Biochemical and Biophysical Research Communications **280,** 164–171 (2001)
doi:10.1006/bbrc.2000.4133, available online at http://www.idealibrary.com on IDEAL®

Molecular Cloning and Characterization of DEC2, a New Member of Basic Helix-Loop-Helix Proteins

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Received December 2, 2000

DEC1 is a basic helix-loop-helix (bHLH) protein related to *Drosophila* Hairy, Enhancer of split and HES, and involved in the control of proliferation and/or differentiation of chondrocytes, neurons, etc. We report here the identification and characterization of human, mouse and rat DEC2, a novel member of the DEC subfamily. DEC2 had high (97%) and moderate (52%) similarities in the bHLH region and the Orange domain with DEC1, respectively. However, DEC2, but not DEC1, had alanine and glycine-rich regions in the C-terminal half. Unlike Hairy, Enhancer of split and HES, DEC2 lacked the WRPW motif for interaction with the corepressor Groucho. The DEC2 gene was mapped to human chromosome 12p11.23-p12.1, mouse chromosome 6 G2-G3 and rat chromosome 4q43 distal–q4, where the conserved linkage homology has been identified among these species. Unlike DEC1, which was broadly expressed in many tissues, DEC2 showed a more restricted pattern of mRNA expression. The DEC subfamily proteins may play an important role in tissue development.

Key Words: **DEC2; Hairy; Enhancer of split; HES; basic helix-loop-helix protein; transcription factor; chromosomal mapping; development.**

Basic helix-loop-helix (bHLH) transcription factors are crucial for the control of proliferation and differentiation during development (1). These proteins, forming homodimers or heterodimers, regulate the expression of target genes through binding to their DNA consensus sequences such as E-box and N-box. We cloned cDNA for a novel bHLH protein, named DEC1, that was isolated from human primary chondrocytes cultured in the presence of dibutyryl cyclic AMP (Bt_2cAMP) (2). The transcript of DEC1 is expressed in a variety of developing and adult tissues (2–4). Bt_2cAMP increased the expression of DEC1 mRNA in many cell types including chondrocytes (Shen *et al.*, unpublished data). Independently, a mouse homologue (Stra13) and a rat homologue (SHARP-2) of DEC1 were identified during the course of searching for the proteins expressed in differentiated neurons, and were shown to be implicated in neurogenesis (3, 4). Stra13 functions as a transcriptional repressor and promotes neuronal differentiation of P19 cells (3). The SHARP-2 mRNA level in the brain increases during development of neurons in embryonic rats and reach a plateau in adult rats (4). Kainic acid increased DEC1 expression in the brain when it caused seizures (4). DEC1 expression may be associated with plasticity of the central nervous system. Recently, Stra13 has been shown to be associated with cell growth arrest and to interact directly with components of the histone deacetylase corepressor complex, which may induce transcriptional repression through deacetylation of histone tails (5). Upon various stimuli such as NGF, kainic acid, PDGF and cAMP, the expression of DEC1/Stra13/SHARP-2 mRNA was rapidly induced within 1 h as an immediate-early gene (3, 4, 6, 7). Thus, DEC1/Stra13/SHARP-2 is likely to be involved in immediate changes adaptive to extracellular stimuli. In addition, retinoic acid, trichostatin A and serum starvation induced Stra13 expression 4-6 h after adding these compounds (3, 5).

The bHLH transcription factors comprise a very large family. The bHLH region of DEC1 exhibits high similarities to that of *Drosophila* Hairy, Enhancer of split (E(Spl)) and HES, which are known as transcrip-

The nucleotide sequences of human and mouse DEC2 have been submitted to GenBank under Accession Nos. AB044088 and AB044090, respectively.

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tional repressors associated with neuronal differentiation (8). However, unlike Hairy/E(Spl)/HES proteins, DEC1 lacks the WRPW motif which is necessary for interaction with the Groucho family members of corepressors. SHARP-1, which is similar to SHARP-2 in amino acid sequence, has also been cloned (4). Although SHARP-1 (253 amino acids) is much shorter than SHARP-2 (411 amino acids), the bHLH region shares remarkable similarity, suggesting that these proteins form a subfamily of bHLH proteins.

