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[SHORT COMMUNICATION]

HNF-1 Regulates the Promoter Activity of the *HP-27* Gene

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ABSTRACT—The hibernation-specific *HP-27* gene is expressed specifically in the liver of the chipmunk, a hibernating species of the squirrel family, and exists as a pseudogene in the tree squirrel, a nonhibernating species. In the promoter region, the chipmunk gene has a potential HNF-1 binding site, and the tree squirrel gene has two base substitutions in the corresponding sequence. In this paper, we investigated the role of HNF-1 in the *HP-27* gene promoter activity. Gel retardation assays with *in vitro*-translated HNF-1 and super-shift assays using HepG2 nuclear extracts and an anti-HNF-1 antibody revealed that HNF-1 bound to the chipmunk gene sequence. HNF-1 also bound to the tree squirrel sequence, but with much lower affinity. In HepG2 cells, HNF-1 activated transcription from the chipmunk *HP-27* gene, but not from the tree squirrel gene. In addition, the tree squirrel-type base substitutions in the HNF-1 binding site greatly reduced the promoter activity of the chipmunk *HP-27* gene. These results indicate that HNF-1 is required for the promoter activity of the chipmunk *HP-27* gene, and that the base substitutions in the HNF-1 binding site are involved in the lack of *HP-27* gene expression in the tree squirrel.

Key words: hibernation, promoter, transcriptional regulation, HNF-1, HP-27

INTRODUCTION

Mammalian hibernation is a unique physiological adaptation that allows life to be sustained under extremely low body temperatures (Kayser 1961; Johansson *et al*., 1967; Swan 1972). Only certain small mammals, primarily in the orders Rodentia, Insectivora, and Chiroptera, can undergo hibernation. In the squirrel family, some species, such as the chipmunk (*Tamias asiaticus*) and the 13-lined ground squirrel (*Citellus tridecemlineatus*), hibernate; other species, such as the tree squirrel (*Callosciurus caniceps*), do not. The chipmunk hibernation-specific protein HP-27 was identified as a component of a 140-kDa complex (Kondo and Kondo 1992). The amount of this complex is drastically lowered in the blood during hibernation. In the chipmunk and the 13-lined ground squirrel, the *HP-27* gene is expressed specifically in the liver and is downregulated during hibernation (Takamatsu *et al*., 1993). Although the tree squirrel has the *HP-27* gene, its mRNA is not expressed (Takamatsu *et al*., 1993). In a previous paper, we showed that the 170-bp 5' flanking sequence of the chipmunk *HP-27* gene contains the promoter for the liver-specific transcription, and that the transcription factors that bind to the region from nucleotides –170 to –140 play important roles in *HP-27* gene transcription (Ono *et al*., 2003). Despite, the 69-bp 5' flanking sequence still retained about 10% of the promoter activity of the 170-bp 5' flanking sequence, which implied that there was a contribution of the HNF-1 binding site from –54 to –40 of the chipmunk *HP-27* gene to the liver-specific promoter activity (Ono *et al*., 2003). In this paper, we investigated the role of HNF-1 in the *HP-27* gene promoter activity.

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MATERIALS AND METHODS

Gel retardation assay

Nuclear extracts were prepared from human hepatoma HepG2 cells as described (Kojima *et al*., 2000). Mouse HNF-1 was synthesized from pcDNA3/HNF-1 (Ono *et al*., 2001) using an *in vitro* transcription/translation system (Promega). HepG2 nuclear extracts or *in vitro*-translated HNF-1 were preincubated with 1 μg of poly (dI-dC) on ice for 5 min in 20 μl of 1 × gel-retardation buffer (12% glyc-

erol, 12 mM HEPES-KOH [pH 7.9], 60 mM KCl, 5 mM $MgCl_2$, 4 mM Tris-HCl [pH 7.9], 0.6 mM EDTA, 5 mM DTT), then mixed with 1 ng of ^{32}P-labeled, double-stranded oligonucleotide probe CM27G-63/-30 (5'-CTTTGTGGTTAAAAATTAGCCAACCTATTG-3'), containing the chipmunk *HP-27* gene sequence from nucleotides -63 to -30, or TS27G-63/-30 (5'-CTTTGTGCTTAAAAATTTGCCAAGCTATTC-3'), containing the tree squirrel *HP-27* gene sequence from nucleotides –63 to –30, and incubated on ice for an additional 30 min. For competition assays, a 10- or 100-fold molar excess of CM27G-63/-30 or TS27G-63/-30 was used as a competitor, and for supershift experiments, a rabbit polyclonal HNF-1 antibody (Santa Cruz Biotechnology) or normal rabbit serum was included in the binding reactions. The samples were subjected to electrophoresis through a nondenaturing 4% polyacrylamide gel at room temperature, then autoradiographed.

