D. melanogaster* and *C. elegans*, respectively.

Table 1. *Percentage sequence identity of some TMS family members with mouse TMS-1*

Species	Accession number	Gene	% Identity
Mus musculus	L26647	TMS-1	100
Mus musculus	AF181685	TMS-2	60
Human	L29441	MUSTETU	78
Human	U49188	Diff33	60
Caenorhabditis elegans	Z14718	tms-1	43
Drosophila melanogaster	AF181686	TMS1d	42
Rattus norvegicus	L20319	TPO1	37
Saccharomyces cerevisiae	Z47746	*TMS1*	29

The sequences were aligned pairwise with mouse TMS-1 (Accession number AF181684) using the program Bestfit.

The amino acid sequence alignment of the different members of the TMS family is depicted in Fig. 1. Two human proteins were identified; the MUSTETU protein is the homologue of mouse TMS-2, and Diff33 is homologous to TMS-1. All proteins encoded by the ORF of the TMS family encode proteins of 400–450 amino acid residues. The hydropathy plots of the TMS proteins are very similar, and the prediction of transmembrane segments for the proteins suggests 11 transmembrane helices. The N termini of the proteins are quite hydrophobic and contain a unique sequence in the first predicted transmembrane helix between amino acid residues 1 and 29. The sequence contains 6–8 cysteine residues, except in yeast Tms1p, which contains only three cysteine residues. C16, C26 and C29 are conserved in all members of the family (the last two are present in positions 20 and 21 in yeast *TMS1*). This unique feature prevents a reasonable prediction of the sidedness of the potential transmembrane helices. Nevertheless, we propose that the cysteine-rich helix 1 is present in the membrane in such a way that the first residue is present at the outer face of the membrane. Alternatively, it would stay at the cytoplasmic surface of the membrane. Apart from the yeast protein, there are few consensus potential glycosylation sites. We propose that the site near amino acid residue 34 is not utilized, either because it is present in the cytoplasm or because is too close to the membrane (Monne et al., 1998). The most likely potential glycosylation site to be utilized is the one between transmembrane segments 8 and 9. This loop faces the lumen according to our model (Fig. 2). Potential phosphorylation sites are present in the loops between transmembrane segments 3 and 4, 5 and 6, and 9 and 10. All these loops face the cytoplasm in our model. All members of the TMS family contain a conserved 9-residue sequence between transmembranes 7 and 8. The conserved sequence is PRSGLLQSS and is known to be a *myc*-type ‘helix–loop–helix’ dimerization domain signature (Suzuki et

