Two murine and human homologs of mab-21, a cell fate determination gene involved in Caenorhabditis elegans neural development

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We report the cloning and genetic characterization of one human and two murine homologs of the mab-21 cell fate specification gene. mab-21 participates in the formation of sensory organs in the male nematode tail, and is essential for other developmental functions elsewhere in the Caenorhabditis elegans embryo. The expanding mab-21 gene family, which is strikingly conserved in evolution, includes two putative Drosophila members. The two mammalian genes, encoding 41 kDa nuclear basic proteins, are expressed in partially overlapping territories in the embryonic brain, eye and limbs, as well as in neural crest derivatives. Recent genetic data implicating mab-21 as a downstream target of TGF-\(\beta\) signaling, together with the distribution of mab-21 transcripts in the mouse embryo, propose these novel genes as relevant factors in various aspects of vertebrate neural development.

INTRODUCTION

The nematode Caenorhabditis elegans represents an ideal model for the study of genetic interactions occurring during embryonic development. In that system, the analysis of genetic mutants has led to the isolation of factors playing key roles in the formation of specific body structures, and the analysis of epistatic interactions has clarified their relationships with other genes and hierarchical status within specific morphogenetic pathways. The identification of the male abnormal 21 (mab-21) mutation (1) and the analysis of the corresponding gene are examples of the power of the nematode as a biological system in molecular embryology. By genetic analysis of spontaneous loss-of-function mutants, this gene has been implicated in cell fate determination events crucial to the correct formation of peripheral sense organs, named sensory rays, within the nematode male tail. Although each ray develops from an identical ray cell sublineage, each ray forms at a distinct site, has a distinct morphology and mediates specific behavioral responses during mating. Additionally, each ray must exhibit a specific cell recognition code, allowing it to assemble into separate organs. Mutations in several genes cause alteration in this combinatorial code, leading to anomalous fusions between different rays. HOM-C/Hox genes play a primary role in these processes, and mab-21 mutations act as dominant modifiers of their effects (2). In particular, mab-21 interacts genetically with an Abd-b homolog, egl-5 (2), and possibly with a Pax6 homolog, namely mab-18 (3).

Homozygous recessive mutations of mab-21 were associated with short-range homeotic transformations, resulting in three cells descended from the sensory ray 6 precursor cell adopting fates of anterior homologs (ray 4), and a fourth, linearly unrelated hypodermal cell transforming into a neuroblast. mab-21 mutations have additional pleiotropic effects affecting body shape, fecundity and motor coordination, suggesting that mab-21 plays developmental roles outside the tail region of male nematodes. The alleles leading to those changes were hypomorphic ones, whilst null alleles resulted in early embryonic lethality.

Recently published work has provided a link between mab-21 and the TGF-\(\beta\) pathway initiated by the cet-1 ligand, and transduced by a receptor heterotrimer containing SMA-6 and DAF-4, and by activator SMAD proteins SMA-2, SMA-3 and SMA-4 (4). cet-1 is virtually identical to another TGF-\(\beta\) ligand gene cloned independently and dubbed dbl-1 (5). The two ligands are homologous to TGF-\(\beta\) proteins of the decapentaplegic (DPF)/bone morphogenetic protein (BMP) class (for review, see ref. 6). Epistatic analysis indicated that the above pathway antagonizes mab-21 function, with mab-21 acting downstream of it, whereas expression data suggested that TGF-\(\beta\) interacts with mab-21 at the post-transcriptional level (4).

A human homolog of mab-21 exists (7), and has been partially characterized, especially with regard to its putative role in human neurologic and psychiatric diseases. The human MAB21L1 gene was cloned from a retinal cDNA library in a search for transcripts containing CAG triplet repeats (7). In MAB21L1, a highly polymorphic CAG trinucleotide repeat was found in the 5\(^\prime\) untranslated region (5\(^\prime\)-UTR), displaying allele variation in normal individuals. Potter (8) analyzed 928 chromosomes from controls and patients with a variety of...
neurologic diseases. The analysis of family pedigrees established that short repeats were transmitted in a Mendelian fashion, whereas longer repeats \((n \geq 45)\) displayed meiotic instability. An additional study \((9)\) confirmed that longer repeats are meiotically unstable and tend to expand, but failed to associate the expanded allele with any phenotypic abnormalities. These authors hypothesized that the CAG repeat in \(MAB21L1\) may behave as a premutation, and that longer expansions may be associated with a clinical phenotype \((9)\).

