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Mouse brain and muscle tissues constitutively express high levels of Homer proteins

Mikhail M. Soloviev, Francisco Ciruela, Wai-Yee Chan and R. A. Jeffrey McIlhinney

Medical Research Council, Anatomical Neuropharmacology Unit, Mansfield Road, Oxford, UK

In order to characterize expression of Homers in mouse brain and peripheral tissues we have developed a coupled reverse transcription (RT)-PCR/restriction digestion approach. This has allowed us to determine the molecular composition and relative levels of the constitutive expression of the Homer-1, -2 and -3 mRNAs across mouse tissues. We report here that mammalian brain constitutively expresses high levels of the Homer-1, -2 and -3 mRNAs. Expression of the Homer-1 mRNAs reaches 66% of the brain total Homer mRNAs expression, followed by Homer-3 mRNA (22%) and Homer-2 mRNAs (12%). Quantitative RT-PCR analysis and the Western blotting using pan-Homer antibody revealed that mouse heart, skeletal muscle and diaphragm constitutively express high levels of the Homer proteins and their mRNAs. We have shown that the molecular profile of expression of Homer-1, -2 and -3 mRNAs in muscle containing tissues resembles that obtained for mammalian brain.

Keywords: Homer; brain; heart; diaphragm; muscle.

Recently, a family of mammalian Homer/Vesl proteins (later in this paper referred to as Homer proteins for simplicity) consisting of three proteins containing postsynaptic density-95, discs large, zona occludens-1 (PDZ)-like domains, have been characterized [1–4]. Homer proteins bind specifically to the C-termini of the metabotropic glutamate receptor mGluR1α and mGluR5 [1,3,4], play a role in their targeting [5] and may therefore contribute to neuronal development and plasticity, memory acquisition and learning. All Homer proteins share homologous N-termini of which the first ≈ 110 amino acids are necessary for binding the group I mGluRs [1]. Amino acid sequence similarity there reaches ≈ 84%. The C-termini of the Homer proteins, except for Homer-1A, are predicted to adopt similar 'coiled-coil' secondary structure [6–8] despite the low amino acid sequence similarity between them. The coiled-coil motif allows Homer proteins to form homo- or heteromultimeric complexes [3,4,9,10]. Alternative splicing of the Homer-1 mRNA results in a very different long untranslated 3′ end of Homer-1A transcript which codes for a very short C-terminus. Unlike all other Homer proteins, the Homer-1A does not contain a coiled-coil domain. Homer-1A/Vesl-1S mRNA was identified by differential cloning strategies among the genes which are rapidly induced in neurons of the hippocampus and cortex by excitatory synaptic activity, following seizure, high-frequency stimulation, or during development [1,2,11]. Following an induction, the expression of Homer-1A is quickly downregulated. This could be due to the presence of the 3′-untranslated AUUUA repeats, which are implicated in the instability of the mRNA and are a common feature of Immediate Early Gene mRNAs [12]. *In situ* hybridization studies of Homer protein mRNAs expression across brain tissues revealed inducible expression of Homer-1A mRNA in cortex, hippocampus and striatum. High levels of constitutive expression of Homer-1B/C mRNA were previously reported in cortex, hippocampus and olfactory bulbs, whereas Homer-2A/B mRNA was expressed in hippocampus, thalamus and olfactory bulbs, and Homer-3 mRNA in cerebellum and hippocampus [4].

We attempted to check and quantify constitutive regional expression of Homer mRNAs in mouse tissues other than brain. We used quantitative reverse transcription (RT)-PCR amplification – the most sensitive method for characterizing and quantifying the levels of expression of an mRNA of interest in different tissues [13,14], especially if the amount of the expressed messenger RNAs is limited or when limited amount of tissue is available [15–17]. To this end we have developed a coupled RT-PCR/restriction digestion approach which has allowed us to compare quantitatively the expression of the Homer mRNAs in different mouse tissues as well as the expression of different Homer mRNAs in each tissue. The results presented here indicate that Homers are constitutively expressed at high level in mouse brain and muscle containing tissues.

