The Hepatocyte Nuclear Factor 3β Stimulates the Transcription of the Human Insulin-like Growth Factor I Gene in a Direct and Indirect Manner*

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Linda A. Nolten, Paul H. Steenbergh‡, and John S. Sussenbach

From the Laboratory for Physiological Chemistry, Utrecht University, Graduate School of Developmental Biology, Universiteitsweg 100, 3584 CG Utrecht, The Netherlands

Promoter 1 (P1) of the human insulin-like growth factor I (IGF-I) gene is most active in adult liver. In this study we show that HNF-3β, a member of the winged helix protein family of liver-enriched transcription factors, has a strong stimulatory effect on the activity of P1. Transient transfection experiments in combination with bandshift and DNase I footprinting analysis revealed the presence of two HNF-3 binding sites in the proximal promoter region of P1. Both binding sites, which are well conserved in evolution, are required for maximal transactivation. Studies employing HNF-3 mutant constructs indicated that IGF-I expression is also regulated indirectly by HNF-3β as a consequence of enhanced expression of HNF-1α. This liver-enriched transcription factor has previously been shown to transactivate P1. Thus, HNF-3β regulates the expression of the human IGF-I gene via two distinct mechanisms.

Human insulin-like growth factor I (hIGF-I) is a small basic polypeptide of 70 amino acids, which plays an important role in postnatal development. hIGF-I mediates the growth and differentiation promoting effects of growth hormone (1). Growth hormone is the main regulator of IGF-I gene expression and regulates its expression at the transcriptional level (2). IGF-I is expressed by many tissues during development; however, postnatally expression is mainly restricted to liver tissue (3). During puberty and adulthood, the liver is the main endocrine source of circulating IGF-I.

The hIGF-I gene is a complex transcriptional unit. The gene consists of six exons together spanning more than 90 kilobase pairs of genomic sequence (4–6). Expression of the gene gives rise to three classes of mRNA, due to alternative splicing and alternative usage of two poly(A) signals. All three classes render the same mature IGF-I polypeptide. In addition, the hIGF-I gene harbors two alternatively used promoters, P1 and P2, located upstream of exon 1 and exon 2, respectively. Differential usage of the promoters has been observed both in vitro and in vivo (7). In hepatocytes, promoter P1 is the stronger promoter yielding about 80% of all IGF-I transcripts and may be considered to function as a liver-specific promoter, at least postnatally. From studies on other promoters of genes that are highly expressed in liver, it has become apparent that multiple liver-enriched transcription factors work together in a concerted action to achieve high expression levels in adult liver (8–11). Four families of liver-enriched transcription factors have been described to date, i.e. the C/EBP, HNF-1, HNF-3, and HNF-4 families. The expression of the transcription factors belonging to these families is not totally restricted to liver tissue. In all probability, it is the combination of several cis-acting elements, present in the promoter regions of liver specifically expressed genes, to which liver-enriched trans-acting factors of different families can bind, that confers liver specificity and strong stimulation of gene expression.

Previously, we have described the involvement of two families of liver-enriched transcription factors in the regulation of hIGF-I gene expression (12, 13). Members belonging to the bZIP family of liver-enriched transcription factors (C/EBPα and C/EBPβ) enhance basal transcription directed by P1 up to 12-fold. Transactivation of P1 by these factors is dependent on the presence of an high affinity C/EBP binding site located at position −119 in P1 (12). HNF-1α, a liver-enriched member of the helix-loop-helix family of transcription factors, is also able to transactivate P1 and was shown to be an even stronger transactivator than C/EBP family members. Bandshift experiments revealed that two HNF-1 binding sites are present in P1. The upstream HNF-1 binding site, located between −282 and −270, has a slightly higher binding affinity for HNF-1α than to the downstream HNF-1 binding site, located between −119 and −107. Site-directed mutagenesis experiments revealed that the latter, downstream binding site is more important for transactivation of P1 by HNF-1α (13).

