Divergent homeobox gene *Hex* regulates promoter of the Na\(^+\)-dependent bile acid cotransporter

LEE A. DENSON, SAUL J. KARPEN, CLIFFORD W. BOGUE, AND HARRIS C. JACOBS

Department of Pediatrics, Yale University, New Haven, Connecticut 06520-8064

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Denson, Lee A., Saul J. Karpen, Clifford W. Bogue, and Harris C. Jacobs. Divergent homeobox gene *Hex* regulates promoter of the Na\(^+\)-dependent bile acid cotransporter. *Am J Physiol Gastrointest Liver Physiol* 279: G347–G355, 2000.—The divergent homeobox gene *Hex* is expressed in both developing and mature liver. A putative *Hex* G347–G355 promoter through this site. Successive 5′-deletions of the *ntcp* promoter in a luciferase reporter construct transfected into Hep G2 cells confirmed a *Hex* response element (HRE) within the *ntcp* promoter (nt −733 to −714). Moreover, p-CMHex transactivated a heterologous promoter construct containing HRE multimers (p4xHRELUC), whereas a 5-bp mutation of the core HRE eliminated transactivation. A dominant negative form of *Hex* (p-Hex-DN) suppressed basal luciferase activity of p-4xHRELUC and inhibited activation of this construct by p-CMHex. Interestingly, p-CMHex transactivated the HRE in Hep G2 cells but not in fibroblast-derived COS cells, suggesting the possibility that *Hex* protein requires an additional liver cell-specific factor(s) for full activity. Electrophoretic mobility shift assays confirmed that liver and Hep G2 cells contain a specific nuclear protein that binds the native HRE. We have demonstrated that the liver-specific *ntcp* gene promoter is the first known target of *Hex* and is a useful tool for evaluating function of the *Hex* protein.

Transcriptional regulation; liver

Homeobox genes constitute a highly conserved family of transcription factors characterized by the presence of a 60-amino acid motif known as the homeodomain (30, 32, 44). These genes fall into two categories. One group, *Hox* genes, are tightly clustered on four chromosomes and are identified by similarities in the homeodomain with homologous *Drosophila* genes involved in determination of body pattern (31, 42). The remaining homeobox genes are referred to as divergent homeobox genes and are scattered throughout the genome. These genes tend to have more limited domains of expression and, where evaluated, have been shown to have tissue-specific function (see, e.g., Refs. 6, 19, and 26).

The divergent homeobox gene, *Hex*, was originally identified from hematopoietic tissue (3, 22). However, we (4) and others (46, 47) have recently provided evidence that *Hex* is expressed at high levels in the developing fetal liver, including the epithelium of the bile duct and gallbladder, and in the mature liver. It is also expressed in Hep G2 cells, a human hepatoma cell line (22, 46). This pattern of expression strongly suggests a role for *Hex* both in hepatobiliary development and in the expression of genes in the mature liver. Tanaka et al. (46) recently reported that a fusion protein consisting of *Hex* with the DNA binding domain of Gal4 functions in Hep G2 cells as a repressor with a reporter plasmid containing five copies of the Gal4 binding site. Although that group did not test with a putative *Hex* target, their findings are consistent with *Hex* functioning as a transcriptional regulator.

Although one report identified a core binding sequence for *Hex* consisting of 5′-ATTAA-3′ (12), no known DNA targets of *Hex* (and of most other homeobox genes) have been identified. We identified a potential *Hex* binding site in the promoter of the sodium-dependent bile acid transporter gene (*ntcp*). The *ntcp* gene, which is expressed exclusively in hepatocytes, codes for the major transporter of bile acids across the basolateral surface of the hepatocyte (33). Both cDNA (18) and genomic clones (24) for rat *ntcp* have been reported. Analysis of these clones has greatly facilitated our understanding of the complex molecular mechanisms regulating the enterohepatic circulation of bile acids. However, these mechanisms are still not completely understood.

Bile flow is dependent on the synthesis, transport, and recirculation of bile acids (reviewed in Refs. 36 and 45). We reported previously (24) that the minimal promoter of *ntcp*, from nucleotide −158 to +47 relative to the transcription start site, regulated basal and an unidentified factor, footprint B binding protein (24). However, our data indicated that regions upstream of the minimal *ntcp* promoter participated in the regulation of *ntcp* gene expression.

