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Molecular Brain Research 118 (2003) 91–101
MOLECULAR BRAIN RESEARCH
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Research report

The upstream regulatory region of the gene for the human homologue of the adhesion molecule TAG-1 contains elements driving neural specific expression in vivo

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Accepted 31 July 2003

Abstract

Cell adhesion molecules (CAMs) of the immunoglobulin superfamily (IgSF) exhibit restricted spatial and temporal expression profiles requiring a tight regulatory program during development. The rodent glycoprotein TAG-1 and its orthologs TAX-1 in the human and axonin-1 in chick are cell adhesion molecules belonging to the contactin/F3 subgroup of the IgSF. TAG-1 is expressed in restricted subsets of central and peripheral neurons, not only during development but also in adulthood, and is implicated in neurite outgrowth, axon guidance and fasciculation, as well as neuronal migration. In an attempt to identify the regulatory elements that guide the neuronal expression of TAG-1, we have isolated genomic clones containing 4 kb of the *TAX-1* upstream sequence and used them to drive the expression of the LacZ reporter gene in transgenic mice. We demonstrate that this sequence includes elements not only sufficient to restrict expression to the nervous system, but also to recapitulate to a great extent the endogenous pattern of the TAG-1 expression in the developing CNS.

Theme: Cellular and Molecular Biology
Topic: Gene Structure and Function: general

Keywords: Transgenesis; Regulatory sequences; Cell adhesion molecules; Immunoglobulin superfamily; TAG-1/axonin-1

1. Introduction

Cell adhesion molecules (CAMs) belonging to the immunoglobulin superfamily (IgSF) participate in a number of critical processes during the development of the nervous system. Throughout embryogenesis, they mediate neurite extension, axon guidance, migration, fasciculation and synapse formation, and in the adult, they participate in synaptic plasticity. IgSF proteins exhibit a restricted spatial and temporal expression profile, which requires a tight regulatory program. For this reason, they have become a focus for both in vitro and in vivo investigations in an attempt to identify neural specific regulatory elements responsible for this complex pattern of expression [10]. Previous evidence has revealed the regulatory role that Pax proteins play in the expression of the *N-CAM* and *L1* genes [18,29]. Moreover, the restricted expression of the mammalian L1 and its avian ortholog NgCAM is governed by the presence of multiple neural restrictive silencer elements (NRSE) [19–21]. Thyroid hormone has also been shown to regulate the expression of the *L1* gene in vivo [1]. Finally, structural analysis of other CAM

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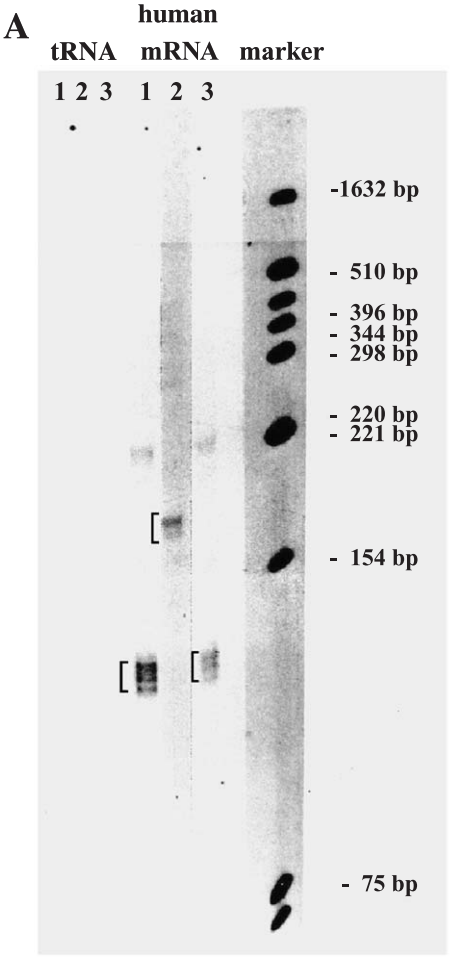
doi:10.1016/j.molbrainres.2003.07.004

promoters, such as these of the *F3* and *NB-3* genes, pointed to the presence of putative binding sites for a number of ubiquitous as well as neuronal specific transcription factors [5,27].

The rodent glycoprotein TAG-1 [9,11,12] and its orthologs TAX-1 in the human [15,48] and axonin-1 in chick [37,43] are cell adhesion molecules belonging to the contactin/F3 subgroup of the IgSF [45]. Other members of the subgroup known so far are contactin/F3/F11 [4,13,38], BIG-1, BIG-2, [55] NB-2 and NB-3 [22,33]. These proteins have common structural features: six Ig-like domains and four FN III-like repeats followed by a carboxyl terminal GPI moiety through which the molecules are bound to the cell membrane.

Although contactin/F3 subgroup proteins are functionally related, they exhibit different, developmentally regulated expression patterns. TAG-1, in particular, is expressed in restricted subsets of central and peripheral neurons [9,23], not only during development but also in adulthood, and is implicated in neurite outgrowth, axon guidance and migration [5,8,26]. Recent evidence has also implicated TAG-1 in the establishment of axon–glia interactions [46].

We have previously cloned the cDNA for the human ortholog of TAG-1 (TAX-1) and investigated some of the functional aspects of the protein, [35,48,49]. This report attempts to characterize some of the regulatory elements underlying its expression. We have isolated genomic clones containing 4 kb of the *TAX-1* upstream sequence and used them to drive the expression of the LacZ reporter gene in transgenic mice. We demonstrate that the upstream portion we employed includes elements not only sufficient to restrict expression to the nervous system, but also to recapitulate to a great extend the endogenous pattern of the TAG-1 expression in the developing CNS.



