The activation function-1 of hepatocyte nuclear factor-4 is an acidic activator that mediates interactions through bulky hydrophobic residues¹

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The hepatocyte nuclear factor-4 (HNF-4) contains two transcription activation domains. One domain, activation function-1 (AF-1), consists of the extreme N-terminal 24 amino acids and functions as a constitutive autonomous activator of transcription. This short transactivator belongs to the class of acidic activators, and it is predicted to adopt an amphipathic α -helical structure. Transcriptional analysis of sequential point mutations of the negatively charged residues (Asp and Glu) revealed a stepwise decrease in activity, while mutation of all acidic residues resulted in complete loss of transcriptional activity. Mutations of aromatic and hydrophobic amino acids surrounding the negatively charged residues had a much more profound effect than mutations of acidic amino acids, since even a single mutation of these residues resulted in a dramatic decrease in transactivation, thus dem-

onstrating the importance of hydrophobic residues in AF-1 activity. Like other acidic activators, the AF-1 of HNF-4 binds the transcription factor IIB and the TATA-binding protein directly *in vitro*. In addition, the cAMP-response-element-binding-protein, a transcriptional adapter involved in the transactivation of a plethora of transcription factors, interacts with the AF-1 of HNF-4 and co-operates in the process of transactivation by HNF-4. The different protein targets of AF-1 suggest that the AF-1 of HNF-4 may be involved in recruiting both general transcription factors and chromatin remodelling proteins during activation of gene expression.

Key words: Co-activators, nuclear receptors, transcriptional activation.

INTRODUCTION

Hepatocyte nuclear factor-4 (HNF-4), a member of the superfamily of nuclear hormone receptors, was originally identified by its importance in the regulation of liver-specific genes and is also expressed in the pancreas, kidney, stomach, skin and intestine [1–3]. HNF-4 is a crucial regulator of several metabolic pathways, including those for glucose and lipid homeostasis and mutations in HNF-4 impair insulin secretion and cause Type 2 diabetes mellitus [4]. In addition, HNF-4 is a key transcriptional activator for apolipoprotein gene expression [5–9]. It was recently shown that fatty acyl-CoA thioesters bind to HNF-4 and modulate its transcriptional activity [10]. HNF-4 also plays a vital role in development. In mice, HNF-4 transcripts have been detected as early as day 4.5 and its knock-out impairs gastrulation and is embryonic lethal [3,11].

In a previous study, we performed a systematic analysis of the functional domains of HNF-4 that are involved in DNA binding, dimerization, and transactivation [12]. We have found that HNF-4 contains two activation functions (AFs), designated AF-1 and AF-2, located in the A/B and D/E regions respectively, which activate transcription in a cell type-independent manner. In most cell types examined, AF-1 and AF-2 synergize for full HNF-4 activity. The AF-1 consists of the extreme N-terminal 24 amino acids and functions as a constitutive autonomous activator of transcription [12]. Like many other transcriptional activators

the AF-1 of HNF-4 is rich in acidic amino acids, and secondary-structure analysis predicts it will form an amphipathic α -helix. Acidic activator domains were first described in yeast GAL4 and GCN4 activator proteins [13,14]. In the GAL4 protein, transcriptional activation is mediated by acidic amino acid residues and activation closely correlates with net negative charge. These results led to the hypothesis that acidic amino acids are crucial for transactivation. However, several studies with many acidic activation domains indicated the importance of hydrophobic amino acids for activation, whereas single acidic amino acid substitutions had little or no effect [15–18]. Furthermore, many acidic activators were predicted to form amphipathic α -helices [19]. Remarkably, when helix-incompatible amino acids were substituted into the predicted α -helical structure of the VP16 activator, activation was retained at wild-type levels suggesting that an α -helical structure is not required for activation by VP16 [15]. In addition, from studies with the yeast transcriptional activator, GCN4 [20], it was proposed that acidic activators can also act as unstructured acidic ‘blobs’ [21]. Structural studies employing CD have suggested yet another model for the activation domains of GAL4 and GCN4 that can adopt β -sheet conformations [22]. Further studies of acidic activation domains will help to identify conserved amino acid residues that are important for the activation functions of these proteins.