HNF-3α, HNF-3β, and HNF-3γ belong to a third family of liver-enriched transcription factors. All three proteins are expressed in adult liver tissue, HNF-3α and HNF-3β being more abundant than HNF-3γ (14). In addition, HNF-3α and HNF-3β are expressed in intestine and lung, whereas HNF-3γ is expressed in intestine and testis, but is absent from lung (15, 16). In hepatocytes, HNF-3 transcription factors function as activators of liver-specific gene expression (17). HNF-3α was originally identified as a protein that binds specifically to sequences proven to be functionally important in the liver-specific expression of the transthyretin (TTR) and α1-anti-trypsin genes (18). Other target sites for HNF-3 proteins have been described in the promoter/enhancer region of the genes encoding α-fetoprotein (19), albumin (20), tyrosine aminotransferase (21), phosphoenolpyruvate kinase (22), transferrin (23), and aldolase B (24) and of the transcription factors HNF-1α (25) and HNF-3β (26). HNF-3 binds to DNA as monomers via a divergent helix-turn-helix motif, referred to as the winged helix motif (27–29).

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‡ To whom correspondence should be addressed: Laboratory for Physiological Chemistry, Utrecht University, Stratumum, Universiteitsweg 100, 3584 CG Utrecht, The Netherlands. Tel.: 31-30-2538986; Fax: 31-30-2538035; E-mail: steenbergh@med.ruu.nl.

The abbreviations used are: hIGF-I, human insulin-like growth factor I; IGF-I, insulin-like growth factor I; GST, glutathione S-transferase; PCR, polymerase chain reaction; TTR, transthyretin; P1 and P2, promoters 1 and 2, respectively.
Although the DNA binding domain of HNF-3α, HNF-3β, and HNF-3γ is more than 90% conserved, a large range of DNA sequences can be recognized by these proteins. Subtle changes in the highly conserved DNA binding domain and in a more variable 20-amino acid region located adjacent to the DNA recognition helix may contribute to DNA binding specificity (30, 31). Recently, the sequence 5′-WRRRYMAAYA-3′ has been proposed as the consensus recognition and binding site for HNF-3 family members (32).

Aim of this study is to investigate whether the members of the HNF-3 family of liver-enriched transcription factors contribute to the expression level of the hIGF-1 gene in adult liver.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The reporter constructs containing P1-derived promoter fragments of different lengths cloned in front of the firefly luciferase reporter gene, which were used in transient transfection experiments, have been described previously (12). The mutant reporter construct HNF-1 DB mut, which extends from the Asp718 site at position −733 to the Asp1500 GAGGAGTAATTTAGCAGTGACAGTGAGATTAGCGAA-3′, a PCR fragment from which the wild type P1-HNF1II site at position −9, a 65-mer extending from +65 to +1 was synthesized. This oligonucleotide contains a single point mutation, A → G at position +9 in the lower strand. The HNF-3 binding site was generated by ligating in 5′-protruding ends at the NcoI site using [32P]dCTP and Klenow DNA polymerase. Typically, 106 cpm of probe was added to a 20-μl reaction mix containing 1 μg of poly (dI-dC) competitor DNA. DNA-protein complexes were separated on a 5% (37.5:1 (w/w) acrylamide:bisacrylamide) polyacrylamide gel. Nase I footprinting samples were analyzed using a 10% (19:1 (w/w) acrylamide:bisacrylamide) polyacrylamide, 8 m urea sequencing gel. Gels were dried and autoradiographed at −80 °C using Fuji XR films.

**RESULTS**

**HNF-3β Stimulates IGF-I Gene Expression**

HNF-3β activates IGF-I promoter P1 (Fig. 1A). Addition of increasing amounts of expression vector encoding full-length HNF-3β, up to 1 μg, resulted in a maximal 50-fold activation of promoter activity compared to the basal level of the same reporter construct. Addition of an expression vector encoding either full-length HNF-3α or full-length HNF-3γ did not result in a significant enhancement of the basal transcriptional level. Only when large amounts of expression vector were added to the cells, a small, maximally 7-fold, enhancement of the basal level of transcription was observed (Fig. 1A). Co-transfection experiments were also performed in two different cell lines, HepG2 cells (human hepatoma cell line) and COS-7 cells (monkey kidney cells). Similar results as described for Hep3B cells were obtained. In HepG2 cells addition of an expression vector encoding HNF-3β results in strong transactivation of P1, although to a slightly lesser extent than in Hep3B cells. HNF-3α and HNF-3γ are not able to enhance P1 promoter activity to a significant level (Fig. 1B). In COS-7 cells the same pattern was observed, although lower levels of transactivation were achieved, which required higher amounts of HNF-3 expression vector (Fig. 1C). These data indicate that HNF-3β is a very
strong transactivator of IGF-I promoter P1 and that HNF-3α and HNF-3γ do not enhance P1 activity, or to a much lesser extent.