We hypothesized that *Hex* would transactivate the *ntcp* promoter via a putative *Hex* response element (HRE) located in the 5′-upstream region from −733 to −714. The HRE contains an ATTAA core at −725 to
In the studies reported here, we demonstrate that Hex is indeed a regulator of the ntcp promoter via Hex binding to the HRE. These data document the ntcp promoter as the first known target of Hex and provide a target for evaluation of the Hex protein.

**EXPERIMENTAL PROCEDURES**

**Materials and supplies.** DNA restriction enzymes, calf intestinal alkaline phosphatase, T4 polynucleotide kinase, Vent DNA polymerase, and T4 DNA ligase were purchased from New England Biolabs. Taq DNA polymerase and dNTPs were purchased from Boehringer Mannheim. Radiolabeled nucleotides and nylon filters were purchased from Amer-sham. Tumor necrosis factor-α and interleukin-1β were purchased from R&D Systems (Minneapolis, MN). Cell culture media and fetal calf serum were purchased from GIBCO-BRL (Gaithersburg, MD). Luciferase and β-galactosidase assay kits were obtained from Promega (Madison, WI). A lactate dehydrogenase kit was obtained from Sigma (St. Louis, MO). Routine biochemicals were purchased from Fisher and U. S. Biochemical.

**Oligonucleotides.** All oligonucleotides were synthesized by the DNA Synthesis Lab, Critical Technologies Program, at Yale University on a model 3948 ABI DNA Synthesizer. The sequences of double-stranded oligonucleotides used in gel mobility shift assays and single-stranded oligonucleotides for plasmid construction are shown in Table 1.

**Plasmid construction and sequence analysis.** A full-length mouse Hex cDNA was prepared making use of a partial restriction map of the Hex gene and a partial Hex cDNA clone kindly provided by Dr. Leanne Wiedemann (Leukemia Research Fund Centre, Institute of Cancer Research, London, UK). Standard screening techniques were used to isolate genomic clones from a λFIX II library (kindly provided by Dr. Adrian Hayday, Yale University School of Medicine, New Haven, CT). Filters were hybridized and washed using a DNA restriction enzymes, calf intestinal alkaline phosphatase, T4 polynucleotide kinase, Vent DNA polymerase, and T4 DNA ligase were purchased from New England Biolabs. Taq DNA polymerase and dNTPs were purchased from Boehringer Mannheim. Radiolabeled nucleotides and nylon filters were purchased from Amer-sham. Tumor necrosis factor-α and interleukin-1β were purchased from R&D Systems (Minneapolis, MN). Cell culture media and fetal calf serum were purchased from GIBCO-BRL (Gaithersburg, MD). Luciferase and β-galactosidase assay kits were obtained from Promega (Madison, WI). A lactate dehydrogenase kit was obtained from Sigma (St. Louis, MO). Routine biochemicals were purchased from Fisher and U. S. Biochemical.

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<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
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<tr>
<td>Rnttcp−710</td>
<td>5′-gcttcctatctcgttgtaatttcagag-3′</td>
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<tr>
<td>Rnttcp+47</td>
<td>5′-ggtgaagttcaagacagacagtct-3′</td>
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<tr>
<td>HM5-Bam</td>
<td>5′-GATCTTAGGAAAGGCCGTCCACTATG-3′</td>
</tr>
<tr>
<td>HM5-Bgl</td>
<td>5′-GATCTTAGGACCCTCTGAGCCTATG-3′</td>
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<tr>
<td>AP-1</td>
<td>5′-GCCAGGTGCACGCTGAGCCT-3′</td>
</tr>
<tr>
<td>p-HEXFLAG</td>
<td>5′-GCCACCATGCGAATTCCGACGAGGG-3′</td>
</tr>
<tr>
<td>5′ Primer</td>
<td>5′-TTTATACGTCTGTGCTCTTGCCTATG-3′</td>
</tr>
<tr>
<td>3′ Primer</td>
<td>5′-TTTATACGTCTGTGCTCTTGCCTATG-3′</td>
</tr>
<tr>
<td>p-HEXDN</td>
<td>5′-TTTATACGTCTGTGCTCTTGCCTATG-3′</td>
</tr>
<tr>
<td>5′ Primer</td>
<td>5′-ATGAGCCCTTTGCTCCAGCAGAGG-3′</td>
</tr>
<tr>
<td>3′ Primer</td>
<td>5′-TTTATACGTCTGTGCTCTTGCCTATG-3′</td>
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Lowercase letters indicate a restriction site for cloning.