In a search for novel regulatory genes involved in embryonic brain development, our group has isolated the first murine homolog of \(mab-21\). The gene, initially labeled \(Mab21\), has been renamed \(Mab21\), in keeping with the approved human nomenclature. In a recent expression study \((10)\), we described the distribution of the corresponding transcript during embryogenesis. \(Mab21\) is expressed starting early in development at several neural and extraneural sites. More recently, we have cloned a second member of this family \((Mab21l2)\) in the murine and human genomes.

The present study describes the expanding \(mab-21\) gene family. In particular, we present the nucleotide and peptide sequences of murine \(Mab21\) and \(Mab21l2\), and of human \(MAB21L2\), and describe the phylogenetic relationships among the new and known family members, as well as their map localizations. Furthermore, we describe the comparative distribution profiles of the two murine transcripts, and analyze the subcellular distribution of the corresponding proteins.

RESULTS

Cloning and sequence analysis of \(mab-21\) homologs in human and mouse

In recent years, to isolate genes expressed within restricted domains along the antero-posterior axis of the embryonic CNS, we devised a PCR-based differential screening protocol \((11)\) subsequently utilized by our group and by others \((12–16)\) in various developmental and differentiation studies. In that study, we compared RNAs extracted from various segments along the AP axis of the mouse brain at embryonic day 13 \((E13)\). Among others, we isolated and cloned one band \((band 270)\) corresponding to a transcript mostly restricted to the basal forebrain, midbrain and hindbrain. Sequencing and database analysis identified clone 270 as the mouse homolog of \(C.elegans\) \(mab-21\) \((1;\) GenBank accession no. U19861). An additional human homolog, cloned independently \((7)\), was found in the database, containing a triplet repeat in its \(5^\prime\)-UTR \((CAGR1\), subsequently renamed \(MAB21L1)\) \((GenBank accession no. U38810)\). This gene was mapped by others to chromosome 13q13 \((7)\). We used clone 270 to screen an E11.5 whole embryo cDNA library \((Stratagene)\), and isolated three phage clones spanning a 1.8 kb contig, containing a 1.08 kb coding sequence preceded by in-frame stop codons. Whilst we provisionally dubbed the mouse gene \(Mab21\) \((10)\), the same gene was eventually renamed \(Mab21\) by the Mouse Nomenclature Committee based on homology to the human 13q13 gene \((7)\).

Later, through the analysis of the mouse EST database, we gathered evidence of a second mouse gene, that we cloned from the same E11.5 whole embryo cDNA library, using an EST sequence \((GenBank accession no. W64940)\) as a probe, and named \(Mab21l2\) \((GenBank accession no. AF149877)\). Moreover, in the human EST database, we found one cDNA sequence \((GenBank accession no. AA679158)\) homologous to the 5′ region of \(Mab21l2\). By PCR-screening with two primers designed from this sequence, we isolated four PI artificial chromosome \((PAC)\) clones \((860J22, 869K18, 869L18 and 1173I17)\) containing the human ortholog of mouse \(Mab21\), provisionally named \(MAB21L2\). We directly sequenced one of these genomic \(PAC\) clones \((860J22)\) with walking primers derived from the human EST clone \((GenBank accession no. AA679158)\). As a result, we assembled a 2050 bp genomic contig spanning the entire coding region \((1.08\) kb) and 865 bp upstream of it, containing several in-frame stop codons. Differently from \(MAB21L1\), the 5′-UTR of \(MAB21L2\) does not contain a CAG triplet repeat.

In this study, we have not characterized the promoter regions of vertebrate \(mab-21\) genes. The only genomic sequence analyzed by our group \((human MAB21L2)\) does not exhibit significant features of any recognizable promoter elements.

