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Gene Structure and Chromosomal Location of a Human bHLH Transcriptional Factor DEC1·Stra13·SHARP-2/BHLHB2[1]

Masao Teramoto,*,† Kazuko Nakamasu,* Mitsuhide Noshiro,* Yoichi Matsuda,‡,§ Osamu Gotoh,‖ Ming Shen,*,¶ Shinichi Tsutsumi,*,# Takeshi Kawamoto,* Yukihide Iwamoto,† and Yukio Kato*,[2]

Department of Biochemistry, Hiroshima University School of Dentistry, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8553; †Department of Orthopaedic Surgery, Graduate School of Medical Science, Kyushu University, Fukuoka 812-8582; ‡Chromosome Research Unit, Faculty of Science, and §Laboratory of Cytogenetics, Division of Bioscience, Graduate School of Environmental Earth Science, Hokkaido University, Sapporo 060-0810; ‖Saitama Cancer Center Research Institute, Saitama 362-0806; ¶First Hospital, Norman Bethune University of Medical Sciences, Changchun 1300-21, People's Republic of China; and #Department of Orthopaedic Surgery, Gunma University Faculty of Medicine, Maebashi 371-8511

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DEC1/BHLHB2 is a novel cAMP-inducible basic helix-loop-helix (bHLH) transcriptional factor isolated from human chondrocyte cultures by the subtraction method [Shen *et al.* (1997) *Biochem. Biophys. Res. Commun.* 236, 294–298]. DEC1 seems to be involved in controlling the proliferation/differentiation of some cell lineages. We determined the structure of the human DEC1 gene and its chromosomal locus. Phylogenetic analysis and comparison of the gene structure showed that the DEC1 protein is a member of a new subgroup of the proline bHLH protein family that diverged earlier than other proline bHLH proteins including HES, hairy and E(spl). The human DEC1 gene spans approximately 5.7 kb and contains 5 exons. The putative promoter region contains multiple GC boxes but no TATA box. A primer extension study showed multiple transcriptional initiation sites. In the 5′-flanking region of the DEC1 gene, several transcriptional factor binding sites, including a cAMP-responsive element (CRE), were found using the transcription factor database. The DEC1 gene locates at Chromosome 3p25.3–26 by the FISH method. This is the first study to determine the genomic structure of the DEC1 gene subgroup.

Key words: bHLH, cAMP, chondrocyte, DEC1, gene structure.

In the course of exploring novel genes involved in the proliferation and differentiation of chondrocytes, we cloned a novel cAMP-inducible gene named DEC1 using the subtraction method from human embryonic epiphysial chondrocytes treated with dibutyryl cAMP (*1*). DEC1 appears to be a transcriptional factor because it has a bHLH domain. DEC1 shows relatively high homology (40–45%) to rat HES1 (*2*), HES2 (*3*), HES5 (*4*), *Drosophila* hairy (*5*), and Enhancer of split m7 [E(spl) m7] (*6*) in the bHLH domain (*1*). The HES/hairy/E(spl) subgroup, whose members are involved in the control of neuronal differentiation, have a "WRPW domain" in the C-terminal region to which corepressors bind (*7*), but DEC1 lacks the "WRPW domain."

Subsequent to our study, two groups reported mouse Stra13 (*8*) and rat SHARP-2 (*9*), which were orthologous to human DEC1. SHARP-1, cloned from rat brain, is similar but not orthologous to DEC1/SHARP-2. NGF and kainic acid induce SHARPs in PC12 cells and *in vivo* brain, respectively (*9*). SHARPs may be involved in the adaptive changes of mature CNS neurons (*9*). Stra13 was identified as a retinoic acid–inducible gene from P19 embryonal carcinoma cells (*8*). Stra13 expression in NIH3T3 cells is associated with growth arrest, and the bHLH protein represses the transcription of Stra13 and c-Myc through histone deacetylase- and TFIIB-dependent mechanisms, respectively (*10*). The overexpression of Stra13 in P19 cells and NIH3T3 cells promotes neuronal differentiation and suppresses cell proliferation, respectively (*8*, *10*).

