Praja1, a novel gene encoding a RING-H2 motif in mouse development

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As part of a cloning strategy to identify genes involved in early mouse liver development we have isolated *Praja1*, a gene with similar sequences to the Drosophila melanogaster gene goliath (gl) which is involved in the fate of mesodermal cells ultimately forming gut musculatures, fat body, and the heart. Prajal is a 2.1 kb gene encoding a putative 396 amino acid ORF and includes a COOHterminal RING-H2 domain. Using the Jackson Laboratory BSS panel, we have localized Praja1 on chromosome X at 36 cM, which may be a candidate gene for mouse sla (sex linked sideroblastic anemia), near the X inactivation center gene, Xist. Northern blot analysis demonstrated three transcripts (3.1, 2.6 and 2.1 kb) in mRNA from adult mouse tissues brain, liver, and kidney as well as in mRNA from developing mouse embryos (days 7, 11, 15 and 17 post coitus, p.c.). In vitro transcription/translation yielded a product with an M_r of 59 kD. Immunohistochemical staining of in vitro liver explant cultures using a heterologous antibody against praja1 demonstrated cytoplasmic staining of cuboidal cells that have hepatocyte morphology and organization. The presence of the RING-H2 domain, a proline-rich region at the COOH-end, and regions rich in acidic amino acids, leads to the hypothesis that the Praja1 product is possibly involved in mediating protein-protein interactions, possibly as part of a protein sorting or transport pathway. This is strengthened by the similarity of Prajal to rat Neurodap1, whose product has been shown to localize to the endoplasmic reticulum and golgi in brain.

Keywords: *Praja1* gene; PRAJA1 protein; RING-H2 domain; molecular cloning; liver development; chromosome localization; explant cultures

Introduction

The molecular mechanisms underlying hepatocyte differentiation are not well understood. Identifying the genes underlying the control of liver development will provide powerful tools for understanding liver development and may allow induction of liver differentiation for therapeutic purposes. As part of a strategy to clone such genes, we isolated a new RING-H2 finger gene, *Praja1*. RING-H2 fingers, a type of zinc finger are similar to RING fingers except that Cys4 is replaced by His (Freemont, 1993; Lovering *et*

al., 1993). Here we show that *Praja1* possesses a RING-H2 motif near the COOH-terminal. The RING-H2 motif is similar to that of the Drosophila melanogaster gl gene (Bouchard and Cote', 1993), and to the rat Neurodap1 gene (Nakayama et al., 1995). *Praja1*, which localizes to chromosome X, is expressed in mouse brain, liver, and kidney. The presence of the RING-H2 motif, plus the acidic, hydrophilic nature of the translation product, lead us to hypothesize that *Praja1* may play a role in protein transport.

Results and discussion

Isolation and sequence analysis of a novel gene Praja1

As part of the analysis of genes involved in liver development and function, we utilized a pair of primers based on sequence of a cDNA from a subtractive library and amplified the 3' end of a previously undescribed gene. Using the amplified sequence (CH7) as a probe to screen a mouse embryonic cDNA library we isolated two overlapping clones, Praja1-5 and Praja1-6. Sequence analysis of the consensus overlap region revealed an open reading frame (ORF) of 396 amino acids, with a predicted size of 44.22 kD. Hydropathy analysis (Kyte and Doolittle, 1982; not shown) shows that the translation product is highly hydrophilic, with no hydrophobic leader or membranespanning regions. The translated product is also predicted to be very acidic, with a pI of 4.52 and containing 17.7% acidic residues. The putative ATG start codon indicated in Figure 1 was selected because it is the first upstream ATG that is in-frame with the ORF, and is preceded 21 bp upstream by a TAG stop codon. The context of this ATG, however, is only a weak fit to the consensus Kozak recognition sequence GCCACCatgG in that it does not have an A purine at -3 nor a G at +4 (reviewed by Kozak, 1996). Sequence analysis of the amino acid translation revealed the presence of a COOH-terminal RING-H2 motif, which is a zinc finger variant (Freemont, 1993). Figure 2 shows an alignment of the RING-H2 motif of Prajal with those of several other RING-H2-containing proteins.

