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Hepatocyte Nuclear Factor 3β Contains Two Transcriptional Activation Domains, One of Which Is Novel and Conserved with the *Drosophila* Fork Head Protein

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The hepatocyte nuclear factor 3 (HNF-3) gene family is composed of three proteins (α, β, and γ) that are transcription factors involved in the coordinate expression of several liver genes. All three proteins share strong homology in their DNA binding domains (region I) and are able to recognize the same DNA sequence. They also possess two similar stretches of amino acids at the carboxyl terminus (regions II and III) and a fourth segment of homology at the amino terminus (region IV). Furthermore, the HNF-3 proteins demonstrate homology with the *Drosophila* homeotic gene fork head in regions I, II, and III, suggesting that HNF-3 may be its mammalian homolog. In order to define HNF-3β protein domains involved in transcriptional activation, we have used a reporter gene, whose transcription is dependent on HNF-3 binding, for hepatoma cell cotransfection assays with expression vectors that produced different truncated HNF-3β proteins. A position-independent activation domain which contained conserved regions II and III was identified at the carboxyl terminus of the HNF-3β protein (amino acids 361 to 458). Moreover, site-directed mutations that altered the sequences within regions II and III demonstrated their importance to transactivation. The region II-III domain does not possess amino acid sequences in common with other transcription factors and may define a novel activation motif. HNF-3β amino-terminal sequences defined by conserved region IV also contributed to transactivation, but region IV activity required the participation of the region II-III domain. Region IV is abundant in serine amino acids and contains two putative casein kinase I phosphorylation sites, a feature similar to protein motifs described for the transcription factors Pit-1/GHF-1 and HNF-1.

Cellular differentiation during mammalian development is accompanied by the differential expression of tissue-specific genes (27). The identification of genes responsible for homeotic or developmental mutations in *Drosophila melanogaster* has shown that temporal cascades of transcription factors are involved in establishment of tissue-specific expression patterns (36, 55). The existence of homologies between the homeotic proteins and mammalian transcription factors suggests that similar regulatory cascades are employed during mammalian development (20, 55). Since transcriptional initiation is the main regulatory step in the expression of tissue-specific genes, an understanding of transcriptional control will provide insight into mechanisms of cellular differentiation (27, 45). Tissue-specific gene transcription is maintained through the recognition of promoter sites by transcription factors which display restricted cellular activity (27). The functional characterization of these tissue-specific factors will identify protein regions involved in transcriptional activation and may provide insight into their regulatory mechanisms (3, 16, 25, 35, 48, 50, 57, 62). The analysis of promoter regions regulating the expression of tissue-specific transcription factors may identify their position in the hierarchy of the developmental regulatory cascade (2, 8, 31, 41, 42, 49, 58, 61, 63).

The analysis of transcriptional initiation has been the focus of considerable research because it is a major regulatory step in gene expression. The initiation complex is composed of an array of basal transcription factors (for example, TFII-A, -B, -D, -E, -F, and -G) that usually assemble at the TATA box sequence with RNA polymerase II (for reviews, see references 27, 45, and 52). In addition to the TATA sequences, promoter regions include multiple DNA binding motifs that bind sequence-specific transcription factors and elevate the rate of transcriptional initiation. The functional analysis of a number of transcription factors has demonstrated that these proteins are modular in structure and consist of domains that can function independently of one another. These analyses have identified regions which are involved in specific DNA recognition (DNA binding domains) and distinct amino acid residues that participate in the stimulation of transcriptional initiation (activation domains). The activation domains function at various positions with respect to the DNA binding motif within the protein and can confer activation potential upon the DNA binding domain of an unrelated transcription factor. Several of these domains consist of acidic amino acid residues or are rich in either glutamine or proline. In addition, there are other activating domains which do not contain any of these amino acid features, thus suggesting the existence of other families of activation motifs (55). Although their mechanism of transcriptional activation remains elusive, there are indications that the latter activation motifs may function by interaction with basal transcription factors either directly or through coactivator proteins (14, 39, 53, 59).

The characterization of the promoters of liver-specific genes suggests that several genes are coordinately regulated by a limited number of transcription factors which demon-

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strate cell-type restriction (10, 11, 13, 21, 46). The requirement for the combinatorial action of liver-enriched factors to achieve promoter activity may be responsible for the maintenance of hepatocyte-specific expression (6, 9, 18, 38). In addition, *cis*-acting DNA elements which bind negatively acting factors may also play a role in the cell-specific expression patterns (23, 37, 54). The liver factors which have been cloned include liver factor B1/hepatocyte nuclear factor 1 (LF-B1/HNF-1), a member of the POU-homeobox gene family (1, 15); the leucine zipper dimerization family members C/EBPα (34), DBP (47), a third protein that is referred to by several different names (C/EBPβ, LAP, interleukin-6-DBP [IL-6DBP], and C/EBP-related protein 2 [CRP-2] [5, 12, 51, 66]), C/EBPγ (5), CRP-1, and CRP-3 (66); HNF-3, which shares homology with the *Drosophila* homeotic gene fork head (32, 33); and HNF-4, which belongs to the steroid hormone superfamily (57). The availability of cDNA clones encoding these proteins has allowed identification of amino acid residues involved in DNA binding and transcriptional activation (5, 16, 32, 35, 48, 50, 57, 66).

The transcription factor HNF-3 was first discovered as a protein required for transthyretin (TTR) promoter activity and was subsequently shown to participate in the coordinate expression of other liver genes (4, 10, 13, 26, 40). The cloning of HNF-3 cDNAs revealed the expression of three different hepatocyte genes (α, β, and γ), which is consistent with the protein-DNA complexes observed with liver nuclear extracts (32, 33). These three HNF-3 proteins demonstrated 95% homology in their DNA binding domains (region I) and possessed conserved residues at the carboxyl terminus (regions II and III) and the amino terminus (region IV). Unlike many DNA-binding proteins, the HNF-3 protein appears to bind as a monomer to DNA through a still-uncharacterized protein motif. In addition, the HNF-3 DNA binding motif may be utilized by a larger gene family, since a brain cDNA clone that contains significant sequence conservation with the HNF-3 DNA binding domain has been isolated by low-stringency hybridization (60). Furthermore, the HNF-3 proteins also share homology with a *Drosophila* homeotic fork head protein in regions I, II, and III, thus suggesting that these conserved sequences may play a role in their transcriptional activity (33, 64, 65).