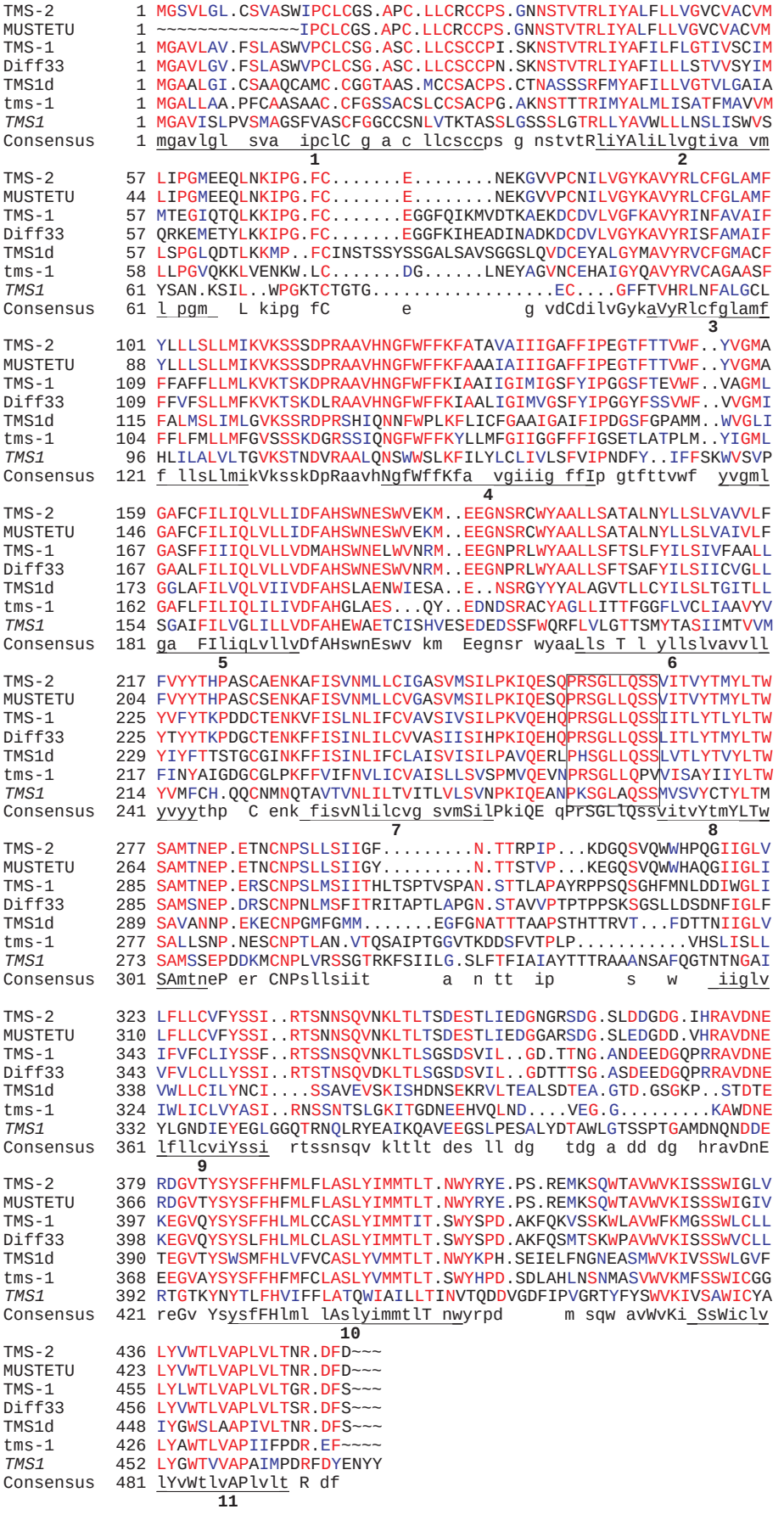

Fig. 1. Amino acid alignment of the TMS family members. The amino acid sequences of mouse TMS-1 (L26647 and present study) and TMS-2 (present study), human MUSTETU (L29441) and Diff33 (U49188), *Saccharomyces cerevisiae TMS1* protein (Z47746), *Caenorhabditis elegans* tms-1 (Z14718) and the *Drosophila melanogaster* TMS1d (present study) were aligned using the program Pileup. The predicted transmembrane domains are underlined and numbered. Red letters indicate identical amino acid residues, blue letters indicate conservative substitutions and black letters indicate different residues. The box between transmembrane segments 7 and 8 indicates the conserved sequence of the *myc*-type helix–loop–helix dimerization domain.

