Title page

Full title: Characterization of novel alternative splicing variants of the mouse *MCF-2 (DBL)* proto-oncogene

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Running title: Characterization of mouse *MCF-2* proto-oncogene splicing variants

Abstract

MCF-2 (*DBL*) proto-oncogene is a prototype guanine nucleotide exchange factor (GEF) that modulates Rho GTPases such as Rho, Rac and Cdc42. Although the partial sequence of mouse *MCF-2* has been determined, its full-length cDNA and biochemical functions had not been elucidated. We isolated the complete mouse *MCF-2* cDNA and obtained recombinant functional protein. Homology between the mouse and human *MCF-2* (*DBL*) cDNAs is 75.08% identity and between the mouse and human amino acid sequences 74.52% identity. Analysis of tissue distribution showed that mouse *MCF-2* mRNA is expressed in brain, kidney, intestine and testis. The brain-specific transcript is an alternatively spliced derivative that omits the 48bp exon 11. A similar alternatively spliced mRNA product is also found in humans (*DBL*). Guanine nucleotide exchange activities of the testis-expressed mouse Mcf-2 and human Dbl were analyzed using RhoA, Rac1 and Cdc42 as substrates. RhoA and Cdc42 were activated similarly by both gene products, but Rac1 was activated only by the mouse product. The brain-specific Mcf-2 gene product, and its human counterpart, was less active than the respective testis-specific products. This indicates that the element encoded by the 48 bp exon missing in the brain transcripts is necessary for full GEF activity. This report provides fundamental data on the structure of Mcf-2, which regulates a variety of cellular signaling pathways.

Key words

MCF-2 (*DBL*) proto-oncogene; guanine nucleotide exchange factor (GEF); Small G protein; and Rho family GTPase

Introduction

The *MCF-2* (*DBL*) proto-oncogene is a prototype guanine nucleotide exchange factor (GEF) for small G proteins including Rho family GTPase. Dbl converts Rho family proteins from the GDP-bound to the GTP-bound form. The exchange of G TP for GDP induces a conformational change in the GTPase, allowing its effector domain to interact with downstream molecular targets and thereby induce a wide range of cellular responses (1). In humans, Rho family GEFs including Trio, Fgd1 and Vav play important roles in the maturation and organization of skeletal muscle and neurons (2), the morphogenesis and proliferation of B and T cells and cytokine production (3,4). Mutations in some GEF genes are associated with human disease (5). Although the mouse homologue of human Dbl, designated Mcf-2, is important for intracellular signal transduction, only the partial cDNA sequence of *MCF-2* had been reported (6) and its biochemical characteristics are still unclear. Here we report isolation of the complete *MCF-2* cDNA and characterization of its alternatively regulated spliced product in the brain. We also obtained recombinant Mcf-2 protein and compared its guanine nucleotide exchange activity with the previously identified human Dbl.

Materials and Methods

Mouse *MCF-2* cDNA cloning

To isolate the 5'- and 3'- flanking regions of the previously reported partial mouse *MCF-2* cDNA, RACE (rapid amplification of cDNA ends) was performed using a 5'- and 3'-adapter-ligated mouse brain cDNA library (Marathon-ready cDNA amplification kit; Clontech Inc., Palo Alto, CA).

For 3' RACE, the primary PCR was performed using a gene-specific sense primer M1 (5'-GTCTCCCAAATTGGACAATAGCTTGGAT-3') and the Marathon adapter antisense AP1 (5'-CCATCCTAATACGACTCACTATAG-3') in a 25 μl reaction containing 1×LA PCR Buffer II (Takara-Bio Inc. Kyoto, Japan), 2.5mM $MgCl_2$, 400μM each dNTP, 0.05 unit/μl of Takara LA Taq polymerase (Takara), and 2.5 μl of cDNA at 94˚C for 30sec and at 68˚C for 4min for 40 cycles. The PCR product was used as a template for the secondary PCR, where M1 and the nested adapter antisense AP2 (5'-ACTCACTATAGGGCTCGAGCGGC-3') primers were used to amplify the 3' region cDNA. The 5' RACE was performed likewise using gene-specific antisense M2 (5'-GCATTGAGTCAAGTGCCTCCT-3') and the Marathon adapter sense AP1 primers for the first round PCR. Gene-specific nested antisense M3 (5'-GCAAATAGGAATCCAGACCATAGTCGATG-3') and nested adapter sense AP2 were used for the second PCR. The PCR products were subjected to electrophoresis on 1.25% agarose gel and in the case of the 3' product was cloned into pT7 Blue vectors (Novagen Inc., Madison, WI). The 5' product was sequenced directly. The resulting full-length

sequence was used to search the NCBI database for homologous genes using the BLASTN program.

