cDNA cloning and expression of rat homeobox gene, *Hex*, and functional characterization of the protein

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We isolated two cDNA clones of rat Hex, a homeobox protein, studied its expression in rat liver and various cells, and characterized the protein. The levels of Hex mRNA were only slightly increased in liver of rats refed with a high-carbohydrate diet or after partial hepatectomy. Whereas the expression of Hex mRNA was detected in hepatocytes isolated from adult rat liver and also in highly differentiated hepatoma cells, no Hex mRNA was detected in poorly differentiated hepatoma cells. Hex mRNA was also detected in liver from embryo aged 15 days. Expression of Hex was increased in F9 cells during differentiation into visceral endoderm cells by treatment with retinoic acid. This stimulation occurred prior to an increase in the level of α-fetoprotein mRNA. When fusion-protein expression vectors of GAL4 DNA-binding domain and *Hex* were co-transfected with luciferase reporter plasmid, with or without five copies of the GAL4-binding site, into HepG2 cells, the luciferase activities were decreased in concentration- and GAL4-binding site-dependent manners. This repression did not require the presence of the homeodomain, which is located between the amino acid residues 137 and 196. Its repression domain was mapped between the residues 45 and 136 in the proline-rich N-terminal region. In addition, the homeodomain was responsible for DNA-binding of Hex. These results indicate that Hex functions as a transcriptional repressor and may be involved in the differentiation and/or maintenance of the differentiated state in hepatocytes.

Key words: differentiation, hepatocyte, Hex mRNA, transcriptional repressor.

INTRODUCTION

A number of homeobox genes have been found to be expressed in haematopoietic cells [1,2] and it has been suggested that they may play a role in the molecular control of cellular differentiation and lineage specificity during haematopoiesis [3]. Haematopoietically expressed homeobox (*Hex*) gene, which is also referred to as proline-rich homeobox (*Prh*) gene, is one such gene. *Hex* cDNA has been cloned in mouse [4], human [5], chicken [6] and *Xenopus* [7]. Hex is expressed in a range of multipotent haematopoietic progenitor cells and cell lines, and is down-regulated during terminal cell differentiation [8]. It has been postulated that Hex is involved in haematopoietic cell differentiation in the early stages by acting as a transcriptional regulator. However, its transcriptional role and target genes have never been established. In addition, Hex expression has been observed in several adult tissues containing liver, lung, spleen, thymus and pancreas [4–6]. However, levels of expression are not consistent between reports. We have detected high levels of Hex mRNA in mouse liver [9]. Thus Hex may also be involved in differentiation of these tissues.

The rat L-type pyruvate kinase (L-PK) gene is expressed in the liver, kidney, small intestine and pancreatic-β cells [10,11]. The cell type-specific regulatory region of the L-PK gene is located within 170 bp upstream of the transcription-initiation site and consists of three *cis*-acting elements, designated L-I, L-II and L-III respectively [12]. Whereas hepatocyte nuclear factor 1 (HNF1) binds to the L-I element, both HNF4 and nuclear factor-1 family members bind to the L-II element [13]. Upstream stimulating factor and an unidentified protein (L-III-element-binding protein or L-IIIBP) bind to the L-III element, which is also referred to as the carbohydrate-response element [12,14,15].

In this study, we attempted to clone L-IIIBP using the yeast one-hybrid system developed by Wang and Reed [16] and, as a result, obtained the rat *Hex* homologue as an artifact. Since the physiological significance of Hex in the liver has never been examined, we carried out an investigation of Hex expression in the rat liver under various conditions and cell lines by Northern-blot analysis. Moreover, we clarified the transcriptional activity of Hex and mapped its functional domains of transcriptional repression and DNA binding.

MATERIALS AND METHODS

Materials

The SUPERSCRIPT™ plasmid system was purchased from Gibco/Bethesda Research Laboratories. The Dye terminator cycle sequencing kit was obtained from PE Applied Biosystems. Megaprime DNA labelling system, Hybond N nylon membrane, pGEX-2T, pGEX-5X-1, glutathione–Sepharose 4B and molecular-mass markers were purchased from Amersham Pharmacia Biotech. Plasmids pT7-blue T and pBluescriptII SK (+) were obtained from Novagen and Stratagene, respectively. [α-^{32}P]dCTP and [γ-^{32}P]ATP (both 111 TBq/mmol) were from Du Pont New England Nuclear. Dual luciferase assay kit,

Abbreviations used: L-PK, L-type pyruvate kinase; L-IIIBP, L-III-element-binding protein; DBD, DNA-binding domain; RA, all-*trans*-retinoic acid; CAT, chloramphenicol acetyltransferase; GST, glutathione S-transferase; EMSA, electrophoretic mobility shift assay; E, embryonic age.

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The nucleotide sequence data reported in this paper appear in the DDBJ, EMBL and Genbank nucleotide sequence databases with accession number D86383.

pGL3–Control and pRL–SV were obtained from Promega. Qiagen plasmid kit and anti-glutathione S-transferase (GST) antibody (SC-138) were purchased from Qiagen and Santa Cruz Biotechnology respectively.