B

AP-2	GGCTGGCCT	-1232
	CGCCCCGCGGCC	-257
	GGCCCCCGAGCG	-106
	TGGCTCCGGGC	-17
	CACCCGCCACCG	42
Brn-2 (r)	TGGGATTTCTGATTTG	-463
E-box	CATCTG	-1203
	CATTTG	-717
ets-1 (r)	CTCTTCCTC	-444
GRE (r)	AGATCA	-771
	AGATCAGA	-1156
NF-kB	GGGATTTC	-429
NRSE	GGCTCGCTCCCTGGTCCTTGA	-206
PBS (r)	GGAGCA	-1042
	GGAGCA	-1031
Sp1	CCGCCC	-342
	GGCGGG	-272
	CCGCCC	-235

Fig. 1. The *TAX-1* upstream sequence. (A) For the mapping of the transcriptional start site of the TAX-1 gene, three different pieces of upstream sequence were subcloned and used for the production of radiolabeled antisense RNA. All three probes were hybridized with 0.5 μg poly(A)$^+$RNA from human brain, or 40 μg tRNA, and gave multiple RNase protection bands that corresponded to the same four nucleotides upstream of the *TAX-1* cDNA (lanes 1–3, brackets). (B) Putative binding sites for several transcription factors found in the 1.4 kb upstream sequence for the TAX-1 gene. The names of the transcription factors are indicated in the first column, while the sequence of their binding sites and their position in the 1.4 kb upstream sequence for the TAX-1 gene, in the second and third column, respectively.

2. Materials and methods

2.1. Isolation of human TAG-1 genomic clones

A portion of the *TAG-1* cDNA [12] was used as probe to isolate several genomic clones from a human EMBL4 genomic library [31] kindly supplied by Dr. Michaelidis. Portions of the clone that contained the 5′ most end of the human *TAG-1* cDNA were further subcloned and analyzed.

2.2. Computer analysis of the upstream sequence and mapping of the transcriptional start site

A total of 4 kb of genomic *TAG-1* DNA has been sequenced and deposited in Genbank under accession number X92681. This sequence includes 1.4 kb of upstream region, the first four exons, and partially intronic sequence. The upstream sequence was analyzed using the GCG software packaged and searched using the TRANSFAC database [16,50], in combination with MatInspector [36], for putative transcription factor binding sites. For the mapping of the transcriptional start site three different pieces of upstream sequence were subcloned and used for the production of radiolabeled antisense RNA [39]. RNase protection assay was carried out using standard procedures [39]. All three probes gave multiple RNase protection bands that corresponded to the same four nucleotides upstream of the *TAX-1* cDNA.

2.3. Preparation of the transgenic construct and generation of transgenic lines

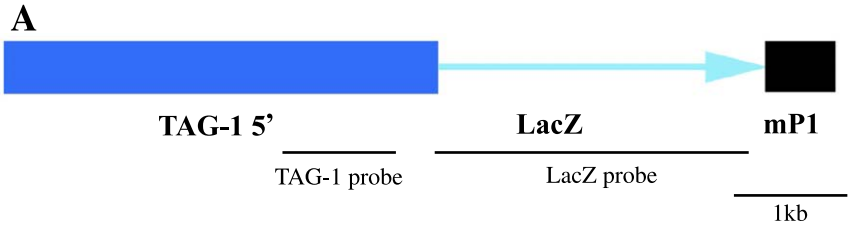
The transgenic construct was prepared as follows: a fragment containing 4 kb of upstream sequence was cloned into vector pnlacFZ [30], which contained the LacZ gene followed by a fragment containing the intron and the polyA signal from the mouse protamine gene. Transgenic mice were obtained by microinjection of the purified transgene insert at the Transgenic Facility of the Institute of Molecular Biology and Biotechnology. Potential founders were identified by Southern blotting screening of genomic DNAs obtained from tail biopsies [17] using as probes either the LacZ portion of the transgene or a fragment corresponding to the 1350 bp *Nco*I–*Sac*I portion of the human *TAG-1* upstream portion.

2.4. Analysis of transgenic animals

LacZ expression was detected by histochemical staining on 100-μm vibratome (postanatal and adult animals) or 25-μm cryostat (embryos) sections as described [28,34]. In vitro spectrophotometric assay of LacZ was performed as described [28,34].

2.5. In situ hybridization

In situ hybridization experiments were performed on 14-μm cryostat or 250-μm vibratome sections according to



B

	E14.5	E17.5	P4	P9	P13	P16	P19	Adult
Olfactory cortex	-	-	±	±	±	±	±	-
Brain	-	±	++	+++	+++	+++	+++	+
Cerebellum	-	-	++	++	++	++	++	±
Eyes	-	-	±	±	±	±	±	-
Kidney	-	-	-	-	-	-	-	-
Liver	-	-	-	-	-	-	-	-
Heart	-	-	-	-	-	-	-	-
Lung	-	-	-	-	-	-	-	-
Spleen	-	-	-	-	-	-	-	-
Intestine	-	-	-	-	-	-	-	-

