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A role for HNF-3 in the regulation of the HNF-1 gene of the Atlantic salmon

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Abstract Hepatocyte nuclear factors -1 (HNF-1) and -3 (HNF-3) are hepatocyte-enriched transcription factors that are central to the establishment and maintenance of the liver phenotype in vertebrates. In the present study we demonstrate that, in the Atlantic salmon, asHNF-3 regulates the expression of the gene for asHNF-1. Multiple putative binding sites for asHNF-3 were identified within the 5′ flanking region of the HNF-1 gene using a computer-based algorithm, and these were confirmed to be functional by electrophoretic mobility shift assays. In transient transfection assays it was shown that co-expression of asHNF-3 leads to a decrease in the promoter activity of the 5′ flanking region of the asHNF-1 gene.

Keywords Hepatic Nuclear Factor-1/-3 · Transcriptional network · *Salmo salar*

Introduction

The generation and maintenance of cellular differentiation in higher organisms involves the coordinate expression of defined subsets of genes. The stimulation or repression of genes is modulated largely through interactions between DNA and *trans*-acting factors. Transcription factors present in the hepatocyte include some that are globally expressed and others that are enriched in the liver. The latter class includes HNF-4, a nuclear hormone receptor; C/EBP, which contains a basic leucine zipper; HNF-6, which contains a single Cut domain and a novel homeodomain; HNF-1/vHNF-1, which is classified as containing a variant homeodomain; and HNF-3, characterised as a winged helix/forkhead isoform (Cereghini 1996; Lemaigre et al. 1996). These liver-enriched transcription factors coordinately regulate the expression of genes that are transcribed preferentially in the liver, such as transthyretin and transferrin. They also act together in networks that help define and maintain the adult liver phenotype. Our knowledge of the complicated processes of cross-regulation and autoregulatory loops involved in such networks is constantly expanding (Rastegar et al. 2000).

HNF-1 expression, control of which is exerted at the transcriptional level, occurs in differentiated hepatocytes, and not in cells that do not express the entire set of liver functions (Tronche et al. 1994). vHNF-1, a distinct protein with a high degree of sequence homology to HNF-1, is expressed in dedifferentiated cells, suggesting that it functions at an earlier stage of liver differentiation (Cereghini et al. 1992). Expression of HNF-1 occurs relatively late, at the time of liver organogenesis, and HNF-1 gene disruption does not perturb the development of organs in which it is normally expressed (Pontoglio et al. 1996).

Within a 110-amino acid domain the HNF-3 proteins share a high degree of identity to a region in the product of the developmentally important *Drosophila* homeotic gene *forkhead* (Weigel and Jäckle 1990). This DNA binding domain, termed a winged helix for its three-dimensional structure upon binding, directs monomeric recognition of the HNF-3 consensus binding site 5′-A(A/T)TRTT(G/T)RYTY-3′ in a number of genes that are expressed specifically or preferentially in the

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liver (Overdier et al. 1994). The essential role of HNF-3 proteins during embryogenesis has been demonstrated by the profound effects of partial and complete HNF-3β knockouts on morphogenesis in the mouse (Ang and Rossant 1994) and by studies of the spatial and temporal pattern of protein expression during development in many species (Monaghan et al. 1993; Sasaki and Hogan 1993).

We have recently cloned cDNAs for both HNF-1 and HNF-3 from the liver of the Atlantic salmon (Deryckere et al. 1994; Stenson et al. 2000). A comparison of the deduced amino acid sequence of asHNF-3 with homologues from other species indicates greatest identity to zebrafish forkhead-2, whilst sequence alignment with known mammalian HNF-3 family members indicated closest identity to rat HNF-3γ (Stenson et al. 2000). asHNF-3 was shown to be expressed early in salmon development and continues to be expressed in liver, pancreas and intestine in the adult fish (Stenson et al. 2000). We have also determined that the asHNF-3 protein binds to a functional HNF-3 consensus binding site from the rat transthyretin gene (Stenson et al. 2000). asHNF-1 is the most divergent member of the HNF-1 family isolated to date. asHNF-1 shows essentially the same degree of homology to rat HNF-1 and vHNF-1 sequences (Deryckere et al. 1994), suggesting that the gene duplication event that gave rise to mammalian HNF-1 and vHNF-1 may have occurred after the divergence of the teleost and tetrapod lineages.