In this study, we have focused on the HNF-3β protein, and we report here on the localization of two transcriptional activation domains with a cotransfection assay with HNF-3β reporter and expression plasmids. In addition to the requirement of the DNA binding domain, we show that the HNF-3β carboxyl terminus, which includes conserved regions II and III, will function as a position-independent transcriptional activation domain. This is particularly interesting because it represents a region which is conserved both among the HNF-3 family members and in their *Drosophila* homolog fork head. We also define an activation domain in the conserved residues at the amino terminus (region IV), which requires the participation of the carboxyl-terminus regions II and III for its activity. Region IV contains several putative casein kinase I phosphorylation sites which may be important in the activity of this domain.

MATERIALS AND METHODS

Construction of HNF-3β deletion mutations and amino acid changes by site-directed mutagenesis. For construction of both HNF-3β expression and reporter plasmids, we employed molecular biology protocols outlined in the work of Sambrook et al. (56). To express the HNF-3β protein in cotransfection experiments, we used the cytomegalovirus (CMV) immediate-early promoter to drive the expression of the HNF-3β cDNA. We inserted the HNF-3β cDNA as an *Eco*RI fragment (1.6 kb) in a region containing multiple restriction enzyme cloning sites (derived from pGem1 [Promega]). These sites were nested between the CMV promoter and transcriptional termination sequences obtained from the simian virus 40 early region (Fig. 1A). This HNF-3β cDNA fragment contained the 5′ untranslated region and the entire coding region and terminated 35 nucleotides 3′ to the translational stop codon (33). The basal TATA box-chloramphenicol acetyltransferase (CAT) gene (19) plasmid (TATA-CAT) was made by the addition of *Bgl*II linkers onto an *Rsa*I-*Sma*I fragment which contained the CMV promoter TATA sequence (Fig. 1A). The TATA sequence was inserted 5′ to the CAT gene in a plasmid that contained additional cloning sites upstream. The HNF-3 reporter constructs were made by inserting tandemly ligated HNF-3 binding sequences (TTR promoter, −111 to −85) (10) into an *Xba*I site located adjacent to the TATA sequence. The HNF-3 reporter constructs consisted of either four copies (4× HNF-3–TATA–CAT) or six copies (6× HNF-3–TATA–CAT) of the HNF-3 binding sites next to the TATA sequence driving expression of the CAT gene.

Deletions that removed carboxyl-terminal sequences were made from the 3′ end of the HNF-3β cDNA with BAL 31 exonuclease. We ligated an *Xba*I linker onto the deleted end, and the deleted HNF-3β cDNA was inserted as an *Eco*RI-*Xba*I fragment into the CMV expression vector that contained translational termination codons in all three frames 3′ to the site of insertion. The end point of the deletions was determined by dideoxy sequencing of double-stranded plasmids (22) with Sequenase (United States Biochemical) and primers specific for the expression plasmid. Amino-terminal deletions were also made with BAL 31 exonuclease, sequenced, and then inserted into one of three different CMV expression vectors containing the initiating methionine codon in the appropriate reading frame. We constructed internal deletions by using primers to amplify HNF-3β region II and III sequences by the polymerase chain reaction. These polymerase chain reaction products were fused in frame with HNF-3β carboxyl-terminal deletions with a common *Xba*I site (5′ end of primers). The polymerase chain reactions were performed with the Vent DNA polymerase, buffers provided by the manufacturer (New England Biolabs), specific HNF-3β primers, a primer specific for the simian virus 40 small-t-antigen region, and the CMV HNF-3β plasmid as a template. The sequences of the primers include (amino acids [aa] 361 to 366) GAGTCTAGA GAG GCC CAC CTG AAG CCC, (aa 396 to 401) GCGTCTAGA CAG CCC CAC AAA ATG GAC, (aa 427 to 432) GCGTCTAGA CCA GTC ACG AAC AAA GCC, and (simian virus 40 small-t-antigen) GTTTGTCCAATTAT GTCA, where the *Xba*I site is underlined and the HNF-3β codons are indicated by spaces.

Specific amino acid alterations in HNF-3β region II and III sequences were introduced by oligonucleotide-directed mutagenesis as described by Kunkel (30) and described previously (9, 49). The amino acid substitutions were identified by DNA sequencing. The mutagenesis primers included (region III) GCT GCA GAC ACT TCC G(AT)C (GT)AC (GA)AG GGA GTG TAC TCC and (region II) GCC TTC AAC CAC CCC G(AT)G (GA)AG G(AG)C AAC AAC CTC ATG TC. The MacVector program (IBI[International Biotechnologies Inc.]) was used to generate secondary structure predictions for the HNF-3β 361-to-458 sequences by employing the

