Differential expression of chicken dimerization cofactor of hepatocyte nuclear factor-1 (DcoH) and its novel counterpart, DcoH α

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We have used differential display PCR to study altered gene expression in immortalized chicken embryo fibroblasts (CEFs) that have been established in our laboratory. This technique resulted in the cloning of a novel counterpart of the previously cloned chicken dimerization cofactor of hepatocyte nuclear factor (HNF)-1 (cDcoH), which was identified as cDcoH α . The steady-state mRNA levels of cDcoH α were up-regulated in all immortal CEFs tested compared with primary CEF cells. cDcoH and cDcoH α showed opposite patterns of mRNA expression due to differential regulation of transcription rates, but not mRNA half-lives, in primary and immortal CEFs. Expression of cDcoH α increased in the late G1 and early S phases of the cell cycle, while cDcoH mRNA increased in the late S and G2/M phases. In contrast with consistent expression of both genes in primary quiescent cells, cDcoH mRNA, but not cDcoH α mRNA, was

dramatically decreased in primary senescent cells. The highest levels of cDcoH α mRNA were found in the kidney, liver, heart and ovarian follicles, while the major tissues expressing cDcoH were hypothalamus, kidney and liver. cDcoH and cDcoH α probes did not cross-hybridize to human hepatocyte mRNA. When transfected into human HepG2 cells, both cDcoH and cDcoH α showed similar functional activity as measured by increased expression of a reporter gene, as well as α -fetoprotein and albumin genes that both contain HNF-1 binding elements in their promoters. Our results suggest that the novel chicken DcoH α might function as a transcriptional cofactor for HNF-1 in specific cellular-environmental states.

Key words: chicken embryo fibroblast, DF-1, hepatocyte nuclear factor-1, immortalization, senescence.

INTRODUCTION

Normal human and avian cells have rarely been shown to be capable of spontaneous immortalization [1,2]. However, we have recently established a number of non-virally and non-chemically immortalized chicken embryo fibroblasts (CEFs), including the spontaneously immortalized cell line, DF-1 [2,3], and several immortal CEFs derived from specific tissues. Our immortal CEFs should serve as excellent models for the analysis of altered gene expression associated with cell growth, senescence and immortalization. Therefore we have used differential display PCR (DD-PCR) to isolate genes up-regulated in immortal CEFs. We have cloned and characterized a novel counterpart of chicken dimerization cofactor of hepatocyte nuclear factor (HNF)-1 (cDcoH). DcoH is a bi-functional protein, serving as a transcriptional cofactor [4,5] for HNF-1, which regulates tissue-specific expression of a variety of genes, such as α -fetoprotein, albumin and phenylalanine hydroxylase [6–8], and as a pterin carbinolamine dehydratase (PCD) involved in the regeneration of tetrahydrobiopterin [9,10].

HNF-1 is composed of HNF-1 α and HNF-1 β , a family of transcription factors that contain homeodomains [11,12]. The DcoH has been shown to stabilize HNF-1 dimers bound to their consensus-binding element [13], which results in increased transcriptional activity [4,5]. Structural studies have suggested that DcoH appears as a double saddle-like tetrameric structure that binds tightly to the minimal region of HNF-1 α dimerization

domain and is similar to the TATA-binding transcription factor [14–16]. Moreover, it has been demonstrated that DcoH can bind macromolecules, such as proteins and nucleic acids [17].

The tetrahydrobiopterin regenerated by the PCD enzymic activity of DcoH is known to be an essential cofactor for aromatic amino acid hydroxylases [18], which are involved in the synthesis of neurotransmitter precursors, such as dopamine, noradrenaline, adrenaline and serotonin by the metabolism of phenylalanine, tyrosine and tryptophan. Tetrahydrobiopterin has been shown to increase the growth rates of PC12 pheochromocytoma cells, and transformed fibroblasts and C6 glioma cells [19]. Although the loss of tetrahydrobiopterin is directly associated with hyperphenylalaninaemia or phenylketonuria [20], a lack of PCD/DcoH function is shown to be related with a rare, mild, form of hyperphenylalaninaemia [21]. Therefore it seems possible that other factor(s) might be involved in the regeneration of tetrahydrobiopterin.

In the present study, we compare altered expression patterns of cDcoH and its novel counterpart, cDcoH α , in primary and immortal CEFs and characterize their function as cofactors to increase HNF-1 α transcriptional activity.

MATERIALS AND METHODS

Cells and culture conditions

All cell culture reagents were purchased from Life Technologies (Rockville, MD, U.S.A.). All primary and immortal CEFs, as

Abbreviations used: CEF, chicken embryo fibroblast; BCEFi, immortal breast-derived CEF; BoCEFi, immortal bone-derived CEF; HCEFi, immortal heart-derived CEF; SkCEFi, immortal skin-derived CEF; VCEFi, immortal vector-derived CEF; DD-PCR, differential display PCR; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HNF, hepatocyte nuclear factor; DcoH, dimerization cofactor of HNF-1; cDcoH, chicken DcoH; PCD, pterin carbinolamine dehydratase; RT-PCR, reverse transcriptase PCR.