A fusion protein between the carboxy terminus of Hex and glutathione S-transferase (GST) was prepared in pGEX-2T (Pharmacia, Piscataway, NJ). Primers were prepared to amplify a sequence from amino acid 128 (serine) through the termination codon of Hex. A BamHI site was incorporated into the 5′ primer, and an EcoRI site was incorporated into the 3′ primer. The PCR product was digested with these two enzymes and cloned into the matching sites in pGEX-2T. The sequence was confirmed, as was the appropriate frame (pGST-Hex). Another plasmid was prepared consisting of the same segment of Hex except that a FLAG epitope tag was incorporated into the end of the 3′ primer as above. This PCR product was cloned into the pcBR2.1 vector (Invitrogen). Sequencing confirmed that there were no PCR errors. The insert from this clone was isolated after digestion with EcoRI and then cloned into the EcoRI site of pcDNA3 to produce a dominant negative Hex; this plasmid is referred to as HEDXN. Primer sequences are given in Table 1.

Two plasmids, p-1237LUC and p-758LUC, consisting of ntcp regulatory regions driving luciferase in pSV0AL have been described previously (24). These two plasmids consist of genomic sequence in the ntcp regulatory region starting at −1237 and −758 relative to the transcription start site for ntcp and ending at +47. The third plasmid, p-710LUC, was prepared from p-1237LUC by PCR. For this purpose, a 5′ primer, Ratnttcp-710, was synthesized (Table 1) starting at −710 relative to the transcription start site and included a Hind III site on the 5′ end. The 3′ primer, RatLuc2, was described previously (24). This primer ends within the Luciferase coding region of p-1237LUC. The PCR was performed with Vent DNA polymerase (New England Biolabs, Beverly, MA) according to the manufacturer’s instructions, digested with HindIII, and cloned into the HindIII site of pSV0AL. Orientation was confirmed, and the insert was sequenced.

We constructed a plasmid containing four copies of HRE inserted into pGL3-Promoter (Promega). We produced these by synthesizing the partially complementary oligonucleotides HRE-Bgl and HRE-Bam (Table 1), which were hybrid-
ized together, ligated, and digested with *Bgl* II and *Bam*H I. Only head-to-tail concatamers resisted enzymatic digestion. After digestion, the product was ligated into the *Bgl* II site of pGL3-Promoter. Colonies were picked and digested to identify concatamers. A clone containing four copies of *HRE* was sequenced to confirm the head-to-tail arrangement as well as the correct sequence and the correct orientation (p-4HRELUC). We constructed a similar plasmid containing four tandem copies of HM5 (see Fig. 1 and Table 1) (p-4HM5LUC).

All plasmid sequences were verified by the Keck Biotechnology Center, Yale University, using an ABI automated DNA sequencer model 373A. Promoter sequence search comparisons were performed via Lasergene programs (DNASTAR, Madison, WI).

**Cell lines, nuclear extracts, and electrophoretic mobility shift assays.** The procedures for these methods were the same as described previously (24). Briefly, Hep G2 cells (human hepatoblastoma) and COS cells were purchased from American Type Culture Collection and maintained in MEM supplemented with 10% FBS and penicillin-streptomycin-glutamine. Cells were plated in 12-well plates (10⁵ cells/well) for transient transfections and luciferase expression assays, 6-well plates for immunohistochemical localization of *Hex* by the FLAG epitope tag, or 10-cm plates (10⁶ cells/plate) for isolation of nuclear extracts and RNA. They were grown until they were ~75% confluent before transfection with the calcium phosphate-DNA coprecipitation technique as described previously (24).

