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Transcriptional regulation of the apoAI gene by hepatic nuclear factor 4 in yeast

Hans A.Fuernkranz, Yisheng Wang, Sotirios K.Karathanasis and Paul Mak*
Department of Cardiovascular Molecular Biology, Lederle Laboratories, 401 N. Middletown Road, American Cyanamid Co., Pearl River, NY 10965, USA

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ABSTRACT

Hepatocyte Nuclear Factor 4 (HNF-4), a liver-enriched orphan receptor of the nuclear receptor superfamily, is required for the expression of a wide variety of liver-specific genes including apoAI. To explore the possibility that site A of the apoAI gene enhancer might also be the target for HNF-4 without the interference of endogenous mamalian cell proteins that also bind to site A, we tested the ability of HNF-4 to activate transcription from site A in yeast cells. Electrophoretic mobility shift assays (EMSA) and Scatchard plot analysis demonstrated that yeast produced HNF-4 binds to site A with an affinity two times higher than that of yeast produced RXRα. Mapping analysis indicated that the 5′ portion of site A containing two imperfect direct repeats (TGAACCCTTGACC) and the sequence of the trinucleotide spacer (CCT) between these imperfect repeats are critical determinants for selective binding and transactivation by HNF-4. Similar observations were obtained when these mutated versions of site A were evaluated by transient cotransfection assays in CV1 cells. We conclude that the unique structural determinants of site A in conjunction with the differential binding affinity of HNF-4 for site A may play a fundamental role in apoAI gene regulation.

INTRODUCTION

Gene activation in higher eukaryotes requires the assembly of sequence-specific transcription factors on enhancers, which in turn dictate the level of transcription by RNA polymerase II. The high level of specificity required to regulate eukaryotic gene expression during development is in part determined by the modular components of transcription factors and their *cis*-acting DNA elements. The final assembly of transcription initiation complexes involves either direct or indirect interactions between enhancer bound transcription complexes and the components of the basal transcriptional apparatus. The expression of the gene coding for apolipoprotein AI (apoAI), the major protein constituent of HDL, is controlled by a powerful liver specific enhancer located between nucleotides −222 to −110 upstream of the apoAI gene transcription start site (+1) (1). Activation of apoAI gene transcription requires synergistic interactions between transcription factors bound to three distinct sites, sites A (−214 to −192), B (−169 to −146), and C (−134 to −119) within this enhancer (2). Among the transcription factors implicated in apoAI gene regulation are members of the nuclear receptor superfamily (3) including retinoid X receptor (RXRα) and the orphan receptor, apolipoprotein AI regulatory protein (ARP-1) (4,5). We have previously shown that RXRα binds to apoAI site A and activates the nearby basal promoter in response to 9-cis retinoic acid (4) while ARP-1 also binds to site A but represses the ligand-dependent RXRα transcriptional activation (6). Retinoic acid receptor (RARα) homodimers do not bind to site A but RXRα/RARα heterodimers bind to site A with high affinity and activate transcription in response to both 9-cis retinoic acid and all-trans retinoic acid (7).

Hepatocyte Nuclear Factor 4 (HNF-4), a new member of the nuclear receptor superfamily, is required for the expression of a wide variety of genes involved in cholesterol metabolism and gluconeogenesis (8,9). In particular, HNF-4 is an important regulator of apolipoprotein genes including apoAI, apoB and apoCIII (10−12). Although HNF-4 contains significant sequence similarity to the mammalian RXRα receptor, it does not heterodimerize with any of the nuclear receptor superfamily nor does it respond to 9-cis-RA. In fact, HNF-4 has not been associated with a specific ligand and is therefore classified as an orphan receptor (9).

Although HNF-4 has been implicated in the regulation of apoAI, it remains to be determined if apoAI site A is indeed the responsive element for HNF-4. We have previously demonstrated that RXRα homodimers function as ligand-dependent transcriptional activators in yeast cells, which are devoid of endogenous RAR or other factors that bind to site A (13). In this report, we further utilize this genetic tool for a systematic analysis of the following: 1) reconstitution of HNF-4 function in yeast, 2) mutation analysis of site A, and 3) comparison of the responsiveness of several mutated site A to HNF-4 in yeast and mammalian cells.