A 1100-bp DNA fragment corresponding to the 5′ flanking region of the HNF-1 gene has been cloned previously and shown to contain the minimal asHNF-1 promoter (Deryckere et al. 1996). DNase footprinting and gel retardation analysis of the 5′ proximal 400-bp region indicates the presence of binding sites for several transcription factors, namely Sp1, COUP-TFs and HNF-4 (McNair et al. 2001). We have utilised a computer algorithm to search for putative HNF-3 consensus sites further upstream in the asHNF-1 5′ flanking region. Here we report the identification and characterisation of three such sites. Gel retardation analysis indicates that asHNF3 protein can bind to these three sites, while co-transfection studies performed in liver-derived HepG2 cells suggest a negative role for asHNF-3 in regulation of the expression of the asHNF-1 gene.

Materials and methods

In vitro transcription-translation of the asHNF-3 cDNA

The TNT Coupled Reticulocyte Lysate system (Promega) was used as recommended by the manufacturer. Briefly, 1 μg of pGem3z containing the full-length asHNF-3 cDNA sequence immediately downstream from the T7 RNA polymerase promoter was added directly to the TNT lysate and incubated in a 50-μl reaction volume for 1–2 h at 30°C. In order to confirm the presence, and estimate the size, of the asHNF-3 translation product, a parallel reaction incorporating [^{35}S]methionine (1000 Ci/mmol) was carried out, and analysed by electrophoresis on an 8% SDS-polyacrylamide gel and subsequent autoradiography. This allowed the non-radiolabelled asHNF-3 protein to be used directly for gel shift analyses. Plasmids containing no insert were prepared in the same way and used as negative controls.

Electrophoretic mobility shift assays (EMSA)

The following sequences were used in the binding studies: the HNF-3I site (5′-ATATATTGAAAAATAAATATGCTAGG-3′), the HNF-3II site (5′-TCATGATAAATGTTAAGCTACAACA TC-3′), the HNF-3III site (5′-GAGGATGCTTTTGTTTGGGT TTTATGG-3′), and the HNF-4 site (5′-GGAGATGGGGACA AAGTTCACAGAAAGCGC-3′).

Complementary oligonucleotides were annealed by incubating equivalent amounts of each single-stranded oligonucleotide at 85°C for 3–5 min in the presence of 50 mM TRIS-HCl pH 7.5, 1 mM spermidine, 10 mM $MgCl_2$ and 5 mM DTT. The annealed oligonucleotides were then allowed to cool slowly (30–45 min) to 40°C and end-labelled with [γ-^{32}P]ATP. Then 1 μl of reticulocyte lysate was incubated with 1 pmol of labelled double-stranded oligonucleotide for 15 min at room temperature in binding buffer (4% glycerol, 10 mM TRIS-HCl pH 7.5, 1 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA) in the presence of 1 μg of poly(dI-dC), 1 μg of calf thymus DNA, 80 mM spermidine (magnesium salt) and 10 mM Na_2HPO_4.

Competition assays were performed by premixing different concentrations of unlabelled competition oligonucleotide with the radiolabelled oligonucleotide prior to the addition of the mix to the binding reactions. Protein-DNA complexes were separated from free probe by non-denaturing electrophoresis in a 6% polyacrylamide gel in 0.25×TBE. Following electrophoresis the gel was fixed for 30 min in 10% methanol/acetic acid, and dried before autoradiography.

Construction of HNF-1 promoter deletions and the HNF-3 expression plasmid

5′ Deletion derivatives of the 5′ flanking region of the asHNF-1 gene were generated by PCR using salmon genomic DNA as template. In each case, the 5′ primer contained a *Kpn*I site to introduce this site at the upstream end of the promoter fragment; a 3′ primer containing a *Hin*dIII site was used in all cases. The *Kpn*I + *Hin*dIII-digested amplicons were ligated to the pGL2-basic vector (Promega) cleaved with *Kpn*I and *Hin*dIII, fusing the promoter construct to the luciferase reporter gene. The pCI-asHNF-3 construct for expression of asHNF-3 was generated by *Eco*RI digestion of the cloned asHNF-3 cDNA in pGEM followed by gel purification and ligation into the pCI vector (Promega). The correct orientation of all plasmid constructs was confirmed by restriction digestion, PCR with vector and insert primers and sequencing into the insert from the vector.

