2098–2104 *Nucleic Acids Research, 1998, Vol. 26, No. 9*

The activation function 2 domain of hepatic nuclear factor 4 is regulated by a short C-terminal proline-rich repressor domain

Valentine P. Iyemere, Neil H. Davies and George G. Brownlee*

Chemical Pathology Unit, Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK

Received January 26, 1998; Revised and Accepted March 19, 1998

ABSTRACT

Hepatic nuclear factor 4 (HNF4) is a transcription factor whose expression is crucial for mouse embryonic development, for liver-specific gene expression and for the prevention of one form of maturity-onset diabetes of the young. Its domain structure has been defined previously and is similar to other members of the nuclear receptor superfamily. A repressor domain has now been localised to a region of 14 amino acids (residues 428–441) near the C-terminus of HNF4 and is sufficient by itself to repress the activity of the activation function 2 (AF2) domain. Multiple mutations within this repressor domain enhance activity. Interestingly, this repressor domain shares homology with a repressor domain in the progesterone receptor. In a detailed mutagenesis study of the AF2 core, we demonstrate that L 366, which is conserved in the AF2 core between HNF4 and a number of orphan nuclear receptors, is essential for the full activity of the AF2 domain. Furthermore, a double mutation of E 363 and L 366 suggests that these residues might act in a cooperative manner.

INTRODUCTION

Hepatic nuclear factor 4 (HNF4) was first isolated as a protein involved in regulating a number of liver-specific genes (1). It shares amino acid sequence homology with the nuclear receptor superfamily. Since no ligand has yet been found for HNF4, it is classified as an orphan nuclear receptor.

Extensive work carried out on nuclear receptors (e.g. oestrogen and retinoic acid receptors) has shown that they have a modular structure, with distinct regions carrying out specific functions (2). The N-terminal region (A/B) contains an activation function 1 (AF1) whose activity can be cell-type as well as promoter-context specific (3). A highly conserved zinc finger domain, required for DNA binding, is present in region C, with region D acting as a hinge (2). The C-terminal half of these receptors contains a long hydrophobic domain (region E), which encodes various functions. These include receptor dimerisation, ligand binding and a ligand-dependent activation function 2 (AF2) (2). The AF2 domain contains a core region of seven amino acids ΦΦXaΦΦa/L (where 'Φ' is a hydrophobic residue, 'X' any residue, 'a' an acidic residue and L = leucine) which is critical for activation and is conserved in many nuclear receptors (4). The extreme C-terminal region of these receptors (region F) varies in length between receptors and its function is unknown. In HNF4, this region is extended and contains a proline-rich domain (1).

HNF4 is expressed in many tissues including liver, kidney, pancreas and intestine (1,5,10). There are a number of isoforms generated by alternative splicing (5). For example, the HNF4α gene in humans encodes three isoforms, α1, α 2 and α4. There is, in addition, a distinct γ subtype encoded by the HNF4γ gene (5). The rat HNF4 isoforms 1-455 and 1-465 are equivalent to human α1 and α2 respectively (1,6). In *Xenopus laevis*, HNF4 is encoded by two distinct (α and β) genes (7).

Deletion of HNF4 is embryonic lethal in mice, since it resulted in the induction of cell death in embryonic ectoderm and impairment of gastrulation of mouse embryos carrying a homozygous (–/–) knockout of the HNF4α gene (8). In the regulation of the factor IX gene, point mutations which disrupt an HNF4 binding site result in haemophilia B (9,10). Recently, heterozygotes carrying mutations in the HNF4α allele were linked to one form of maturity-onset diabetes of the young (MODY1) (11). HNF4 is also involved in the regulation of a number of genes required for both carbohydrate and lipid metabolism as well as erythropoiesis (1,12). These genes include apolipoprotein CIII, pyruvate kinase, α1-antitrypsin and erythropoietin.

Previous work aimed at investigating the C-terminal activation domain of HNF4 in transcription showed that large deletions in the C-terminal region abolished transactivation (12,13). HNF4 was also shown to interact with the basal transcription factor TFIIB (13). Recent evidence has suggested that E363 and L366 of the AF2 core are important for the activity of the AF2 domain (14). However, this study was limited to single mutations at either position in the context of GAL4-HNF4 deletion fusion constructs (amino acids 128–370) which lacked other essential domains (the N-terminal AF1 domain, the DNA binding domain, and the repressor domain in the extended C-terminal region which is characterised in this report).

In this report, we demonstrate by extensive mutagenesis in the context of both full-length HNF4 and full-length HNF4 fused to

*To whom correspondence should be addressed. Tel: +44 1865 275559; Fax: +44 1865 275556; Email: george.brownlee@path.ox.ac.uk

the GAL4 DNA binding domain that Leu 366 of the AF2 core is essential for full AF2 activity. By investigating the effect of a double mutation involving both E363 and L366, we show that these residues may act cooperatively. In addition, we have precisely mapped the location of a repressor domain (initially described in ref. 14 and postulated to lie between residues 371 and 455) to amino acids 428–441 and have defined amino acid residues within this domain which are required for its activity.