Correspondence to M. Soloviev, Medical Research Council, Anatomical Neuropharmacology Unit, Mansfield Road, Oxford OX1 3TH, United Kingdom. Fax: + 44 0 1865 271647, Tel.: + 44 0 1865 271865, E-mail: mikhail.soloviev@pharm.ox.ac.uk

Abbreviations: RT, reverse transcription; PDZ, postsynaptic density-95, discs large, zona occludens-1; mGluR, metabotropic glutamate receptor; GAPDH, glyceraldehyde-phosphate dehydrogenase; PVDF, poly(vinylidene fluoride).

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EXPERIMENTAL PROCEDURES

Design of the pan-Homer primers

A pair of partially degenerate primers for the PCR amplification of the Homer protein cDNAs was designed to anneal to the conservative 5′-fragments of the coding sequences (which codes for ≈ 100 amino acids of the Homer-1, -2 and -3 N-termini) of all of the Homer proteins (Fig. 1) and thus to amplify all corresponding cDNA fragments quantitatively in one reaction. The primer sequences were PanHF [5′-G(AG)GA(AG)CA(AG)CC(AGCT)AT(ACT)-TT-3′] and PanHR [5′-TC(CT)TG(AG)AA(CT)TT(CT)TC-(AGCT)GC(AG)AA-3′].

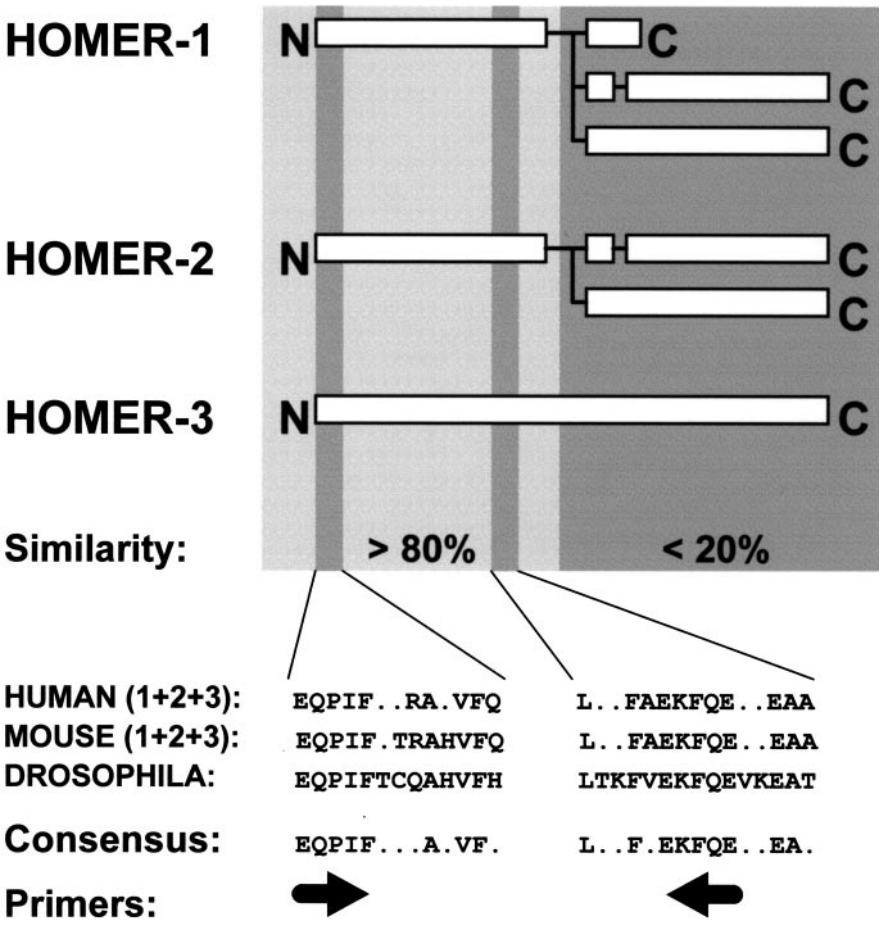

Fig. 1. Structure of the Homer proteins and design of pan-Homer primers. The separate consensus Homer sequences from human, mouse and *Drosophila* are shown. Partially degenerate primers (shown as arrows) were designed to anneal to conservative sequences of all Homer mRNAs (for primer sequences see Experimental procedures).