In the present study we show that the AF-1, present between amino acids 1–24 at the N-terminus of HNF-4, is an acidic

Abbreviations used: AF-1/AF-2, activation function 1/2; β -gal, β -galactosidase; CAT, chloramphenicol acetyltransferase; CREB, cAMP-responsive-element-binding protein; CBP, CREB-binding protein; DTT, dithiothreitol; GST, glutathione S-transferase; HepG2 cells, human hepatoma cells; HNF-4, hepatocyte nuclear factor-4; NP40, Nonidet P40; PBST, PBS containing 0.1% Tween 20, 5% non-fat dry milk and 0.5% BSA; TBP, TATA-binding protein; TFIIB, transcription factor IIB.

¹ This paper is dedicated to the memory of Christos Cladaras, an inspired scientist, who died suddenly on 10 August 1994.

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activator. Mutational analysis of acidic amino acids that eliminate the negative charge, and of aromatic and hydrophobic amino acids to either Ala or the helix-breaker Gly, revealed that bulky hydrophobic residues are critical for AF-1 transactivation. These results, and sequence comparison with other acidic activators, indicated that the AF-1 of HNF-4 is complex and shares significant structural similarities with activation domains found in other well-studied transcriptional activators. Like other acidic activators, the AF-1 of HNF-4 binds multiple protein targets. The different protein targets of AF-1 suggest that the AF-1 of HNF-4 may be involved in recruiting both general transcription factors and chromatin remodelling proteins during activation of gene expression.

EXPERIMENTAL

Plasmid construction

All constructs were generated using PCR. Oligonucleotide-directed mutagenesis was used to introduce mutations into the GAL-CD10 plasmid encoding the N-terminal 49 amino acids of HNF-4 [12]. The PCR-amplified fragments were subcloned into the *Eco*RI/*Bam*HI sites of the pBXG1 vector containing the GAL-4DBD (1–147) [12]. PCR reactions were carried out using as 5' primer oligonucleotides listed in Table 1 and as 3' primer oligonucleotide GAL-49RB: 5'-TCTAGAGGATCCTCAGG-CACTGACACCCAGGCTTGG-3'. All mutations were verified by DNA sequencing. The point mutations are denoted as the amino acid (in one-letter code), its position and the amino acid with which it was replaced, e.g. D2, 5, 11A/E18, 20A indicates that Asp at positions 2, 5 and 11 and Glu at positions 18 and 20 were replaced with an Ala residue. Table 1 shows the sequence of oligonucleotides used as 5' primers and the names of the corresponding plasmids. Underlined letters indicate deviations from the wild-type sequence.

The construction of expression plasmids for HNF-4 and its deletion derivatives, as well as the GAL-HNF-4 chimaeras, have been described previously [12]. The glutathione S-transferase (GST)-CBP (1–771) fusion construct, the wild-type cAMP-response-element-binding-protein (CREB)-binding protein (CBP) and mutant CBP (1–771) [23], the transcription factor IIB (TFIIB) and TATA-binding protein (TBP) cDNAs and pGEX-B plasmids were generously given by Dr Dimitris Thanos (Department of Biochemistry and Molecular Biophysics, Columbia University, New York, NY 10032, U.S.A.).

Cell transfection and CAT assay

Plasmids were transfected into human hepatoma (HepG2) cells and assayed for their ability to promote transcription of the promoterless chloramphenicol acetyltransferase (CAT) gene. HepG2 cells were maintained as stocks in Dulbecco's modified Eagle's medium ('DMEM') supplemented with 10% fetal-calf serum. Transient transfections were performed on 60%-confluent cells in 60 mm diameter dishes using the calcium phosphate coprecipitation method [24]. The transfection mixture contained 10 µg of the reporter plasmid, pG₅CAT, which has five GAL4 DNA-binding sites upstream of the β-globin promoter and the CAT gene [12], 5 µg of the various mutant GAL-CD10 plasmids listed in Table 1, and 3 µg of rous-sarcoma-virus-β-galactosidase (β-gal) plasmid. For the transfection experiments with CBP, 30 mm-diameter dishes were used, and the reporter construct was pG₅CAT (3 µg) and co-transfections were performed with 0.1 µg of GAL-HNF-4, GAL-CD10, or GAL-M3K/Y6N/Y14H/L17P in the absence or presence of 1.25 µg of CBP. In

every case vector DNA was added as added as necessary to achieve a constant amount of transfected DNA. Cells were harvested 42 h later and lysed by freeze-thawing. The β-gal activity of the cell lysates was determined as previously described [25] and the values were used to normalize variabilities in the efficiency of transfection. CAT activities were determined using [¹⁴C]chloramphenicol and acetyl-CoA as described previously [26]. CAT enzyme levels that exhibited more than 60% conversion of acetylated product were diluted and reassayed for CAT activity in the linear range. The CAT values represent the mean ± S.E.M. for five independent transfection experiments. For the CBP experiments, the CAT values represent the mean for at least three independent transfections each carried out in duplicate.

