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Mastermind is a putative activator for Notch

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During signaling by the Notch receptor, Notch's intracellular domain is cleaved, moves to the nucleus and associates with a DNA-binding protein of the CSL class (CSL for CBF1, Suppressor of Hairless (Su(H)), LAG-1); as a result, target genes are transcriptionally activated (reviewed in [1,2]). In *Caenorhabditis elegans*, a glutamine-rich protein called LAG-3 forms a ternary complex with the Notch intracellular domain and LAG-1 and appears to serve as a transcriptional activator that is critical for signaling [3]. Although database searches failed to identify a LAG-3-related protein, we surmised that Notch signaling in other organisms might involve an analogous activity.

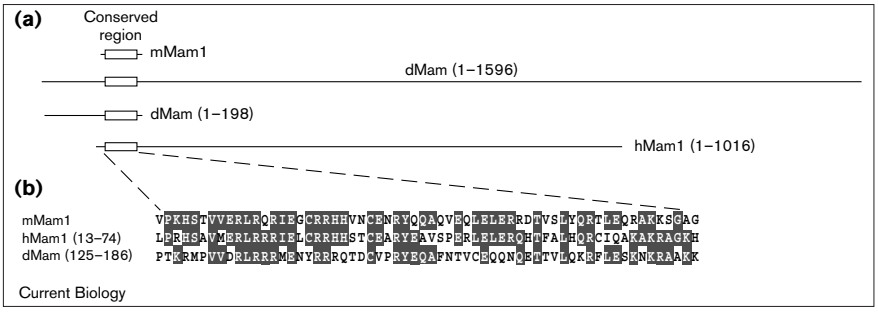
To search for a LAG-3-like activity in mice, we used a modified yeast two-hybrid screen similar to that used to identify LAG-3 [3]. Briefly, we used a complex bait to screen a library of mouse cDNAs fused to the Gal4 activation domain (Clontech). That bait included mouse CBF1 fused to the Gal4 DNA-binding domain (GD) as well as the intracellular domain of mouse Notch1. The bait proteins were co-expressed from a pBridge vector. Out of 6 million transformants, we recovered one positive with similarity to *Drosophila* Mastermind and human KIAA0200 (Figure 1a). We focused on this clone because *Drosophila* Mastermind is known to be critical for Notch signaling (reviewed in [2]) [4,5]. We call the murine ortholog of Mastermind mMam1, and the human one hMam1. The mMam1 fragment recovered in the two-hybrid screen consisted of 62 amino acids and included a conserved region present in both fly and human Mastermind proteins (Figure 1).

To explore the idea that Mastermind might have a role similar to LAG-3 in Notch signaling, we conducted a series of two-hybrid assays (Figure 2). We first showed that mMam1 bound mCBF1–GD in the presence of either Notch1 or Notch3, but not in their absence (Figure 2a). We next asked whether *Drosophila* Mastermind might participate in a similar complex in flies. We made a fusion protein carrying the Gal4 activation domain and the amino-terminal 198 amino acids of fly Mastermind (dMam (1–198), Figure 1a; henceforth called dMam), which includes the conserved region of Mastermind that is critical for complex formation among mouse components. We found that dMam bound Su(H) strongly in the presence of the fly Notch intracellular domain, but not in its absence (Figure 2b).

We next explored the interchangeability of proteins from different species. Remarkably, the fly protein, dMam, interacted with murine Notch1 or Notch3 and murine CBF1 (Figure 2c), and mMam1 interacted with fly Notch and Su(H) (Figure 2d). In contrast, *C. elegans* LAG-3 did not form a complex with either murine or fly components (Figure 2e), and mMam and dMam did not complex with worm components (Figure 2f). We conclude that both fly and murine Mastermind proteins form a ternary complex with either fly or murine receptors and CSL proteins. This interchangeability underscores the similarity between the fly and murine Notch pathways. Although murine Mastermind is not described, a full-length cDNA sequence for human Mastermind is available. Comparison of human and fly Mastermind sequences reveals only one short region of significant similarity that is limited to 60 amino acids at the amino terminus (Figure 1). Therefore, despite a low overall sequence similarity between mouse and *Drosophila* Mastermind proteins, the region crucial for complex formation is conserved.