Quantitative RT-PCR analysis of Homer mRNAs expression

Poly(A)$^+$RNAs were isolated from mouse liver, spleen, lung, heart, kidney, stomach, upper and lower colon, diaphragm, skeletal muscle and total mouse brain preparations. Equal amounts of each tissue were processed in parallel under necessarily the same conditions using Pharmacia Quick Prep kit. cDNAs were obtained using random hexamer primer and SuperScript II M-MLV Reverse Transcriptase (GibcoBRL) according to the manufacturers protocol. An excess of each of the forward (PanHF) and the reverse (PanHR) primers (200 pmol per 100 μL reaction), five units of *Taq* polymerase and 1 μL of first strand reaction mix (taken without purification) were used for each amplification. All reactions were assembled using the same master mix containing all the reaction components. Amplification conditions were 1 cycle of 5 min at 96 °C, 30 s at 45 °C, 10 min at 72 °C, and 30 cycles of 1 min at 96 °C, 20 s at 45 °C and 1 min at 72 °C followed by 10 min at 72 °C. The described conditions and the number of amplification cycles used resulted in a linear amplification of the 310 b.p. cDNAs as was checked in the initial control experiments using the mouse brain derived mRNA preparation, the richest source of the Homer transcripts (Fig. 2A, left). Following the amplification, equal amounts of the amplified products were analysed by electrophoresis in 3% agarose gels containing 0.5 $\mu g \cdot mL^{-1}$ ethidium bromide. All cDNA fragments obtained were subsequently gel-purified and cloned. A number of individual clones obtained from each of the cloned 310 b.p. cDNA bands was sequenced to confirm the identity of the amplified products. The amount of cDNAs obtained from each tissue was quantified by direct measurements of the gel fluorescence of the 310 b.p. fragments using a 'GelDoc 1000' digital imaging system and 'MultiAnalist' software from Bio-Rad Laboratories Inc.

Amplification of the mouse glyceraldehyde-phosphate dehydrogenase (GAPDH) was performed using primers G1F (5′-GGAGCCAAACGGGTCATCATCTC-3′) and G2R (5′-GAGGGGCCATCCACAGTCTTCT-3′) and the same cDNA samples as were used for analysis of expression of the Homer mRNAs. The amplification conditions were 1 cycle of 5 min at 95 °C, 1.5 min at 96 °C, 1 min at 70 °C, 3 min at 72 °C, and 24 cycles of 45 s at 96 °C, 45 s at 70 °C and 45 s at 72 °C followed by 10 min at 72 °C. The described conditions and the number of amplification cycles fall within a linear range of amplification which for the 233 b.p. mouse GAPDH cDNA fragment extends to 26–28 cycles (Fig. 2A, righthand panel). Homer mRNA expression levels in different mouse tissues were normalized to either the expression level of GAPDH mRNA in each tissue or to the amount of original tissue used for mRNA preparation.

Western blotting

The pan-Homer antisera VHr20 used in this study was raised against the recombinant glutathione S-transferase fusion protein containing Homer-1A sequence, a conservative N-terminal domain of Homer proteins. The immunization of rabbits, affinity purification and characterization of the antisera were performed as described previously [5,18]. Prior to SDS/PAGE [19] adult mouse tissues were dissected, washed in cold