Linkage analysis places *Praja1* on mouse chromosome X. A restriction fragment length polymorphism for *Praja1* was identified using CH7 as a probe on a Southern blot containing DNA from the two parental (C57B16/J and SPRET/Ei) strains digested with several restriction enzymes (*TaqI*, *Bg1II*, *Eco*RI, *Eco*RV, *Hind*III, *HincII*, *KpnI*, *PstI*). For every enzyme used, C57B16/J had only a single restriction fragment, while two fragments were always observed within the SPRET/Ei lane. A polymorphism obtained using TaqIwas used to type the inheritance of the C57B1/6J allele in the BSS panel. There are two Spretus bands S1 and S2 and one C57B1/6J band B1. After comparison of the *Praja1* genotypes to other genes typed within the database, we determined that *Praja1* maps to mouse chromosome X at about the 36 cM position (Figure 3). The S1 band is the *Praja1* allele on X chromosome of SPRET/Ei. The S2 TaqI fragment appears in every backcross animal. Since all males from the backcross contain this allele, it is not localized to the X chromosome. Since females also have the S2 band, it is not Y-linked. Therefore S2 is an autosomal locus

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88	AAG	TA	ATI	ATC	сті	TAC	GCP	TAG	тта	TCA	TCA	CCA	CAA	AAA	AAG	ATI	CCA	ACC	TTT	тсс	ACA	GAA	ста	TTA	TGA	ттт	тта	TTI	TATAT
175	GAA	TG	FAT	GTA	573	ATT 58,	ATI W4	ATA 774	TGA 2	ACI	ccī	АТА	ATG	ATC	ACC	TTI	ACA	TA1	тся	CAT	TTT	CTT	ААТ	ТАА	TAG	1TT	AGC	CGC	GTCC
262	GGF	GG.	rec	GAC	AGC	тст	GCA	GCT	cca	IAGC	GCG	CGA	CTA	acc	AGA	AAG	TTT	CAG	GCC	ATC	CAT M	GAG S	CCA H	Q.CCA	IGGA E	AAG R	GAT I	TGC A	CAGC S
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958 1045 1132 1219 1306 1393 1480 1567 1654	ATC I ATG M GAI D ATG M CTT L GAG B TGC C TAC TGA	CCT P CCTC L CGC G CCT P CCT P CCCT P CCCT P CCCT P CCCT P CCCT P CCCT P CCCT C CCT C C C C	TIG N BGA D TIT L BAC T SAC T SAC C C AG A TG	GCT L TGG G GGG G GGG G GGC A GGT M GAG GTT	TCG R AAAA N AGT V CCT L CCT L CCT L GTT F CCC F GTG GTG	CTA Y CAA N GGCC A GGCC A GGCC A SCTI E CCCC P TTG' TAC	CAA N CAA N CGA E ACA H CAC T T CAC P F CCC P F TCC AAT	TGA E CCTT L AGC A CTT L CCGA E CCCC P TAA GAC	GAA N GGA E CAT I GGA E AGA D CCA E AGA I CCA	TGA B AGA D CTC S GTC S TCA H CCA H CCA TAC.	AAG S IGA D CTA Y ICT L IGG G CTA Y AAA	CAG S CTC S CGT V CGC A TTC F SGCC GAA	CAG S CAG S GGA D CGT V V AGT V V CCA U V AGT T CA CGT CGT CGT CGT CGT CGT CGT CGT CGT CGT	CGA E CGT V TCC P cl TGA D GGG G G CAAV K GCT K AGT TCC	G G G S A G S C C C C C C C C C C C C C C C C C C	TGA D CGA E GTT P CH CGA E CGA C CGT CGT CGT	TAA'N AGAAD CCTCL 7 AGTU 7 AATU M CGTU AAAT	TGA B CCT L CAC T SGC A STG STC S STC S AGT AGA	GTC S CGA E CTA Y CAA Y CAA CAG CAT I CAG CAA SGT.	TAC T AGT V CAT M CCC P CTG CTG CTG CTG AAGA	CCA H GGA D GGC A A A CC P TAT I GGT TTG A A GGT	TGA E CTG W TCT L AGC C A C C TCA Q TCA Q TAC: SGG4	GCT L BAGS S GA S S C C S AAG S C C S AATT S AATT S AATT	CAT I CCT L AGA E CAG K CAG S CCC S CCC CCT AGG	ACA Q GTT F GCG ⁴ R GGA E CGA E TGG ⁴ G TGT ⁴ TGT ⁴ AAA	GCC P TGA D TCT L SAG S S ATA' Y CAC' T CCG S ATA' Y	TGG G G G G G G G G C A T G C C T G C C C C C C C C C C C C C C	GAT M GTT F CCA Q TGA D GAA K CCCC P CCCC	GTTC F TGCC A GGCA A TGCC A GGGG G AGTG V ACAA CATG TGCT