Both human genes \((MAB21L1\) and \(MAB21L2)\) as well as their murine counterparts present putative coding regions of 1077 nucleotides preceded by in-frame stop codons. The putative initiation codon is located in an acceptable context for initiation of translation \((17)\), with a dATP at position –3, preceded by several dCTPs, and a purine \((A)\) at position +4. We transcribed and translated in vitro both murine cDNAs and obtained single protein products for each cDNA, whose sizes were compatible with the sizes of the deduced peptide sequences \((41\) kDa). Likewise, in western analysis performed in adult neural tissues, a single 41 kDa band is detected by an anti-peptide antibody raised against the 14 amino acid C-terminal peptide of \(MAB21L1\). This antibody crossreacts with \(in\) \(vitro\) translated \(MAB21L2\) \((data\ not\ shown)\).

Given the possibility that additional \(mab-21\) family members exist in the murine and human genomes, we searched human and murine EST databases using the BLASTN and TBLASTN programs. This search failed to identify any additional \(mab-21\) homologs in the corresponding genomes. Albeit suggestive, these results cannot be taken as formal, conclusive evidence that only two mammalian \(mab-21\) paralogs exist.

In summary, the expanding \(mab-21\) family includes at least two mouse genes and two human genes \((Fig. 1)\). The phylogenetic tree constructed with the four mammalian members of the family and with \(C.elegans\) \(mab-21\), as well as the pairwise comparisons shown in Table 1 reveal that \(Mab21l1\) and \(Mab21l2\), probably ensuing from an ancestral duplication, are roughly equidistant from their \(C.elegans\) homolog.

Finally, by analyzing the \(Drosophila\) genome EST database, we identified five partial cDNA sequences assembling into two distinct clusters. Cluster 1 contains three cDNAs \((GenBank accession nos AI134107, AA941125 and AI531549)\) while cluster 2 contains two cDNAs \((GenBank accession nos AI533533 and AI532715)\). These contigs identify two new fruitfly members of the \(mab-21\) family. Their conceptual translations yield two distinct peptides \((93\ and\ 41\ residues,\ respectively)\) clearly homologous to the N-terminus of known \(MAB-21\) proteins. Albeit incomplete, these cDNA sequences suggest that two, possibly redundant, \(mab-21\)-like paralogs are present in the \(Drosophila\) genome, providing a tentative explanation of why this developmental gene was not identified in the
course of various mutagenesis screens conducted in the fly (18).

**Protein sequence analysis**

Both human and mouse genes encode 359 amino acid proteins, with predicted molecular weights of 41 kDa. The conceptually translated proteins are mostly hydrophilic and have predicted isoelectric points of 9.45. A search for similarities to known functional domains found no hits, except for several putative serine phosphorylation sites. Two of these are found perfectly conserved from man to nematode, that fits the consensus binding sequence for the SH2 domain of SEM-5 (residues 79–81) (19,20) (box 1 in Fig. 1).

**Mab21l1 and Mab21l2 map to mouse chromosome 3**

By haplotype and linkage analysis of the BSS backcross DNA panel generated and maintained at the Jackson Laboratory (Bar...
Harbor, ME) (21), we mapped the two mouse genes to murine chromosome 3 (Fig. 2). Although syntenic, the two genes are not clustered, being separated by an 11 cM genetic interval, with \( Mab21l1 \) centromeric to \( Mab21l2 \).

Mapping of \( Mab21l1 \) and \( Mab21l2 \) was made possible by the existence of \( TaqI \) and \( MspI \) polymorphisms, respectively, between the parental DNAs (C57BL/6JEi and SPRET/Ei). Such polymorphisms allowed BS heterozygotes to be distinguished from SS homozygotes among the N2 progeny of the cross. With the probes used in these experiments, the polymorphism consisted of a single SPRET/Ei-specific \( TaqI \) or \( MspI \) band, and of two C57BL/6Ei-specific \( TaqI \) or \( MspI \) fragments. The human homolog of \( Mab21l1 \) has been mapped to 13q13 (7) (confirmed in the present study, see below). Our data provide the first evidence of synteny between human 13q and murine chromosome 3.

\( MAB21L2 \) maps to human chromosome 4q31

By PCR-screening of the CEPH YAC library and by FISH, we obtained the physical map of \( MAB21L2 \) to human chromosome 4q31, a chromosomal region of known synteny to mouse chromosome 3. We isolated four human 4q31 YACs (931B5, 921C3, 769H11 and 970F2) belonging to the WC4.6 contig positive for \( MAB21L2 \). Since YACs 710G2 and 799F1, belonging to the same contig, are negative for the gene, we conclude that the physical domain containing \( MAB21L2 \) is bracketed by STS markers D4S1606 and RPS3A1.