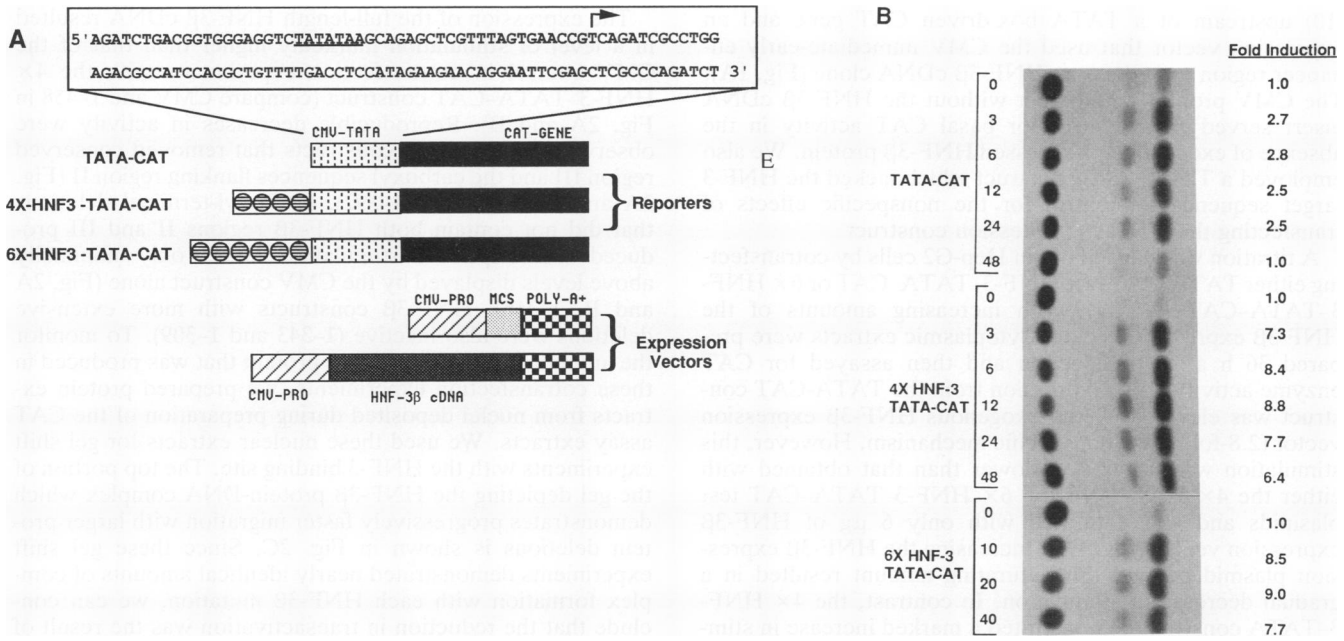


FIG. 1. Reporter plasmids that require HNF-3 binding for expression are stimulated by increasing amounts of HNF-3β expression vector. (A) Schematic representation of the HNF-3 reporter and HNF-3β expression constructs used in a Hep-G2 cell (29) cotransfection assay. The TATA sequence (underlined) from the CMV promoter (sequence shown at top) was placed upstream of the CAT gene and used as the basal promoter plasmid. The HNF-3 reporter constructs inserted either four or six copies (indicated by four or six circles) of the HNF-3 DNA recognition sequence (TTR promoter, −111 to −85) (10) upstream of the TATA-CAT region. Also shown are the HNF-3β expression vectors which used the CMV immediate-early promoter (PRO) region to drive expression of HNF-3β and contained simian virus 40 small-t-antigen sequences which provided both a splice site and a polyadenylation signal (POLY-A+). The CMV promoter construct which contained multiple cloning sites (MCS) was included as a control for expression levels without exogenously added HNF-3β. (B) Titration of HNF-3β expression vector in cotransfection assay. Increasing amounts (shown on the left, in micrograms) of HNF-3β expression plasmid were cotransfected with a constant amount (40 μg) of the indicated HNF-3 reporter plasmid into Hep-G2 cells, and CAT enzyme activity in transfected cytoplasmic extracts was measured 36 h later (19). Shown is the fold stimulation of the reporter plasmid by the HNF-3β expression vector cotransfection compared with that by the CMV control plasmid (lanes 0; 10 μg). Note that the 4× HNF-3– and 6× HNF-3–TATA–CAT constructs exhibited maximal stimulation with 12 and 20 μg of HNF-3β expression plasmid, respectively.

Robson-Garnier algorithm (17). This included the HNF-3β sequences which were substituted for the mutation created in regions II and III, and therefore we derived a correlation between structural prediction and function.

Cell lines, DNA cotransfection, and gel shift assays. Human hepatoma (Hep-G2) cells (29) were maintained as monolayer cultures and grown in Ham's F12 medium supplemented with 7% heat-inactivated fetal calf serum, 0.5× minimal essential medium amino acids, 100 U of penicillin-streptomycin per ml (GIBCO Laboratories), and 0.5 U of insulin per ml. During transfection experiments, Hep-G2 and HeLa cells were grown in Dulbecco modified Eagle medium supplemented with 7% heat-inactivated fetal calf serum, 1× nonessential amino acids, and 0.5 U of insulin per ml. 4× HNF-3– or 6× HNF-3–TATA–CAT reporter plasmids (40 μg) (Fig. 1A) were cotransfected with CMV HNF-3β expression constructs (15 μg) into Hep-G2 or HeLa cells (100-mm-diameter dish) by a modification of the calcium phosphate coprecipitation method as described by Chen and Okayama (7). Included was a CMV β-galactosidase construct (1 μg) to monitor transfection efficiencies (gift from Terry Van Dyke, University of Pittsburgh). Incubation of cells for 16 to 20 h with calcium phosphate-precipitated DNA was followed by two rinses with phosphate-buffered saline and addition of fresh medium. Cellular protein extracts were made 24 to 36 h later by three cycles of freeze-thawing, nuclei were removed by centrifugation, and protein concentration was determined by Bio-Rad protein assay. After normalization for equal amounts of protein, cellular extracts were used to measure CAT enzyme activity as described by Gorman et al. (19) and β-galactosidase enzyme activity was determined as described by Miller (44). The nuclei from transfected cells were used for the preparation of protein extracts and for gel shift assays with the TTR HNF-3 binding site (−111 to −85) as described previously (10). This gel shift assay allowed us to monitor the amount of HNF-3β protein produced during the cotransfection assay through visualization of the HNF-3β protein-DNA complex. CAT activity was quantified by densitometer scans of autoradiographs as described previously (9) and normalized to the β-galactosidase enzyme activity. HNF-3β transactivation levels were presented as a ratio of mutant to wild-type levels in which activation by the wild-type HNF-3β protein was set at 1.0. The transactivation values are derived from at least two separate experiments.

RESULTS

HNF-3β cotransfection assay. To identify the HNF-3β protein domains required for transcriptional activation, we developed a cotransfection assay which could respond to exogenously expressed HNF-3β protein. This assay consisted of cotransfection of a reporter plasmid that contained multiple copies of the HNF-3 DNA recognition sequence

(10) upstream of a TATA box-driven CAT gene and an expression vector that used the CMV immediate-early enhancer region to express an HNF-3β cDNA clone (Fig. 1A). The CMV promoter construct without the HNF-3β cDNA insert served as a control for basal CAT activity in the absence of exogenously expressed HNF-3β protein. We also employed a TATA-CAT construct which lacked the HNF-3 target sequence to control for the nonspecific effects of transfecting the HNF-3β expression construct.