In this study, we report the genomic structure of the DEC1 gene, including the region upstream of the gene, the location of the DEC1 gene on the chromosome, and the phylogenetic tree, including DEC1-related genes. The structures of HES and hairy genes have already been reported, but there has been no study reporting the structure of DEC1 subgroup genes.

MATERIALS AND METHODS

Isolation of the Genomic DNA Library and DNA Sequence Analysis—Genomic DNA was isolated from human white blood cells using a previously described procedure (*11*). Genomic DNA was partially digested with *Mbo*I and ligated into the *Bam*HI site of EMBL3 phage vector

[1] DEC1 was designated as BHLHB2 by the human gene nomenclature committee (http://www.gene.ucl.ac.uk/nomenclature/), the sequence data reported in this paper have been entered in the DDBJ/EMBL/GenBank data base under the accession number of AB043885.

[2] To whom correspondence should be addressed. Tel/Fax: +81-82-2575629, E-mail: ykato@hiroshima-u.ac.jp

(Strategene), packaged and transfected into host *Escherichia coli* XL1-Blue MRA(P2) (*12*). The libraries were plated and screened with a ^{32}P-labeled 1.7 kb probe (D1p-T3), a DEC1 cDNA fragment from −210 to 1493 (*1*), using the plaque hybridization method (*11*). Positive clones were isolated, digested with appropriate enzymes and subcloned into pBluescript II SK(−) phagemid vector (Strategene).

The subcloned genomic DNAs were digested with the proper restriction endonucleases to ligate them into the pBluescript II SK(−) phagemid vector. DNA sequencing was performed by the method of Sanger *et al.* (*13*) using an ABI Prism 310 autosequencer (Perkin-Elmer).

Southern Hybridization Analysis—Total genomic DNA (15 μg) was extracted from human placenta (Clontech), and the isolated genomic clones (0.5 μg) were digested with *Bam*HI, *Hin*dIII, *Pst*I, and *Sal*I, and subjected to agarose gel electrophoresis (*14*). The separated DNA fragments were transferred to NYTRAN® 0.45 nylon membranes (Schleicher & Schwell). The membranes were hybridized with a ^{32}P-labeled probe, D1p-T3, in hybridization solution containing 6× SSC, 5× Denhardt's, 10 mM EDTA, 1% SDS, and 0.5 mg/ml sonicated salmon sperm DNA at 68°C. The membranes were washed with 0.1× SSC containing 0.5% SDS and exposed to X-ray film.

Determination of the Transcription Initiation Site—The location of the cap site of the DEC1 mRNA was determined by a primer extension method (*15*). Total RNAs were prepared from HeLa cells using an ISOGENE kit (Nippon Gene), and poly(A)$^+$ RNA was enriched with Oligotex™-dT30 (Nippon Roche). Primers DEC1-50 (5′-ATCTTCGGCTTTGAGATGCAGCTTCAGTTG-3′, corresponding to −173 to −143, 0.5 pmol) and DEC1-52 (5′-TTTGAAATCCCCTGGGCTGCTGGAATCTTC-3′, corresponding to −149 to −119, 0.5 pmol) were end-labeled with ^{32}P and T4 polynucleotide kinase, annealed to 2.0 μg of poly(A)$^+$ RNA prepared from HeLa cells, and extended with 200 units of SuperScript II (Life Technologies). The size of the extended radioactive DNA was analyzed on a 6% acrylamide gel, and sequencing ladders were primed from the same synthesized oligonucleotides.

Chromosome Preparation and In Situ Hybridization—The direct R-banding FISH method was used for the chromosomal assignment of the human DEC1 gene. Preparation of R-banded chromosomes and FISH were performed as described by Takahashi *et al.* (*16*, *17*). The chromosome slides were prepared using mitogen-stimulated lymphocytes. R-band staining was performed by exposing the Hoechst 33258-stained chromosome slides to UV light. The human 1.7 kb cDNA D1p-T3 was labeled with biotin 16-dUTP (Roche Diagnostics) following the manufacturer's protocol. The labeled probe was denatured and hybridized on the denatured chromosomes at 37°C overnight. After washing with 50% formamide in 2× SSC at 37°C, and in 2× SSC and 1× SSC at room temperature, the slides were incubated with anti-biotin antibody (Vector Laboratories) and stained with fluoresceinated donkey anti-goat IgG (Amersham Pharmacia Biotech). Excitations at wavelength 450–490 nm (Nikon filter set B-2A) and near 365 nm (UV-2A) were used for observation.

