1476–1484 *Nucleic Acids Research, 1997, Vol. 25, No. 8*

The bifunctional DCOH protein binds to HNF1 independently of its 4-α-carbinolamine dehydratase activity

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Received February 13, 1997; Accepted March 5, 1997

ABSTRACT

HNF1 is a liver enriched atypical homeoprotein isolated from vertebrates which is involved in the transcriptional activation of liver, kidney, intestine and pancreas specific genes. HNF1 contains an N-terminal dimerisation and a POU-like domain both essential together with the homeodomain for DNA specific recognition. Using the yeast two-hybrid system we searched for proteins interacting with HNF1. We repeatedly obtained cDNA clones encoding DCOH/4-α-carbinolamine dehydratase, an enzyme involved in the oxidation of aromatic amino acids that was shown to bind to and stabilise HNF1 dimers. Using the yeast system, we show that the enzymatic activity of DCOH is not essential for HNF1 binding and that the HNF1 dimerisation domain is sufficient for DCOH binding. Furthermore we demonstrate that both proteins co-localise in co-transfected cells.

INTRODUCTION

For many genes, transcription initiation is a major regulatory step that controls their expression. It requires the assembly of a large nucleoprotein complex, including the RNA polymerase II and general transcription factors, and can be activated by transcription factors bound to specific DNA sites in promoters and enhancers. Hepatic nuclear factor 1 (HNF1, also referred to as LFB1, HNF1-α and HP1) is one such transcription activator. This protein, so far only found in vertebrates, is preferentially expressed in liver, kidney, intestine and pancreas. In liver it is involved in transcription activation of many specific genes, such as serum proteins (α-1-antitrypsin, albumin, α-fetoprotein, β-fibrinogen, etc.), or enzymes [phenylalanine hydroxylase, alcohol dehydrogenase, aldolase B, etc. (1)]. HNF1 is dimeric and binds to a pseudo-palindromic site on DNA. It can also form heterodimers with vHNF1 (also called LFB3 and HNF1-β), a related protein which binds to the same cognate sites on DNA. Functional mapping studies have shown that the dimerisation domain lies in the 31 extreme N-terminal residues, and is mainly α-helical. The binding of HNF1 to its cognate site on DNA is achieved by a tripartite domain comprising the dimerisation domain, an atypical homeodomain and a POU related domain (2–4). The transcription activation domains have been mapped to the C-terminal part of the protein (5–8). No distinct short activation motif has been identified in HNF1, only the high frequency of serines, threonines and glutamines is a conserved property among the activating domains.

As of today, little is known about the molecular targets of HNF1 in the transcription machinery. Only one protein has been described to bind to this factor. This small (11 kDa) protein named DCOH (dimerisation cofactor of HNF1) was first isolated by co-purification with HNF1 from liver nuclear extracts (9). It has been shown to stabilise the HNF1 homodimers (or the heterodimers it forms with vHNF1) by preventing exchange of monomers of HNF1 between dimers (10,11). In addition, it was claimed that DCOH increases gene activation by HNF1 in transient transfection assays (9,12). Two DCOH molecules bind to a dimer of HNF1. This interaction is only observed if HNF1 protein is present when DCOH is being synthetised (R. Ficner and D. Suck, personal communication). Later, in an apparently unrelated field, the same DCOH gene was shown to encode an enzyme involved in recycling of tetrahydrobiopterin, a cofactor essential for aromatic amino acid hydroxylases. It bears the 4-α-carbinolamine dehydratase activity. Alone, DCOH forms homotetramers, which have been crystallised and for which the three-dimensional structure has been determined (13,14). No clear link has so far been established between the enzymatic activity of DCOH and its ability to form hetero-tetramers with HNF1. However, one should mention in this context the results of the recent inactivation of the HNF1 gene by homologous recombination in mouse (15). While the transcription of target genes like albumin or α-1-antitrypsin was reduced only 2–4-fold in homozygous mutant mice, that of PAH (phenylalanine hydroxylase, the major consumer of tetrahydrobiopterin in mammals) was totally abolished. This result may be fortuitous or, conversely, reinforce the link between HNF1, DCOH and PAH. The expression of DCOH itself was only partially reduced in these mice.

In an attempt to isolate molecular partners of HNF1, we have used this transcription factor as a bait in a yeast two-hybrid screen applied to a human liver cDNA library. The fact that HNF1 *per se* is able to activate transcription in yeast was expected to be a major obstacle to its use in this system. However, we have adapted the working conditions so as to be able to detect over-activation

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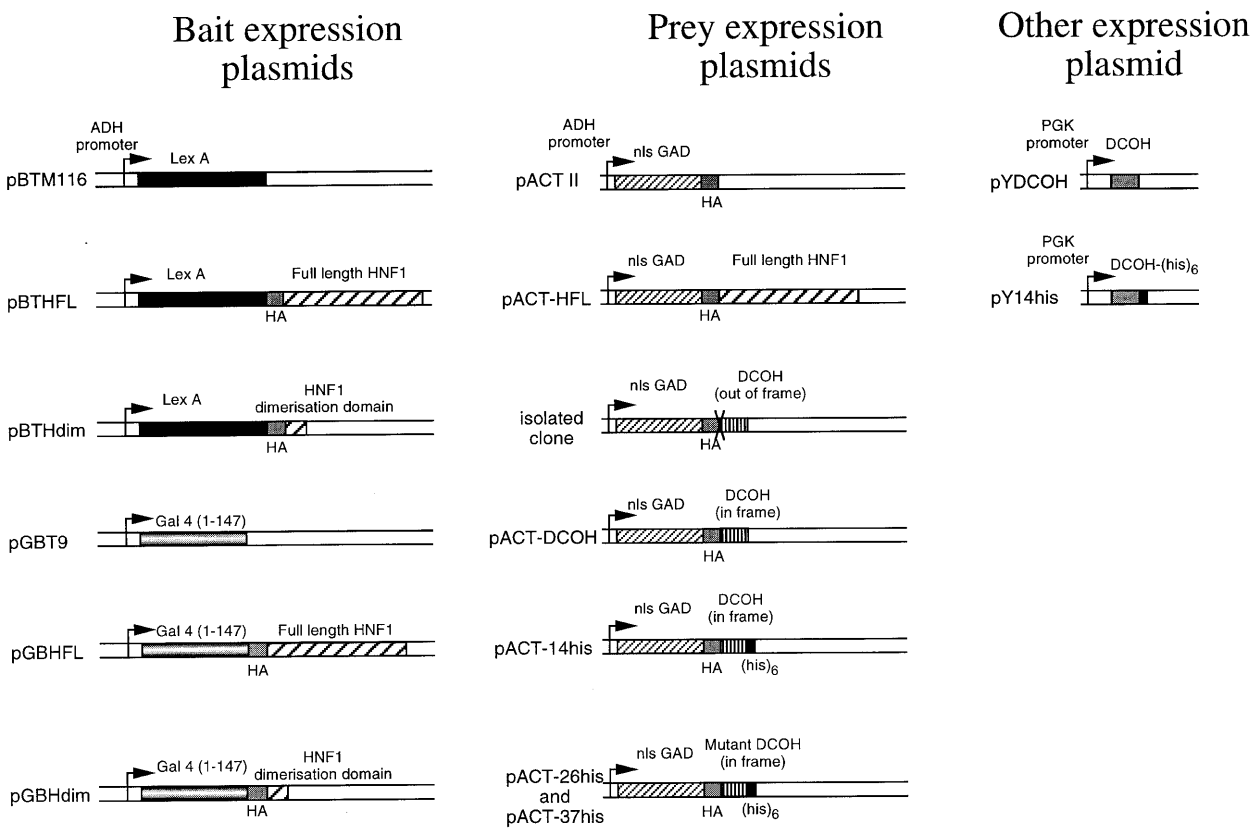