To identify new members of the DEC subfamily of bHLH proteins, we searched the expressed sequence tags (EST) data bank. In the present study, we cloned a novel bHLH protein, human DEC2 (hDEC2) and its mouse homologue. We also analyzed the expression patterns of the DEC2 mRNA by Northern blotting and reverse transcriptase (RT)-polymerase chain reaction (PCR). In addition, we determined the genomic position of the DEC2 gene by fluorescence *in situ* hybridization (FISH).

MATERIALS AND METHODS

cDNA cloning of human DEC2. We searched the human EST data bank using amino acid and nucleotide sequences of DEC1 as the query sequence. The 5′- and 3′-rapid amplification of cDNA ends (RACE) were performed using LA *Taq* polymerase (Takara, Kyoto, Japan) and the human chondrocyte MATCHMAKER cDNA library (Clontech, Palo Alto, CA). Gene specific primers were designed from sequences of the EST Clone AA996006 for 5′-RACE (5′-GCAAGTGGTTGATCAGCTGGACACA-3′) and 3′-RACE (5′-ATTCAGTCCGACTTGGATGCGTTCCA-3′), respectively. PCR products were cloned into a TA cloning vector pGEM-T Easy (Promega, Madison, WI). The nucleotide sequence was determined using the BigDye terminator cycle sequencing kit (PE Applied Biosystems, Redwood, CA) with an ABI Prime 310 DNA sequencer (PE Applied Biosystems). Three independent isolates were sequenced for each PCR product, because *Taq* DNA polymerase may misincorporate nucleotides.

cDNA cloning of mouse DEC2. Based on the hDEC2 sequence, we found the mouse DEC2 (mDEC2) homologue by EST database searching. To obtain cDNA clones encoding an entire protein, RT-PCR was performed using skeletal muscle and brain poly(A)$^+$ RNA as a template with a forward primer (5′-AAAATCTCTCCAGGCGACCGT-3′) derived from AA013582 and a reverse primer (5′-AGCCTGTCGAGCATCGCTTA-3′) derived from AI844147. The amplified product was separated on agarose gel, and the DNA of the expected size (1.4 kb) was isolated and sequenced.

RNA purification. Total RNA was extracted from various tissues of three mice by the guanidine thiocyanate/cesium trifluoroacetate method (9). Poly(A)$^+$ RNA was isolated using Oligotex-dT30 (Nippon Roche, Tokyo, Japan).

Northern blot analysis. Approximately 1.5 μg of poly(A)$^+$ RNA from mouse tissues was electrophoresed on 1% agarose–formaldehyde gel, transferred onto Nytran nylon membrane (Schleicher & Schuell, Dassel, Germany), and immobilized by UV crosslinking. PCR fragments (0.6 kb) containing the 5′ coding region of human and mouse DEC2 were labeled with [^{32}P]dCTP using random primer labeling kit (Amersham Pharmacia Biotech, Uppsala, Sweden) and used as probes. The prepared membrane blotting mouse RNA and Human Multiple Tissue Northern Blot (Clontech) were hybridized with each probe in ExpressHyb hybridization solution (Clontech) at 68°C for 1 h, then washed in 0.1× SSC and 0.1% SDS at 50°C, and subjected to autoradiography. BioMax X-ray films (Eastman Kodak, Rochester, NY) were exposed with an intensifying screen at −80°C. Subsequently, a *Bam*HI–*Pst*I fragment (0.7 kb) of the DEC1 cDNA and β-actin cDNA were used for the hybridization of the membrane blotting human RNA, and Stra13 cDNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were used for the hybridizaton of the membrane blotting mouse RNA as probes.

RT-PCR analysis. First-strand cDNA synthesis was performed using the Superscript preamplification system (Gibco BRL, Rockville, MD) with oligo(dT) primer and 1 μg of total RNA from various mouse tissues. The cDNAs were amplified using Advantage KlenTaq polymerase mix (Clontech) with a forward primer (5′-AAGCGAGACGATACCAAGGATACC-3′) and a reverse primer (5′-ATTCTGTAAAGCAATTATC-3′). PCR was performed at 94°C for 1 min, at 94°C for 30 s and at 68°C for 1 min (27 cycles), followed by a 3-min extension at 68°C. PCR products were separated by 1.2% agarose gel electrophoresis, and transferred onto a nylon membrane. The membrane was hybridized with a [^{32}P]dCTP-labeled probe of mouse DEC2 cDNA in hybridization buffer consisting of 6× SSC, 0.5% SDS, 10 mM EDTA, 5× Denhardt's solution and 100 μg/ml of denatured salmon sperm DNA at 68°C overnight, then washed in 0.1× SSC and 0.5% SDS at 50°C. Autoradiography was performed with an intensifying screen at −80°C.

