J. Biochem. **135**, 217–223 (2004)
DOI: 10.1093/jb/mvh025

Identification of the Transactivating Region of the Homeodomain Protein, Hex

Shinya Kasamatsu[1], Ayuko Sato[1], Taichi Yamamoto[1], Vincent W. Keng[1], Hiroshi Yoshida[1], Yuka Yamazaki[1], Masafumi Shimoda[2], Jun-ichi Miyazaki[2] and Tamio Noguchi[*,1]

[1]Department of Applied Molecular Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa-ku, Nagoya 464-8601; and [2]Division of Stem Cell Regulation Research, Osaka University Medical School, Suita, Osaka 565-0871

Received October 29, 2003; accepted December 1, 2003

The homeodomain-containing protein Hex acts as an activator as well as a repressor of transcription in animals. While its repression domain has been mapped to the amino-terminal region, the activation domain has never been identified. Here, we show that the homeodomain and the acidic carboxyl-terminal region are necessary for full activation of the *sodium-dependent bile acid cotransporter* gene promoter in a cell type-independent manner, suggesting that the carboxyl-terminal region comprising residues 197 to 271 functions as the activation domain. In addition, we observed that a Hex mutant without this activation domain acts as a dominant-negative mutant as to the transactivating function of Hex.

Key words: dominant-negative mutant, Hex, sodium-dependent bile acid cotransporter, transcriptional activator.

Abbreviations: Ntcp, sodium-dependent bile acid cotransporter; GFP, green fluorescent protein; PCR, polymerase chain reaction.

Homeobox genes comprise one of the most important gene families involved in organogenesis and cell differentiation during embryonic development (*1*, *2*). They are recognized by a highly conserved approximately 60 amino acid motif called the homeodomain, which can mediate DNA binding (*3*). Most human homeobox genes are found in four distinct genomic clusters of 9 to 11 homeobox genes called *HOXA, HOXB, HOXC* and *HOXD,* which are located on chromosomes 7, 17, 11, and 2, respectively (*4*). These genomic clusters are expressed along the anterior to posterior body axis according to their positions in the *HOX* cluster. Homeobox genes outside of these clusters are called orphans.

Hex (haematopoietically expressed homeobox) is one of the above orphans. *Hex* cDNA was first identified in haematopoietic cells of chicken, mouse and man, and has also been isolated from various species (*5–10*). The human and mouse genes have been mapped to chromosomes 10 and 19, respectively (*7*, *11*). The expression pattern of *Hex* is both tissue- and developmental stage-specific. It has been reported that *Hex* is expressed in the liver, spleen, lungs, thyroid gland, and vascular smooth muscle cells in adult animals (*8*, *12–14*). We and others have shown that *Hex* plays an essential role in progenitor cells committed to the hepatic endoderm, B cell development, haematopoietic differentiation of the monocyte lineage, forebrain formation and thyroid development (*15–17*). Generally, homeodomain proteins can bind to A/T-rich specific sequences. Hex can bind to sequences containing 5′-TAAT-3′, 5′-CAAG-3′, or 5′-ATTAA-3′ (*6*, *18*). So far, only a few genes whose expression is modulated by Hex have been reported. Genes whose expression is repressed by Hex include *goosecoid* (*19*), involved in early embryonic anterior-posterior development, and *thyroglobulin* (*18*), expressed in the thyroid gland. On the contrary, genes whose expression is activated by Hex include *GATA-1*, *fli-1*, and *flk-1*, which are involved in early endothelial and blood cell differentiation of zebrafish (*10*). *SMemb*, a nonmuscle isoform of the myosin heavy chain, is also activated by Hex (*14*). Furthermore, Denson *et al*. have shown that the expression of *sodium-dependent bile acid cotransporter* (*ntcp*) was triggered by Hex through direct interaction with a putative Hex-binding site located in the *ntcp* promoter from nucleotides –733 to –714 relative to the transcription start site, and that this activation only occurred in hepatocytes (*20*).

Thus, Hex can function as either a transcriptional repressor or a transcriptional activator. However, the molecular basis for these dual functions has never been clarified. We previously found that Hex can act as a transcriptional repressor using Hex-GAL4 DNA binding domain fusion proteins, and mapped its repression domain to residues 45 to 136 in the proline-rich amino-terminal region (*8*). In the present study, we identified the region of Hex responsible for transcriptional activation using the *ntcp* promoter as a Hex-acting site. In addition, we produced a dominant-negative mutant as to the transactivating function of Hex.

[*]To whom correspondence should be addressed. Fax: +81-52-789-4121, E-mail: tnoguchi@agr.nagoya-u.ac.jp

MATERALS AND METHODS

Materials—A dye terminator cycle sequencing kit was obtained from PE Applied Biosystems. A dual luciferase reporter gene assay kit, pGL3-Basic and pRL-SV40 were obtained from Promega. Qiagen Midi-plasmid and Qiaex gel extraction kits were purchased from Qiagen. Horseradish peroxidase-conjugated secondary antibodies, ECL reagent, Hyperfilm and [α-^{32}P]dCTP (111 TBq/mmol) were purchased from Amersham Pharmacia Biotech. pSTBlue-1 and pBluescript II SK+ were purchased from Stratagene. Lipofectamine 2000 transfection reagent and TRIZOL reagent were obtained from Invitrogen Life Technologies.