Figure 1. Plasmids used in this study. All baits are in the same 2µ plasmid context, bearing a trp1 cassette. All prey vectors are in the same 2µ plasmid context, bearing a leu2 cassette. pYDCOH and pY14his are in the same 2µ plasmid context as pEMBLye30/2, bearing a leu2 cassette. The lengths of the coding sequences are not to scale.

resulting from interactions of HNF1 with relevant partners. We report here the isolation of DCOH as a relevant partner of HNF1 in our system. Furthermore, we used our *ex vivo* system to study the possible link between the enzymatic activity of DCOH and its binding to HNF1. We show that substitution mutants of DCOH known to have impaired 4-α-carbinolamine dehydratase activity still bind to HNF1, just as the wild-type protein. Finally, it had previously been shown that the dimerisation domain of HNF1 was necessary for the binding of DCOH *in vitro*. However, it was not excluded that other domains of the protein could be essential for this interaction. We show here that the dimerisation domain of HNF1 is sufficient for binding to DCOH in yeast and *in vitro*.

MATERIALS AND METHODS

Cloning of a human liver cDNA library in a two-hybrid prey vector

After extraction of total RNA from normal human liver by a hot phenol procedure (16), polyA$^+$ RNA was purified using an Oligotex mRNA kit (Qiagen). Oligo-d(T) primed cDNA synthesis was performed with 5 µg polyA$^+$ RNA using a Zap cDNA kit (Stratagene) which generated oriented cDNA fragments with an *Eco*RI site overhang at their 5′ ends and a *Xho*I site overhang at their 3′ ends. A size selected cDNA fraction (>600 bp) was then ligated to pACTII (17) vector DNA digested at the *Eco*RI and *Xho*I sites and dephosphorylated which allowed cloning of the cDNAs in a sense orientation with respect to the Gal4 activation domain coding sequence of the vector. The *Escherichia coli* strain DH10B (Gibco BRL) was transformed with the ligated DNA by electroporation which generated a library containing 1.5×10^6 independent transformants. The bacteria were scraped from the plates and pooled in LB medium with antibiotics. Several aliquots of the pooled transformants were frozen in medium containing 7% DMSO and the remainder of bacteria was used to extract plasmid DNA using a Qiagen plasmid kit. For library amplification, bacteria from a frozen aliquot were plated at a density of 5×10^4 colony forming units per plate (15 cm diameter) and plasmid DNA prepared as indicated above.

The quality of the library was checked by preparing the plasmid DNA from 10 randomly picked independent transformants. Digestion at the *Eco*RI and *Xho*I cloning sites indicated that eight of the 10 cDNA clones contained a detectable insert, the insert size ranging from 800 to 3300 bp.

Plasmids

The HNF1 containing baits were prepared as follows. The HNF1 coding sequence was cloned in two steps into vector pAS1-CYH2 (17,18). First the *Nco*I 335 bp fragment of the HNF1 ORF was cut from pRHP (7) and cloned into *Nco*I cut pAS1-CYH2, yielding intermediate vector pAS1-HSI. Then the 1856 bp long *Sac*I/*Bgl*II fragment was cut from pRHP and cloned into *Sac*I/*Bam*HI cut pAS1-HSI, yielding vector pAS1-HFL. Then the *Eco*RI/*Xho*I fragment was cut from pAS1-HFL. It contains sequences of the HA epitope tag followed by the HNF1 ORF. This fragment was cloned into *Eco*RI/*Sal*I cut pBTM116 (19) or pGBT9 (20) yielding bait expression vectors pBTHFL and pGBHFL (Fig. 1).

The HNF1 dimerisation domain containing baits were prepared as follows. The *Nco*I/*Xma*I 98 bp fragment of the HNF1 ORF was cut from pRHP (7) and cloned into *Nco*I/*Xma*I cut pAS1-CYH2, yielding intermediate vector pAS1-Hdim. Then the *Eco*RI/*Xho*I fragment was cut from pAS1-Hdim and cloned into *Eco*RI/*Sal*I cut pBTM116 (19) or pGBT9 (20) yielding bait expression vectors pBTHdim and pGBdim. Prey expression vector pACT-HFL was obtained by cutting the HNF1 ORF out of pAS1-HFL, an *Nde*I/*Xho*I fragment, and cloning it into *Nde*I/*Xho*I cut pACTII vector (Fig. 1). Prey expression vector pACT-DCOH was obtained by cutting with *Nco*I an out-of-frame isolated clone containing the complete DCOH ORF, and self ligating this vector, putting DCOH back in-frame. Prey expression vectors pACT-14his, pACT-26his and pACT-37his were obtained by cutting the *Nde*I/*Bam*HI fragment out of pHDH14, pHDH26 and pHDH37 (21), respectively, and cloning it into *Nde*I/*Bam*HI cut pACTII vector, thus yielding in-frame fusions with the Gal4 activation domain (Fig. 1). Expression vector pYDCOH was obtained by cutting the complete DCOH ORF fragment out of an out-of-frame isolated clone (i.e. a *Bam*HI/*Bgl*II fragment) and cloning it into pEMBLye30/2 (22) (Fig. 1). Expression vector pY14his was obtained by cutting the *Bgl*II/*Bam*HI fragment from vector pACT-14his and cloning it into *Bgl*II cut vector pEMBLye30/2. Mammalian expression vector pCG-DCOH was obtained by cloning the human DCOH ORF from isolated clone 1 (*Bam*HI/*Bgl*II fragment) into the *Bam*HI site of pCG (23).