RESULTS AND DISCUSSION

Phylogenetic Relationships—Many bHLH proteins are structurally related to each other, especially in the bHLH domain. For example, the homology is approximately 80% among myogenic bHLH proteins (*18*, *19*). A phylogenetic tree (Fig. 1) shows the relationship between DEC1 and other bHLH proteins that show high homology to DEC1.

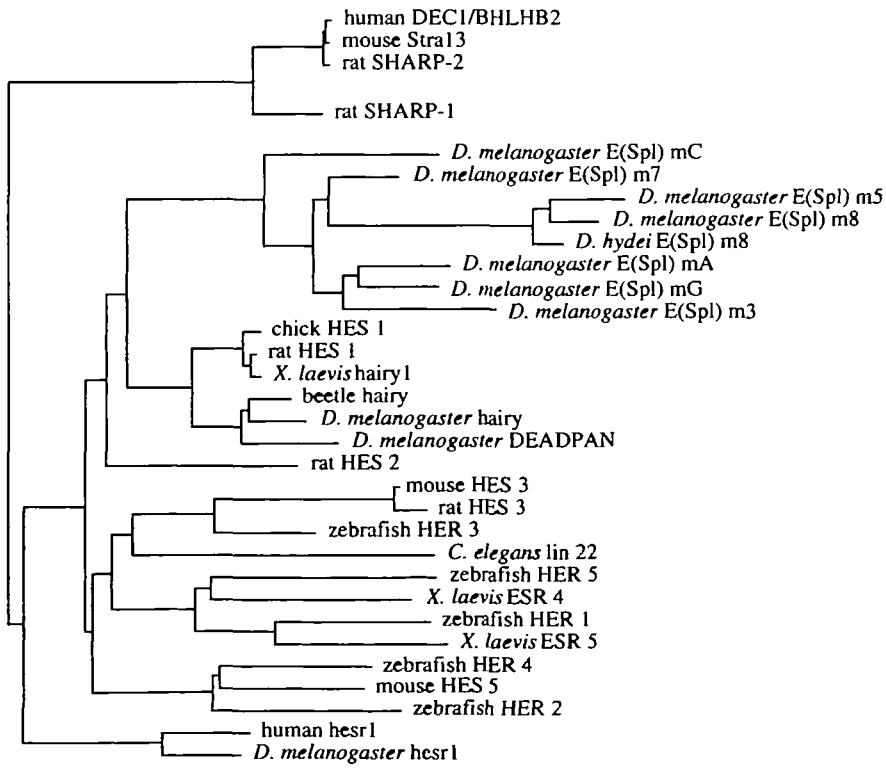


Fig. 1. **Phylogenetic tree of bHLH proteins showing high homology to DEC1.** The topology was calculated from the values for amino acid sequence divergence. Amino acid sequences were taken from the databases. *D. melanogaster, Drosophila melanogaster; D. hydei, Drosophila hydei; X. laevis, Xenopus laevis; C. elegans, Caenorhabditis elegans.*

This tree includes DEC1, hairy, E(spl), HES, and other members of the proline bHLH protein family. The amino acid sequence data for these proteins were multiply aligned by the doubly nested randomized iterative refinement (DNR) method (*20*) to construct a detailed phylogenetic tree for these bHLH proteins. The phylogenetic tree was constructed from the global alignment by the neighbor-joining method (*21*) as described previously (*22*). Although DEC1 orthologous proteins show high similarities to hairy, E(spl), and HES proteins in the bHLH region, this topology also indicates that DEC1 orthologous proteins form another group that is different from the group comprising hairy, E(spl), and HES.