MCF-2 mRNA expression analysis

First-strand cDNA was synthesized from the total RNA of 12 different mouse organs (OriGene Technologies Inc., Rockville, MD) using an RT-PCR kit (Applied Biosystems Co., Foster city, CA). PCR was carried out in a 50 μl reaction containing 250 ng of the first-strand cDNA, 2.5 mM $MgCl_2$, 400μM each dNTPs, 1×LA buffer (Takara), 2.5 U LA Taq DNA polymerase (Takara), 200 nM each sense and antisense primers, at 94˚C for 20sec, 47˚C for 30sec, 72˚C for 2min for 30 cycles. The ß-actin-specific forward (5'-ATTGTGATGGACTCCGGAGA-3')

and reverse (5'-AAGAAGGAAGGCTGGAAAAG-3') primers, the primers specific to *MCF-2* MM1AS (5'-ATGATATCGAAAGCCTGC-3') and MS2AS (5'-AAAATCATCCTTCCTTTCCAG-3') were used to identify mRNA in organs. The PCR products were subjected to electrophoresis.

Expression of recombinant mouse *MCF-2*

Full-length cDNA of the mouse *MCF-2* was isolated using PCR carried out in a 25 μl reaction containing 1 μl of cDNA template of mouse brain and testis (Clontech), 2.5 mM $MgCl_2$, 400μM each dNTPs, 1×LA buffer (Takara), 1.25 U LA Taq DNA polymerase (Takara), 200 nM each sense and antisense primers, and 50 ng of first-strand cDNA at 94˚C for 30sec, 52˚C for 30sec, 72˚C for 2min for 35 cycles. Primers were mF1 (5'-ATGGCAGTTGCAAATCCCCC-3') and mR1

(5'-ATGGGCTGAGAAGCAGGCAG-3'). The RT-PCR product was inserted into the *Hind*III/*Xba*I site of the pRc/CMV expression vector (Invitrogen, Carlsbad, CA). The resulting plasmid was introduced into COS7 cells using the Lipofectamine Plus reagent (Gibco BRL, Grand Island, NY), and protein was extracted after 48h.

Assay for guanine nucleotide exchange reaction

We transfected vectors containing the mouse brain- or testis-derived *MCF-2* (pRc/Mcf-2br or pRc/Mcf-2te) or wild-type human *DBL* (pRc/Dblwt)(7) into COS7 cells. Cell lysates were extracted after 48h of culture. Recombinant RhoA, Rac1, or Cdc42 (Cytoskeleton Co. Fillmore St., Denver Co.) was incubated with 1μM (525GBq/mmol) of [^{3}H]GDP (Amersham Biosciences Corp. Piscataway, NJ) in a solution containing 10mM Tris-HCl, pH7.5, 3mM $MgCl_2$, 9.6mM EDTA, 0.6mM DTT and 1mM DDPC at 25˚C for 20min to generate the respective [^{3}H]GDP-bound Rho protein. The reaction was terminated by adding $MgCl_2$ to a final 13.2mM. Cell lysates from COS7 cells (10μg) expressing each recombinant protein were reacted with [^{3}H]GDP-bound RhoA, Rac1 or Cdc42 in a 100μl solution containing 10μM GDP, 39mM Tris-HCl (pH7.5), 0.75mM DDPC, 50μM GTP, 9.6mM $MgCl_2$ and 30μM DTT at 25˚C for 20min. The reaction was stopped by adding ice-cold buffer (20mM Tris-HCl pH8.0, 25mM $MgCl_2$, 100mM NaCl), after which the reaction mix was applied onto nitrocellulose membranes (Millipore, Bedford, MA). Filters were dried and solubilized in an Ultima Gold MV liquid scintillator cocktail (Packard instrument, Research parkway, Meriden), followed by measurement

of radioactivity in a liquid scintillation counter.