Plasmid DNAs for transfection were purified using Qiagen Maxiprep columns. Transfection efficiency was controlled by co-transfecting a pEF-1a-CAT vector.

Transient transfection of cultured cells and measurement of luciferase and CAT activities

Human hepatoma cells (HepG2) were maintained as a monolayer culture in Dulbecco's Modified Eagle's Medium (DMEM) containing L-glutamine (BioWhittaker) supplemented with 10% fetal calf serum (BioWhittaker) and streptomycin (50 mg/ml) (GibcoBRL-Life Technologies) at 37°C under 5% CO_2.

For the transfections 2×10^5 cells were seeded overnight in 60-mm dishes and the medium was changed 4 h before transfection was carried out. For each reaction, a mixture containing 5 μg of the HNF-1 promoter-luciferase constructs, 250 ng of PEF-1a-CAT and, in the case of co-transfections, 500 ng of expression plasmid (pCI-asHNF-3) was used to transfect the HepG2 cells by the calcium-phosphate method. In brief, an equal volume of 2×HEPES-Buffered Saline pH 7.0 (50 mM HEPES, 1.5 mM Na_2HPO_4,

300 mM NaCl) and 200 µl of 250 mM $CaCl_2$ was added to the DNA, and the suspension was incubated at room temperature for 20 min before dropwise addition to the cells. After overnight incubation the cells were washed twice with Phosphate-Buffered Saline (PBS) and fresh medium was then added. Cells were harvested 24–48 h later. For the luciferase assay (Brasier and Ron 1992) 350 µl of luciferase buffer (25 mM glycylglycine, 10 mM $MgSO_4$, 2 mM ATP) was added to 5–10 µl of cleared cell lysate. The luciferase activity of the sample was measured immediately upon injection of luciferin mix (20 mM glyclyglycine, 2 mM luciferin) using a Moonlight 1500 luminometer (Analytical Luminescence Laboratory) over the course of 10 s. CAT activity was measured with the CAT-ELISA kit (Boehringer Mannheim), following the protocol supplied, and using 5–10 µl of cell lysate in each assay reaction. Luciferase activity was expressed as the number of light units emitted per µg of reporter plasmid after taking the average of duplicate wells. For normalisation of the transfection efficiency, the luciferase activity of the constructs was corrected for the control CAT activity.

Results

Localisation of putative HNF-3 consensus binding sequences in the 5′ flanking region of the asHNF-1 gene

Three putative HNF-3 binding sites were detected upstream from the asHNF-1 transcriptional start site using the computer algorithm MatInspector V2.2 (Quandt 1995) (Fig. 1 and see Table 1).

Gel retardation analysis

Gel mobility shift assays demonstrated that the putative HNF-3 consensus sequences were capable of binding to asHNF-3 synthesised in vitro. Three protein products were observed upon in vitro transcription and translation (IVT) of asHNF-3 cDNA (data not shown). The calculated molecular weight (35.8 kDa) for the product of the full-length asHNF-3 cDNA agrees well

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AAGCTTTGATGTGACATCTATGTCTGATCTAATCACAGGAGGTAAAGCAC -1013

TTTCAATGAGAGAGAGGAAGGAATGAATGAGAAAAGGGTTGGACAAAGGG -963
                HNF-3(III)
GTGGGAGGATGCTTTTGTTTGGGTTTTATGGCCAGATTGTCTACTGCTCT -913