The bHLH domain is 95% conserved between rat SHARP-1 and SHARP-2, and 100% among human DEC1, mouse Stra13 and rat SHARP-2 (*8*, *9*). The bHLH domain of these proteins shows high homology to those of hairy, E(spl), and HES proteins, but other structural features are distinct. In DEC1 subgroup proteins, histidine 49 and arginine 50 in the basic region differ from lysine and proline that are conserved at the corresponding positions in the hairy, E(spl), and HES families (see Fig. 4). This conserved proline residue in hairy, E(spl), and HES proteins affects the DNA-binding specificity (*23*, *24*). Based on the alignment analysis of DEC1 and other most closely related proteins, such as mouse Stra13, rat Sharp1 and Sharp2, this proline residue in the basic region is located a further two amino acid residues toward the N-terminus than that in hairy, E(spl), and HES proteins (Fig. 4) (*1*, *8*). This difference may change the DNA-binding specificity (*23*, *24*). HER, ESR, lin22, and hesr1 proteins have not been reported to have high homology to DEC1 (*25–27*). The proline residue in the basic region of these proteins, except hesr1, is located at the same position as the proline residue in HES/hairy/E(spl) proteins. Despite the similarity to hairy/E(spl) proteins, hesr1 does not have the proline residue in the basic region (*26*). These findings indicate the divergence of this tree and suggest that the proline residue in the basic region may be important for distinguishing the function of these proteins.

Isolation of Clones Containing the Human DEC1 Gene— Six positive clones were isolated from 3.6×10^6 plaques by screening EMBL3 genomic libraries using D1p-T3 as a probe, and subjected to restriction mapping analysis. Four

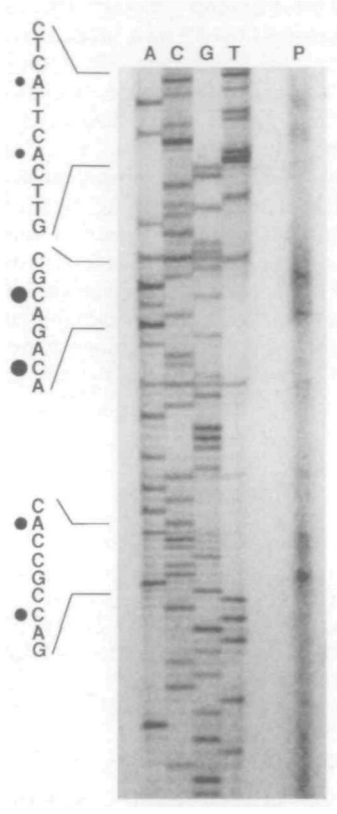
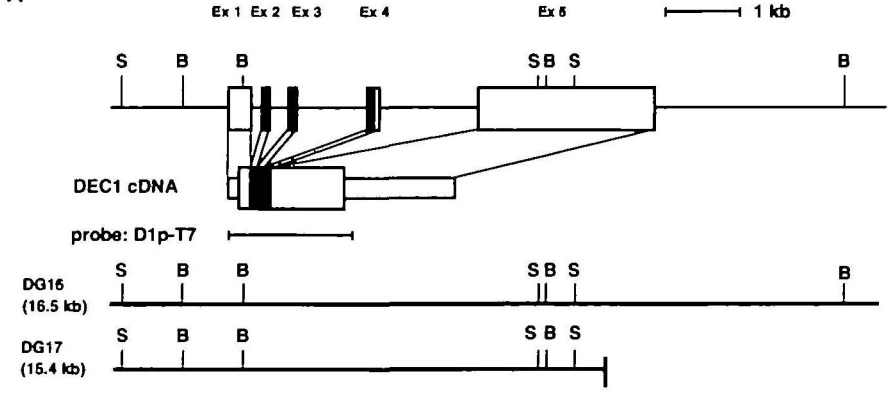


Fig. 3. **Determination of the transcriptional initiation site.** The nucleotide sequences around the start site sequence are represented by the 5′ to 3′ orientation from top to bottom. The large closed circles indicate the major initiation sites for transcription and the small closed circles indicate the minor sites. P lane, HeLa poly(A)$^+$ RNA; A, C, G, and T lanes, DNA ladder obtained by sequencing the DEC1 plasmid clone with primer DEC1-52.