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well as HepG2 cells, were grown in Dulbecco's modified Eagle's medium (DMEM) enriched with 10% (v/v) fetal calf serum (FCS), 1% (v/v) antibiotic/antimycotic solution and 1% (w/v) L-glutamine. The chicken-transformed hepatocyte cell line, LMH, was grown in DMEM enriched with 6% (v/v) FCS, 10% (v/v) tryptone phosphate broth and 1% (v/v) antibiotic/antimycotic solution. Primary CEF cells normally senesce and enter a crisis stage between passages 17 and 20. The spontaneously immortalized DF-1 cell line [2,3] was derived from *ev-0* Leghorn layer embryos (Avian Disease and Oncology Laboratory, East Lansing, MI, U.S.A.) and has been in culture for over 5 years (> passage 300). All additional immortal CEFs (BCEFi, immortal breast-derived CEF; HCEFi, immortal heart-derived CEF; SkCEFi, immortal skin-derived CEF; BoCEFi, immortal bone-derived CEF; and VCEFi, immortal vector-derived CEF) and their primary CEF counterparts were cultured using identical conditions as above. BCEFi, HCEFi and SkCEFi cells were derived from specific pathogen-free avian supply ('SPAFAS') embryos (SPAFAS Inc., Preston, CT, U.S.A.). The VCEFi and BoCEFi cells were established from *ev-0* embryos. Primary *ev-0* CEFs (presenescent passage 3 and senescent passage 17) were seeded as above and allowed to grow until they became approx. 80% confluent. Primary passage 8 *ev-0* cells were made quiescent by allowing cells to reach approx. 80% confluency, and then re-feeding with DMEM enriched with 0.2% FCS for 72 h. Cell-cycle arrest was determined by [³H]thymidine labelling. After 72 h, a second set of quiescent cultures was serum stimulated by re-feeding for 5 h with DMEM containing 20% (v/v) FCS. To confirm phenotypic senescence, primary cells (passage 17) were seeded as above and re-fed every 3 days for 2 weeks. Cells were stained with 5-bromo-4-chloroindol-3-yl β -D-galactopyranoside (X-Gal) at pH 6.0 to detect senescence-associated β -galactosidase (also known as LacZ), a histochemical marker of replicative senescence [22].

Total RNA isolation

Total RNA was isolated using TRIzol® (Life Technologies) from different cell passages of primary *ev-0* CEFs (presenescent passage 3, quiescent passage 8, serum-restimulated passage 8 and senescent passage 17), immortal DF-1 cells (passages 45 and 185), all exponentially growing immortal CEFs and their primary counterparts, and HepG2 and LMH cells, as well as various tissues of 18-week-old White Leghorn chickens.

DD-PCR

Differentially expressed genes were identified using the procedure of Liang and Pardee [23]. To characterize differentially expressed products, cDNA fragments of interest were eluted from the DNA sequencing gel and re-amplified using the same primers as in the initial DD-PCR reaction. Reamplified cDNA products were blunt-ended, phosphorylated and cloned into the *EcoRV*-digested pBluescript SK(−) vector (Stratagene, Inc., La Jolla, CA, U.S.A.).

cDNA library screening and sequence analysis

A λ ZAPII (Stratagene) cDNA library (constructed using total cellular RNA from passage 65 DF-1 CEFs) was screened using the cDcoH α PCR fragment as a probe. A total of five positive clones were obtained from a primary screening of 1×10^5 plaques, and three clones were subcloned by *in vivo* excision and both strands were subjected to automated DNA sequencing in the Advanced Genetic Analysis Center (University of Minnesota, St. Paul, MN, U.S.A.).

Northern-blot analysis

RNA (20 μ g) from different cell passages was separated by electrophoresis on a 1% (w/v) agarose gel containing formaldehyde, and blotted on to a positively charged nylon membrane (Schleicher and Schuell, Keene, NH, U.S.A.). Reamplified cDNAs were labelled using a Prime-It II Random Primer Labeling Kit (Stratagene). The membrane was hybridized with a [α -³²P]dCTP-labelled cDcoH α fragment derived from DD-PCR, cDcoH, human DcoH, α -fetoprotein and albumin cDNA (amplified by specific primers as described below) in a hybridization solution containing 50% (v/v) deionized formamide, $5 \times$ SSPE (where $1 \times$ SSPE corresponds to 150 mM NaCl, 10 mM sodium phosphate monobasic and 1 mM EDTA, pH 7.4), 1% (w/v) SDS, $10 \times$ Denhardt's solution (where $1 \times$ Denhardt's solution corresponds to 0.02 g of Ficoll 400, 0.02 g of polyvinylpyrrolidone and 0.02 g of BSA dissolved in 100 ml of water) and 200 μ g/ml sonicated salmon sperm DNA at 42 °C for 16 h. Equal RNA loading was determined by re-probing the membrane with [α -³²P]dCTP-labelled chicken glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or by staining the gel with ethidium bromide.

Figure 1 Comparison of nucleotide and amino acid sequences of cDcoH and cDcoH α .

(A) The nucleotide sequence of cDcoH α is compared with the coding region of cDcoH [26], and identical nucleotides are shaded. Translation start and termination sites are shown in bold. The polyadenylation signal is underlined. (B) Comparison of the putative amino acid sequence of cDcoH α with cDcoH [26], human DcoH (hDcoH) [4] and *Xenopus* DcoH (xDcoH) [25]. The amino acids that are identical between cDcoH α and other DcoHs are shaded. The amino acids critical for PCD enzymic activity are shown in bold and are underlined.