Chromosomal mapping. The direct R-binding FISH method was used for chromosomal assignment of the DEC2 gene to human, mouse and rat chromosomes. Preparation of R-banded chromosomes and FISH were performed as described by Takahashi *et al.* (10) and Matsuda *et al.* (11) for human, and mouse and rat, respectively. The 1.0-kb human and 0.8-kb mouse DEC2 cDNA fragments were labeled by nick translation with biotin-labeled 16-dUTP (Roche Diagnostics). The hybridized probes were reacted with goat anti-biotin antibodies (Vector Laboratories, Burlingame, CA) and then stained with fluoresceinated donkey anti-goat IgG (Nordic Immunology) at a 1:500 dilution for 1 h at 37°C. The hybridization signals were visualized with Nikon filter sets B-2A and UV-2A. Kodak Ektachrome ASA100 films were used for microphotography.

RESULTS AND DISCUSSION

cDNA cloning of human and mouse DEC2. Searching the human DNA database of EST retrieved a clone (GenBank Accession No. AA996006), which was one candidate for a new member of the DEC subfamily. AA996006 contained a novel sequence similar to, but distinct from, the DEC1 protein sequence. To determine the entire cDNA sequence of this clone, we performed 5′- and 3′-RACE with human chondrocyte cDNA using primers derived from AA996006. The PCR products were isolated and sequenced as described under Materials and Methods. The largest open reading frame encoded a protein of 482 amino acids with a calculated molecular weight of 50.5 kDa. The sequence (AACATGG) surrounding the first ATG agreed with the Kozak consensus sequence (12). In the 3′-untranslated region, potential polyadenylation signals and a poly(A) tail were excluded (data not shown). Sequence analysis revealed that the full-length cDNA