Fig. 2. The construct used for the production of transgenic mice. (A) Approximately 4.0 kb of *TAX-1* upstream sequence were fused with the LacZ reporter gene followed by the intron and polyA signal from the mouse protamine gene. The two probes used for the identification of the transgenic mice are indicated. One fragment corresponds to 1.4 kb of the TAX-1 promoter region and the other is the 3.0 kb LacZ insert. (B) Expression of the transgene is restricted to the nervous system as evidenced by somatic tissue screening using in vitro spectrophotometric assay of LacZ (ONPG assay). For each age, at least two mice from the PromII transgenic line were used (−: OD_{420} < 0.1, ±: OD_{420} ~ 0.1, +: OD_{420} 0.1–0.4, ++: OD_{420} 0.4–0.8, +++: OD_{420} > 0.8).

published protocols [2,40]. The TAG-1 specific probe was produced as follows: a 650-bp PCR product was obtained using as template P7 mouse cerebellar cDNA (primers start from position 2704 to 2728 and 3347 to 3319 of the rat *TAG-1* published cDNA sequence) [12]. The fragment was subcloned into pBS and the antisense probe was obtained by *Hin*dIII digestion and T3 polymerase, while the sense with *Xho* digestion and T7 polymerase.

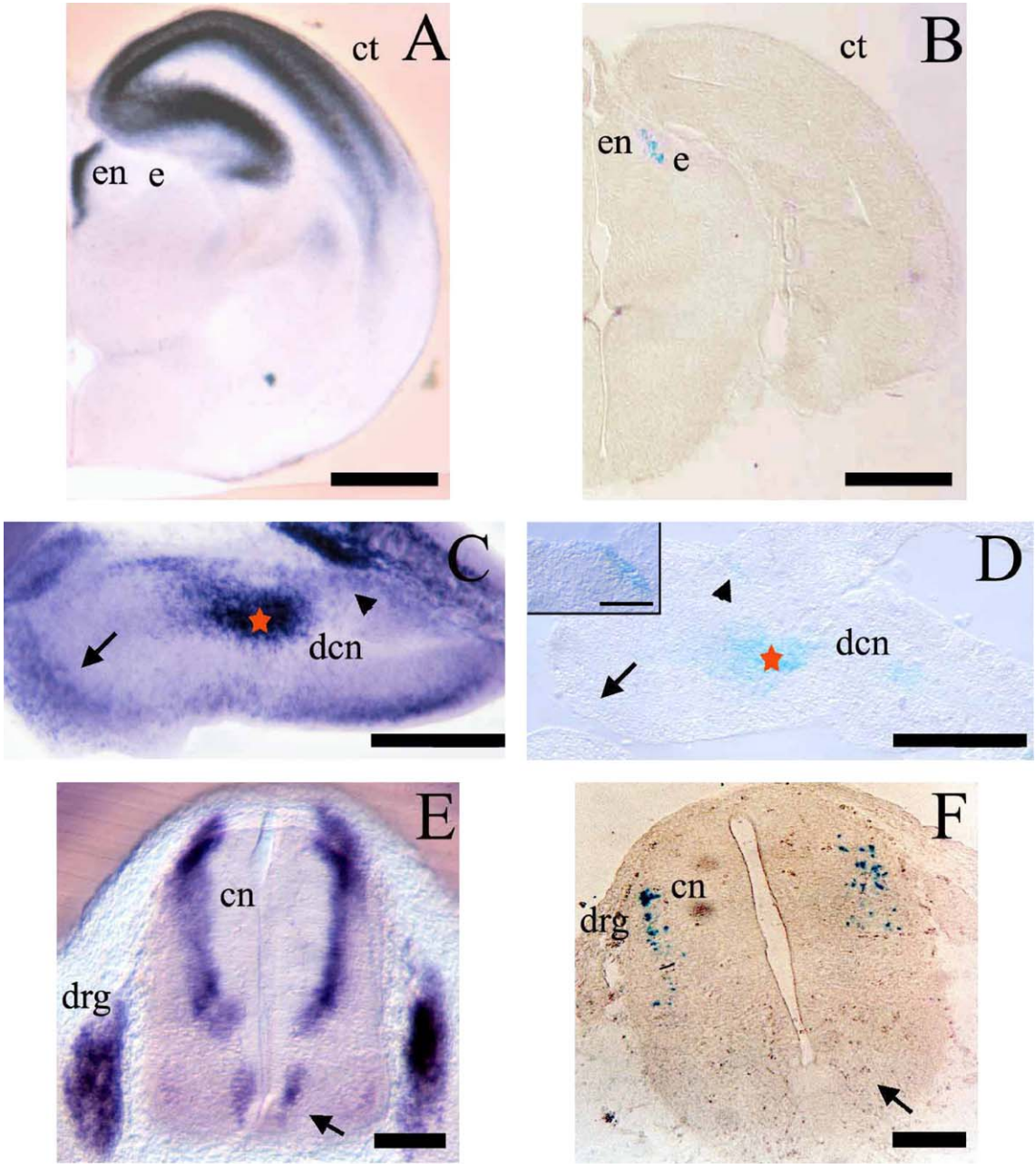


Fig. 3. Transgene and endogenous mRNA expression during embryonic development. (A, C, E) In situ hybridization for TAG-1 and (B, D, F) LacZ staining for the transgene, during embryonic development. (A) Coronal section of an E15.5 wt embryo showing TAG-1 positive cells in the cortex (ct) and the epithalamic neuroepithelium (en). (B) Transgene expression in epithalamic nuclei (e), but not in the cortex, in a coronal section from an E15.5 PromII transgenic embryo. (C) Expression of TAG-1 mRNA in the cerebellar primordium of an E14.5 wt embryo, showing staining in deep nuclei (star), the forming EGL (arrowhead) and the differentiating Purkinje cells layer (arrow). (D) LacZ expression in deep nuclei (star) and the forming EGL (arrowhead) of the cerebellar primordium, from an E14.5 PromII embryo. Inset: Higher magnification of the LacZ positive cells found in the forming EGL. (E) In situ hybridization for TAG-1 in the spinal cord of an E12.5 wt embryo reveals staining in the commissural and faintly in laterally located motor neurons as well as in the DRG. Arrow indicates postmitotic migrating neurons. (F) LacZ staining is found only in the commissural neurons of an E12.5 PromII embryo. Scale bars: A–D: 500 μm, E, F: 187.5 μm, inset: 100 μm.