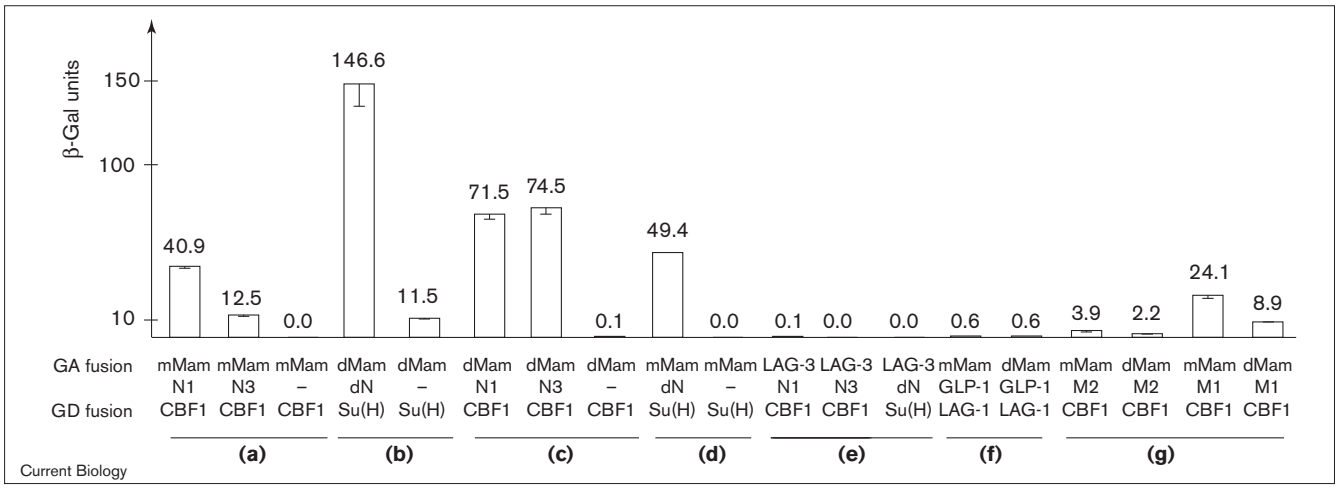
Finally, we examined the importance of the receptor's ankyrin repeats for complex formation. In *C. elegans*, formation of the ternary complex is dependent on the ankyrin repeats of the Notch-related receptor GLP-1 [3]. To ask whether the same situation holds for the murine complex, we used two missense mutants, M1 and M2, each of which bears amino-acid substitutions in the

Figure 1



Conserved region of Mastermind. **(a)** Mastermind proteins. mMam1 shows the fragment recovered in a modified two-hybrid screen for proteins that interact with murine Notch1 and murine CBF1; dMam (1–1596), full-length fly Mastermind; dMam (1–198), fragment of fly Mastermind used for yeast assays; hMam1(1–1016), full-length human Mastermind. The only conserved region between fly and human Mastermind is shown by the rectangle. It consists of only 60 amino acids and resides at the amino terminus. **(b)** Comparison of amino acid sequences of the conserved region; identical amino acids are shaded.