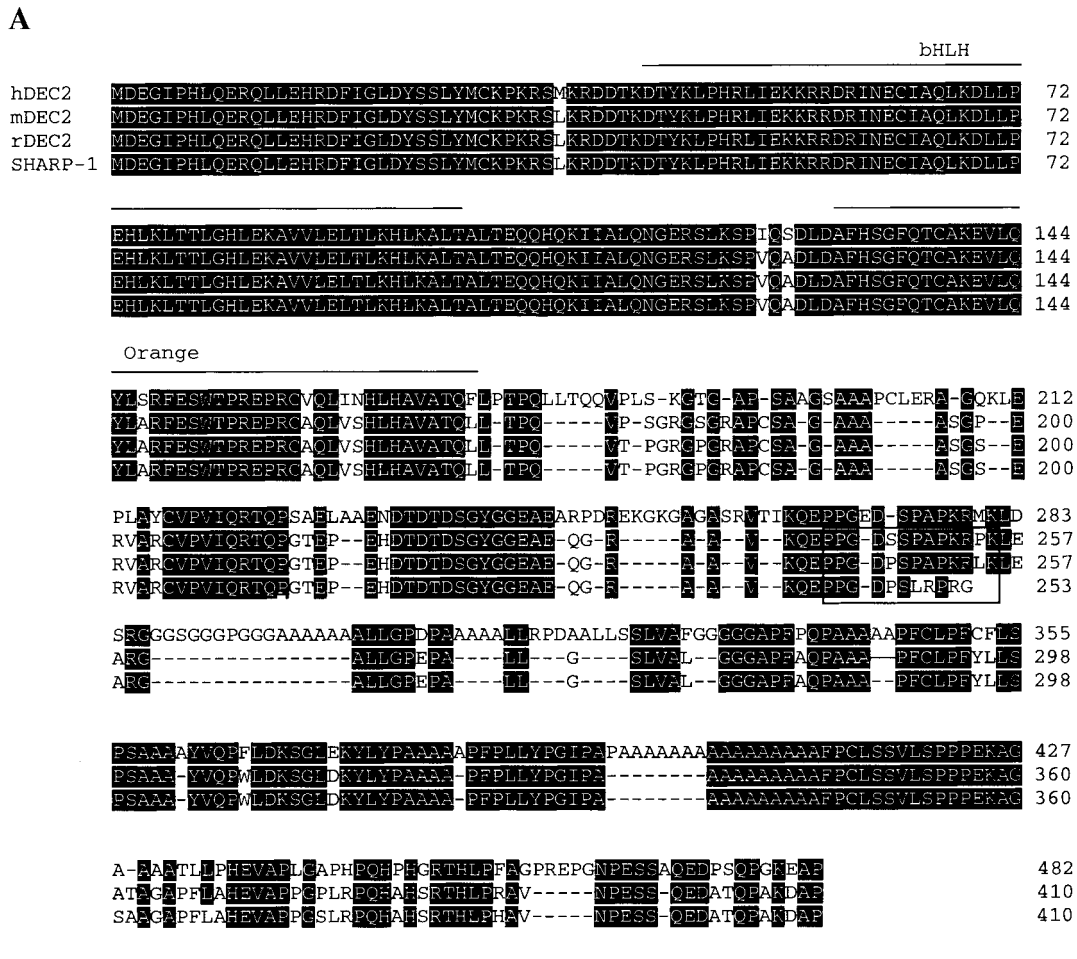

FIG. 1. Sequence comparison between DEC2 and SHARP-1. (A) Amino acid sequence alignment of hDEC2, mDEC2, rDEC2, and SHARP-1. The amino acid residues are numbered on the right. The amino acid sequences for hDEC2 and mDEC2 were deduced from their cDNA sequences, while the amino acid sequence for rDEC2 was deduced from the partial sequence of the cloned cDNAs and the SHARP-1 nucleotide sequences. The bHLH and Orange domains are indicated with bars above the alignment. The nucleotide sequence of the boxed area is shown in B. (B) Nucleotide sequence alignment of mDEC2, rDEC2, and SHARP-1. The nucleotide residues are numbered on the right. Identical nucleotides are indicated by dots. A termination codon is indicated by an asterisk. The deduced amino acid residues for mDEC2 and SHARP-1 are shown above and below the alignment, respectively. The underlined cytidine is present in mDEC2 and rDEC2 but not in SHARP-1. Amino acid residues identical among human, mouse, and rat DEC2 are shown in black. Hyphens represent gaps introduced to maintain the alignment.

encoded a novel bHLH protein (DEC2) closely related to DEC1 (see below).

In addition, we searched the mouse EST data bank using the nucleotide sequence of hDEC2 as the query to identify the mouse cDNA homologue of DEC2. Two EST clones (GenBank Accession Nos. AA013582 and AI844147) showed similarities to the hDEC2 protein sequence, and also contained the sequences of the putative 5′- and 3′ non-coding regions of mDEC2, respectively. The cDNA fragments encoding the entire DEC2

FIG. 2. Amino acid sequence alignment of hDEC2 and DEC1. The amino acid residues are numbered on the right. The bHLH and Orange domains are indicated above the alignment. Amino acid residues identical between hDEC2 and DEC1 are shown in black background, and less conserved residues are shaded in gray. Hyphens represent gaps introduced to maintain the alignment.

protein were obtained by RT-PCR amplification with primers derived from AA013582 and AI844147. We isolated and sequenced five independent clones as described under Materials and Methods. The open reading frame of mDEC2 encoded a protein of 410 amino acids with a calculated molecular weight of 44 kDa.

As shown in Fig. 1A, the N-terminal region of DEC2 was highly conserved between human and mouse. The amino acid sequence in the bHLH region was completely conserved, and that in the Orange domain, which is conserved among Hairy/E(Spl)/HES proteins (13), was 89% identical. In contrast, the middle and C-terminal regions showed sequence divergence, and required a number of gaps to maintain the alignment. mDEC2 was shorter than hDEC2 by 72 amino acids, in which many alanine and glycine residues were present. Overall sequence identity was 73% between human and mouse DEC2 proteins, whereas DEC1 and its mouse homologue Stra13 shared 90% amino acid identity.