3. Results

3.1. Characterization of the upstream sequence of the TAX-1 gene

The cloning of *TAX-1* genomic fragments revealed that the *TAX-1* gene covers about 40 kb of DNA and the genomic organization agrees with what has been reported previously [25] and is similar to that of the avian gene ortholog *axonin-1* [14]. The upstream sequence that was cloned is 4 kb. Of that, 1.4 kb was sequenced, and this sequence, along with a portion of the genomic organization, has been deposited in the databank under accession number X92681. The transcriptional start site was mapped by RNase protection (Fig. 1A) to a region 82–88 nt upstream of the TAX-1 cDNA previously cloned by our laboratory [48]. The RNase protection assay mapped four consecutive sites, separated by a single nucleotide. The most distal one was designated +1 in the sequence.

Analysis of the upstream sequence did not show the presence of any TATA or CCAAT boxes within the first 200 bp upstream of the start of transcription, in agreement with what has been reported for *axonin-1* [14] and the *NB-3* gene as well [27]. Computer analysis revealed a number of putative binding sites for several transcription factors that might regulate TAX-1 expression (Fig. 1B). Most putative sites were identified by comparison of the sequence against the TRANSFAC database of transcription factors [16,50]. Those include, among others, two E-boxes (recognized by b-HLH transcription factors) at nt −1203 and −717, one NF-kB binding site at nt −429, several Sp1 sites, two GRE boxes at nt −1156 and −771, one binding site for ets-1 at nt −444, and several AP-2 binding sites. Most of these elements are commonly found in promoter regions of other cell adhesion molecules such as F3 [5] and NB-3 [27].

In addition to binding sites that correspond to global transcriptional regulators, some are shared, presumably, only by genes whose expression is restricted to the nervous system. One sequence motif of particular interest is located at nt −1053 and −1042 (in the reverse orientation) and corresponds to the element identified in the *N-CAM* promoter that is recognized by *Pax* gene products [10,29]. The element that is most interesting, however, is a sequence (NRSE) that is a putative binding site for the neural restrictive silencer factor and has been identified in a number of neurally restricted genes [41]. Functional NRSE have been reported modulating NgCAM developmental expression [21] as well as for the *L1* locus where they act as both repressors and enhancers to fine tune postnatal and adult gene expression [19,20]. We identified four putative NRSE in the genomic *TAX-1* sequenced portion. One is located in the 5′ upstream sequence (at nt −206), one is in the 5′ UTR, one in the known sequence of intron 1, and one in intron 3 (nt 1458, 1590 and 3490, respectively, in the sequence deposited in Genbank) [47]. Three of these sequences are in the reverse orientation and one in the forward direction. Note that all the NRSE identified for *L1* are in the reverse orientation compared to those found in most of the other genes [21].

Finally, comparison of the 1.4-kb upstream sequence of the *TAX-1* gene with the known upstream sequence of *axonin-1* [14] reveals a conserved region of 50 nucleotides with 94% identity, in the proximal promoter region of both genes (nt 1077–1126 and 1317–1366 in the sequences deposited in Genbank for axonin-1 and TAX-1, respectively).

Preliminary in vitro experiments showed that the *TAX-1* upstream sequence cloned included elements able to direct basal levels of gene expression in HeLa and COS cells that do not normally express TAG-1 [47], suggesting that the cloned portion includes elements characteristic of a basic promoter.

3.2. In vivo analysis of the TAX-1 upstream sequence

Since there are no significant discrepancies between the human and the rodent expression profile of TAG-1 [24], we investigated whether the 4-kb upstream sequence of the *TAX-1* gene that we have cloned can produce a neuronal restricted expression pattern, and if so, to what degree this pattern recapitulates the endogenous developmental profile of TAG-1. We thus inserted the 4-kb fragment of the promoter of the *TAX-1* gene in the pnlacFZ plasmid (containing a promoterless LacZ gene upstream of a fragment of the intron and the polyA sequence of the mouse protamine gene), and this construct was used to generate transgenic mice (Fig. 2).

Seven founders were obtained, named PromI–VII. Two of the founders, PromIII and PromV, did not transmit the transgene to any of their progeny, and thus, no lines were established. When tested for LacZ expression by histochemical staining, founder PromIII did not express the transgene whereas founder PromV exhibited neuronal LacZ staining, mostly in the hippocampal and cortical regions, in a pattern similar to the remaining lines as discussed below. Five founders established lines, which were bred to homozygosity, and analyzed for LacZ expression in embryonic, postnatal, and adult stages.