Promoter-reporter plasmid constructs

To construct chipmunk-type promoter-reporter plasmids, pCM27G-63/luc and pCM27G-32/luc, the 5' flanking sequences were generated by PCR using a chipmunk *HP-27* genomic clone (Ono *et al.*, 2003) as the template, digested with *Hind*III at nucleotide +84, and subcloned into the *Sma*I and *Hind*III sites of the promoterless firefly luciferase expression vector pGV-B (Nippon Gene). The numbers –63 and –32 in the construct names indicate the 5' end of the 5' flanking sequence. For a tree squirrel promoter-reporter plasmid, pTS27G-63/luc, the corresponding sequence was amplified by PCR using a tree squirrel *HP-27* genomic clone as the template, and was subcloned into pGV-B. Three mutant constructs, pCM27G-170m-1/luc, pCM27G-170m-2/luc, and pCM27G-170m-3/luc, which carry both or either of the tree squirrel-type base substitutions at nucleotides –53 and –43, were constructed by PCR from pCM27G-170/luc (Ono *et al.*, 2003).

DNA transfection and luciferase assay

HepG2 cells were maintained in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum. The cells were plated at 1.0×10^5 cells per 20-mm dish, and after 20 hr were transfected with 0.75 μg of a firefly luciferase promoter-reporter plasmid and 30 ng of a *Renilla* luciferase internal control plasmid, pRL-TK (Promega), using FuGENE 6 (Roche Diagnostics). Where denoted, the indicated amounts of the mouse HNF-1 expression constructs pcDNA3/HNF-1 were cotransfected. After 40 hr, the luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega).

RESULTS AND DISCUSSION

The 170-bp 5' flanking sequence of the chipmunk *HP-27* gene contains the entire promoter region necessary for its liver-specific transcription (Ono *et al.*, 2003). Despite, the 69-bp 5' flanking sequence still retains about 10% of the promoter activity. The sequence from nucleotides –54 to –40 of the chipmunk *HP-27* gene shows an 87% identity with the consensus HNF-1 binding sequence (Fig. 1) (Tronche and Yaniv, 1992). We first examined whether HNF-1 could bind to this chipmunk sequence by gel retardation assays using a double-stranded oligonucleotide probe CM27G-63/-30 containing the sequence from nucleotides –63 to –30 of the chipmunk *HP-27* gene. Incubation of *in vitro*-translated HNF-1 with the probe CM27G-63/-30 resulted in the formation of a protein-DNA complex (Fig. 2A, lane 3), and a similar DNA-protein complex formation was observed with the HepG2 nuclear extracts (Fig. 2A, lane 5). When an anti-

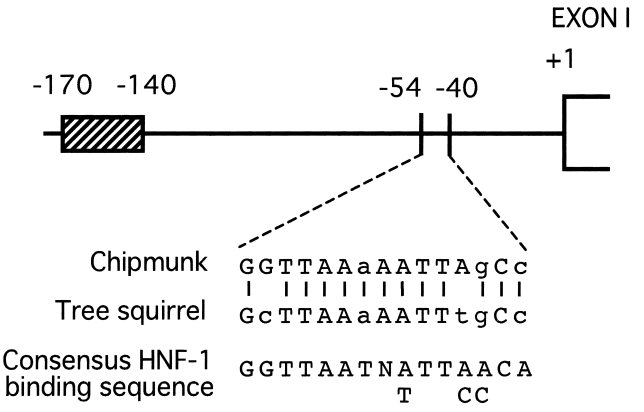

Fig. 1. Alignment of the potential HNF-1 binding sequences from the chipmunk and tree squirrel *HP-27* genes with the consensus HNF-1 binding sequence. Nucleotides that are different from the consensus HNF-1 binding sequence are indicated by lower-case letters. Identical nucleotides between the chipmunk and the tree squirrel *HP-27* genes are indicated by vertical lines. The region essential for the promoter activity of the chipmunk *HP-27* gene (nucleotides –170 ~ –140) is indicated by a shaded box.