Oligonucleotides

All oligonucleotides were synthesized with a DNA synthesizer. Nucleotide sequences of sense and antisense *Bam*L-III and *Hex* oligonucleotides, which contained the L-III element of the L-PK gene and the putative binding sequence of Hex respectively, were as follows: sense *Bam*L-III, 5′-GATCCGCCACGGGGCACTCCCGTGG-3′; antisense *Bam*L-III, 5′-GATCCCACGGGGAGTGCCCCGTGGCG-3′; sense *Hex*, 5′-CTAGCATGTAGGCAATTAAAGTTATGATC-3′; and antisense *Hex*, 5′-CTAGGATCATAACTTTAATTGCCTACATG-3′.

The following PCR primers were used to generate various *Hex*-deletion mutants. GBT-9 was derived from the DNA-binding domain (DBD) of GAL4, a transcription factor of yeast. Other primers were derived from rat *Hex* cDNA. Their nucleotide sequences were as follows: GBT-9, 5′-TCATCGGAAGAGAGTAG-3′; *Hex*-6, 5′-TATCCCGGGACTGAAGCAGGAGAATCCT-3′; *Hex*-7, 5′-ATACCCGGGTCTGCACAAAAGGAAAGGC-3′; *Hex*-79, 5′-ATAGAATTCGCCTTCTCGCATCACCCC-3′; *Hex*-Pro, 5′-ATTGGATCCCTACAGCGACCTCTGCAC-3′; *Hex*-C, 5′-GGAGGATCCGATGAGTTGGACAGTTTGGA-3′; *Hex*-3, 5′-GCCGGATCCTGACTGTCATCCAGCATTA-3′; *Hex*-5, 5′-CCTTCAGTGCAGAGGTCGCTGTA-3′; and *Hex*-91, 5′-TAATCTAGAGCAGCGGCCAGCGCGGCG-3′.

Cloning and characterization of rat *Hex* cDNA

We attempted to clone cDNA for L-IIIBP using the yeast one-hybrid system [16]. yWAM2 cells, a yeast strain, pRS315HIS and pPC86 were kind gifts from Dr. R. R. Reed (Johns Hopkins University, Baltimore, MD, U.S.A.). Rat liver cDNA was synthesized using the SUPERSCRIPT™ plasmid system and directionally cloned into the *Sal*I/*Not*I sites of pPC86, which allows expression of fusion protein with the activation domain of the yeast transcription factor GAL4. The cDNA library contained 7.7×10^5 independent clones.

Five copies of *Bam*L-III oligonucleotides were inserted into the *Bam*HI site upstream of the *gal1*-minimal promoter and the HIS3 gene of pRS315HIS. The resultant plasmid, pHISL-III, was used as a reporter plasmid in the yeast one-hybrid system. yWAM2 cells were transformed with pHISL-III as described in [17]. The resultant transformant was used for screening the rat liver cDNA library [16]. Two independent positive clones, y9-10 and y9-18, were obtained. The sizes of their inserts were 1059 and 1619 bp, respectively. Plasmid DNAs were prepared from both clones as described in [18] and used for transformation in *Escherichia coli*. These cDNA inserts were isolated by digestion with *Sal*I and *Not*I, and subcloned into the *Sal*I/*Not*I sites of pSPORT1. The complete nucleotide sequences of the resultant two clones, p9-10 and p9-18, were analysed using the Dye terminator cycle sequencing kit and are shown in Figure 1.

These two clones have identical sequence, including an open reading frame for 271 amino acids, except in the 3′ non-coding region. A homology search was carried out on the sequence using the BLAST search program of the National Center for Biotechnology Information Webs. It was found that the protein coding sequence was very similar to those of *Hex*/*Prh* cDNA, a homeobox-containing protein, in mouse, human and chicken [4–6], indicating that these clones encode the rat Hex homologue protein and will be called rat Hex hereafter. However, further analyses revealed that Hex was not L-IIIBP, since bacterially expressed Hex fused to GST did not bind to ^{32}P-labelled L-III oligonucleotide (results not shown) and co-transfection of the *Hex* expression plasmid did not affect expression from the chloramphenicol acetyltransferase (*CAT*) reporter gene, containing five copies of the L-III element, in HepG2 cells (results not shown).