Figure 2 Expression of cDcoH and cDcoH α in primary and immortal CEFs

(A) Differential expression of the 0.9 kb cDcoH α transcript from different passages (p) of primary and immortal DF-1 cells. (B) cDcoH α expression in BCEFI, HCEFI and their primary cell counterparts. RNA loading was determined by probing with chicken GAPDH cDNA. Consistent results were obtained using RNA prepared from a second independent experiment. (C) The RNA blot prepared from immortal (Im) CEFs and their primary (Pr) counterparts at approx. 80% confluency was hybridized with the cDcoH cDNA probe, using the hybridization and wash conditions described in the Materials and methods section. The cDcoH probe showed a specific single 1.3 kb transcript. (D) After stripping the cDcoH probe, the blot was re-hybridized with the cDcoH α cDNA probe, and washed under high stringency conditions as described in the Materials and methods section. The cDcoH α probe showed a specific single 0.9 kb transcript. Equal loading of RNA was determined by reprobing the membrane with a chicken GAPDH cDNA. The steady-state mRNA levels of cDcoH and cDcoH α visualized in (C) and (D) were quantified using the NIH image program, and values normalized to GAPDH mRNA levels are presented as relative expression levels (means $\pm$ S.D.). For both (C) and (D): lane 1, primary CEF passage 5; lane 2, immortal BoCEFI; lane 3, immortal DF-1; lane 4, immortal VCEFI; lane 5, primary skin CEF passage 5; lane 6, immortal SkCEFI; lane 7, primary breast CEF passage 4; lane 8, immortal BCEFI; lane 9, primary heart CEF passage 6; lane 10, immortal HCEFI. Consistent results were obtained using RNA prepared from a second independent experiment. Despite the high nucleotide sequence similarity, there was no evidence that the cDcoH and cDcoH α transcripts cross-hybridized to the other respective probe.

Nuclear run-on assays

Nuclei were prepared from approximately 1×10^8 primary passage 5 and immortal DF-1 passage 245 CEFs. The *de novo* synthesized transcripts were labelled with [α - 32 P]UTP as previously described [24]. Two membranes were blotted with equal amounts of the PCR-amplified cDcoH and cDcoH α cDNA. GAPDH and luciferase genes, amplified by PCR, were used as positive and negative controls respectively. Hybridization conditions for nuclear run-on transcription rates were identical with those used in the Northern-blot analysis, and specific hybridization signals were analysed by the NIH image program.

mRNA half-life analysis

To determine the half-lives of cDcoH and cDcoH α mRNAs, primary passage 5 and immortal DF-1 passage 245 CEFs were allowed to grow to approx. 80% confluence in DMEM containing 10% (v/v) FCS, and were then treated with 5 μ g/ml actinomycin D. Total RNA was isolated at increasing time points after treatment (0, 1, 3, 6, 10 and 16 h). The half-lives of cDcoH and cDcoH α mRNAs were determined by semi-quantitative reverse transcriptase PCR (RT-PCR) with their specific primer pairs and then quantified using the NIH image program.

Figure 3 Transcription rates and half-lives of cDcoH and cDcoHα mRNAs in primary and immortal CEFs

(A) *De novo* synthesized [32 P]UTP-labelled transcripts from primary CEFs and immortal DF-1 cells were used to probe PCR-amplified cDNA fragments (cDcoH, cDcoHα, GAPDH and luciferase) to determine transcription rates. Quantitative analysis of cDcoH and cDcoHα transcription activity was carried out using the NIH image program, and values are displayed as relative rates of synthesis (means $\pm$ S.D.). (B) The half-lives of cDcoH and cDcoHα mRNAs were determined by RT-PCR using total cellular RNA isolated from primary CEFs and immortal DF-1 cells treated with actinomycin D (ActD) at various time points. Each cDcoH and cDcoHα transcript half-life was quantified using the NIH image program, and values are plotted as the percentage of mRNA remaining (means $\pm$ S.D.).

Cell-cycle analysis

Primary CEFs were allowed to reach approx. 80 % confluency in the presence of 10 % (v/v) FCS, and were subsequently growth arrested with medium containing 0.2 % FCS for 72 h. The DNA content of trypsin-treated cells was determined by staining with propidium iodide using a Cellular DNA Flow Cytometric Analysis kit (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.). Cell-cycle analysis was carried out using a FACScan instrument, and data were analysed using a Cell Quest software program (Becton Dickinson, San Jose, CA, U.S.A.).

