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DNA binding and transcription activation specificity of hepatocyte nuclear factor 4

James D. Fraser*, Veronica Martinez, Rose Straney and Michael R. Briggs

Ligand Pharmaceuticals Inc., 10255 Science Center Drive, San Diego, CA 92121, USA

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ABSTRACT

Hepatocyte nuclear factor 4 (HNF-4) is an orphan intracellular receptor that appears to be a key factor in the regulation of energy metabolism. In order to gain greater understanding of the binding and activation requirements of HNF-4, we performed genetic analysis of the *apoCIII* promoter, a promoter that has previously been shown to be highly sensitive to HNF-4-induced transcription. We identified two elements within the *apoCIII* promoter that bind HNF-4, either of which are sufficient to confer promoter induction in response to HNF-4. These two elements are both direct repeat-like in nature, but they differ significantly in motif sequence and the repeats are separated by either 1 or 2 nt. Therefore, to better define the DNA sequence recognition requirements of HNF-4, we utilized PCR-based binding site selection. HNF-4 selected for direct repeat-like elements with either 1 or 2 nt between the repeats. Surprisingly, the strongest selection was for a core motif that included the nucleotides between the repeats. Mutation of the nucleotide between the repeats resulted in a 6-fold reduction in affinity, indicating that the interaction between HNF-4 and the intervening nucleotide(s) is critical for high affinity binding.

INTRODUCTION

Hepatocyte nuclear factor 4 (HNF-4) is a highly conserved member of the steroid/thyroid superfamily of ligand-dependent transcription factors that are expressed in liver, kidney, intestine and pancreas (1,2). No ligand has been identified at present and therefore HNF-4 is referred to as an orphan member of the intracellular receptor family (3–5). While it is not clear whether or not an activity-modulating ligand exists, HNF-4 is capable of activating transcription in tissue culture cells under normal culture conditions (6–9). While its true overall role and level of regulation remains to be elucidated, HNF-4 does appear to be a critical component of several metabolic pathways, including glucose and lipid homeostasis (7–11). Apolipoprotein gene expression in particular appears highly sensitive to HNF-4 activity in that HNF-4 binding sequences have been identified in the promoter region of the *apoAI*, *apoAIV*, *apoB* and *apoCIII* genes (7–14).

Of these genes, *apoCIII* appears to be one of the most sensitive to HNF-4 modulation. Over-expression of HNF-4 in hepatic cell lines results in a 5- to 10-fold increase in the reporter activity of *apoCIII* promoter constructs while co-transfection of a dominant negative mutant of HNF-4 results in an 80% loss of activity (7,8,15,16). These findings have recently been extended to the endogenous *apoCIII* gene with similar results (16). Therefore, we chose the *apoCIII* promoter for genetic analysis of the requirements for HNF-4 binding and gene activation. The DNA binding domain of the intracellular receptors is highly conserved among family members and utilizes two zinc finger motifs that serve as interfaces in both protein–DNA and protein–protein interactions (17). HNF-4 utilizes an unusual amino acid in the ‘knuckle’ of the first zinc finger and binds DNA as a homodimer. At present, there is no evidence of HNF-4 forming heterodimers with other members of the intracellular receptor family (1,18). It has previously been shown that HNF-4 binds to sequences containing direct repeats of the hexamer AGGTCA separated by one base (DR1) (1). In these studies we examine the contribution from two quite distinct sequence motifs in the apoCIII promoter, either of which is sufficient for a near maximal response to HNF-4 co-transfection. The differences between these two response elements and the heterogeneity of the reported HNF4 binding sequences, along with the desire to be better able to predict possible HNF-4 response elements, encouraged us to better define the DNA sequence requirements for HNF-4 binding. The results of binding site selection and analysis of the affinity of HNF-4 for these sequences indicate that HNF-4 recognizes DNA sequence elements with several distinct features which may contribute to specificity.