Because of the apparent discrepancy between the mapping position of murine and human \( Mab21l1 \), while reproducing the mapping assignment in mouse, we decided to confirm the mapping of human \( MAB21L1 \), previously assigned by others to 13q13 through linkage in CEPH families and radiation hybrid mapping (7). Several human 13q13 YACs (748EG, 881F2, 927A3, 955F1, 802G8 and 903C7) belonging to the WC13.2 contig were positive for \( MAB21L1 \), while YACs 772B4 and 931A6 were negative. Therefore, the physical domain containing \( MAB21L1 \) is delimited by STS markers W15283 and IB237, from the WC13.2 contig. The map assignments for both human genes were confirmed by cytogenetic means: fluorescence in situ hybridization (FISH) done using the 860J22 PAC clone (\( MAB21L2 \)-positive) and the 802G8 and 748E4 YACs (\( MAB21L1 \)-positive) as DNA probes, confirmed their localization to 4q31 and 13q13, respectively.

\( Mab21l2 \) is expressed in the CNS and neural crest in midgestation embryogenesis

An initial analysis of \( Mab21l1 \) expression has been described previously (10). In the present paper, we characterize the distribution of the \( Mab21l2 \) transcript in depth. Three previously published images (Figs 4A and C, and Fig. 5A) of
**Mab21l1** transcript distribution in embryogenesis are re-used here, with permission, for cross-reference (10). We assayed the expression of Mab21l2 in mouse development by various means. By whole-mount in situ hybridization (Fig. 4) of E9.5 embryos, the Mab21l2 transcript (A′) was clearly visible in the dorsal midbrain, even in the posterior portion of the mesencephalon, where Mab21l1 is expressed at lower levels (A). Mab21l2 signal was also detected in the retinal primordium of the optic cup. Neither Mab21l1 nor Mab21l2 signal could be detected in the alar telencephalon, at this or later stages.

At E10 (Fig. 4B and B′), the two genes are expressed to different temporo-nasal extents in the embryonic retina and, correspondingly, to different anteroposterior extents in the mesencephalon. Mab21l1 (Fig. 4B) covers the temporal aspect of the retina and the anterior two thirds of the alar midbrain, whereas Mab21l2 (B′) is expressed in the temporal and nasal aspect of the retina, and throughout the length of the dorsal midbrain.

At E10.5 (Fig. 4C and C′) Mab21l1 and Mab21l2 transcripts could be revealed in the same territories expressing the two genes earlier on. In the eye, Mab21l1 expression keeps marking the temporal aspect of the retina, while a new expression domain appears in the lens. Extraneural sites of Mab21l2 expression include the branchial region, limb buds and intestine. In the limb buds, Mab21l2 (Fig. 4C′) marks the apical region that does not express Mab21l1 (C).

To compare Mab21l2 to Mab21l1 transcript distribution in detail, we performed radioactive in situ hybridizations of mouse tissue sections at developmental stages ranging from E10.5 to E14.5 (Fig. 5). Strong signal was seen at all sites observed by whole-mount hybridization. High expression levels could be observed in the alar mesencephalon (arrowheads in Fig. 5A–C and A′–C′) and in the rhombencephalic basal plate. At the lumbar level, Mab21l2, as well as Mab21l1, is expressed in the spinal cord. Outside the brain, the optic cup and presumptive lens also express Mab21l1 and Mab21l2 at high levels (Fig. 5E and E′). Peripherally, signal was observed for both genes in the olfactory epithelium (data not shown).

Other expression sites included the four branchial arches (that contain neural crest derivatives) and the limbs.

The main differences between the two expression profiles consisted of a strong Mab21l1 signal in the posterior third of the tectum where the Mab21l1 transcript is less abundant (arrows in Fig. 5A′–D′), and in the presumptive gut wall (Fig. 5A′ and B′) where Mab21l1 is not expressed. The intestinal wall contains post-synaptic parasympathetic ganglia of neural crest origin. On the other hand, Mab21l2 expression could not be detected in the genital ridge, where Mab21l1 mRNA is present (Fig. 5A).