RT-PCR

DNase I-treated RNA (3 μ g) was converted into cDNA with Superscript II reverse transcriptase (Life Technologies) following the manufacturer's instructions. RT-PCR was conducted with a portion (1 μ l) of the reverse transcriptase reaction using the following specific primers: cDcoH, 5'-ATGGCAGGTAAAGC-CCACAGG-3' (forward primer) and 5'-GTCCAACGTCGA-AGAGAGAGGACT-3' (reverse primer); cDcoHα, 5'-ATGTC-TTCCCAGTCCCATTGGCTGAC-3' (forward primer) and 5'-TTATACAGAGGCAGCAGCCTTGTC-3' (reverse primer); human α -fetoprotein, 5'-GTTGCAGAGGAGATGTGCTG-3' (forward primer) and 5'-CTGAGACAGCAAGCTGAGGA-3' (reverse primer); human albumin, 5'-TGCAGATCCTCATG-AATGCT-3' (forward primer) and 5'-GGTTCAGGACCAC-GGATAGA-3' (reverse primer). After denaturing at 95 °C for 1 min, cDcoH, cDcoHα, human α -fetoprotein and human albumin fragments were amplified by 23 cycles (verified to be in the linear range) using *TaKaRa Ex Taq*TM (TaKaRa Biomedicals, Shiga, Japan) at 95 °C for 30 s, 65 °C for 1 min and 72 °C for 45 s for each amplification cycle. Chicken and human GAPDH mRNAs were amplified by 18 cycles (verified to be in the linear range) to normalize the RT-PCR reaction. All RT-PCR products were subjected to agarose gel electrophoresis fractionation followed by transfer on to positively charged nylon membranes, and verified by hybridization to their respective cDNA probes, using identical conditions as described above.

Genomic Southern-blot analysis

Genomic DNA was obtained from chicken CEFs using lysis buffer [50 mM Tris/HCl (pH 8.0), 0.5 % SDS, 50 mM EDTA

and 50 μ g/ml proteinase K] at 56 °C for 1 h, followed by the addition of 10 μ g of DNase-free RNase A (Roche Molecular Biochemicals) at 37 °C for 30 min. Genomic DNA was extracted using the phenol/chloroform/ethanol precipitation method. For genomic Southern-blot analysis, 15 μ g of DNA was digested with *Hind*III, separated by electrophoresis, blotted and probed with cDcoH followed by cDcoHα.

Transfection and reporter gene assay

The pcDNA3.1 vector (Invitrogen, Carlsbad, CA, U.S.A.) was used to construct both cDcoH and cDcoHα expression plasmids (pcDNA3.1-cDcoH and pcDNA3.1-cDcoHα). The HNF-1-luciferase reporter plasmid (pGL3-hPHABOX-1) was constructed in the pGL3 luciferase reporter vector (Promega, Madison, WI, U.S.A.) using double-stranded oligonucleotides corresponding to HNF-1 consensus binding sequences (PHABOX-1, 5'-CTCATGGATTATGATTAACCTCAACCTT-3'; derived from the human phenylalanine hydroxylase gene [8]). Human HepG2 cells were transfected with pcDNA3.1 (control), pcDNA3.1-cDcoH or pcDNA3.1-cDcoHα expression plasmids using the standard calcium phosphate precipitation method. After antibiotic selection, total RNA from stable transfectants was isolated to determine the functional activity of cDcoH and cDcoHα by monitoring the expression of HNF-1 regulated genes, such as α -fetoprotein and albumin [6,7].

For reporter gene assays, pGL3-hPHABOX-1 plasmid (10 μ g) and pcDNA 3.1 *LacZ* plasmid (2 μ g) were transiently co-transfected into stable pcDNA3.1, pcDNA3.1-cDcoH or pcDNA3.1-cDcoHα transfectants. The pcDNA 3.1 *LacZ* plasmid was used to normalize transfection efficiency. Reporter gene activity was measured 24 h after transfection. Following cell lysis with TRIzol[®], total RNA was isolated and treated with RNase-free DNase I to eliminate possible genomic or transfected plasmid DNA contamination. Expression levels of luciferase mRNA were determined by semi-quantitative RT-PCR using identical methods to those described for RT-PCR. All luciferase, GAPDH and *LacZ* genes were amplified with their specific primers at 95 °C (30 s), 65 °C (1 min) and 72 °C (45 s) for 16 cycles. The following specific primers were used: luciferase, 5'-GTGTCG-CTCTGCCTCATAGAACTGCCTGCG-3' (forward primer) and 5'-GTCTCCAGAATGTAGCCATCCATCCTTGTC-3' (reverse primer); *LacZ*, 5'-ATCTTCCTGAGGCCGATACT-GTCGT-3' (forward primer) and 5'-GCACATCTGAACTTC-

AGCCTCCAGT-3' (reverse primer). The relative expression levels of the luciferase reporter gene were normalized using the expression levels of the GAPDH and *LacZ* genes.

Data documentation and analysis

All image data were processed by the Eagle Eye® II still video system (Stratagene) and analysed with the NIH Image software program.

RESULTS AND DISCUSSION

Isolation of a novel cDcoH counterpart, cDcoH α

Cellular immortalization may be the result of altered expression of genes involved in cell proliferation. Comparison and identification of genes differentially expressed in primary and immortal cells could be important for understanding the mechanism(s) of immortalization. To isolate such genes, we performed DD-PCR to compare the expression patterns of a variety of genes in primary presenescent (passage 3), presenescent quiescent (passage 8), presenescent quiescent/serum stimulated (passage 8) and senescent (passage 17) CEFs, as well as in passages 45 and 185 of the immortal DF-1 cells. The DD-PCR cDNA fragments up-regulated in immortal cells were used as probes to screen a cDNA library prepared from immortal DF-1 cells. Among the initial 108 DD-PCR cDNA fragments, sequence analysis identified one of the clones as a novel counterpart of DcoH. The cloned fragment had nucleotide similarities with the majority of DcoH genes identified from *Xenopus* [25], chicken [26] and mammals [4] (Figure 1). Therefore we designated the novel cDcoH counterpart as cDcoH α .