Yeast strains and transformation

We have used yeast strains L40 (19) and Y190 (17). When these strains did not contain any HNF1 expression plasmids, they were trasformed as described (24). Expression of an HNF1 containing bait in these strains resulted in strong flocculation, thus reducing secondary transformation efficiency. To perform the secondary transformation, bait containing yeast cells were grown in liquid selective medium up to a concentration of 3×10^6 cells/ml under intensive agitation. The cells were then transferred to a complete medium and were grown for two additional generations. Yeasts were collected and further processed as described (24).

Isolation of yeast plasmids

Isolated clones were grown on solid medium, and scraped off the Petri dish. The cells were resuspended in 200 µl of breaking buffer (100 mM NaCl, 10 mM Tris–HCl pH 8.0, 1 mM EDTA, pH 8.0, 2% Triton X-100, 1% sodium duodecyl sulfate). After addition of 300 mg of 425–600 µ glass beads (Sigma), and 200 µl of buffered (25:24:1) phenol/chloroform/isoamyl alcohol mixture, the cells were broken by vortex agitation for 3 min. After centrifugation at 13 000 r.p.m. for 5 min, the aqueous phase was kept, and used for electroporation of *E.coli* MC1066 strain (generous gift of E. J. Miller and L. Prakash) by standard techniques. The electroporated bacteria were plated onto selective medium in order to isolate the prey expression plasmid.

Screening of the library

Strain L40 containing bait expression plasmid pBTHFL was transformed with the pACT II human liver cDNA library. Transformants were plated onto 160 10 cm circular nylon membranes lying on top of selective solid medium. After 36 h of growth at 28°C, the membranes were plunged into a liquid nitrogen bath for 10 s. The membranes were then brought back to room temperature, and were laid onto Whatman paper (3mm) soaked with β-galactosidase assay buffer (40 mM NaH_2PO_4, 60 mM NaH_2PO_4, 1 mM $MgCl_2$, 50 mM β-mercaptoethanol) containing 1 mg/ml of X-gal (USB). As they turned blue, colonies were picked and re-plated onto solid medium. The membranes were left on the staining buffer soaked paper until all colonies turned blue.

β-Galactosidase assay

The liquid β-galactosidase assays were performed by standard methods. Briefly, 5 ml of a mid-exponential liquid culture of yeast was centrifuged and resuspended in 500 µl of β-galactosidase buffer. Chloroform (40 µl) was added and the cells were vortexed for 1 min. Then 250 µl of β-galactosidase assay buffer complemented with 4 mg/ml of ortho-nitro-phenol-galactopiranoside was added. The incubation took place at 37°C, and activity was measured by optical density at 420 nm divided by time of incubation and number of cells.

Yeast protein extracts

Total protein extracts from yeast were obtained by a standard glass beads method. Briefly, yeasts grown in liquid medium were pelleted and resuspended in one cell pack volume of buffer O-low (100 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 50 mM $(NH_4)_2SO_4$, 1 mM PMSF, 10% glycerol) together with one cell pack volume of glass beads (300–600 µm Sigma). After vigorous vortexing at 4°C, the cell debris and beads were pelleted, and the crude extract was centrifuged at 100 000 *g* for 30 min. The pellet was discarded and the clarified supernatant kept. Final concentration of proteins was measured by Bradford assay (25) and adjusted to 20 mg/ml.

Purification of nickel binding proteins

The poly-histidine tagged proteins were purified on Ni-NTA agarose columns (Qiagen) from yeast extracts as recommended by the manufacturer. Briefly, 5 ml of each extract was loaded on a 2 ml Ni-NTA column, which was then rinsed with 20 ml rinsing buffer 1 (buffer O-low supplemented with 10 mM imidazole), then with 20 ml of rinsing buffer 2 (buffer O-low supplemented with 20 mM imidazole). The Ni^{2+} binding proteins were eluted with elution buffer (buffer O-low supplemented with 300 mM NaCl and 180 mM imidazole) in a final volume of 250 µl. For clones over-expressing poly-histidine tagged DCOH, the final protein concentration was 370 ng/µl, as measured by Bradford assay.

Anti-DCOH polyclonal antibody preparation

The sequence coding for human DCOH was isolated from the prey plasmid of clone number 1 (an *Nco*I–*Eco*RI fragment) and was cloned into pGEX-BNAME (7) in-frame with glutathione-S transferase, yielding bacterial expression vector pGEX-DCOH. A total of 30 mg GST-DCOH fusion protein was produced and purified on glutathione coupled beads, essentially as described previously (7). Rabbit polyclonal antibodies were raised against this fusion protein. Histidine-tagged human DCOH was produced with expression plasmid pHDH14 and purified on a Ni-NTA agarose column (Qiagen) as recommended by the manufacturer. Antibodies specific for human DCOH were purified from 2 ml of serum using a total of 30 mg of DCOH$(his)_6$ covalently coupled to a Affi-Gel 10 column (Bio-Rad) and concentrated to a final volume of 2 ml.

Immunoprecipitations

Prey plasmids from each of the six clones that were sequenced were introduced into yeast strain L40 bearing bait plasmid pBTHFL. As controls, empty prey plasmid pATC II or in-frame DCOH prey plasmid pACT-14his were also introduced into that same strain. Double transformants were grown in liquid medium and total proteins were extracted. A total of 1 mg of each extract was incubated at 4°C for 12 h in RIPA buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% Triton X-100, 0.1% sodium deoxycholate) with 50 µl of protein A–sepharose gel (Pharmacia Biotech) pre-coated with 25 µl of purified rabbit anti DCOH antibodies. After three rinses in 1.5 ml RIPA buffer, the protein A–sepharose gels were mixed with an equal volume of 2× SDS loading buffer (20% glycerol, 4% SDS, 125 mM Tris–HCl pH 6.8, 280 mM β-mercaptoethanol, 10 mg/l bromophenol blue), heated for 5 min at 95°C, centrifuged and the supernatant was analysed by SDS–PAGE followed by western blot.

Western blots

For nickel binding proteins, a total of 5 µl of each Ni-NTA agarose eluate was loaded on separate lanes of a 15% SDS polyacrylamide gel. The gel was run and blotted onto a nitrocellulose membrane (Biorad). The membrane was stained with Ponceau solution (5g/l Ponceau S, 1% acetic acid), and a digitised image of the membrane was kept. The membrane was then rinsed, blocked and incubated with affinity-purified anti LexA antibody number 13 (26). The western blot was revealed using ECL (Amersham).