*To whom correspondence should be addressed

MATERIALS AND METHODS

Chemicals and buffers

Restriction enzymes, T4 polynucleotide kinase and T4 DNA ligase were purchased from New England Biolabs. Calf intestinal alkaline phosphatase was from United States Biochemical Corporation; *Taq* polymerase from Perkin-Elmer Cetus; radioinert steroids, adenine sulfate, o-nitrophenyl β-D-galactopyranoside and uracil from Sigma; casamino acids, yeast nitrogen base and dextrose from Difco; glassperlen from B.Braun Melsungen AG. 9-cis-retinoic acid was synthesized by Lederle Laboratories.

Homogenization buffer contained 10 mM Tris, 0.1 mM EDTA, 2 mM dithiothreitol and 10% glycerol, pH 8.1 at room temperature (TEDG buffer). Transcription buffer for β-galactosidase assay contained 0.12 M sodium phosphate (dibasic), 0.04 M sodium phosphate (monobasic), 10 mM potassium chloride, 1 mM magnesium sulfate and 0.27% 2-mercapto-ethanol, pH 7.0 at room temperature.

Yeast strains

The *Saccharomyces cerevisiae* strain used was BJ2168 (MATa, prc1-407, prb1-1122, pep4-3, leu2, trp1, ura3-52) (14). Expression of HNF-4 was under the control of the CUP1 promoter as described previously (15). Growth and transformation of yeast cells were performed according to standard procedures (16).

Construction of plasmids (mammalian)

Expression vectors. The vector, pMT2-HNF4 expressing HNF-4 has been described previously (12).

Reporter plasmids. All reporter constructs were made by cloning different double-stranded oligonucleotides into a *Bam*HI site inserted at nucleotide position −41 upstream of the apoAI gene transcription start site (+1) in a previously described construct in which the apoAI gene promoter region −41 to +397 is fused to the bacterial chloramphenicol acetyltransferase (CAT) gene. Each of these single stranded oligonucleotides was synthesized with a 5′ GATC overhang to facilitate cloning into the *Bam*HI site of the reporter plasmid. The nucleotide sequence (5′ to 3′) of each of these oligonucleotides containing the entire site A (A), or the mutated versions of site A (A5′, A3′ or M2) is listed as below. The underlined sequences represent the imperfect direct repeats of site A.

5′-GATCACTGAACCCTTGACCCCTGCCCTGC-3′ (A)
5′-GATCACTGAACCCTTGACCCCTG-3′ (A5′)
5′-GATCCTTGACCCCTGCCCTGC-3′ (A3′)
5′-GATCACTGAACTGATGACCCCTG-3′ (M2)

Transient transfection assays

Maintenance of CV-1 cells, transfection of plasmid DNA into these cells, CAT and β-galactosidase assays have been described previously (16). The transfection mixtures contained 5 μg of each reporter plasmid, 5 μg of the β-gal expression vector pRSV-β-gal (16). The amount of pMT2-HNF-4 expression vectors used was 2 μg each and the final amount of plasmid DNA in the transfection mixture was adjusted to a total of 13−17 μg. Whole cell extracts containing HNF-4 were made by multiple freeze-thawing of the cells.

Yeast expression vector

Two unique restriction sites (*Eag*I and *Bss*HII) were inserted at the 5′ and 3′ ends of the HNF-4 coding sequence (8) for insertion into the high copy yeast expression vector (YEp351), which has been modified to carry the CUP1 promoter (15,17) and a synthetic linker containing *Eag*I and *Bss*HII restriction sites down stream of the ubiquitin gene as described previously (15,17). This was achieved by PCR-mediated mutagenesis. After complete digestion with *Eag*I and *Bss*HII, the expression vector was ligated to PCR amplified HNF-4 cDNA. The resultant receptor expression vector (YEp_cHNF-4) was used to transform the yeast strain, BJ2168, using the lithium acetate protocol (16). Transformants were selected by leucine auxotrophy.

Construction of yeast reporter plasmids

Oligonucleotides containing one or two copies of either the apoAI gene site A (A) or its mutated versions, A5′, A3′ and M2 were cloned into the unique *Xho*I site of the yeast reporter plasmid PC2 as described previously (15,17). The resulting reporter plasmids (YEpA) contained either site A or mutated site A upstream of the iso-1-cytochrome c (CYC1) promoter, which is fused to the β-galactosidase gene of *Escherichia coli*. These plasmids were used to transform yeast cells expressing the HNF-4 (YEp_cHNF4). The final double transformant yeast strain (YEp_cHNF4/YEpA) was selected by leucine and uracil auxotrophy.