Of these lines, PromI stained for LacZ only in choroid plexus in postnatal and adult animals and was not further analyzed. In the other four lines, expression was restricted to the nervous system as evidenced by somatic tissue screening

Table 1
Expression profile of *TAX-1* transgene during embryonic development

	E12.5	E15.5–E17.5
Telencephalon	–	–
Diencephalon		
Habenulla	–	+
Thalamus	–	+
Hypothalamus	–	+
Mesencephalon	–	+
Cerebellar primordium	–	+
Medulla	+	+
Pons	–	+
Spinal cord	+	+

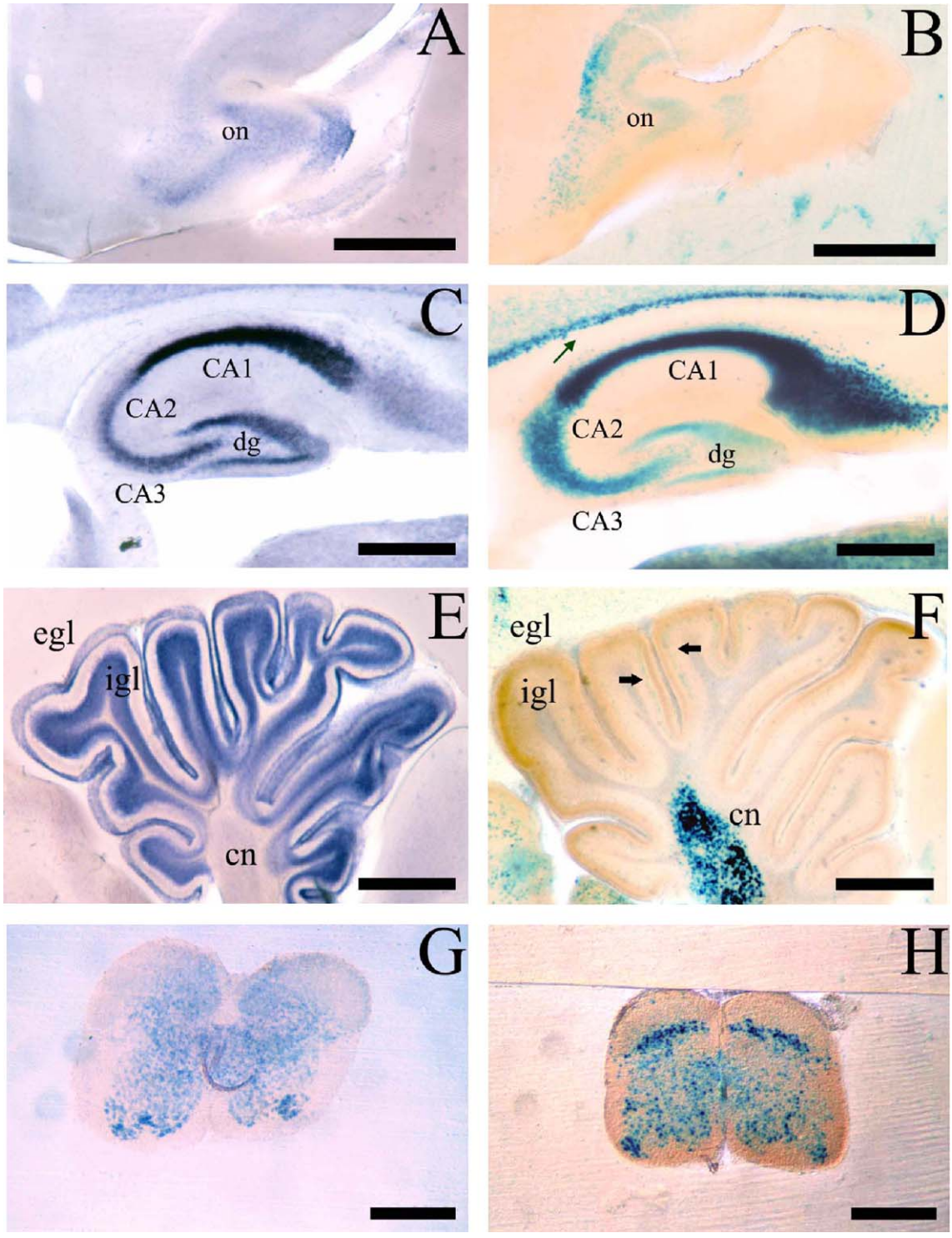


Fig. 4. Transgene and endogenous mRNA expression during postnatal development. (A, C, E, G) In situ hybridization for TAG-1 and (B, D, F, H) LacZ staining for *TAX-1* transgene, during postnatal development. Sagittal sections from P4 wt (A) and transgenic (B) PromII mouse embryos, showing TAG-1 mRNA and LacZ staining respectively, in the olfactory nucleus (on). (C) In situ hybridization for TAG-1 in the hippocampus of a P5 mouse, showing expression in the CA1, CA2, CA3 hippocampal subfields as well as in the dentate gyrus (dg). (D) The same expression profile is revealed by the transgene in the hippocampal formation of a P5 PromII transgenic mouse. Arrow indicates intense LacZ staining in layer VI of the cerebral cortex. Expression profiles of endogenous mRNA (E) and the transgene (F) in the cerebellum of P5 wt and PromIV transgenic mouse respectively. TAG-1 mRNA is expressed both in the external (egl) and internal (igl) granule layers, while in the transgenic mouse, transgene expression is found only in the upper igl. Strong transgene staining but not TAG-1 expression is also observed in the cerebellar nuclei (cn). (G, H) Expression in the postnatal spinal cord. Scale bars: A–H, 500 μm.

using whole mount LacZ staining or a spectrophotometric assay (Fig. 2B). Of all the lines analyzed, PromII showed the strongest and most extensive staining. Despite the fact that PromIV, PromVI and PromVII exhibited fewer labeled cells (see Section 4), particularly postnatally, the overall spatiotemporal distribution pattern of LacZ was found to be consistent in all the lines examined. Thus, the observed patterns of transgene expression were independent of the integration site. Because of this, the description of the transgene expression to follow will reflect findings corresponding to all four transgenic lines.