A plasmid containing β-galactosidase driven by a RSV promoter (p-RSV[βGAL]) was cotransfected with luciferase reporter plasmids to control for transfection efficiency. Luciferase and β-galactosidase assays were done according to the recommendations of the manufacturer (Promega). Nuclear extracts from Hep G2 cells and rat liver for electrophoretic mobility shift assays (EMSA) were prepared by standard techniques (20, 43). Nuclear and cytosolic protein aliquots were stored at -70°C until used. Band intensity was measured by densitometry using the Personal Densitometer and ImageQuant software from Molecular Dynamics (Sunnyvale, CA).

EMSA was done as previously described (24). Briefly, 5 μg of nuclear extracts were incubated with 2 μg of poly(dI-dC)·poly(dI-dC) in a 20-μl reaction containing (in mM) 25 HEPES (pH 7.6), 50 KCl, 0.5 dithiothreitol, 0.5 EDTA, and 5 MgCl2 and 10% glycerol at room temperature for 10 min. 32P-end-labeled oligonucleotide (2 × 10⁴ cpm) was added, and the binding reaction was allowed to proceed for a total of 30 min. The entire sample was then electrophoresed through a non-denaturing 6% polyacrylamide gel in 0.25 Tris-borate-EDTA buffer at 12 V/cm for 2–3 h. Competing nonlabeled oligonucleotides were added to the binding mixtures along with the labeled oligonucleotide at 50- to 100-fold excess.

**Immunohistochemistry.** Immunohistochemistry was carried out by plating Hep G2 (or COS) cells in 6-well plates containing a sterile coverslip. Transfections were done with pcDNA3 (control) or HEXFLAG1 as above, after which the cells were fixed with methanol. After rebidysis in PBS, nonspecific protein binding was blocked by incubating the fixed cells for 1 h at room temperature in 30% goat serum-70% PBS. Cells were then incubated for 1 h at room temperature in a 1:2,000 dilution of a mouse monoclonal anti-FLAG antibody (Sigma) prepared in PBS containing 10% goat serum and 2% BSA. After five washes at room temperature with PBS, cells were incubated at room temperature for 30 min with a 1:2,000 dilution of a Cy3-tagged goat anti-mouse antibody (Amersham) in the same solution. Cells were again washed in PBS and once in distilled water and mounted on glass slides with Crystal Mount (Biomedia, Foster City, CA). Light and fluorescent micrographs were taken on an Olympus IX70 microscope fitted with a charge-coupled device camera. Light and fluorescent micrographs were combined in Adobe Photoshop. We then printed light micrographs and combined light and fluorescent micrographs.

**Statistics.** All statistical comparisons were done by a Student’s t-test. All measurements, including EMSA, were done with an n ≥ 3.

**Animals.** Adult male Sprague-Dawley rats (Camm Research Institute, Wayne, NJ) weighing 250–275 g were used for these experiments. The Yale Animal Care and Use Committee approved study protocols. Animals received humane care in compliance with the National Research Council’s criteria as outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Institutes of Health (NIH Pub. no. 86-23, revised 1985).

**RESULTS**

*Hex* transactivates ntcp promoter via an upstream element. Figure 1A shows a schematic drawing of the ntcp promoter with known and putative regulatory regions specified, including the HRE located between nucleotides −733 and −714. Figure 1B provides the sequence of the HRE and the 5-bp mutation in the core binding sequence of *Hex* producing the mutated HRE, HM5. We used a standard 5′ deletion approach to evaluate the ability of *Hex* to regulate the ntcp promoter. Three plasmids, p-1237LUC, p-758LUC, and p-710LUC, were tested. The putative core element of the *Hex* binding site from nucleotides −725 to −721 is eliminated from the shortest construct. Cotransfection of either p-1237LUC (not shown) or p-758LUC into Hep G2 cells with p-CMHex resulted in an almost twofold increase in luciferase activity relative to the test plasmids alone (Fig. 2A). In contrast, luciferase expression was not different when Hep G2 cells were transfected with p-710LUC (background) compared with cotransfection with p-CMHex and p-710LUC.