Transactivation of P1 by HNF-3β Is Not Primarily Mediated by HNF-1α Induction—Previously we have shown that promoter P1 of the human IGF-I gene harbors two HNF-1 binding sites, both of which are required and sufficient for maximal transactivation of P1 by HNF-1α (13). Since HNF-3 is known to be a regulator of HNF-1α expression (25), we examined whether activation of P1 promoter activity by HNF-3β occurs via up-regulation of HNF-1α expression and subsequent binding to and activation of P1. To this end, transient co-transfection experiments were performed to compare a wild type P1 reporter construct (Asp-733) and a mutated P1 reporter construct, were used as negative control constructs. As expected, expression of HNF-3β does not influence the activity of both of these constructs (Fig. 3). These results indicate that the HNF-3-responsive element in P1 is located between the PvuII site at position −65 and the AciI site at position +54.

P1 Region from −65 to +54 Harbors Two HNF-3 Binding Sites—To confirm that HNF-3β binds to the region in P1 between −65 and +54 relative to the transcription start site, bandshift experiments were performed. The DNA-binding domain of HNF-3β fused to the glutathione S-transferase domain (GST-HNF-3β) was overexpressed in Escherichia coli and purified using Gl matrix. As a positive control for HNF-3β/DNA complex formation, a double-stranded oligonucleotide (TTR-S) harboring the high affinity HNF-3 binding site from the TTR promoter was synthesized (18). Incubation of end-labeled TTR-S with bacterially overexpressed GST-HNF-3β rendered a stable complex (Fig. 4). Subsequently, a DNA fragment extending from the PvuII site at −65 to the NcoI site at +54 was end-labeled and used as a probe. Incubation of this probe with
bacterially expressed GST-HNF-3β resulted in the formation of two complexes with different electrophoretic mobility (Fig. 4). The complex with the highest mobility runs at a position in the gel similar to the complex formed with TTR-S, indicating that this complex represents a single GST-HNF-3β protein bound to the P1 fragment. The position of the complex with lower mobility suggests that two HNF-3 proteins are bound to the same probe. These data indicate that HNF-3β is capable of binding to the region in P1 extending from −65 to +54 and suggest that this region harbors two HNF-3 binding sites. To determine the position of both of the HNF-3 binding sites in P1 more precisely, truncations of the PvuII-NcoI fragment were made and tested in similar bandshift experiments. 5′-Truncation up to the MboII site at −10 (−10 to +54) resulted in the loss of the lower mobility complex, indicating that one HNF-3 binding site is located between position −65 and −10 (Fig. 4). Further truncation of this fragment to the EarI site at position +9 (+9 to +54) completely abolishes binding of HNF-3β to the probe. However, incubation of the reciprocal fragment (−65 to +9) with GST-HNF-3β resulted in the formation of only one complex (Fig. 4), suggesting that the second HNF-3 binding site is located around the EarI site at position +9.

To establish the precise position of the HNF-3 binding sites in P1, DNase I footprinting experiments were performed with the PvuII-NcoI fragment labeled at the NcoI site as probe. Incubation of the PvuII-NcoI fragment with bacterially overexpressed GST-HNF-3β showed protection of two regions in P1 extending from −34 to −18 and +2 to +18 (Fig. 5A). Both of the protected regions show induction of a DNase I-hypersensitive site within the area protected upon HNF-3 binding, at position −31 in the upstream region and position +9 in the downstream region. Comparison of the nucleotide sequence of the protected regions with the HNF-3 consensus binding sequence 5′-WRR-RYMAYA-3′, as described by Roux (32), revealed that both of the footprints encompass potential HNF-3 binding sites. The upstream HNF-3 binding site 5′-TAGATAAATG-3′ located in the upper strand from −32 to −23 shows one mismatch at position 10 and the downstream HNF-3 binding site 5′-TTAGCACAAAC-3′ located in the lower strand between +14 and +5 has one mismatch at position 2 compared to the consensus binding sequence (Table I).