MATERIALS AND METHODS

Plasmids and transfections

A genomic fragment spanning nt –1415 to +24 of the human *apoCIII* promoter generously provided by Dr Bart Staels was subcloned into the *Sma*I site of pGL2 (Promega) to create p1400CIII-luc. The –810 to +23 *apoCIII* reporter vector was created using 5′-ACGAGAGAATCAGTCCTGGT-3′ and 5′-TGCCTCTAGGGATGAACT-3′ as primers for PCR from human genomic DNA to create a fragment containing from –810 to +23 of the *apoCIII* promoter. The product was then ligated into Bluescript KS– via the *Bam*HI and *Spe*I sites. The *apoCIII* promoter fragment was sequenced and then the *Bam*HI–*Spe*I

*To whom correspondence should be addressed. Tel: +1 619 550 7781; Fax: +1 619 550 7876; Email: jfraser@ligand.com

fragment was ligated into pGL2 digested with *Bgl*II and *Sma*I to create p810CIII-luc. The *apoCIII* promoter mutants were created using crossover PCR (19). The pCIII(–78)-luc mutant changed the wild-type sequence of –79 to –72 from TGACCTTT to CAGTTCCC. The pCIII(–740)-luc mutation changed –737 to –724 from GGGTCCAGAGGGCA to GAACTCAGGAAGTG. The construct pCIII(DM)-luc contains mutations at both the –78 and –740 sites. The multimer constructs of each HNF-4 binding sequence were generated by synthesizing oligonucleotides containing three copies of the indicated HNF-4 binding element each separated by 10 nt. The oligonucleotides were synthesized in order to have 5′ *Hin*dIII and 3′ *Sal*I overhangs and the annealed oligos were ligated into *Xho*I and *Hin*dIII-digested pTK-2.

The construction of the expression vectors pC-HNF4α1 and pC-Δ111HNF4α1 was previously described (16). All PCR-created constructs were sequenced. Transient transfections were performed as previously described (16). Each transfection included RSV-βgal and all luciferase values were normalized to β-galactosidase values. Each result is representative of at least three independent transfections.

Electrophoretic mobility shift assays

Nuclear extracts were prepared from adenovirus-infected HepG2 cells as previously described (16). Protein concentrations were determined using the Bradford method (20). DNA–protein binding assays were done by incubating nuclear extracts at 4°C in reaction buffer containing 10 mM HEPES (pH 7.8), 40 mM KCl, 0.5 mM $MgCl_2$,1 mM dithiothreitol, 10% glycerol, 5 µg BSA; 1 µg poly(dI·dC) was added as a non-specific competitor and 0.5 ng ^{32}P-labeled (5000–20 000 c.p.m.) specific probe. The –78 oligo refers to an oligonucleotide containing nt –98 to –59 of the *apoCIII* promoter and the –78M oligonucleotide is identical to the –78 oligonucleotide except that the sequence TGACCT T TGCCCA is changed to CAGTTC C CGCCCA. The –740 oligo refers to an oligonucleotide corresponding to nt –749 to –712 of the *apoCIII* promoter and the –740M oligonucleotide is identical to the –740 oligonucleotide except that the sequence GGGTCC AG AGGGCA is changed to GAACTC AG GAAGTG. The final volume of each binding reaction was 20 µl. Complexes were separated on 6% polyacrylamide gels with 22.5 mM Tris–borate (pH 8.0) and 1 mM EDTA buffer.