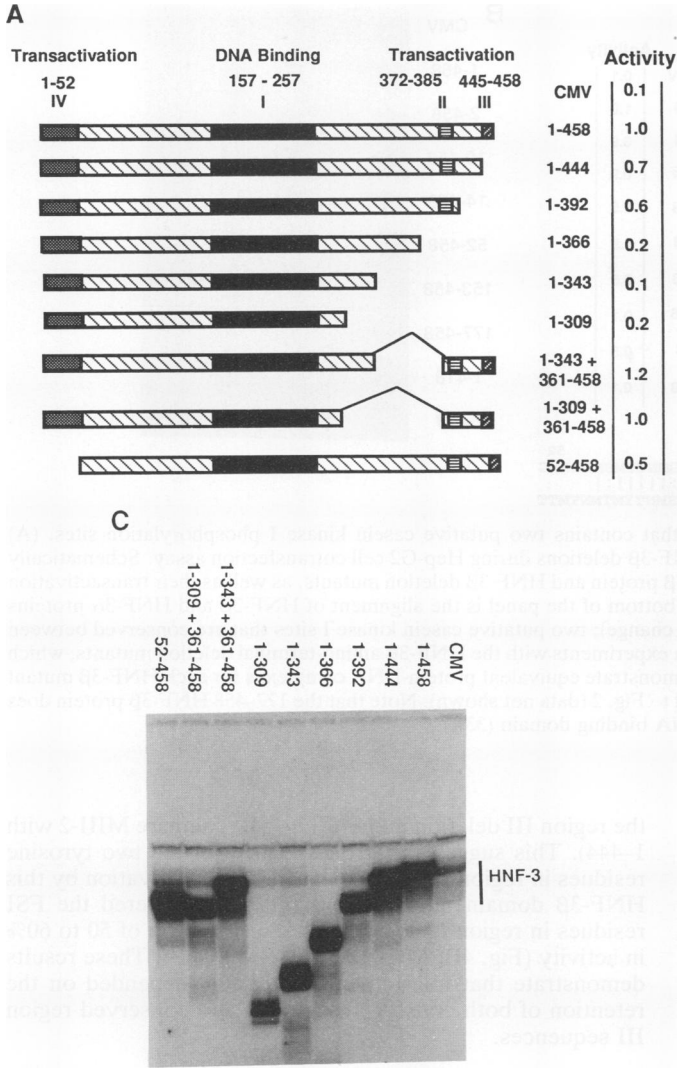
A titration was carried out in Hep-G2 cells by cotransfecting either TATA-CAT, 4× HNF-3–TATA–CAT or 6× HNF-3–TATA–CAT (40 μg), with increasing amounts of the HNF-3β expression vector. Cytoplasmic extracts were prepared 36 h after transfection and then assayed for CAT enzyme activity (19). Expression from the TATA-CAT construct was elevated by the exogenous HNF-3β expression vector (2.8-fold) by a nonspecific mechanism. However, this stimulation was severalfold lower than that obtained with either the 4× HNF-3– or the 6× HNF-3–TATA–CAT test plasmids and was saturated with only 6 μg of HNF-3β expression vector (Fig. 1B). Increasing the HNF-3β expression plasmid beyond this saturating amount resulted in a gradual decrease in stimulation. In contrast, the 4× HNF-3–TATA construct demonstrated a marked increase in stimulation (8.8-fold) at 12 μg of HNF-3β expression plasmid and did not display decreases when the cotransfected HNF-3β was increased above saturating levels (Fig. 1B). The 6× HNF-3–TATA–CAT construct also resulted in large stimulation of CAT enzyme activity by exogenous HNF-3β, which saturated at even greater amounts of cotransfected HNF-3β expression plasmid. Although Hep-G2 cells contain HNF-3 proteins, we found that the HNF-3 reporter constructs were quite responsive to the expression of additional HNF-3β protein in the cotransfection experiments. These results suggest that the HNF-3 binding sites were contributing significantly to transcriptional stimulation and provided a reliable assay to evaluate mutants within the HNF-3β coding region.

HNF-3 and *Drosophila* fork head conserved regions II and III define an HNF-3β transcriptional activation domain at the carboxyl terminus. Schematically shown in Fig. 2A is a diagram of the wild-type HNF-3β gene product (1–458) in which the boxes represent the locations of the conserved amino acid regions between the HNF-3 family members and the *Drosophila* fork head protein (33, 64). These include the DNA binding domain (region I), two 14- to 20-amino-acid stretches in the carboxyl terminus (regions II and III), and region IV, which is conserved only among the HNF-3 family members. In order to evaluate the contribution of regions II and III of the HNF-3β protein, BAL 31 deletions were made from the 3′ end of the cDNA and placed in a CMV expression vector which contained termination codons in all three frames 3′ to the site of cDNA insertion. HNF-3β mutant constructs (15 μg) (illustrated in Fig. 2A) were cotransfected with 4× HNF-3–TATA–CAT (40 μg) into Hep-G2 cells, and cytoplasmic extracts were assayed for CAT enzyme activity. A CMV–β-galactosidase construct (1 μg) was included in these experiments to normalize the CAT activity for differences in transfection efficiency. Normalized HNF-3β mutant CAT enzyme activity is expressed as a fraction of wild-type activity (Fig. 2A). Basal CAT expression in the absence of exogenously added HNF-3β protein is provided by the CMV vector cotransfection. In addition to the DNA binding domain, the results described below provide evidence supporting the requirement of conserved HNF-3 regions II and III for transcriptional activation.

The expression of the full-length HNF-3β cDNA resulted in a level of stimulation markedly higher than that of the CMV control in cotransfection experiments with the 4× HNF-3–TATA–CAT construct (compare CMV and 1–458 in Fig. 2A and B). Reproducible decreases in activity were observed with HNF-3β constructs that removed conserved region III and the carboxyl sequences flanking region II (Fig. 2A and B, 1–444 and 1–392). Carboxyl-terminal deletions that did not contain both HNF-3β regions II and III produced transcriptional stimulation levels only minimally above levels displayed by the CMV construct alone (Fig. 2A and B, 1–366). HNF-3β constructs with more extensive deletions were also inactive (1–343 and 1–309). To monitor the amount of HNF-3β mutant protein that was produced in these cotransfection experiments, we prepared protein extracts from nuclei deposited during preparation of the CAT assay extracts. We used these nuclear extracts for gel shift experiments with the HNF-3 binding site. The top portion of the gel depicting the HNF-3β protein-DNA complex which demonstrates progressively faster migration with larger protein deletions is shown in Fig. 2C. Since these gel shift experiments demonstrated nearly identical amounts of complex formation with each HNF-3β mutation, we can conclude that the reduction in transactivation was the result of the removal of critical amino acid residues and not of protein instability. Furthermore, these decreases were not due to the removal of the nuclear targeting sequences, because all of the HNF-3β mutants displayed DNA binding activity in nuclear extracts prepared from transfected hepatoma cells (Fig. 2C).