Fig. 2. **Genomic structure of the human DEC1 gene.** (A) Restriction enzyme map of a genomic clone of human DEC1. The thick line represents the human DEC1 gene locus, with transcription proceeding from left to right. The DG16 and DG17 clones isolated from human genomic libraries were used to construct an entire map of the DEC1 gene. Restriction enzyme sites shown in the figure are as follows: B, *Bam*HI; S, *Sal*I. Open boxes and closed boxes in the constructed gene represent exons, and closed boxes indicate the bHLH region. The wide box on the DEC1 cDNA shows the coding region. The DEC1 cDNA used for hybridization is shown as a 1.7 kb probe D1p-T3. kb, kilobase pairs; Ex, exon. (B) Nucleotide sequence of exon/intron boundaries in the human DEC1 gene. The sizes of exons and their flanking splice donor and acceptor sequences are shown. The exonic sequences are shown in uppercase; the intron sequences are shown in lowercase. bp, base pairs.

A



B

Exon No	length (bp)	splice acceptor	splice donor
1	289		CGGAGACCTACCAGGgtaagttggc
2	70	cttcctgcagGATGTACCCTGCCCA	AGCGAGGACAGCAAGgtaagcaagt
3	108	cgtcttgcagGAGACCTACAAATTG	CATCTCAAACTTACAgtaagtgaga
4	124	ttccttccagACTTTGGGTCACTTG	GTGGTTTACAAGCTGgtgagtgctg
5	2345	ctttccccagGTGAGCTGTCAGGGA	

of the six isolated clones showed *Bam*HI and *Sal*I restriction enzyme sites, at least partly, overlapping one another. The entire structure of the DEC1 gene was finally constructed using clones DG16 and DG17 (Fig. 2A). The entire structure of the DEC1 gene was confirmed by Southern blot analysis of total human genomic DNA and the isolated genomic clones using D1p-T3 as a probe (data not shown).

Transcriptional Initiation Site—To define the start site for transcription, primer extension was performed using poly(A)$^+$ RNA from HeLa cells. Hela cells express DEC1 mRNA at high levels (Shen *et al.*, unpublished observation). When template mRNA preparations were primed with the DEC1-52 oligonucleotide, two major extended fragments ending at nucleotide C were observed at 261 and 257 nucleotides upstream from the ATG translation initiation codon (Fig. 3). Several minor extended fragments were also observed around the major fragment as indicated in Fig. 3. When the mRNA templates were primed with DEC1-50 oligonucleotide, the extended fragments localized to the same positions determined with DEC1-52 primer (data not shown), confirming the reproducibility of the multiple transcriptional initiation sites.

In the region preceding the putative cap site, no TATA(A/T)A sequence, the expected TATA box, was observed. Instead, the 5′-flanking region is rich in GC nucleotides, and the sequences GGGCGG or CCGCCC, possible GC box sites (SP1 sites), were found at positions −342, −333, −300, and −296 (Fig. 5 box). Multiple copies of the GC box in the transcriptional initiation region without a TATA box have been found predominantly in ubiquitously expressed genes, and this structure is considered to be characteristic of housekeeping genes (*28*), although DEC1 does not appear to be a house-keeping gene.

Organization of the Human DEC1 Gene—As shown in Fig. 2A, the functional human DEC1 gene spans approximately 5.7 kb and contains 5 exons. Exons 1 through 5 consist of 289, 70, 108, 124, and 2,345 bp, respectively (Fig. 2B). Nucleotide sequences of all exon/intron boundaries follow the typical GT/AG rule (Fig. 2B) (*29*). Flanking sequences of the 5′- and 3′-splicing sites in the introns are good in accordance with the splice junction consensus sequences proposed by Mount (*30*). The nucleotide sequences of the exons are almost identical to those of the reported DEC1 cDNA (*1*), except for two bases in the non-coding region in exon 5. These differences may represent variation among human individuals. All introns are located within the protein coding region. Of four introns, only intron 3 is located within the bHLH domain; introns 1 and 2 are located between the N-terminal and the bHLH domain, and intron 4 is located at the C-terminal of the bHLH domain. The positions of the introns in the human DEC1 gene differ from those in the mouse HES-1 (*31*), HES-2 (*32*), HES-3 (*33*), and HES-5 genes (*34*). The exon-intron boundaries are preserved among mouse HES genes. HES-1, HES-2, and HES-3 have three introns (*31–33*): introns 1 and 2 located within the bHLH region, and intron 3 located at the C-terminal of the bHLH domain. HES-5 has two introns: intron 1 within the bHLH region, and intron 2 at