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   1 GCGGAGCAGCCATGCAGTTCCCGCACCCGGGGCCCGCGGCTGCGCCCGCCGTCGGAGTCC
               M  Q  F  P  H  P  G  P  A  A  A  P  A  V  G  V  P   17
  61 CGCTGTACGCGCCCACGCCGCTGCTGCAGCCCGCTCACCCGACGCCTTTCTACATCGACG
      L  Y  A  P  T  P  L  L  Q  P  A  H  P  T  P  F  Y  I  D  D   37
 121 ACATCCTGGGTCGCGGGCCCGCCGCTCCCACGCCCACTCCCACGCTGCCGTCCCCCAACT
      I  L  G  R  G  P  A  A  P  T  P  T  P  T  L  P  S  P  N  S   57
 181 CCTCCTTCACCAGCCTCGTGTCCTCCTACCGGACCCCGGTGTACGAGCCCACGCCGGTCC
      S  F  T  S  L  V  S  S  Y  R  T  P  V  Y  E  P  T  P  V  H   77
 241 ACCCCGCCTTCTCGCATCACCCCGCCGCCGCGCTGGCCGCTGCCTATGGCCCCGGTGGCT
      P  A  F  S  H  H  P  A  A  A  L  A  A  A  Y  G  P  G  G  F   97
 301 TCGGAGGCCCTCTGTACCCGTTCCCGCGGACGGTGAACGACTACACGCACGCCCTACTCC
      G  G  P  L  Y  P  F  P  R  T  V  N  D  Y  T  H  A  L  L  R  117
 361 GCCACGACCCCCTGGGCAAGCCCTTGCTCTGGAACCCCTTCCTACAGCGACCTCTGCACA
      H  D  P  L  G  K  P  L  L  W  N  P  F  L  Q  R  P  L  H  K  137
 421 AAAGGAAAGGCGGTCAAGTGAGGTTTTCCAACGACCAGACCATCGAGCTGGAGAAGAAGT
      R  K  G  G  Q  V  R  F  S  N  D  Q  T  I  E  L  E  K  K  F  157
 481 TCGAGACTCAGAAATACCTCTCCCCACCTGAGAGAAAGCGTCTGGCCAAGATGTTGCAGC
      E  T  Q  K  Y  L  S  P  P  E  R  K  R  L  A  K  M  L  Q  L  177
 541 TCAGCGAGAGACAGGTCAAAACCTGGTTTCAGAATCGCAGAGCTAAATGGAGACGACTGA
      S  E  R  Q  V  K  T  W  F  Q  N  R  R  A  K  W  R  R  L  K  197
 601 AGCAGGAGAATCCTCAAAGCAATAAAAAGGATGAGTTGGACAGTTTGGACACTTCCTGTG
      Q  E  N  P  Q  S  N  K  K  D  E  L  D  S  L  D  T  S  C  D  217
 661 ACCAGGGCCAAGACTTGCCCAGTGAACAGAATAAAGGTGCCTCTTTGGACAGTTCTCAGT
      Q  G  Q  D  L  P  S  E  Q  N  K  G  A  S  L  D  S  S  Q  C  237
 721 GTTCACCCTCCCCAGCCTCTCCGGAAGACCCTGAATCAGAGATCTCAGAGGATTCCGACC
      S  P  S  P  A  S  P  E  D  P  E  S  E  I  S  E  D  S  D  Q  257
 781 AGGAAGTGGACATCGAGGGTGATAAAGGCTATTTTAATGCTGGATGACAGTCATCGGCCG
      E  V  D  I  E  G  D  K  G  Y  F  N  A  G  *                 271
 841 TGACCATGTTCAGAGACTGGACTTTAGAATACCGTTCTGCTACAGACAAACTGGGAAATT
 901 CTGAGAGAAAGAAGTACCATTCTTCTAGCGTTTTGAAAATATTTGGCACGGCGTGCATTG
 961 AAACCATACTTTTGGTTTAGCGTACGTTTTCCGTACCGGTGTTAAGTTGTTTTGATAAAG
1021 TGGCTAAATGTGACTTCGTTTTTAAAAAGTGAATTCTTTCTATTTGTGAGAAGTAATTTG
1081 AACTTTTGCCTGAAGCTTAAACTATAACTTTAAAGGTTTTGGGCGGCTGTTCTCGAATGA
1141 CTTTTCCCAAATGTGTAGCTGTTCCCTCGACTTCAGAGGTGCACTTAGAAAGGGGGCCAG
1201 CATTTGGGGATCCATGGAATGCTTTCAAAGAGCACACTTGGTTTTTACGTGACACCTTAC
1261 ATTGCTGCCTTAACTCCAAAGCCGGTTCAGAGCACTTGGCCCCTGGTGTCTGTTCTCATA
1321 AGCAAGATAGTTGATAAGATTCTTACAATCTTGTTTTGCTCGGCCAGTGCCTCTGACCCT
1381 GGTATTAAGGGATGACGCAGTCTCTACAGGCAGGGAAACTGACTCACTTGTGACTGATCT
1441 CACATGAACTAGAAATAGCAGGACAATTAAATGTAAGTAGATTGTAGATAGTGTGTTTTA
1501 TAGAAACCGTGTCTATAGCATGTGTATAGAATTATTCGTTGTAAAAAAAACCCTCCCCAA
1561 ATGTTTGATTTTTTTTTTTAATTTAATGAATTAACTTTCATATAAAAAAAAAAAAAAAA
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Figure 1 Nucleotide sequence and the deduced amino acid sequence of rat Hex

The nucleotides (numbered on the left) and amino acids (on the right) are numbered from the 5′ end of the cDNA and from the methionine residue, respectively. Putative polyadenylation signals are underlined. The arrow indicates the polyadenylation site of p9-10. A stop codon is indicated by an asterisk. The double-underlined region is homologous to the homeodomain.