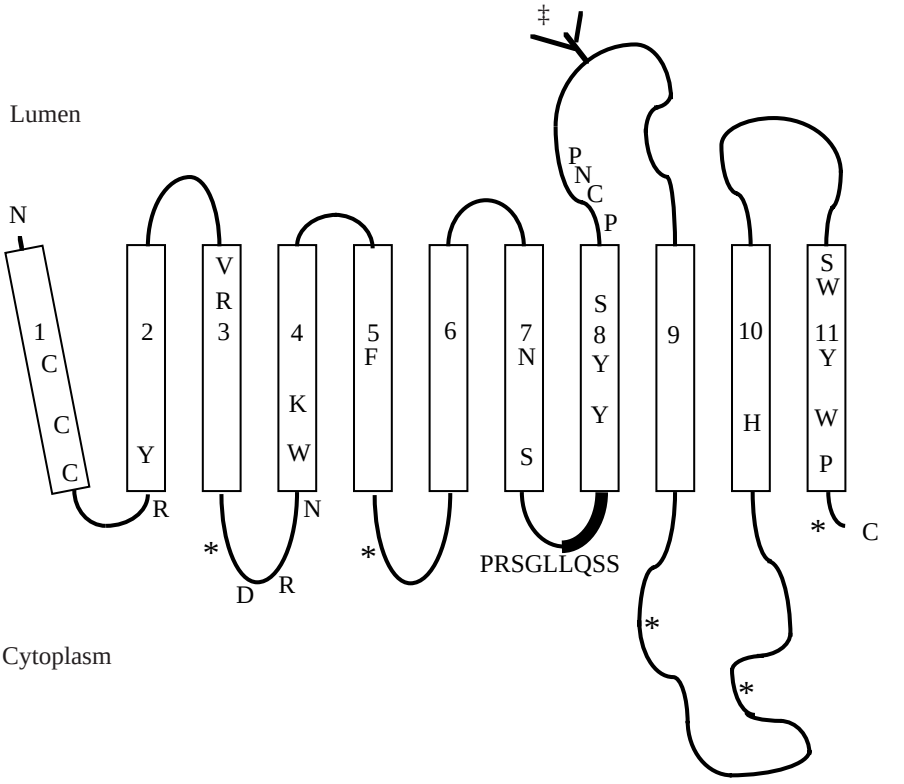

Fig. 2. The proposed membrane topography of the TMS family members. The proposal is based on hydropathy plots of each family member, on a consensus glycosylation site (‡) (excluding the yeast protein) and on potential phosphorylation sites (*). The sequence of the *myc*-type helix–loop–helix dimerization domain is also shown in a proposed cytoplasmic loop between transmembrane segments 7 and 8. The positions of some highly conserved amino acids situated in the transmembrane domains are indicated.

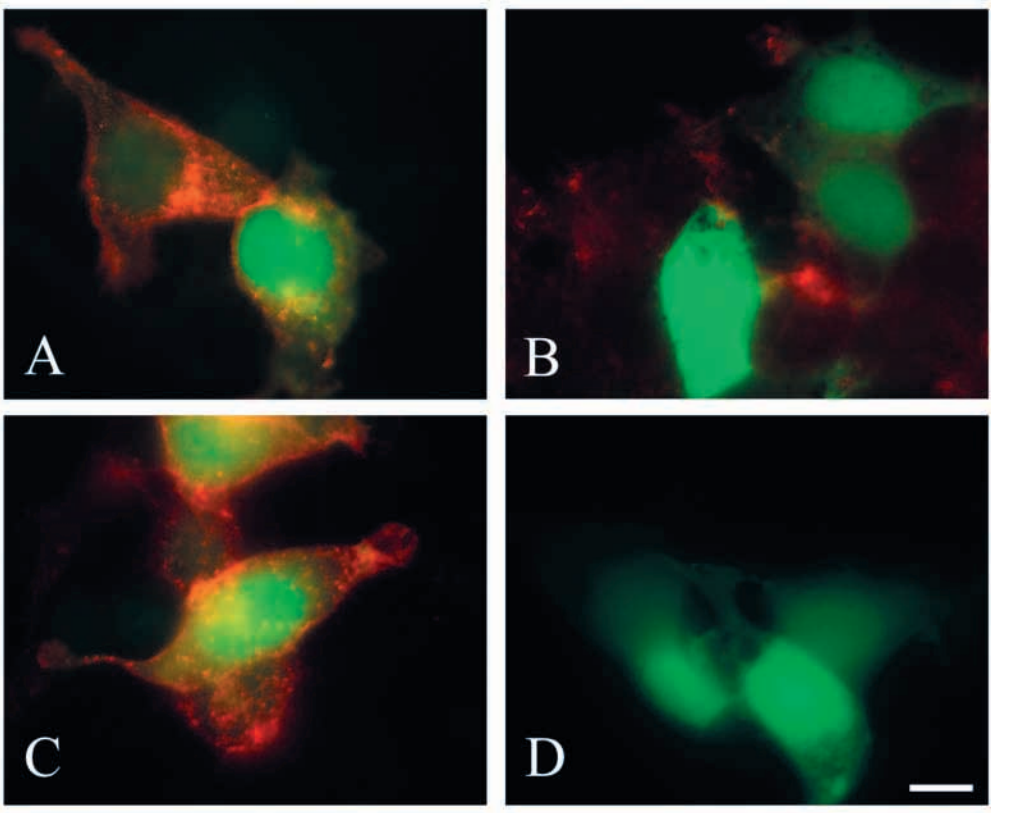

Fig. 3. Subcellular localization of mouse epitope-tagged TMS-1 and TMS-2 proteins. HEK 293 cells were transiently cotransfected with pEGFP and either TMS-1- or TMS-2-containing plasmids (A and C, respectively), or pcDNA plasmid as a control (B and D). Cells were analyzed 48 h after transfection by immunostaining for the HA-tagged protein (TMS-1) and the Flag-tagged protein (TMS-2). A secondary *cy*3-conjugated goat anti-mouse IgM antibody was used for staining the cells. (A) Cells expressing GFP (green) and HA-tagged TMS-1 (red). (B) Control cells expressing only GFP. (C) Cells expressing GFP (green) and Flag-tagged TMS-2 (red). (D) Control cells expressing only GFP. The control cells were treated in identical fashion to the TMS-expressing cells. Scale bar, 10 μm.