The HNF-3β functional analysis suggests that the amino acids encompassing regions II and III define a transcriptional activation domain. To test this hypothesis, internal amino acid deletions were made by joining the HNF-3β sequences spanning regions II and III (361 to 458) with two inactive HNF-3β mutants that contained extensive deletions of the carboxyl terminus (309 to 343). The HNF-3β region II and III sequences were sufficient to restore wild-type transactivation to both of these deletion mutants in the cotransfection assay (Fig. 2A and B, 1–343 + 361–458 and 1–309 + 361–458). Gel shift experiments demonstrated that nuclear extracts derived from these transfection experiments produced protein-DNA complexes which migrated more slowly, as was consistent with additional molecular weight contributed by the 361-to-458 sequences (Fig. 2C). Furthermore, these internal deletions demonstrate that the region II and III (region II-III) sequences were able to activate transcription of the HNF-3 target gene when placed at various positions within the HNF-3β protein. The region II-III sequences do not possess amino acid sequences in common with other activation domains and may define a novel activation motif.

HNF-3 conserved region IV defines a second activation domain at the amino terminus. The experiments described above demonstrated that transactivation of the HNF-3 reporter plasmid required HNF-3β region II-III sequences (Fig. 2A). To determine the role of conserved region IV in HNF-3 transcriptional activation (Fig. 3), we prepared a series of 5′ deletion constructs and inserted them in a CMV expression vector that contained the translational initiation codon in the appropriate reading frame (see Materials and Methods). These HNF-3β deletion plasmids were cotransfected into Hep-G2 cells with the 4× HNF-3–TATA–CAT construct to determine their transcriptional activity. Removal of the first 14 residues of the HNF-3β amino terminus resulted in minimal loss of activity (Fig. 3), but deletion of the remainder of region IV caused an approximately 50%



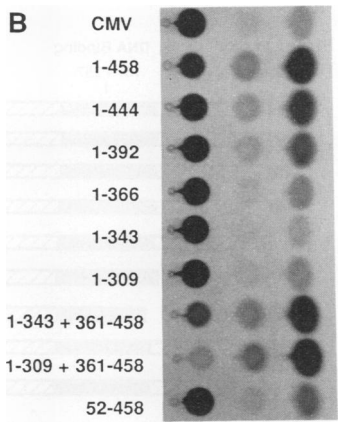


FIG. 2. HNF-3β deletions define two activation domains, one of which is position independent and contains conserved regions II and III. (A) Definition of HNF-3β transactivation domains and summary of transcriptional activities produced by various HNF-3β deletions tested in a Hep-G2 cell cotransfection assay. Schematically shown is the HNF-3β protein and indicated are the sequences conserved among the HNF-3 family. These include region I (DNA binding domain) and regions II, III, and IV, whose involvement in transcriptional activation is demonstrated in this figure (33, 65, 66). Also shown are the HNF-3β deletion mutants and their transcriptional activity, which is expressed as a ratio of mutant to wild-type levels. Different HNF-3β deletions (15 μg) were cotransfected with the 4× HNF-3–TATA–CAT construct (40 μg) into Hep-G2 cells, and CAT enzyme activity (see panel B) was assayed as described in the legend to Fig. 1. CAT enzyme levels were normalized for differences in transfection efficiency by including the CMV–β-galactosidase plasmid (1 μg) as an internal control. In addition, CAT enzyme levels that exhibited more than 60% conversion of acetylated product were diluted and reassayed for CAT activity in the linear range. The CMV promoter vector was included as a guide for CAT enzyme levels produced without HNF-3β expression (activity ratio, 0.1). The carets represent internal deletions in HNF-3β, which demonstrate position-independent activation by the region II and III sequences. (B) CAT enzyme assays from cotransfection experiments with the HNF-3β mutants, which were used to tabulate the results shown in panel A. (C) Gel shift assay with Hep-G2 transfected extracts. Gel shift assays demonstrate equivalent protein-DNA complexes for each HNF-3β mutant tested in a Hep-G2 cotransfection assay. The top portion of the gel shift assay depicts the HNF-3 protein-DNA complex with an oligonucleotide containing the HNF-3 DNA binding site (TTR promoter, −111 to −85) (10) and nuclear extracts prepared from Hep-G2 cells transfected with HNF-3β mutants. The protein-DNA complexes formed by all of the HNF-3β mutants demonstrated equivalent protein expression. Also indicated is the position of the endogenous HNF-3 protein-DNA complexes.

reduction in transactivation (Fig. 2 and 3, 52–458). No further decreases occurred until the DNA binding domain was disrupted (Fig. 3; compare 153–458 and 177–458). The sequences necessary for region IV activity contain an abundance of serine and tyrosine residues as well as two potential casein kinase I phosphorylation sites (DWSS and EGYS) (28). These are features that are found in the activation domains of transcription factors Pit-1/GHF-1 (25, 62) and HNF-1 (48). Deletion of either region III or region IV caused equivalent reductions in activity (Fig. 3; compare 52–458 with 1–418), whereas removal of both sequences inactivated the HNF-3β protein (52–418). These results show that in addition to the DNA binding domain, HNF-3β requires at least two of the other three conserved sequences for activity (region II, III, or IV).