Western-blot analysis

COS-1 cells were transfected with the different GAL-fusion constructs (as shown below) in Figure 2. Protein extracts corresponding to approx. 0.5×10^6 cells/lane were combined with $5 \times$ loading buffer [0.3 M Tris/HCl (pH 6.8)/10% (w/v) SDS/50% (v/v) glycerol/25% (v/v) β-mercaptoethanol/0.05% Bromophenol Blue] in a total volume of 50 µl and separated on SDS/12%-PAGE gel. Proteins were transferred to an Immobilon-P membrane (Millipore, Bedford, MA, U.S.A.) by electro-blotting. Membranes were preincubated in PBS containing 0.1% Tween 20, 5% non-fat dry milk and 0.5% BSA (PBST) for 1 h at 25 °C. Subsequently, they were incubated with the primary anti-GAL4 rabbit polyclonal antibody (Upstate Biotechnology, Waltham, MA, U.S.A.) at a dilution of 1:1000 in PBST, for 1 h at 25 °C. Membranes were washed with PBS containing 0.1% Tween 20 and incubated with the secondary antibody, goat anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology Inc.) at a dilution of 1:50000 in PBST for 1 h at 25 °C. Membranes were washed in PBS containing 0.1% (v/v) Tween 20 and once in PBS, and proteins were revealed by exposure to ECL[®] (enhanced chemiluminescence) reagent (Amersham International) according to the manufacturer's specifications.

In vitro transcription and translation

³⁵S-labelled proteins were produced from cDNA templates cloned downstream of T7 RNA polymerase promoter using the TNT Quick-coupled-reticulocyte-lysate system (Promega).

Preparation and expression of GST-fusion proteins

Fresh transformants of the *Escherichia coli* strain BL21 containing the plasmid for the GST-fusion protein were grown in Luria-Bertani ('LB') media supplemented with ampicillin (50 µg/ml) at 37 °C until a D_{550} of 0.5 was reached. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM. After incubation for 2–3 h, cells were harvested by centrifugation at 4000 g for 10 min. Cell pellets were resuspended in 1/20 of the original culture volume in PBS containing 10% glycerol, 1% Nonidet P40 (NP40), 1 mM dithiothreitol (DTT), 100 mM EDTA, 0.1 mM benzamidine, 2 µg/ml aprotinin, 1 µg/ml pepstatin and 1 µg/ml leupeptin, and the cells were lysed by sonication on ice. Cell debris was removed by centrifugation at 10000 g for 20 min, and the supernatant was incubated with glutathione-Sepharose 4B beads or glutathione-agarose beads equilibrated with for 2 h in PBS containing 10% glycerol, 1% NP40, 1 mM DTT, 10 mM EDTA, 0.1 mM benzamidine, 2 µg/ml aprotinin, 1 µg/ml pepstatin and 1 µg/ml

Table 1

Sequences of mutated oligonucleotides used to generate the point mutations in the AF-1 domain of HNF-4. The underlined nucleotides show the positions of the mutations.

Construct	5' Primer
D2A D5A	5'-GATATCGAATTCATGG(A/C)CATGGCTG(A/C)CTACAGTCTGCCTTG-3'
D2N D5N D2, 5/N	5'-GATATCGAATTCATG(G/A)ACATGGCT(G/A)ACTACAGTGC-3'
D11A E20A D11A/E20A E18A/E20A	5'-GATATCGAATTCATGGACATGGCTGACTACAGTCTGCCTTG(A/C)CCCAGCCTACACCACCCTGG(A/C)GTTT(A/C)GTTT(A/C)GAATGTGCA-3'
D11N E18Q E20Q D11N/E18Q E18Q/E20Q	5'-GATATCGAATTCATGGACATGGCTGACTACAGTCTGCCTTG(G/A)ACCCAGCCTACACCACCCTG(G/C)AGTTT(G/C)AGAATGTGCA-3'
D2, 5, 11A/E18, 20A	5'-GATATCGAATTCATGGCCATGGCTGCCTACAGTCTGCCTTGCCCCAGCCTACACCACCCTGGCGTTTGCGAATGTGCA-3'
D2, 5, 11N/E18, 20Q	5'-GATATCGAATTCATGAACATGGCTAACCTACAGTCTGCCTTGAAACCAGCCTACACCACCCTGCAGTTTCAGAATGTGCA-3'
Y6G Y6A	5'-GATATCGAATTCATCGACATGGCTGACG(G/C)CAGTCTGCCTTGGA-3'
L10G L10A	5'-GATATCGAATTCATGGACATGGCTGACTACAGTCTGCCG(G/C)CGACCCAGCC-3'
Y14G Y14A	5'-GATATCGAATTCATGGACATGGCTGACTACAGTCTGCCTTGACCCAGCCG(G/C)CACCACCCTGGA-3'
L17G L17A	5'-GATATCGAATTCATGGACATGGCTGACTACAGTCTGCCTTGACCCAGCCTACACCACC(G/C)CGAGTTTGAAAATGT-3'
M3K/Y6N/Y14H/ L17P	5'-GATATCGAATTCATGGACAAGGCTGACAACAGTCTGCCTTGACCCAGCCACACACCACCCGGAGTTTG-3'