For immunoprecipitations, a total of 20 µl of each reaction was loaded per lane on a 15% SDS–polyacrylamide gel. The gel was run and blotted as described previously. Once blocked, the membrane was incubated with monoclonal anti-HA epitope antibody 12CA5 (27). The western blot was revealed using ECL (Amersham).

Immunofluorescence

pCG-DCOH and pRFHE (7) driving the expression of DCOH and VSV epitope tagged HNF1 were transfected into C33 cells as described previously (7). Purified polyclonal rabbit anti-DCOH antibody was used to detect DCOH while P5D4 monoclonal antibody (28) was used to visualise VSV epitope tagged HNF1. Images were acquired on an Axiophot (Zeiss) microscope with a slow scan CCD camera C4880 (Hamamatsu), and processed as described previously (29). Briefly, at 100× magnification, optical tomography of transfected cells was performed with a piezo-electric controled motion of the objective. A total of 512 sections in each channel (FITC, Texas Red, DAPI) formed raw three-dimensionnal images with a 120 × 120 × 120 nm^3 cubic voxel size. Using fluorescent beads (100 nm size, Molecular Probes) point-spread functions were identically acquired in the same three channels. The raw images were deconvolved with the respective point-spread functions by the method of Jansson and van Cittert (30,31), yielding high resolution three-dimensional images.

RESULTS

Construction of the bait and preys for the two-hybrid screen

In an attempt to isolate molecular partners of HNF1 in a yeast two-hybrid screen, we cloned the complete HNF1 coding sequence into a two-hybrid bait expression plasmids. This vector, pBTHFL, drives the expression of a chimeric bait protein containing the bacterial LexA repressor, an epitope tag from the influenza virus hemaglutinin, and HNF1 (Fig. 1; see Materials and Methods). A human liver cDNA library was orientedly cloned into the two-hybrid prey vector pACTII (17). The resulting plasmid drives the expression of a chimeric protein comprising a short nuclear localising signal, the Gal4 C-terminal activation domain, the same HA epitope tag and the translation product of one of three frames from the cDNA (Fig. 1; see Materials and Methods).

The bait expression vector was introduced into yeast strain L40 (19) which bears the reporter genes lacZ and His3 under the control of eight and four LexA binding sites, respectively. As could be expected, this bait activated the transcription of both lacZ and His3 reporter genes by itself. As a consequence, the colorimetric detection of β-galactosidase activity always yielded a high basal level when this activating bait was present. We hoped that interacting molecules linked to a genuine yeast activation domain would increase this activity. A secondary transformation was performed with the human liver cDNA library cloned downstream of the Gal4 activation domain (Fig. 1; see Materials and Methods). A total of 4×10^6 transformants were plated onto Nylon membranes lying on solid medium. At this stage, the only selection applied to the transformants was for the presence of both the bait expression vector and a prey expression plasmid. After growth, the colorimetric detection of β-galactosidase activity was performed as described (in Materials and Methods), and clones were isolated as they turned blue, in a timely ordered manner. The assay was stopped when all colonies turned blue due to the basal activity induced by the bait alone.

Clones isolated with the two-hybrid system fall in three groups

During the first hour of the assay, 18 ‘early’ blue clones were isolated. One ‘middle’ clone turned blue during the second hour, and nine ‘late’ clones were isolated during the third hour, before background became too high. The positive clones were grown on solid medium and their respective prey expressing vectors were isolated as described (see Materials and Methods). In order to eliminate possible false positives, we double checked these preys. Strain Y190 (17) bears the same two reporter genes (lacZ and His3) under the control of Gal4 binding sites. We constructed an HNF1 containing bait for this strain. This vector, pGBHFL, drives the expression of a chimeric protein containing the Gal4 DNA binding domain (residues 1–147) fused to the same epitope tag as in pBTHFL, and HNF1 (see Materials and Methods). The preys isolated during the primary screen were introduced into strains L40 and Y190 together with either an empty bait (pBTM116 and pGBT9, respectively) or the HNF1 containing bait (respectively pBTHFL and pGBHFL). Only the last four ‘late’ clones were false positives (Fig. 2). All 18 ‘early’ preys gave a strong signal when HNF1 was in the bait, and no signal when an empty bait was used. The ‘middle’ prey gave a weaker, yet specific signal, while five of the ‘late’ preys gave a signal just above background.

Clones coding for DCOH are out-of-frame

The five ‘late’ preys bear the very same sequence, which encodes the human serum albumin precursor. We regarded these clones as ‘biological background’ in the experiment (see Discussion). The ‘middle’ prey bears the sequence of an unknown gene. The sequence of the translation product of this gene does not bear any

Table 1. Sequence of the 5′ end of various DCOH cDNA isolated with the two-hybrid screen

Clone Number	*Polylinker* insertion site ↓	Met[1]	Frameshift
1	*atc cga att cgg cac gag* CCG CTG GCC ACC TGC TGC CGC CCG CAC GCG CC**A TG**G CTG GCA…		+2
10	*atc cga att cgg cac gag* TGC CGC CCG CAC GCG CC**A TG**G CTG GCA…		+2
2	*atc cga att cgg cac gag* GCG CC**A TG**G CTG GCA…		+2
4	*atc cga att cgg cac gag* GCG CC**A TG**G CTG GCA…		+2
12	*atc cga att cgg cac gag* TG GCA…		+1
5	*atc cga att cgg cac gag* GCA…		+2

Codons are shown according to the frame of the Gal4 activation domain, and HA epitope tag upstream of the cloning site. The natural translation initiation codon of DCOH is in bold letters.