Fig. 1. Schematic presentation of the ntcp promoter and *Hex*-related sequences. A: a schematic of the ntcp promoter is shown with binding sites for putative and confirmed factors regulating ntcp. The sequence of the potential *Hex* binding site between nucleotides −733 and −714 is given with the 5 core nucleotides in capital letters and underlined. B: the sequence of the segment called *Hex* response element (HRE) is given 5′ to 3′ along with the mutated *Hex* binding site produced by changing the 5 core nucleotides (HM5). Changes are shown as underlined capital letters.
Also, transfection of p-710LUC alone produced luciferase activity that was only 75% of that found with p-758LUC (Fig. 2A). This reflects activation of luciferase from endogenous Hex via binding at a site in p-758LUC that was deleted in preparing p-710LUC. These data suggest the presence of a HRE between nucleotides −758 and −710.

Ntcp promoter sequence from −733 to −714 confers Hex responsiveness to a heterologous promoter in Hep G2 cells but not in COS cells. p-4xHRELUC was used to evaluate whether the HRE was sufficient to transactivate a heterologous promoter in Hep G2 cells. The parent vector, pGL3-Promoter, has a minimal SV40 promoter driving expression of a luciferase reporter gene. Transfection of p-4xHRELUC into Hep G2 cells increased luciferase activity >11-fold compared with vector alone (Fig. 2B). In contrast, transfection of p-4xHRELUC into COS cells did not significantly increase luciferase activity compared with vector alone (Fig. 2B). These data support the existence of a liver-enriched factor that can bind and transactivate via the HRE.

Cotransfection of Hep G2 cells with pGL3-Promoter and p-CMHex did not increase luciferase activity compared with cells transfected with pGL3-Promoter alone (Fig. 3). p-4xHRELUC activity was enhanced approximately twofold when cotransfected into Hep G2 cells with p-CMHex compared with transfection with p-4xHRELUC alone (Fig. 3). In contrast, there was no stimulation of expression of luciferase from p-4xHM5LUC when cotransfected with p-CMHex into Hep G2 cells compared with transfection with vector alone. We also cotransfected p-758LUC with p-CMHex and p-4xHRELUC with p-CMHex into COS cells. We detected no increase in luciferase activity compared with cells transfected with either vector alone (Fig. 3). These results indicate that Hex is capable of stimulating gene expression from the ntcp promoter via the HRE in Hep G2 cells but not COS cells. It is likely that Hep G2 cells contain a cofactor(s) necessary for Hex function that is not present in COS cells.

To further evaluate this interaction, we cotransfected Hep G2 cells with a series of plasmids and p-HEXDN (Fig. 4). The protein produced from p-HEXDN contains the DNA binding domain of Hex but lacks most of the amino-terminal domain and would be expected to act as a dominant negative. There was no effect of p-HEXDN on pGL3-Promter. In con-
Hep G2 cells and rat liver contain proteins that bind specifically to the HRE. Because liver and Hep G2 cells are known to express Hex (4, 22, 25, 37, 47), we evaluated liver and Hep G2 cells for the presence of a protein capable of binding to the HRE. An EMSA was done using the HRE (double stranded) and HM5 (double stranded) as probes. Nuclear extracts from both Hep G2 cells and rat liver contained a protein(s) that specifically bound the labeled HRE (Fig. 6). Although an excess of the nonlabeled HRE competed successfully with the labeled HRE for binding, this was not the case for HM5 or for an oligonucleotide containing the unrelated activating protein-1 (AP-1) binding site. Together, these data indicate that rat liver and Hep G2 cells contain a protein that binds specifically to the HRE.

In vitro expressed Hex binds to the HRE in the ntcp promoter. To determine whether Hex specifically recognized the HRE, a fusion protein was created between GST and the carboxy-terminal portion of Hex including the homeodomain starting from amino acid 128. GST and the GST-Hex fusion proteins were isolated from bacteria using glutathione-coated Sephadex beads (Pharmacia). Polyacrylamide gel electrophoresis documented that single bands of the appropriate molecular weights were obtained for both isolates (not shown). GST alone was unable to bind to HRE and served as a negative control in the binding assay. In contrast, binding of the GST-Hex fusion protein to HRE was

![Relative Luciferase Activity](Image)