DNA binding site selection

In order to select for high affinity binding sites for HNF-4, we synthesized by PCR a mixture of 70 base oligonucleotides using as template the random oligomer 5′-CGCGGATCCTGCAGCT-CCAGN$_{30}$GTCGACAAGCTTCTAGAGCA-3′ and the forward and reverse primers 5′-CGCGGATCCTGCAGCTCGAG-3′ and 5′-TGCTCTAGAAGCTTGTCGAC-3′ respectively, as has previously been described (21). Before amplification, the forward primer was end-labeled using polynucleotide kinase and [γ-^{32}P]ATP. The primary amplification reaction was carried out using 20 pmol random oligomer and 100 pmol each end-labeled forward primer and reverse primer for three cycles of 1 min at 94°C, 2 min at 52°C and 3 min at 72°C. As a control we also amplified a 70 base oligomer containing the same primers and the upstream HNF-4 binding site from the *apoCIII* promoter (5′-CGCGGATCCTGCAGCTCCAGATCAGTGGGTCCAGA-GGGCAAAATAGGGAGGTCGACAAGCTTCTAGAGCA-3′). Between 5 and 10 pmol double-stranded mixed oligomer and *apoCIII* control were incubated with ~0.1 ng baculovirus-expressed HNF-4 for 10 min. The complexes were separated by electrophoresis through a 5% polyacrylamide gel in 0.5× TBE. A band with the same mobility as the HNF-4–*apoCIII* element complex was excised from the random oligomer lane and the bound DNA was eluted (0.5 M NH_4 acetate, 1 mM EDTA pH 8.0). The bound DNA was ethanol precipitated and amplified using 100 pmol each end-labeled forward primer and reverse primer for 20 cycles. The selection protocol was repeated five times and the products were cloned into Bluescript SK+. Colonies containing inserts were subjected to sequence analysis.

DNA binding analysis

To determine dissociation constants (K_d) for the various HNF-4 binding site oligonucleotides, mobility shift analysis was performed with increasing amounts of oligonucleotide with set levels of baculovirus-expressed HNF-4. To quantitate the bound and free oligonucleotide, the bands were analyzed with a Phosphorimager (Molecular Dynamics). Specific counts were determined by subtracting background. Data were analyzed by the method of Scatchard (22).

RESULTS

Identification of HNF-4 response elements in the *apoCIII* promoter

The *apoCIII* promoter has previously been shown to be activated by HNF-4 (7,8,15). Moreover, co-transfection of a truncated, dominant negative mutant of HNF-4 in hepatic cell lines results in a 70–80% decrease in *apoCIII* promoter activity, demonstrating that endogenous HNF-4 is necessary for normal *apoCIII* expression (16). In order to identify and characterize the HNF-4 response elements contained within the *apoCIII* promoter, we made a series of truncations and mutations starting with a 1.4 kb 5′ promoter fragment (p1400CIII-luc). Maximal activation by HNF-4 appeared to be mediated by sequences contained within the first 810 bp of the promoter, as 5′ truncations of the p1400CIII-luc construct down to one containing 810 bp (p810CIII-luc) did not result in loss of basal or HNF-4-induced promoter activity, while further truncation did (Fig. 1 and data not shown). This region contains a previously identified DR1-like element between –71 and –82 that binds HNF-4 (1,7). Gel shift analysis with an oligonucleotide corresponding to this region (oligo –78) demonstrated that this sequence binds HNF-4 with high affinity and that mutation within the DR1-like sequence results in the loss of HNF-4 binding (Fig. 2). However, mutation of the –78 element in the p810CIII-luc construct did not decrease basal activity and failed to eliminate HNF-4-induced promoter activation (Fig. 3). This result suggested that there must be an additional HNF-4 response element or elements contained within p810CIII-luc.

To identify other potential HNF-4 response elements, the region of the *apoCIII* promoter contained within p810CIII-luc was scanned for additional direct repeat-like sequences. Located between –720 and –733 is the sequence (GGGTCC AG AGGGCA) with a 2 bp spacer. A double-stranded oligonucleotide corresponding to this region (oligo –740) was specifically bound by HNF-4 (Fig. 4). Competition with an oligonucleotide containing mutations within the repeat failed to compete for HNF-4 binding, indicating that the repeat-like sequence was