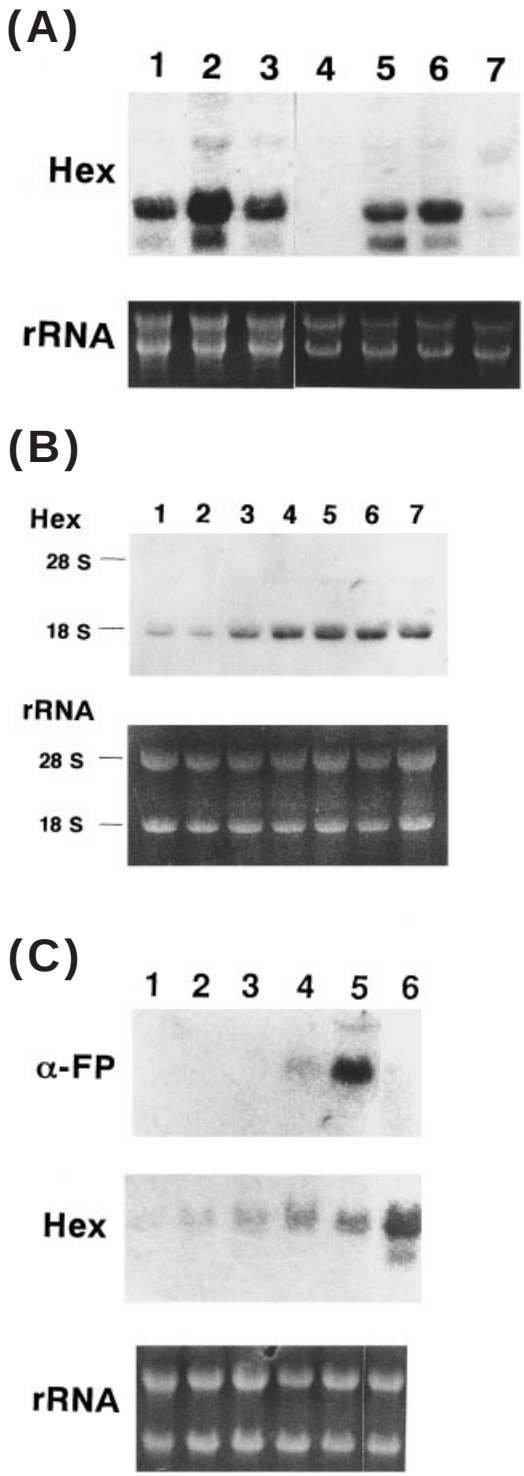

Figure 2 Northern-blot analysis of Hex mRNA

Total RNA (10 μg) were loaded on to denatured agarose gels. The bottom of each panel shows staining with ethidium bromide. (**A**) Expression of Hex mRNA in liver and hepatoma cell lines. Lane 1, fasted liver; lanes 2 and 3, rat liver refed with high-carbohydrate diet for 6 and 16 h, respectively; lane 4, dRLh-84 cells; lane 5, MH_1C_1 cells; lane 6, hepatocytes; lane 7, HepG2 cells. (**B**) Expression of Hex mRNA in developing and regenerating liver. Lane 1, fetal liver (day 15); lane 2, fetal liver (day 20); lane 3, control liver (time 0); lane 4, regenerating liver (8 h); lane 5, regenerating liver (1 day); lane 6, regenerating liver (2 days); lane 7, regenerating liver (4 days). (**C**) Expression of α-fetoprotein mRNA (top) and Hex mRNA (middle) during endoderm differentiation of F9 cells. Lane 1, untreated F9 cells; lanes 2–5, F9 cells treated with 7.5×10^{-8} M RA after (lane 2) 1 day, (lane 3) 2 days, (lane 4) 4 days and (lane 5) 6 days; lane 6, rat liver.

Plasmids

pLcat62′ was constructed as previously described [12]. Five copies of the *Bam*L-III oligonucleotide were inserted into the *Bam*HI site of pLcat62′. pGM-4 was a gift from Dr. P. Monaci and Dr. A. Nicosia (Instituto di Ricerche di Biologia Moleculare, Rome, Italy) and was used as previously described [13]. The p9-18 clone was digested with *Sal*I and *Bam*HI and an insert was ligated into the *Sal*I/*Bam*HI sites of pGM-4 to produce pRSV–*Hex*.

pSG424 and 5 × GAL4-E1bCAT vectors were kindly provided by Dr. R. Stein (Vanderbilt University, Nashville, TN, U.S.A.) [19,20]. The pSG424 plasmid is an expression vector of the DBD of GAL4. The 5 × GAL4–E1bCAT plasmid contains five copies of the GAL4-binding site, a TATA box from the adenovirus E1b promoter, and the CAT reporter gene. A 106 bp *Pst*I/*Xba*I fragment of the 5 × GAL4–E1bCAT plasmid was inserted into *Pst*I/*Xba*I sites of pBluescriptII SK(+). It was digested with *Xba*I and blunt-end ligated by Klenow reaction. After digestion with *Kpn*I, the insert was ligated into the *Kpn*I/*Sma*I sites of the pGL3–Control vector to produce a 5 × GAL4–GL3Control vector.

A 1185 bp *Sma*I/*Bam*HI fragment of p9-18 was inserted into pUC19 *Sma*I/*Bam*HI sites. Subsequently, the *Sma*I/*Xba*I fragment of this plasmid was ligated into the *Sma*I/*Xba*I sites of the pSG424. This construct was designated pSG–*Hex*(6–271), which expresses GAL4–DBD fused to amino acids 6–271 of Hex. PCR reactions were carried out using pSG–*Hex*(6–271) as a template and the following combinations of primers: GBT-9 and *Hex*-5, GBT-9 and *Hex*-91, *Hex*-79 and *Hex*-3, *Hex*-7 and *Hex*-3, and *Hex*-6 and *Hex*-3. The products were subcloned into pT7-blue T vector, and resultant plasmids were digested with *Sma*I and *Xba*I or *Eco*RI and *Xba*I. After isolation by agarose-gel electrophoresis, these inserts were ligated into the *Sma*I/*Xba*I sites or *Eco*RI/*Xba*I sites of the pSG424, to produce pSG–*Hex*(6–136), pSG–*Hex*(6–91), pSG–*Hex*(79–271), pSG–*Hex*(134–271), and pSG–*Hex*(200–271), respectively. pSG–*Hex*(6–271) was digested with *Bst*XI and *Bam*HI, blunt-end ligated, and self-ligated to produce pSG–*Hex*(6–153). pSG–*Hex*(6–136) was digested with *Apa*I and self-ligated to produce pSG–*Hex*(45–136).