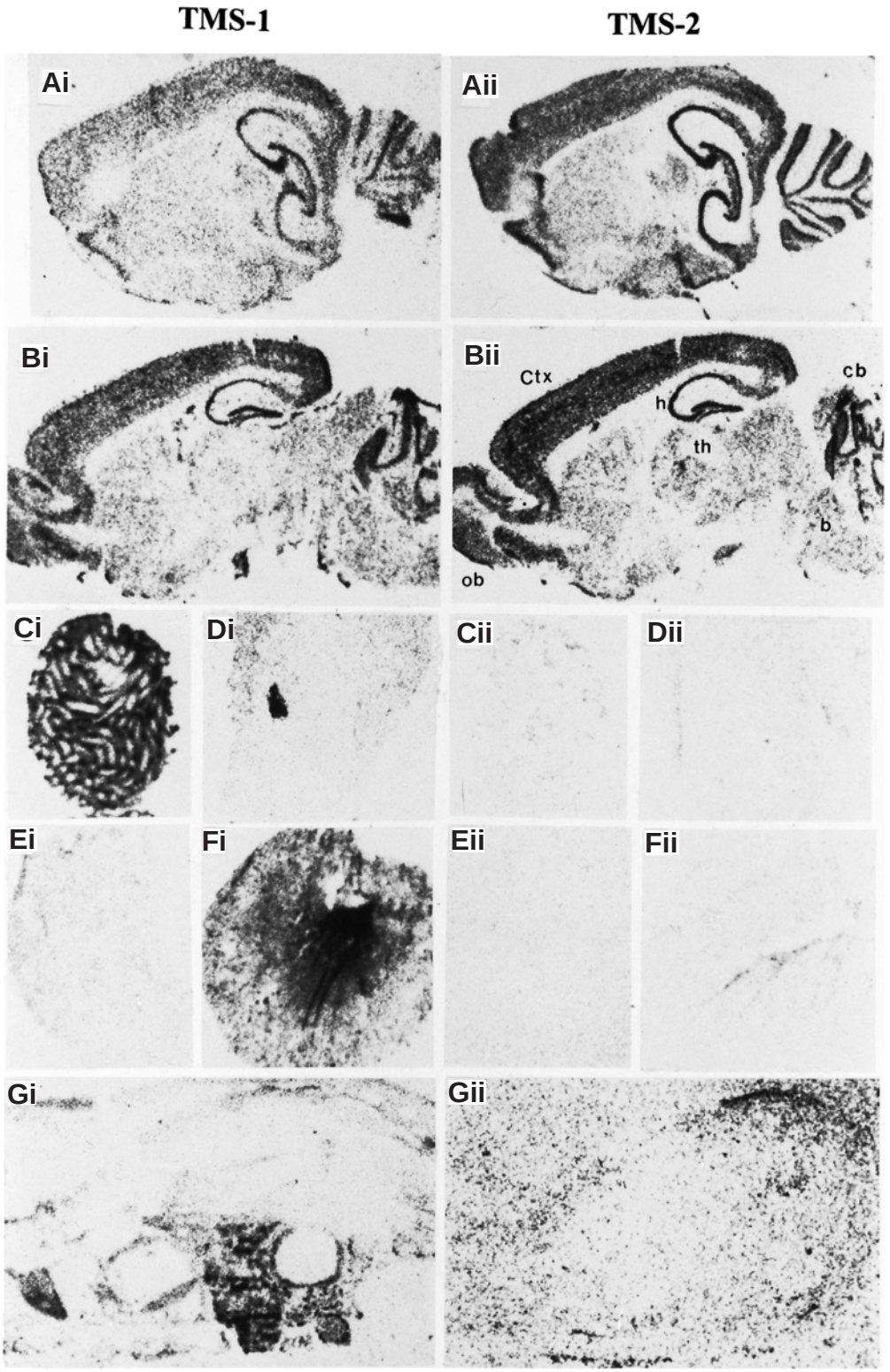

Fig. 4. Regional distribution of TMS-1 and TMS-2 mRNA in mouse brain and peripheral tissues. The experiment was performed as described in Materials and methods. (Ai,ii,Bi,ii) Parasagittal brain sections. (Ci,ii) Testis. (Di,ii) Liver (the dark spot on the liver stained with TMS-1 was identified as a tumour). (Ei,ii) Heart. (Fi,ii) Kidney. (Gi,ii) Sagittal section of a 1-day-old mouse. Brain regions are indicated in the sagittal section (Bii) of the adult mouse brain. b, brainstem; cb, cerebellum; Ctx, cortex; h, hippocampus; ob, olfactory bulb; th, thalamus.

al., 1998). The significance of this amino acid sequence in membrane proteins is not known.