958 1045 1132 1219 1306 1393 1480 1567 1654 1741	ATC I ATG M GAT D ATG M CTT L GAG B TGC C TAC TGA GCT	CCT P CTC G G G G G G G G G G G G G C C C C C	TIG N BAC T L BAC T BAC T BAC C C AG A GA	GCT L TGG G GGG G GGG A GGG T I M GAG GTT TTG	TCG R AAAA N AGT V CCT L CCT L CCT E GTT F CCCC GTG GTG CAG	CTA Y CAA N GGCC A GGCC A GGCC V SCT V SCT P TTG TAA	CAA N CAA N CGA E ACA H CAC T T CC P T CC P T T C C P	TGA E CCTT L AGC A CTT L CCA C C C C C C C C C C C C C C C C C	GAA N GGA E CAT I GGA B AGA D CCA H GCT L ACT ACA	TGA E AGA D CTC S GTC S GTC S CTCA H CCA H CCA H TAC. TAA.	AAG S IGA D CTA Y ICT L IGG G CTA Y AAA Y AAA	CAG S CTC S CGT V CGC A TGC A TGC A CGC A CGC A A AAA	CAG S CAG S GGA D CGT V AGT V AGT V CCA H AAGC TCA CTG	CGA E CGT V TCCC P CA CA CA CA CA CA CA CA CA CA CA CA CA	SGG G SAG S CAG CAG CAG CAG CAG CAG CAG	TGA D CGA E GTT F CH CGA E GGA E CGT CGT CGT CGT GAA	TAA' N AGAA D CCT L 7 AAGT V AAAT V AAAT V AAAT V AAACA	TGA E CCT L CAC T SGC A STG STC S STC S AGA AGA	GTC S CGA E CTA Y CAA N CTG C C C C C C C C C C C C C C C C C C	TAC T AGT V CAT M CCC P CTG CTG CTG AGA	CCA H GGA D GGC P TAT I GCT L GGT TTG' AAG	TGA E CTG W TCT L AGC. A C C C C TCA Q TAC. ITG. 3GG& ICTV	GCT L GAG S GGA E S GGA S C C GAA K ATT SAA. STA	CAT I CCT L AGA E CAA K CAG S CCC S CCC CCC S AGG S	ACA Q GTT F GCG R GGA E CGA G TGT G TGT TGT TGT TGT	GCC P TGA D FCT L SAG S SAG S SAG T CCG S SAT T CCG S SAT	TGG G G G G G G G G C AT I C T G C A AAA I I C T G C A AAA I I C T G G G G G G G G G G G G G G G G G G	GAT M GTT F CCA Q CCA D GAA K CCCC P CCCC P CCCC CTT CTT	GTTC P TGCC A GGCA A TGCC A GGGG G G GGGG G A ACAA CATG TGCT A CCT
958 1045 1132 1219 1306 1393 1480 1567 1654 1741 1828	ATC I ATG M GAI D ATG ATG C TGC C TGC C TAC TGC GCT GTT	CCT P CCTC G G G G G G CCT P G G CCT P CCCT P CCCT P CCCT P CCCT C CCT C C C C	TIG N SGA D TIL SAC T SAC T SAC SAC SAC SAC SAC SAC SAC SAC SAC SAC	GCT L TGG G GGG G GGC A GGC T I GAC T TG GAG GTT TTG CAG	TCG R AAAA N AGT V CCT L CCT L CCT L CCT E GTT F CCC GTG GGT GGT	CTA Y CAA N GGCC A GGCC A GGCC V GCCC P TTG TAC	CAA N CAA N CGA E ACA T CAC T T CAC T F CAC T F CAC T T CAA T T CAA ACC T T CAA ACA T T CAA ACA T T CGA A CAA CAAA CAAA CAA CAA CAAA CAAA CAAA CAAA CAA CAAA CAAA CAAA CAA CAAA CAAA CAAA CAAA CAA CAAA CAAA CAA CAAA CAA CAAA CAAA CAA CAA CAA CAA CAA CAA CAA CAA CAA CAA CAA CAA CAA C	TGA E CCTT L AGC A CTT L CGA E C C C C C C C C C C C C C C C C C C	GAA N GGA E CAT I GGA E AGA H GCT L CCA H GCT L CCA T ACA GCT	TGA E AGA D CTCC S GTCC S GTCC S GTCCA H CTA H CTA TAC. TAC. TAC.	AAG S IGA D CTA D CTA IGG G CTA IGG CTA IGG CTA IGG CTA	CAG S CTC S CGC CGC A TGC A TGC A A AAA S CGC A TTT F GCC CGA A AAA	CAG S CAG S GGA D CGT V AGT V AGT V AGT CCA CCA CCA CCA CCA CCA CCA CCA CCA CC	CGA E CGT V TCC P cli TGA D GGG G G CAAV K GCT K GCT K	SGG G SAG S S CA S CA S CA S CA S CA S C	TGA D CGA E GTT P CH CGA E GGA E C CGT C CGT C CGT CGT CGT	TAA' N AGA D CCTP L 7 AGT V AAT V AAT V AAT I TTA	TGA E CCT L CAC T 3GC A 3TG S C S TC S S C S TC S C S C S TC S C S	GTC S CGA E CTA Y CAA N CTG C C C C C C C C C C C C C C C C C C	TAC T AGT V CAT M CCC P CTG P CTG P CTG AGA	CCA H GGA D GGC A C P TAT I GCT I GGT TTG' I GCA I	TGA E TCTG W TCTG L AGC C TCA Q TCA Q TAC: ITTG: IGTG	GCT L GAG S SGA E SGA E SGA S CTG C SAA K TT SAA STA	CAT I CCT L AGA E CAG K CAG S CCC S CCC S CCC S CCC S CCC S CCC S CCC S CCC S CCC S CCC S CCC S CCC S CCC S CCT L AGA S AGA S S S CCT S S CCT S S CCT S S CCT S S CCT S S CCT S S CCT S S CCT S S CCT S S CCT S S CCT S S CCT S S CCT S S CCT S S CCT S S CCT S S C C AGA S S S S CCT S S C C A S S S S C C S S S S CCT S S S S C C S S S S	ACA/ Q GTT F GCG' R GGA/ E G G G TGT/ TGT/ TGT/ TGT/ TGT/ TTA/	GCC P IGA D ICT L SAG S S ATA Y CAC T CCG SAT T CCG SAT T CCG	TGG G G G G G G G CAT I CAT I C C C C C C C C C C C C C C C C C C	GAT M GTT F CCA Q CCA D GAA K CCC P CCC P CCC FTC CTT CTTG	GTTC F TGCC A GGCA A TGCC A G G G G G G G G G C A G G G G C A C A