Oligonucleotides and Plasmids—All oligonucleotides were synthesized with a DNA synthesizer. The sequences of the NLS/S and NLS/AS oligonucleotides, which contain the nuclear localization signal from simian virus 40 large T antigen (*21*) and a multi-cloning site, are as follows: NLS/S, 5′-AATTACCATGGCTCCGAAGAAGAAGCGGAAGGTAGGTACCCTCGAGGAGCTCGCTAGCCCCGGGAATTCTTAGATAAGTAAT-3′, and NLS/AS, 5′-AATTATTACTTATCTAAGAATTCCCGGGGCTAGCGAGCTCCTCGAGGGTACCTACCTTCCGCTTCTTCTTCGGAGCCATGGT-3′.

The following oligonucleotides were used as primers for polymerase chain reaction (PCR) to amplify DNA fragments to construct various plasmids: ntcp-783Bam/S, 5′-GGGGGATCCTGGTCAGTATTCAACAGTC-3′; ntcp+46 Bam/AS, 5′-GGGGGATCCTCAGGACAGAGTTTGTG GACA-3′; Hex131Bam/S, 5′-ATTGGATCCCTACAGCGACCTCTGCAC-3′; Hex197Bam/AS, 5′-AGGATCCTTCA GTCGTCTCCATTTAGCTC-3′; Hex131Nhe/S, 5′-GGCTAGCCTACAGCGACCTC TGCA-3′; Hex216Eco/AS, 5′-CGAATTCACAGGAAGTGTCCAAACTGTC-3′; Hex235Eco/AS, 5′-CGAATTCAGAACTGTCCAAAGAGGCAC-3′; Hex254-Eco/AS, 5′-CGAATTCATCCTCTGAGATCTCTGATTCAG-3′; Hex196/AS, 5′-CAGTCGTCTCC ATTTAGCTCTG-3′; HexID1/S, 5′-CGCAGAGCTAAATGGAGACGACTG CAGTGTTCACCCTCCCCA-3′; HexID2/S, 5′-CGCAGAGCTAAATGGAGAC GACTGGACCAGGGCCAAGACTTG-3′; Hex197Nhe/S, 5′-GGCTAGCAAGCAGGAG AATCCTCAAAGC-3′, Hex2/S, 5′-TGCAGTTCCCGCACCCG-3′, and Hex271/AS, 5′-ACCGGTACGGAAAACGTAC-3′.

The rat *ntcp* promoter region spanning –783 to +46 was amplified by PCR using rat genomic DNA as a template with the ntcp-783Bam/S and ntcp+46Bam/AS primers containing *Bam*HI sites. The PCR product was digested with *Bam*HI, and then ligated into the *Bgl*II site of pGL3-Basic to produce reporter plasmid pNLuc783.

We used pCAGGS as the expression plasmid. This plasmid contains the chicken β-*actin* promoter and cytomegalovirus enhancer, β-*actin* intron and bovine β-*globin* poly-adenylation signal (*22*). The NLS/S and NLS/AS oligonucleotides containing the nuclear localization signal were annealed and the resulting fragment was ligated into the *Eco*RI site of pCAGGS to produce pCAG-NLS. This vector was used for the expression of Hex deletion mutants to ensure their nuclear transport. An *Eco*RI fragment containing the entire coding sequence of Hex was isolated from p9–18 (*8*) and ligated into the *Eco*RI site of pCAGGS to generate pCAG-Hex. Fragment Hex 131–271 was obtained by *Bam*HI digestion of previously reported pGST-Hex131–271 (*8*) and subcloned into the *Bam*HI site of the pBluescript II SK+ vector. The resulting plasmid was then digested with *Spe*I/*Eco*RI and fragment Hex 131–271 was inserted into the *Nhe*I/*Eco*RI sites of pCAG-NLS to produce pCAGN-Hex131–271. Fragment Hex 131–197 was obtained by PCR using p9-18 as a template, and the Hex131Bam/S and Hex197-Bam/AS primers. The amplified PCR product was subcloned into pSTBlue-1 and the resulting plasmid was digested with *Bam*HI in order to isolate Hex 131–197, which was then inserted into the *Bam*HI site of pBluescript II SK+ vector. Then, the *Spe*I/*Eco*RI Hex 131–197 fragment was isolated and inserted into the *Nhe*I/*Eco*RI sites of pCAG-NLS to produce pCAGN-Hex131–197. Hex2-197 and Hex 197–271 fragments were obtained by PCR with the Hex2/S and Hex197Bam/AS primers, and Hex197Nhe/S and Hex271/AS primers, respectively, using p9–18 as a template. These PCR products were subcloned into pSTBlue-1, and the resulting plasmids were digested with *Kpn*I/*Nhe*I and *Nhe*I/*Eco*RI, respectively. The two fragments were ligated into the *Kpn*I/*Nhe*I sites and *Nhe*I/*Eco*RI sites of pCAG-NLS to generate pCAGN-Hex2–197 and pCAGN-Hex197–271, respectively. The Hex 131–196/217–271 and Hex 131–196/236–271 fragments were obtained by PCR through two steps. In the primary PCR, reactions were performed with p9-18 as a template with the following combinations of primers: Hex131Nhe/S and Hex196/AS, HexID2/S and Hex271/AS, and HexID1/S and Hex271/AS. The products were named Hex131–196, Hex217–271, and Hex236–271, respectively. In the secondary PCR, reactions were carried out with the combination of Hex131–196 and Hex217–271 or Hex131–196 and Hex236–271 as templates, and primers Hex131Nhe/S and Hex271/AS. The products were subcloned into pSTBlue-1 and the resulting plasmids were digested with *Nhe*I/*Eco*RI. Then, these inserts were ligated into the *Nhe*I/*Eco*RI sites of pCAG-NLS to produce pCAGN-Hex131–196/217–271 and pCAGN-Hex131–196/236–271. Hex 131–271 carboxyl-terminal region deletion mutants were obtained by PCR using p9-18 as a template with the following combinations of primers: Hex131Nhe/S and Hex216Eco/AS, Hex131Nhe/S and Hex235Eco/AS, and Hex131Nhe/S and Hex254Eco/AS. The PCR products were subcloned into the pSTBlue-1 acceptor vector and the resulting plasmids were digested with *Nhe*I/*Eco*RI. After isolation by agarose gel electrophoresis, these inserts were ligated into the *Nhe*I/*Eco*RI sites of pCAG-NLS to produce pCAGN-Hex131–216, pCAGN-Hex131–235 and pCAGN-Hex131–254. All plasmids were confirmed by DNA sequencing.