Figure 1 Characteristics of the AF-1 domain of HNF-4

Left panel: schematic representation of an α -helix formed by the N-terminal sequence of HNF-4 as predicted by the algorithms of Chou and Fasman [27] and Garnier et al. [28]. The numbers indicate positions of amino acids from the N-terminus. The four hydrophobic residues targeted for mutagenesis are shown in open boxes and the acidic residues targeted for mutagenesis are shown in **bold** letters. Right panel: the amino acid sequence containing AF-1 is shown in single-letter code for hHNF-4 α _{1,2}, hHNF-4 α ₄, rHNF-4 α ₂, mHNF-4 α ₇, hHNF-4 γ , and xHNF-4. Species designations are as follows: h, human; r, rat; m, mouse; x, *Xenopus*. Numbers indicate positions of amino acids. The grey-shaded box highlights the region of homology within the AF-1 domain.

leupeptin. The coupled proteins were washed several times with the PBS buffer and were stored at 4 °C. The purity of proteins bound to the beads was identified by SDS/PAGE.

Protein-binding assays using GST-fusion proteins

For the binding assays, 5–50 μ l of GSH-agarose beads containing approx. 0.5–1 μ g of the fusion protein were incubated at 4 °C in 0.5 ml of buffer containing 150 mM NaCl, 20 mM Tris/HCl, pH 7.5, 0.25 % BSA, 0.25 % NP40, 1 mM DTT and 0.5 mM PMSF. For the binding assay of GST-CBP1, incubation was performed in 0.35 ml of 150 mM sodium acetate, pH 7.0, 25 mM Hepes (pH 7.2), 2 mM EDTA, 0.25 % BSA, 0.1 % NP40,

5 mM DTT and 1 mM PMSF (GST-CBP1). Equal amounts of *in vitro* translated protein were added, and binding was allowed to proceed for 2 h at 4 °C. The beads were washed three times with the same buffer and then once with the same buffer lacking BSA. The bound proteins were eluted with 50 μ l of SDS/PAGE-loading buffer and were resolved by electrophoresis. The proteins were revealed by autoradiography.

RESULTS

Characteristics of the AF-1 domain of HNF-4

In a previous study we showed the presence of a potent activator domain in region A/B of HNF-4, spanning residues 1–48

Construct	Amino Acid Sequence
Gal-CD10	MDMADYSAALDPAYTTLEFENVQV
D2A	-A-----
D2N	-N-----
D5A	---A-----
D5N	---N-----
D2,5N	-N--N-----
D11A	-----A-----
D11N	-----N-----
E20A	-----A-----
D11A/E20A	-----A-----A-----
E18A/E20A	-----A-----A-----
E18Q	-----Q-----
D11N/E18Q	-----N-----Q-----
D11N/E20Q	-----N-----Q-----
E18Q/E20Q	-----Q-----Q-----
D2,5,11A/E18,20A	-A--A-----A-----A-----A-----
D2,5,11N/E18,20Q	-N--N-----N-----Q-----Q-----
Y6G	----G-----
Y6A	----A-----
L10G	-----G-----
L10A	-----A-----
Y14G	-----G-----
Y14A	-----A-----
L17G	-----G-----
L17A	-----A-----
M3K/Y6N/Y14H/L17P	--K--N-----H--P-----

Figure 2 Targeted mutagenesis of the AF-1 domain

A series of constructs were made containing the AF-1 domain of HNF-4, and mutants of this domain fused in-frame with the yeast GAL-4DBD (1–147). The single-letter amino acid sequence is shown for the wild-type AF-1 domain (GAL-CD10) with the residues targeted for mutation underlined. Underneath is the series of mutants named as described in the Materials and methods section. For each mutant, only the amino acids that have been changed are shown.