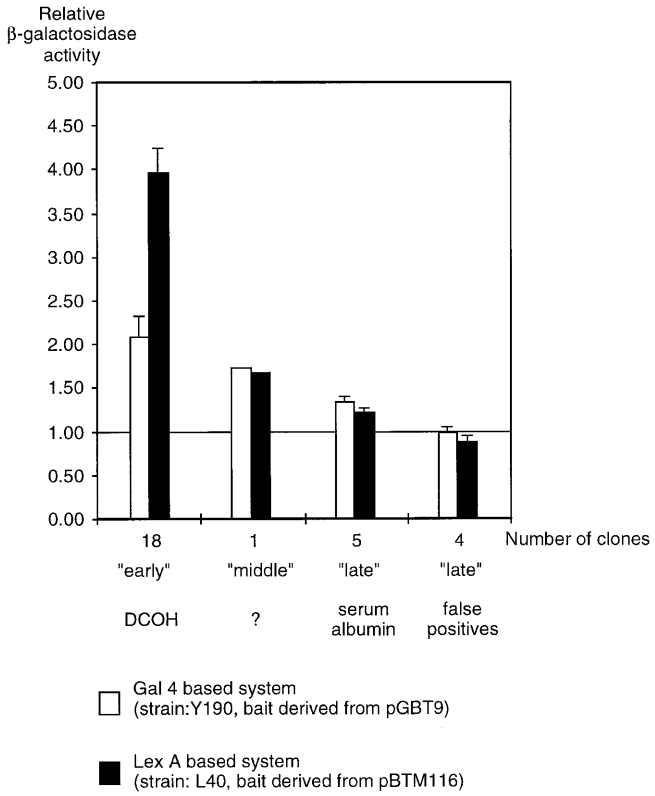

Figure 2. *Ex vivo* binding of HNF1 and the different preys isolated in the two-hybrid screen. The interactions are shown as the β-galactosidase activity measured on liquid cultures (see Materials and Methods). The preys are tested both in the Gal4 based system (strain Y190, baits derived from pGBT9), and the LexA based system (strain L40, baits derived from pBTM116). The β-galactosidase activity resulting from the interactions was normalised twice, first by the level obtained with an empty prey vector (i.e. pACT II) and the same HNF1 containing bait, second with an empty bait vector (pGBT9 or pBTM116) and the same prey. The number of clones isolated in each group and the corresponding gene are indicated below the histogram. For each time group, the mean activity and standard deviation measured with all clones are plotted.

resemblance to any known protein in data banks. This prey was therefore kept for further studies. By cross hybridisation (not shown), we determined that all 18 ‘early’ clones bear a cDNA encoding the same protein. Six of these preys were sequenced and proved to contain the coding sequence of DCOH bearing five different 5′ ends. Curiously, in none of these plasmid was DCOH in-frame with the Gal4 activation domain. Since the putative translation products of frames +1 and +2 of the DCOH ORF present in these clones are very short (7 and 9 amino acids long, respectively), we did not believe that HNF1 would fortuitously bind to both of them. Nevertheless as both frame shifts +1 and +2 were represented among these preys (Table 1), and DCOH is known to bind to HNF1 (9), we went on with the study of these clones.

We first examined whether internal translational start sites were leading to the expression of DCOH alone, without the Gal4 activation domain. Synthesis of free DCOH could perhaps stabilise the LexA–HNF1 fusion protein and increase its transcriptional activity. We therefore constructed pYDCOH, a vector expressing DCOH alone and introduced it into strains L40 and Y190 bearing an HNF1 containing bait. However, no signal higher than background could be detected. We concluded that DCOH alone does not enhance the activation of the reporter genes by the HNF1 containing baits. We next examined if, despite the frameshifts, a fusion between the Gal4 activation domain and DCOH was produced with low efficiency. It is well established that in both yeast and higher eukaryotic cells the translation machinery can overcome frameshifts with low to moderate frequency (32–34). We thus introduced the DCOH ORF back in-frame with the Gal4 activation domain in the same two-hybrid prey expression vector. The resulting plasmid, pACT-DCOH, was introduced into strains L40 and Y190. In both strains, this vector alone induced slowed growth (the generation time was roughly tripled, not shown). When an HNF1 containing bait was added to these yeasts, a strong induction of the lacZ reporter was detected, while no signal was visible with an empty bait. We therefore concluded that a fusion protein between the Gal4 activation domain and DCOH was, indeed, produced in the ‘early’ clones although the two corresponding ORFS are out-of-frame.

To confirm that such fusions are being produced, we used direct immunodetection. To that end, we prepared and purified anti-human DCOH rabbit polyclonal antibodies. With the antibodies we performed an immunoprecipitation with extracts from yeasts bearing both the pBTHFL bait vector and each one of the out-of-frame prey plasmids. The proteins immunoprecipitated were separated on an SDS–PAGE, blotted and probed with a monoclonal anti-HA epitope antibody. Figure 3 shows that the anti-HA epitope antibody detects proteins of ~30 kDa present in various amounts depending on the clone and the frameshift. These proteins had the size expected for an in-frame fusion. No such protein was detected

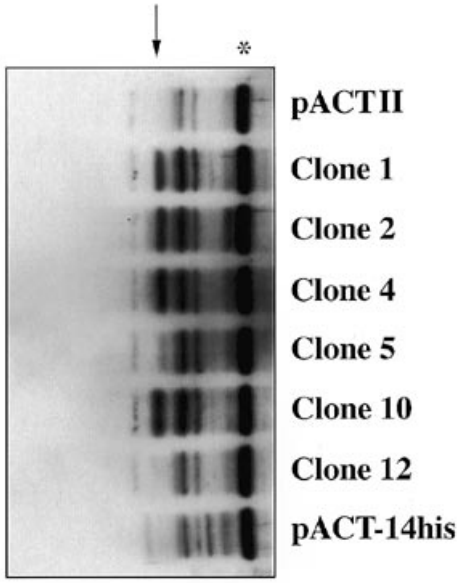

Figure 3. Detection of fusion proteins encoded by out-of-frame sequences from prey plasmids. Western blot of immunoprecipitates from extracts of strain L40 bearing both bait plasmid pBTHFL and one prey plasmid (one for each lane). The immunoprecipitation was performed with purified anti-DCOH polyclonal antibodies while the western blot was probed with anti-HA epitope monoclonal antibody. The arrow indicates the position of the fusion proteins encoded by the prey plasmids (size of ~30 kDa). The yield of the in-frame fusion protein from plasmid pACT-14his was reproducibly low, caused by the instability of the corresponding strain. The asterisk indicates the position of rabbit immunoglobulin heavy chain.

in the negative control (lane 'pACT II'). The protein produced by the in-frame construct is slightly shorter as predicted from the shorter linker sequence in this plasmid (pACT-14his). In the prey expression plasmids, the HA epitope coding sequence and the DCOH ORF are separated by the frameshifts. However, since these proteins were detected by both the purified anti-DCOH polyclonal antibodies and an anti-HA monoclonal antibody, we conclude that the translation machinery of yeast overcomes these frameshifts (with a variable efficiency depending on the clone) and leads to the production of a fusion between the Gal4 activation domain, the HA epitope tag and human DCOH.