Figure 1 Nucleotide sequence of *Praja1*. Arrows indicate 5U ends of homologous est clones, named in boldface. The 5' end of CH7, the original PCR product, is similarly indicated; it continues to the 3' end. The amino acid sequence designates the open reading frame. The RING-H2 finger is indicated by the shaded box. The clear box at 3UTR indicates primer DI utilized to probe the Northern blots

that contains sequence homology to the *Praja1* probe sequence and further experiments are necessary to determine its location in the SPRET/Ei genome (BSB cross).

At present Prajal may be a candidate gene for mouse sideroblastic anemia (sla) (Falconer and Isaacson, 1962), and mutational studies are underway to determine this. Other genes mapping to this general region include moesin (Msn), androgen receptor (Ar), interleukin-2 receptor gamma (IL-2R), X-linked zinc finger protein (Zfx), and tabby (Ta). This area is also 1.1 ± 1.1 cM from the Xist locus. Further studies are needed to determine if Prajal is not expressed on inactivated X-chromosomes and if it plays a role in Xinactivation. The synteny and conserved gene order between mouse and human X chromosomes (Herman et al., 1996) allows comparison with human disease genes in the region. Human diseases in this region with mesodermal involvement include the recently cloned anhidrotic ectoderm dysplasia (eda), and sideroblastic anemia with spinocerebellar ataxia (asat).