The DcoH was originally isolated from mouse liver tissue [4] and shown to be identical with the PCD gene [9,10]. Interestingly, the novel cDcoH α also has a highly conserved motif (His-His-Pro/His) for PCD enzymic activity [27], as shown in Figure 1(B). The nature of the highly similar nucleotide sequence and the completely conserved PCD motif of both cDcoH and cDcoH α suggest that the novel cDcoH α may exert similar activities to that of cDcoH.

Differential regulation of cDcoH and cDcoH α mRNA expression in primary and immortal CEFs

Northern-blot analysis revealed that the steady-state levels of cDcoH and cDcoH α mRNAs from primary and immortal CEFs were differentially regulated (Figure 2). The expression of cDcoH α increased in the immortal DF-1 cells compared with primary presenescent, quiescent, quiescent/serum stimulated and senescent cells (Figure 2A). Furthermore, elevated cDcoH α expression levels were detected in all of the immortal CEFs assayed (Figures 2B and 2D). Interestingly, when expression levels of cDcoH and cDcoH α mRNAs were compared, quite different patterns were obtained from primary and immortal CEFs. In contrast with the up-regulated expression of cDcoH α in immortal CEFs, cDcoH was down-regulated in all of the immortal CEFs (Figures 2C and 2D).

To further define whether the differential expression pattern was regulated by altered *de novo* mRNA synthesis or by degradation [28], the transcription rates and half-lives of cDcoH and cDcoH α in primary and immortal CEFs were measured using *in vitro* nuclear run-on assays and actinomycin D treatment respectively. As shown in Figure 3(A), the *de novo* transcription rate of cDcoH in primary CEFs was 3.4-fold higher than that observed in immortal DF-1 cells, whereas the cDcoH α mRNA synthesis rate was 3.7-fold higher in immortal DF-1 cells com-

Figure 4 Cell-cycle dependent expression of cDcoH and cDcoH α mRNAs

(A) For analysis of the expression patterns of cDcoH and cDcoH α in different phases of the cell cycle, primary passage 8 CEFs were growth-arrested then stimulated with 20% (v/v) FCS. Cell-cycle analysis of primary CEFs was performed using FACScan and the Cell Quest software program. Abbreviations used: C, control [for exponentially growing cells in DMEM containing 10% (v/v) FCS]; Q, quiescent [for growth-arrested cells in DMEM containing 0.2% FCS for 72 h]. (B) Expression of cDcoH and cDcoH α in different stages of the cell cycle was determined by semi-quantitative RT-PCR. (C) The cDcoH and cDcoH α mRNA levels from three independent experiments were quantified using the NIH image program, and values normalized to GAPDH mRNA levels are plotted as relative expression levels (means $\pm$ S.D.).

pared with primary CEFs. In contrast with the altered transcription rates, both cDcoH and cDcoH α have similar mRNA half-lives in primary and immortal CEFs (approx. 8 h) as confirmed by actinomycin D treatment (Figure 3B). Therefore the altered expression of cDcoH and cDcoH α mRNAs in primary and immortal CEFs seems to be the result of transcriptional, rather than post-transcriptional, regulation.

Cell-cycle dependent expression of cDcoH and cDcoH α

To further characterize cDcoH and cDcoH α mRNA expression, we determined the expression levels of both genes at different

Figure 5 Serum-independent, senescence-dependent, alteration of cDcoH and cDcoH α expression

(A) Comparison of the steady-state expression levels of both cDcoH and cDcoH α in proliferating primary CEFs (passage 8), growth-arrested primary CEFs (passage 8) at 100% confluency, and growth-arrested primary CEFs (passage 8) grown in 0.2% FCS for 1, 2 and 3 days respectively. cDcoH, cDcoH α and GAPDH mRNA levels were determined by semi-quantitative RT-PCR. (B) Expression levels of cDcoH and cDcoH α in different primary CEF passages (young-presenescent passage 3, and senescencing passages 17 and 18) were compared using semi-quantitative RT-PCR. The cDcoH and cDcoH α mRNA levels from three independent experiments were quantified using the NIH image program, normalized to GAPDH mRNA levels and are plotted as relative expression levels (REL; means $\pm$ S.D.).

points in the cell cycle of primary CEFs. Primary passage 8 cells were synchronized by reducing serum concentration to 0.2% FCS for 72 h. In this growth state, the majority of primary CEFs (94%) were arrested in the G0/G1 phase of the cell cycle (Figure 4A). Primary CEFs arrested at the G0/G1 stage entered the S and G2/M phases at 10 and 20 h after serum stimulation respectively (Figure 4A).

Expression of chicken c-Myc mRNA (*c-myc* is an early serum response gene [29]) was monitored to verify serum stimulation of primary quiescent passage 8 CEFs. The expression of both cDcoH and cDcoH α mRNAs was shown to be similar in control (rapidly growing cells) and serum-deprived quiescent cells, whereas the expression of both cDcoH and cDcoH α mRNAs decreased in the early stages of G1 following serum stimulation (Figures 4B and 4C). This observation suggests that the expression of both cDcoH and cDcoH α mRNAs might be repressed by certain growth factor(s) found in serum. However, the expression of cDcoH α mRNA increased in the late G1 and S phases, and decreased dramatically in G2/M phases, while cDcoH mRNA remained elevated in the G2/M phases (Figures 4B and 4C).