MATERIALS AND METHODS

Reporter plasmids and GAL4-HNF4 fusion constructs

Reporter plasmids pCAT4 and pAPF1-HIV-CAT (1,15) were used to determine the transactivation activities of GAL4-HNF4 fusion constructs and native HNF4 constructs, respectively. pCAT4 is a reporter plasmid with four GAL4 response elements, an inverted CCAAT box, and a TATA element controlling the CAT reporter gene in the pBluescript backbone; pAPF1-HIV-CAT contains two HNF4 response elements in a direct repeat, and the –57 to +80 of the HIV LTR controlling the CAT reporter gene. To monitor transfection efficiency, the internal control plasmid pGLtkLUC (16) was used. The plasmid pGAL4 1-147 (17) contains the DNA-binding domain (DBD) of the yeast GAL4 protein driven by the immediate-early human cytomegalovirus (CMV) promoter. The GAL4-HNF4 full-length fusion constructs, and all C-terminal deletion constructs of HNF4 were constructed by PCR amplification of the appropriate fragment of HNF4 derived from the plasmid, pCB6HNF4 (16), using *Pfu* polymerase (Stratagene). After purification of the PCR products, a *Bam*HI/*Kpn*I restriction digest was carried out, followed by an in-frame ligation with the GAL4-DBD using the *Bam*HI/*Kpn*I restriction sites in pGAL4 1-147.

PCR mutagenesis

All GAL4-HNF4 full-length fusion proteins carrying a point mutation at E 363 or L 366, and the repressor domain mutants, were constructed by PCR-based mutagenesis using *Pfu* polymerase (Pritlove, unpublished data). Native (as opposed to GAL4-HNF4 fusion constructs) full-length HNF4 constructs with point mutations at E 363 or L 366 were constructed by removing the GAL4-DBD (GAL4 1-147) from the appropriate GAL4-HNF4 fusion construct by a *Hin*dIII/*Bam*HI restriction digest. The fusion construct GAL4-HNF4 1-372+428-441 was made by first amplifying the pGAL4-HNF4 1-372 fragment, followed by a ligation step to insert a double-stranded oligonucleotide of the repressor domain of HNF4 (residues 428–441). The sequence of all constructs were verified by fluorescent dideoxy sequencing.

Cell culture, transient transfections, enzymatic and gel mobility shift assays

HeLa and HepG2 cells were cultured using 60 mm dishes in minimal essential medium (MEM) supplemented with 10% foetal calf serum. Transient transfections were carried out using the standard calcium-phosphate precipitation method (10). Briefly, a total of 15 µg of DNA was transfected, consisting of 10 µg of the reporter plasmid, 2 µg of the expression plasmid, 2 µg of the internal control plasmid and 1 µg of salmon sperm DNA (Sigma). Sixteen hours after transfection, the cells were washed twice with PBS and fresh medium added. Cells were harvested 48 h post-transfection.

	Φ	Φ	X	a	Φ	Φ	a/L	
360	L	L	Q	**E**	M	L	L G G S	rHNF4α
360	L	L	Q	**E**	L	L	L G G T	xHNF4β
360	L	L	Q	**E**	M	L	L G G E	dHNF4
395	L	I	R	D	M	L	L S G S	hARP-1
384	L	I	R	D	M	L	L S G S	hEAR-2
402	L	I	R	D	M	L	L S G S	COUP-TF1
524	L	I	R	D	M	L	L S G N	dSvp
401	L	F	L	**E**	V	F	E D Q E	hTRα
455	F	L	M	**E**	M	L	E A P H	mRXRα
409	L	I	Q	**E**	M	L	E N S E	mRARα
418	L	V	L	**E**	V	G	E N I S	hVDR
539	L	L	L	**E**	M	L	D A H R	hER

Figure 1. Alignment of the amino acid sequence around the activation function 2 (AF2) core of HNF4 from different species with a number of orphan nuclear receptors (above) and receptors with known ligands (below). 'Φ' represents a hydrophobic residue, 'X' any amino acid residue and 'a' an acidic residue. The invariant glutamic acid (E) conserved between all nuclear receptors with known ligands and HNF4 is highlighted in bold. The leucine (L) conserved between HNF4 and a number of orphan nuclear receptors is underlined. r, rat; x, *X.laevis*; d, *Drosophila*; h, human; m, mouse. The definitions of the symbols, ARP1, EAR, COUP-TF1, Svp, TR, VDR, RAR, RXR and their accession numbers can be found in ref. 2.

Luciferase assays were carried out according to the manufacturer using a luciferase assay kit (Promega) and quantitated using a Wallac LKB 1250 luminometer. Chloramphenicol acetyl transferase assays were performed by standard methods (10) to determine the reporter activity of each construct. TLC plates were quantitated using a Storm phosphorimager and analysed using the Image Quant software. Gel mobility shift assays were performed essentially as described (17) using a DNA probe containing a single GAL4 binding site, and the same HeLa cell extracts that were used to determine the relative transactivation activities of the constructs in Figure 2.

Statistics

P values were calculated using student's *t*-test when comparing the mean and standard deviation of mutants. When comparing mutants with the full-length fusion protein or native wild-type full-length HNF4 protein, *P* values were calculated from z values.