To confirm that both of the HNF-3 binding sites in P1 are capable of binding HNF-3β, double-stranded oligonucleotides harboring these binding sites were synthesized and cloned into the polylinker of pUC18. Fragments containing the HNF-3 binding sites were subsequently excised using EcoRI and HindIII, end-labeled, and used as probes in bandshift experiments. Incubation of both probes with increasing amounts of bacterially expressed GST-HNF-3β resulted in distinct complex formation (Fig. 5B). These data indicate that both of the potential HNF-3 binding sites in P1 are able to bind HNF-3β, also in different contexts of flanking sequences.

**Endogenously Expressed HNF-3 from Rat Liver Binds to P1**—The different family members of the HNF-3 family of transcription factors are expressed at high levels in adult liver. Bandshift experiments were performed to demonstrate that the HNF-3 binding sites in P1 are able to form complexes with the HNF-3 proteins expressed in adult liver. Incubation of nuclear extract derived from adult rat liver with labeled double-stranded oligonucleotides harboring either the HNF-3 binding site from the TTR promoter or the downstream HNF-3 binding site from P1 resulted in the formation of three distinct complexes (Fig. 6). The relative positions of these complexes in the gel suggest that all three HNF-3 variants bind to the region.
in P1 extending from −3 to +23. To confirm the identity of these complexes, the same double-stranded oligonucleotides were incubated with nuclear extract derived from COS-7 cells overexpressing HNF-3α, HNF-3β, or HNF-3γ (Fig. 6). Incubation of TTR-S, the double-stranded oligonucleotide harboring the HNF-3 binding site from the TTR promoter, resulted in clear complex formation of this probe with HNF-3α, HNF-3β, or HNF-3γ as indicated in Fig. 6. The two additional complexes are due to nonspecific binding of endogenously expressed COS-7 proteins and are also formed with extract from untransfected COS-7 cells. Incubation of a double-stranded oligonucleotide harboring the downstream HNF-3 binding site in P1 resulted in a similar binding pattern. However, the TTR-S site yields a stronger complex with HNF-3β and HNF-3γ, whereas the HNF-3 binding site in P1 has the highest affinity for HNF-3β. The differences in binding affinity of HNF-3α, HNF-3β, and HNF-3γ for the HNF-3 binding site in P1 correlate well with the transient cotransfection experiments, which indicated that HNF-3β is a strong transactivator of P1 and that HNF-3α and HNF-3γ do not, or do to a much lesser extent, enhance P1 activity.

Both of the HNF-3 Binding Sites Are Required for Maximal Transactivation—To establish the contribution of each of the HNF-3 binding sites in transactivation of P1 by HNF-3β, mutant reporter constructs were made in which either one or both of the HNF-3 binding sites were mutated. Single point mutations were introduced in both of the HNF-3 sites. In the upstream HNF-3 site, an A → G point mutation was introduced at position −27 (5′-TAGATAAATG-3′) was altered to 5′-TAGATTTATTG-3′ and in the downstream HNF-3 binding site an A → G point mutation was introduced at position +9 (5′-TTAGCAAAAACA-3′) was altered to 5′-TTAGCAAAAACA-3′; see also Table I). To prove that the introduction of these point mutations into the HNF-3 binding sites completely abolishes HNF-3β binding, bandshift experiments were performed. PvuII-NcoI fragments, excised from the wild type or mutant reporter constructs, containing either the wild type HNF-3 binding sites or their mutated versions were tested for complex formation with HNF-3β (Fig. 7). Addition of increasing amounts of GST-HNF-3β to the PvuII-NcoI fragment harboring two intact HNF-3 binding sites resulted in formation of two complexes as described. Introduction of a single point mutation in either of the HNF-3 binding sites (mut +9 or mut −27) resulted in the loss of the lower mobility complex, indicating that only one HNF-3 binding site is intact and capable of binding. Point mutations in both of the