Plasmids to produce GST–Hex fusion proteins in *E. coli* were constructed as follows. *Hex* deletion mutants were produced by PCR reaction using primer combinations of *Hex*-Pro and *Hex*-3 for *Hex*(130–271), *Hex*-C and *Hex*-3 for *Hex*(206–271), and *Hex*-7 and *Hex*-4 primers for *Hex*(134–199), respectively. The products, *Hex*(130–271) and *Hex*(206–271), were then digested with *Bam*HI, and inserted into the *Bam*HI site of pGEX-2T to produce pGST–*Hex*(130–271) and pGST–*Hex*(206–271), respectively. The product of *Hex*(134–199) was digested with *Sma*I and *Bam*HI, and ligated into the *Sma*I/*Bam*HI sites of pBluescriptII SK (+). The *Sma*I/*Not*I fragment of this plasmid was ligated into the *Sma*I/*Not*I sites of pGEX-5X-1 to produce pGST–*Hex*(134–199). All plasmids were confirmed by sequencing.

Animals and treatment

Male Sprague–Dawley rats (body mass 170–190 g) were used for experiments. For studies to determine the effects of high-carbohydrate diet, rats were starved for 48 h and then refed with a diet containing 50 % dextrin, 31 % glucose and 10 % casein for 6 and 16 h. Partial hepatectomy (70 %) was performed under ether anaesthesia and rats were killed at indicated times after surgery. Pregnant female rats were killed on 15 and 20 days of gestation and fetal livers were collected.

Cells and cell culture

Both HepG2 cells, a human hepatoma cell line, and MH_1C_1 cells, a rat hepatoma cell line, were obtained from the American Type Culture Collection. Both dRLh-84 cells, a rat hepatoma cell line, and F9 cells, a mouse teratocarcinoma cell line, were supplied by the Health Science Research Resources Bank (the former Japanese Cancer Research Resources Bank). All cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37 °C in a 5% CO_2 incubator.

For induction of differentiation into visceral endoderm cells, F9 cells were treated with 7.5×10^{-8} M all-*trans*-retinoic acid (RA) for indicated times in aggregation culture on bacterial Petri dishes. Rat hepatocytes were prepared by collagenase perfusion of the liver as previously described by Seglen [21].

Isolation of total RNA and Northern-blot analysis

Total RNA was isolated from rat tissues and cells by the acid guanidine/phenol/chloroform method [22]. Various total RNAs (10 μg) were electrophoresed on 1% agarose/formaldehyde gels. After staining with ethidium bromide to assure that equal amounts of RNA had been loaded, they were blotted on to nylon membranes. After the membranes had been crosslinked by UV irradiation, they were hybridized with labelled probes, washed and exposed to Kodak X-AR film at -80 °C with an intensifying screen as previously described [23]. The intensity of bands on autoradiograms were determined by densitometry. Mouse α-fetoprotein cDNA was a gift from Dr. S. M. Tilghman (Princeton University, Princeton, NJ, U.S.A.) [24]. A 700 bp *Sac*II/*Eco*RI fragment of *Hex* cDNA and a 900 bp *Hin*dIII fragment of mouse α-fetoprotein cDNA were labelled with [α-^{32}P]dCTP using the Megaprime DNA labelling system and used as probes.

DNA transfection and reporter assays

All plasmids used were prepared using the Qiagen plasmid kit. Transfection was performed by the calcium phosphate method [25]. The CAT reporter system was used and CAT assays carried out as described previously [26].

For transfection assays using the dual luciferase reporter system, HepG2 cells were cultured at a density of 2×10^5 cells/well in a 24-well plate for 16 h before transfection. $5 \times$GAL4–GL3Control (0.3 μg), 6 ng of pRL–SV and various amounts of effector plasmids were co-transfected into HepG2 cells. The total DNA amount (0.506 μg) was adjusted by the addition of pBluescriptII SK (+). Medium was changed 4 h after transfection. Cells were harvested after 48 h, and firefly and *Renilla* luciferase activities were determined according to the manufacturer's protocols. The ratio of relative light of firefly luciferase to that of *Renilla* luciferase was calculated to produce a value of relative luciferase activity.

Preparation of GST–Hex fusion protein and electrophoretic mobility shift assays (EMSAs)

BL21(DE3) cells, an *E. coli* strain, were transformed with GST or GST–*Hex* mutant expression vectors. Induction and preparation of these fusion proteins were described previously [13]. The purified fusion proteins were analysed by 12% SDS/PAGE. Purified fusion proteins (60 ng) were incubated for 30 min on ice with ^{32}P-labelled *Hex* oligonucleotide (0.1 ng) and 1 μg of poly(dI–dC) in 4% ficoll/1 mM $MgCl_2$/20 mM Hepes/NaOH (pH 7.9)/1 mM dithiothreitol/50 mM NaCl in a total volume of 20 μl [6]. Competition assays were performed by the addition of a 500-fold molar excess of unlabelled oligonucleotide to the binding mixture. Supershift assays were carried out by pre-incubating the fusion proteins with 1 μl of anti-GST antibody for 30 min on ice before adding ^{32}P-labelled *Hex* oligonucleotide. After the binding reaction, the mixture was subjected to 6% PAGE in 45 mM Tris/45 mM boric acid/1 mM EDTA at 200 V for 60 min, and the gel was dried and exposed to X-ray film.