RESULTS

Mapping the C-terminal activation function 2 (AF2) of HNF4

Analysis of the amino acid sequence of rat HNF4 identified a consensus amino acid sequence (ΦΦXaΦΦa/L, amino acid residues 360–366) found in a number of orphan and ligand nuclear receptors (Fig. 1). This conserved sequence, known as the activation function 2 (AF2) core, is known to form an amphipathic α-helix required for ligand-dependent activation of nuclear receptors, e.g. RARγ(18). The sequence alignment (Fig. 1) revealed that the Leu at position 366 (the last residue in the AF2 core) in HNF4 is conserved in a number of orphan receptors (Fig. 1, underlined L), whereas an acidic residue (an E or D) is found at the corresponding position in a number of nuclear receptors with known ligands. In addition, the extreme C-terminal region of HNF4, specifically amino acids 400–447, is rich in proline residues and was previously proposed to contain a proline-rich activation domain (1).

In order to determine if the proline-rich region contained an activation domain, and establish the role of the conserved amino acid sequence between 360 and 366, GAL4-HNF4 fusion

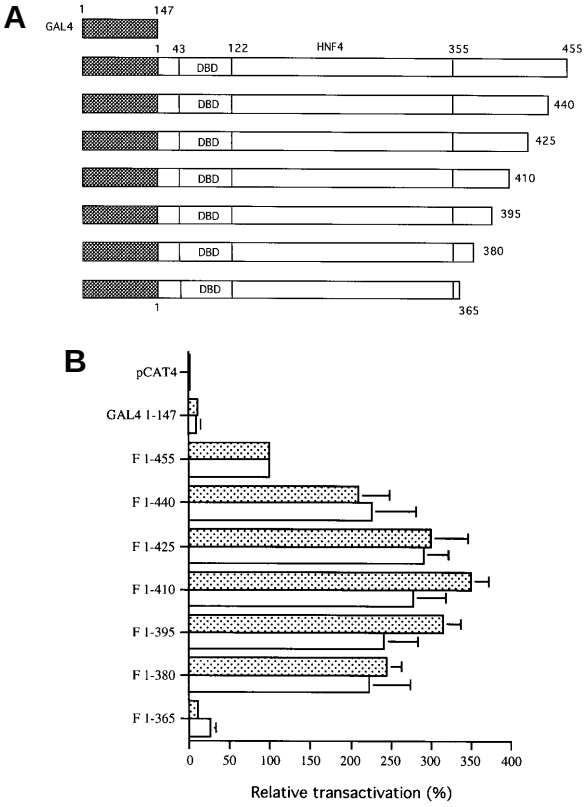

Figure 2. Mapping the C-terminal activation function 2 (AF2) of HNF4. (**A**) Schematic representation of the GAL4 DBD, the full-length HNF4 fusion and HNF4 C-terminal deletion fusions from 366 to 455 at 15 amino acids intervals. (**B**) Transactivation in HeLa (dotted histogram) and HepG2 (blank histogram) cells, relative to the full-length HNF4 fusion (which is set at 100% in all experiments). The mean of three independent transfection experiments is shown, ± standard deviation. In the context of GAL4-HNF4 fusion constructs, 'F' is used to represent GAL4 1-147. pCAT4 (see Materials and Methods) is a transfection control.

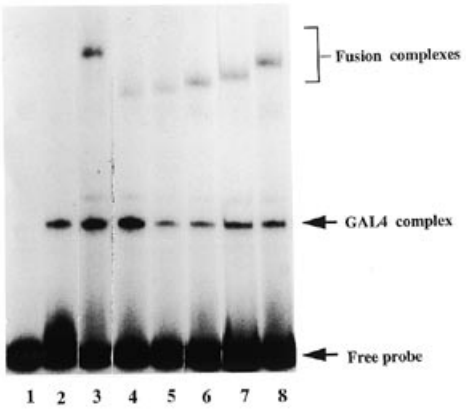

Figure 3. A gel mobility shift assay using HeLa cell extracts to determine the protein expression levels of the full-length and C-terminal deletion fusion constructs shown (except F 1-425) in Figure 2. Lane 1, HeLa cell extract from cells transfected with the reporter (pCAT4) and internal control (pGLtkLUC) plasmids; lane 2, GAL 1-147; lane 3, F 1-455; lane 4, F 1-365; lane 5, F 1-380; lane 6, F 1-395; lane 7, F 1-410; lane 8, F 1-440. The position of the gel shifted 'fusion complexes' is shown, free probe and the position of a presumptive degradation products comigrating with the GAL4 1-147 (the DNA binding domain of GAL4) are indicated by arrows.

proteins were constructed with successive 15 amino acid deletions from the C-terminus of HNF4 up to residue 366 (Fig. 2A). Unexpectedly, deletions up to residue 381 produced fusion proteins with higher activities than the full-length fusion protein in both HeLa and HepG2 cells (Fig. 2B, F 1-440 to F 1-380). For example, the activity of deletion F 1-410 was three and half times that of the full-length fusion protein in HeLa cells. However, when amino acids 366–380 were deleted (Fig. 2B, F 1-365) the activity decreased to 12% of full-length fusion protein. These results suggested there was a repressor domain spanning residues 380–455. However, there was no evidence of a proline-rich activation domain between residues 400 and 447 as previously suggested (1). The fact that transactivation was reduced to near background level when residues 366–380 were deleted suggested that Leu 366, conserved in a number of orphan receptors and the last residue in the consensus sequence ΦΦXaΦΦa/L (Fig. 1), is important. To exclude the possibility that the increased activities of the C-terminal deletion fusions was due to higher levels of protein expression in some constructs than others, a gel mobility shift assay was carried out as described in Materials and Methods. As shown in Figure 3, gel shifted fusion complexes of all constructs were present in comparable amounts, with insufficient variation in yield to account for the difference in activity observed in Figure 2. Thus, no further gel shift analysis of protein levels was carried out on further constructs in this report.