In vitro expression produces a protein product larger than the predicted size. Figure 4a shows an autoradiogram of the *in vitro* transcription/translation product of *Praja1*. The product, which ran as a band of $M_r = 59$ kD, is larger than the predicted ORF size of 44.22 kD (Figure 4a). One possible explanation is that the expression product is very acidic and acidic proteins such as granins are known to give anomalously high M_r on SDS–PAGE (Huttner *et al.*, 1991). Of note, expression of the fusion protein also gave a high M_r of 85 kD on SDS–PAGE–compared to the predicted M_r of 73.22 kD (Figure 4b, and c).

Utilizing an antibody to a 12 amino acid peptide, corresponding to a unique part of *praja1* (aa 145–157), we examined the expression of *prajal* in embryonic liver explant cultures. Mouse embryonic liver explants were cultured in our laboratory, at day 10-10.5 when the liver bud is 0.2 mm (Figure 5). When cultured in the complete absence of mesodermal derivatives, hepatic endoderm deteriorates rapidly. Only two out of 15 such liver explants survived. Hematoxylin and eosin staining showed a necrotic endoderm with no apparent signs of hepatic differentiation (see Figure 5b). When associated with the surrounding mesoderm particularly cardiac mesoderm (en bloc dissections), the endodermal cells had proliferated and invaded the mesodermal strands. Cuboidal cells with hepatocyte morphology were seen to be organized in cords separated by sinusoids with pseudo-lobule formation (see Figure 5a,c,d). On hematoxylin staining, the cuboidal cells had large lightly staining nuclei and



Figure 2 Alignment of RING-H2 motifs from *Praja1* with those of several other proteins, as found in a BLAST search of non-redundant GenBank CDS translations plus SwissProt plus PIR databases. Alignment was performed using the GCG program PILEUP. Dark and light boxes indicate identical and conservative substitutions, respectively. Genes and organisms are: Neurodap1, rat; C0166.4, F55A11.3, S15788, Cenorabditis elegans; gl, D. melanogaster; X95455, Gallus; YOL013.c, Saccharomyces

scant cytoplasm. All 15 out of 15 cultures from en bloc dissections were completely viable. These studies confirm prior explant studies demonstrating the necessity of surrounding mesoderm for liver formation (Houssaint 1980; Le Douarin 1975). Immunoperoxidase staining by antibody against *praja1* resulted in the labeling of the cytoplasm of the cuboidal cells (Figure 6a). Further studies will be required to define the role of *praja1* in the liver explants.

Praja1 transcripts are present in embryonic and in mouse tissues (Figure 7a and b). Northern blot analysis of RNA from adult mouse showed expression of 3.1, 2.6, and 2.1 kb transcripts in liver, brain, and kidney, and an additional 2.3 kb transcript in testis (Figure



Figure 3 Localization of *Praja1* on mouse chromosome X with map figure from The Jackson BSS backcross showing localization of Prajal on mouse Chromosome X. The map is depicted with the centromere towards the top. Loci mapping to the same position are listed in alphabetical order. Centimorgans with standard errors are indicated to the left between loci. Raw data from The Jackson Laboratory were obtained from the World Wide Web address http://www.jax.org/resources/documents/ cmdata. Other genes indicated with human orthologous position in bold are: Fln 1(actin binding protein, non-muscle filamin) Xq28, Ctsh-rs 1 (Cathepsin h), Mpp1 (membrane protein, palmitolated 1), Ahch (Mouse Homologue of DAX-1), Dmd (dystophin) Xp21.3-p21.2, Egr5 Ors (Early growth response-5, M36386). Human disease genes in the corresponding human syntenic region that are candidates for having Prajal mutations include anhidriotic ectodermal dysplasia (EDA Xq12.2-13.1) and sideroblastic anemia with spinocerebellar ataxia (ASAT Xq13)

7a). The *Praja1* protein is unlikely to be a membrane receptor, since it lacks a hydrophobic transmembrane domain. The uniform hydrophilicity suggests a soluble protein. The Prajal RING-H2 motif is shown aligned with those from several other proteins in Figure 2. RING fingers are generally thought to function in protein-protein interactions (Borden and Freemont, 1996; Saurin et al., 1996). To cite a specific example, if either of the two cysteines that comprise the Zn^{2+} binding site of the RING finger of acute promyelocytic leukemia proto-oncoprotein PML are mutagenized, then the nuclear multiprotein complex, or so-called nuclear bodies, fail to occur (Borden et al., 1995). The authors conclude that the PML RING domain, and probably other RING finger domains, are involved in protein-protein interactions.