**Mab21l1 and Mab21l2 remain expressed in the adult cerebellum and eye**

The Mab21l1 and Mab21l2 probes used for northern analysis were obtained from the 5′-UTR regions of both cDNAs in order to maximize specificity. Furthermore, washes were conducted at high stringency conditions (final wash: 15 min at 0.1x SSC, 0.1% SDS, 65°C). In such experimental conditions, no cross-hybridization was observed between Mab21l1 and Mab21l2. Northern analysis performed on adult mouse tissues (Fig. 6) revealed that none of 13 extraneural adult tissues examined (thymus, lung, heart, liver, spleen, intestine, kidney, adrenals, ovary, uterus, testis, prostate and muscle) expresses detectable levels of either Mab21l1 or Mab21l2 (data not shown). However, the two genes are expressed in the adult cerebellum and eye, with lower levels in the adult forebrain. Based on the amounts of actin transcript, used for normalization, it seems clear that both genes are expressed at the highest levels in the adult eye. Comparatively, Mab21l1 is expressed more abundantly in the cerebellum than Mab21l2.

In the three adult tissues that express the two genes, complex banding patterns could be observed. The Mab21l1-specific probe detects a doublet in RNA extracted from cerebellum and eye, consisting of a 3.0 kb band and a 2.85 kb band. Only the upper band of the doublet is found in forebrain RNA. The Mab21l2-specific probe detects a complex pattern in northern analysis, consisting of at least three bands in eye and cerebellum.
Figure 4. Comparison of Mab21l1 and Mab21l2 expression in the mouse embryo. Whole-mounts, whole-mount in situ hybridization of mouse embryos at 9.5 (A and A'), 10 (B and B'), and 10.5 days post-coitum (C and C'), with the Mab21l1 (A–C) and Mab21l2 (A'–C') probes. bra, branchial arches; hl, hindlimb bud; fl, forelimb bud; in, intestine; mb, midbrain; or, optic recess. The arrowhead indicates retinal expression domain; the arrow points to the hindlimb bud. 

DISCUSSION

The present paper introduces and describes three new mammalian (one human and two murine) members of the expanding gene family homologous to mab-21, a nematode gene involved in cell fate determination events required for the formation of sensory structures in the male nematode tail. Mapping experiments indicate that Mab21l1 and Mab21l2 are not clustered either in the human or in the mouse genome, and that the ancestral duplication originating the second paralog led to the generation of fully independent transcribed and cis-acting regulatory sequences.

In addition to cloning two full coding mouse cDNAs and a complete coding region in the human genome, our group, by database analysis, has also identified two potential mab-21 homologs in the Drosophila genome, that are clearly distinct from each other. This preliminary finding suggests that the developmental function encoded by mab-21 may be overspecified in Drosophila, which in turn may explain why no mab-21 mutants have been identified in the numerous mutagenesis screens conducted in that organism.

In the mouse embryonic neuraxis, both mab-21 genes are expressed in the dorsal half of the spinal cord, in a domain reminiscent of Pax3 transcript distribution (22). In vertebrae, this region contains mostly prospective sensory neurons and other neural crest precursor cells. Caenorhabditis elegans mab-21 is required for the proper patterning of sensory rays in the male tail, a process requiring dynamic changes in cell adhesion, cell fate determination and migration properties. In vertebrates, neural crest cells go through analogous changes in shape, motility and adhesive features while initiating and carrying out their migration.

In the nematode, mab-21 engages in genetic interactions with the Ahd-B homolog egl-5. Vertebrate Hox genes homologous to egl-5 include paralogs 9–13, specifying the posterior trunk and prospective lumbo–sacral segments of the axial skeleton, as well as the limbs (23,24). Besides being expressed in the spinal cord, Mab21l1 is expressed in two parallel proximodistal strips along the limb axis, whereas Mab21l2 is found in a broader domain including the apical region of the limb bud, that expresses several Hox genes (24).

Furthermore, anteriorly, mab-21 gene expression peaks at the level of the rhomboencephalon and midbrain. In the hindbrain, the mab-21 transcripts are distributed early (E12.5) in the rhombic lip, a region from which granule cell precursors migrate tangentially to form the external germinal layer of the developing cerebellum (25,26). In this mitotic territory, granule cells keep proliferating, differentiate further, and enter their radial, centripetal migration through the molecular layer to form the internal granular layer in the mature cerebellum, where expression of Mab21l1 and Mab21l2 is maintained through adulthood.
In the midbrain, \textit{mab-21} genes are transcribed throughout the thickness of the ventricular wall, and at higher levels in the mantle layer. \textit{Mab21l1} is expressed mostly in the anterior two thirds of the tectum, whereas \textit{Mab21l2} covers the entire length of the tectum.