Figure 2



(a–g) Ternary complexes as detected by yeast two-hybrid assay. Activity of the β-galactosidase reporter (β-Gal) was assayed in triplicate for each experiment; standard deviations are shown at the top of each bar. To take into account differences in the self-activation by different bait proteins, the β-galactosidase activity was normalized by subtracting β-galactosidase activity of the corresponding bait proteins in the presence of the Gal4 activation domain. GA fusion, fusion protein with the Gal4 activation domain; GD fusion, fusion protein with the Gal4 DNA-binding domain. M1 and M2, mNotch1 with either of two mutations in the fourth ankyrin repeat [6]; in yeast, M2 and M1 were expressed at levels comparable to that of the wild-type protein (data not shown). The fragment of mMam1 comprised the 62 amino acids shown in Figure 1a; other protein fragments used were Notch1 (1744–2193), CBF1 (1–526), Notch3 (1665–2109), dNotch (1763–2224), Su(H) (110–594), dMam (1–198), LAG-1 (199–673), GLP1 (788–1171), LAG-3 (1–490). See text for further explanation.

fourth ankyrin repeat of mNotch1 [6]. Consistent with results in *C. elegans*, both M1 and M2 compromised interactions among Notch1, CBF1 and either mMam1 or dMam (Figure 2).

What is the role of Mastermind in Notch signaling? Previous studies suggested a role in transcriptional control. In *Drosophila*, Mastermind is a nuclear protein [7] and is bound to chromatin [8]. Furthermore, in *Drosophila*, Mastermind acts downstream of Notch in signaling [9]. The amino-acid sequences of both human and fly Mastermind proteins are rich in glutamine and proline (see below), a common feature in transcriptional activators [10]. In the work reported here, we provide a physical link between Mastermind and the major CSL transcription factor of the Notch pathway. We also show that the interaction of both mMam and dMam with the Notch intracellular domain and CBF1 relies on the receptor's ankyrin repeats (Figure 2g). These repeats are essential for Notch signaling and the transcriptional response. In *C. elegans*, point mutations in the ankyrin repeats severely compromise signaling by the Notch-related receptor GLP-1 [11]. In tissue culture cells, the M1 and M2 point mutations abolish receptor function [6] and compromise the activation of transcription by Notch signaling [12,13]. The simplest explanation for all these findings is that Mastermind functions as a transcriptional activator for Notch signaling.

We note important parallels between LAG-3 in *C. elegans* and Mastermind in *Drosophila* and mammals. First, all of these proteins form a ternary complex with an intracellular fragment of Notch and a CSL DNA-binding protein. Second, mutations in the fourth ankyrin repeat of the receptor compromise ternary complex formation for *C. elegans* [3] and mouse proteins, as we report here. Third, all three proteins are rich in glutamine and proline: 27.6% in LAG-3, 29.4% in dMam and 22% in hMam1. Fourth, LAG-3 and Mastermind function downstream of Notch in *C. elegans* [3] and *Drosophila* [9], respectively. We propose that LAG-3 and Mastermind perform analogous functions as activators for Notch.

What is the evolutionary relationship between LAG-3 and Mastermind? An intriguing idea is that LAG-3 and Mastermind share a common ancestor. The conservation in amino-acid sequence between Mastermind orthologs is much lower than is found for other components of the pathway: whereas hMam1 and dMam share similarity only in a stretch of 60 amino acids within a much larger protein (Figure 1), Notch and CSL proteins show high similarity (44.8% and 74.5% identity for hNotch1/dNotch and hCBF1/Su(H), respectively) over most of their length between these same species. It therefore seems plausible that the absence of similarity between LAG-3 and Mastermind may reflect a high rate of amino-acid substitution in these proteins rather than a distinct evolutionary origin.