GGGAAAGTGAAAACCCAACCTTAGGTTAGGTTACCTGGGGGAGAAAAAGA -863

CCCAGGAAGAGGGTCAGAGGAAAGGTTAGGTGACTGTCAGGGACAGCAGA -813

AATATTCAAGCGAGCAGAGAGAAACAGAACCCAAAGTCAGTGAACGCTAG -763

TAGGTGTTCTCTGGCATCTAAAATCTACCTCTATCATACCCTGTCTTTCT -713

AGAATGATGCAGTAGTGCCCTCTAGTGCTGTGCAGGACAACAGCTACTTT -663

CAATTTCTCTGTCCAATTCATTCCAAACCTCTCAAAACACACCTATCAGG -613
        HNF-3 (II)
TTTTCATGATAAATGTTAAGCTACAACATCCATTCCATTAGGCCTTTACA -563

TTACAATTGATATGAAATATAGAATAGTATAGTTATACTAGCCACAAATT -513

ACATCAAGGCAGTCTAAAATATGCATTTTGCTGTTATCCTTTTTGGACCC -463

TAAAATGAATGCCTATAAGTGTCATGGAGAATTGTGTCGGTTTAGAATAT -413
        HNF-3 (I)
ATTGAAAAAATAAATATGCTAGGTTTTAATGTGCGTATTAAGATGCAGTTA -363

GCTTATTTTTGTAACCTCACATATGTATTACATTTGTGTGTTATACAGGA -313
                                              COUP TF
GGCTGTATGAATGTGCAGTTTATTTTCATTTATAGTTTGACTGCACTTTG -263
                                    HNF-4
CTCACTGTTAGGGATGGACGGAGATGGGGACAAAGTTCACAGAAAGCGCA -213
          Sp1
TGGGGGAGAGGGGGAGGGAAAAAAAGACCTTTTAAAGCATAACAGCAGGG -163

TGGAGGGCTAAAACCAGACA CTCAGCTCAGAACGAGACGAGGAGAAAAA -113

TCAAACGGGGGAAAATGAAAGCAAACTTTCTCTATTCCTAAACTGAAGGA  -63

CAGAAATGCAATGATTCCAGAAGCCAATTGCTCTGTTTTTTTGTGTAAGC  -13
          (+1)
GCATGACTGCAGTGTGTCTGTGTTGGTGTGTGAGTGTGTGTTTGTGACCT  +37
  COUP TF
GTGGCCTGCGTAGTCTGTGTGCTGTGTTGTGAGGTAAAGATTAGGCAACA  +87

GCGTAATGGAGGGAGAGGAGAGGAAAGGAGAAGCAGGGCCCGGCCCTGGT +137
     Met
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Fig. 1 Sequence of the asHNF-1 promoter. Putative binding sites for liver-enriched transcription factors known to play a role in HNF-1 expression are *underlined*

Table 1 Comparison of the three HNF-3 consensus sequences in the 5′ flanking region of the salmon HNF-1 gene with known consensus binding sites

Species	Gene	Sequence (5′→3′)[a]	Position	Reference
Consensus		VAWTRTTKRYTY		
Salmon	HNF-1 (site I)	G**C**ATATTTATTT	–396 to –407	Deryckere et al. (1996)
	HNF-1 (site II)	**TA**CTATTTAC**AA**	–597 to –608	
	HNF-1 (site III)	**TT**TTGTTTG**GG**T	–951 to –940	
Salmon	Transferrin	AA**G**TGTTTGC**AG**	–294 to –283	Kvingedal (1994)
Human	Transferrin	C**T**TTGTTTGCTT	–60 to –71	Auge-Gouillou et al. (1993)
Mouse	HNF-1	C**TC**TGTTTAC**A**T	–17 to –28	Kuo et al. (1992)
Rat	Transthyretin	GATTATTGACTT	–93 to –104	Costa et al. (1989)
	Transthyretin	**TC**ATATTTGT**G**T	–143 to –134	
	Transthyretin	A**C**ATGTTTG**AA**C	–1925 to –1936	Samadani et al. (1996)
High-affinity sites		G**T**TTGTTTAC**A**C	SAAB[b]	Overdier et al. (1994)
		TGTTGTTTATTT		
		AAATATT**A**GCTT		

[a]Abbreviations used are: V = A/C/G, W = A/T, K = G/T, Y = C/T, R = G/A. Variations from the consensus sequence are *highlighted in bold and underlined*