(construct GAL-CD10) [12]. Algorithms by Chou and Fasman [27] and Garnier et al. [28] predicted that the region spanning residues 1–24 has the potential to adopt an α -helical structure whereas the region spanning residues 25–48 is unstructured (Figure 1, left panel). We previously demonstrated that the unstructured region is dispensable for the transcriptional activity of AF-1 and that 100 % of this activity resides within the first 24 amino acids [12]. Interestingly, this region is most highly conserved among human, rat and *Xenopus* HNF-4 α and human HNF-4 γ (Figure 1, right panel). Furthermore, within the α -helix, hydroxylated and acidic amino acids are interrupted by a hydrophobic amino acid (Leu or Val) every seven residues (Figure 2).

Negatively charged amino acids influence AF-1 activity of HNF-4

To investigate whether the hydrophobic residues or the α -helical structure in HNF-4 are involved in the activation of AF-1, we introduced substitution mutations within the N-terminal half of the A/B domain of HNF-4 in construct GAL-CD10 (Figure 2). The presence of five negatively charged residues within this region indicated that it might fall within the acidic class of activation domains. Therefore, by creating single amino acid substitutions of acidic amino acids and combinations of multiple acidic amino acid substitutions, the importance of these residues and the net negative charge of the AF-1 activation domain was tested. We have mutated acidic residues (Asp 2, 5, 11 and Glu

Figure 3 The role of negatively charged amino acids in the AF-1 of HNF-4 transcriptional activity

The reporter plasmid pG₅CAT was co-transfected into HepG2 cells with pRSV- β -gal plasmid and effector plasmids expressing the AF-1 of HNF-4 (GAL-CD10) and the indicated point mutants. Cells were harvested 48 h later and assayed for CAT and β -gal activities. The normalized CAT values (CAT/ β -gal) are shown as the percentages of the activity obtained with the pG₅CAT reporter construct transfected with GAL-CD10. Results are means $\pm$ S.E.M. for five independent experiments.

Figure 4 Bulky hydrophobic residues are critical for AF-1 transcriptional activity

The reporter plasmid pG₅CAT was co-transfected into HepG2 cells with pRSV- β -gal plasmid and effector plasmids expressing HNF-4 AF-1 and the indicated point mutants. The normalized CAT values (CAT/ β -gal) are shown as the percentages of the activity obtained with the pG₅CAT reporter construct co-transfected with GAL-CD10. Results are means $\pm$ S.E.M. for five independent experiments.

Figure 5 Western-blot analysis of GAL-CD10 and its substitution mutants fused to GAL4-DBD (1–147)

COS-1 cells were transfected with the indicated constructs. Protein extracts corresponding to approximately 0.5×10^6 cells/lane were analysed by SDS/12%-PAGE, followed by transfer to an Immobilon-P membrane. GAL-fusion proteins were detected using an anti-GAL4 rabbit polyclonal antibody as described in the Materials and methods section. Arrows indicate the GAL-4DBD (1–147) and GAL-fusion proteins.

18, 20) to either Ala or the corresponding Asn or Gln (Figure 2). These mutants were analysed for their ability to transactivate the pG₅CAT reporter. As shown in Figure 3, analysis of 17 such mutants revealed that acidic amino acids are important for this activation domain. Generally, transcriptional activity correlated with net negative charge. Replacement of increasing numbers of acidic residues with uncharged residues led to a progressive decrease in transcriptional activation. Single amino acid substitutions at positions 2, 5, 11, 18 and 20 had an average activity of 40.44% and varied in the range from 18.0% (D11N) to

69.0% (E20Q), indicating that the location of the charged residue was also important. Double amino acid substitutions displayed activity that was in the range from 10.0 (D11N/E20A) to 32.0% (E18A/E20A), with an average of 16.16%. Removal of all five negatively charged amino acids in the AF-1 activation domain (D2, 5, 11A/E18, 20A and D2, 5, 11N/E18, 20Q) inactivated the transactivation potential to 3.0% of the parental construct. Single mutations to either Ala or an uncharged residue with a similar side chain (for example, Glu to Gln) had similar effects on the activity; D2A and D2N gave very close values as well as the mutants D5A and D5N, D11A and D11N, D11A/E20A and D11N/E20Q (Figure 3). Therefore the negative charge, but not the side chains of the residues, at these positions contributed to the transactivation potential.