Results and Discussion

Cloning of full-length mouse *MCF-2* cDNA

We cloned the complete cDNA of the mouse *MCF-2* proto-oncogene. Previous analysis of the partial cDNA showed that mRNA expression occurred in the adrenal gland, brain, cerebellum, ovary and testis (6). Therefore, we amplified the cDNA from a mouse brain cDNA library. We obtained and sequenced both the 3'- and 5'- fragments, and were able to determine the full-length *MCF-2* cDNA of approximately 3.5kb length (Registered as GenBank/EMBL/DDBJ Acc.No. **AB052945**).

Homology of the mouse *MCF-2* cDNA coding region to the human *DBL* cDNA is 75.08%. Queries in BLAST revealed that the homology of reported partial sequence of mouse *MCF-2* cDNA to the Dbl homology (DH) and pleckstrin homology (PH) domains of the human *DBL* proto-oncogene is 77.64%. The homology of the mouse cDNA to d ifferent regions of the human *DBL* cDNA is as follows: +1 ~ +1272 (exons1 – portions of 10), 85%, +1475 ~ +2445 (portions of exons11 - 21), 84%, +3026 ~ +3186, 84%, +3373 ~ +3450, 87%. The amino acid sequence homology between mouse and human Dbl is 74.52% (Fig.1).

Tissue distribution of *MCF-2* mRNA expression

Although previous studies reported that *MCF-2* was expressed only in brain, adrenal grand, cerebellum, ovary and testis (6), our RT-PCR analysis showed that mouse *MCF-2* mRNA was expressed in brain, kidney, intestine, and testis (Fig.2A). We had previously found that human *DBL* mRNAs were expressed in brain, heart,

kidney, testis, placenta, stomach and peripheral blood (7). Thus, mouse *MCF-2* showed similar distribution to the human in the case of brain and kidney and testis but differed in other tissues. We had found a brain-specific alternatively spliced product in humans missing exon11, and in mouse we identified a brain-specific alternatively spliced product missing 48bp of exon11 (The full-length cDNA sequence containing this 48bp exon was registered as Acc.No. **AB101616**) (Fig.2B). We had previously isolated 4 splicing variants of human Dbl (7), indicating there also might exist other splicing variants of mouse Mcf-2.

Biological activity of Mcf-2

Each recombinant Mcf-2 protein was expressed in COS7 cells. Mouse Mcf-2 was with almost the same molecular weight as human Dbl proto-oncogene (Data not shown). Since Dbl activates RhoA and Cdc42 and possibly Rac1 in cell culture and *in vitro* biochemical analyses (8-10), the GEF activity for RhoA, Rac1, and Cdc42 was assayed by measuring dissociation of [^{3}H] GDP from each Rho family member (Fig.3). Testis-specific Mcf-2 showed GEF activity toward Cdc42 and RhoA>Rac1, while the brain-specific Mcf-2 was less active. Human brain-specific Dbl was inactive in this assay, whereas the full-length species containing the additional 48 bp was highly active. These results indicate that the 48bp exon 11, which was specifically excised out in the brain transcript, is required for full activity. Previously, Hirsch et al. generated a Mcf-2 KO mouse and reported that although they did not display major abnormalities, they exhibited decreased dendrite growth (11). This report and our results indicate that the GEF activity of brain-type Mcf-2 is normally weak, yet this gene is especially important for nervous system function. We also previously identified

4 human Dbl splicing variants and found that their distribution and function was variable (7). This strongly suggests that there may also be some splicing variants of mouse Mcf-2, and these may be functionally differentiated in several tissues.

The present description of the mouse Mcf-2 proto-oncogene provides a molecular basis to study in more detail whether and how *MCF-2* expression is involved in the regulation of cellular signal transduction and in the pathogenesis of various diseases.

References

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Figure legends

Fig. 1 Comparison of human Dbl and mouse Mcf-2 amino acids sequences

Asterisk indicates identical amino acid residues.

Fig. 2 *MCF-2* mRNA expression in various mouse tissues

MCF-2 mRNA expression was analyzed by RT-PCR. (A) Portions of cDNA were amplified from total RNA of 12 organs by RT-PCR using *MCF-2*-specific primers MM1AS and MS2AS, and ß-actin specific primers. Lane 1: brain, 2: heart, 3: kidney, 4: spleen, 5: thymus, 6: liver, 7: stomach, 8: intestine, 9: muscle, 10: lung, 11: testis, 12: skin (B) cDNA structures of brain form and testis form of *MCF-2*. Alternatively spliced 48bp exon11 that encodes an additional 16 amino acids is shown in bold letters (Full-length cDNA sequence contained 48bp exon11 was registered as Acc.No. **AB101616**).