3.3. Embryonic expression

The earliest transgene expression is detected at E12.5, and it is restricted to very few areas, such as the spinal cord (commissural neurons) (Fig. 3F), the vestibulocochlear ganglion and the lateral medulla oblongata. In the following days (E13–E14), LacZ cells appear in the pontine and mesencephalic tegmentum, around the neuroepithelium of cerebral aqueduct and the cerebellar primordium (cerebellar nuclei and external granular layer) (Fig. 3D). From E15, LacZ cells appear in the diencephalon (habenula (Fig. 3B), lateral thalamus, lateral hypothalamus, subthalamus), while at E16–E17, labeled cells are also present in the medullary reticular formation, vestibular nuclei, cochlear nuclei, facial nucleus, lateral lemniscus, trigeminal nuclei, rostral pons, periaqueductal gray matter (PAG), deep layers of superior colliculus, inferior colliculus, red nucleus and occulomotor nucleus. An overview of the expression profile of *TAX-1* transgene during embryonic development is found in Table 1.

3.4. Postnatal and adult expression

Through all postnatal ages examined (P0–P17), LacZ staining is generally the same, persisting in all the regions described to express the transgene at late embryonic stages (E17.5). In addition, it is detected in the olfactory bulbs, the cerebral cortex and the hippocampal formation. In particular, LacZ expressing cells are observed in the following structures of the nervous system: In the forebrain, transgene expression is present in the anterior and less in the posterior olfactory nucleus (Fig. 4B), in layers II–III and VI of the cerebral cortex, in CA1, CA3, CA2 pyramidal layers and dentate gyrus of the hippocampal formation (Fig. 4D), in the septum (medial, lateral and triangular septal nuclei) and in the basal ganglia (bed nucleus of stria terminalis); in the diencephalon, transgene expression is detected in the epithalamus (habenula), in the thalamus (reunions, anteromedial, centromedial etc nuclei), in the metathalamus (dorsolateral and medial genuculate nuclei) and hypothalamus (lateral hypothalamic area and dorsomedial hypothalamic nucleus); LacZ staining is also observed in several mesencephalic (inferior colliculus, superior colliculus, red, occulomotor, tegmental nuclei), cerebellar (external granular layer, internal granular layer, dentate, interposed, fastigial nuclei) (Fig. 4F) and pontine structures (pontine, reticular, vestibular, cochlear, etc. nuclei). Finally, LacZ positive cells are dispersed in most regions of medulla oblongata and all over the gray matter of the spinal cord (Fig. 4H).

Upon entering adulthood, transgene expression is altered. Analysis of adult animals (2 or 3 months old) revealed a remarkably restricted LacZ staining, compared to the postnatal stage (Table 2). The main differences are observed in the diencephalon and mesencephalon, where expression of the transgene turns off completely, with the exception of the PromII transgenic line, where it persists. Minor changes are also detected in the cerebral cortex, where LacZ positive cells are fewer but still found in the II/III and VI layers. It is noteworthy that neuronal structures such as the olfactory bulbs, the hippocampal formation (Fig. 5B) and the cerebellum (Fig. 5D) continue to express the transgene upon adulthood, in agreement with the endogenous expression profile.

3.5. The transgene expression partially recapitulates the endogenous TAG-1 pattern

Although the expression pattern of TAG-1 protein has been characterized in detail [51,53,54], little information is

Table 2
Expression profile of *TAX-1* transgene during postnatal development and in the adulthood

	Postnatal	Adult
Olfactory cortex		
Anterior olfactory nucleus	+	+
Posterior olfactory nucleus	±	±
Hippocampus		
CA1	+	+
CA2	±	±
CA3	+	±
Dentate gyrus	±	±
Cerebral cortex		
Layer I	–	–
Layers II/III	+	+
Layer IV	–	–
Layer V	–	–
Layer VI	+	+
Diencephalon		
Habenulla	+	–[a]
Thalamus	+	–[a]
Metathalamus	+	–[a]
Hypothalamus	+	–[a]
Mesencephalon		
Superior colliculus	+	–[a]
Inferior colliculus	+	–[a]
Cerebellum		
Granule layer	+	+
Purkinje layer	–	–
Cerebellar nuclei	+	+
Pons	+	±
Medulla	+	±
Spinal cord	+	+

[a] Indicates that transgene expression is only seen in PromII and not in the other three lines.