Cell Culture and DNA Transfection—HeLa, COS-7 and Hep G2 cells, obtained from the American Type Culture Collection, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37°C in a 5% CO_2 incubator.

All plasmids used for transfection were prepared using the Qiagen Midi-plasmid kit. Transfection was performed by the calcium phosphate method (*23*) or with Lipofectamine 2000 according to the manufacturer's protocol. For reporter assays, cells were cultured at 20–30% density in 6-well plates for 24 h before transfection. One microgram of pNLuc783, 20 ng of pRL-SV40 and various amounts of effector plasmids were co-transfected into

each cell type. The total amount of DNA was adjusted by the addition of pCAGGS. The medium was changed at 5 h after transfection and cells were harvested after 48 h. Firefly and *Renilla* luciferase activities were determined according to the manufacturer's protocols. Firefly luciferase activity (relative light units) was normalized as to *Renilla* luciferase activities.

Construction of and Infection with Adenovirus Vectors—A dominant-negative mutant of Hex was isolated from pCAGN-Hex2-197 by digestion with appropriate restriction enzymes and ligated into the expression cassette of the pAFC3 cosmid (J. Miyazaki, unpublished), which is a derivative of pALC cosmid (*24*). This cassette bicistronically expresses any protein and green fluorescent protein (GFP) driven by the chicken β-*actin* promoter and cytomegalovirus enhancer. The resultant cosmid was used to produce a recombinant adenovirus vector of the Hex mutant (Adv-dnHex) as described previously (*24*). Adenovirus expressing GFP only (Adv-Blank) was used as a control.

Hepatocytes were isolated from male Sprague-Dawley rats by the collagenase perfusion method and cultured as previously described (*25*, *26*). The experiment was approved by the Animal Experiment Committee, Nagoya University, and the rats were treated in accordance with the guidelines. Adenovirus infection was performed at 3 h after plating. Hepatocytes were incubated for 1 h in a reduced volume of culture medium containing the virus at a multiplicity of infection (MOI) of 5 before changing to fresh medium. The cells were incubated for 48 h.

Western Blot Analysis—Anti-Hex antibodies were produced in a rabbit, according to the standard protocols, against a synthetic short peptide corresponding to the carboxyl-terminal end of the rat Hex sequence, CQEVDIEGDKGYFNAG. A cysteine residue was added to the amino-terminal for linking to the keyhole limpet hemocyanin. The peptide was injected subcutaneously into the rabbit once every fortnight. The collected antiserum was purified by affinity column chromatography.

Transfected whole cell lysates were separated by 15% SDS–polyacrylamide gel electrophoresis, followed by transfer to membranes. The membranes were incubated for 1 h at room temperature with anti-Hex antibodies, and then washed three times with phosphate-buffered saline containing 0.1% Tween 20 before being probed with horseradish peroxidase-conjugated secondary antibodies. Blots were developed using ECL reagent and exposed to Hyperfilm, according to the manufacturer's protocols.

Isolation of Total RNA and Northern Blot Analysis—Total RNA was prepared from hepatocytes using TRIZOL reagent. Total RNA (15 μg) was subjected to 1% agarose/formaldehyde gel electrophoresis, blotted onto a membrane and then hybridized with ^{32}P-labeled cDNA probes as previously described (*8*). The cDNAs for ntcp and 36B4, a ribosomal protein, were obtained by PCR using rat liver cDNA as a template. The sense and antisense primer pairs used were as follows: *ntcp,* 5′-GCTGCTTATCATGCTCTCAC-3′, 5′-CTAATTTGCCATCTGACCAG-3′; *36B4*, 5′-GGGCAAGAACACCATGATGCGC-3′, 5′-GTTCAGCAGAGTGGCTTCGCTGG-3′.