Fig. 3 Guanine nucleotide exchange activity of mouse Mcf-2 and human Dbl

Guanine nucleotide exchange activity was measured by the dissociation of [^{3}H]GDP from RhoA, Rac1 and Cdc42. The results indicate the relative amount of bound [^{3}H]GDP. The data are expressed as the mean ± SD of 3 independent experiments.

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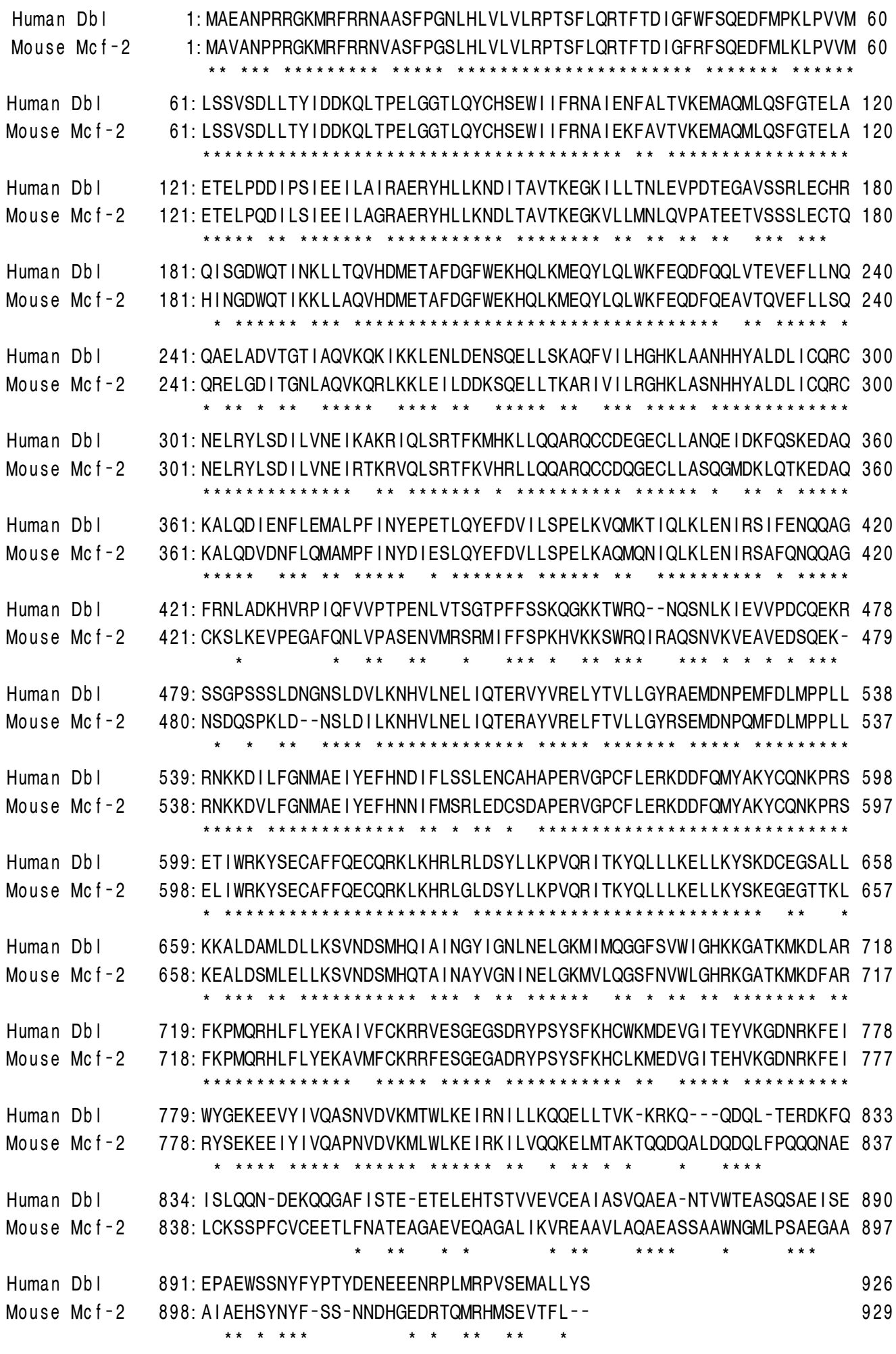