HNF-1 antibody was added to the binding reaction, the protein-DNA complex was super-shifted (Fig. 2A, lanes 4 and 6). The faster migrating bands in lane 5 were considered to represent nonspecific protein binding because they were not competed out by an excess of unlabeled probe (data not shown). On the other hand, the corresponding tree squirrel *HP-27* gene sequence differs from the chipmunk sequence by two nucleotides, and shows a 73% identity with the consensus HNF-1 binding sequence (Fig. 1). When incubated with *in vitro*-translated HNF-1, the tree squirrel-type probe TS27G-63/-30 formed a similar protein-DNA complex to that observed with CM27G-63/-30, but not as effectively as CM27G-63/-30 (Fig. 2B, lanes 2 and 4). Similarly, the complex formed between HNF-1 and the probe CM27G-63/-30 was competed by CM27G-63/-30, but not as effectively by TS27G-63/-30 (Fig. 2C, lanes 2–5). From these results, we concluded that HNF-1 binds to the 5' flanking sequence of the chipmunk *HP-27* gene from nucleotides –63 to –30, and that the two base substitutions in the corresponding tree squirrel *HP-27* gene sequence disturb HNF-1 binding.

We next investigated whether HNF-1 could activate transcription from the chipmunk and tree squirrel *HP-27* gene promoters in HepG2 cells. Upon cotransfection of pcDNA3/HNF-1, the luciferase activities of pCM27G-63/luc increased in a dose-dependent manner (Fig. 3). In comparison, the promoter activities of pCM27G-32/luc, which lacks the potential HNF-1 binding site, and a tree squirrel-type promoter-reporter construct pTS27G-63/luc were hardly affected by HNF-1. These results indicate that HNF-1 can activate transcription of the chipmunk *HP-27* gene, but not of the tree squirrel *HP-27* gene.

Because the 63-bp 5' flanking sequence of the chipmunk *HP-27* gene retained only 10% of the promoter activity of the 170-bp 5' flanking sequence, we then investigated the effect of HNF-1 on the promoter activity in the context of the