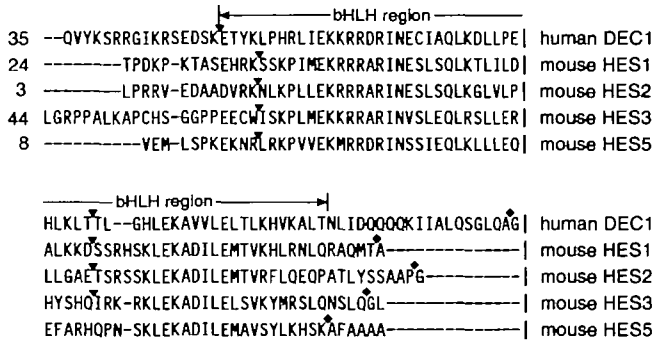


Fig. 4. **Comparison of exon–intron boundaries between human DEC1 and mouse HES proteins.** The amino acid sequences of human DEC1, mouse HES-1, HES-2, HES-3, and HES-5 are compared with the bHLH region. The locations of introns are indicated by triangles and lozenges: (▼) intron lies between the codons for the flanking amino acids; (◆) intron lies between the first and second nucleotide of the following amino acid. Numbers on the left correspond to the amino acid positions of each protein.

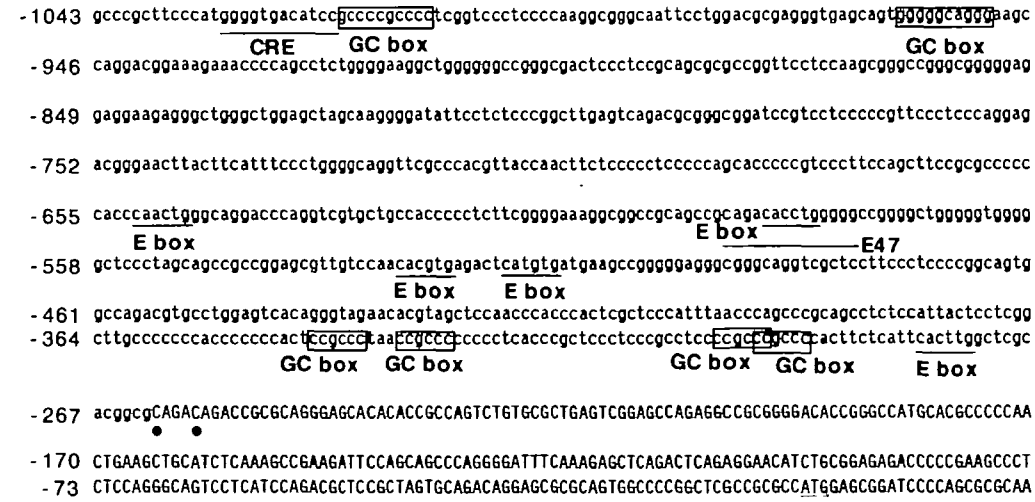


Fig. 5. **The 5′-flanking region of the human DEC1 gene.** The nucleotide of the first methionine codon is numbered as +1 and the ATG codon is double underlined. GC boxes are boxed and two major transcriptional initiation sites are indicated by dosed circles below the sequence. Consensus sequences for the binding of transcriptional factors are underlined below the sequence.