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Mouse Mcf-2 61:LSSVSDLLTYIDDKQLTPELGGTLQYCHSEWIIFRNAIEKFAVTVKEMAQMLQSFGTELA 120
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Mouse Mcf-2 361:KALQDVDNFLQMAMPFINYDIESLQYEFDVLLSPELKAQMQNIQLKLENIRSAFQNQQAG 420
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Mouse Mcf-2 480:NSDQSPKLD--NSLDILKNHVLNELIQTERAYVRELFTVLLGYRSEMDNPQMFDLMPPLL 537
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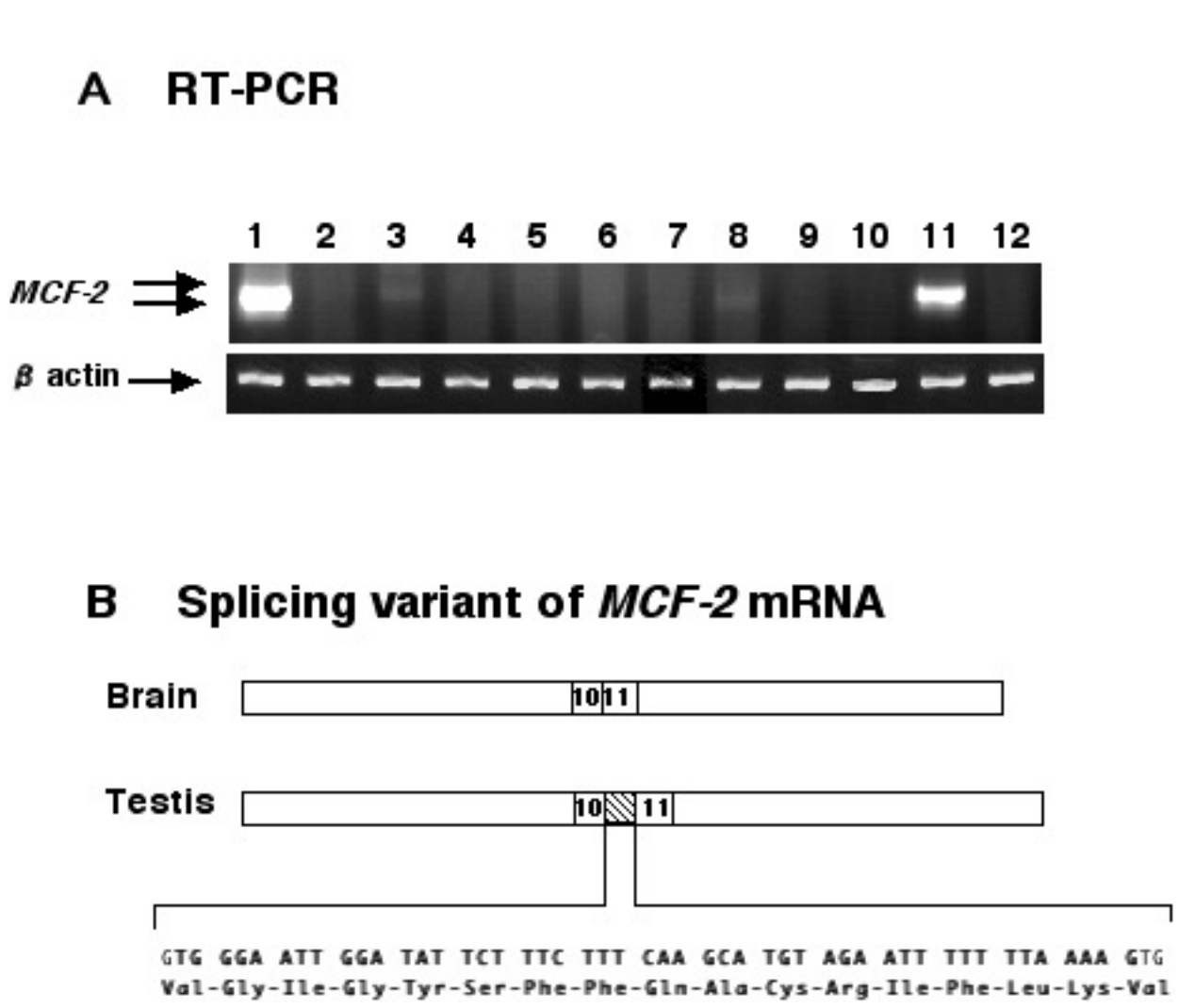