Serum-independent, age-dependent, alteration of cDcoH and cDcoH α expression

After reducing serum levels or contact inhibition, most cells withdraw from the cell cycle, maintain an unduplicated DNA content and persist in a quiescent state where macromolecular synthesis is dramatically reduced [30]. To confirm the growth arrest-independent expression of cDcoH and cDcoH α , primary passage 8 CEFs were subjected to cell-cycle arrest by serum reduction or contact inhibition. As shown in Figure 5(A), the steady-state expression levels of both cDcoH and cDcoH α mRNAs were not altered by either low serum concentrations (0.2% FCS) or contact inhibition. These results indicate that the expression of both cDcoH and cDcoH α mRNAs appears not to be regulated by specific states of cell growth.

Normal senescent cells inevitably arrest with a G1 DNA content similar to quiescent cells. However, senescent cells irreversibly lose their ability to enter S phase in response to mitogen stimulation [31,32]. Interestingly, cDcoH mRNA was markedly down-regulated in primary senescing CEFs at passage

17 and even more dramatically at passage 18, whereas cDcoH α mRNA was continuously expressed in senescing cells (Figure 5B). Therefore the senescence-specific down-regulation of cDcoH mRNA might be another distinguishable characteristic marker between quiescent and senescent cells.

Genomic DNA analysis, tissue distribution and interspecies expression of cDcoH and cDcoH α

The unique nature of the cDcoH and cDcoH α genes was confirmed by genomic Southern-blot analysis (Figure 6A). Both genes displayed specific differences in the major hybridization bands after genomic DNA restriction-enzyme digestion, strongly suggesting that cDcoH and cDcoH α genes are structurally unique and distinct. As expected, due to the high sequence similarity of the two cDNA sequences, there were several minor genomic bands that cross-hybridized with the two probes.

Expression patterns of both cDcoH and cDcoH α were examined in various tissue types by Northern-blot analysis. DcoH was originally isolated from mammalian liver and was highly expressed in the liver and kidney [4,33]. As expected, cDcoH was up-regulated in the liver, kidney and, interestingly, in the hypothalamus (Figure 6B). This result suggests that the synthesis of various neurotransmitters, such as dopamine and serotonin, might be regulated by tetrahydrobiopterin regenerated by PCD/DcoH enzyme function in the hypothalamus [18]. In contrast with the tissue-specific expression of cDcoH, cDcoH α was detected in the majority of chicken tissues analysed, and was shown to have highest expression in the kidney, liver, heart and ovarian follicles (Figure 6B). The expression of cDcoH α mRNA in various tissues (where co-transcriptional activity for HNF-1 might not necessarily be needed) suggests that the cDcoH α gene might have other plausible biological function(s), such as the recycling of an essential cofactor of several other metabolic enzymes through regeneration of tetrahydrobiopterin in a variety of tissue environments.

To address the expression of the novel DcoH α gene in other species and notably in mammals, we prepared total RNAs from human HepG2 and chicken-transformed LMH hepatocytes, and performed Northern-blot analysis (Figure 6C). The cDcoH mRNA was highly expressed in the LMH cell line compared with DF-1 cells, and cDcoH α mRNA was detected at similar levels in

Figure 6 Genomic DNA analysis and tissue distribution of cDcoH and cDcoH α

(A) Chicken genomic DNA (15 μ g) was digested with *Hind*III. DNA membranes were hybridized with full-length cDcoH and cDcoH α cDNA probes. Lambda-DNA digested with *Hind*III was used as a DNA size marker (kb). (B) Northern-blot analysis of RNA from various chicken tissues was used to determine the tissue distribution patterns of cDcoH and cDcoH α . RNA loading and integrity were monitored by staining the RNA blot with Methylene Blue (results not shown). (C) Total cellular RNAs from immortal DF-1 cells (lane 1), chicken transformed LMH hepatocytes (lane 2) and human HepG2 hepatocytes (lane 3) were hybridized to cDcoH, cDcoH α and human DcoH (hDcoH) cDNA probes. The arrows indicate specific signals hybridized to their corresponding probes. RNA loading and integrity were monitored by staining the RNA gel with ethidium bromide (EtBr).

both DF-1 and LMH cells. Both cDcoH and cDcoH α probes did not cross-hybridize with human HepG2 mRNA. Additional Northern-blot analysis clearly showed that the human DcoH cDNA probe hybridized only to total cellular RNA derived from human HepG2 cells, but not chicken DF-1 or LMH cells. Even though both chicken and human DcoH probes displayed high nucleotide sequence similarities, they did not cross-hybridize to each other's endogenous DcoH mRNA. Although we did not detect a possible human DcoH α counterpart using Northern-blot analysis, the existence of a human DcoH α cannot be ruled out.