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References

1. Artavanis-Tsakonas S, Rand MD, Lake RJ: **Notch signaling: cell fate control and signal integration in development.** *Science* 1999, **284:**770-776.
2. Artavanis-Tsakonas S, Matsuno K, Fortini ME: **Notch signaling.** *Science* 1995, **268:**225-232.
3. Petcherski AG, Kimble J: **LAG-3 is a putative transcriptional activator in the *C. elegans* Notch pathway.** *Nature* 2000, **405:**364-368.
4. Lehmann R, Jimenez F, Dietrich U, Campos-Ortega J: **On the phenotype and development of mutants of early neurogenesis in *Drosophila melanogaster*.** *Wilhelm Roux's Archiv Dev Biol* 1983, **192:**62-74.
5. Xu T, Rebay I, Fleming RJ, Scottgale TN, Artavanis-Tsakonas S: **The Notch locus and the genetic circuitry involved in early *Drosophila* neurogenesis.** *Genes Dev* 1990, **4:**464-475.
6. Kopan R, Nye JS, Weintraub H: **The intracellular domain of mouse Notch: a constitutively activated repressor of myogenesis directed at the basic helix-loop-helix region of MyoD.** *Development* 1994, **120:**2385-2396.
7. Smoller D, Friedel C, Schmid A, Bettler D, Lam L, Yedvobnick B: **The *Drosophila* neurogenic locus *mastermind* encodes a nuclear protein unusually rich in amino acid homopolymers.** *Genes Dev* 1990, **4:**1688-1700.
8. Bettler D, Pearson S, Yedvobnick B: **The nuclear protein encoded by the Drosophila neurogenic gene *mastermind* is widely expressed and associates with specific chromosomal regions.** *Genetics* 1996, **143:**859-875.
9. Schuldt AJ, Brand AH: **Mastermind acts downstream of Notch to specify neuronal cell fates in the *Drosophila* central nervous system.** *Dev Biol* 1999, **205:**287-295.
10. Mitchell PJ, Tjian R: **Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins.** *Science* 1989, **245:**371-378.
11. Kodoyianni V, Maine EM, Kimble J: **Molecular basis of loss-of-function mutations in the *glp-1* gene of *Caenorhabditis elegans*.** *Mol Biol Cell* 1992, **3:**1199-1213.
12. Jarriault S, Brou C, Logeat F, Schroeter EH, Kopan R, Israël A: **Signaling downstream of activated mammalian Notch.** *Nature* 1995, **377:**355-358.
13. Kato H, Taniguchi Y, Kurooka H, Minoguchi S, Sakai T, Nomura-Okazaki S, Tamura K, Honjo T: **Involvement of RBP-J in biological functions of mouse Notch1 and its derivatives.** *Development* 1997, **124:**4133-4141.

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A ubiquitous family of putative gap junction molecules

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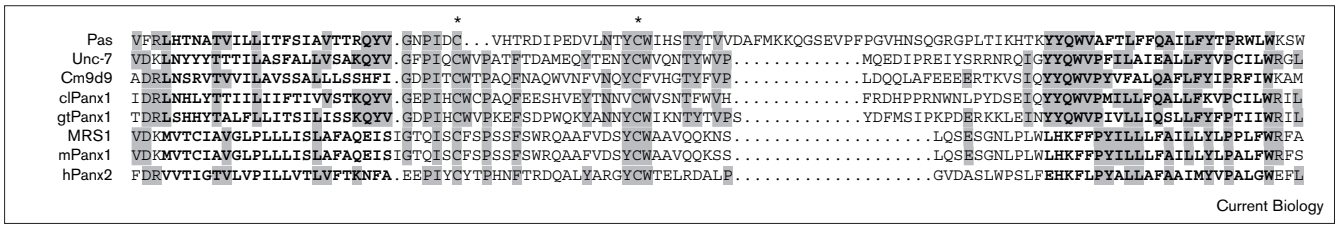
Gap junctions are one of the most common forms of intercellular communication. They are composed of membrane proteins that form a channel permeable for ions and small molecules connecting the cytoplasm of adjacent cells. Although gap junctions provide similar functions in all multicellular organisms, vertebrates and invertebrates are believed to use unrelated proteins for this purpose [1–3]. The family of gap junction molecules called connexins is well-characterized in vertebrates, but no homologs of these proteins have been found in invertebrates [1–5]. In turn, only gap junction molecules with no sequence homology to connexins have been identified so far in insects and nematodes [3–7]. It was suggested that these are specific invertebrate gap junction proteins, and they were thus named innexins (invertebrate analog of connexins) [3]. Here, we demonstrate the presence of innexin homologs in different taxonomic groups, including vertebrates.