Figure 5. Comparison of \textit{Mab21l1} and \textit{Mab21l2} expression in the mouse embryo. Tissue sections, mRNA \textit{in situ} hybridization of embryonic tissue sections at days 10.5 (A and A'), 12.5 (B, B', D, D', F and F') and 14.5 days post-coitum (C, C', E and E') with \textit{Mab21l1} and \textit{Mab21l2} probes. All sections are sagittal, rostral is on the left, except (E and E'), coronal sections of the right eye, and (F and F'), coronal sections of the caudal spinal cord. Arrows, posterior portion of the mesencephalon; arrowheads, alar mesencephalon; bra, branchial arches; cb, cerebellum; fl, forelimb bud; gr, genital ridge; in, intestine; l, lens; max, maxilla; mb, midbrain; r, retina; rh, rhomboencephalon; sc, spinal cord; t, telencephalon. (A) is reprinted from \textit{Mechanisms of Development}, 79, Mariani, M. \textit{et al}., \textit{Mab21}, the mouse homolog of a \textit{C.elegans} homeotic regulator, participates in cerebellar, midbrain and eye development, pp. 131–135, © 1998, with permission from Elsevier Science (10).

Figure 6. \textit{Mab21l1} and \textit{Mab21l2} remain expressed in the adult CNS and retina. Northern blot analysis of \textit{Mab21l1} and \textit{Mab21l2} transcript levels (A) conducted on total RNAs extracted from adult mouse forebrain, cerebellum and eyes. Actin mRNA levels (B) are shown for normalization. See text for discussion of banding patterns.

Figure 7. MAB-21 proteins localize mainly in the nucleus. Cellular immunofluorescence analysis of NIH 3T3 cells transfected with \textit{Mab21l1} (A) and (B) and \textit{Mab21l2} (C) and (D) cDNA constructs encoding N-terminally myc-tagged mouse MAB-21 proteins. The transfected cell (B) displays dual localization to the nucleus and cytoplasm. Similar distributions were observed in cells expressing high levels of the MAB21L2 protein. Fixed cells were immunostained with a monoclonal anti c-myc antibody. Cells shown in the figure are representative of >100 observations.

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of the dorsal midbrain. The temporal half of the retinal ganglionic layer projects retinotectal fibers to the anterior half of the tectum, whereas the nasal side of the retina projects fibers to the posterior half of the tectum (for review, see ref. 27). Thus, the territories expressing Mab21l1 in the retina correspond to those expressing it in the tectum. Likewise, Mab21l2, being expressed further ventrally in the retina, covers a greater extent of the tectum along the anteroposterior axis. It is therefore tempting to speculate that mab-21 genes and their combinatorial code may play a role in the proper retinotectal migration of ganglion cell axons.

mab-21 genes are also expressed in the forebrain. In the diencephalon, the main site of expression observed in early embryogenesis corresponds to the optic recess/cup which gives rise to the prospective retina and lens.

In regard to expression sites observed outside the central nervous system, those in the branchial arches and dorsal root ganglia are consistent with a possible involvement of vertebrate mab-21 in the specification/differentiation of neural crest derivatives. The striking expression levels of Mab21l2 in the embryonic gut may reflect a role in the maturation of post-synaptic neurons in parasympathetic ganglia, that derive from the cephalic and caudal crest. Extraneural signal for Mab21l1 is also present in the genital ridge, a territory specified by gradients of homeobox gene expression (28).

Both mammalian mab-21 homologs described in this study encode 41 kDa proteins containing putative protein kinase sites, to suggest that MAB-21 proteins may be part of a signaling cascade, and their function may be modulated post-translationally, via serine/threonine or tyrosine phosphorylation. Recent data obtained in the nematode by epistatic analysis suggest that MAB-21 function is antagonized by TGF-β signaling at the post-transcriptional level (4). Thus, the TGF-β cascade may inactivate MAB-21 proteins by phosphorylating them, or by regulating molecular interactions of MAB-21 with other intracellular members of the pathway.