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**Table I**

| Species     | Sequence                  | Reference |
|-------------|---------------------------|-----------|
| Upstream HNF-3 binding site (−30) |                 |           |
| Human       | 5′-TAGATAAATG-3′          | (5)       |
| mut −27     | ------------------------- | (50)      |
| Chicken     | ------------------------- | (51)      |
| Pig         | ------------------------- | (52)      |
| Rat         | ------------------------- | (53)      |
| Salmon      | 5′-GACACCTCA-3′           | (54)      |
| Sheep       | 5′-TAGATAAATG-3′          | (54)      |
| Downstream HNF-3 binding site (+10) |                 |           |
| Human       | 5′-TTAGCAAAAACA-3′        | (5)       |
| mut +9      | ------------------------- | (50)      |
| Chicken     | ------------------------- | (51)      |
| Pig         | ------------------------- | (52)      |
| Rat         | ------------------------- | (53)      |
| Salmon      | 5′-GACACCTCA-3′           | (54)      |
| HNF-3 consensus binding sitea | 5′-WASHINGTON-3′ | (32)      |

*The abbreviations used are: W, A or T; R, A or G; Y, C or T; M, A or C.*
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**Fig. 6. Bandshift experiment with rat liver nuclear extract.** Two double-stranded oligonucleotides TTR-S (T) and P1–3/+23 (P1) were incubated with nuclear extracts derived from COS-7 cells (indicated by −), COS-7 cells overexpressing HNF-3α (α), HNF-3β (β), HNF-3γ (γ), or nuclear extract from adult rat liver. The positions of complexes containing HNF-3α (466 amino acids), HNF-3β (458 amino acids), and HNF-3γ (358 amino acids) are indicated. Complexes resulting from nonspecific binding of COS-7 proteins are indicated by arrowheads. Free probe is not shown.

HNF-3 binding sites (mut +9/-27) completely abolished all HNF-3β binding to the PvuII-NcoI fragment.

Subsequently, the mutant reporter constructs were tested in transient co-transfection experiments in Hep3B cells for transactivation by HNF-3β in comparison to the wild type reporter construct (Asp-733). Transactivation of wild type reporter construct by HNF-3β was set to 100%. Mutation of either the upstream or the downstream HNF-3 binding site reduced transactivation by HNF-3β to 30–40% (Fig. 8A). Enhancement of the activity of the HNF-3 double mutant was even further impaired, resulting in only 20% activation relative to the wild type construct. Surprisingly, activation was not abolished completely. The residual 20% activation relative to the wild type reporter construct still represents a 5-fold induction of basal activity. As a control, transient transfection experiments were performed with the same reporter constructs to show that these constructs, despite their mutations close to the region of the transcription start site, are still activated by C/EBP family members and by HNF-1α. Comparison of the wild type P1 reporter construct and HNF-3 mutant reporter constructs showed no difference in transactivation by either LAP (C/EBPβ) or HNF-1α, indicating that the mutations do not interfere with basal transcription machinery complex formation (Fig. 7B).

**HNF-1 Induction by HNF-3β Contributes to Transactivation of P1 by HNF-3β**—Since the HNF-1 DB mut reporter construct, lacking functional HNF-1 binding sites, is somewhat less activated by HNF-3β than the wild type reporter construct (Fig. 2) and since the basal transcription level of the HNF-3 double mutant reporter construct can still be enhanced by HNF-3β (Fig. 8A), we examined the potential contribution of HNF-1α to the transactivation of P1 by HNF-3β. To this end, the same mutations as described for the Asp-733 reporter construct were introduced in the HNF-1 DB mut reporter construct resulting in P1 reporter constructs with mutations in both of the HNF-1 binding sites and one or both of the HNF-3 binding sites. Transient co-transfection experiments with these reporter constructs and an expression vector encoding HNF-3β showed a similar transactivation pattern as described for Asp-733-derived constructs. However, the level of transactivation by HNF-3β was lower for all constructs and no enhancement of basal transactivation was observed with the reporter construct harboring mutations in both of the HNF-1 and both of the HNF-3 binding sites (Fig. 5C). These experiments suggest that the residual transactivation observed in co-transfection experiments with the HNF-3 double mutant is due to induction of the expression of HNF-1α by HNF-3β and subsequent binding and activation of P1 by HNF-1α.