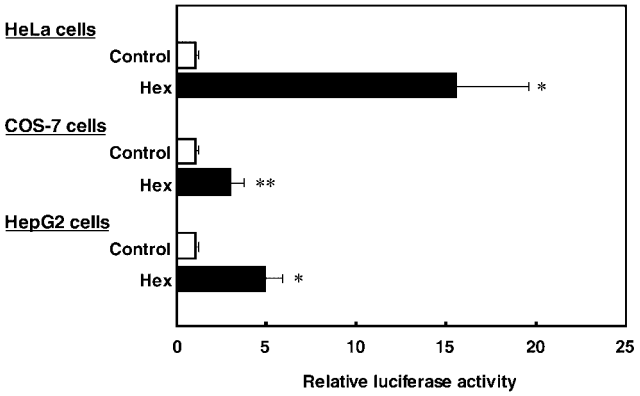

Fig. 1. **Hex activates the *ntcp* promoter in a cell type-independent manner.** Reporter plasmid pNLuc783 was transfected into HeLa, COS-7 and Hep G2 cells with 1 μg of either pCAGGS or pCAG-Hex. Luciferase activities are expressed relative to that in control cells transfected with pCAGGS, which is set at 1. Data are the means ± SD for four experiments. *$p < 0.01$ compared to control. **$p < 0.05$ compared to control.

STATISTICS

Statistical analysis was performed by Student's *t*-test or Welch's *t*-test. Statistical significance was defined as $p < 0.05$.

RESULTS

Cell Type-Specificity of Transcriptional Activation by Hex—Reporter (pNLuc783) and effector plasmids (pCAG-Hex or pCAGGS) were co-transfected into the HeLa, COS-7, and Hep G2 cell lines, and then transient expression of the luciferase gene was examined. Of the cell lines tested, only Hep G2 hepatoma cells express *Hex* (*8*, *27*). In agreement with this, the *ntcp* promoter activity in HeLa and COS-7 cells was 2% of that in Hep G2 cells. The *Hex* expression resulted in significant stimulation of the promoter activity in all cell lines tested, although differences in the stimulation level were observed among the cell lines (Fig. 1). These results indicate that there is essentially no cell type-specificity of *ntcp* promoter activation by Hex.

Identification of Hex Transcriptional Activation Domain—The pNLuc783 reporter plasmid and various expression vectors of full-length or mutant constructs of Hex (Hex, Hex131–271, Hex131–197, and Hex2-197), in the indicated amounts, were co-transfected into HeLa cells. pCAGGS was used as a control. Hex131–271 was nearly as active as full-length Hex, while Hex131–197 and Hex2-197 hardly stimulated the *ntcp* promoter (Fig. 2B). These results indicate that the homeodomain alone has no activity and that the carboxyl-terminal segment of Hex (amino acids residues 197 to 271) may participate in the transcriptional activation of *ntcp*.

In order to investigate the carboxyl-terminal segment of Hex, a series of carboxyl-terminal deletion mutants (Hex131–271, Hex131–216, Hex131–235, and Hex131–254) were individually co-transfected in various amounts with the reporter plasmid. As shown in Fig. 2C, Hex131–271 transactivated the *ntcp* promoter by about 5-fold at 0.25 μg, but Hex131–216, Hex131–235, and Hex131–254