To identify the critical residue(s) in the region between residues 359 and 380, fusion proteins with deletions at three amino acid intervals between residues 359 and 380 were constructed and tested in the transient transfection assay in HeLa cells (Fig. 4). Deleting amino acids 369–380 had no effect on the activity of the C-terminal activation domain (Fig. 4B, compare F 1-380 and F 1-368). Deleting the next three amino acids, 366–368, resulted in a significant decrease in activity to background level (Fig. 4B, F 1-365). Two further deletions, F 1-362 and F 1-359, also had background activity. We conclude that the region between amino acids 365 and 368 is critical for transactivation. Leu (L) 366 is the last amino acid residue in the proposed amphipathic α-helix formed by the AF2 core (Fig, 1; 18) and thus may be an important residue.

To investigate the significance of L366 in more detail, point mutations of L 366 to A, D, Q and I were made both in the context of GAL4-DBD full-length HNF4 fusion proteins (Fig. 5A) and in the context of native full-length HNF4 (Fig. 5C). All mutations at L366 resulted in decreased activity compared to wild type GAL4-DBD-HNF4 fusion protein or native HNF4 (Fig. 5B and D). In the context of native HNF4 (Fig. 5D), the activity of mutant L 366 I (which of all the mutants differed least in activity from the wild-type), was still statistically significantly different ($P < 0.01$) from wild-type in a z value test (Materials and Methods). When the activities of mutants 366 A and Q were compared to that of mutant 366 I, they were also found to be statistically significantly different ($P < 0.01$ in both cases). However, the transactivation activities of mutants 366 D and I were not statistically significantly different ($P > 0.05$) in a student's *t*-test. Comparing the activities of the same set of point mutants at L 366 in the context of the GAL4-HNF4 fusion (Fig. 5B) shows that mutants 366 Q and D were statistically significantly different from I ($P < 0.01$ in both cases), although no significant difference was seen between 366 I and A ($P > 0.05$) or between mutants 366 A, D and Q. These results show that L 366 is critical for full transactivation.

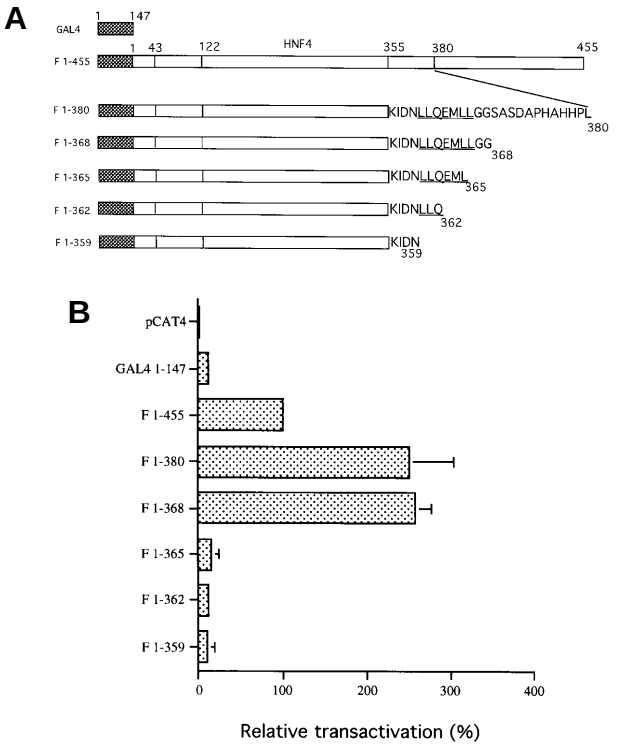

Figure 4. Determining the critical amino acid residues in the AF2 core of HNF4 in HeLa cells. (**A**) Schematic representation of the GAL4 DBD, the full-length HNF4 fusion and C-terminal deletion fusions from 359 to 380 at three amino acid intervals. Intermediate constructs between 368 and 380 are not shown since the activities of these constructs were not significantly different to fusion F 1-368 (results not shown). (**B**) Transactivation relative to the full-length HNF4 fusion set at 100% (see legend to Fig. 2B for further details).

Position 4 (E 363 in HNF4) of the consensus AF2 core is an acidic residue in both orphan and nuclear receptors with known ligands (Fig. 1). From the crystal structure of the ligand binding domain of RARγ bound to *all-trans* retinoic acid, this position is critical for the formation of a salt bridge between helix 4 and 12 (18), essential for AF2 activity. Thus, we investigated the point mutant 363 E→A in HNF4 in the context of both a GAL4-HNF4 fusion protein (Fig. 5B) and in native HNF4 (Fig. 5D). Transactivation was statistically significantly lower than that of the wild-type fusion protein in both cases ($P < 0.001$ and $P < 0.0001$, respectively). These results indicate that the E at 363 is also important for full transactivation.