DCOH mutants for 4-α-carbinolamine dehydratase activity still bind to HNF1

DCOH was first cloned as a protein co-purifying with HNF1 (9). However, in two independent studies, the sequencing of the purified 4-α-carbinolamine dehydratase necessary for the recycling of tetrahydrobiopterin led to the very same gene (35,36). The link between this enzymatic activity of DCOH and its specific binding to HNF1 is still not completely elucidated (see Discussion). In an attempt to better understand this link, we investigated the effect on binding to HNF1 of mutations that affect the enzymatic activity of DCOH. We used two such mutants, 'C81→S' and 'C81→R' that have been previously characterised biochemically (21). The exact sequences that have been used by Köster *et al* (21) were introduced into the two-hybrid prey expression plasmids. The resulting vectors, pACT-14his, pACT-26his and pACT-37his drive the expression of fusions between the Gal4 activation domain and wild-type DCOH, mutants 'C81→S' and 'C81→R', respectively. All three

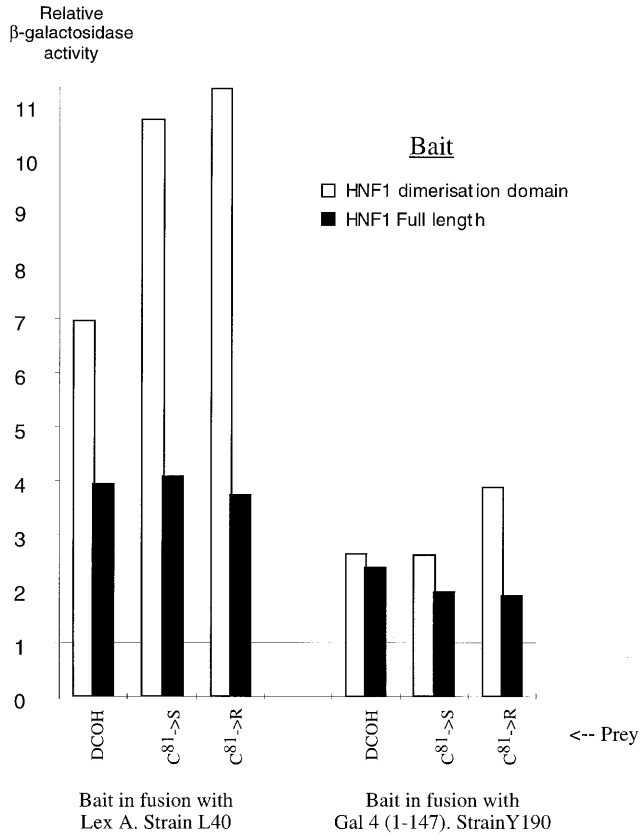

Figure 4. *Ex vivo* binding of wild-type or substitution mutants of DCOH to HNF1 or to the dimerisation domain of HNF1. Both the LexA based system (left) and the Gal4 based system (right) are shown. The β-galactosidase activity was normalised as in Figure 1.

bear six additional histidine residues at their C-termini, just where they were biochemically characterised (21). As shown in Figure 4, both mutants bind HNF1 just as wild-type DCOH. Besides, the poly-histidine tag does not seem to affect these protein–protein contacts.

The dimerisation domain of HNF1 is sufficient for binding to DCOH

Previous *in vitro* results (9) have shown that the dimerisation domain of HNF1 is necessary for binding to DCOH. However, it is not known whether this domain is specifically recognised or if other regions of HNF1 need to be in a dimeric form in order to bind to DCOH. In an attempt to elucidate this issue, we have constructed baits containing only this part of HNF1. Plasmids pBTHdim and pGBHdim drive the expression of chimeric proteins containing the dimerisation domain of HNF1 fused at the C-terminus of LexA and the Gal4 DNA binding domain, respectively. These baits were introduced into strains L40 and Y190, together with vector pACT-14his, pACT-26his or pACT-37his, or with the empty prey vector pACTII. Figure 4 shows the resulting β-galactosidase activities in these strains. A clear signal is observed with wild-type DCOH and both mutants. We therefore conclude that the dimerisation domain of HNF1 is sufficient for binding to DCOH or to the two C81 substitution mutants *ex vivo* (i.e. in *Saccharomyces cerevisiae*). Interstingly, when these enzymatically inactive DCOH mutants are expressed in fusion

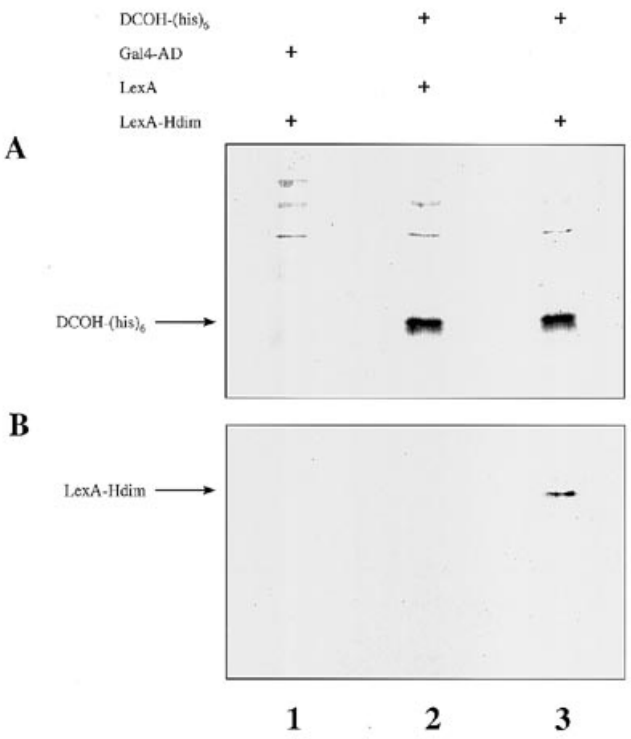

Figure 5. Direct interaction between the HNF1 dimerisation domain and DCOH evidenced by co-purification of LexA–HNF1 dimerisation domain fusion with poly-histidine tagged wild-type DCOH. Ponceau S staining (**A**) and western blot (**B**) performed with anti-LexA purified antibodies. The 15% SDS–PAGE was loaded with Ni^{2+} binding fractions of protein extracts from L40 yeast clones expressing: LexA–HNF1 dimerisation domain fusion and Gal4 activation domain alone (lane 1), wild-type LexA and poly-histidine tagged wild-type DCOH (lane 2) and LexA–HNF1 dimerisation domain fusion and poly-histidine tagged wild-type DCOH (lane 3).