Immunoblot analysis

Yeast cells carrying the HNF-4 expression plasmids were grown in synthetic complete (SC) medium. For receptor induction under the CUP1 promoter, 100 μM of cupric sulfate was added to the medium when the cell density reached an OD of 0.8−1.0 at 600 nm. After 2 h of copper induction, cells were harvested and yeast extracts were prepared according to standard protocols (15). Protein samples were electrophoresed on 10% SDS−PAGE,

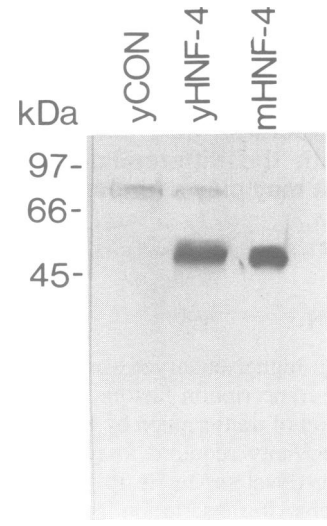

Figure 1. Immunoblot analysis of yeast-produced HNF-4. Aliquots of yeast extracts (50 μg) prepared from yeast cells without (yCON) or with the HNF-4 expresson plasmids (yHNF-4) under induced conditions (+Cu) were resolved by 10% SDS−PAGE. Extracts (25 μg) containing CV1 cell-produced HNF-4 were used as positive control (mHNF-4). Proteins were transfered onto a nitrocellulose membrane, which was probed with a polyclonal antibody raised against a 12 amino acid peptide of HNF-4. Molecular weight standards are indicated in kilodalton (kDa).

electroeluted onto nitrocellulose and probed with a polyclonal antiserum raised against a 12 amino acid peptide of HNF-4 (18).

Electrophoretic mobility shift assays (EMSA)

The procedures for EMSA have been described previously (4,12). Briefly, double-stranded oligonucleotides corresponding to site A were labeled with ^{32}P-dATP by filling in reaction using Klenow enzyme. The labeled fragments (30,000 cpm/reaction) were incubated with cell extracts (30 μg). Bound and free DNAs were separated on a 6% non-denaturing gel. To determine the dissociation constants (K_d) of RXRα or HNF-4 for site A, a constant amount of yeast extracts containing these transcription factors was incubated with an increasing concentration (0.25 to 15 nM) of ^{32}P-labeled probe (site A) as described previously (12). The protein bound and free radioactivity were determined from the autoradiogram using a betascope. The data were converted into Scatchard plots (18) to obtain the K_d values.

Transcription assays

Double transformant yeast strains ($YEp_cHNF4/YEpA$) were grown in synthetic complete medium (without leucine and uracil). When cell density reached late-log phase (OD = 0.8), 100 μM of cupric sulfate was added to the medium for 4 h. Subsequently, yeast extracts were prepared and assayed for β-galactosidase activity as described previously (15,17).

RESULTS

HNF-4 expression in yeast

The HNF-4 cDNA was cloned into a high copy yeast expression plasmid under the control of a regulated promoter, CUP1 which can be induced by cupric sulfate as described previously (15). Production of HNF-4 in yeast transformants was confirmed by Western blot analysis using a HNF-4 specific antibody (8). Extracts prepared from yeast transformants under induced conditions (+Cu) contain an immunoreactive polypeptide with an apparent molecular weight of 54 kDa (Fig. 1, yHNF-4) comigrating with the mammalian (CMT cells)-produced HNF-4 (mHNF-4). There was no specific immunoreaction between the antiserum and the yeast control extracts containing the mouse androgen receptor (17) (yCON). The 54 kDa band was also observed in extracts prepared from the yeast strain carrying the HNF-4 expression plasmid under non-induced conditions (-Cu) (data not shown), indicating that the CUP1 promoter is leaky as we have described previously (15).

HNF-4 binds to site A of the apoAI promoter

In vitro binding of HNF-4 to site A was evaluated by electrophoretic mobility shift assays (EMSA) as described previously (12). The results in Figure 2 show that a distinct retardation complex (bottom arrow) was detected when the ^{32}P-labeled site A probe was incubated with yeast extracts (yHNF4) prepared from the yeast strain carrying the HNF-4 expression vector under inducing conditions (lane 2). This retardation complex could be supershifted to form a slower migrating complex (top arrow) by incubating the reaction mixtures with anti-HNF-4 polyclonal antiserum (lane 3). For comparison, retardation complex formation and supershifting were also observed for HNF-4 produced in CMT cells (lanes 4 and 5) but not in control yeast extracts (yCON) (lane 8) under the same binding conditions. The complex observed for HNF-4 (complex 1) is specific for site A since it is competed by 100-fold molar