Using PCR with degenerate primers, we cloned sequences homologous to innexins from mollusc central nervous system and flatworm whole-animal cDNA (Figure 1). This finding is important because it refutes the hypothesis that innexin proteins could represent a specific feature of recently postulated Ecdysozoa clade ('moulting animals', including among others arthropods and nematodes but not molluscs and flatworms) [3,8]. Moreover, a database search using BLAST [9] for homology matches to the new mollusc and flatworm sequences revealed similarity to two human proteins: MRS1, function unknown, predicted from cDNA sequence submitted by G.B. Bolger and M.R. Steele (GenBank accession number AF093239) and a novel protein similar to MRS1 recently predicted from chromosome 22 DNA sequence (hPanx2 in Figure 1, accession number AL022328). A PSI-BLAST search [9] unambiguously detected the same two proteins even when seeded by one of the original innexin sequences, the Unc-7 gap junction protein from *Caenorhabditis elegans*: with an *E*-value inclusion threshold of 0.01, the two human homologs were detected with expectation (*E*) values of 10^{-5} at the first iteration. In reciprocal searches initiated by the human homologs, the *C. elegans* innexins were detected with *E* values of 10^{-9} in the second iteration.

It can be argued that the presence of four (compositionally biased) transmembrane domains is a possible source of error while searching for homologous sequences. Theoretically, seeding BLAST searches with transmembrane region containing sequences may result in retrieval of similar membrane proteins that are, nevertheless, not homologous. However, in the case of innexins and related vertebrate sequences, because of the presence of a relatively well-conserved region containing two conserved cysteine residues just carboxy-terminal to the first transmembrane sequence, there is sufficient similarity outside the transmembrane regions to indicate homology: a PSI-BLAST search seeded by the hPanx2 sequence of the first putative extracellular loop flanked by only four amino acids from adjacent transmembrane regions with the *E*-value inclusion threshold of 0.05 revealed similarity to Unc-7 with *E* values of 10^{-12} in the second iteration.

Several sequences homologous to innexins were also detected among

Figure 1



The region of the highest similarity shared by predicted amino acid sequences of all putative gap junction pannexin family proteins include first two transmembrane domains and the stretch between them. An alignment of the insect (Pas, L13306) and nematode (unc-7, Z19122 and cm9d9, U59213) innexins (see [6]) is supplemented with protein sequences from the mollusc *Clione limacina* (clPanx1, AF207818), the flatworm *Girardia tigrina* (gtPanx1, AF207819), mouse (mPanx1, AF207817) and two from human (MRS1, AF093239; and hPanx2, AL022328). Predicted transmembrane domains are in bold. Invariant cysteines are indicated with an asterisk. Residues conserved in four or more family members are shaded.

human, mice and chicken expressed sequence tags (ESTs). Using this information, we cloned murine cDNA encoding a hypothetical 'innexin' molecule from a fetal brain sample. Proteins predicted from vertebrate sequences display statistically significant similarity to invertebrate innexins, including conservation of invariant cysteines and the locations of the four putative transmembrane domains [3–6]. Thus, the list of animal phyla with identified innexin family members extends to Platyhelminthes, Nematoda, Arthropoda, Mollusca and even Chordata, which makes the 'innexin' name inappropriate. Given the apparent ubiquitous distribution of this protein family in the animal kingdom we suggest that they should be called pannexins (from the Latin pan — all, throughout and nexus — connection, bond).

The identification of this new type of human putative gap junction proteins may be significant for medicine. A sequenced tagged site (STS, GenBank accession number G43027, alias stSG3927) [10] identical to the fragment of the human pannexin MRS1 places this gene nearby the centromeric border of the q21 band on chromosome 11, between genes encoding melatonin receptor 1B (locus MTNR1B) and vitamin D3 receptor-interacting protein DRIP80 (locus WI-15663). This region has been suggested to be the site of gene(s) predisposing to several major mental disorders including schizophrenia and a rare form of Charcot–Marie–Tooth disease (CMT4B) [11,12]. It is interesting to note that the more common X-linked form of the latter disease (CMTX) is caused by deficiency in a gap junction protein of the connexin family (Cx32) [1,2], suggesting that the pannexin MRS1 could be a good candidate for CMT4B.