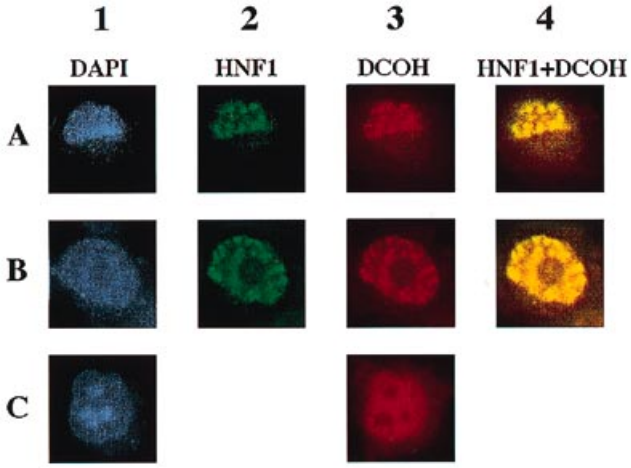

Figure 6. Immunolocalisation of DCOH and HNF1 in C33 transfected cells. Panels A1–4 and B1–4 show an optical section of cells expressing both DCOH and HNF1, while panels C1 and C3 show an optical section of a cell expressing DCOH alone. A1, B1, C1: DNA DAPI staining. A2 and B2: detection of epitope-tagged HNF1 by P5D4 monoclonal antibody. A3, B3 and C3: detection of DCOH by purified polyclonal antibody. A4 and B4: superposition of signals of panels A2–A3 and B2–B3, respectively.

with the Gal4 activation domain, they do not induce slowed growth. There is thus no selection pressure for lower levels of expression of these fusions, which could explain the higher signal observed with these mutants relative to wild-type DCOH.

In order to biochemically confirm the direct interaction between DCOH and the HNF1 dimerisation domain, we prepared three yeast strains, each bearing a pair of expression plasmids. Strain 1 expressed both the LexA–HNF1 dimerisation domain fusion and the Gal4 activation domain alone (vectors pBTHdim and pACT2). Strain 2 expressed both wild-type LexA and the poly-histidine tagged wild-type DCOH (vectors pBTM116 and pY14his). Strain 3 expressed both the LexA–HNF1 dimerisation domain fusion and the poly-histidine tagged wild-type DCOH (vectors pBTHdim and pY14his). Ni^{2+} binding fractions were purified from protein extracts of strains 1, 2 and 3. Figure 5A shows that, as expected, poly-histidine tagged wild-type DCOH is purified only from strains 2 and 3. The western blot shown in Figure 5B was performed with an affinity purified polyclonal antibody recognising the N-terminal 87 residues of LexA (generous gift from Manfred Schnarr). The LexA–HNF1 dimerisation domain fusion was specifically co-purified with the poly-histidine tagged wild-type DCOH. Indeed, lane 1 shows that the LexA–HNF1 dimerisation domain fusion alone has no affinity for Ni^{2+}, and lane 2 shows that wild-type LexA is not co-purified with the poly-histidine tagged wild-type DCOH. Lane 3 shows that LexA can be purified on the Ni^{2+} column only when fused to the HNF1 dimerisation domain and co-expressed with the poly-histidine tagged wild-type DCOH. This demonstrates that DCOH and the dimerisation domain of HNF1 are physically associated in a purifiable complex.

HNF1 co-expression modifies intranuclear localisation of DCOH in transfected cells

In vertebrates, DCOH is expressed in a number of organs or cell types not all of them expressing HNF1 (9,12,37,38). In order to better understand the biological significance of the specific binding of DCOH to HNF1, we compared the nuclear localisation of DCOH in the presence or absence of HNF1. We used human C33 cells which do not express HNF1 nor DCOH. Transfection with mammalian expression plasmids pCGDCOH or RFHE led to the expression of wild-type human DCOH or rat HNF1 tagged with an epitope from VSV (7). In our conditions, the amount of DCOH produced is in excess relative to HNF1, as is believed to occur *in vivo*. Purified polyclonal rabbit anti-DCOH and monoclonal P5D4 (28) antibodies were used to localise DCOH and HNF1. Transfection of pCG-DCOH alone in C33 cells was followed by three-dimensional high resolution imaging. As shown in Figure 6C, in the absence of HNF1, DCOH is mostly nuclear, is evenly distributed in the nucleus and is excluded from the nucleoli. Figure 6, panels A and B, shows optical sections of three-dimensional high resolution images of doubly transfected cells. HNF1 can only be detected in the nucleus with a punctuated pattern (ref. 7 and results not shown). DCOH loses the uniform nuclear labelling pattern and accumulates in distinct intranuclear areas. Panels 4A and 4B of Figure 6 show that in the nucleus both proteins co-localise in distinct strongly labelled areas. The intensity of HNF1 versus DCOH signals were plotted for each voxel of the nucleus. The strong linear correlation obtained confirmed that both proteins are co-localised in the nucleus of co-transfected cells (results not shown). Although we have not yet characterised the subnuclear domains where HNF1 and DCOH preferentially co-localise, our results strongly suggest that both

proteins do interact in the nucleus of mammalian cells. Moreover, the fact that the intranuclear localisation of DCOH changes when HNF1 is present confirms that both proteins interact *in vivo*.

DISCUSSION

The primary aim of this work was to identify molecular partners of HNF1. It resulted in the isolation of three distinct classes of cDNA. One of them was the human serum albumin precursor. Although it is known that HNF1 is involved in transcriptional regulation of the albumin gene (39–45), we do not believe that the binding of HNF1 to the serum albumin precursor is meaningful. The signal observed is very weak, while the messenger RNA of albumin is abundant [up to 10% of total polyA$^+$ RNA in liver (46)]. HNF1 is a nuclear transcription factor while albumin is secreted into circulating fluids. There is therefore little chance that these two proteins meet *in vivo*. The fact that the complete full length precursor was isolated and not shorter clones only bearing the sequence encoding serum albumin could be due to the fact that HNF1 binds to the N-terminus of the precursor which is cleaved and absent in secreted serum albumin. Nevertheless, albumin is known for its colloidal properties and it is not surprising that it can bind weakly and unspecifically to another protein (44). The interaction between HNF1 and albumin may be fortuitous and may define a 'biological background' just above the technical background of the screen.

The second class involved a single isolate with a short ORF that corresponded to an EST present in data banks. Since this EST sequence was isolated from human endothelial cells, it is possible that blood vessels present in the human liver biopsy from which our cDNA library was prepared contributed this messenger RNA. Due to this consideration and to the relatively low signal observed in two-hybrid with this sequence, this clone was kept for further studies.

Since HNF1 is able to form homo- or heterodimers with vHNF1, also present in the liver, the fact that none of these two genes was isolated with the screen is puzzling. In order to elucidate this paradox, we constructed pACT-HFL, an HNF1-containing prey expression vector. When introduced into strain Y190 or L40, this construct by itself proved to be lethal. We believe that this high toxicity explains the absence of HNF1-containing preys among the isolated clones. It should be recalled that the dimerisation domain of HNF1 is at the extreme N-terminus of the protein, and since the library contains cDNAs that were oligo dT primed, any prey containing this dimerisation domain would necessarily contain the entire HNF1 ORF and would thus be lethal. That, at least partly, explains why the two-hybrid approach was not exhaustive in the quest for HNF1 molecular partners.