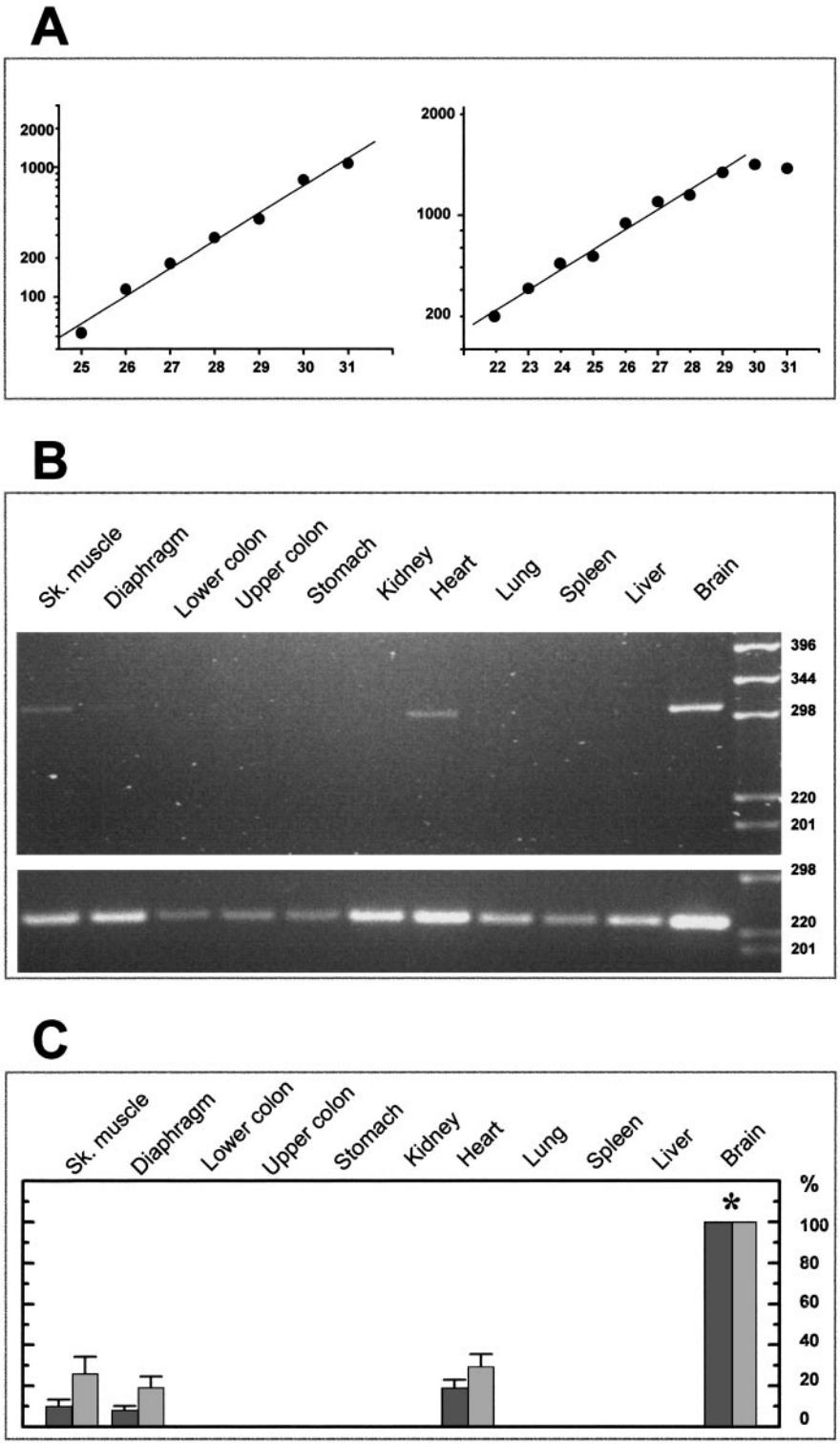

Fig. 2. Quantitative RT-PCR analysis of Homer mRNAs expression. (A) RT-PCR amplification of the 310 b.p. pool of Homer cDNAs (left) and GAPDH cDNA (right) from mouse brain. The vertical axis indicates fluorescence of the amplified products (arbitrary units), the horizontal axis indicates the number of amplification cycles, including the first longer cycle (see Experimental procedures). Linear range of amplification extends to 31 cycle for Homer amplifications and to 26–28 cycles for GAPDH amplification (the lower cycle number threshold is due to higher abundance of the GAPDH transcript compared to that of Homers). (B) RT-PCR amplification of the 310 b.p. pool of Homer cDNAs (top) or GAPDH 233 b.p. fragment (bottom) from adult mouse tissues. DNA molecular size markers (GibcoBRL) are in b.p. (C) Direct measurements of the gel fluorescence of the 310 b.p. fragments (total Homer mRNAs expression level) shown as percentage (± SEM, $n = 5$) to that obtained for mouse brain (*) which is taken as 100%. The data are normalized to either the expression level of GAPDH mRNA in each tissue (light gray bars) or to the amount of original tissue used for mRNA preparation (dark gray bars). The latter represents absolute levels of Homer mRNA expression in various mouse tissues shown as percentage of Homer mRNA level in brain.