We next determined the effect of a double mutation of E363A/L366A in the context of a GAL4 full-length HNF4 fusion (Fig. 5A, F E363A/L366A). Unexpectedly, the activity of the double mutant was comparable to that of the single mutants at either position (Fig. 5B, F E363A/L366A compared to F L366A or F E363A), suggesting that E363 and L366 may act cooperatively in regulating the activity of the AF2 domain.

The AF2 activity of HNF4 is regulated by a repressor domain near the C-terminus

A repressor domain near the C-terminus of the progesterone receptor (PR) was recently shown to inhibit the activity of PR in the absence of progesterone (19). Alignment of the PR repressor domain sequence (residues 917–928) with the entire HNF4 protein sequence identified a potential 12 amino acid long sequence homology (Fig. 6A, boxed region) near the C-terminus of HNF4 (residues 430–441). This potential repressor region showed 40% identity to the sequence of the repressor domain of PR, which is also near its C-terminus. This homology and our previous results defining a repressor domain between residues 380 and 455 (see above), prompted us to investigate the effect of fusing a short region of HNF4 containing residues 430–441 downstream of a high activity C-terminal deletion mutant. Thus, when a 14-long peptide (residues 428–441) of HNF4 was fused in-frame downstream of GAL4-HNF4 1-372 (Fig. 6B), its activity was reduced from 300 to 22% of the full-length fusion protein [Fig. 6C, compare F 1-372+428-441 to F 1-455 ($P < 0.001$)]. This suggested the peptide 428–441 may function as a repressor domain in isolation.

The role of this repressor domain was further investigated by mutagenesis in the context of the full-length HNF4 fusion protein. Two constructs with multiple point mutations (a run of five or six alanine residues) in the proposed repressor domain (Fig. 6A) were constructed (Fig. 7A). The activity of the N-terminal mutant [YKLLPG→AAAAAA, (427–432)] increased 2.3 times when compared to the wild-type full-length fusion (Fig. 7B). The C-terminal mutation [ITTIV→AAAAA, (434–438)] showed an increase in activity which was 3.5 times that of the wild-type full-length fusion protein (Fig. 7B). Both mutants are significantly different from wild-type ($P < 0.01$). These results confirm the presence of a repressor domain between amino acids 428 and 441.

DISCUSSION

In this report we have studied the C-terminal activation region of HNF4, concentrating our attention on two short regions. These are firstly, the well-known AF2 core, which is conserved in nuclear receptors (4) and secondly, a novel short repressor domain. These regions were studied either by deletion analysis or by site-directed mutagenesis of HNF4 fused to the GAL4 DNA binding domain. Cotransfection of such fusion constructs with a chloramphenicol acetyl transferase reporter plasmid containing upstream GAL4 binding sites into either HeLa or HepG2 cells transiently, was used to study the activities of the deleted or mutated constructs (Materials and Methods).

Previous studies of the C-terminal activation domain of HNF4 suggested that the AF2 core (residues 360–366) was important for activity (12–14). Furthermore, a mutation of L366→E, in an HNF4 deletion fusion construct (amino acids 128–370) suggested that residue 366 was important for the activity of the AF2 domain (14). However, this result is preliminary because it was studied on a HNF4 deletion construct which lacked three essential domains, the N-terminal AF1 domain, the DNA binding domain and the C-terminal repressor domain (see below). It is arguable that the results might not be typical of intact HNF4. Another potential criticism of a previous study (14) is that the conclusions as to the importance of particular amino acid residues of the AF2 core particular were based on studies of a single point mutant at each position, 363 and 366, which might not be representative had other mutants been studied. Here we set out to study multiple mutations at position 366 of the AF2 core and to study a double mutant involving both positions 363 and 366 to gain further insight into the role of the AF2 core.

The results of successive C-terminal deletions (Fig. 2) and fine deletion mapping (Fig. 4) initially suggested that L366 might be