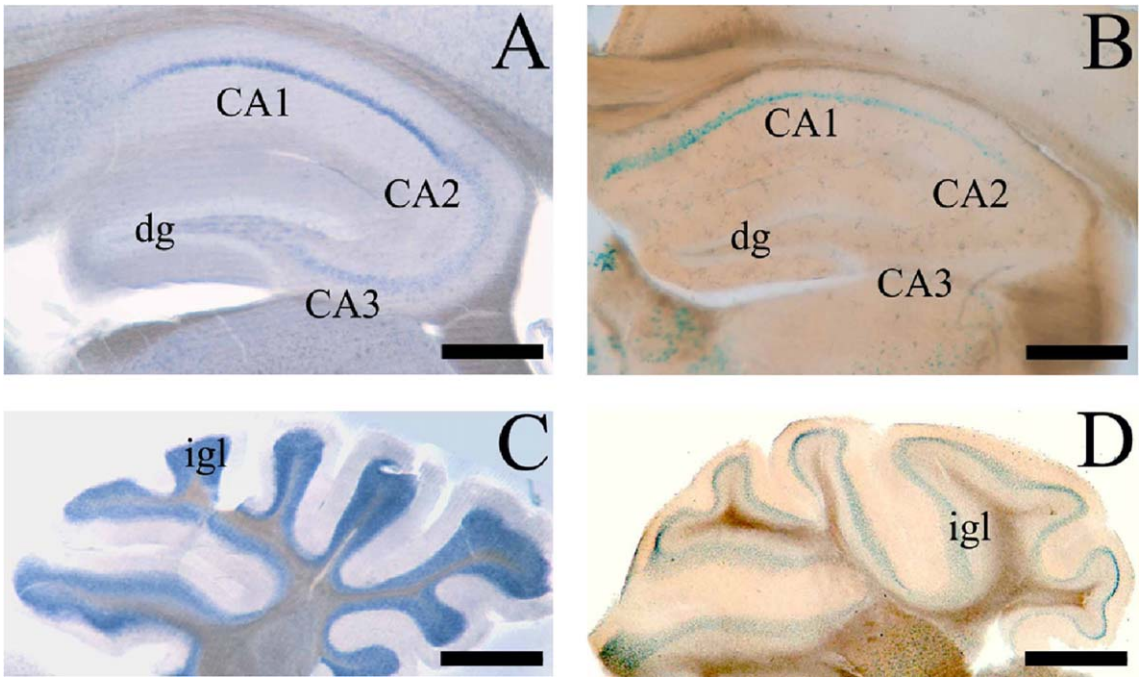


Fig. 5. Transgene and endogenous mRNA expression during adulthood. (A, C) In situ hybridization and (B, D) LacZ staining in adult wt and transgenic mice, respectively. (A) TAG-1 positive cells in the CA1 and less in CA2 and CA3 subfields of the hippocampal formation of a wt animal. (B) The transgene is expressed mainly in the CA1 subfield (PromVII). (C) In situ hybridization for endogenous mRNA in adult cerebellum of a wt mouse shows signal only in the internal granule layer (igl). (D) Transgene expression (PromVII) is also observed in the igl of an adult transgenic mouse, but only in the upper part. Furthermore, the transgene continues to be expressed in the cerebellar nuclei, but at lower intensity than in postnatal animals. Scale bars: A–D, 500 μm.

available for the TAG-1 mRNA expression profile. So far, all published studies have been focused mostly in postnatal developmental stages and in the adult [51,55], or in certain structures of the CNS during embryonic development [8,26]. In order to be able to compare transgene expression with the endogenous, we have performed in situ hybridization for TAG-1 mRNA, from E12.5 through the adult stage.

At embryonic stages E12.5–E17.5, TAG-1 mRNA signal is distributed in regions that are characterized by migrating neurons and developing axons. The earliest expression is detected at E12.5 and is restricted to a few areas, such as the vestibulocochlear ganglion, the lateral medulla, the preplate zone of the cerebral cortex, the spinal cord (commissural neurons) and the dorsal root ganglia (DRG) (Fig. 3E). At later developmental stages, TAG-1 mRNA signal is expanded to other regions of the CNS. At E14.5, for example, TAG-1 expressing cells are found in the marginal and lower intermediate zone of the cerebral cortex, in the hippocampal formation (Fig. 3A), diencephalon (Fig. 3A), mesencephalon, cerebellar promordium (deep nuclei, forming EGL, differentiating zone for migrating Purkinje cells) (Fig. 3C), pons and spinal cord.

Comparison of transgene expression to the endogenous reveals two major differences. First, while transgenic mice lack labeling in the cerebral cortex and the hippocampal formation (compare Fig. 3A with Fig. 3B), normal mice exhibit dense TAG-1 labeling in these areas from E13.5–E14 onwards. Secondly, we have not observed any LacZ staining in the dorsal root ganglia of transgenic embryos, as would be expected based on the endogenous pattern (compare Fig. 3E with Fig. 3F).

At early postnatal stages (P0–P5) TAG-1 mRNA expression is remarkably restricted. TAG-1 expressing cells are found mainly in the anterior olfactory nucleus and less in the posterior olfactory nucleus of the olfactory bulbs (Fig. 4A), in CA1, CA2, CA3 pyramidal layers and in the dentate gyrus of the hippocampal structure (Fig. 4C), in the external granule layer and less in the internal granular layer of the cerebellum (Fig. 4D) and, finally, in the spinal cord (Fig. 4G). Weaker signal is observed in the cortex (mainly in the VI layer), as well as in the brainstem (data not shown). At later developmental stages (P6–P17) and in the adulthood, TAG-1 mRNA expression is less intense but present in the olfactory bulbs, the hippocampus (Fig. 5A) and in the cerebellum (Fig. 5C) where it is observed mainly in the internal granular layer. Our observations are in agreement with previous studies [52,55].