$NaCl/P_i$ and homogenized in SDS/PAGE sample buffer. Transfer to poly(vinylidene difluoride) (PVDF) membranes was as described in [20]. After blocking with 5% (w/v) dry milk in $NaCl/P_i$ containing 0.05% Tween20 ($NaCl/P_i$-T), the membranes were washed and incubated overnight at 4C with the VHr20 antibody (2–4 μg/mL) in $NaCl/P_i$-T-milk. Homer immunoreactive bands were detected with swine horseradish peroxidase conjugated antirabbit antibody using chemiluminescence detection (Pierce).

The coupled RT-PCR – restriction digestion analysis of Homer mRNAs expression

Pan-Homer amplifications were carried out using cDNAs derived from the Homer-positive mouse tissues as described above. Immediately following the amplifications, the PCR products were treated with the RsaI restriction endonuclease, chosen to yield different restriction maps for the amplified Homer-1, Homer-2 and Homer-3 cDNA fragments and blunt

ended cDNAs, suitable for cloning. The digested cDNAs were analysed by electrophoresis in 4% agarose gels containing 0.5 μg/mL ethidium bromide. All of the obtained cDNA fragments were of the expected lengths, their identity was confirmed by cloning and sequencing. To determine the relative abundance of each of the individual Homer cDNAs gel fluorescence of the digested cDNA fragments was measured as described above. Abundance of the Homer-1 cDNA was determined by measuring the fluorescence of the 240 b.p. fragment, the Homer-2 cDNA – of the 310 b.p. fragment and the Homer-3 – of the 182 b.p. fragment. As the ethidium bromide fluorescence is proportional to the amount of the DNA, the fluorescence measured was normalized relative to the length of each of the cDNA fragments to allow calculation of the corresponding molar ratios.

RESULTS AND DISCUSSION

The high level of amino acid sequence similarity between the N-termini of the three known Homer proteins (Fig. 1) has allowed us to design pan-Homer oligonucleotide primers (PanHF and PanHR), able to amplify all corresponding cDNA fragments quantitatively in one reaction. The RT-PCR using these primers resulted in ample amplification of a pool of 310 b.p. cDNAs from mouse brain but also, unexpectedly, from the muscle containing tissues such as heart, skeletal muscle and diaphragm (Fig. 2B). Because all the mRNAs were purified from equal amounts of tissues and the cDNAs were synthesized and linearly amplified in parallel under exactly the same condition, the amounts of the 310 b.p. cDNAs in each lane on the Fig. 2B represents the total amounts of the Homer mRNAs in the original preparations. Analysis of the intensities of these cDNA fragments revealed that heart, skeletal muscle and diaphragm contain significant total amounts of the Homer transcripts (respectively 19%, 10% and 8%, relative to the level of Homer mRNAs in mouse brain, Fig. 2C). The amplification condition used have yielded no detectable signal from either of liver, spleen, lung, kidney, stomach, upper or lower colon. Thus Homer mRNAs are either not constitutively expressed there or their levels are below the detection limits of the linear range RT-PCR amplification used here. Our results indicate for the first time that muscle containing tissues express high levels of Homer mRNAs, comparable to those in mammalian brain.

We further confirmed our findings by Western blotting experiments using the pan-Homer antiserum. The VHr20 antibody produced strong staining in brain, heart, diaphragm and skeletal muscle (Fig. 3). The apparent molecular weight of the stained proteins (46–48 kDa) is in agreement with the calculated masses of 'long' Homer-1B/C, -2A/B and -3. The intensity of staining obtained with the pan-Homer antiserum followed closely the profile of Homer mRNAs expression described above (Fig. 2B,C). No staining corresponding to the 'short' Homer-1A was detected in either of the tissues tested. This is in agreement with the previous studies which indicated transient character of Homer-1A expression [1,2]. In the only other study on the distribution of Homers in tissues other than brain [4] various anti-Homer antisera were employed, which yielded immunoreactive bands in brain, somewhat weaker staining in kidney and liver (using anti-Homer-1 antibody), liver (with anti-Homer-2 antibody) and strong staining in lung (with anti-Homer-3 antibody). We cannot exclude the possibility that Homer subtype-specific antisera may reveal the presence of low level expression of specific Homer members in tissues, which might go undetected by our pan-Homer antiserum. Nevertheless our data suggest that the level of expression of Homer proteins in these tissues must be significantly lower than that in brain, heart, diaphragm and skeletal muscle.