RESULTS

Expression of rat Hex mRNA in liver under various conditions and in various cell lines

To explore possible roles of Hex in the liver, we determined the levels of Hex mRNA under various conditions by Northern-blot analysis. First, we examined the effect of high-carbohydrate diet in the liver. As shown in Figure 2(A), two bands of Hex mRNA were observed: a major band of 2 kb and a minor band of 1.4 kb in length. When rats were starved and refed with a high-carbohydrate diet for 6 and 16 h, the levels of Hex mRNA in the liver increased only 1.7-fold after 6 h and returned to the control level after 16 h (Figure 2A). Next, we determined the levels of Hex mRNA in various cells and cell lines. The highest level of Hex mRNA was detected in freshly isolated hepatocytes. Whereas the Hex mRNA was also detected in highly differentiated hepatoma cell lines, such as MH_1C_1 cells and HepG2 cells, no Hex mRNA was detected in poorly differentiated hepatoma cell lines, such as dRLh-84 cells (Figure 2A). Furthermore, we investigated the levels of Hex mRNA in developing and regenerating liver. As shown in Figure 2(B), Hex mRNA was detected in fetal liver from embryonic age (E) 15 and E 20, but its levels were lower than those of normal adult liver. After 70% hepatectomy, the remaining liver starts to regenerate immediately. The level of Hex mRNA increased only slightly (1.6-fold) in the regenerating liver by 4 days after partial hepatectomy. These results suggested that expression of Hex mRNA may be related to the liver differentiation and/or maintenance of the differentiated state in hepatocytes.

Next, we examined this possibility using the F9 cell differentiation system. When F9 cells are treated with RA in suspension culture, they differentiate into visceral endoderm cells. In these cells, many marker molecules, including α-fetoprotein, are expressed during the differentiation [24,27]. As shown in Figure 2(C), low levels of Hex mRNA were observed in untreated F9 cells. After the addition of RA to the culture medium, the levels of Hex mRNA gradually increased during the cells' differentiation into visceral endoderm. The levels of Hex mRNA reached a maximum on around days 4 and 6, but levels were lower than those of the liver. On the other hand, α-fetoprotein mRNA was first detected on day 4 and increased on day 6.

Functional analysis of Hex in transcription

To analyse the transcriptional role of Hex, we carried out co-transfection experiments. pGL3–Control vector is an expression vector of the firefly luciferase gene under the control of the SV40 promoter and enhancer. We constructed the $5 \times$GAL4–GL3Control reporter plasmid, in which five copies of the GAL4-binding site were inserted upstream of the SV40 promoter in the pGL3–Control vector. We also prepared effector plasmids, pSG424 and pSG–*Hex*(6–271), which express GAL4–DBD alone or amino acids 6–271 of Hex fused to GAL4–DBD, respectively. When $5 \times$GAL4–GL3Control and various amounts of pSG–*Hex*(6–271) were co-transfected into HepG2 cells, luciferase activity was decreased in a concentration-dependent manner (Table 1). Maximal inhibition was obtained with 0.1 μg of pSG–*Hex*(6–271). In contrast, pSG424 did not show any effect

Table 1 Transcriptional activity of Hex

The indicated amounts of the expression plasmid were transfected into HepG2 cells with pRL-SV and reporter plasmid 5 × GAL4–GL3Control. The luciferase activity was normalized with respect to the activity of the *Renilla* luciferase. Data are expressed as the percentage of luciferase activity relative to the value of cells transfected with 0.2 μg of pSG424 and are means ± S.E.M.

Amount of pSG–Hex(6–271) plasmid (μg)	Relative luciferase activity (%)
0	100
0.001	98.7 ± 4.4
0.005	66.9 ± 3.7
0.01	58.5 ± 3.0
0.025	39.3 ± 2.7
0.05	30.1 ± 2.1
0.1	21.3 ± 1.1
0.2	18.9 ± 1.4

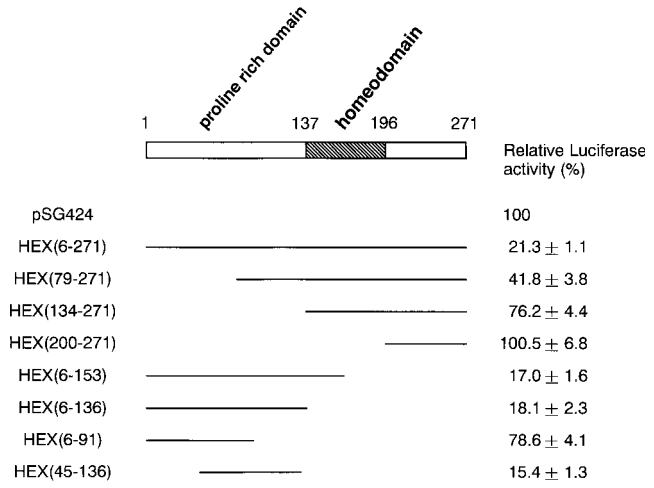

Figure 3 Determination of repressor domain of Hex

5 × GAL4–GL3Control was co-transfected into HepG2 cells with 0.1 μg of effector plasmids expressing the various deletion mutants fused to GAL4–DBD. The normalized firefly luciferase activity was calculated as the percentage of the activity obtained with the 5 × GAL4–GL3Control reporter plasmid co-transfected with 0.1 μg of pSG424. Data are shown as the means ± S.E.M.