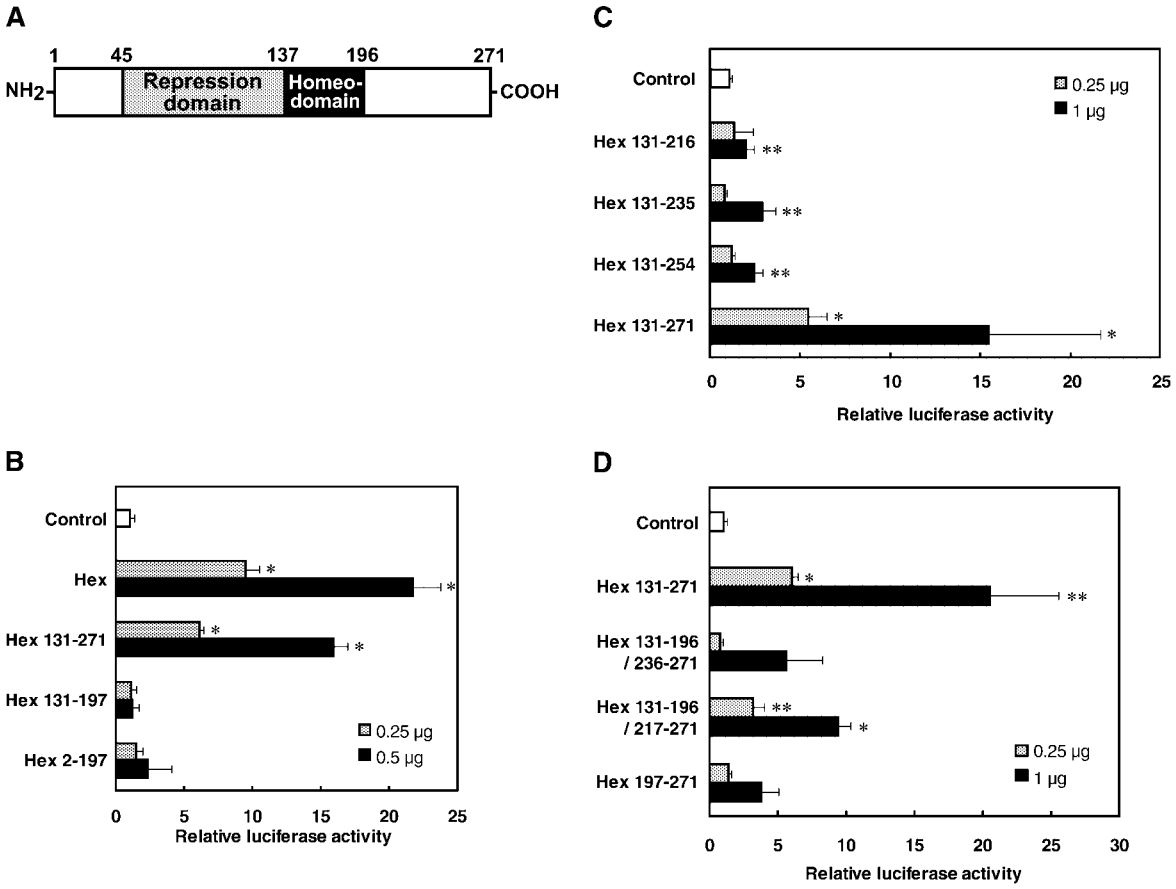

Fig. 2. **Identification of the transcriptional activating domain of Hex.** (A) Schematic representation of full-length Hex. (B, C, D) HeLa cells were co-transfected with reporter plasmid pNLuc783 and the indicated amounts of the various expression plasmids of Hex shown at the left. Luciferase activities are expressed relative to that in control cells transfected with pCAGGS, which is set at 1. Data are the means ± SD for three to four experiments. *$p < 0.01$ compared to control. **$p < 0.05$ compared to control.

exhibited little activity at this dose. At 1 μg, these mutants showed weak activity. These results indicate that the carboxyl-terminal (amino acid residues 255 to 271) of Hex is necessary for its transactivating activity.

In addition, various internal deletion mutants (Hex-131–196/236–271, Hex131–196/217–271, and Hex197–271) were prepared from Hex131–271. When compared with the activity of Hex131–271, internal deletion of Hex, from amino acid residue 197 to 216 (Hex131–196/217–271), reduced the promoter activity by about 50% and further deletion up to 235 (Hex131–196/236–271) resulted in an about 75% reduction in activity (Fig. 2D). Exclusion of the homeodomain from Hex131–271 greatly reduced the activity. The reduced activity of mutants containing the homeodomain is not due to reduced binding to the *ntcp* promoter, since deletion of the carboxy-terminal region, from residue 200 to 271, did not affect the DNA binding activity of the homeodomain (*8*). Thus, the homeodomain (residues 137 to 196) and carboxyl-terminal segment (residues 197 to 271) are necessary for full activation of the *ntcp* promoter.

Expression of Various Hex Mutants—In order to confirm the expression of the various Hex mutants used in Fig. 2D, the four Hex mutants were individually transfected into HeLa cells. Total cell lysates were subjected to Western blot analysis using anti-Hex antibodies. As shown in Fig. 3, the four Hex mutants were detected as bands corresponding to the expected sizes, while the untransfected cell lysate gave no protein band. Since all

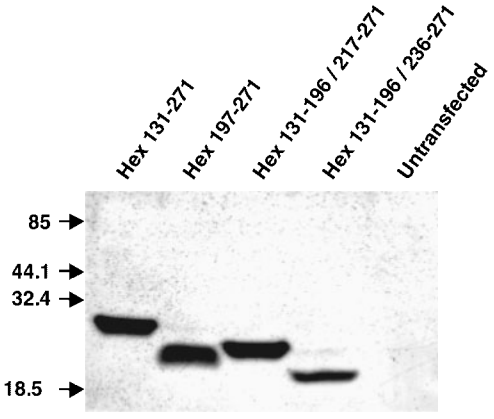

Fig. 3. **Western blot analysis of various Hex mutants.** The various deletion mutants of Hex expression vectors shown at the top were transiently transfected into HeLa cells. Cell extracts were separated by 15% SDS–polyacrylamide gel electrophoresis, followed by transfer to a polyvinylidene difluoride membrane. Rabbit anti-Hex IgG and HRP-conjugated donkey anti-rabbit IgG were used to detect the Hex mutant proteins.

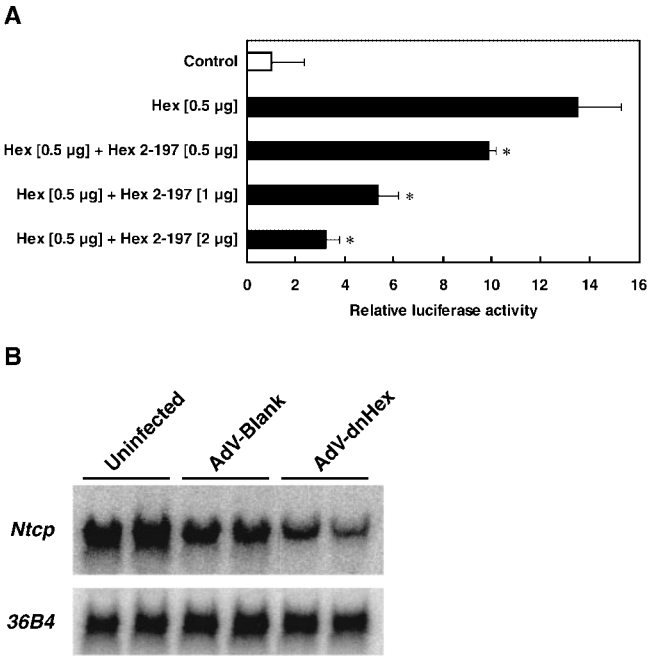

Fig. 4. **Hex2-197 functions as a dominant-negative mutant.** (A) Hep G2 cells were co-transfected with reporter plasmid pNLuc783, pCAG-Hex and increasing amounts of pCAGN-Hex2-197. Luciferase activities are expressed relative to that in control cells transfected with pCAGGS, which is set at 1. Data are the means ± SD for three to six experiments. *$p < 0.01$ compared to pCAG-Hex. (B) Rat hepatocytes were infected with the adenovirus vectors shown. After 48 h, total RNA was isolated, and the levels of ntcp and 36B4 mRNAs were determined by Northern blot hybridization. Data are representative of two experiments.