Protein sequences throughout regions II and III are important for HNF-3β transactivation. The contribution of the amino acids that lie between regions II and III was evaluated by joining the HNF-3β sequence 396 to 458 or 427 to 458 in frame with various carboxyl-terminal deletions. The 396-to-458 sequences increased the activity of mutants containing either a portion or the whole of region II to wild-type levels (Fig. 4, 1–392 + 396–458 and 1–379 + 396–458). In contrast, addition of the 427-to-458 residues to these same deletion mutants demonstrated only 45% of wild-type levels, representing little restoration of activity (Fig. 4A, 1–392 + 427–458 and 1–379 + 427–458; compare with Fig. 2A). These experiments illustrate that region III alone is not sufficient for activation and requires additional sequences that are located between regions II and III to achieve activity (for the amino acid sequence, see Fig. 5A). The 396-to-458 segment provided a substantial increase in transactivation (55%) to HNF-3β deletion mutants that were missing both regions II and III (Fig. 4A). In contrast, the 427-to-458 segment elicited a lower level of stimulation of the same inactive deletion mutants (30%), further illustrating the transcriptional involvement of the residues that are located between regions II and III (Fig. 4). We also showed that the 361-to-458 sequences could restore activity to several other HNF-3β

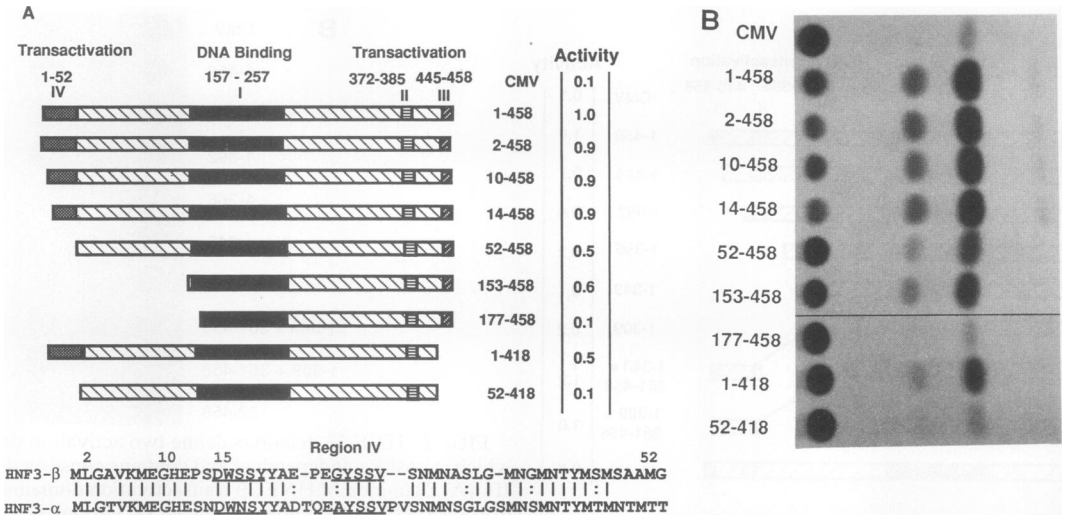


FIG. 3. Region IV defines a second HNF-3β activation domain that contains two putative casein kinase I phosphorylation sites. (A) Summary of transcriptional activities produced by amino-terminal HNF-3β deletions during Hep-G2 cell cotransfection assay. Schematically shown are the locations of conserved regions I to IV within the HNF-3β protein and HNF-3β deletion mutants, as well as their transactivation ratios (mutant/wild-type activity; see legends to Fig. 1 and 2). At the bottom of the panel is the alignment of HNF-3β and HNF-3α proteins at the amino-terminus region IV sequences (| , identity; :, conserved change); two putative casein kinase I sites that are conserved between these proteins are underlined (28). (B) CAT assays from cotransfection experiments with the HNF-3β amino-terminal deletion mutants, which were used to tabulate the results shown in panel A. Gel shift assays demonstrate equivalent protein-DNA complexes for each HNF-3β mutant tested in the cotransfection assay performed as described in the legend to Fig. 2 (data not shown). Note that the 177–458 HNF-3β protein does not interact with the HNF-3 site because it is missing part of the DNA binding domain (33).

carboxyl deletions which still retained the DNA binding domain, further confirming that the HNF-3β region II-III sequences could stimulate transcription in various positions within the protein (Fig. 4). Each of the transfected nuclear extracts produced protein complexes with sizes that were consistent with the predicted size of a particular HNF-3β expression construct (data not shown). Thus, we have identified a position-independent transactivation domain which requires the retention of HNF-3β residues 361 to 458 to attain full activity.

This region II-III activation domain encompasses amino acid sequences which not only are conserved among the HNF-3β family members but also extend to the *Drosophila* homeotic gene fork head. In order to evaluate the contribution of conserved regions II and III to transactivation, we constructed site-directed mutations which altered the properties of three amino acids (Fig. 5A). We chose to target the YYQ residues in region III because the removal of five carboxyl amino acids from region III (1–453) had no effect on HNF-3β activity (data not shown). The mutations in regions II and III are illustrated in Fig. 5A, which also depicts the alignment of the 359-to-458 region between HNF-3β and HNF-3α. These HNF-3β site-directed mutants were analyzed for transactivation with the 4× HNF-3–TATA–CAT reporter construct in Hep-G2 cells (Fig. 5B). The wild-type HNF-3β expression plasmid (1–458) and a mutant which lacked region III (1–444) were included for comparison, and in these sets of experiments, the removal of region III elicited greater decreases in transactivation. HNF-3β region III site-directed mutations which maintained one of the two tyrosines (445 and 446) produced reductions of 25 to 40% in transcriptional activation relative to that of wild-type HNF-3β (Fig. 5B, MIII-1 and MIII-5). The mutant MIII-2, which altered both of the tyrosines, exhibited a more dramatic reduction (80%) which was equivalent to activation by the region III deletion mutant (Fig. 5B; compare MIII-2 with 1–444). This suggests that the integrity of the two tyrosine residues in region III is important for transactivation by this HNF-3β domain. HNF-3β mutants which altered the FSI residues in region II demonstrated a reduction of 50 to 60% in activity (Fig. 4B, MII-1, MII-2, and MII-3). These results demonstrate that full activation potential depended on the retention of both conserved region II and conserved region III sequences.