Sequence comparison between DEC2 and SHARP-1. A protein database search blast revealed a very high similarity between the N-terminal half of DEC2 and SHARP-1 (85–95% identity). However, SHARP-1 did not contain the amino acid sequence corresponding to the C-terminal half of DEC2 (Fig. 1A). Comparison of nucleotide sequences among DEC2 and SHARP-1 suggested that although nucleotide sequences were similar throughout their entire cDNA length, one nucleotide (cytidine in nucleotide 746) was deleted in the coding region of SHARP-1. This deletion led to a frame-shift of the following amino acid sequence and the truncation of the C-terminal half (Figs. 1A and 1B). To examine the deletion observed in SHARP-1, we cloned DNA fragments containing the critical region by PCR amplification from rat skeletal muscle cDNA and genomic DNA. As in the case of the human and mouse DEC2 sequences, one extra cytidine was present in the nucleotide sequences of both rat cDNA and genomic DNA without other differences from the SHARP-1 sequence. Consequently, as shown in Fig. 1A, the amino acid sequence of rat DEC2 (rDEC2) determined in the present study exhibited a significant homology with mDEC2 over their entire length (amino acid sequence identity was 97%). The difference in sequence of SHARP-1 by Rossner's study was probably due to a sequencing error or a minor frame-shift mutant of their cDNA clone, although we could not detect such a deletion mutant among the human, mouse and rat clones presently examined.

Comparison of primary structure of hDEC2 and hDEC1. The amino acid sequence of hDEC2 was compared with that of hDEC1 (Figs. 2 and 3). The homology between these proteins was 42% in total and 97% in the bHLH region, which is involved in DNA binding and protein dimerization. The high homology in the bHLH domain suggested that these proteins form a subfamily within the bHLH protein family. The Or-

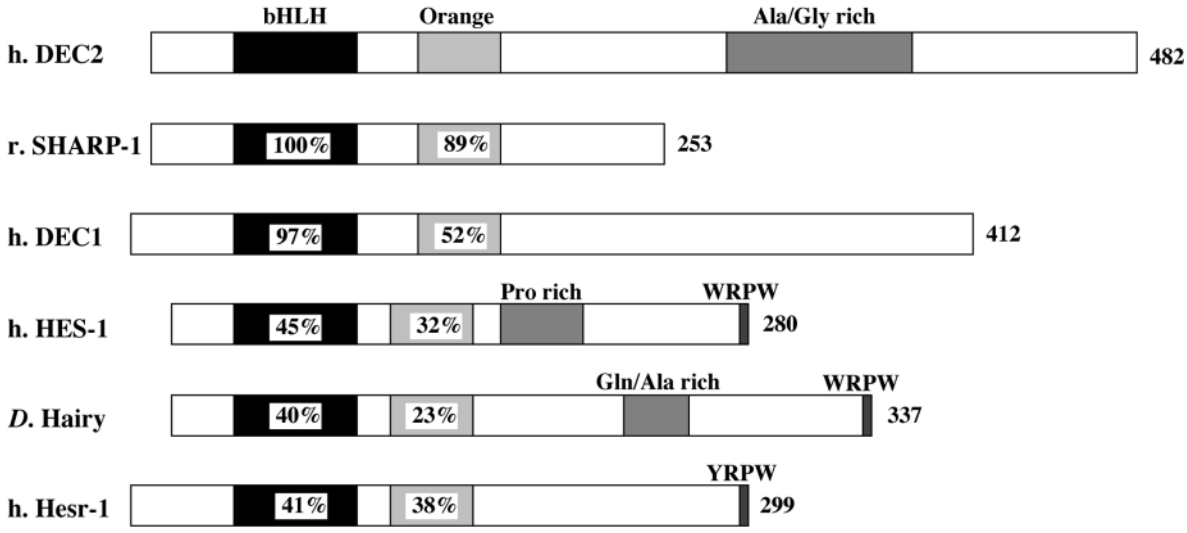

FIG. 3. Schematic representation of the structures of DEC2 and related bHLH factors. Percentages indicate amino acid identity in the bHLH region or the Orange domain between the respective proteins and DEC2. The numbers of amino acids are shown on the right. h., human; r., rat; *D., Drosophila*.

ange domain, the region following the bHLH domain, was moderately conserved (amino acid sequence identity was 52%). Besides the bHLH and Orange domains, there was several conserved amino acid sequences. These motifs showed no apparent similarity to any other known proteins so far examined. A recent study demonstrated that histone deacetylase, Sin3A, and nuclear receptor corepressor, which are involved in the control of gene transcription (14, 15), could interact with the Stra13 C-terminal region, which was also required for the transcriptional repression activity of Stra13 (3, 5). Although it is not known whether DEC2 also functions as a transcriptional repressor, the conserved regions in the C-terminal half may be involved in the common function of DEC proteins. However, DEC2 contained the alanine and glycine-rich regions that were absent in DEC1. This feature may distinguish the function of DEC2 from that of DEC1. The Ala/Gly rich domains are also present in other transcription proteins such as BFP/ZNF179 (16), Oct-6 (17), and Msx-1 (18).