Our data suggest that mammalian MAB-21 proteins are localized predominantly, albeit not exclusively, in the nucleus. Indeed, the analysis of transfected cells expressing high levels of MAB-21 proteins clearly reveals that a quota of the protein localizes in the cytoplasm. This may be due either to saturation of the nuclear localization machinery because of the high intracellular protein levels, or to a regulated entry in the nucleus. The latter, in turn, may be linked to specific stages in the cell cycle, or modulated by signaling pathways, possibly through protein–protein interactions or post-translational modifications.

Although mosaic analysis in C. elegans provides genetic evidence of a non-cell-autonomous activity of the mab-21 gene (1), we have found no evidence to support the notion that the corresponding proteins, devoid of a canonical leader peptide, are secreted by the cell. Thus, their likely role is in the context of pathways transducing signals from the membrane to the nucleus, or vice versa.

In summary, the expanding mab-21 family contains two murine and two human homologs, as well as likely homologs in Drosophila melanogaster. The gene family exhibits striking phylogenetic conservation from man to nematode. Genetic and functional analysis in vivo and in vitro is underway to determine the role of these factors, and their mode of interaction with regulatory pathways active in CNS development.

MATERIALS AND METHODS

General procedures

Standard molecular techniques, including nucleic acid purification, restriction analysis, gel electrophoresis, DNA ligation, cloning, subcloning, dye-sequencing, probe radiolabeling, northern and Southern analysis, and library screening were performed according to established protocols (29). Automated sequencing with Dyedexx primers or Dyedexx terminators was performed on an ABI 373 machine. Hybridizations of northern and Southern blots (Pall) were performed at 65°C in 125 mM sodium phosphate (pH 7.2), 250 mM NaCl, 7% SDS, 10% PEG. Filters were washed at 65°C to final stringencies of 0.2× SSC for 10 min. Phage plaque hybridizations and subsequent washes were carried out under comparable stringency conditions.

Experimentation on animal tissue conformed to the stipulations of the Institutional Animal Care and Use Committee of the San Raffaele Scientific Institute, Milan, Italy.

Tissue preparation and RNA extraction

Preparations of embryonic CNS were done as follows: under a dissection microscope, brain tissue was separated from surrounding mesoderm and non-neural ectoderm. Embryonic hindbrain, midbrain and forebrain territories from two CD1 litters were separated and pooled. Fresh tissue preparations were lysed in guanidinum isothiocyanate. RNA extraction was carried out on a cesium chloride gradient (29).

RNA fingerprinting

Clone 270 was derived through a modification (11) of the RNA fingerprinting protocol (30) from the RNAs extracted as detailed above. RNA fingerprinting was conducted as follows: a reverse transcription reaction was carried out using a (dT)16 primer on total RNAs extracted by the cesium chloride method (29) and digested with 4 IU DNase I/kg total RNA. Radioactive PCR reactions, in duplicate, were performed from 1 μl of each RT reaction in 50 μl final volume with an arbitrary 12mer (DR34 sequence: 5′-GAC GAG GCT GGA) (final concentration 4 μM). PCR conditions were 3 min at 94°C, 2 min at 80°C at which Taq polymerase was added (hot start), followed by 35 cycles of 1 min at 94°C, 1 min at 50°C, 30 s at 72°C, with a final elongation step of 5 min at 72°C. [α-32P]dCTP (0.1 μl) was added to each reaction. Amplified products were separated on a 5% denaturing acrylamide gel and visualized by autoradiography. Differentially displayed bands were cut from the gel and eluted. The bands were reamplified using the same 12mer primers and blunt-end cloned into pBluescript II SK+ (Stratagene, La Jolla, CA) as described (31).

Sequence analysis

Data bank searches (GenBank, GenEMBL, SwissProt and PIR) were run through the BLAST server (32). Additional sequence analysis and contig assembly was done using the MacVector program (Oxford Molecular, Oxford, UK) and the Sequencher program (Gene Code, Ann Arbor, MI), respectively.
Genetic mapping was performed on 96 DNAs, corresponding to the parental genotypes 94 N2 progeny of a (C57BL/6j × SPRET/Ei)F1 × SPRET/Ei (BSS) backcross generated and distributed by the Jackson Laboratory (Bar Harbor, ME) (21). A TaqI RFLP was identified, as described above. Its segregation was followed, and linkage analysis performed, with the MapManager v.2.6 program (Jackson Laboratory) (33).