Comparison of transgene expression to the endogenous reveals that the transgene is rather temporally misregulated, especially in the first postnatal days (P0–P5) (Table 3). In the cerebral cortex and the hippocampus, LacZ staining appears for the first time during postnatal development, in contrast to the endogenous, in which the mRNA is detected from embryonic stages and in the cortex ceases postnatally. In addition, expression in the thalamus, hypothalamus and mesencephalon persists after birth, again in contrast to the

Table 3
Comparison of the endogenous *TAG-1* with the transgenic LacZ expression, during postnatal developmental stages and in the adulthood

	Postnatal		Adult	
	Endogenous	LacZ	Endogenous	LacZ
Olfactory cortex				
Anterior olfactory nucleus	+	+	+	+
Posterior olfactory nucleus	±	±	±	±
Hippocampus				
CA1	+	+	+	+
CA2	±	±	±	±
CA3	+	+	±	±
Dentate gyrus	+	+	–	±
Cerebral Cortex	±	+	–	±
Diencephalon	–	+	–	–
Mesencephalon	–	+	–	–
Cerebellum				
Granule layer	+	+	+	±
Purkinje layer	–	–	–	–
Cerebellar nuclei	–	+	–	+
Pons	±	+	–	±
Medulla	±	+	–	±
Spinal cord	+	+	+	+

endogenous. Overall, the postnatal stage appears as a transitory state where the expression of the transgene is not very tightly regulated. However, upon entering the adult stage most neuronal populations acquire a transgene expression pattern that is most reminiscent of the endogenous distribution (with the exception of PromII; see Table 2). Table 3 summarizes the two expression patterns in the various brain regions through the postnatal and adult stages.

4. Discussion

We have isolated 4 kb of the human *TAG-1* (*TAX-1*) upstream regulatory sequences. Using RNase protection analysis, we identified four transcription initiation sites within the *TAX-1* locus. Analysis of the *TAX-1* promoter revealed that *TAX-1* lacks the TATA and CCAAT transcription initiation motifs, in agreement with the avian gene ortholog (*axonin-1*), and possesses a number of putative *cis*-regulatory elements for several transcription factors. The most interesting are: (a) a sequence motif which corresponds to the element identified in the *N-CAM* promoter that is recognized by *Pax* gene products [10,29], (b) a sequence that is a putative binding site for the neural restrictive silencer factor and has been identified in a number of neurally restricted genes [41], such as NgCAM [21] and L1 [19,20]. Apart from the *cis*-regulatory elements described in this study, it is very likely that more binding sites are present in most upstream sequences of the 4-kb *TAX-1* promoter, which we have cloned. We cannot also exclude the possibility that intronic regulatory elements, as in the case of L1, NgCAM [10] and F3, [6], are present in the uncloned portion of intron 1 that covers at least 10 kb. Such elements are likely to be present in the first intron of the *TAX-1* gene since a transgene inclusive of the first exon largely recapitulates the endogenous pattern of TAX-1 [3], although in this study, there is no information about the expression profile of this transgene in the CNS (apart from P8 cerebellum) during development, as in our study.

Analysis of the 4-kb *TAX-1* upstream sequence in transgenic mice reveals *cis*-regulatory elements that restrict LacZ expression to the nervous system. Transgene expression in the four lines examined exhibits similar spatial and temporal regulation, showing that the observed patterns of transgene expression were independent of the integration site. Nevertheless, it is noteworthy that within each line and age examined, not all sections exhibited the same number of LacZ-stained cells (data not shown). This can be attributed to the variegation phenomenon observed in LacZ transgenic animals [32]. The variegation observed could also be due to the lack of particular enhancer elements that act to increase the proportion of expressing cells and suppress the variability [44].

The spatial distribution of the *TAX-1* transgene agrees in general with the endogenous pattern, although it seems to be temporally loosely regulated. Certain areas of the CNS, such as the spinal cord, diencephalon and mesecenphalon display a persistence of transgene expression past the time point when the endogenous expression ceases. Overall, the differences between the endogenous and transgenic expression patterns can be summarized in three points: (1) Persistence of the transgene expression past the embryonic stage and in the adult, in which endogenous gene expression is not detectable, in neuronal populations of the diencephalon, mesencephalon and spinal cord. This suggests absence of a repressive element responsible for downregulating expression once it is induced. (2) Ectopic transgene expression in certain neuronal populations. The most likely explanation is the absence of NRSE such as those identified for the L1 gene [19,20]. (3) The delay in the initiation of expression in the cortex. The transgene is not expressed embryonically and turns on in the postnatal stages and persists in the adult, when the endogenous expression ceases. Again, a lack of appropriate *cis*-regulatory sequences is hypothesized. However, one point that should be considered is the report of the silencing action LacZ exerts on regulatory sequences with ubiquitous regulation [7]. Even though TAG-1 expression in vivo is very restricted and not ubiquitous, it is possible that what we observe with the limited transgene expression might be partly due to a LacZ effect. Finally, in addition to the necessary elements missing from our transgene construct, it is possible that there might be subtle differences in the transcription factors guiding TAG-1 expression in the mouse versus the human.

Despite the limitations described here regarding the reproduction of the endogenous pattern, the obtained transgenic lines are of particular interest as they display staining in specific neuronal populations. Besides analyzing the regulatory elements required for proper expression, transgenic animals for reporter genes can also be used to study

particular neuronal networks providing neuroanatomists with novel experimental tools [42].

In summary, we have cloned and characterized a portion of the upstream sequence of *TAX-1* that harbors enough regulatory information to restrict neuronal expression in vivo and to produce a spatiotemporal pattern that is consistent among the various lines. In addition, some of the elements modulating TAX-1 expression in particular cell types are also present, but their penetrance is dependent on the integration site, suggesting the absence of context-independent enhancers from our sequence. What appears to be missing is a number of regulatory elements that fine-tune the endogenous expression in the various cell types. A detailed functional characterization of the *TAX-1* locus might result in a more concise understanding of the gene's in vivo regulation and, thus, lead to better tools for cell restricted expression.

Acknowledgements

We thank Dr. Clio Mamalaki and Kostas Kourouniotis for help with the generation of transgenic animals. We also thank Katerina Athanassaki for secretarial help. This work has been supported by the European Union [contract BIO4CT980329] to D.K. and intramural IMBB funds.

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