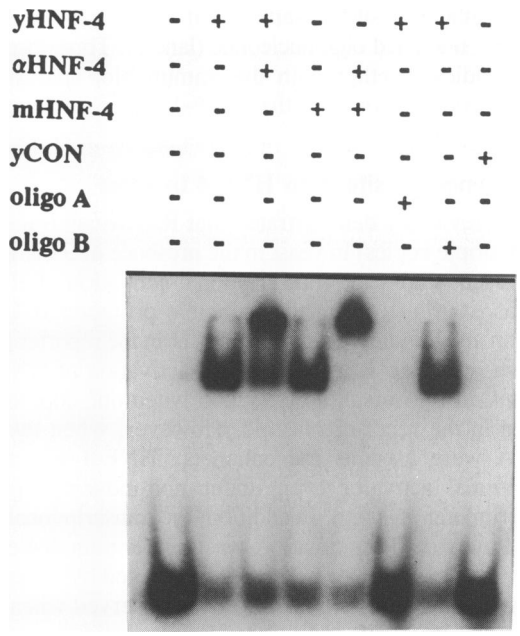

Figure 2. Electrophoretic mobility shift assays (EMSA) of HNF-4. Extracts (30 μg) from yeast cells producing HNF-4 (yHNF-4) or from control yeast cells (yCON) were analyzed by EMSA as described in Materials and Methods. Extracts (5 μg) containing CV1-produced HNF-4 were used as positive controls (mHNF-4). For supershift, 1 μl of polyclonal antibody (αHNF-4) was added to the binding reactions (lanes 3 and 5). Binding specificity was determined by adding 100 fold-molar excess of unlabaled oligo A (lane 6) or unlabeled oligo B, which contains a sequence encoding a protein-binding site of Epstein Barr virus DNA (lane 7). Retardation and supershift complexes are indicated by the bottom and top arrows respectively.

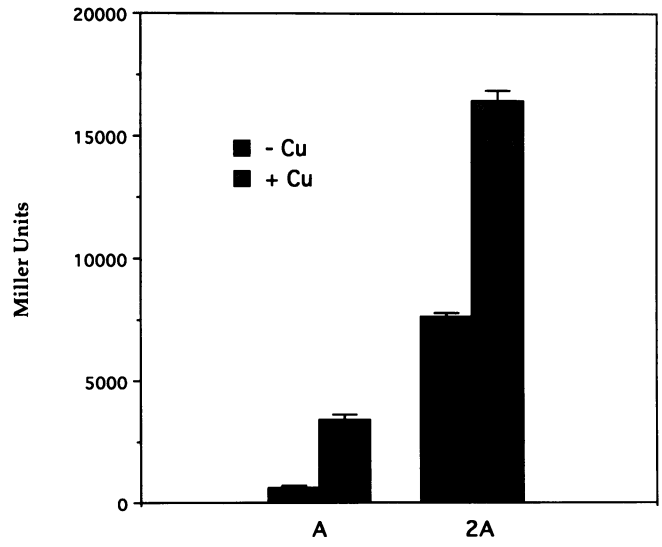

Figure 3. Transcriptional activation of HNF-4 in yeast. Yeast strains carrying the HNF-4 expression plasmid and the reporter plasmids containing single copy (A) or two copies (2A) or site A were grown overnight at 30°C min synthetic complete media in the absence or presence of copper (Cu). Induction of β-galactosidase enzyme (Miller Units) was measured in yeasat extracts as described in Materials and Methods. Values represent three separate experiments with standard errors of the mean indicated.

excess of unlabeled site A (lane 6) but not by a 100-fold molar excess of an unrelated oligonucleotide (lane 7). Thus, these DNA-binding studies together with the immunoblot analysis in the previous section demonstrate that HNF-4 can be stably expressed in yeast.

Responsiveness of site A to HNF-4 in yeast

We have previously demonstrated that RXRα can transactivate site A (multiple copies) in yeast in the presence of 9-cis-RA (13). In the present study, the transcriptional activation of HNF-4 in yeast was examined using the inducible promoter (CUP1) for expression and the same response element in the reporter plasmid. As shown in Figure 3, transcriptional activation of HNF-4 was dependent on cupric sulfate induction when one copy of site A was used in the reporter plasmid. However, when two copies of site A were used as the enhancer, HNF-4 was a potent transcriptional activator even under non-inducing conditions (CUP1 promoter is leaky). In addition, the transcriptional activity mediated by two copies of site A was greater than that expected by a simple summation of the activity exhibited by a single copy of site A. Similar effects have also been observed when HNF-4 was replaced by RXRα (data not shown).