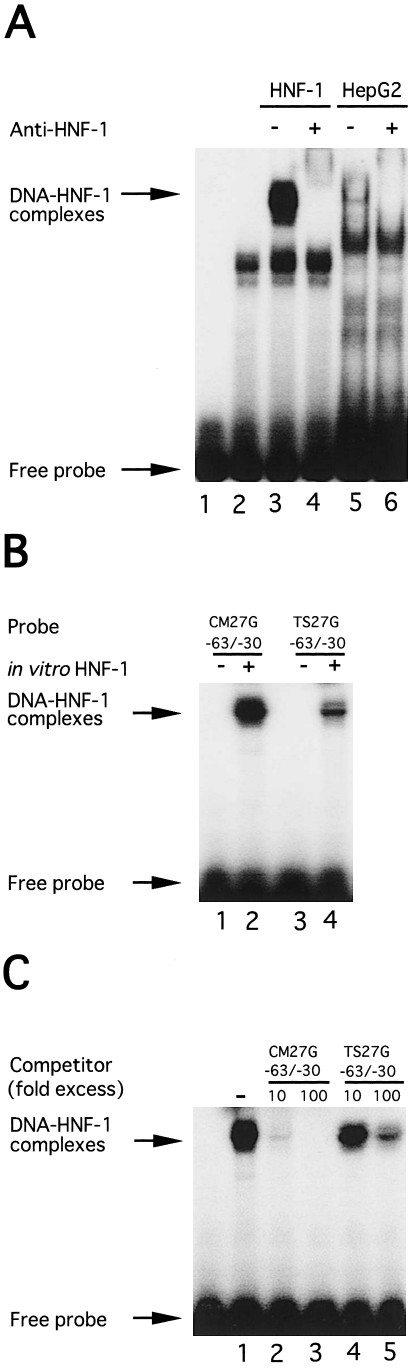

Fig. 2. Demonstration of HNF-1 binding to the *HP-27* gene promoter. (A) Double-stranded, end-labeled oligo nucleotide CM27G-63/-30 containing the chipmunk *HP-27* gene sequence from nucleotides –63 to –30 was incubated with *in vitro*-translated HNF-1 (lanes 3 and 4), or nuclear extracts from HepG2 cells (lanes 5 and 6) in the presence of anti-(HNF-1) Ig (lanes 4 and 6) or normal rabbit serum (lanes 3 and 5). Lane 1 lacks nuclear extracts. In lane 2, the *in vitro* transcription-translation reaction of an empty pcDNA3 vector was used. (B) A gel-retardation assay was carried out using the oligonucleotides CM27G-63/-30 (lanes 1 and 2) and TS27G-63/-30 (lanes 3 and 4) and an *in vitro* transcription-translation reaction with pcDNA3/HNF-1 (lanes 2 and 4) or pcDNA3 (lanes 1 and 3). (C) A gel-retardation assay was performed in which ^{32}P-labeled CM27G-63/-30 was incubated with *in vitro*-translated HNF-1 in the absence (lane 1) or presence of a 10- or 100-fold molar excess of unlabeled CM27G-63/-30 (lanes 2 and 3) or TS27G-63/-30 (lanes 4 and 5) as a competitor.

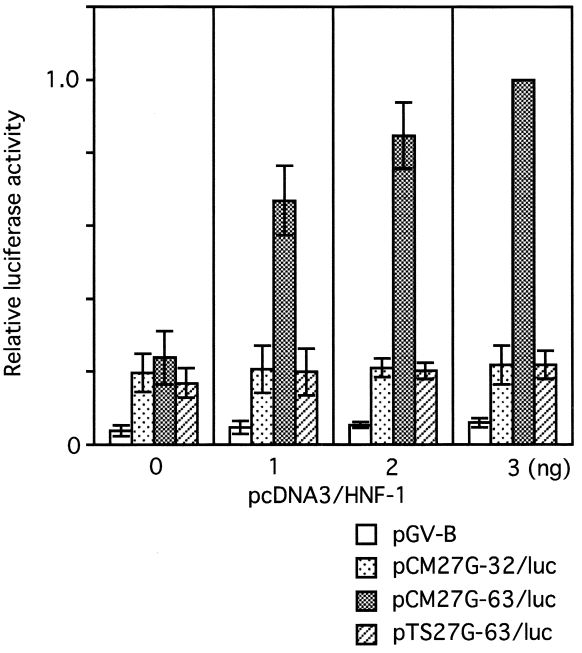

Fig. 3. HNF-1 activates transcription from the chipmunk *HP-27* gene promoter but not from the tree squirrel *HP-27* gene promoter. HepG2 cells were transfected with 0.75 μg of a promoter-reporter construct together with pRL-TK as a control for transfection efficiency. Where denoted, the indicated amounts of a mammalian expression construct for mouse HNF-1 (pcDNA3/HNF-1) were also transfected. Each firefly luciferase activity was normalized to the *Renilla* luciferase activity and is shown as the fold increase compared with that of pCM27G-63/luc plus 3 ng of pcDNA3/HNF-1. The data represent the mean ± SE from six separate experiments.

entire 170-bp promoter (Ono *et al*., 2003). To this end, we made three mutant constructs from pCM27G-170/luc: pCM27G-170m-1/luc, pCM27G-170m-2/luc, and pCM27G-170m-3/luc. The first construct carries both the tree squirrel-type base substitutions, at nucleotide –53 and –43, in the HNF-1 binding site; the last two each carry one mutation, at nucleotide –53 or –43, respectively (Fig. 4). These mutant constructs were transfected into HepG2 cells, and those luciferase activities were compared with that of pCM27G-170/luc (Fig. 4). The promoter activity of the double mutant, pCM27G-170m-1/luc, was 70% less, and those of the single mutants, pCM27G-170m-2/luc and pCM27G-170m-3/luc, were each 50% less than the promoter activity of pCM27G-170/luc. These results indicate that the binding site for HNF-1 in the chipmunk *HP-27* gene from –54 to –40 is required for effective transcriptional activation. On the other hand, in the case of the tree squirrel *HP-27* gene, its low affinity for HNF-1 is likely to be insufficient for effective transactivation by HNF-1.