In Praja1, aside from the RING-H2 finger, the stretch of thirty-four COOH-terminal amino acids just past this motif (Figure 1) is especially rich in proline residues (17.6%); and, as stated, the protein in general is very acidic. Proline-rich domains are found in several mammalian transcription factors, such as that at the COOH-terminus of transcription factor CTF (Mitchell and Tjian, 1989). Proline-rich regions and also acidic regions are likely to function in contacting other proteins (Mitchell and Tjian, 1989). A BLAST search of the proline-rich COOH-terminus revealed no significant matches to any protein in the available databases. However, when considering the Praja1 sequence as a whole, the rat Neurodap1 gene has the highest similarity; the alignment is presented in Figure 8.

Neurodap1 is expressed abundantly in rat brain, with much smaller amounts in heart and skeletal muscle (Nakayama et al., 1995). Though Prajal likewise shows greatest expression in brain, unlike Neurodap1 it also expresses in liver and kidney (Figure 7a). The subcellular localization of Neurodap1 was shown to be concentrated around the endoplasmic reticulum (ER) and golgi of the cerebral cortex and facial nucleus, and especially in the postsynaptic density region of axosomatic synapses (Nakayama et al., 1995). Based on its subcellular localization, plus the presence of the RING-H2 finger, the authors concluded that Neurodap1 is probably linked to a secretory or protein sorting pathway. *Praja1* does differ from Neurodap1 in several respects, however. In addition to being expressed in some different tissues than Neurodap1, Prajal encodes for a product that is smaller (44.22) kD vs 77.9 kD for Neurodap1. The difference in size is at the N-terminus of the proteins. The largest transcript we observed for Prajal was 3.1 kb (Figure 7a), whereas Neurodap1 exists as a single 4.3 kb transcript on Northern blots of rat brain mRNA (Nakayama et al., 1995).

In light of the fact that BRCA1, which possesses a RING finger, has an acidic pI, and is a secretory protein, also has properties of the granin family of proteins (Jensen *et al.*, 1996), we examined *Praja1* for a granin signature. We found no region in the *Praja1* translation that gave a perfect match to the consensus E[N/S]LX[A/D]X[D/E]XEL, though two regions matched five of the seven conserved residues. We were also unable to demonstrate the presence of clear coiled-coils, which are present in Praja1 and liver development L Mishra et al

BRCA1 and proteins with the previously-mentioned tripartite structures. In these respects, *Praja1* is more similar to Neurodap1 than to proteins such as BRCA1. Also, though the RING-H2 finger in *Praja1* shows much similarity to that from the *D. melanogaster* goliath (g1) protein (Bouchard and Cote', 1993) (Figure 2), the goliath protein possesses an alkaline pI (8.9) and no sequence similarity to *Praja1* outside of the RING-H2 finger. Though the exact function of *Praja1* must await further testing, the RING-H2 motif plus acidic and proline-rich regions, and similarity to Neurodap1, lead us to conclude that *Praja1* is most likely involved in protein-protein interactions, possibly in a protein sorting or secretory pathway.



cDNA preparation and 3'-RACE PCR

RNA was isolated from livers of day 11 p.c. embryonic mice (ICR, Harlan Sprague-Dawley) using guanidine thiocyanate (Chomczynski and Sacchi, 1987). Poly(A)⁺ mRNA was isolated from total RNA using Dynabeads, as per manufacturer's instructions. First strand cDNA was made from poly(A)⁺ mRNA using the Promega Reverse Transcriptase System and the 3'-RACE primer 5'-GACTCGAGTCGA-CATCGA-T17 (Frohman, 1990). The 3'-RACE primer was also used as the reverse primer in the PCR reaction. The forward PCR primer, originally designed to amplify a conserved region of a clone 145/PH, was 5'-CTCAAG-CAGGTCCTGGCACA. The PCR reaction mix contained cDNA from about 10 ng of poly(A)⁺ mRNA, 25 pmol of



b

Glutathione Transferase :: Praja-1 Protein Fusions



Figure 4 (a) *In vitro* transcription/translation product of *praja1*, and luciferase control. Numbers at arrows indicate molecular masses of products in kD; numbers at left indicate molecular weight markers. (b) Diagram showing construct for *PRAJA1* fusion protein. (c). Top arrow indicates GST-*praja1* fusion protein. Bottom arrow indicates the GST control