[b]These sequences were isolated from a pool of oligonucleotides by sequential *s*election *a*nd *a*mplification of *b*inding sites

with the molecular mass estimated for the largest translation product based on SDS-PAGE analysis. The apparently truncated protein forms may be generated by a combination of factors, including premature termination of translation, degradation or alternative initiation codon usage. The last possibility is suggested by the presence of two potential internal ATG codons at sites 69–71 and 188–190, which would generate hypothetical proteins of 35 kDa and 30 kDa, respectively, corresponding to the sizes of the truncated proteins synthesised in vitro (Stenson et al. 2000). The most distal ATG site is positioned within the boundaries of the DNA-binding domain, and its use would generate a protein devoid of the binding function. The IVT system may also yield other, smaller, proteins that do not occur in vivo.

Following incubation of the asHNF-3 proteins with oligonucleotides representing the putative consensus sites, either one prominent (sites II and III) or a pair of complexes with similar mobilities (site I) were observed (Fig. 2). Longer exposure times did not reveal any other retarded complexes. In the presence of a 50- or 100-fold molar excess of the corresponding unlabelled oligonucleotide, formation of the complexes was prevented – implying that the binding of asHNF-3 protein is sequence-specific. In no case did the incubation of the asHNF-3 proteins with an oligonucleotide sequence derived from HNF-4 generate any retarded complexes (see Fig. 2C, lane 5). In addition, the presence of an excess of unlabelled HNF-4 site oligonucleotide did not affect binding to the HNF-3 sites (see Fig. 2C, lane 6), indicating that binding of asHNF-3 to all three sites was specific.

Differences in the efficiency of formation of the various retarded complexes may be result from deviations from the HNF-3 consensus binding sequence (Table 1). Such alterations have previously been observed and have been shown to play a role in modulating binding specificity (Samadani et al. 1996). Differences may also be due to the presence of the shorter recombinant protein products. The significance of smaller proteins cannot be determined in this context; however, previous observations using salmon nuclear extracts and a high-affinity transthyretin HNF-3 consensus site did not indicate the binding of any other shorter products, and Northern analysis indicated that only one liver-enriched asHNF-3 mRNA transcript is present in vivo (Stenson et al. 2000).