Like the *HP-27* gene, another hibernation-specific gene, *HP-25,* is expressed in a liver-specific manner in the chipmunk, and exists as a pseudogene in the tree squirrel (Takamatsu *et al*., 1993; Kojima *et al*., 2000; Kojima *et al*., 2001). HNF-4 plays an essential role in the promoter activity of the chipmunk *HP-25* gene (Kojima *et al*., 2000). In con-

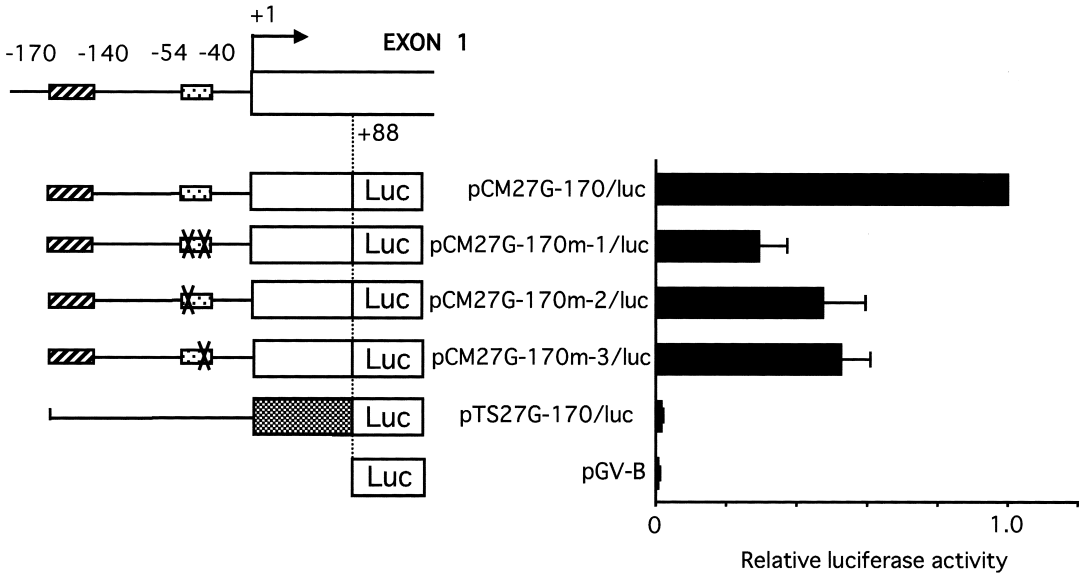

Fig. 4. The HNF-1 binding site is required for the chipmunk *HP-27* gene promoter activity. HepG2 cells were transfected with 0.75 μg of the chipmunk or tree squirrel wild-type promoter-reporter construct (pCM27G-170/luc or pTS27G-170/luc) or a mutant construct (pCM27G-170m-1/luc, pCM27G-170m-2/luc, or pCM27G-170m-3/luc) together with pRL-TK as a control for transfection efficiency. Each firefly luciferase activity was normalized to the *Renilla* luciferase activity and is shown as the fold increase compared with that of pCM27G-170/luc. The data represent the mean ± SE from eight separate experiments. In the diagram, ×s indicate the tree-squirrel-type base substitutions at nucleotides –53 and –43.

trast, the tree squirrel *HP-25* gene has a base substitution in the corresponding HNF-4 binding site, which abolishes the binding of and transactivation by HNF-4; this mutation is the likely cause of the lack of *HP-25* gene expression in the tree squirrel (Kojima *et al.*, 2001). Similarly, the tree squirrel *HP-27* gene has several base substitutions that hinder the binding of the transcription factors required for the promoter activity of the chipmunk *HP-27* gene (Ono *et al.*, 2003). Collectively, the tree squirrel *HP-25* and *HP-27* genes have mutations in the important transcription factor binding sites.

The results presented in this paper have revealed that the binding of HNF-1 to the proximal promoter region from nucleotides –54 to –40 is required for the chipmunk *HP-27* gene promoter activity. In the previous paper, we demonstrated that the transcription factors that bind to the sequence from nucleotides –170 to –140 are essential for the liver-specific promoter activity of the chipmunk *HP-27* gene (Ono *et al.*, 2003), and to further understand the transcriptional regulation of the *HP-27* gene, the transcription factors that bind to this region must be identified.

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