Supplementary material

Supplementary methodological material is available at http://current-biology.com/supmat/supmatin.htm.

References

1. Bruzzone R, White TW, Paul DL: **Connections with connexins: the molecular basis of direct intercellular signaling.** *Eur J Biochem* 1996, **238:**1-27.
2. White TW, Paul DL: **Genetic diseases and gene knockouts reveal diverse connexin functions.** *Annu Rev Physiol* 1999, **61:**283-310.
3. Phelan P, Bacon JP, Davies JA, Stebbings LA, Todman MG, Avery L, *et al.*: **Innexins: a family of invertebrate gap-junction proteins.** *Trends Genet* 1998, **14:**348-349.
4. Phelan P, Stebbings LA, Baines RA, Bacon JP, Davies JA, Ford C: ***Drosophila* Shaking-B protein forms gap junctions in paired *Xenopus* oocytes.** *Nature* 1998, **391:**181-184.
5. Barnes TM. **OPUS: a growing family of gap junction proteins?** *Trends Genet* 1994, **10:**303-305.
6. Starich TA, Lee RY, Panzarella C, Avery L, Shaw JE: **eat-5 and unc-7 represent a multigene family in *Caenorhabditis elegans* involved in cell-cell coupling.** *J Cell Biol* 1996, **134:**537-547.
7. Landesman Y, White TW, Starich TA, Shaw JE, Goodenough DA, Paul DL: **Innexin-3 forms connexin-like intercellular channels.** *J Cell Sci* 1999, **112:**2391-2396.
8. Aguinaldo AM, Turbeville JM, Linford LS, Rivera MC, Garey JR, Raff RA, Lake JA: **Evidence for a clade of nematodes, arthropods and other moulting animals.** *Nature* 1997, **387:**489-493.
9. Altschul S, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ: **Gapped BLAST and PSI-BLAST: a new generation of protein database search programs.** *Nucleic Acids Res* 1997, **25:**3389-3402.
10. Wang DG, Fan JB, Siao CJ, Berno A, Young P, Sapolsky R, *et al.*: **Large-scale identification, mapping, and genotyping of single-nucleotide polymorphisms in the human genome.** *Science* 1998, **280:**1077-1082.
11. St Clair D, Blackwood D, Muir W, Carothers A, Walker M, Spowart G, Gosden C, Evans HJ: **Association within a family of a balanced autosomal translocation with major mental illness.** *Lancet* 1990, **336:**13-16.
12. Bolino A, Brancolini V, Bono F, Bruni A, Gambardella A, Romeo G, *et al.*: **Localization of a gene responsible for autosomal recessive demyelinating neuropathy with focally folded myelin sheaths to chromosome 11q23 by homozygosity mapping and haplotype sharing.** *Hum Mol Genet* 1996, **5:**1051-1054.

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Supplementary material

A ubiquitous family of putative gap junction molecules

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Supplementary materials and methods

To obtain the complete coding sequence of the putative gap junction molecules from a mollusc, highly representative amplified cDNA samples were prepared from 20 isolated individual 2A type motoneurons from the pedal ganglia of *Clione limacina* using a procedure for amplifying a representative cDNA sample from microamounts of starting material [S1]. RNA isolation yielded approximately 10–20 ng of total RNA per sample. This amount was sufficient to generate amplified samples with representation of no less than 10^7 as estimated from the cycling parameters. Flatworm *Girardia tigrina* whole-animal and mouse fetal brain amplified cDNA were prepared using a SMART cDNA amplification kit (Clontech). *Clione* and *Girardia* cDNA samples were subject to PCR amplification with degenerate primers: sense #1 5′-TCTGCTAAGCA(G/A)TA(T/C)GT(C/T)GG-3′, or sense #2 5′-TCTGCTAAGCA(G/A)TA(T/C)GT(A/G)GG-3′ (both corresponding to amino acids KQYVG in the single-letter amino acid code) and antisense, 5′-AAGCCGACCCA(T/C)TG(G/A)TA(G/A)TA-3′ (corresponding to amino acids YYQWVP). The cycling parameters were: denaturation, 95°C, 10 sec; annealing, 50°C, 30 sec; extension, 72°C, 40 sec (1 cycle); denaturation, 95°C, 5 sec; annealing, 56°C, 30 sec; extension, 72°C, 40 sec (27 cycles).