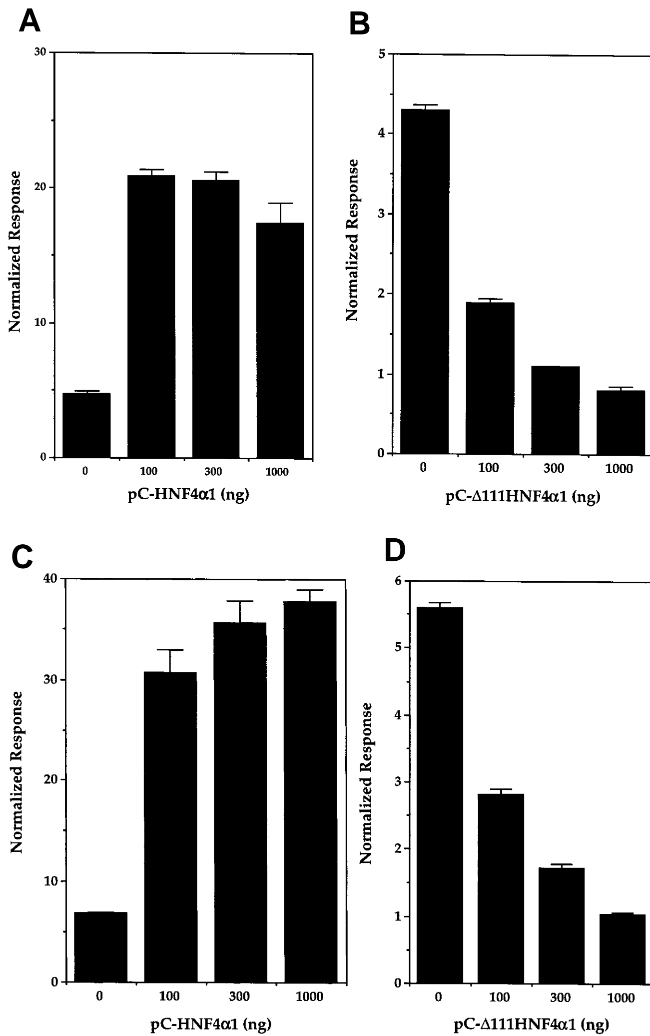

Figure 1. HNF-4 regulates *apoCIII* promoter activity and the HNF-4 response element(s) is located within the –810 to +23 region of the promoter. HepG2 cells were transiently transfected with p1400CIII-luc and the indicated dosage of (**A**) pC-HNF4α1 or (**B**) pC-Δ111HNF4α1. HepG2 cells were transiently transfected with p810CIII-luc and the indicated dosage of (**C**) pC-HNF4α1 or (**D**) pC-Δ111HNF4α1. The transfections received 5 µg reporter vector and the indicated quantities of expression vector per 1.5×10^6 cells. The error bars represent the standard deviation: each condition was performed in triplicate and is representative of at least three independent experiments.

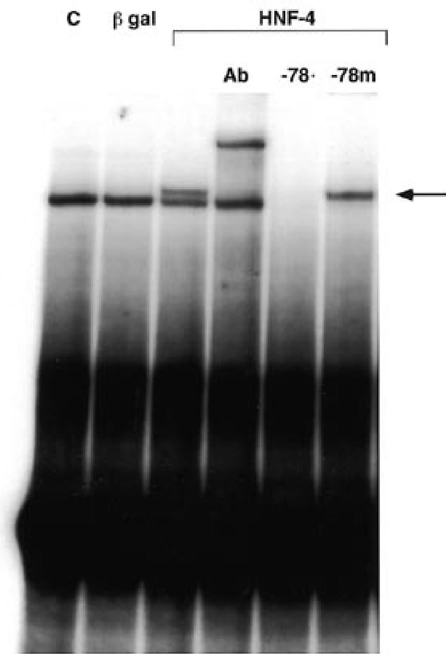

Figure 2. Binding of HNF-4 to the –78 region of the *apoCIII* promoter. Gel mobility assays were performed using an oligonucleotide corresponding to –98 to –59 of the *apoCIII* promoter. Nuclear extracts were from uninfected (C), AdβGal-infected (βGal) or AdHNF4α1-infected HepG2 cells (HNF-4). The lane labeled Ab contains 1 µl HNF-4 antiserum and supershifts the HNF-4-specific band. Competition for HNF-4 binding was performed in the presence of a 50-fold molar excess of unlabeled oligonucleotide –78 or –78M (see Materials and Methods). An arrow indicates the HNF-4-specific complex; the prominent band at the bottom of the figure is free probe.