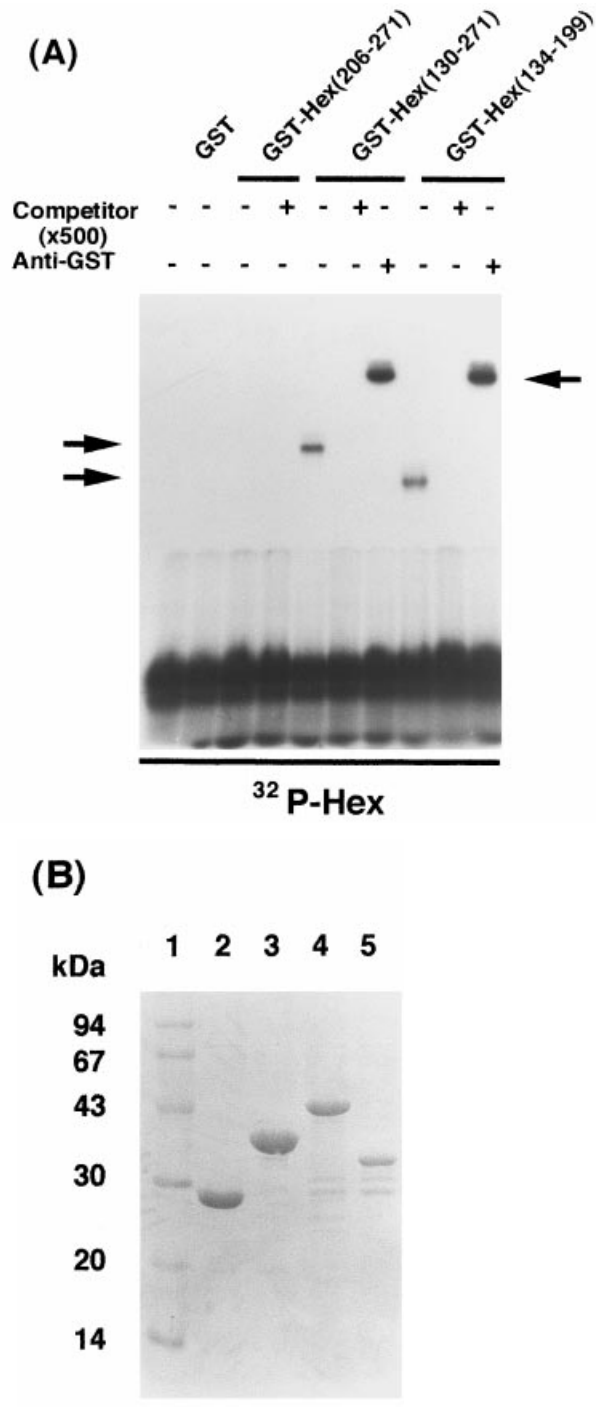

Figure 4 Determination of DBD of Hex

(**A**) Various purified Hex deletion mutants fused to GST (60 ng) were incubated with ^{32}P-labelled *Hex* oligonucleotide for 30 min on ice. Anti-GST antibody (1 μl) was added to the binding mixture. Then, protein–DNA complexes were separated on a 6% polyacrylamide gel. The arrows on left and right indicate Hex–DNA complexes and supershifted complexes with the anti-GST antibody, respectively. (**B**) Purified GST and GST–Hex mutants were separated by electrophoresis on a 12% SDS/polyacrylamide gel and the gel was stained by Coomassie Brilliant Blue R-250. Lane 1, molecular-mass markers; lane 2, GST; lane 3, GST–Hex(206–271); lane 4, GST–Hex(130–271); lane 5, GST–Hex(134–199).

on luciferase expression (results not shown). In addition, when pSG–*Hex*(6–271) was co-transfected with pGL3–Control lacking five copies of the GAL4-binding site, luciferase activity was not changed (results not shown). These results show that GAL4–Hex fusion protein represses luciferase expression by binding specifically to the GAL4-binding sites, indicating that Hex functions as a transcriptional repressor.

We mapped the repression domain of *Hex* by co-transfection of the 5 × GAL4–GL3Control with the effector plasmids, which express GAL4–DBD fusion protein with various *Hex* deletion mutants (Figure 3). Relative luciferase activity without effector plasmid is shown as 100%. pSG–*Hex*(6–271), containing nearly the full length *Hex* cDNA decreased luciferase activity by about 80%. We also analysed C-terminal deletion constructs. Both pSG–*Hex*(6–153) and pSG–*Hex*(6–136) repressed luciferase gene expression by a similar amount as pSG–*Hex*(6–271), whereas pSG–*Hex*(6–91) decreased luciferase activity only by 21%. Next, we analysed the successive N-terminal deletion constructs of *Hex*. pSG–*Hex*(79–271) repressed luciferase activity by about

58%, indicating that pSG–*Hex*(79–271) has partial repressor activity. In contrast, pSG–*Hex*(134–271) and pSG–*Hex*(200–271) decreased luciferase gene expression by only less than 30%. Finally, when the N- and C-terminal deletion mutant, pSG–*Hex*(45–136), was co-transfected, the luciferase activity was decreased by about 85%. These results indicate that the homeodomain of Hex, which is located between amino acids 137 and 196, is not required for transcriptional repression, and that a region from amino acids 45 to 136 of Hex is responsible for the repressor activity.