The PCR products, about 200 bp long, were cloned and sequenced. The obtained sequences indicated a high degree of similarity to known innexin proteins from *Drosophila* and *Caenorhabditis*. The gene-specific primers for RACE (5′-GAGTTTCTTTCTTTCATGGG-3′ and 5′-TCGGTACCCAACAATGAATC-3′ (nested) for *Girardia* 5′-RACE; 5′-GATTCATTGTTGGGTACCGA-3′ and 5′-GATGAAAGAAAGAAACTCGA-3′ (nested) for *Girardia* 3′-RACE; 5′-GAGTCGTAGGGTAGGTTCCA-3′ and 5′-CACCAGCAGTGAATTGGTTC-3′ (nested) for *Clione* 5′-RACE; 5′-GAACCAATTCACTGCTGGTG-3′ and 5′-TGGAACCTACCCTACGACTC-3′ (nested) for *Clione* 3′-RACE) were designed according to the sequence of the obtained fragments; RACE was performed as described [2]. The longest *Clione* and *Girardia* 3′-RACE and 5′-RACE fragments were cloned and sequenced. *Clione* and *Girardia* gene-specific primers (5′-GCTTAAACGTTAATTACTCTC-3′ and 5′-ATAAGGCTTTAAAACAAAAATTCATTAAC-3′ for *Clione* and 5′-GCACAGATTTGAAAAAAATTGTC-3′ and 5′-AAAACACATAACATTATTATTTTGTTTC-3′ for *Girardia*) were synthesized for the 3′-most parts of the longest 3′-RACE fragments and to the 5′-most parts of the longest 5′-RACE fragments. PCR with these primers produced 2819 bp and 1494 bp products from *Clione* and *Girardia,* respectively, containing 5′ and 3′ untranslated regions and single open reading frame (ORF) of 426 amino acid residues in *Clione* and 439 amino acid residues in *Girardia.* These were registered in GenBank as clPanx1, AF207818, and gtPanx1, AF207819.

To clone the murine MRS1 homolog, the corresponding expressed sequence tag (EST) database was screened with MRS1 nucleotide sequence. Gene-specific primers were designed according to the sequences of the ESTs with high similarity to MRS1 (GenBank accession numbers AA930980, AA387579 and AI428680); primers were 5′-AGGTAGACCCACGGCAATACA-3′ and 5′-AGACTTTGTCAAGTTCCTCCAT-3′ (nested) for 5′-RACE and 5′-CCTCGGCGCACTCTTCTGG-3′ and 5′-GCTCAAGTCATTGTAGCCTTC-3′ for cloning the inner part of the gene.

Further cloning procedure was as described for *Clione* and *Girardia* using murine fetal brain cDNA (Clontech) and yielded a sequence with ORF of 448 amino acid residues.

Supplementary references

S1. Lukyanov KA, Diachenko L, Chenchik A, Nanisetti A, Siebert PD, Usman NY, *et al.*: **Construction of cDNA libraries from small amounts of total RNA using the suppression PCR effect.** *Biophys Biochem Res Comm* 1997, **230:**285-288.

S2. Chenchik A, Diachenko L, Moqadam F, Tarabykin V, Lukyanov S, Siebert PD: **Full-length cDNA cloning and determination of mRNA 5′ and 3′ ends by amplification of adaptor-ligated cDNA.** *Biotechniques* 1996, **21:**526-534.