DECs form a subfamily within bHLH proteins. Among bHLH proteins, Hairy/E(Spl)/HES proteins and Hesr/Hey/HRT proteins showed the highest similarities to DEC/Stra13/SHARP proteins. The similarity was 40–45 and 41–42%, respectively, in the bHLH region, and 14–32 and 33–42%, respectively, in the Orange domain (Fig. 3). Hairy/E(Spl)/HES proteins are categorized as repressive bHLH proteins containing a proline residue in the basic region (19). A proline residue is also found in the basic region of DECs, although the position of the proline residue is different from that of Hairy/E(Spl)/HES proteins. Another critical motif (WRPW sequence) for transcriptional repression has been identified in the C-terminus of Hairy/E(Spl)/HES proteins (20), and similar sequences (YRPW, YQPW and YHSW) are also present in the C-terminus of Hesr/Hey/HRT proteins (21–23). However, this motif was absent in DEC1 and DEC2 proteins. Thus, DEC proteins are distantly related to Hairy/E(Spl)/HES and Hesr/Hey/HRT proteins.

Tissue distributions of DEC2 mRNA. To determine the expression pattern of DEC2, we performed Northern blot analysis of human and mouse tissues. As shown in Fig. 4A, hDEC2 mRNA was detected as a single band at 3.6 kb, which was identical with the length of the isolated cDNA containing an additional 100 bp of the poly(A) tail. DEC2 was expressed in the skeletal muscle and brain at high levels, in the heart and pancreas at moderate levels, and in the placenta and lung at low levels. Only a faint band of DEC2 was detected in the liver and kidney. Similar findings were obtained with the mouse RNA blot (Fig. 4B). However, DEC1 and Stra13 mRNAs were expressed in all tissues examined, and at particularly high levels in the skeletal muscle and lung.

We also investigated the expression of DEC2 mRNA by RT-PCR using total RNA from multiple tissues (Fig. 4C). The expression of DEC2 mRNA was detected in all tissues examined, and the expression pattern was almost similar to that of Northern blotting. Thus, DEC2 and DEC1 mRNAs were ubiquitously expressed, but the expression level of DEC2 was more varied tissue-dependently than that of DEC1. DEC2 unlike DEC1 was expressed in the liver and kidney at very low