Expression and subcellular localization of TMS-1 and TMS-2 in HEK 293 cells

Epitope-tagged TMS-1 and TMS-2 were expressed in HEK 293 cell lines by transient transfection with the appropriate constructs in suitable plasmids (see Materials and methods). The expressed proteins were detected by western blotting and found to be distributed in the cellular membranes (not shown). The subcellular localization of TMS-1 and TMS-2 recombinant protein in HEK 293 cells was therefore analyzed.

An HA C-terminus epitope-tagged construct for TMS-1 and a Flag epitope-tagged TMS-2 were transiently cotransfected with pEGFP plasmid. The immunofluorescence revealed that TMS-1 is predominantly present in the plasma membrane (Fig. 3A). The red fluorescence in Fig. 3B is the background with the control plasmid. The fluorescence pattern at the plasma membrane was punctate, suggesting an association with regionally restricted membrane forms or caveolae. TMS-2 exhibits a similar pattern of staining (Fig. 3C). As shown in Fig. 3, almost all TMS-positive cells were also GFP-positive, suggesting that the cells had acquired both the TMS and GFP plasmids. This result supports the feasibility of cotransfection as a method of transient coexpression of exogenous genes in the same cell. This method allowed us easily to follow and examine the morphology in live cells expressing TMS proteins. The cells expressing TMS genes showed no significant morphological changes under the conditions studied.

Tissue distribution of TMS-1 and TMS-2 mRNAs

The distribution of ESTs encoding TMS proteins in the various mouse libraries indicated their presence in the brain. In addition, TPO1 has been shown to be present in rat oligodendrocytes (Krueger et al., 1997). Therefore, we followed the distribution of TMS-1 and TMS-2 mRNAs in various regions of the mouse brain as well as in peripheral tissues. As shown in Fig. 4, *in situ* hybridization in frontal and sagittal sections of mouse brain revealed almost identical overlapping signals for the two mRNA species. The signal for both TMS-1 and TMS-2 clearly corresponds to that of the glutamatergic areas of the central nervous system (CNS), with a high level of mRNA expression being associated with the hippocampus (dentate gyrus and Ammon's horn fields CA1–CA4), the olfactory bulb, the cerebral cortex and the granule cell layer of the cerebellum. Both transcripts were colocalized and were thus expressed in identical cell populations. Both mRNAs were distributed throughout the CNS and were mostly, if not exclusively, neuronal. A high expression level was found in many types of neuronal populations such as Purkinje cells in the cerebellum, brainstem and spinal motoneurons, locus coeruleus and raphe nuclei (Table 2 and unpublished data). Higher magnifications of the grain distribution of both TMS-1 and TMS-2 mRNA in the CA3 hippocampal field are shown in Fig. 5. Although complete overlap between the two mRNA was observed, the hybridization signals for TMS-2 were much greater than those for TMS-1. The cellular distribution of TMS-2 in cortical layers I–IV of mouse brain is shown in Fig. 6. Virtually every neuron expresses the transcript, which is particularly enriched in pyramidal cell layer III. The developmental onset of TMS-1 and TMS-2 expression was determined in sections of a 1-day-old mouse (Fig. 4G). No signal was detected in the CNS; however, dorsal root ganglia appear to show low levels of expression of both TMS-1 and TMS-2 mRNA. High levels of expression of TMS-1 were also found in thymus, kidney, liver and testis. Fig. 7 shows a higher magnification of the cellular distribution of TMS-2 in the dorsal root ganglion of a 1-day-old mouse. Neuronal populations of the dorsal root ganglia are thought to use glutamate together with substance P and other neuropeptides as their neurotransmitters. In contrast to the CNS, the peripheral tissue was devoid of TMS-2 mRNA expression, but TMS-1 was expressed in some tissues: it was highly expressed in testis in a cell population surrounding the tubulus seminipherus as well as in kidney, mainly in the kidney medulla region. No mRNA signal was detected in heart or liver; however, we found that a small tumour in the liver appeared to express very high levels of TMS-1 mRNA (Fig. 4Di). TMS-2 mRNA was not detected in any of the above-mentioned tissues.