Differential binding affinities of RXRα and HNF-4 for site A

The relative potency of a transcriptional activator is determined partly by the affinity for its binding site on the DNA. Since the DNA-binding domain of HNF-4 is very similar to that of RXRα with 60% amino acid identity, the binding affinities of these transcription factors for site A were compared by Scatchard analysis. The top panels of Figure 4A and 4B are the autoradiograms of the retardation complexes formed with increasing amounts of radiolabeled oligo A probe for RXRα and HNF-4 respectively. Scatchard plot analyses of these data revealed K_d values of 11.0 nM for yRXRα and 4.7 nM for yHNF-4. Thus, these binding kinetic studies show that HNF-4 binds to site A with a higher affinitiy than RXRα.

Distinct sequences within the apoAI site A confer responsiveness to HNF-4 in yeast and mammalian cells

ApoAI site A consists of three imperfect TGACC direct repeats separated by a trinucleotide spacer (CCT) at the 5′ portion of site A and a dinucleotide spacer (CC) at the 3′ portion. In order to further establish the role of site A in HNF-4 induced transactivation, several mutants of site A were constructed and analyzed for transactivation by HNF-4 in yeast and mammalian cells. The yeast strain carrying the HNF-4 expression plasmid was transformed with reporter plasmids containing one copy of site A or various mutated versions of site A and the resultant double transformant yeast strains were analyzed for transcriptional activity under non-induced or induced conditions. The mutated versions of site A used for these experiments were the two direct repeats separated by the trinucleotide spacer at the 5′ portion of