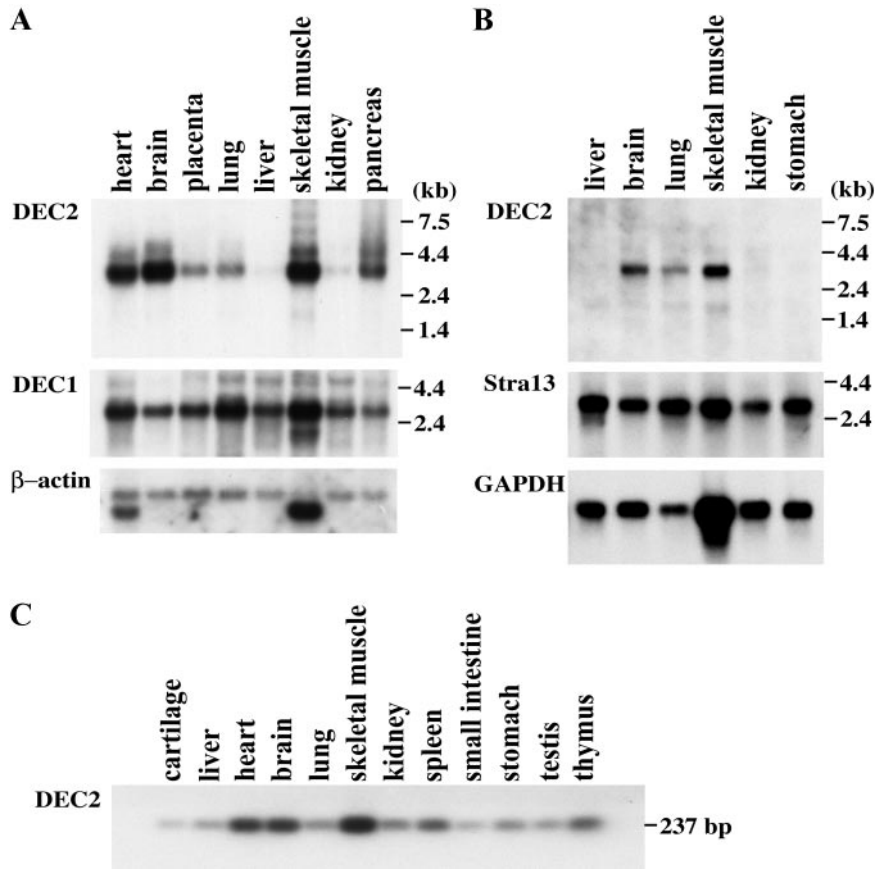

FIG. 4. Tissue distribution of the DEC2 mRNA. (A) Northern blot analysis using Human Multiple Tissue Northern Blot (Clontech). Each lane contains 2 μg of RNA from the tissues. The sizes of the hDEC2 and DEC1 mRNAs were estimated to be approximately 3.6 and 3.1 kb, respectively. (B) Northern blot analysis of mouse tissues. Approximately 1.5 μg of poly(A)$^+$ RNA derived from various mouse tissues was applied to each lane. The sizes of the mDEC2 and the Stra13 mRNAs were estimated to be approximately 3.4 and 3.0 kb, respectively. (C) RT-PCR analysis of mouse tissues. The expected PCR product of mDEC2 (237 bp) is shown.

levels. These findings suggest that DEC2 and DEC1 may have distinct roles in tissue development.

Chromosomal localization of DEC2. Chromosomal location of the DEC2 gene was determined in human, mouse and rat by the direct R-banding FISH using cDNA fragments as probes. The DEC2 gene was localized to human chromosome 12p11.23-p12.1, mouse chromosome 6 G2-G3 and rat chromosome 4q43 distal–q44 (Fig. 5). They were mapped in the region where the conserved linkage homology has been identified among the three species (24, 25). Previous studies demonstrated that the DEC1 gene is localized on human chromosome 3p26 (26) and mouse chromosome 6 E-F1 (27). Thus, the DEC genes were not clustered within the genome.

Previous studies have demonstrated that human chromosome 12p11.23-p12.1 to which hDEC2 maps links to a variety of tumor types and various diseases. Prostate cancer (28), testicular germ cell tumors (29), acute lymphoblastic leukemia (30) and synpolydactyly (31) have been mapped to 12p12-13, 12p11.2-p12.1, 12p12, and 12p11.2, respectively. Synpolydactyly is a congenital disorder characterized by abnormalities of the distal parts of both upper and lower limbs. In addition to these diseases, phenotypic abnormalities linking to 12p have been reported. Major characteristics of the phenotype of trisomy 12p, Pallister-killian syndrome, include severe psychomotor retardation, generalized hypotonia, round face with prominent cheeks, flat and broad nasal bridge with short nose (32). In addition, deletion of this region has been associated with severe psychomotor retardation and facial dysmorphism (33). Although the phenotypes may be attributed to more than one gene, DEC2 is considered to be an attractive candidate gene for the disease, because the bHLH transcription factor is essential for normal tissue development.

In conclusion, we cloned cDNA for DEC2, a novel member of the DEC subfamily in bHLH proteins, and