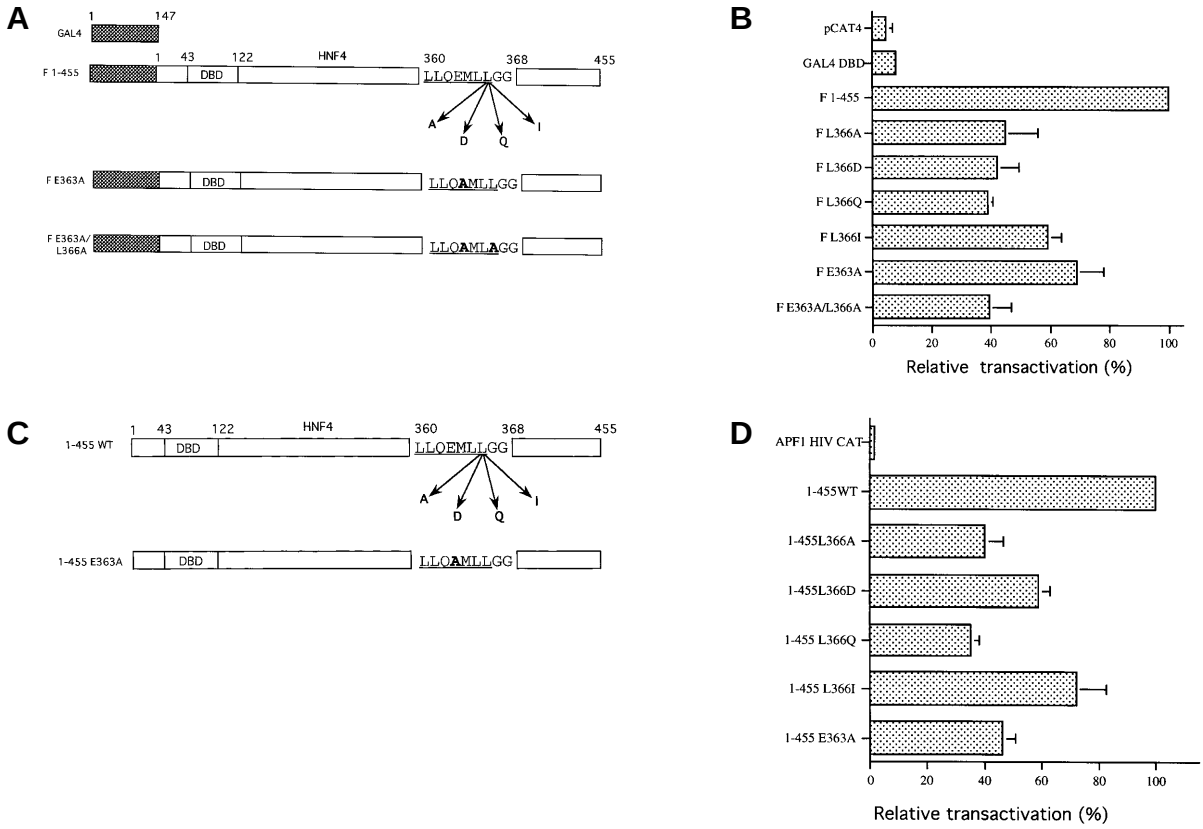

Figure 5. The roles of L366 and E363 in the activity of the C-terminal AF2 domain of HNF4 in HeLa cells. (**A**) Schematic representation of the GAL4 DBD, the wild-type GAL4-HNF4 full-length fusion construct with arrows showing the point mutations of L366→A, D, Q or I. F E363→A shows the GAL4-HNF4 full-length construct with the bolded 363E→A mutation of the AF2 core. F E363A/L366A shows the double mutant (**B**) Relative transactivation (see legend to Fig. 2B for further details). (**C**) Schematic representation of the native full-length HNF4 and mutants studied as in (A) above. WT = wild-type. (**D**) Transactivation relative to wild-type native full-length HNF4 set at 100%. APF1-HIV-CAT is a transfection control (see Materials and Methods). (See legend to Fig. 2B for further details.)

important for the activity of the C-terminal AF2 domain. Therefore, four different point mutations (L→A, D, Q or I) were constructed to definitively assess the role of L 366 in both full-length HNF4 (Fig. 5C and D) and GAL4 full-length HNF4 fusion constructs (Fig. 5A and B). All mutations decreased the transactivation activity, indicating that Leu 366 is required for full activity. However, in contrast to a previous report where the activity of a mutant at 366 was reduced to ~5% of wild-type, we detected activities varying from ~35 to 75% of wild-type. This suggested, surprisingly, that transactivation activity is not absolutely dependent on Leu 366, even although it is conserved in a number of orphan receptors (Fig. 1). Other residues e.g. A, D, Q and I were clearly functional at least in our transient transactivation assay. This result is not an artefact of the fusion of intact HNF4 to the GAL4 DNA binding domain, since essentially similar quantitative results were obtained irrespective of whether full-length HNF4, or full-length HNF4 fused to GAL4 DNA binding domain, were studied (compare Fig. 5B and D). However, the fact that a conservative amino acid substitution 366 (L→I) showed the highest activity in our assay (~60–70%), whereas non-conservative amino acid substitutions 366 (L→A, D, Q) showed lower activities (35–60%) still argues for the functional importance of Leu at 366 (Fig. 5).

E363 is also highly conserved between HNF4 and all nuclear receptors with known ligands, although it is substituted by a D in some orphan receptors (Fig. 1). E363 was selected for mutation because it is known to be involved in the formation of a salt-bridge and is critical for transactivation in RARγ (18). Mutation of E363→A in HNF4 here (Fig. 5) indicated that this residue, like residue 366, was required for full activity. Our result at this position differed from a previous report (where activity was reduced to <5% of wild-type; 14) in that significant residual activity was observed (between 50 and 70% of wild-type; Fig. 5C and D) depending on whether with the full-length HNF4 construct or the GAL4 full-length HNF4 fusion construct was used in the transactivation assay. We attribute this difference to the fact that full-length HNF4 was used in our studies, whereas HNF4 deletion constructs were used in the previous report (14).

It is significant that a double mutant of 363 and 366 also retained ~50% activity in our assay (Fig. 5). The fact that the double mutant failed to demonstrate an additive effect over and above that of the two point mutants assayed separately is consistent with E363 and L366 cooperating to regulate the activity of the AF2 domain. These experiments illustrates both the importance of E363 and L366 of the AF2 core, yet also demonstrate the core is partly redundant at least in our transactivation assay. It remains possible that the redundancy observed in the mutations studied here results from a compensatory effect of the AF1 domain, which is known to transactivate (14) and is present in our full-length HNF4 constructs.

Our initial C-terminal deletion experiments suggested the presence of a repressor activity within residues 380–455 (Fig. 2B, and previously proposed to lie between residues 371 and 455; 14).