the Hex mutants contain the nuclear localization signal of simian virus 40 large T antigen, they are expected to be mostly located in the nuclei. To confirm this, we performed immunohistochemical analysis with anti-Hex antibodies. As expected, Hex and its deletion mutants, including Hex131–271, Hex131–196/236–271, Hex131–196/217–271, and Hex197–271, were mainly detected in the nuclei (data not shown). Furthermore, Hex2–197 and Hex131–254 were also mainly observed in the nuclei, when expressed as fusion proteins with GFP (data not shown).

Hex2–197 Functions as a Dominant-Negative Mutant—Although Hex2–197 contains the homeodomain, it is incapable of activating the *ntcp* promoter. Thus, this mutant could act as a dominant negative mutant as to transactivating function of Hex. To test this possibility, the indicated amounts of Hex and Hex2–197 expression plasmids were co-transfected into Hep G2 cells with pNLuc783. As shown in Fig. 4A, the level of reporter activity induced by Hex decreased depending on the amount of Hex2–197 added. These results suggest that Hex2–197 functions as a dominant-negative mutant *in vivo*. To examine this, we produced a recombinant adenovirus expressing Hex2–197 (Adv-dnHex) and infected primary cultured rat hepatocytes with it. Adv-Blank expressing GFP only was used as a control. Most hepatocytes were infected with these viruses judging from the GFP expression (data not shown). Endogenous *ntcp* expression was analyzed by Northern blot hybridization and the results are shown in Fig. 4B. When the level of ntcp mRNA was normalized as to that of mRNA for 36B4, a ribosomal protein, the values for the control virus (Adv-Blank) and Hex2–197 (Adv-dnHex) were 73 ± 16% (mean ± S.D.) and 34 ± 10% of the uninfected value, respectively. These results indicated that overexpression of Hex2–197 suppressed endogenous *ntcp* expression in hepatocytes and that this mutant functions as a dominant negative mutant *in vivo*.

DISCUSSION

Ntcp expression is strictly limited to hepatocytes (*28*). Denson *et al*. have shown that Hex activates the *ntcp* promoter through a Hex response element (–733 to –714) in Hep G2 hepatoma cells but not in COS-7 cells (*20*). Based on these results, they suggested that Hex only activates *ntcp* transcription in hepatocytes, and that a co-activator(s) is necessary for its hepatocyte-specific activation. In contrast, the present study shows the activation of the *ntcp* promoter by Hex in three different cell lines, although the basal promoter activity was much higher in Hep G2 cells than in HeLa and COS-7 cells, indicating that there is essentially no cell type-specificity of *ntcp* transcriptional activation by Hex. This difference may be due to the different plasmids used to express *Hex*. We used a strong expression plasmid, pCAGGS, which contains the chicken β-*actin* promoter and cytomegalovirus enhancer, β-*actin* intron and bovine β-*globin* poly-adenylation signal (*22*). We observed about 5- and 3-fold stimulation of the ntcp promoter activity by Hex in Hep G2 and COS-7 cells, respectively. However, when Hex was expressed under the control of a simian virus 40 promoter, it failed to stimulate the promoter activity in COS-7 cells. On the other hand, Denson *et al.* found only a 2-fold increase in the activity caused by Hex in Hep G2 cells and no change in COS-7 cells. They used pcDNA3 as a Hex expression plasmid, which contains the cytomegalovirus promoter. In addition, they did not examine the effect of Hex on the *ntcp* promoter in other cell lines such as HeLa cells.

In the current study, we attempted to further elucidate which region of Hex comprises the activator domain. We have shown that both the homeodomain (residues 137 to 196) and the carboxyl-terminal region (residues 197 to 271) are necessary for the full activating function of Hex. Since the homeodomain is responsible for DNA-binding (*8*), the carboxyl-terminal region, which is characterized by its acidic nature, probably functions as an activator domain.

Deletion of the activator domain from the carboxyl-end impaired the activating function of Hex more severely than that from the amino-end. Thus, amino acids residues 255 to 271 are more critical for the function of Hex as a transcriptional activator.

It has been postulated that the transcriptional activation domain interacts with general transcription factors or other co-activators in order to activate transcription (*29*, *30*). It has been reported that the TATA-box binding protein binds to a fragment of Hex containing the homeodomain and carboxyl-terminal region (*31*). Therefore, Hex may interact with this protein via the carboxyl-ter-

minal region and may somehow contribute to the activation of transcription.