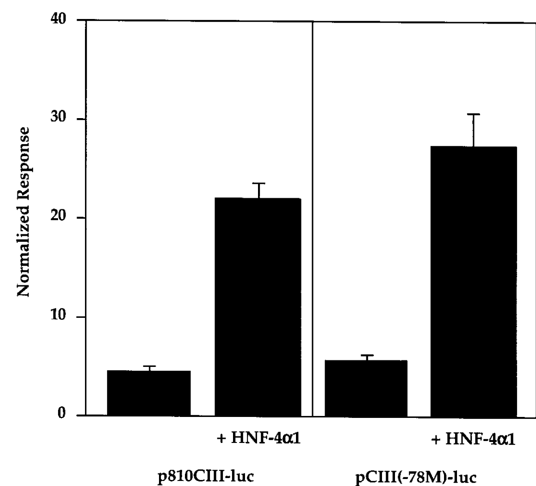

Figure 3. Mutation of the HNF-4 binding element at –78 fails to abrogate HNF-4 activation of the *apoCIII* promoter. HepG2 cells were transiently transfected with either p810CIII-luc or pCIII(–78M)-luc in the presence or absence of pC-HNF4α1. The transfections received 5 µg reporter vector and 100 ng pC-HNF4α1 where indicated per 1.5×10^6 cells. The error bars represent the standard deviation: each condition was performed in triplicate and is representative of at least three independent experiments.

responsible for the HNF-4 binding. Similar to what we observed after mutation of the proximal HNF-4 binding sequence, mutation of the –740 element also did not result in a loss of HNF-4-induced promoter activity by the p810CIII-luc construct (Fig. 5). However, mutation of both the –78 and –740 regions did result in a construct (pDM) that had ~30% of the basal activity of p810CIII-luc when transfected into HepG2 cells and was unresponsive to co-transfection with HNF-4-expressing vectors. These results demonstrate that the *apoCIII* promoter contains two elements responsive to HNF-4-mediated activation, either of which is capable of generating a near maximal response to HNF-4 co-transfection.

The DNA binding sites previously described for HNF-4 are quite disparate in sequence. As our analysis of the *apoCIII* promoter had identified two functional HNF-4 response elements, both of which differ from a canonical repeat motif and having either one or two spacer nucleotides, we decided to better define

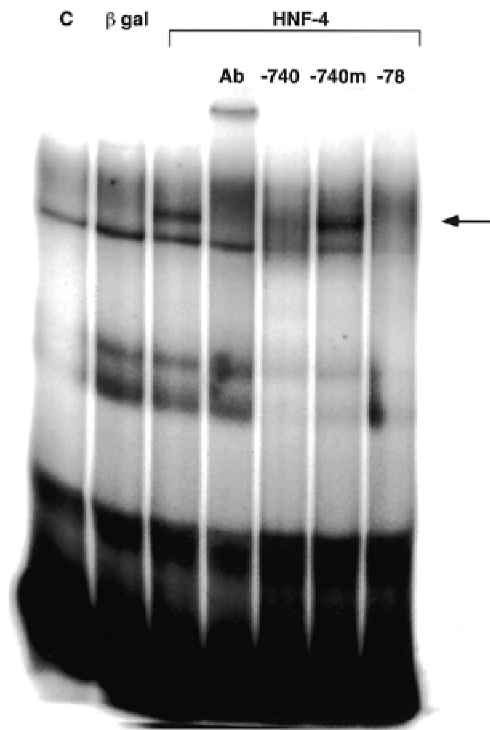

Figure 4. HNF-4 binds to the –740 region of the *apoCIII* promoter. Gel mobility assays were performed using an oligonucleotide corresponding to –749 to –712 of the *apoCIII* promoter. Nuclear extracts were from uninfected (C), AdβGal-infected (βGal) or AdHNF4α1-infected HepG2 cells (HNF-4). The lane labeled Ab contains 1 µl HNF-4 antiserum and supershifts the HNF-4-specific band. Competition for HNF-4 binding was performed in the presence of a 50-fold molar excess of unlabeled oligonucleotides –740, –740M or –78 (see Materials and Methods). An arrow indicates the HNF-4-specific complex; the prominent band at the bottom of the figure is free probe.