The activity of the AF-1 activation domain is critically dependent on bulky hydrophobic and aromatic amino acids

To examine the importance of hydrophobic interactions in transcriptional activation by AF-1, single substitution mutations were introduced in the amino acid residues that comprise the hydrophobic surface of the α -helix (Figure 1, left panel). Substitution mutations were introduced in Tyr-6, Leu-17, Leu-10 and Tyr-14 to either Ala or Gly (Figure 2).

Mutations in either one of these aromatic and hydrophobic amino acids had a much more profound effect than mutation of acidic amino acids (Figure 4). Mutations of Tyr in position 6, Leu in position 10, Tyr in position 14 or Leu in position 17 to the helix-breaker Gly or to another hydrophobic amino acid, Ala, resulted in an activation domain retaining only 4.0–8.0% of wild-type activity. Substitution of all five acidic amino acids was necessary to achieve the same effect. Thus all four of the examined aromatic and hydrophobic residues were equally critical for the transcriptional activity of the AF-1 domain. Interestingly, replacement of these residues by Ala severely compromised AF-1 activity, indicating that hydrophobicity alone is not sufficient for

Figure 6 Interaction of HNF-4 with TFIIB and TBP *in vitro*

In vitro-translated and [³⁵S]methionine-labelled HNF-4 and HNF-4 deletion mutants were incubated with GST, GST-TFIIB or GST-TBP immobilized on GSH-agarose beads. Labelled HNF-4 proteins that were retained on the beads after extensive washing were analysed by SDS/PAGE and autoradiography, together with the *in vitro* translation product (INPUT). HNF-4 and the HNF-4 deletion mutants used are shown schematically on the left of the panel. The letters indicate the domains of HNF-4 and functional domains are marked above. The numbers represent amino acid residues.

AF-1 activity and that Leu and Ala residues were not interchangeable.

The expression levels and integrity of the wild-type AF-1 and its substitution mutants fused to the DNA-binding domain of GAL4 (1–147) in transfected COS-1 cells was monitored by Western-blot analysis using an anti-GAL4 antibody (Figure 5). In general the transfected cells were shown to express wild-type and mutant proteins in comparable amounts, with insufficient variations to account for the observed differences in their transcriptional activation potentials.

Targets for AF-1 HNF-4 transcriptional activation

As a first step towards identifying unique transcriptional targets of AF-1, we have used the immobilized-template assay to determine whether HNF-4 was capable of specifically interacting with the general transcription factor TBP. TBP is a reasonable target for specific interactions with HNF-4, since TBP was shown to be the first step in the assembly of the preinitiation complex [29]. In addition, it was shown that the association of TBP with TFIIB results in the stabilization of the preinitiation complex and the recruitment of RNA polymerase II, suggesting that TFIIB may also be a candidate for interacting with HNF-4 [29]. Glutathione S-transferase (GST) chimaeric fusion proteins containing human TBP or human TFIIB were expressed in *E. coli* and purified on GSH-agarose beads. ³⁵S-labelled HNF-4 protein was produced by *in vitro* transcription and translation, and was incubated separately with control GST beads and GST-TFIIB or GST-TBP containing beads. After washing, the bound protein was eluted and analysed by SDS/PAGE and autoradiography. This analysis showed that ³⁵S-labelled HNF-4 specifically interacted with TFIIB and TBP (Figure 6). Greater than 50% of the input of [³⁵S]HNF-4 was retained on the GST-TFIIB or GST-TBP beads. In the same experiment, HNF-4 was not retained on columns containing only GST, indicating that the HNF-4 interactions with TBP and TFIIB were specific (Figure 6). This experiment identified that one or more of the targets for HNF-4 transactivation are TBP and/or TFIIB. Furthermore, we have tested selective HNF-4 mutants to address specifically whether the AF-1 of HNF-4 interacts with TFIIB or TBP. Sequential-deletion mutants of HNF-4 that remove the AF-2 domain of HNF-4 (Figure 6) retained the ability to interact specifically with GST-TFIIB and GST-TBP (Figure 6). The ability of HNF-4 mutants to interact with TBP and TFIIB indicated that there is at least one interacting domain located within the N-terminal region of HNF-4 that encompasses the AF-1 domain.