We further analysed the relative content of Homer-1, -2 and -3 mRNAs in different tissues. We have discovered that every 'Homer-positive' tissue (Fig. 2B,C and Fig. 3) contains mRNAs coding for all three Homers (Fig. 4A). In each sample tested the Homer-1, Homer-2 and Homer-3 transcripts were coamplified with each other under the same conditions and with the same primers, with the length of the amplified cDNAs being the same as well. Under such conditions different Homer cDNA fragments became ideal endogenous competitor standards for each other and their relative abundance remains the same throughout the amplification. Fluorimetric quantitation of the cDNA fragments corresponding to the individual Homers (separated from each other using restriction digestion followed

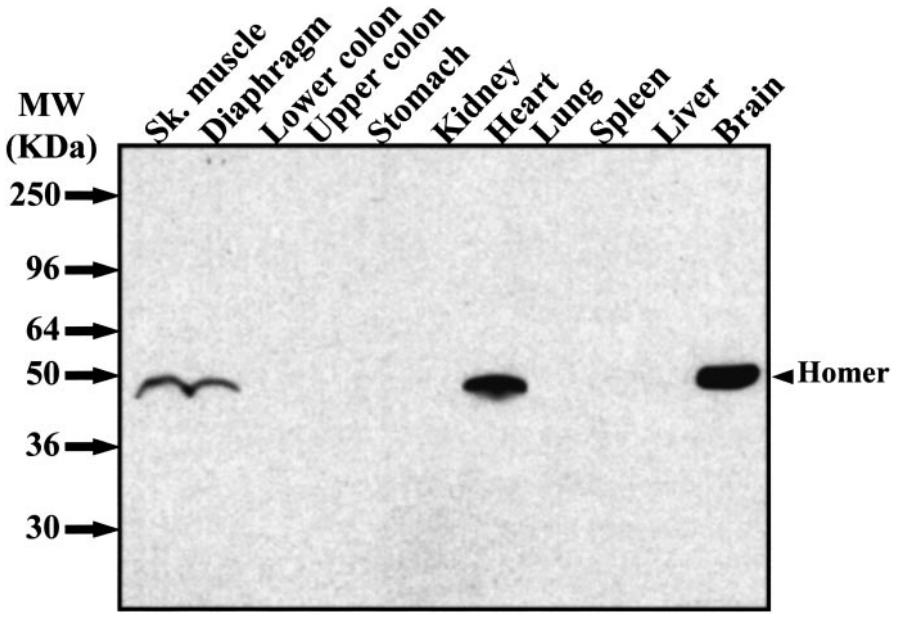

Fig. 3. Immunodetection of Homer proteins in different mouse tissues. Samples (40 μg) were separated on 10% SDS/PAGE and transferred to PVDV membranes for immunoblotting using VHr20 antibody. The arrowhead on the right indicate the position of the constitutively expressed Homer proteins.