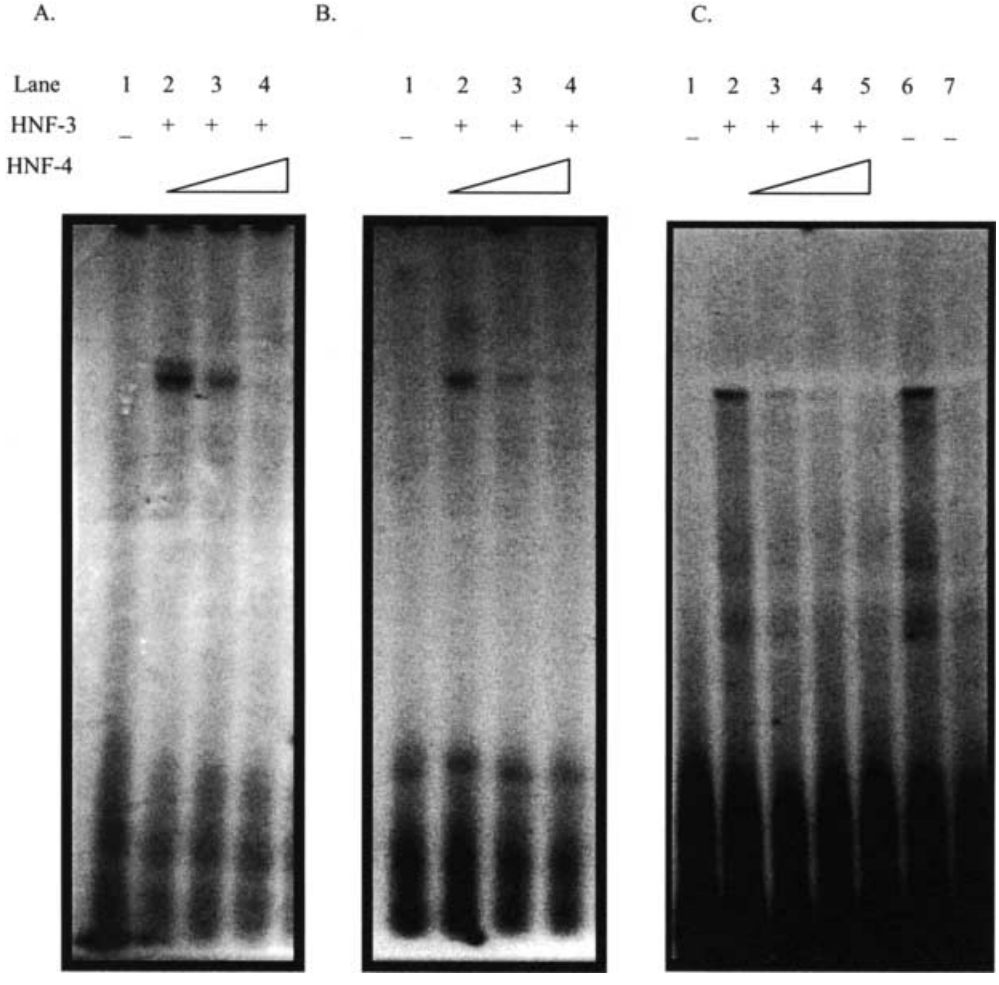

Fig. 2A–C Electrophoretic mobility shift assay of the binding of recombinant HNF-3 protein to putative target sequences site in the 5′ flanking region of the asHNF-1 gene. **A** Site I. **B** Site II. **C** Site III. Lane 1 in each case was loaded with the corresponding radiolabelled consensus HNF-3 sites incubated in the absence of recombinant HNF-3 protein. Radiolabeled HNF-3 oligonucleotides were also incubated with recombinant asHNF-3 protein in the absence (lane 2) or presence of a 50-fold (lane 3) or 100-fold (lane 4) excess of unlabeled HNF-3 oligonucleotides or a 100-fold unlabelled HNF-4 consensus site oligonucleotide (**C**, lane 6). Controls included recombinant asHNF-3 incubated with HNF-4 oligonucleotide (**C**, lane 5) and HNF-4 probe without recombinant asHNF-3 (**C**, lane 7). The results for the controls presented for site III (**C**) are representative of those observed for each site. In all cases the mixtures were analysed on a 5% native polyacrylamide gel

Analysis of the role of asHNF-3 in the regulation of the HNF-1 gene using transient transfection assays

To investigate the role, if any, of HNF-3 in the control of asHNF-1 expression, transient transfection studies, using defined deletion derivatives of the 5′ flanking region of the asHNF-1 gene fused to a luciferase reporter gene, were carried out in HepG2 cells. The promoter fragments were designed so that either site I alone, or sites II and II were present in the constructs. The liver-derived cell line HepG2 was transfected with an asHNF-1 reporter construct in the presence or absence of the HNF-3 expression plasmid (Fig. 3). In keeping with previous observations, a minimal promoter region was defined in construct pGL2-576, consisting of one HNF-3 target sequence (site I) and several sites that bind the nuclear receptors HNF-4 and members of the Coup-TF protein family; removal of the sequence from –576 to –245 bp led to a reduction in activity (Deryckere et al. 1996; McNair et al. 2001). Interestingly, transfection of the larger construct pGL2-1041, produced a lower level of reporter activity than the pGL2-576 construct, indicating the potential presence of at least one negative regulatory element in the distal 5′ flanking region of the asHNF-1 promoter, and that at least one component in the HepG2 cell has the capacity to exert that effect. Overexpression of asHNF-3 in the HepG2 cell line did not significantly alter the activity of pGL2-576. Interestingly, co-transfection of the pGL2-1041 construct with the pCI-asHNF-3 expression plasmid led to a 50% decrease in the activity of the pGL2-1041 construct. The pGL2-1041 construct (with all the sites present) reflects the in vivo situation, and this result thus indicates that asHNF-3 is a repressor of asHNF-1 gene expression in the HepG2 cell, and probably acts through sites II and III.