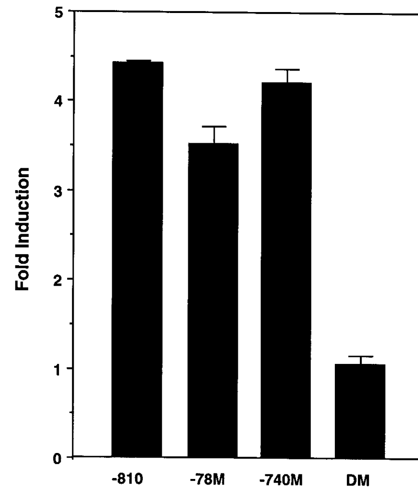

Figure 5. The *apoCIII* promoter contains two HNF-4 response elements, either of which is sufficient for HNF-4-induced promoter activity. HepG2 cells were transiently transfected with the indicated reporter construct in the presence or absence of pC-HNF4α1. The constructs pCIII(–78M)-luc and - pCIII(–740M)-luc contain mutations in the –78 and –740 regions (see Materials and Methods), while pCIII(DM) contains mutations at both sites. The transfections received 5 µg reporter vector and 100 ng pC-HNF4α1 where indicated per 1.5×10^6 cells. The error bars represent the standard deviation: each condition was performed in triplicate and is representative of at least three independent experiments.

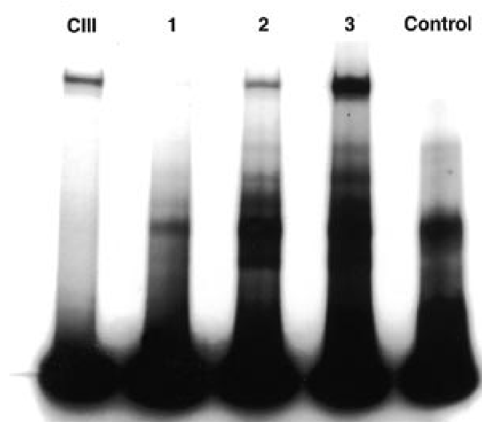

Figure 6. Selection of high affinity binding sites for HNF-4 homodimers. PCR-assisted DNA binding site selection was performed to select HNF-4 binding sequences. Double-stranded oligonucleotides consisting of forward and reverse primers and 30 randomized base pairs end-labeled with [γ-^{32}P]ATP were incubated with HNF-4. Protein–DNA complexes were separated from free DNA by electrophoresis on non-denaturing polyacrylamide gels. Bound oligonucleotides were excised from the gel and amplified using PCR. As a positive migration control we used an oligomer containing the same primers and the upstream HNF-4 binding site from the *apoCIII* promoter (CIII). Lanes 1–3 refer to the first to third rounds of selection. The Control lane was the oligonucleotide pool from the third round of selection with no added HNF-4.

the DNA binding properties of HNF-4 using PCR-based binding site selection. Binding of HNF-4 to the distal *apoCIII* element was used as a marker to localize bound DNA after electrophoresis. Bound oligonucleotides were excised from the region of the gel co-migrating with the *apoCIII* probe, amplified by PCR and subjected to gel shift analysis (Fig. 6). After the fourth and fifth rounds of selection, the bound oligonucleotides were subcloned into Bluescript SK+ and inserts from 28 independent clones were subjected to sequence analysis. Of these, 23 inserts contained direct repeat-like sequences, three contained a single hexamer sequence and two inserts did not display any form of consensus sequence. Of the 23 clones which contained repeats, 19 contained a single nucleotide between the repeats and four contained 2 nt (Table 1). The consensus sequence derived from the DR1-like inserts differed from a classical DR1 in several regards. All 19 clones contained an identical CAAAG sequence for the central 5 nt including the spacer. The four DR2 inserts also contained deoxyadenosine in the spacer, two had a CCAAAG and the other two CAAAAG. In addition, the fourth nucleotide of the 5′ repeat did not appear to be selected for and the initial base could be either deoxygaunosine or deoxyadenosine.