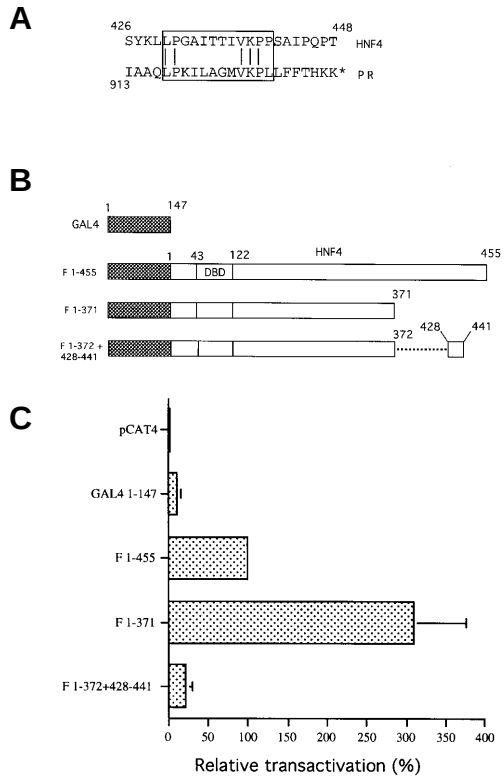

Figure 6. A study of the C-terminal repressor of HNF4. (**A**) Homology (boxed) between the C-terminal repressor domain of PR and a potential repressor domain in the extended C-terminal region of HNF4 (see text). * indicates the C-terminus of PR. Amino acids are numbered in HNF4 above and PR below. (**B**) Schematic representation of the GAL DBD, the full-length fusion (F 1-455), the 1-371 C-terminal deletion fusion and a 1-372 C-terminal deletion fusion containing an in-frame fusion with the proposed repressor domain (428–441) of HNF4. (**C**) Transactivation in HeLa cells relative to the full-length fusion set at 100% (see legend to Fig. 2B for further details).

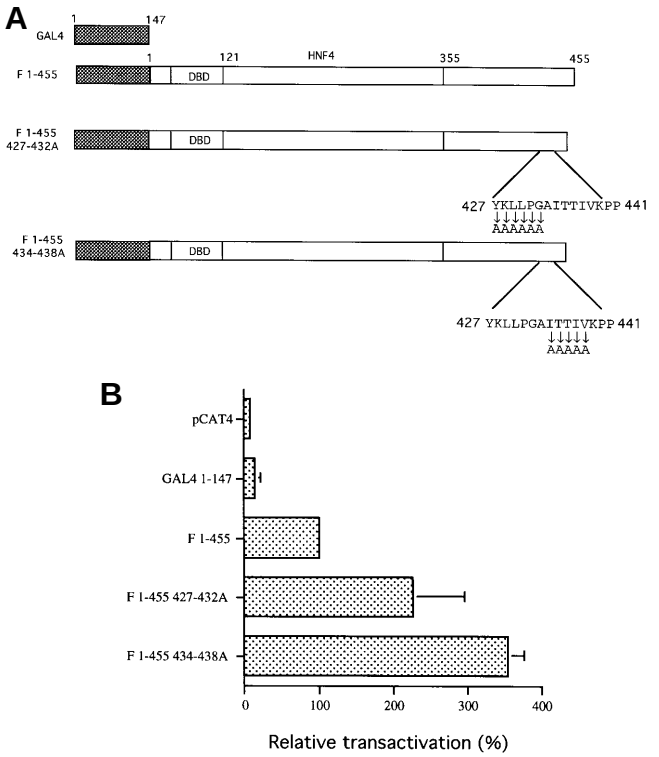

Figure 7. Mutation of amino acids in the repressor domain of HNF4 enhances activity. (**A**) Schematic representation of the GAL 4 DBD, the full-length HNF4 fusion (F 1-455) and two full-length HNF4 fusions with multiple mutations to alanine (as shown) in the repressor domain of HNF4. (**B**) Transactivation in HeLa cells relative to the wild-type full-length HNF4 fusion. (For further details see legend to Fig. 2B.)

By aligning the amino acid sequence of the PR repressor domain (19) with the entire HNF4 sequence, a short region (residues 430–441) showing 40% identity to a previously characterised repressor domain in PR was localised in HNF4 (Fig. 6A). This presumptive repressor domain was first shown to confer repressor activity by itself (Fig. 6C). Thus, when a short peptide from amino acid 428 to 441 containing the HNF4 repressor domain was fused in-frame downstream of a high activity C-terminal deletion fusion construct, transactivation was severely inhibited, decreasing from >300 to 22%. This is unlikely to be the result of decreased protein expression of this particular fusion construct, since a gel shift analysis of a panel of deletion constructs all showed reasonably similar levels of protein expression (Fig. 3) This implied that the peptide containing amino acids 428–441 was sufficient, by itself, to act as a repressor. Further evidence for this repressor domain came from a study of two constructs with multiple point mutations (Fig. 7C).