To support this hypothesis, transient transfection experiments were performed with a reporter construct containing the proximal promoter region of the human albumin promoter (−120 to +4), which harbors an HNF-1 binding site but no HNF-3 binding site (43, 44). Co-transfection experiments in Hep3B cells with this reporter construct and an expression vector encoding full-length HNF-1α resulted in strong (45-fold) transactivation of the albumin promoter (Fig. 8D). Co-transfection with an expression vector encoding HNF-3β, instead of HNF-1α, resulted in a much lower but significant enhancement of the basal transcription level. This level of transactivation of the albumin promoter is in the same range as the enhancement of the P1 HNF-3 double mutant reporter construct by HNF-3β (Fig. 8D). The basal level of the thymidine kinase promoter (TK-Luc), which harbors neither an HNF-1 nor an HNF-3 binding site, was not enhanced by the addition of HNF-3β expression vector (Fig. 3).

**DISCUSSION**

Recently, we have described the involvement of two families of liver-enriched transcription factors in the expression of the hIGF-I gene in adult liver (12, 13). C/EBPα and C/EBPβ (LAP) activate P1 of the hIGF-I gene up to 12-fold via a high affinity binding site located between positions −119 and −111 relative to the major transcription start site (12). HNF-1α is able to enhance basal transcription in Hep3B cells up to 16-fold. Transactivation is dependent on two HNF-1 binding sites located between −282 to −270 and −119 to −107. The latter site completely overlaps the C/EBP binding site (13). Since the transcription of a number of liver-specifically expressed genes has been shown to be regulated by the concerted action of members from different families of liver-enriched transcription factors (8), we investigated whether family members of the HNF-3 family of winged helix DNA binding transcription factors are also involved in the transcriptional regulation of the hIGF-I gene.

Here we show that HNF-3β is a very strong transactivator of P1 in transient transfection experiments in Hep3B, HepG2, and COS-7 cells. Since HNF-3β is able to enhance P1 transcription in both hepatocyte-derived and, to a lesser extent, in non-hepatocyte-derived cell lines, it is likely that transactivation occurs mainly via a direct mechanism and that a co-factor is not absolutely required for the transactivational properties of HNF-3β on P1. Enhancement of basal P1 activity by HNF-3β
revealed a linear relation between the amount of expression vector added and the stimulation of basal transcription. No plateau level was reached using quantities of up to 2 μg of expression vector. HNF-3α and HNF-3γ are not able, or were able to a much lesser extent, to enhance the basal transcriptional level of P1 in the three cell lines tested, suggesting that hIGF-I gene expression is significantly influenced by HNF-3β, but not by HNF-3α or HNF-3γ. Differential transactivation by the different HNF-3 family members is described for other genes as well. Region I in the promoter of the Clara cell secretory protein (CCSP) gene harbors two HNF-3 binding sites. Co-transfection experiments with expression vectors encoding either HNF-3α or HNF-3β had opposite effects. HNF-3α was able to enhance basal activity, whereas HNF-3β had a inhibitory effect on promoter activity (45). In addition, the promoter region of the rat cytochrome P450 2C13 (CYP2C13) gene harbors two HNF-3 binding sites, which show different binding characteristics. Site A preferably binds HNF-3β, while site B can be activated by HNF-3α and HNF-3γ (46).