As mentioned above, Hex can function as either a transcriptional activator or repressor. How is this switch regulated? It is known that other homeodomain proteins such as NK-4 also act as a transcriptional activator or repressor (*32*). When NK-4 interacts with co-activator p300, it functions as an activator. However, when it interacts with co-repressor Groucho, it functions as a repressor. Furthermore, recent research has shown that a co-activator of LIM-homeodomain proteins and a co-repressor of LIM-homeodomain proteins can regulate each other (*33*). This suggests that co-factors can self- or co-regulate, which makes the direction of transcriptional regulation positively or negatively. The context of *cis*-elements of target genes may also be an important factor for recruiting a co-activator or co-repressor. We previously identified the repression domain of Hex using Hex fusion proteins with the GAL4 DNA binding domain (*8*). In this case, the reporter plasmid contained GAL4 binding sites and simian virus 40 enhancer located upstream of the luciferase gene as *cis*-acting elements. On the other hand, the *ntcp* promoter region used in this study has been reported to interact with several transcription factors including Hex and hepatocyte nuclear factor 1 (*28*). Some transcription factor(s) may be necessary for Hex to recruit co-factors. Further research is required to clarify these issues.

We observed that Hex2–197, with the deleted carboxyl-terminal region, functions as a dominant-negative mutant as to the transactivating function of Hex. To confirm this, primary cultured rat hepatocytes were infected with Hex2–197–expressing adenovirus. We found that over-expression of Hex2–197 decreased the level of ntcp mRNA. Since this region contains the DNA binding homeodomain, but not the activation domain, as mentioned above, it is reasonable to speculate that Hex2–197 inhibits the activating function of full length Hex through competitive binding to target genes. Thus, Hex2–197 acts as a dominant-negative mutant *in vivo*, and will be a useful tool for determining the function of Hex. Denson *et al*. found that the region comprising amino acids 128 to 271 of mouse Hex functioned as a dominant negative mutant as to the transactivating function of full length Hex (*20*). The reason for this discrepancy is not known at the present time, although rat Hex was used for this study while they used mouse Hex. The similarity of the amino acid sequences of these two Hex species is very high (97%); six residues are different in the amino acid 128–271 region and only 3 in amino acid 2–197 region (*5*, *8*). Thus, it is unlikely that the discrepancy is due to the species difference.

This work was supported by in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

1. Wright, C.V., Cho, K.W., Oliver, G., and De robertis, E.M. (1989) Vertebrate homeodomain proteins: families of region-specific transcription factors. *Trends Biochem. Sci.* **14**, 52–56
2. Gehring, W.J., Affolter, M., and Burglin, T. (1994) Homeodomain proteins. *Annu. Rev. Biochem.* **63**, 487–526
3. Levine, M., and Hoey, T. (1988) Homeobox proteins as sequence-specific transcription factors. *Cell* **55**, 537–540
4. Acampora, D., D'Esposito, M., Faiella, A., Pannese, M., Migliaccio, E., Morelli, F., Stornaiuolo, A., Nigro, V., Simeone, A., and Boncinelli, E. (1989) The human HOX gene family. *Nucleic Acids Res.* **17**, 10385–10402
5. Bedford, F.K., Ashworth, A., Enver, T., and Wiedemann, L.M. (1993) *HEX*: a novel homeobox gene expressed during haematopoiesis and conserved between mouse and human. *Nucleic Acids Res.* **21**, 1245–1249
6. Crompton, M.R., Bartlett, T.J., MacGregor, A.D., Manfioletti, G., Buratti, E., Giancotti, V., and Goodwin, G.H. (1992) Identification of a novel vertebrate homeobox gene expressed in haematopoietic cells. *Nucleic Acids Res.* **20**, 5661–5667
7. Hromas, R., Radich, J., and Collins, S. (1993) PCR cloning of an orphan homeobox gene (PRH) preferentially expressed in myeloid and liver cells. *Biochem. Biophys. Res. Commun.* **195**, 976–983
8. Tanaka, T., Inazu, T., Yamada, K., Zaw Myint, Keng, V.W., Inoue, Y., Taniguchi, N., and Noguchi, T. (1999) cDNA cloning and expression of rat homeobox gene, *Hex*, and functional characterization of the protein. *Biochem. J.* **339**, 111–117
9. Newman, C.S., Chia, F., and Krieg, P.A. (1997) The X*Hex* homebox gene is expressed during development of the vascular endothelium: overexpression leads to an increase in vascular endothelial cell number. *Mech. Dev.* **66**, 83–93
10. Liao, W., Ho, C.Y., Yan, Y.L., Postlethwait, J., and Stainier, D.Y. (2000) Hhex and Scl function in parallel to regulate early endothelial and blood differentiation in zebrafish. *Development* **127**, 4303–4313
11. Ghosh, B., Jacobs, H.C., Wiedemann, L.M., Brown, A., Bedford, F.K., Nimmakayalu, M.A., Ward, D.C., and Bogue, C.W. (1999) Genomic structure, cDNA mapping, and chromosomal localization of the mouse homeobox gene, *Hex*. *Mamm. Genome* **10**, 1023–1025
12. Keng, V.W., Fujimori, K.E., Zaw Myint, Tamamaki, N., Nojyo, Y., and Noguchi, T. (1998) Expression of Hex mRNA in early murine postimplantation embryo development. *FEBS Lett.* **426**, 183–186
13. Bogue, C.W., Ganea, G.R., Sturm, E., Ianucci, R., and Jacobs, H.C. (2000) *Hex* expression suggests a role in the development and function of organs derived from foregut endoderm. *Dev. Dyn.* **219**, 84–89
14. Sekiguchi, K., Kurabayashi, M., Oyama, Y., Aihara, Y., Tanaka, T., Sakamoto, H., Hoshino, Y., Kanda, T., Yokoyama, T., Shimomura, Y., Iijima, H., Ohyama, Y., and Nagai, R. (2001) Homeobox protein Hex induces SMemb/nonmuscle myosin heavy chain-B gene expression through the cAMP-responsive element. *Circ. Res.* **88**, 52–58
15. Keng, V.W., Yagi, H., Ikawa, M., Nagano, T., Zaw Myint, Yamada, K., Tanaka, T., Sato, A., Muramatsu, I., Okabe, M., Sato, M., and Noguchi, T. (2000) Homeobox gene *Hex* is essential for onset of mouse embryonic liver development and differentiation of the monocyte lineage. *Biochem. Biophys. Res. Commun.* **276**, 1155–1161; erratum: **279**, 739
16. Martinez Barbera, J.P., Clements, M., Thomas, P., Rodriguez, T., Meloy, D., Kioussis, D., and Beddington, R.S.P. (2000) The homeobox gene *Hex* is required in definitive endodermal tissues for normal forebrain, liver and thyroid formation. *Development* **127**, 2433–2445
17. Bogue, C.W., Zhang, P.X., McGrath, J., Jacobs, H.C., and Fuleihan, R.L. (2003) Impaired B cell development and function in mice with a targeted disruption of the homeobox gene *Hex*. *Proc. Natl Acad. Sci. USA* **100**, 556–561
18. Pellizzari, L., D'Elia, A., Rustighi, A., Manfioletti, G., Tell, G., and Damante, G. (2000) Expression and function of the homeodomain-containing protein Hex in thyroid cells. *Nucleic Acids Res.* **28**, 2503–2511
19. Brickman, J.M., Jones, C.M., Clements, M., Smith, J.C., and Beddington, R.S.P. (2000) Hex is a transcriptional repressor