Figure 5 Hematoxylin and eosin staining showed a necrotic endoderm with no apparent signs of hepatic differentiation (b). When associated with the surrounding mesoderm particularly cardiac mesoderm (*en bloc* dissections), the endodermal cells had proliferated and invaded the mesodermal strands. Hepatocytes were seen to be organized in cords separated by sinusoids with pseudo-lobule formation (a,c,d)

each primer, 1 mM dNTP mix, and 2.5 units of AmpliTaq DNA polymerase (Perkin-Elmer) all in 10 mM Tris, 1.5 mM MgCl₂ and 75 mM KCl, pH 9.2 in a final volume of 50 μ l. The temperature program comprised 35 cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min), and extension (72°C, 3 min), followed by an additional 8 min extension. One of the resulting PCR products (CH7) comprised a 725 bp fragment, which was cloned into vector pCRII using the Invitrogen TA Cloning Kit for sequencing and found by sequence analysis to possess a RING-H2 finger. The portion of the final cDNA clones which correspond to CH7 is indicated in Figure 1.

Library screening

The PCR product CH7 was labeled with ³²P-dCTP (3000 Ci/mmol, Amersham) via primer extension using the reverse PCR primer plus AmpliTaq polymerase at 72°C in PCR buffer (Konat *et al.*, 1994). The resulting antisense probe was used to screen plaque lifts of a whole embryonic mouse (day 11 p.c.) cDNA library in vector λ Zap (Stratagene). Positive plaques were picked and purified, and DNA was isolated from lysates using standard procedures (Silhavy *et al.*, 1984). Inserts were excised from λ Zap DNA using *Eco*RI, and were subcloned into





Figure 6 (a) Immunoperoxidase staining by antibody against *prajal* in liver explants demonstrated labeling of the cytoplasm of cuboidal cells with hepatocyte-like (*) morphology. (b) is a control, with no antibody-showing a few pyknotic nuclei

pGEM3Zf(-) (Promega) for sequencing and subsequent manipulations.

DNA sequence analysis

DNA sequence comparisons to existing sequences were performed utilizing BLAST searches in Genbank. Alignments were performed using the GCG program PILEUP.

Chromosomal mapping

Southern blot analysis of genomic DNA from C57BL/6J (B6) and Mus spretus (SPRET/Ei) using ³²P-labeled CH7 as a probe revealed a restriction fragment length polymorphism for the enzyme *TaqI*. This polymorphism was used to follow the inheritance of the *Praja1* gene using the (B6 X SPRET/Ei) X SPRET/Ei backcross panels (BSS) from The Jackson Laboratory Backcross DNA Panel Map Service (Rowe *et al.*, 1994). Linkage and order relative to other markers was determined by minimizing the number of multiple recombinants within each haplotype.

Northern blot analysis of Praja1 expression

Northern blots containing two micrograms of $poly(A)^+$ mRNA from mouse tissues (Clontech) were probed with ³²P-labeled CH7 antisense strand using Express Hyb hybridization solution (Clontech) at 68°C, washed according to manufacturer's instructions, and subjected to autoradiography. A ³²P-labeled β -actin probe supplied with the Northern blots was used as a control to normalize RNA levels in each lane.

In vitro transcription/translation

A transcription/translation-coupled rabbit reticulocyte lysate system (Promega) was used, as per manufacturer's instructions for ³⁵S-methionine labeling. Clones of Prajal in pGEM3Zf(-) plus a luciferase control clone were used with T7-RNA polymerase (sense-direction). Each reaction comprised 12.5 μ l rabbit reticulocyte lysate, 1 ml reaction buffer, 0.5 µl 1 mM amino acid mix minus methionine, 0.5 ml T7-RNA polymerase, and 20 units RNasin, all in 25 ml final volume. After a 90 min incubation at 30°C, products were lysed in SDS/mercaptoethanol treatment buffer and separated on a 10% SDS-polyacrylamide gel according to Laemmli (1970). Proteins were electroblotted onto a BAS-NC membrane (Schleicher & Schuell) using a BioRad Trans-Blot apparatus according to manufacturer's instructions, Labeled products were visualized by autoradiography.