**Fig. 3.** Relative luciferase activity from specific plasmids in Hep G2 and COS cells. Individual plasmids were transfected into Hep G2 cells or COS cells with and without p-CMHex. Luciferase activity is shown for cotransfection of the indicated plasmid with p-CMHex relative to transfection of the plasmid alone. Cotransfection of p4xHRELUC with p-CMHex gave increased luciferase activity compared with p4xHRELUC alone (*P < 0.01). In contrast, cotransfection of p4xHM5LUC with p-CMHex did not increase luciferase activity compared with p4xHM5LUC alone. Also, cotransfection of COS cells with p-CMHex and plasmids that respond to Hex in Hep G2 cells gave only background activity. Thus Hex function requires the 5 mutated core nucleotides in the HRE as well as a cofactor(s) that is present in Hep G2 cells but is missing from COS cells.

Contrast, cotransfection with p-758LUC or p-4xHRELUC and p-HEXDN resulted in a decrease in luciferase activity compared with transfection with p-758LUC or p-4xHRELUC alone. Hep G2 cells were then cotransfected with p-4xHRELUC plus 200 ng of p-CMHex and increasing amounts of p-HEXDN (0–500 ng). A statistical decrease in luciferase activity by cotransfection with p-HEXDN was found with 500 ng of p-HEXDN. These results were likely due to suppression of endogenous and exogenous Hex by binding of HEXDN to the HRE. They support Hex activation of ntcp via the native HRE and indicate that the amino-terminal region of Hex is required for protein-protein interaction.

We used the FLAG epitope tag system to verify that Hex was transported to the nucleus in both Hep G2 cells and COS cells (38). We first demonstrated that the FLAG epitope tag had no effect on cells and COS cells (38). We first demonstrated that the absence of a nuclear cofactor rather than failure of nuclear translocation.
observed (Fig. 6). A 100-fold excess of nonlabeled HRE competed successfully with labeled HRE for binding to the fusion protein. The GST-Hex fusion protein did not bind HM5, nor did the HM5 compete with the fusion protein for binding to HRE. These data indicate that Hex binds specifically to the sequence from −733 to −714 in the ntcp promoter that contains the core consensus sequence for Hex and, along with the reporter gene studies, support a direct effect of Hex on ntcp expression.

DISCUSSION

The first homeobox gene was cloned from Drosophila about 15 years ago (31). Mutations and misexpression studies on clustered (Hox) and divergent homeobox genes have provided significant information on both early embryonic and mature, tissue-specific functional aspects of these genes (see, e.g., Refs. 1, 2, 8, 13, 14, 16, 19, 21, 26, 27, 34, and 41). However, only a few targets
HEX REGULATES NTCP PROMOTER

**Probe**

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**Competitor**

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**Nuclear Extract**

- **Liver**
- **HepG2**
- **Fusion Protein**

Fig. 6. Electrophoretic mobility shift assay (EMSA) with liver and Hep G2 nuclear extracts and the fusion protein. The labeled probe and the nonlabeled competitor used in each EMSA are indicated by a plus (+) sign. The shifted band for each EMSA is shown next to the source of the protein used. A shifted band was obtained with the HRE but not HM5 using each extract. Only specific competitor successfully competed away the shifted band. The first lane contained probe but did not contain nuclear extract and showed no nonspecific binding of probe.

of homeobox genes have been identified (e.g., Refs. 7, 11, 40, and 48). Both mutation/misexpression studies and evaluation of target genes are important for understanding organ development and function.

Because Hex is expressed in the developing hepatobiliary system and subsequently in the mature liver, we speculated that Hex is important in liver-specific function and searched for a potential target for Hex in the liver. The ntcp gene, which produces a protein integral to maintaining bile flow, was found to contain a putative Hex binding site upstream of the minimal promoter (24). We hypothesized that the ntcp promoter would be regulated by Hex and in these studies have identified ntcp promoter as the first known target for Hex. We found a significant but modest twofold stimulation of ntcp promoter activity via sequences between nucleotides −733 and −711. This activity was lost when these nucleotides were deleted, which eliminated a sequence that contained the in vitro determined consensus Hex binding sequence, ATTAA (12).