Determination of the DBD of Hex

We attempted to determine which region of Hex is responsible for DNA binding. Various GST–Hex fusion proteins, GST–Hex(130–271), GST–Hex(206–271) and GST–Hex(134–199), were expressed in *E. coli* and affinity purified with glutathione–Sepharose beads. Purified samples contained mainly GST or its fusion proteins with Hex (Figure 4B). The DNA-binding ability of these mutant proteins were examined by EMSAs using putative Hex-binding sequence reported [6] as a probe (Figure 4A). The band of the probe–protein complex was detected by incubation with GST–Hex(130–271) and GST–Hex(134–199), which contains the Hex homeodomain. These bands disappeared by a 500-fold molar excess of the unlabelled *Hex* oligonucleotide. In addition, supershifted bands were detected by addition of anti-GST antibody. In contrast, no band was detected by incubation with GST and GST–Hex(206–271), which did not contain the Hex homeodomain. These results indicate that Hex binds to the putative DNA sequences via its homeodomain.

DISCUSSION

The rat Hex protein consists of 271 amino acids. The N-terminal region (amino acids 1–136) of Hex contains proline-rich sequences, in which the content of proline is almost 20%. The central region (amino acids 137–196) is a homeodomain, which contains 60 conserved amino acids. The sequence identity of the conserved homeodomain among species was 98%, 100%, 97% and 100% for mouse, human, chicken and *Xenopus*, respectively [4–7]. *Hex* belongs to the unclustered and divergent homeobox genes called orphans, which are different from well-characterized homeobox gene clusters of *HOX* [28]. In this class, *Hex* resembles Hlx/HB24 and Hox11, since their homeodomains share about 55% identity [3,29].

High levels of Hex mRNA are detected in haematopoietic cells and liver. Thus, Hex may play important roles in haematopoietic cells and liver. Most studies have focused on the regulation and expression of Hex mRNA in haematopoietic cells. Recently, Manfioletti et al. [8] reported that Hex expression was lost upon differentiation *in vitro* of leukaemic cells into mature monocyte macrophages and megakaryocytes, whereas it was maintained or up-regulated after induction of maturation to granulocytes and osteoclasts. In contrast, its role has never been investigated in the liver. Here, we demonstrated that the levels of hepatic Hex mRNA were dependent upon the differentiation state: the highest expression was observed in freshly isolated hepatocytes, followed by highly differentiated hepatoma cells such as MH_1C_1 cells or HepG2 cells, and no expression was detected in poorly differentiated hepatoma cells such as dRLh-84 cells. Recently, we have analysed Hex mRNA expression in early mouse development using *in situ* hybridization [9]. Hex mRNA was first detected in the chorion of ectoplacental cavity and weakly at the visceral endoderm of the future yolk sac at E 7.5. Thomas et al. [30] detected Hex transcript in the primitive endoderm at E 4.5 and in the visceral endoderm at E 5.5 by *in situ* hybridization [30]. In the present study, we observed an increase in the level of Hex mRNA during differentiation of F9 cells into visceral endoderm cells by treatment with RA. This occurred prior to an increase in the α-fetoprotein mRNA level. Furthermore, Hex expression was detected in the hepatic anlage at E 9.5 and in the fetal mouse liver at E 12.5 and E 15.5 [9]. Hex mRNA was also detected in fetal rat liver from E 15. In contrast, only slight changes in Hex mRNA levels were observed in the liver after partial hepatectomy or after the feeding of a high-carbohydrate diet. These observations from model systems *in vivo* and *in vitro* suggest that Hex plays important roles in cell differentiation and/or maintenance of the differentiated state of hepatocytes rather than in their growth.

It has not been reported whether Hex is a transcriptional activator or repressor. To clarify this, we transfected a Hex expression plasmid, pRSV–*Hex*, with a luciferase reporter plasmid containing several copies of the putative Hex-binding site ligated to the L-PK gene minimal promoter or the SV40 promoter/enhancer into HepG2 cells or HeLa cells. We observed no changes in luciferase activities (results not shown). This may be due to the presence of large amounts of homeobox proteins other than Hex that bind to the putative Hex-binding site in these cells, as demonstrated by EMSA (results not shown). Therefore, we constructed various deletion mutants fused to GAL4–DBD fusion-protein expression vectors and co-transfected with reporter plasmid into HepG2 cells. Nearly full-length Hex(6–271) showed the repression activity, indicating that Hex functions as a transcriptional repressor. Hex contains two characteristic amino acid motifs. One is the TN domain (TPFSVKDIL) identified in the homeobox transcriptional factor Nk2 family [31], which is known to regulate the heart development. The TN domain is a highly conserved domain in this family, and seems to be involved in transcriptional repression. The other is the Hep motif (SIDxILx) originally identified as an activation domain in both Hox11 and Hlx/HB24 [3,32]. These motifs are located at 32-TPFYIDDIL-40 and 34-YIDDILG-40 respectively. However, neither TN-domain- nor Hep-motif-like sequences were involved in the transcriptional repression of Hex, since Hex(45–136) had nearly complete activity.

As expected, the homeodomain of Hex is responsible for DNA binding and binds to the putative sequence reported previously [6]. However, this sequence also appears to be recognized by other homeobox proteins. Thus the question arises as to how the DNA-binding specificity of Hex is determined. Identification of target genes of Hex should help to answer this question.

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