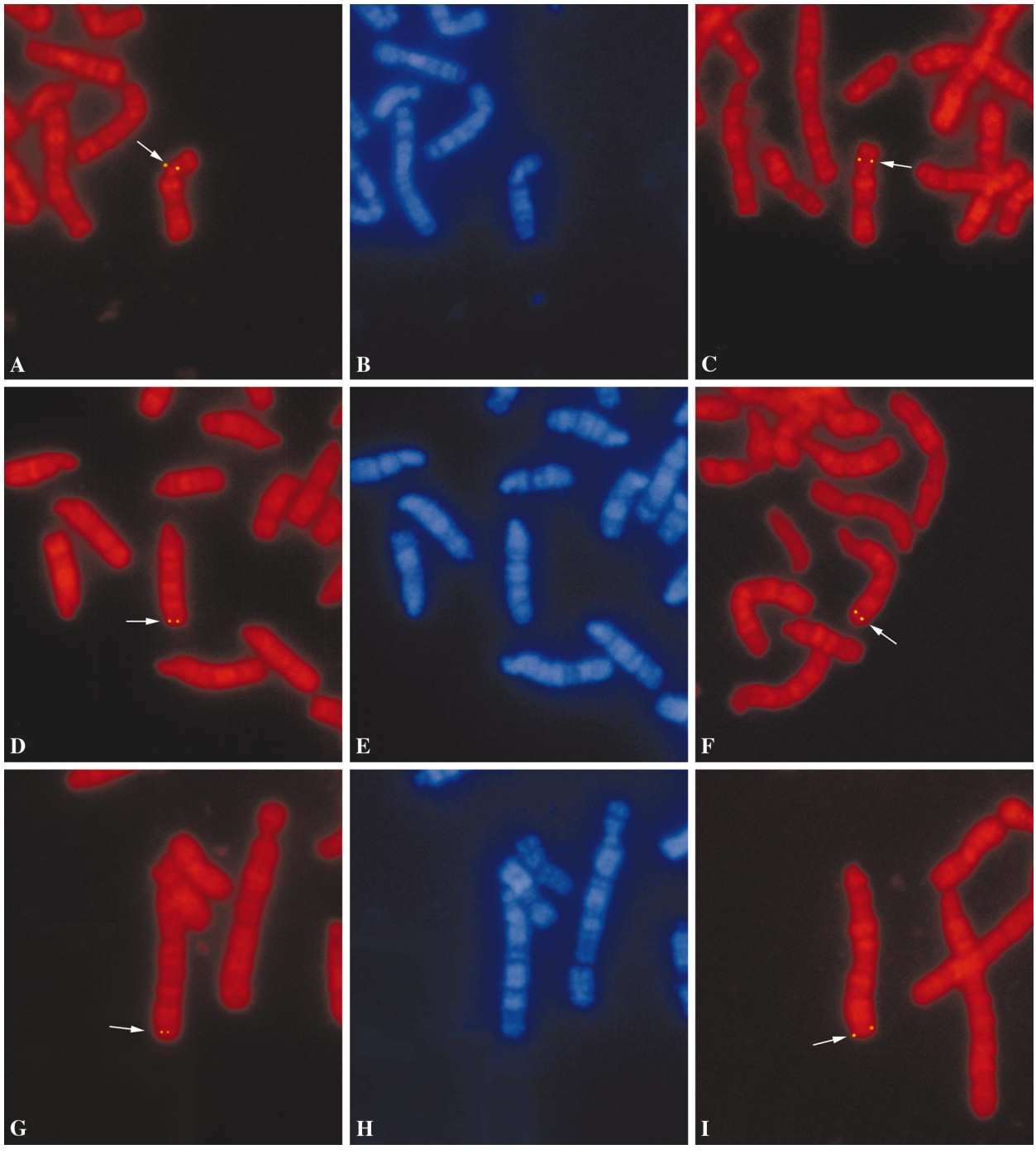

FIG. 5. Chromosomal localization of the DEC2 genes to human (A–C) and mouse (D–F) and rat (G–I) R-banded chromosomes. The human and mouse DEC2 cDNA fragments were used as biotinylated probes. Arrows indicate the hybridization signals. The metaphase spreads were photographed with Nikon B-2A (A, C, D, F, G, I) and UV-2A (B, E, H) filters. R- and G-banded patterns are demonstrated in (A, C, D, F, G, I) and (B, E, H), respectively.

then characterized its structure. DEC2 and DEC1 showed different tissue distributions. Further studies of DEC2 may help to clarify the roles of the DEC subfamily as a transcription factor.

ACKNOWLEDGMENTS

This work was supported by a grant-in aid for science from the Ministry of Education, Science, Sports, and Culture of Japan. We thank Ms. Tomoko Minamizaki and Ms. Mari Yukiiri for technical assistance.

We also thank the Research Center for Molecular Medicine, Faculty of Medicine, Hiroshima University for the use of their facilities.

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