DISCUSSION

The transcription factor HNF-3 participates in the coordinate expression of several hepatocyte-specific genes by binding to their promoter sequences in combinatorial association with a limited number of other liver-enriched regulatory proteins (4, 10, 13, 26, 40). HNF-3 may also play an important role in hepatocyte differentiation, since both HNF-4 and HNF-3 regulate the promoter activity of the liver transcription factor HNF-1 (31). This places HNF-3 in an early position in the hierarchy of transcription factor cascades involved in hepatocyte differentiation. Moreover, characterization of the HNF-3β promoter has identified a cell-specific factor, LF-H3β, which is responsible for its restricted expression and may be important in hepatocyte differentiation (49). In addition to the LF-H3β protein, the HNF-3β promoter also possesses a positive autoregulatory site, which is a property found in many mammalian transcription factors and *Drosophila* homeotic genes involved in cellular determination (2, 8, 36, 42, 55, 58, 61, 63).

In light of the pivotal role HNF-3 plays in hepatocyte gene regulation, we developed a cotransfection assay in Hep-G2 cells to define amino acid residues responsible for HNF-3β transcriptional activation. In the present study, we defined a position-independent activation domain at the HNF-3β car-

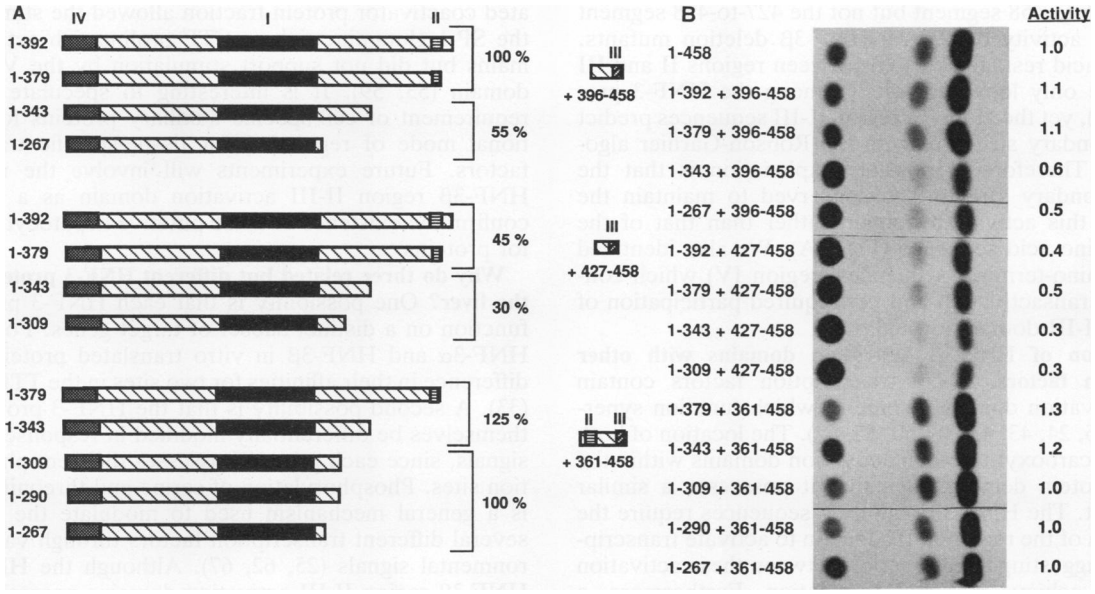


FIG. 4. HNF-3β sequences between regions II and III are required for transactivation. (A) Summary of transcriptional activities produced by internal HNF-3β deletions during Hep-G2 cell cotransfection assays. Schematically shown are internal HNF-3β deletions which were made by using the indicated carboxyl-terminal deletions to create in-frame fusions with region III (aa 427 to 458), region III containing additional amino sequences (aa 396 to 458), or sequences encompassing regions II and III (aa 361 to 458). Also shown is an average of the stimulation elicited by the indicated HNF-3β internal deletions in the cotransfection assay (percentage of wild-type activity). Note that the HNF-3β carboxyl deletion constructs 1–392 and 1–379 provided approximately 60% of wild-type transactivation whereas the 1–343, 1–309, 1–290, and 1–267 deletions were inactive (Fig. 2 and data not shown). (B) CAT assays from cotransfection experiments with the HNF-3β internal deletions. The indicated HNF-3β internal deletion mutants illustrated in panel A were cotransfected with the 4× HNF-3–TATA–CAT reporter in Hep-G2 cells (see legends to Fig. 1 and 2), and their activation levels are indicated (ratio of mutant to wild type). Identical HNF-3 protein expression was observed as detected by gel shift assay as described in the legend to Fig. 2 (data not shown).

boxyl terminus (361–458) which can potentiate the expression of a TATA box-CAT reporter construct containing multimeric DNA recognition sites for the HNF-3 protein. This HNF-3β activation domain required region II and III sequences which are conserved with the HNF-3 family and the *Drosophila* fork head protein. The region II-III sequences do not possess amino acid sequence motifs in common with other transcription factors (e.g., acidic residues or glutamine- or proline-rich sequences) and may belong to a new family of activation domains (27, 45, 55). Furthermore, the sequences contained between regions II and III appear to be critical for transcriptional activation,