**FISH mapping**

Chromosome preparations were hybridized in situ with probes labeled with biotin nick translation, essentially as described (34), with minor modifications. Briefly, 500 ng of labeled probe were used for the FISH experiments; hybridization was performed at 37°C in 2× SSC, 50% (v/v) formamide, 10% (w/v) dextran sulfate, 5 µg COT1 DNA (Boehringer Mannheim) and 3 µg sonicated salmon sperm DNA, in a volume of 10 µl. Post-hybridization washing was three times at 42°C in 2× SSC–50% formamide followed by three washes in 0.1× SSC at 60°C. Biotin-labeled DNA was detected with Cy3-conjugated avidin (Amersham, Uppsala, Sweden). Chromosome identification was obtained by simultaneous DAPI staining that produces a Q-bandining pattern. Digital images were obtained using a Leica DMRXA epifluorescence microscope equipped with a cooled CCD camera (Princeton Instruments, Princeton, NJ), Cy3 and DAPI fluorescence signals, detected using specific filters, were recorded separately as grey scale images. Pseudocoloring and merging of images were performed using the Adobe Photoshop software.

In situ hybridization of mouse tissue sections

Radioactive in situ hybridization (ISH) (35) was carried out as follows: 7 µm paraffin serial sections from a single embryo were placed in four to six adjacent series: two alternative alternative series were used for each probe. Embryos were studied at 10.5, 12.5 and 14.5 days of embryonic development. Slides were deparaffinized in xylene, rehydrated through an alcohol series, treated with 4% paraformaldehyde and proteinase K, acetylated and dehydrated through an ethanol series. One microliter (3 × 10^6 cpm) of Mab21l2 riboprobe, labeled with [35S]UTP (Amersham) in the hybridization mix was added to each slide. Both sense and antisense probes were used but only antisense probes produced a detectable signal (data not shown). Clear differences between expression domains revealed by the Mab21l1 and Mab21l2 probes indicate complete specificity of each probe. Hybridization was carried out overnight at 60°C. Slides were washed under stringent conditions (60°C, 2× SSC, 50% formamide) and treated with RNase A. Autoradiography was performed with Kodak NT/B2 emulsion. Exposure times were 15 days. Sections were examined and photographed on dark and bright fields using a ZEISS Axioskop microscope.

Whole-mount in situ hybridization

E9.5 and E10.5 embryos were isolated and immediately fixed with 4% paraformaldehyde, dehydrated, rehydrated with a methanol series, bleached with 6% hydrogen peroxide and then treated with proteinase K. One microgram of each linearized plasmid was transcribed in vitro in the presence of 0.8 µl 10 mM Dig-11-UTP (Boehringer Mannheim). Both sense and antisense probes were used. Embryos were hybridized overnight at 65°C and washed at the same temperature with 2× SSC, 50% formamide and treated with RNase A. Signal was revealed through an alkaline phosphatase-conjugated antidigoxigenin antibody (Boehringer Mannheim) with BM-purple (Boehringer Mannheim) as substrate. Embryos were examined and photographed on bright fields using a ZEISS SV11 microscope.

**Cell transfection.** Constructs encoding the MAB21L1 or MAB21L2 proteins tagged at the N-terminus with a c-myc-derived epitope (36) obtained by overlap-extension PCR (37) were subcloned into pcDNA3 (Invitrogen, Carlsbad, CA) and used to transfect NIH 3T3 cells by a standard calcium phosphate precipitation protocol (29). Two micrograms of DNA were transfected in each 60 mm dish cell culture. Cells were harvested 24 h after transfection and grown on coverslips for an additional 24 h before the immunofluorescence assay. Transfection efficiency was ~15%.

**Cellular immunofluorescence**

Cells (10^5) grown on glass coverslips were fixed with 3% paraformaldehyde and permeabilized with 0.15% Triton X-100 in PBS for 5 min, and immunostained with the 9E10 anti-myc mAb followed by FITC- or TRITC-conjugated F(ab′)2 fragments of goat anti-mouse IgG (Jackson Immunoresearch Laboratories). Transfected cells were photographed using an Olympus AX70 microscope.

**REFERENCES**