Our screen lead us to isolate preys coding for DCOH. However, none of the plasmids we have sequenced bear the DCOH encoding ORF in-frame with the Gal4 activation domain and the HA epitope tag. It has been claimed that free DCOH was able to increase transcription activation by HNF1 in transient transfection assays in mammalian cells (9,12). Therefore, one could have wondered if internal translational start sites in the isolated prey plasmids were used to synthesise free DCOH which would increase activation by the HNF1 containing baits in yeast. However, the expression of free DCOH has no effect on transcription activation by HNF1-containing baits in yeast. We favor another hypothesis to explain why the DCOH coding sequences have been isolated out-of-frame. Although in-frame fusion between the Gal4 activation domain, the HA epitope tag and DCOH induces a strong signal, they reduced the growth rate of yeast (a phenonemon not observed if DCOH is mutated and has impaired enzymatic activity). We believe that the translation machinery of *S.cerevisiae* is able to perform a compensatory frameshift leading to the synthesis of limited amounts of in-frame fusion between the Gal4 activation domain and DCOH. Such a mechanism has already been described in *S.cerevisiae* (32–34). Detection of this fusion protein by western blotting in crude yeast extracts was difficult. Only after immunoprecipitation, and therefore specific enrichment, were we able to detect this in-frame fusion. Only plasmids driving limited expression of this in-frame fusion could be selected in the two-hybrid screen without growth disadvantage. The truly in-frame fusion driving preys were probably excluded due to too limited growth and absence of visible colonies.

The fact that DCOH and HNF1 co-localise in specific domains of the nucleus of mammalian cells confirms that the specific interaction between these two proteins occurs in their natural cellular context and is not a fortuitous binding detectable in a two-hybrid system. Our present study demonstrates that DCOH is a molecular partner of HNF1 *ex vivo*. Our approach is independent of that of Mendel *et al.* (9) who first cloned DCOH as a protein that co-purifies with HNF1 isolated from liver nuclear extracts. Therefore, our findings confirm that a specific interaction does occur between these two proteins and show that it takes place in specific domains of the nucleus of mammalian cells.

One could wonder whether the enzymatic activity of DCOH is essential for the interaction with HNF1. We show here that naturally occuring substitution mutants of DCOH that have impaired enzymatic activity still bind to HNF1 *ex vivo*. We conclude that DCOH does not need to retain its 4-α-carbinolamine dehydratase enzymatic activity to bind to HNF1. It has been shown previously that in the homotetrameric form of DCOH, residue C81 is exposed to the solvent in solution (21) or in crystals (13,14). We show that substitution of residue C81 by a serine or an arginine does not impair binding between a dimer of DCOH and a dimer of HNF1. Our results thus suggest that the catalytic site in DCOH is not on the surface interacting with HNF1.

Reciprocally, our results show that the dimerisation domain of HNF1 is sufficient for binding to DCOH, or to its substitution mutants. It had been shown that this part of HNF1 was necessary for the binding of DCOH *in vitro* (9), but it could not be ascertained whether this domain was itself directly involved in the interaction or if just the dimerisation of HNF1 was *per se* a prerequisite for this interaction to occur via other parts of HNF1. Our results answer this question by showing a direct contact between the dimerisation domain of HNF1 and DCOH.

This 31 amino acid domain is known to form homodimers *per se*, or heterodimers with full-length HNF1 (5). Its structure is still unknown, but has been suggested to be mostly α-helical (48), and to possibly fold into a four helix bundle (49). Interestingly, the two dimers of DCOH present in the homotetramer of this protein also forms a four helix bundle, where each α-helix is contributed by a different DCOH molecule (13,14). The stoichiometry of the HNF1/DCOH heterotetramers, and the inability of preformed homotetramers of DCOH to bind to HNF1 (9) suggest that the α-helices of one dimer of DCOH can either bind to the dimerisation domain of HNF1 or to another dimer of DCOH. Since the dimerisation domain of HNF1 is mostly α-helical, we speculate that it could take the very place of the α-helices by which one dimer of DCOH contacts another [as first suggested by Ficner *et al.* (13)].

Our data show that one impact of HNF1 on DCOH is a modification of its intranuclear localisation. Since *in vivo* DCOH is present in cells either expressing or not expressing HNF1 or vHNF1 (9,12,34,35), we believe that this recruitment is functional. Presently, the precise role played by DCOH specifically in the nucleus of HNF1 non-expressing cells is unknown. However, our data suggest that interaction with HNF1 redistributes DCOH within the nucleus and alters or redirects its nuclear function to specific domains.

Reciprocally, interaction with DCOH has a functional impact on HNF1. In transient transfection assays, co-transfection of a DCOH expression vector together with limited amounts of HNF1 expression vector has been shown to enhance transcription activation by HNF1 (9,12). It has recently been suggested that this effect is dependent on the enzymatic activity of DCOH (50). Since our results show that mutants of DCOH that have lost enzymatic activity still bind to HNF1, we believe that this activity could play a role at a different step. Indeed, the binding of HNF1 to its cognate site on DNA enables DCOH to be associated to transcription promoters. Since the enzymatic pocket of DCOH is probably not part of the surface interacting with HNF1, the DCOH dimer associated with HNF1 retains its enzymatic activity. Once part of the protein assembly on the promoter, DCOH can bind various pterins it has affinity for (21), and perform catalysis of chemical modifications of its substrates. Therefore, among others, a possible scenario could be that one such reaction, catalysed by DCOH, would be involved in transcription, the substrate pterin being free or covalently linked to part of the pre-initiation complex.

ACKNOWLEDGEMENTS

We are indebted to Dr Beat Thöny for kindly providing wild-type and mutant DCOH vectors pHDH14, pHDH26 and pHDH37, Dr S. J. Elledge for providing yeast strain Y190 and vectors pACTII and pASI-CYH2, Dr J. Y. H. Camonis for making yeast strain L40 and vectors pYEMBL30/2, pGBT9 and pBTM116 available and to Dr M. Schnarr for the generous gift of anti-LexA purified antibodies. We thank Drs E. J. Miller and L. Prakash for the generous gift of *E.coli* strain MC1066, and Dr Ralf Ficner for making DCOH atomic co-ordinates available. We also thank Drs Tanguy Chouard, Benoît Arcangioli and Marc Delarue for fruitful discussions. D.J.D.S. is supported by the Direction de la Recherche et de la Technologie of the DGA. This work was supported by grants from the EEC Biotechnology program, the ARC and the LNFCC.

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