that contributes to anterior identity and suppresses Spemann organiser function. *Development* **127**, 2303–2315
20. Denson, L.A., Karpen, S.J., Bogue, C.W., and Jacobs, H.C. (2000) Divergent homeobox gene *Hex* regulates promoter of the Na^+-dependent bile acid cotransporter. *Am. J Physiol. Gastrointest. Liver Physiol.* **279**, G347–G355
21. Kalderon, D., Roberts, B.L., Richardson, W.D., and Smith, A.E. (1984) A short amino acid sequence able to specify nuclear location. *Cell* **39**, 499–509
22. Niwa, H., Yamamura, K., and Miyazaki, J. (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**, 193–199
23. Sambrook, J. and Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd ed., Cold Spring Harbor Press, Cold Spring Harbor
24. Tashiro, F., Niwa, H., and Miyazaki, J. (1999) Constructing adenoviral vectors by using the circular form of the adenoviral genome cloned in a cosmid and the Cre-*loxP* recombination system. *Hum. Gene Ther.* **10**, 1845–1852
25. Seglen, P.O. (1976) Preparation of isolated rat liver cells. *Methods Cell Biol.* **13**, 29–83
26. Matsuda, T., Noguchi, T., Yamada, K., Takenaka, M., and Tanaka, T. (1990) Regulation of the gene expression of glucokinase and L-type pyruvate kinase in primary cultures of rat hepatocytes by hormones and carbohydrates. *J. Biochem. (Tokyo)* **108**, 778–784
27. Myint, Z., Inazu, T., Tanaka, T., Yamada, K., Keng, V.W., Inoue, Y., Kuriyama, M., and Noguchi, T. (1999) Genomic organization and promoter analysis of a mouse homeobox gene, *Hex*. *J. Biochem. (Tokyo)* **125**, 795–802
28. Karpen, S.J., Sun, A.-Q., Kudish, B., Hagenbuch, B., Meier, P.J., Ananthanarayanan, M., and Suchy, F.J. (1996) Multiple factors regulate the rat liver basolateral sodium-dependent bile acid cotransporter gene promoter. *J. Biol. Chem.* **271**, 15211–15221
29. Ptashne, M. and Gann, A. (1997) Transcriptional activation by recruitment. *Nature* **386**, 569–577
30. Triezenberg, S.J. (1995) Structure and function of transcriptional activation domains. *Curr. Opin. Genet. Dev.* **5**, 190–196
31. Guiral, M., Bess, K., Goodwin, G., and Jayaraman, P.S. (2001) PRH represses transcription in hematopoietic cells by at least two independent mechanisms. *J. Biol. Chem.* **276**, 2961–2970
32. Choi, C.Y., Lee, Y.M., Kim, Y.H., Park, T., Jeon, B.H., Schulz, R.A., and Kim, Y. (1999) The homeodomain transcription factor NK-4 acts as either a transcriptional activator or repressor and interacts with the p300 coactivator and the Groucho corepressor. *J. Biol. Chem.* **274**, 31543–31552
33. Ostendorff, H.P., Peirano, R.I., Peters, M.A., Schluter, A., Bossenz, M., Scheffner, M., and Bach, I. (2002) Ubiquitination-dependent cofactor exchange on LIM homeodomain transcription factors. *Nature* **416**, 99–103