Recently, we have also shown that HNF-4 recruits the CBP co-activator on promoters of genes that contain functional HNF-4 sites [30]. HNF-4 interacts with the N-terminal region of CBP (amino acids 1–771) and the C-terminal region of CBP (amino acids 1812–2441). The two activating functions of HNF-4, AF-1 and AF-2, interact with the N-terminus and the N- and C-terminus of CBP respectively. However, the interaction of HNF-4 AF-1 with the N-terminus of CBP is significantly reduced when the mutated AF-1 domain (M3K/Y6N/Y14H/L17P) is used in the interaction assay, indicating the importance of the hydrophobic residues (Figure 7A). To evaluate the significance of this interaction in AF-1 mediated transcription, transient transfection of HepG2 cells was carried out with the pG₅CAT reporter plasmid and an expression vector for CBP (1–771) and two GAL-HNF-4 fusion proteins. The GAL-fusion constructs contained the N-terminal 49 amino acids of HNF-4 (GAL-CD10), or HNF-4 (1–49) mutated at four sites GAL-M3K/Y6N/Y14H/L17P (Figure 7B). GAL-CD10 activates transcription

Figure 7 CBP activates transcription synergistically with the AF-1 domain of HNF-4

(A) *In vitro* translated and [³⁵S]methionine-labelled GAL-CD10 and GAL-M3K/Y6N/Y14H/L17P were incubated with GST or GST-CBP (1–771) immobilized on GSH-agarose beads. The labelled proteins, retained on the beads after extensive washing, were analysed by SDS/PAGE and autoradiography together with the *in vitro* translation product (INPUT). (B) Transient transfection experiments were performed in HepG2 cells with the G₅CAT reporter plasmid. The effectors GAL-CD10 and GAL-M3K/Y6N/Y14H/L17P are shown schematically at the top of the panel. Transfections with each of these effectors were carried out in the absence (–) or presence (+) of expression vectors for CBP or CBP (1–771). A typical CAT assay is shown. The CAT activity achieved with the reporter alone was set to a value of 1, and other activities are presented relative to this.

from the pG₅CAT promoter by 19.4-fold, and co-transfection with CBP increased the transcriptional activation to 32.4-fold. The GAL fusion containing the mutated AF-1 domain (GAL-M3K/Y6N/Y14H/L17P) was transcriptionally inactive and transfection with CBP did not cause an increase in reporter activity, indicating that CBP does not activate transcription in the presence of mutant AF-1. These results imply that the transcriptional activation by CBP occurs through a functional AF-1 domain. It appears, therefore, that CBP is indeed a target

for the AF-1 domain of HNF-4. The N-terminal mutant CBP (1–771) was not able to co-activate, although this region of CBP interacts strongly with HNF-4 in *in vitro* assays. Therefore the interaction of AF-1 with the N-terminus of CBP is not sufficient to direct transcriptional activation.

DISCUSSION

The activity of AF-1 activation domain is critically dependent on bulky hydrophobic amino acids and net negative charge

We have previously shown that the AF-1 function is located within the 24 N-terminal amino acids of HNF-4 [12]. It can function autonomously and has a potent transcriptional-activation capacity. HNF-4 lacking the AF-1 domain showed a 40% decrease in transactivation capacity, demonstrating the importance of AF-1 in HNF-4 mediated transactivation. The presence of five negatively charged residues within this sequence suggested that it might fall within the acidic class of activation domains. To determine the precise amino acids involved in mediating the activity of this domain, point mutations were introduced into the sequence. Substitutions of single acidic amino acids at five positions within the AF-1 transcriptional activation domain had moderate to significant effects on activation (Figure 3). The decrease in activity was more significant when the number of mutated acidic residues was increased, and the activity was abolished when all five charges were eliminated (Figure 3). Therefore the overall charge was demonstrated to be essential to the transactivation potential. These observations are in agreement with previous mutational analysis of the activation domains from VP16, GAL4 and GCN4, which revealed a positive correlation between the number of acidic amino acids and the transcriptional ability of the acidic activation domains [15,18]. The most important acidic amino acids in AF-1 appear to be Asp-5 and Asp-11, since substitution to either Ala or Asn was associated with significant reduction in transactivation (Figure 3). These results indicate that the transcriptional activity is dependent not only on the net-negative charge, but also on the position of the acidic residue.