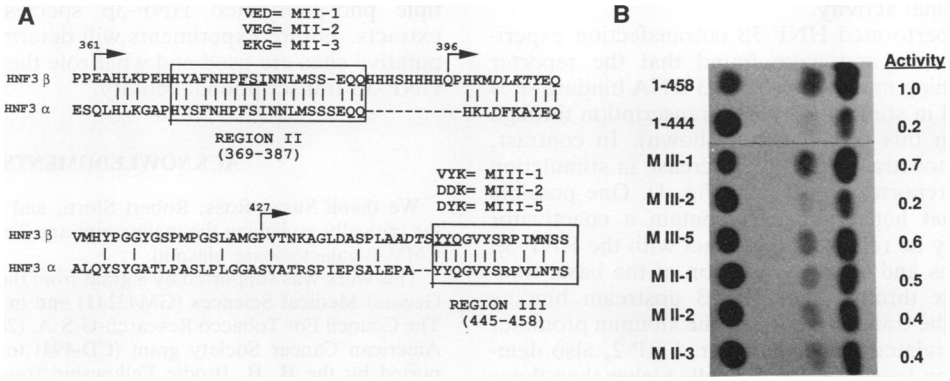


FIG. 5. Conserved sequences within regions II and III are required for transcriptional activation as demonstrated by site-directed mutagenesis. (A) Alignment of HNF-3β (359 to 458) and HNF-3α (377 to 466) region II and III protein sequences (regions are boxed) (32, 33). Also shown are the amino acid residues (underlined) that were targeted for site-directed mutagenesis (30) in region II (MII; FSI) and region III (MIII; YYQ) and the changes that were introduced. Italics indicate the putative casein kinase I phosphorylation sites in the HNF-3β region II-III activation domain (28) (DLKTY and ADTSY). (B) CAT assays from cotransfection experiments with the HNF-3β site-directed mutants in regions II and III. The indicated HNF-3β site-directed mutants (A) were cotransfected with the 4× HNF-3–TATA–CAT reporter in Hep-G2 cells (see legends to Fig. 1 and 2), and cytoplasmic extracts were analyzed for CAT enzyme activity. Indicated are their relative activation levels produced in this cotransfection assay (ratio of mutant to wild type). An HNF-3β region III deletion mutant (1–444) and CMV control (0.07) were included for comparison. Gel shift assays demonstrate equivalent expression of protein-DNA complexes for all site-directed HNF-3β mutants tested in the cotransfection assay (data not shown).

since the 396-to-458 segment but not the 427-to-458 segment rescued the activity of several HNF-3β deletion mutants. The amino acid residues located between regions II and III demonstrate only loose homology among the HNF-3 proteins (Fig. 5), yet the HNF-3α region II-III sequences predict similar secondary structure with the Robson-Garnier algorithm (17). Therefore, a possible explanation is that the HNF-3 secondary structure is conserved to maintain the function of this activation domain rather than that of the primary amino acid sequence (Fig. 5A). We also identified HNF-3β amino-terminal sequences (region IV) which contributed to transactivation and yet required participation of the region II-III domain for activity.

Comparison of HNF-3β activation domains with other transcription factors. Many transcription factors contain several activation domains, some of which function synergistically (16, 24, 43, 45, 48, 50, 55, 62). The location of both amino- and carboxyl-terminal activation domains within the HNF-3β protein demonstrates that it possesses a similar arrangement. The HNF-3β region IV sequences require the contribution of the region II-III domain to activate transcription, thus suggesting an interaction between these activation domains to achieve maximal stimulation. Furthermore, a number of potent transcriptional activation domains possess stretches of acidic amino acid residues (45, 52). In regions that are rich in hydroxylated amino acids, the phosphorylation of serine and threonine by specific protein kinases provides a mechanism to increase the acidity of the sequence in a regulated fashion (67). The HNF-3β region IV sequences are rich in serine and tyrosine, a feature found in the activation domains of the POU-homeobox transcription factors Pit-1/GHF-1 (25, 62) and LF-B1/HNF-1 (48). Like these activation domains, the functionally important HNF-3β sequences possess two putative casein kinase I phosphorylation sites (28). These sites may play a role in regulating both the activity of region IV and its putative interaction with the region II-III activation domain. The *Drosophila* fork head protein does not contain region IV, which suggests that it may have been introduced later in the evolution of the mammalian HNF-3 family (64, 65). Functional analysis of the *Drosophila* fork head protein may determine whether sequences other than the region II-III domain are necessary for its transcriptional activity.

We have also performed HNF-3β cotransfection experiments in HeLa cells and have found that the reporter constructs containing multimeric HNF-3 DNA binding sites are not functional in stimulating basal transcription through the TATA box in this cell line (not shown). In contrast, Hep-G2 cells demonstrate a marked increase in stimulation with the HNF-3 reporter constructs (Fig. 1). One possible explanation is that hepatoma cells contain a coactivator protein which may be required to interact with the HNF-3β activation domains and allow stimulation of the basal transcription complex through the HNF-3 upstream binding sites. Moreover, the transactivation of the albumin promoter by C/EBP and a related family member, CRP-2, also demonstrates activation levels in Hep-G2 cells higher than those in HeLa cells (66). Several studies have indicated that transcriptional activation can arise from different mechanisms. The acidic domain of herpes simplex regulatory protein VP16 demonstrated a direct interaction with the basal transcription factors to achieve activation (39). The stimulation of in vitro transcription by binding of the SP-1 protein to its recognition site upstream of the TATA box has demonstrated the requirement of a coactivator protein in addition to cloned TFII-D protein (14). This TFII-D-associated coactivator protein fraction allowed the stimulation by the SP-1 glutamine-rich or CTF proline-rich activation domains but did not support stimulation by the VP16 acidic domain (53, 59). It is interesting to speculate about the requirement of cell-specific auxiliary proteins for an additional mode of regulation for tissue-specific transcription factors. Future experiments will involve the use of the HNF-3β region II-III activation domain as a reagent to confirm the existence of these putative hepatocyte coactivator proteins.

Why do three related but different HNF-3 proteins exist in the liver? One possibility is that each HNF-3 protein may function on a distinct subset of target genes. For example, HNF-3α and HNF-3β in vitro translated proteins show a difference in their affinities for two sites in the TTR promoter (33). A second possibility is that the HNF-3 proteins could themselves be differentially modified in response to various signals, since each protein possesses different phosphorylation sites. Phosphorylation of serine and threonine residues is a general mechanism used to modulate the activity of several different transcription factors through various environmental signals (25, 62, 67). Although the HNF-3α and HNF-3β region II-III activation domains possess some homology, the HNF-3β protein contains two putative casein kinase I phosphorylation sites which are not conserved between the two proteins (401-DLKTY and 441-ADTSY [Fig. 4A]) (28). In addition, the HNF-3β protein possesses a unique stretch of histidines which may provide a more basic property to the HNF-3β activation domain. The phosphorylation of these putative sites in the HNF-3β activation domain could alter this basic property and may provide a means to regulate its activity separately from that of the HNF-3α protein. Furthermore, the HNF-3β protein also possesses two additional casein kinase II phosphorylation sites (328-SALSPPE and 293-SASE) (28), which lie between the DNA binding domain and the region II-III activation domain. These putative phosphorylation sites may play a regulatory role and allow the HNF-3β and HNF-3α proteins to respond to different signals transduced in response to environmental stimuli. Preliminary experiments using two-dimensional isoelectric focusing sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels have suggested multiple phosphorylated HNF-3β species in liver nuclear extracts. Future experiments will determine which of these putative sites are used and what role they play in regulating HNF-3β transcriptional activity.

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