Functional activity of cDcoH and cDcoH α in HepG2 cells

DcoH has been shown to maximize transcriptional activity of HNF-1 in cultured hepatocytes and liver tissues by the stabilization of homo- or hetero-dimer formation of HNF-1 and stabilization of HNF-1–DNA complexes [11–13]. To assess this

functional activity, we transiently transfected a reporter gene (pGL3-hPHABOX-1) that contained the HNF-1 consensus binding element (derived from human phenylalanine hydroxylase gene [8]) into HepG2 cells that had previously been stably transfected with pcDNA3.1 control vector, pcDNA3.1-cDcoH or pcDNA3.1-cDcoH α expression plasmids. As shown in Figure 7(A), expression of luciferase mRNA (reporter gene) increased 2.5-fold in both stable transfectants derived from transfection with pcDNA3.1-cDcoH or pcDNA3.1-cDcoH α expression plasmids compared with pcDNA3.1 control transfection.

Finally, we have determined the expression levels of endogenous α -fetoprotein and albumin genes that are transcriptionally regulated by HNF-1 [6,7] by overexpressing cDcoH and cDcoH α in HepG2 cells. Consistently, both α -fetoprotein and albumin mRNAs increased 2.2–2.7-fold in HepG2 cells transfected with cDcoH and cDcoH α compared with control vector-transfected cells (Figure 7B). However, there were no evident differences in the expression levels of α -fetoprotein and albumin mRNAs

Figure 7 cDcoH and cDcoH α functionally increase HNF-1 transcriptional activity

(A) Stable HepG2 transfectants derived from the introduction of pcDNA3.1, pcDNA3.1-cDcoH or pcDNA3.1-cDcoH α expression plasmids were transiently transfected with the pGL3-hPHABOX-1 reporter plasmid (10 μ g) and the pcDNA3.1 *LacZ* plasmid (2 μ g). *LacZ* was amplified by PCR to normalize transfection efficiency. Luciferase mRNA levels were determined by semi-quantitative RT-PCR as described in the Materials and methods section. Luciferase mRNA levels in the stable pcDNA3.1 transfectants were set to 1. The normalized values shown are relative expression levels. Results are presented as means $\pm$ S.D. (B) Total RNA was prepared from stable HepG2 transfectants derived from the introduction of pcDNA3.1, pcDNA3.1-cDcoH or pcDNA3.1-cDcoH α expression plasmids. The expression of exogenously transfected cDcoH and cDcoH α genes, as well as HNF-1 regulated genes (α -fetoprotein and albumin), was determined by semi-quantitative RT-PCR. Consistent results were also obtained from two additional independent experiments. Abbreviation used: h, human.

between cDcoH and cDcoH α transfected cells. Together, these results suggest that the novel cDcoH α gene also may have a functional role as a cofactor for HNF-1 transcriptional activity, since the cofactor activity of cDcoH α appears to be similar to that of cDcoH.