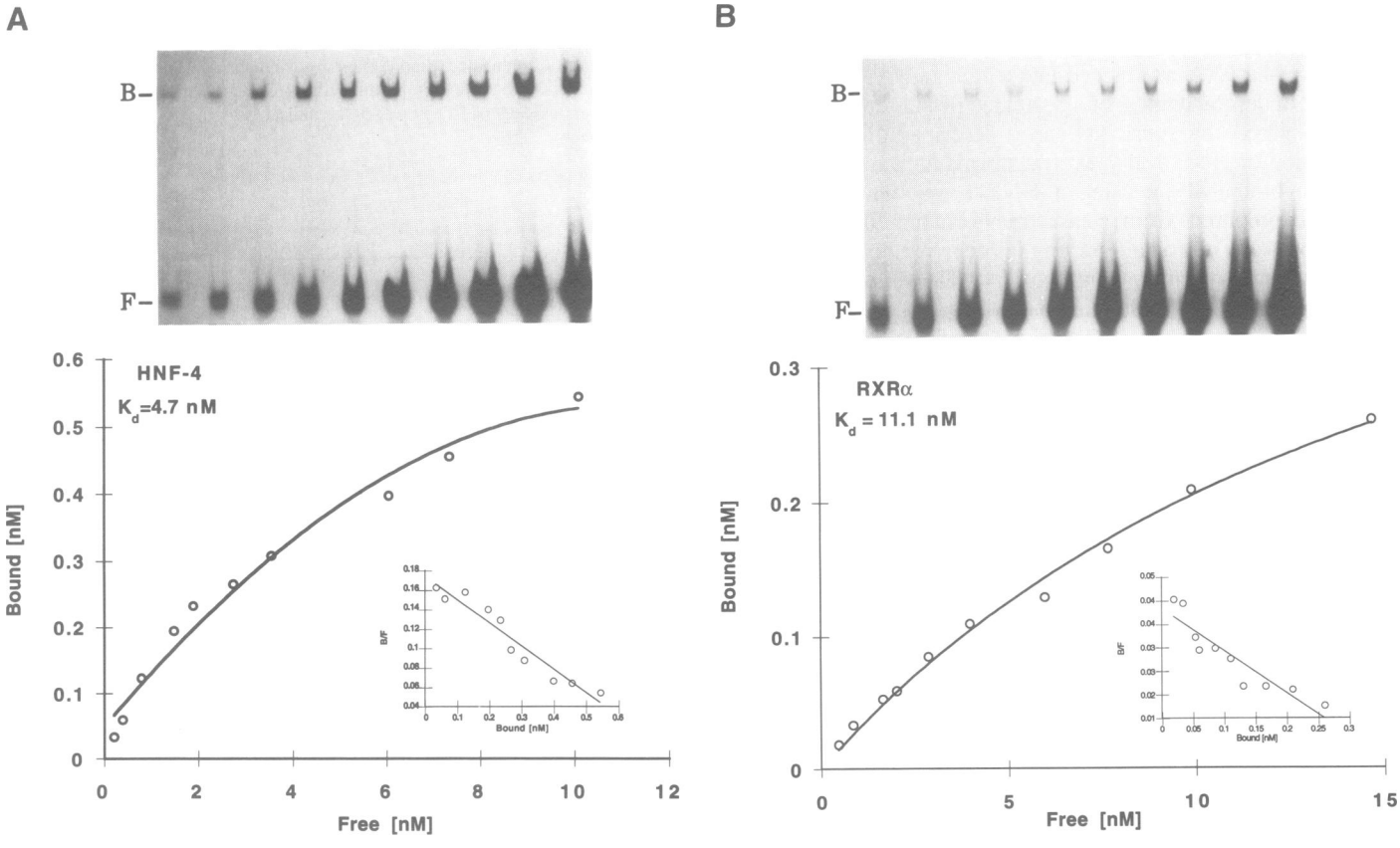

Figure 4. Binding affinities of HNF-4 and RXRα for site A. A constant amount of yeast extracts (10 μg) containing yHNF-4 or yRXRα (13) were incubated with an increasing concentration (0.15 to 10 nM) of ^{32}P-labeled oligo A as described previously (6). The protein bound (B) and free (F) radioactive oligo A were separated by EMSA and determined from the autoradiograms (top panels) shown in **A** and **B**. The bound radioactivity in the retardation complexes were used to construct binding saturation curves (**A** and **B**; bottom panels) and the data were converted into Scatchard plots (inserts) to obtain K_d values. Three separate experiments gave similar K_d values.

site A (A5′), the two direct repeats separated by the dinucleotide spacer at the 3′ portion of site A (A3′) and the mutated version of A5′ with the trinucleotide spacer changing from CCT to TG-A (M2). As shown in Figure 5, transcriptional activation as measured by induction of β-galactosidase was not apparent in these yeast strains under non-induced conditions except for mutant A5′. However, when the CUP1 promoter was induced by copper, highest reporter enzyme induction was detected in the yeast strains containing the mutant, A5′, followed by wild type site A. The mutant, A3′ was less responsive to HNF-4 whereas M2 mutant was essentially inactive.

In order to compare the responsiveness of site A and its mutants to HNF-4 in mammalian cells, we performed transient transfection assays using CV1 cells. When these reporter constructs were cotransfected with the HNF-4 expression vectors in CV1 cells, high levels of CAT activity were observed for the reporter construct containing A5′ followed by the entire site A (A) whereas the constructs containing A3′ and M2 were less responsive to HNF-4 (Fig. 6). Thus, these mammalian transcription data correlate very closely to those observed for the yeast cells, suggesting that the differential transcriptional activity exhibited by distinct sequences within apoAI site A is mediated by HNF-4 per se. Furthermore, these data suggest that the A5′ portion of site A containing the two direct repeats separated by the trinucleotide (CCT) is the predominant target for HNF-4 transactivation and the nucleotide sequence of the trinucleotide spacer in the A5′ plays an essential role in HNF-4 induced transactivation.

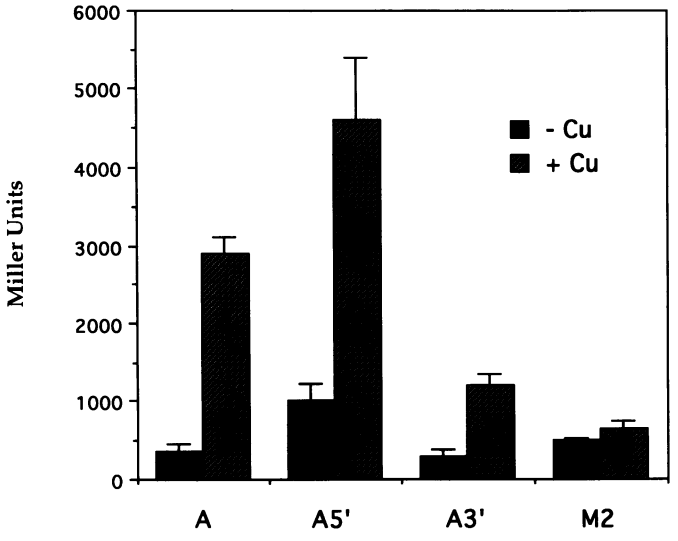

Figure 5. Mutational analysis of apoAI site A in yeast. Yeast strains carrying HNF-4 expression plasmid and the reporter plasmids containing single copy of site A or mutated versions of site A (A5′, A3′ and M2) were grown in synthetic complete media in the absence or presence of copper (Cu). Induction of β-galactosidase enyzyme in Miller units was measured in yeast extracts. Values represent three separate experiments with standard errors of the mean indicated.