It had been previously noted that alignment of the amino acid sequences of several transcriptional activation domains, belonging to different classes, revealed an intriguing pattern of bulky hydrophobic residues [15]. Aligning the sequence of AF-1 of HNF-4 in the same manner showed that it fits this pattern (Figure 8). Aromatic and hydrophobic residues of HNF-4 can be aligned with hydrophobic residues of VP16, SP1 and GAL4, and four of the five acidic amino acids of HNF-4 can be directly aligned with acidic amino acids of VP16 and GAL4, as well as glutamine residues in domain A of SP1 (Figure 8). Previous mutagenesis studies of some of these activation domains demonstrated the importance of positionally conserved hydrophobic amino acids for transcriptional activation. This study also delineated the critical role of aromatic residues at Tyr-6 and -14

and hydrophobic residues at Leu-14 and -17, signifying the importance of aromatic and hydrophobic residues in mediating AF-1 transactivation. These residues fall within the pattern previously observed for other transcriptional activators. Extensive mutagenesis studies were performed on the VP16 activation domain and several of these hydrophobic residues were substituted [16]. Our present results support the conclusion that this pattern is an indicator of amino acids important to the activation function. However, substitution of certain hydrophobic residues of VP16 (in positions analogous to those of HNF-4) for Ala had a much less drastic effect than the other positions, while in the present study all four of the tested residues were equally important (Figure 4).

Multi-target protein interactions of AF-1 and the potential significance of secondary structure

A general feature of acidic activators is their ability to interact with both TFIIB and TBP [31,32]. Functional activators have no structure or sequence in common, and activators from within the same group can activate transcription by a different cellular target or pathway. Consistent with this hypothesis is our finding that AF-1 interacts with the general transcription factors TFIIB and TBP (Figure 6), and also with the co-activator CBP [30] which enhances AF-1-dependent transactivation (Figures 7A and B). Recently a role for amphipathic α -helices in the interaction of proteins in transcriptional regulatory complexes has been defined [33–39]. The interfaces between the proteins in these complexes involve an amphipathic α -helix containing a conserved hydrophobic motif which makes contact with a complementary hydrophobic groove on the surface of the second protein. This type of interaction has been observed for complexes containing p53-MDM2 [33], VP16-TAFII31 [34] and CREB-CBP [35], as well as between several nuclear hormone receptors and their co-activators [39–39], and it is assumed to provide a general mechanism for the assembly of these complexes.

Secondary-structure predictions of AF-1 indicate that amino acids 1–18 would encompass an α -helix which, from helical-wheel analysis, would be organized with hydrophobic amino acids in positions 6, 10, 14 and 17 aligned on one face of the helix (Figure 1, left panel). Structural studies by CD, using a synthetic peptide encompassing these amino acids, revealed that this portion of HNF-4 exists as a random coil in aqueous solution at pH 5.15. Upon addition of the hydrophobic solvent trifluoroethanol, a region of this peptide adopted an α -helical conformation (results not shown). It can be anticipated that a co-activator binding to the AF-1 domain of HNF-4 has an effect similar to that of a hydrophobic solvent, thus allowing the formation of a stable secondary structure. We propose that the α -helix in the HNF-4 AF-1 domain is involved in such an interaction with its transcriptional targets. This is consistent with the fact that even conservative replacements of hydrophobic residues in the helix have a severe effect on the ability of the AF-1 domain to activate transcription (Figure 4 and 7).

The point mutations described in this study that impair this transcriptional activation domain should eventually allow us to identify which components of the transcriptional machinery are contacted by each of the critical residues, and thereby bring us closer to an understanding of how HNF-4 activates transcription.

Our results are in agreement with the study by Green et al. [40] on the critical structural elements of the AF-1 of HNF-4.

HNF-4	M	D	M	A	D	Y	S	A	A	L	D	P	A	Y	T	T	L	E	F	E	N	V	Q	V	L	T
VP-16	L	D	G	E	D	V	A	M	A	H	A	D	A	L	D	D	F	D	L	D	M	L	G	D	G	D
SP1 (A)	L	Q	N	Q	Q	V	L	T	G	L	P	G	V	M	P	N	I	Q	Y	Q	V	I	P	Q	F	Q
GAL4	L	D	F	M	P	R	D	A	L	H	G	F	D	W	S	E	E	D	D	M	S					

Figure 8 Amino acid sequence alignments of the activation domains of HNF-4, VP16, GAL4 and SP1 (A)

Bulky hydrophobic residues are boxed. Acidic residues and glutamine residues are in bold letters ([15]; the present study).

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