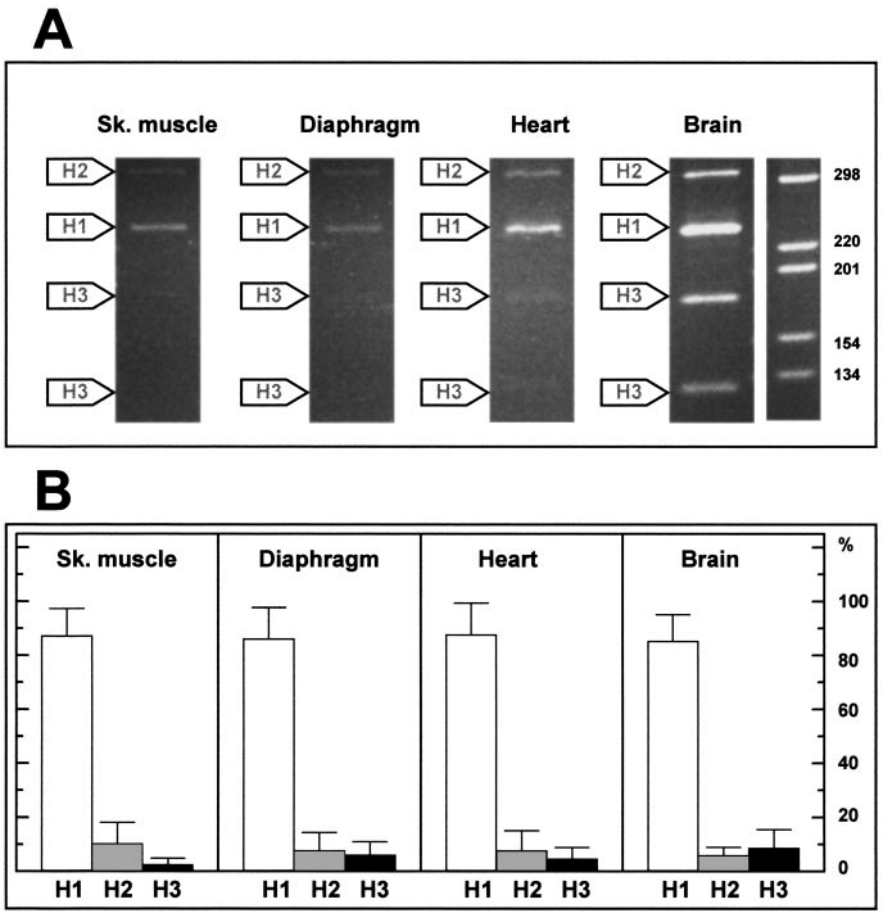

Fig. 4. The coupled RT-PCR/restriction digestion analysis of the Homer mRNAs expression. (A) Pan-Homer amplifications were carried in parallel under the same conditions (as described in the Experimental procedures) using cDNAs derived from the Homer-positive mouse tissues and were subjected to RsaI restriction digestion. H1, H2 and H3 indicate corresponding Homer-1, -2 and -3 cDNA fragments separated on 4% agarose gel. (B) The relative abundance of the individual Homer cDNAs in 'Homer-positive' tissues is expressed as percentage (± SEM, $n = 4$) of the total Homer signal for each such tissue.

by electrophoresis) has allowed us to estimate their relative molar ratios and therefore the relative abundance of the corresponding Homer mRNAs in the original preparations. Whilst the relative amounts of different Homers varies slightly between tissues which express them, their overall expression profiles resemble that obtained for the mouse brain (Fig. 4B, right panel). This may suggest the importance of a balanced expression of different Homers for their function(s). This also strongly indicates that the role which Homers play in brain [3–5,10,21] can be similar to the role they may play in the heart or in the muscle. This seems especially likely as we have recently discovered that a natural Homer target, the metabotropic glutamate receptor mGluR1, is expressed in skeletal muscle (M. M. Soloviev, F. Ciruela & R. A. J. McIlhinney, unpublished data), although its functional role there yet remains to be uncovered. Group I metabotropic glutamate receptors have been also described in rat heart [22]. Moreover, Homer proteins may also have other targets in muscle tissues, like ryanodine or IP3 receptors or other yet unidentified proteins which possess Homer-binding motifs.

We have not attempted to discriminate between alternatively spliced forms of Homer-1 and Homer-2 proteins which, except for Homer-1A, differ marginally by the deletion or insertion of a 12 amino acid fragment (Homer-1B/C) or a 11 amino acid fragment (Homer-2A/B). These differences have not yet been shown to result in any functional differences between Homer-1B and Homer-1C or between Homer-2A and Homer-2B. The mRNA coding for the 'short' Homer-1A was earlier shown not to be expressed constitutively in either brain [1] or peripheral tissues [2]. In accord with those observations, the corresponding 'short' Homer-1A protein could not be detected by immunoblotting in either of the tissues tested (Fig. 3). Therefore, the data presented here describe the quantitative analysis of the tissue distribution and the relative levels of expression of the constitutively expressed 'long' 46–48 kDa Homer proteins. Moreover, the expression of Homers in all of the *muscle* containing tissues tested in this paper justifies a more detailed characterization of the Homers, their function and their interacting proteins in these tissues.

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