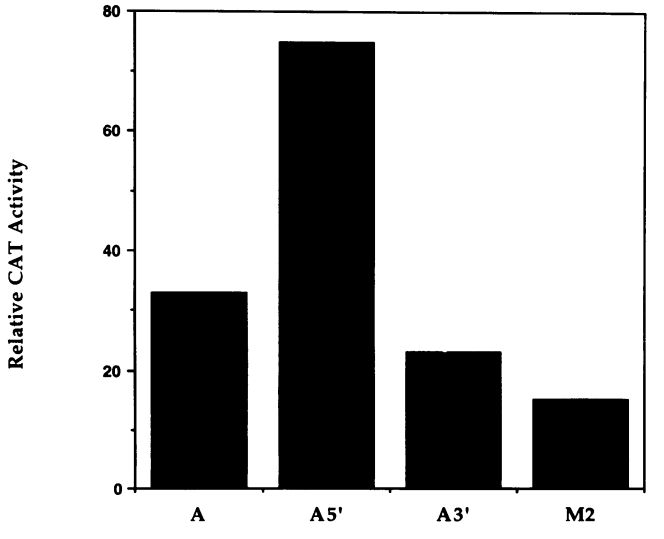

Figure 6. Mutational analysis of apoAI site A in mammalian cells. The reporter constructs containing either wild type site A (A) or mutated versions of site A (A5′, A3′ or M2) were tested for their responsiveness to HNF-4 by transient transfection assays in CV1 cells as described in Materials and Methods. The CAT activity was expressed relative to the normalized CAT activity of the construct in the absence of cotransfected receptor expression vectors (Relative CAT Activity). The data shown represent the average of two independent experiments.

Binding of HNF-4 to site A mutants

The binding of HNF-4 to the mutated versions of site A was further examined by EMSA (Fig. 7). At 100-fold molar excess, the entire site A and A5′ competed efficiently for HNF-4 binding (lanes 3 and 5) whereas A3′ competed less efficiently (lanes 7). M2 could not compete for HNF-4 binding at 10- or 100-fold molar excess (lanes 8 and 9). It is apparent that HNF-4 binds to A5′ with higher affinity than the entire site A as reflected by their ability to compete for the labeled probe at 10-fold molar

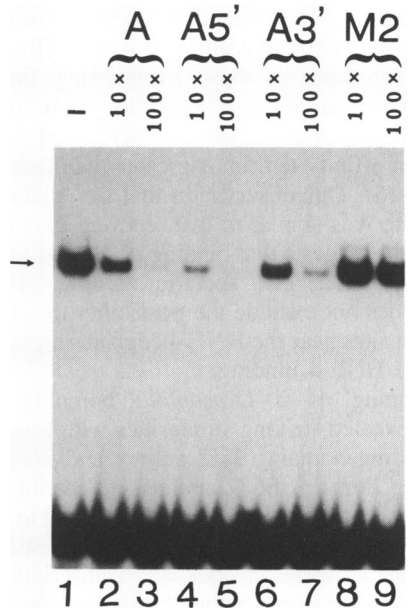

Figure 7. Binding of HNF-4 to site A mutants. Extracts (20 μg) containing yHNF-4 were incubated with ^{32}P-labeled oligo A in the absence (lane 1) or presence of unlabeled site A (lanes 2 and 3), A5′ (lanes 4 and 5), A3′ (lanes 6 and 7) or M2 (lanes 8 and 9) and analyzed by EMSA as described previously. All unlabeled oligo A or site A mutants were present at 10 or 100 fold-molar excess in the binding reactions.

excess (lanes 2 and 4). These binding data further support the results of our transcription experiments indicating that the structural determinants of site A dictate its ability to bind and respond functionally to HNF-4.