This result did not distinguish between direct binding of Hex to this small region of DNA and stimulation via a secondary effect. However, liver and Hep G2 cells, both of which are known to express Hex mRNA, produce a protein that binds specifically to the HRE. A fusion protein between GST and the portion of the Hex protein containing the DNA binding domain binds to the same site. This is consistent with binding of an endogenous protein(s) from liver and Hep G2 cells to this region of DNA. A 20-mer from the ntcp promoter centered on this site drives expression of a reporter gene under control of a heterologous promoter in Hep G2 cells when cotransfected with a plasmid expressing a Hex cDNA. Furthermore, a shortened form of Hex capable of DNA binding, HEDN, functioned as a dominant negative when cotransfected with this plasmid. Finally, an identical reporter plasmid, p-HM5LUC, except for mutation of the core Hex binding sequence, is not activated by Hex. Together, these data allow us to conclude that Hex regulates the ntcp promoter directly by binding to its consensus sequence between nucleotides −725 and −720. Furthermore, it is possible that Hex plays a contributing role in overall ntcp activity in vivo, albeit not as a primary transcriptional activator.

Equally interesting and worthy of further study is the fact that in fibroblast-derived COS cells, Hex was not capable of activating a reporter gene in a plasmid containing a minimal viral promoter and four copies of the HRE. This suggests the presence of a factor(s) that interacts with Hex, is necessary for mediating transactivation of target genes, and is found in Hep G2 cells but not in COS cells. Although this factor may well be tissue specific, our data indicate that this factor is not required for nuclear transport of Hex. Cofactors interacting with other homeobox genes and required for their specificity and activity have been described previously (see, e.g., Refs. 9, 15, 17, 23, 28, 35, 39, and 49).

The Drosophila protein exd functions as a cofactor with homeobox proteins at different times in development. This is particularly interesting to us because Hex shows a very early pattern of expression, suggesting a role in embryogenesis as well as a later, organ-specific pattern (4, 22, 25, 47). The cofactor required for Hex function in Hep G2 cells may well be required for Hex function early in development. We are currently conducting studies to determine the identity of this cofactor.

Our results differ significantly from a recently reported study suggesting that Hex might function as a transcriptional repressor (46). These authors constructed various chimeric fusion proteins utilizing different portions of Hex. These were tested against an artificial but specially designed reporter construct. In contrast, using a native, liver-specific promoter we clearly show that Hex activates the ntcp promoter in the same cell type. We also tested for Hex activation of the ntcp gene in COS cells and found no evidence of activation (or repression) despite demonstrating that Hex protein translocates to the nucleus in both cell types. The advantage of our assay system is that it relies on binding of the native Hex protein to a native target site in the ntcp promoter. Nevertheless, there are other potential explanations for the differences between our results, including the possibility that Hex functions as a repressor or as an activator depending
on the target gene and circumstances. For instance, Zhu and Kuziora (50) found in their analysis of functional domains of the Deformed homeobox protein that mutant Deformed proteins influenced the regulation of other homeotic genes in a manner distinct from that of the native protein. In fact, they found that removal of two transcriptionally active domains, the acidic region and the C tail, converted a chimeric protein from a strong activator to a repressor of the Distal-less element while having little effect on the activation of the empty spiracles element. It is also possible that the Hex-GAL4 fusion protein used by Tanaka et al. (46) does not interact with the liver-enriched cofactor(s) that we have demonstrated is required for target gene activation. Recent reports by Li et al. (29) and Choi et al. (10) provide strong evidence that the interaction of homeodomain proteins with tissue- or cell type-specific cofactors can influence their transcriptional activity, whereby any one homeobox protein can function as either a repressor or activator. Further studies are required to answer this question.

In summary, we have identified the ntcp promoter as the first known target gene for Hex. Furthermore, we provide evidence for the existence of an activating cofactor of Hex present in Hep G2 cells that is absent from COS cells. Hex joins the small group of liver-enriched transcription factors (e.g., HNF-1, HNF-3β, HNF-4, HNF-6, and C/EBP) known to regulate liver-specific gene promoters. These experiments provide an important tool for evaluation of the Hex protein and a strong basis for further investigations into the hepatobiliary role of Hex.

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