It is interesting to note that in the experiment where the isolated repressor domain was fused to F 1-372 (Fig. 6B), the AF2 core was intact; hence an activity closer to the that of the full-length fusion (100%) might have been expected, whereas an activity of 22% was observed (Fig. 6C). This suggests the repressor domain had a more potent inhibitory effect when fused closer to the AF2 core, than if it were in its normal location. Perhaps the close proximity of the repressor domain to the AF2 core may restrict the accessibility of potential coactivator proteins to the AF2 core, either by binding a corepressor protein or simply by masking critical residues in the AF2 core. In the case of the PR repressor domain, the repressor domain was shown to mediate its function through an unknown corepressor (19). Experiments in an attempt to determine if the HNF4 repressor domain functioned through a corepressor were unfortunately inconclusive (data not shown).

It is obviously interesting to know how the repressor domain in HNF4 functions and if it is required to regulate the activity of HNF4 *in vivo*. An attractive, but speculative mechanism might involve the phosphorylation of threonine residues in the sequence ITTI (residues 434–437). A corepressor might bind to the repressor domain in the phosphorylated state and dissociate in the dephosphorylated state. Thus mutations which disrupt these threonine residues would prevent phosphorylation, thus preventing repression and thereby enhancing the activity of HNF4.

In summary, our characterisation of the AF2 domain in the C-terminal region of HNF4 has given new insights into the function of residues 363 and 366. By extensive mutagenesis we have shown that L366 is essential for full AF2 activity. An E363A/L366A double mutant suggested that E363 and L366 may act cooperatively to regulate the activity of the AF2 domain. We have also shown that the activity of the AF2 domain is regulated by a repressor domain located within a proline-rich region. We have localised this repressor domain to within amino acid residues 428–441 of HNF4, and showed it was necessary and

sufficient for repressor activity in transient transfection studies in HeLa cells. Furthermore, multiple point mutations within this repressor domain in the context of the full-length HNF4 fusion construct abolished repressor activity. The repressor domain shares homology with a recently reported C-terminal repressor domain in the progesterone receptor (19).

ACKNOWLEDGEMENTS

This work was supported by the MRC (VPI) and the Wellcome Trust (NHD & GGB) grant number 049537/Z/96. We thank Dr S. Murphy for helpful discussions and the provision of pGAL4 1-147, Dr M. Busslinger for pCAT4 and Dr Y. Talianidis for pCB6HNF4.

REFERENCES

1 Sladek, F.M., Zhong, W., Lai, E. and Darnell, J.E., Jr (1990) *Genes Dev.*, **4**, 2353–2365.
2 Kastner, P., Mark, M. and Chambon, P. (1995) *Cell*, **83**, 859–869.
3 Nagpal, S., Friant, S., Nakshatri, H. and Chambon, P. (1993) *EMBO J.*, **6**, 2349–2360.
4 Danielian, P.S., White, R., Lees, J.A. and Parker, M.G. (1992) *EMBO J.*, **3**, 1025–1033.
5 Drewes, T., Senkel, S., Holewa, B. and Ryffel, G.U. (1996) *Mol. Cell. Biol.*, **3**, 925–931.
6 Hata, S., Tsukamoto, S. and Osumi, T. (1992) *Biochem. Biophys. Acta*, **1131**, 211–213.
7 Holewa, B., Zapp, D., Drewes, T., Senkel, S. and Ryffel, G.U. (1997) *Mol. Cell. Biol.*, **2**, 687–694.
8 Chen, W.S., Manova, K., Weinstein, D.C., Duncan, S.A., Plump, A.S., Prezioso, V.R., Bachvarova, R.F. and Darnell, J.E. (1994) *Genes Dev.*, **8**, 2466–2477.
9 Crossley, M., Ludwig, M., Stowell, K.M., DeVos, P., Olek, K. and Brownlee, G.G. (1992) *Science*, **257**, 377–379.
10 Naka, H. and Brownlee, G.G. (1996) *Br. J. Haematol.*, **92**, 231–240.
11 Yamagata, K., Furuta, H., Oda, N., Kaisaki, P.J., Menzel, S., Cox, N.J., Fajans, S.S., Signorini, S., Stolffel, M. and Bell, G.I. (1997) *Nature*, **384**, 458–460.
12 Galson, D.L., Tsuchiya, T., Tendler, D.S., Huang, L.E., Ren, Y., Ogura, T. and Bunn, H.F, (1995) *Mol. Cell. Biol.*, **4**, 2135–2144.
13 Malik, S. and Karathanasis, S.K. (1996) *Mol. Cell. Biol.*, **4**, 1824–1831.
14 Hadzopoulou-Cladaras, M., Kistanova, E., Evagelopoulou, C., Zeng, S., Cladaras, C. and Ladias, J.A.A. (1997) *J. Biol. Chem.*, **272**, 539–550.
15 Braselmann, S., Graninger, P. and Busslinger, M. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 1657–1661.
16 Morgan, G.E., Rowley, G., Green, P.M., Chisholm, M., Giannelli, F. and Brownlee, G.G. (1997) *Br. J. Haematol.*, **98**, 79–85.
17 Murphy, S. (1997) *Nucleic Acids Res.*, **11**, 2068–2076.
18 Renaud, J.P., Rochel, N., Ruff, M., Vivat, V., Chambon, P., Gronemeyer, H. and Moras, D. (1995) *Nature*, **378**, 681–689.
19 Xu, J., Nawaz, Z., Tsai, S.Y., Tsai, M.J. and O'Malley, B.W. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 12195–12199.