Table 2. *Distribution of TMS mRNA in selected regions of mouse brain*

Brain region	Staining intensity
Olfactory bulb	
Granule cell layer	8
Mitral cell layer	9–10
White matter	2
Striatum	
Caudate/putamen	3
Accumbens	3
Ventral pallidum	1
Diagonal band	5
Corpus callosum	0–1
Anterior commissure	1
Cortex	
Frontal	8
Parietal	8
Occipital	8
Pyriform	9
Hippocampus	
CA3	10
CA1–CA2	10
Dentate gyrus	10
Subbiculum	6
Thalamic nuclei	2–4
Deep mesencephalic nuclei	4
Inferior colliculus	5
Superior colliculus	5–6
Lateral hypothalamic area	1
Mammillary nuclei	6
Cerebellum	
Granule cell layer	8
Purkinje cell layer	8–9
Molecular cell layer	4
Central gray area	6
Pontine reticular nuclei	1
Medullary reticular fields	2–3
Vestibular nuclei	3

A semiquantitative analysis of the intensity of the mRNA signals. The Nissl-counterstained sections were examined with brightfield and darkfield optics using a Leica Leitz DMRB microscope and image analyzer.

Data are expressed as arbitrary units derived from optical density measurements of autoradiographic films.

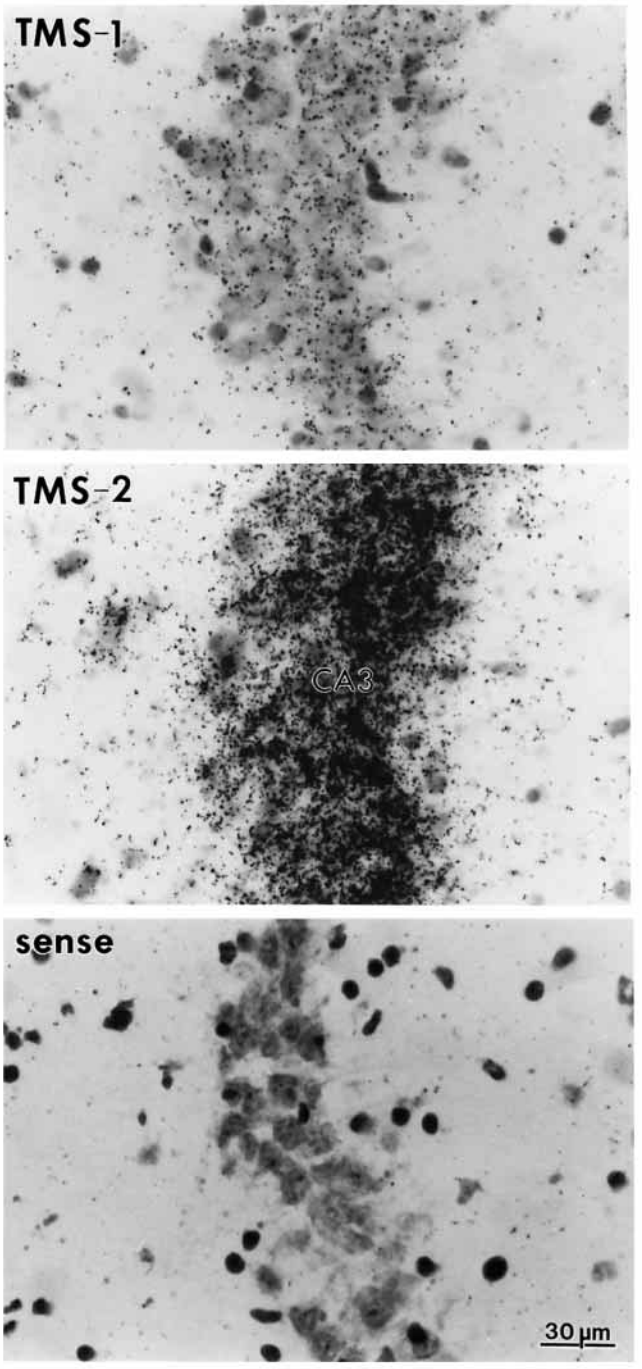

Fig. 5. Cellular distribution of TMS-1 and TMS-2 mRNA in the CA3 hippocampal field of mouse brain. Hybridization and detection were performed as described in Materials and methods. The sense probe (TMS-2) shows no signal.