We have shown that HNF-3β regulates IGF-I gene expression via two different mechanisms. Direct transactivation is observed through binding of HNF-3β to two binding sites in P1 and subsequent enhancement of P1 activity, and indirect activation occurs through the induction of the transcription factor HNF-1α, which in turn can bind and transactivate IGF-I P1. The two HNF-3 binding sites are located in the proximal part of the promoter region and both adhere to the HNF-3 consensus binding sequence 5′-WRRRYMAAYA-3′ (32) at 9 out of 10 nucleotides. This consensus binding sequence contains the core sequence 5′-RTAAAYA-3′, which is present in the binding sites of four human fork head homologs (FREAC, fork head-related activators; Ref. 31), indicating that the right-hand part of the consensus binding sequence is more important for recognition and binding of proteins belonging to the fork head family. The mismatch to the consensus sequence in the upstream HNF-3 binding site in P1 resides in the more conserved part of the consensus binding sequence, at position 10, whereas the downstream site has a mismatch at position 2, which seems less important for binding. However, bandshift experiments with fragments, in which either one or both HNF-3 binding sites
were mutated, it was established that both HNF-3 binding sites in P1 possess equal binding affinity for HNF-3β. Moreover, transient co-transfection experiments revealed that mutant reporter constructs, in which one of the HNF-3 binding sites was mutated, could be activated by HNF-3β to a similar extent, approximately 30–40% of the wild-type construct. In conclusion, the two HNF-3 binding sites in P1 have similar binding affinities for HNF-3β and are equally important for transactivation of P1 by HNF-3β. DNase I footprinting experiments showed the induction of a DNase I-hypersensitive site within the regions protected by HNF-3β. The induction of DNase I-hypersensitive sites is characteristic of an HNF-3 binding site (32, 47). The two DNase I-hypersensitive sites (one on each strand) are staggered by 3 base pairs, as has been shown for the TTR site and sites recognized by FREACs (31, 32). Because the HNF-3 binding sites in P1 of the IGF-I gene are located on different strands, the footprint analysis (as shown in Fig. 5) shows DNase I-hypersensitive sites at different positions within the protected regions.

Comparison of the HNF-3 binding sites present in the promoter of the human IGF-I gene with the homologous promoter regions of other species reveals that both sites are well conserved among mammalian and avian species (Table I). The corresponding sequences in the pig and chicken genes are completely identical to the human sequence, whereas in sheep and rat one of the potential HNF-3 binding sites reveals a single mismatch. In the sheep promoter, the upstream HNF-3-binding site contains four consecutive A nucleotides instead of three and in the downstream HNF-3-binding site in the rat, a G is present at position 8 instead of an A, suggesting that only the upstream HNF-3-binding site is capable of HNF-3 binding. The salmon promoter, however, shows a number of mutations in both of the HNF-3 binding sites at positions that seem to be important for binding. The upstream HNF-3-binding site contains a C at position 7, which in all HNF-3 binding sites described so far is an A. The nucleotide sequence corresponding to the position of the downstream HNF-3-binding site shows both an aberrant G at position 6 and a T at position 10. The deviation at position 6 in the salmon promoter is identical to the mutation we introduced in the downstream HNF-3-binding site to abolish HNF-3 binding, suggesting that HNF-3 is not able to bind to this site.

The promoter region of the HNF-1α gene contains an HNF-3-binding site that contributes to the high level of expression of HNF-1α in hepatocytes (25). Transient transfection experiments suggest that HNF-1α expression can be induced in Hep3B cells by HNF-3β overexpression. IGF-I P1 harbors two HNF-1 binding sites that are important in the regulation of IGF-I gene expression (13). HNF-1 can activate both binding sites, which contain an HNF-1 binding site that contributes to the high level of expression of the IGF-I gene (13). Furthermore, HNF-3β regulates the transcription of the liver-enriched transcription factor HNF-1α and is also involved in the establishment and maintenance of its own expression in hepatocytes via a positive autoactivation loop (25, 26). P1 of the hIGF-I gene harbors two HNF-3 binding sites. Since the HNF-3 family of transcription factors plays such a dominant role in the regulation of expression of liver-specifically expressed genes, HNF-3β might be the most important regulator of IGF-I gene expression. In conclusion, the two HNF-3 binding sites in the proximal promoter region of P1 are located in close proximity to the transcription start site, suggesting that HNF-3 may be involved in recruitment of the basal transcription machinery to the IGF-I promoter. Since P1 does not harbor typical proximal promoter elements like a CCAATT or TATA box, nor an initiator element (48), HNF-3 could fulfill this role in the regulation of IGF-I P1 transcription. Since HNF-3 induces a bend of 13° in the DNA upon bending (27), simultaneous binding of two HNF-3 proteins to the region of initiation of transcription might induce a distortion in the DNA, thereby facilitating initiation of transcription directed by RNA polymerase II.

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