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REFERENCES

- Sedivy, J. M. (1999) Can ends justify the mean?: telomeres and the mechanisms of replicative senescence and immortalization in mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 9078–9081
- Himly, M., Foster, D. N., Bottoli, I., Iacovoni, J. S. and Vogt, P. K. (1998) The DF-1 chicken fibroblast cell line: transformation induced by diverse oncogenes and cell death resulting from infection by avian leukosis viruses. *Virology* **248**, 295–304
- Schaefer-Klein, J., Givol, I., Barsov, E. V., Whitcomb, J. M., VanBrocklin, M., Foster, D. N., Federspiel, M. J. and Hughes, S. H. (1998) The EV-O-derived cell line DF-1 supports the efficient replication of avian leukosis-sarcoma viruses and vectors. *Virology* **248**, 305–311
- Mendel, D. B., Khavari, P. A., Conley, P. B., Graves, M. K., Hansen, L. P., Admon, A. and Crabtree, G. R. (1991) Characterization of a cofactor that regulates dimerization of a mammalian homeodomain protein. *Science (Washington, D.C.)* **254**, 1762–1767
- Sourdive, D. J., Transy, C., Garbay, S. and Yaniv, M. (1997) The bifunctional DCOH protein binds to HNF1 independently of its 4- α -carbinolamine dehydratase activity. *Nucleic Acids Res.* **25**, 1476–1484
- Feuerman, M. H., Godbout, R., Ingram, R. S. and Tilghman, S. M. (1989) Tissue-specific transcription of the mouse α -fetoprotein gene promoter is dependent on HNF-1. *Mol. Cell. Biol.* **9**, 4204–4212
- Courtois, G., Baumhueter, S. and Crabtree, G. R. (1988) Purified hepatocyte nuclear factor 1 interacts with a family of hepatocyte-specific promoters. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7937–7941
- Lei, X. D. and Kaufman, S. (1998) Identification of hepatic nuclear factor 1 binding sites in the 5' flanking region of the human phenylalanine hydroxylase gene: implication of a dual function of phenylalanine hydroxylase stimulator in the phenylalanine hydroxylation system. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 1500–1504
- Lazarus, R. A., Benkovic, S. J. and Kaufman, S. (1983) Phenylalanine hydroxylase stimulator protein is a 4a-carbinolamine dehydratase. *J. Biol. Chem.* **258**, 10960–10962
- Davis, M. D. and Kaufman, S. (1991) 7-Tetrahydrobiopterin is an uncoupled cofactor for rat hepatic phenylalanine hydroxylase. *FEBS Lett.* **285**, 17–20
- Tronche, F. and Yaniv, M. (1992) HNF1, a homeoprotein member of the hepatic transcription regulatory network. *Bioessays* **14**, 579–587
- Medel, D. B., Hansen, L. P., Graves, M. K., Conley, P. B. and Crabtree, G. R. (1991) HNF-1 α and HNF-1 β (vHNF-1) share dimerization and homeo domains, but not activation domains, and form heterodimers *in vitro*. *Genes Dev.* **5**, 1042–1056
- Hansen, L. P. and Crabtree, G. R. (1993) Regulation of the HNF-1 homeodomain proteins by DcoH. *Curr. Opin. Genet. Dev.* **3**, 246–253
- Kim, J. L. and Burley, S. K. (1995) PCD/DCoH: more than a second molecular saddle. *Structure (London)* **3**, 531–534
- Rose, R. B., Bayle, J. H., Endrizzi, J. A., Cronk, J. D., Crabtree, G. R. and Alber, T. (2000) Structural basis of dimerization, coactivator recognition and MODY3 mutations in HNF-1 α . *Nat. Struct. Biol.* **7**, 744–748
- Johnen, G. and Kaufman, S. (1997) Studies on the enzymatic and transcriptional activity of the dimerization cofactor for hepatocyte nuclear factor 1. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 13469–13474
- Rhee, K. H., Stier, G., Becker, P. B., Suck, D. and Sandaltzopoulos, R. (1997) The bifunctional protein DCOH modulates interactions of the homeodomain transcription factor HNF1 with nucleic acids. *J. Mol. Biol.* **265**, 20–29
- Nichol, C. A., Smith, G. K. and Duch, D. S. (1985) Biosynthesis and metabolism of tetrahydrobiopterin and molybdopterin. *Annu. Rev. Biochem.* **54**, 729–764
- Anastasiadis, P. Z., States, J. C., Imerman, B. A., Louie, M. C., Kuhn, D. M. and Levine, R. A. (1996) Mitogenic effects of tetrahydrobiopterin in PC12 cells. *Mol. Pharmacol.* **49**, 149–155
- Scriver, C. R., Kaufman, S. and Woo, S. L. C. (1989) The hyperphenylalaninemias. In *The Metabolic Basis of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly, W. S. and Valle, D., eds.), pp. 495–546. McGraw-Hill, New York
- Citron, B. A., Kaufman, S., Milstien, S., Naylor, E. W., Greene, C. L. and Davis, M. D. (1993) Mutation in the 4a-carbinolamine dehydratase gene leads to mild hyperphenylalaninemia with defective cofactor metabolism. *Am. J. Hum. Genet.* **53**, 768–774
- Dimri, G. P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E. E., Linskens, M., Rubelj, I., Pereira-Smith, O. et al. (1995) A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 9363–9367

- 23 Liang, P. and Pardee, A. B. (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* (Washington, D.C.) **257**, 967–971
- 24 Madisen, L., Krumm, A., Hebbes, T. R. and Groudine, M. (1998) The immunoglobulin heavy chain locus control region increases histone acetylation along linked c-myc genes. *Mol. Cell. Biol.* **18**, 6281–6292
- 25 Pogge von Strandmann, E. and Ryffel, G. U. (1995) Developmental expression of the maternal protein XDcH, the dimerization cofactor of the homeoprotein LFB1 (HNF1). *Development* (Cambridge, U.K.) **121**, 1217–1226
- 26 Bossow, S., Riepl, S. and Igo-Kemenes, T. (1998) Characterization of the chicken and rat DCoH gene domains using an improved ligation-mediated PCR method. *Biol. Chem.* **379**, 1359–1365
- 27 Lei, X. D. and Kaufman, S. (1999) Characterization of expression of the gene for human pterin carbinolamine dehydratase/dimerization cofactor of HNF1. *DNA Cell Biol.* **18**, 243–252
- 28 Bernstein, P. L., Herrick, D. J., Prokipcak, R. D. and Ross, J. (1992) Control of c-myc mRNA half-life *in vitro* by a protein capable of binding to a coding region stability determinant. *Genes Dev.* **6**, 642–654
- 29 Eilers, M., Schirm, S. and Bishop, J. M. (1991) The MYC protein activates transcription of the α -prothymosin gene. *EMBO J.* **10**, 133–141
- 30 Sherr, C. J. (1994) G1 phase progression: cycling on cue. *Cell* (Cambridge, Mass.) **79**, 551–555
- 31 Campisi, J. (1996) Replicative senescence: an old lives' tale? *Cell* (Cambridge, Mass.) **84**, 497–500
- 32 Stein, G. H. and Dulic, V. (1995) Origins of G1 arrest in senescent human fibroblasts. *Bioessays* **17**, 537–543
- 33 Hauer, C. R., Rebrin, I., Thony, B., Neuheiser, F., Curtius, H. C., Hunziker, P., Blau, N., Ghisla, S. and Heizmann, C. W. (1993) Phenylalanine hydroxylase-stimulating protein/pterin-4 α -carbinolamine dehydratase from rat and human liver. Purification, characterization, and complete amino acid sequence. *J. Biol. Chem.* **268**, 4828–4831

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