DISCUSSION

The general notion that the basic mechanisms of RNA polymerase II transcription between yeast and mammals are very similar is supported by the successful reconstitution of many mammalian nuclear receptor function in yeast cells (13,15,17,19–21). The biochemical and transcriptional studies in this report clearly demonstrate that HNF-4 can function as a transcriptional activator in yeast via apoAI site A. Although HNF-4 is a ligand-independent transactivator of the reporter containing a single copy of site A, HNF-4 is a potent ligand-independent transactivator in yeast as reflected by the synergistic activation of multiple copies of site A. Such synergism between multiple hormone response elements has been observed for other members of the nuclear receptor superfamily (22) and can be attributed by the cooperative binding of transcription factor dimers to two or more *cis*-acting elements. Alternatively, efficient transcription of the yeast basal promoter by HNF-4 may require more efficient recruitment of other activators or coactivators to enhance the contact between the enhancer bound transcription complexes and the basal transcription machinery. Thus, synergistic activation of the apoB promoter required both HNF-4 and C/EBP (a leucine zipper) whereas the transcriptional activation of HNF-4 on apoCIII promoter was enhanced by additional unknown factors bound to an upstream element of the promoter (8,9). Saturation analysis indicated that yHNF4 binds to apoAI site A with an affinity two times higher than that of yRXRα. The K_d value reported here for yRXRα (11.0 nM) is slightly higher than that reported for mammalian cell produced RXRα (8.8 nM). This discrepancy might be due to yet unidentified proteins endogenous to mammalian cells that heterodimerize with RXRα. In fact, the dissociation constants of these *trans*-acting factors can be modulated by heterodimerization with their partners. For example, heterodimeric versions of RXRα and ARP-1 bind to site A with an affinity ten times greater than that of RXRα or ARP-1 alone (6). Our observation that the binding affinity of yHNF4 for site A is similar to that reported in mammalian cell studies strongly suggests that binding of HNF-4 to site A is not influenced by other cell specific factors. However, this observation does not exclude the possibility that factors bound to the adjacent sites near the HNF-4 cognate responsive element may influence HNF-4 binding.

Recent cloning of a *Drosophila* homolog of HNF-4, HNF-4(D), revealed striking similarities with rodent HNF-4 in the DNA-binding domain (91% amino acid identity) and the ligand binding domain (66% amino acid identity) (23). This conservation suggests that HNF-4 might respond to a ligand. The transcriptional activity of HNF-4 observed in yeast might argue that HNF-4 is a ligand-independent transcription factor or alternatively, HNF-4 does respond to a ligand, which is present in yeast. The ability of HNF-4 to activate transcription in different cell types including HepG2, CV-1, Hela and yeast suggests that the ligand for HNF-4 if present is ubiquitous in nature.

The apoAI site A is a complex *cis*-acting element, which responds to several members of the steroid/thyroid receptor superfamily (2,3). The core motif of this response element consists of three imperfect direct repeats separated by two spacers. Our deletion analysis of site A revealed that 5′ portion of site A (Mutant A5′) composed of the two TGACC imperfect direct repeats separated by the trinucleotide spacer (CCT) responds more efficiently to HNF-4 than the entire site A. Transcriptional activity was dramatically reduced when the first imperfect repeat at the 5′ end was deleted (mutant A3′). Interestingly, changing the trinucleotide spacer (CCT) of A5′ mutant to TGA (mutant M2) rendered the DNA element unresponsive to HNF-4. While it has been demonstrated that the number of nucleotides in the spacer is an important structural determinant for different response elements, i.e. 3, 4, 5 rule (24,25), our data demonstrate the functional significance of the nucleotide composition within the spacer. In fact, the functional importance of the tridnucleotide spacer (CCT) of the A5′ mutant is indicated by its remarkable conservation among human (26), rat (27) and rabbit (28). The above transcriptional data obtained from yeast cells are further supported by the *in vitro* DNA binding studies and more intriguingly, are remarkably similar to those observed in CV1 cells. Since site A can be occupied by a large number of nuclear receptors including RXRα, ARP-1, EAR1, EAR3 and several heterodimers, the issue of selectivity is of fundamental importance in regard to specific signal transduction pathways. Based on our current observations, it is highly suggestive that the nucleotide sequence in the spacer between the repeats of the hormone responsive element may be an important determinant of nuclear receptor recognition.

Taken together, we conclude that site A is an important integral point of signal transduction by several members of the nuclear receptor superfamily (3) and has preserved critical structural requirements for selective binding and transactivation by HNF-4. This unique structural determinant of site A in conjunction with the differential binding affinities by various members of the nuclear receptor superfamily may play a fundamental role in apoAI gene regulation in liver.

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