To analyze the effects of these differences, we determined the dissociation constants (K_d) of baculovirus-expressed HNF-4 for a canonical DR1 with a deoxythymidine spacer (oligonucleotide A), a canonical DR1 with the selected deoxyadenosine (oligunucleotide B), a canonical DR2 with two deoxyadenosines as the spacing nucleotides (oligunucleotide C) and an oligonucleotide with deoxyadenosine as the spacer nucleotide and deoxyguanosine

Table 1. Consensus sequences binding to HNF-4α1

Clone	Sequence
DR2	
4.3	CCTC AGGTCC AA AGGTCG TA
4.8	TC AGGTCA AA AGGTCA CAAAGG
5.7	AGG GGGGCA AA AGGTCC TTAGT
5.11	G GGGTCC AA AGTTCA ACCG
DR1	
4.1	CCGA GGATCA A AGTCCA ACGA
4.2	CCAA TGGACA A AGCCTA C
4.4	TTG GGGACA A AGGTCA TCGA
4.5	CCAC AGTCCA A AGTGTG CAAC
4.6	AGGGCA A AGGTCA GGCG
4.7	ATCG GGGTCA A AGGTCG
4.10	T GGACCA A AGTCCA CGCG
5.1	TC AGGGCA A AGTACA GC
5.2	AGCA CGGTCA A AGTTCA TCTA
5.4	AT AGGGCA A AGGTCA GAG
5.5	CG AGGGCA A AGTCAA CAG
5.6	G GAGCCA A AGGTCG CAAG
5.8	GATG GGGTCA A AGGTCG A
5.9	TC AGGACA A AGGTCA CGAA
5.12	CACG GGGTCA A AGGTCG GA
5.13	GGAG GGTACA A AGGTCA TGTG
5.14	TA AGGGCA A AGGGCA GGTG
5.16	GGCG CGGTCA A AGGTCG TA
5.17	CTA GGGGCA A AGGTCA TTC

Consensus

	-7	-6	-5	-4	-3	-2	-1	S	1	2	3	4	5	6	7
A	5	7	1	2	4	0	19	19	19	0	0	1	1	13	3
C	3	2	0	0	3	19	0	0	0	0	1	4	16	0	6
G	8	9	18	15	6	0	0	0	0	19	12	2	0	6	5
T	2	1	0	2	6	0	0	0	0	0	6	12	2	0	5
(C)	N	G/A	G	G	N	C	A	A	A	G	G/T	T	C	A/G	N

Table 2. Affinity of HNF-4α1 for selected DR1 sequences (nM)

SEQUENCE	Exp.1	Exp.2
A) AGGTCA T AGGTCA	1.4	1.6
B) AGGTCA A AGGTCA	0.23	0.26
C) AGGTCA AA AGGTCA	1.7	1.4
D) GGGGCA A AGGTCA	0.34	0.40

Exp. 1 and 2 refer to two independent experiments.

Figure 7. *Trans*-activation of constructs containing multimers of the HNF-4 binding sequences. HepG2 cells were transiently transfected with 5 µg indicated reporter construct containing three copies of the respective binding sites upstream of the thymidine kinase promoter and 0, 10, 30 or 100 ng pC-HNF4α1 per 1.5×10^6 cells. The error bars represent the standard deviation: each condition was performed in triplicate and is representative of at least three independent experiments.

substituted for the first four bases of the initial repeat (oligonucleotide D). The results of the dissociation analysis demonstrated that the intervening nucleotide plays a significant role in HNF-4 binding as the single T→A substitution in oligonucletide B increased the affinity of HNF-4 binding by 6-fold (Table 2). In agreement with the relative number of clones recovered, HNF-4 binds the DR1 with 6-fold greater affinity than the DR2 oligonucleotide. As suggested by the consensus sequence derived from the binding site analysis, substitution of deoxyguanosines at the first and fourth nucleotides of the first repeat did not markedly alter the affinity of HNF-4.