Fig.1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MCF-2
β actin
B Splicing variant of MCF-2 mRNA
Brain
10 11
Testis
10 11
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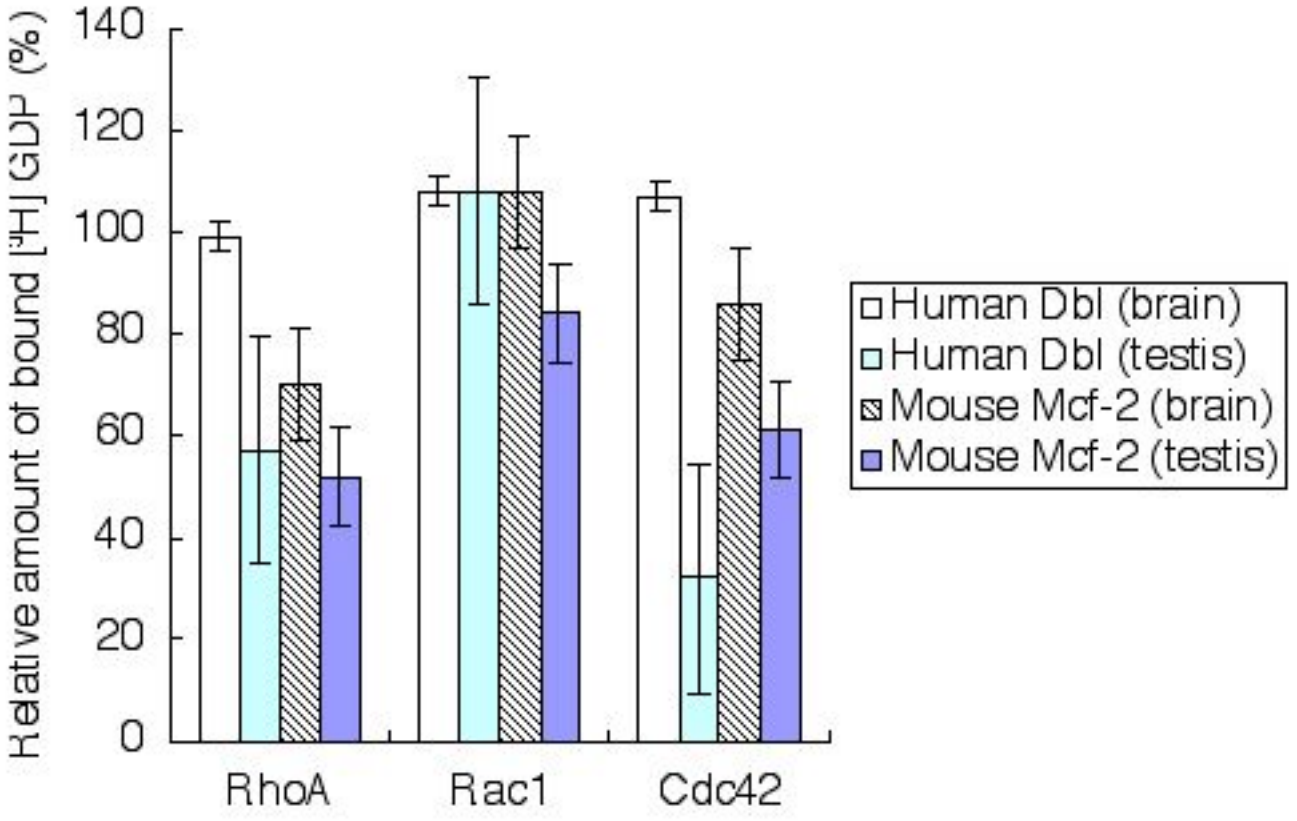
Fig. 2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120
100
80
60
40
20
0
RhoA
Rac1
Cdc42
Human Dbl (brain)
Human Dbl (testis)
Mouse Mcf-2 (brain)
Mouse Mcf-2 (testis)

Fig. 3