Protein fusions of *Prajal* to glutathione transferase (GST) were constructed as follows: A 1.2 Kbp cDNA fragment was amplified by PCR with oligomers 5'-ACGAATTCATGAGC-CACCAGGAAAGGATTGCC-3' and 5'-TGCGGCCGCTA-GACTGTTACGACGAGCCTTGG-3'. The PCR reaction mix contained 10 ng template DNA (clone Praja-I), 25 pmol of each primer, 1 mM dNTP mix and 2.5 units of AmpliTaq DNA polymerase (Perkin-Elmer) all in 60 mM Tris, 12.5 mM (NH4)2 SO4, 1.5 mM MgCl2, pH 8.5 in a final volume of 50 μ l. The temperature program comprised 30 cycles of denaturation (94°C, 1 min), annealing (60°C, 1 min), and extension (72°C, 1 min), followed by an additional 8 min extension. The resulting PCR product, which was 1200 bp was digested with EcoRI and NotI and cloned into the pGEX-5X-1 EcoRI/NotI site (Pharmacia, Uppsala, Sweden). The plasmid thus obtained directed the synthesis of a GST-fused protein carrying Prajal. This GST-Prajal fusion protein was produced in E. coli with IPTG induction and affinity-purified with glutathione-sepharose 4B beads as instructed by the supplier of the vector.

Liver explant cultures

Mouse embryos were obtained from Harlan ICR mice. The age of the embryos was determined by days post appearance of the vaginal plug (day 0). The embryos were further characterized by the number of somites. Isolation of mouse hepatic endoderm, liver buds and mesoderm (en bloc dissection) was as follows: during the 10th day of gestation, the liver bud becomes evident as a thickening of the ventral wall of the foregut, near the origin of the yolk stalk. This ventral endoderm was then either taken alone and cultured, or alternatively with the surrounding mesoderm: the portion of the embryo between the otocyst and the umbilical region. Organ culture: Embryos were placed onto nucleopore filters in a humid chamber as described (Houssaint, 1980) and cultured for 48 h or 96 h. Microscopy: The explants were fixed as in the in situ hybridization protocols, and RNA isolated as described above. 7 mm sections were stained with hematoxylin, eosin and periodic acid schiff (PAS) for glycogen, an indicator of differentiated hepatocytes.

Immunohistochemical characterization

Antibody to a peptide corresponding to amino acids 145– 157 (CLRRKYRSREQPQS) of *praja1* (COVANCE), was used for immunohistochemical localization in liver explant cultures. Embryos were fixed and embedded into paraffin, sectioned and immunostained using indirect immuno histochemistry according to protocols routinely used









Figure 7 (a) Expression of $poly(A)^+$ mRNA transcripts hybridizing to the *Praja1* probe CH7, which contains the RING-H2 motif plus 3U-UTR, in (a) adult mouse tissues, and (b) whole mouse embryos at the indicated days p.c. Lower panels show hybridization with β -actin standard



Figure 8 PILEUP alignment of *Praja1* and rat Neurodap1 proteins. Dark and light boxes indicate identical and conservative substitutions, respectively

(Schevach, 1991). 8 μ m sections were deparaffinized in xylene, the tissue rehydrated in graded alcohols, and rinsed in PBS. The sections were initially treated with a protease

(0.1% Trypsin in 0.05 M PBS) and incubated at 37°C for 30 min. Endogenous peroxide was then removed using 3% hydrogen peroxide. Sections were blocked in PBS containing 5% goat serum for 30 min at room temperature. Sections were then incubated overnight at 4°C in a Humidor with diluted rabbit anti-mouse antibody directed against the PRAJA1 peptide. All further steps were done at room temperature. Six 5 min rinses with PBS-S were performed after each successive step. After incubation in the primary antisera, slides were washed six times for 5 min in 1 × PBS at room temperature. Sections were incubated with a second antibody (diluted in 0.05 M PBS in 1% serum) for 30 min at room temperature.

After rinses the substrate was added as follows: Insoluble Peroxidase substrate DAB (Sigma Fast). 100-150 microliter substrate solution was added to cover the entire tissue on the slide. Color development was monitored under microscope. After rinsing in distilled water for 5 min staining was performed with Harris hematoxylin solution modified (Sigma) for 1 min, followed by a rinse in distilled water for 5 min. Sections were dehydrated by passage through distilled water, then graded alcohol concentrations and finally xylene. Coverslips were mounted using DPX (Fluka labs) or Permount (Fischer scientific), before observation. For the negative controls only the primary antibody diluting solution was added, without any antibody.

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