To address whether these sequences were all functional HNF-4 response elements, we created constucts containing three copies of the A, B and D sequences with the thymidine kinase promoter linked to luciferase as well as constructs containing three copies of the two sites found in the *apoCIII* promoter. When arranged as multimers, all five sequences were activated by HNF-4 and showed roughly similar fold inductions upon co-transfection with increasing amounts of pC-HNF4α1 (Fig. 7).

DISCUSSION

The studies described in this manuscript were initiated with the idea of gaining a greater understanding of the binding and activation requirements of HNF-4 via genetic analysis of the *apoCIII* promoter. A sequence element that specifically bound

HNF-4 had previously been identified between –87 and –66 of the human *apoCIII* promoter (1,7). However, in contrast to a previous report (7), mutation of this element such that it no longer bound HNF-4 failed to decrease either basal promoter activity or HNF-4-induced activity in cell lines containing endogenous HNF-4 (Fig. 3 and data not shown). Since these results indicated that there were additional HNF-4-responsive elements, we scanned *apoCIII* for addditional direct repeat-like sequences. Located between –720 and –733 is a repeat-like sequence with a 2 bp spacer (GGGTCC AG AGGGCA) that had previously been identified in the analysis of *apoAI* expression by intestinal cells (12). This sequence also binds HNF-4, although with 2- to 3-fold lower affinity than does the more proximal DR1-like element (data not shown). While mutation of both elements was required to abrogate the HNF-4-induced increase in promoter activity after co-transfection, it is probable that the two sites are responsive to changes in HNF-4 activity and or interactions with other transcription factors with physiological levels of HNF-4.

Since our analysis of the *apoCIII* promoter had identified two functional elements, one DR1-like and one DR2-like, both of which differed significantly from a canonical direct repeat, we decided to identify the highest affinity binding sequences for HNF-4. Of the 28 clones that were sequenced, 19 were DR1-like and four were DR2-like. The relative number of clones identified and binding affinity analysis indicates that HNF-4 has higher affinity for the DR1-like sequences but HNF-4 clearly can bind and activate DR2 elements; this capability may allow for HNF-4-specific responses versus other DR1 binding intracellular receptors such as the peroxisome proliferator-activated receptor family. In the DR1-like sequences, HNF-4 strongly selected for a core sequence of CAAAG that was absolutely conserved in all 19 clones. This core includes the spacer nucleotide which was also selected for in the DR2-like clones, where both spacer nucleotides were deoxyadenosines, suggesting that the spacer sequence strongly influences HNF-4 binding. In agreement with the binding site selection, we found that mutation of the spacer sequence resulted in a 6-fold difference in affinity for HNF-4. Previous studies with thyroid and retinoid X receptors have demonstrated that the sequence of the spacing base pairs can influence binding due to minor groove interactions and this may be the case for HNF-4 as well (23–25). The selected sequences also differed from canonical repeats in that the 5′ hexamer of the core recognition sequence was enriched in G at the first nucleotide and was random at the fourth. The 3′ hexamer was identical to the canonical repeat sequence. All of the identified HNF-4 binding sequences proved to be functional HNF-4 response elements when incorporated in artificial constructs containing three copies of the binding site and a minimal promoter. Understanding the functional impact of specific base changes and spacing will require more rigorous analysis.

In this paper we describe DNA binding preferences and transcription activation by HNF-4 bound to various response elements. HNF-4 is expressed in several tissues involved in energy metabolism, including liver, pancreas and intestine, and potential HNF-4 response elements have been identified in the promoter regions of a number of genes necessary for lipid and glucose homeostasis (7–11). The recent discovery that a nonsense mutation in the HNF-4α gene results in an autosomal dominant, early onset form of non-insulin-dependent diabetes melitus (MODY1) indicates that HNF-4 is a critical regulator of metabolic output (10). The characterization of the DNA binding properties of HNF-4 described in these studies should help in the identification of HNF-4-regulated genes and perhaps give hints as to how specificity is generated with respect to other intracellular receptors that also bind to DR1 or DR2 sequences.

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