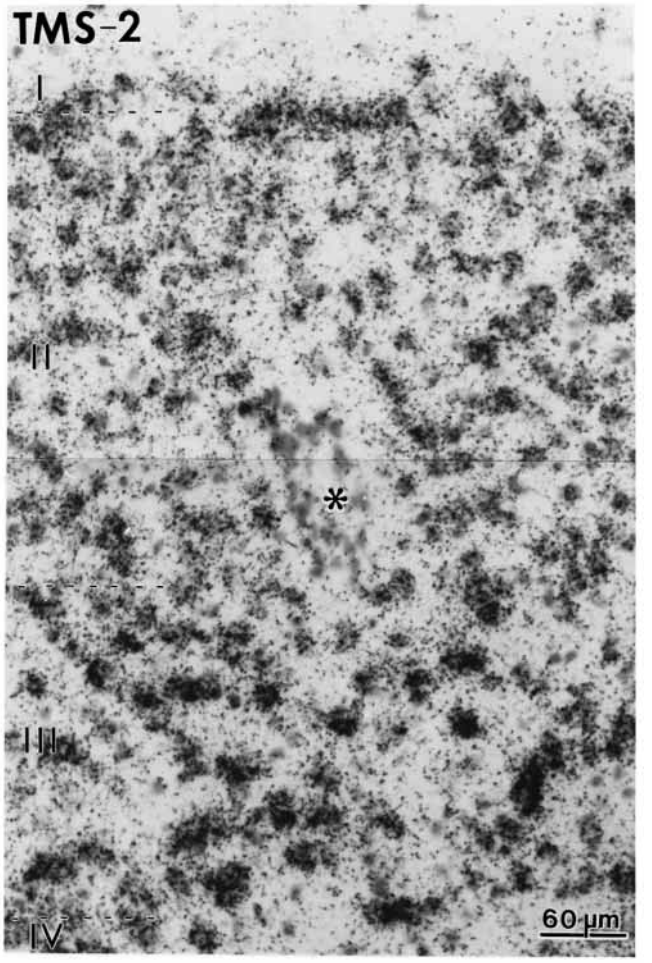

Fig. 6. Cellular distribution of TMS-2 mRNA in cortical layers I–IV of mouse brain. Hybridization and detection were performed as described in Materials and methods. The asterisk indicates a blood vessel.

Discussion

There are several approaches to predicting the events that led to the emergence of life on Earth and the subsequent evolution of all known living organisms from a common origin. One of the most fruitful approaches is to analyze conserved families of proteins and to extrapolate backwards from what we know about the present forms of life to the events that happened at the dawn of existence. Every vital life process is catalyzed by a minimal number of gene products, some of which are highly conserved through evolution. Intuitively, it was expected that the more conserved the protein, the more necessary its function is to sustain vital processes.

Recent studies that generated null mutations in several conserved and non-conserved proteins have revealed that extant living organisms are as dependent on the functionality of recently evolved proteins (non-conserved) as they are on highly conserved gene products. Moreover, vital protein complexes such as F- and V-ATPases, and complexes that are involved in DNA replication, in RNA translation and in respiration and photosynthesis, contain highly conserved subunits alongside non-conserved subunits (Nelson, 1992; Gelles and Landick, 1998; Makiniemi et al., 1999). While, in some instances, the non-conserved proteins are not necessary for the activity of the protein complex, in others each individual subunit is vital. Thus, null mutations in genes