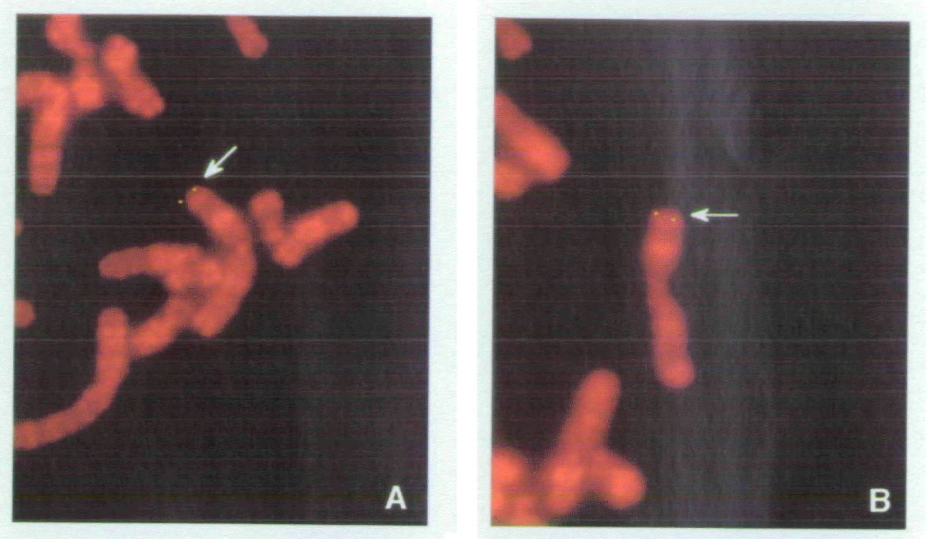


Fig. 6. **Fluorescence *in situ* hybridization of the DEC1 gene.** (A, B) The chromosomal location of the DEC1 gene on R-banded chromosomes using a 1.7 kb cDNA fragment D1p-T3 as a biotinylated probe was determined. The hybridization signals are indicated by arrows. The signals localize to chromosome 3p25.3-p26. Metaphase spreads were photographed with a Nikon B-2A filter.

the C-terminal of the bHLH domain (*34*). Intron 3 of the human DEC1 gene locates at the same position as intron 2 of HES-1, HES-2, and HES-3 (Fig. 4). However, the positions of the other introns of the DEC1 gene differ from those of mouse HES genes. These exon-intron boundaries suggest that DEC1 differs from the HES group in the features of its gene structure.

5′-Flanking Region of the Human DEC1 Gene—The sequence of approximately 1 kb of the human DEC1 gene 5′-flanking region demonstrates the presence of several putative binding sites for known transcriptional factors (Fig. 5). Homology search of the transcriptional factor sequence databases showed the occurrence of some consensus transcription regulatory elements, including CRE in the 5′-flanking region of the human DEC1 gene (*35*, *36*). Two CREs (TGAGGTC and TGACATC) were found at −1030 (Fig. 5) and −2489 (data not shown). The DEC1 gene was isolated by the subtractive hybridization method after the addition of dibutyryl cAMP (*1*). In primary rabbit chondrocyte cultures, dibutyryl cAMP induces the expression of DEC1 mRNA within one hour (Shen *et al.*, manuscript submitted). DEC1 gene transcription may be controlled *via* CREs in the 5′-flanking region.

There are several E-boxes, including E47 binding sites, in the 5′-flanking region (*37*, *38*). Boudjelal *et al.* reported that Stra13 is unable to bind to either the E-box or the N-box (*8*), which are known consensus motifs for bHLH proteins (*4*, *38*). However, Dear *et al.* reported that mouse eip1, which may be identical to mouse Stra13, interacts with E47 and can bind to the E-box (*39*). Accordingly, DEC1 may form a heterodimer with E47 and regulate its own expression by binding to the E-box sequences.

Chromosomal Localization of the Human DEC1 Gene—The chromosomal assignment of the human DEC1 gene was made by direct R-banding FISH using a 1.7 kb cDNA fragment as a probe. The DEC1 gene was localized to Chromosome 3p25.3-p26 (Fig. 6). In another study using a mouse Stra13 cDNA probe, the DEC1 gene was localized to human chromosome 3p26 (*40*). In addition, the mouse Stra13 gene was localized to the sub-telomeric region of mouse chromosome 6 (*41*), a region that is syntenic with human chromosome 3 (*42*). These findings are in good accordance with those of the present study.

The deletion of chromosome arm 3p has been detected in hematologic malignancies: 3p26 in myelodysplastic syndrome (MDS), 3p26 and 3p25 in acute lymphoblastic leukemia (ALL), 3p26 and 3p25 in chronic lymphoproliferative disorder (CLD), 3p26 in Hodgekin's disease (HD), 3p26 in non Hodgekin's lymphoma (NHL) (*43*). DEC1 could be involved in these diseases.

In conclusion, we have determined the structure and transcriptional initiation site of DEC1 gene. This information will be useful for studies on the regulation of DEC1 gene expression and the physiological and pathological roles of DEC1.

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