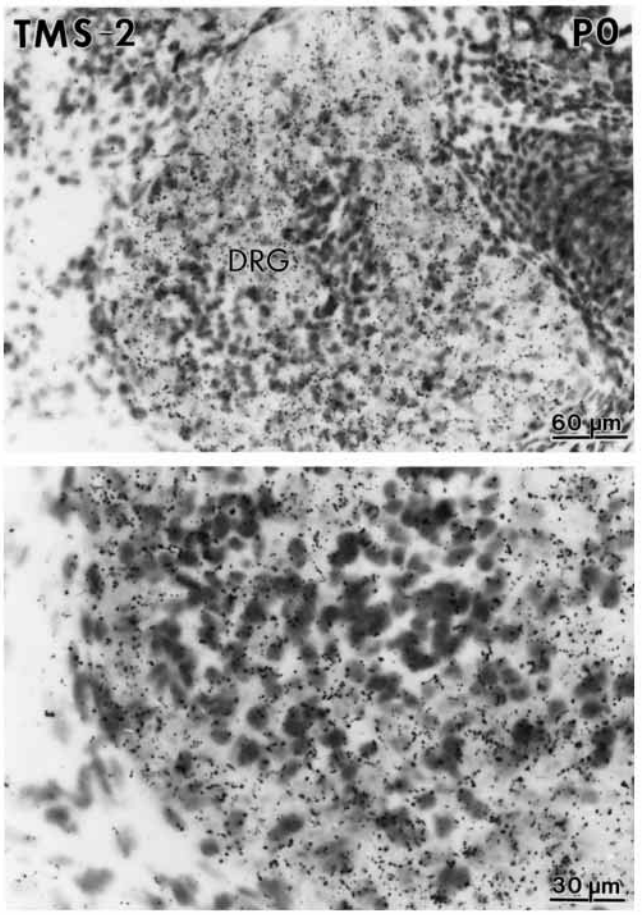

Fig. 7. Cellular distribution of TMS-2 mRNA in the dorsal root ganglion (DRG) of a 1-day-old mouse (PO). Hybridization and detection were performed as described in Materials and methods. The lower panel is at higher magnification.

encoding conserved proteins in all eukaryotes can have no apparent phenotype, yet the high level of conservation of these proteins suggests a genetic pressure that is not quite understood. The TMS family of proteins investigated in this study is one example of such a phenomenon. We interrupted the only gene (*TMS1*) encoding a member of this family in *S. cerevisiae* and the null mutant exhibited no apparent phenotype. This behaviour may be due to an alternative metabolic or transport pathway that takes over in the absence of Tms1p or a physiological adaptation that takes place during the development of the daughter cells. Nevertheless, it would be interesting to determine why this protein was conserved through eukaryotes and what kind of function it has in the living organisms that resulted in such conservation. We think that the answer to this problem may be hidden in the unique structure of the TMS proteins. In the absence of a useful phenotype, the problem cannot be solved at this stage.

We identified two mouse cDNAs encoding TMS-1 and TMS-2 with a common as well as a distinct distribution. While TMS-2 was expressed almost exclusively in the brain, expression of TMS-1 was detected in peripheral tissues such as testis and kidney. In addition, a small tumour that developed in the liver expressed high levels of TMS-1 (Fig. 4Di). In the brain, the expression of TMS-1 and TMS-2 overlapped and, even at the cellular level, could not be differentiated. High-magnification *in situ* hybridization revealed the presence of a high grain density in neurons. The brain regions that expressed TMS-1 and TMS-1 highly are rich in glutamatergic neurons (Jursky et al., 1994), and the high-resolution experiments indicated that, in some areas, almost all the neurons stained for TMS-2 mRNA (Fig. 6). Numerous attempts to detect glutamate uptake into cells expressing transfected TMS-2 *in vitro* were unsuccessful. If TMS-2 is a vesicular glutamate transporter, the failure to detect glutamate uptake may be due to the extremely low affinity of glutamate transport into synaptic vesicles (Lewis and Ueda, 1998).

A rat cDNA encoding TPO1 that exhibits 37 % identity with TMS-1 and 40 % identity with TMS-2 has been cloned, and its expression has been analyzed (Krueger et al., 1997). Membrane proteins encoded by the same genes in different rodents usually exhibit over 80 % identity in their amino acid sequences (Liu et al., 1992a,b). Indeed, the amino acid sequence of human MUSTETU is 96 % identical to mouse TMS-2, leaving little doubt that they are the same gene product. Human Diff33 shares 78 % identity with mouse TMS-1, suggesting that they are the same gene product that has undergone more rapid divergence than TMS-2 and MUSTETU. Because of the relatively low levels of identity of the amino acid sequences, we suggest that the TPO1 protein belongs to the family but is not the TMS-1 or the TMS-2 homologue; it may represent a third gene product that has not yet been identified in mouse libraries. The rat cDNA was detected by northern analysis in the lung, liver and brain and was expressed at relatively high levels in cultured oligodendrocytes (Krueger et al., 1997). Expression of genes in cultured cells frequently does not reflect their expression *in situ*. The very high levels of expression of TMS-1 in liver tumours support this notion. A search in the EST databank revealed several ESTs in libraries obtained from different tumours as well as other rapidly growing tissues such as human placenta and mouse testicular tumours (Lebel and Mes-Masson, 1994). Several genes are induced in these tissues, and the presence of mRNA encoding TMS family members gives no clue to their function. However, the primary cells utilized for detecting the expression of TPO1 may exhibit better correlation with the *in situ* situation. We intend to generate multiple gene knockouts in yeast cells and to use these mutants to determine the function of Tms1p. We hope that this will also shed light on the function of the other family members in mammalian brain and peripheral tissues.

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