The constructs pGL2-576 and pGL2-1041 were also transfected into HeLa cells (data not shown). pGL2-576 gave less activity than observed in HepG2 cells, indicating a minimal requirement for other liver-specific factors not found in HeLa cells. However, pGL2-1041 exhibited the same degree of induction of reporter activity over the basal level as observed in HepG2 cells (approximately 60%), and upon co-transfection with asHNF-3, the reporter activity was reduced to basal levels (unpublished observations). This suggests a potential negative regulatory role for asHNF-3 in controlling the expression of asHNF-1 in this cell type also.

Different isoforms of HNF-3 have been shown to have antagonistic effects upon gene expression. For example, HNF-3α has been shown to have a positive effect upon expression of the lung protein CC10, whereas HNF-3β has a negative effect (Hellqvist et al. 1996). HNF-3 has also been shown to have a negative effect upon HNF-1α in embryoid bodies, a model system in which gene expression parallels that in the liver (Duncan et al. 1998), and can block the activation of protein C by HNF-6 (Spek et al. 1998).

The effect of asHNF-3 on the asHNF-1 gene is interesting, given that asHNF-3 expression may precede that of HNF-1 during salmon development (Cereghini et al. 1992; Stenson et al. 2000). Regulation of the asHNF-1 gene by asHNF-3 may prevent ectopic

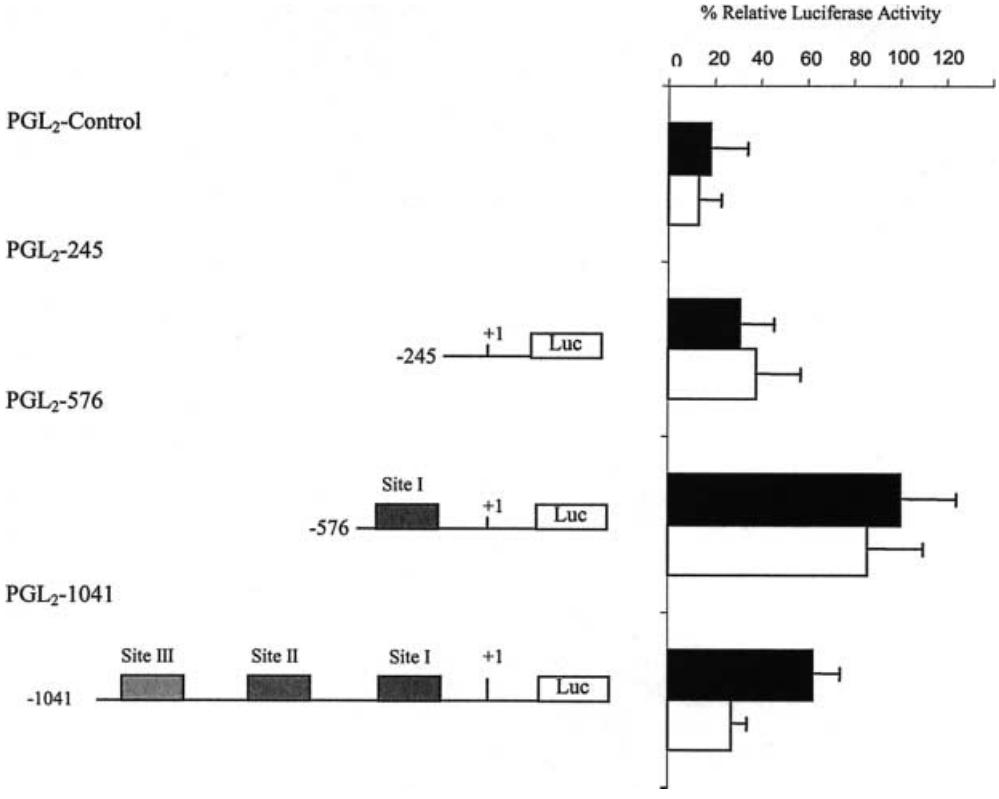

Fig. 3 Co-transfection of deletion derivatives of the 5′ flanking region of the asHNF-1 gene, fused to the luciferase gene as a reporter, together with the asHNF-3 expression plasmid. HepG2 cells were transiently transfected with various 5′ deletion constructs of the asHNF-1 gene, either on their own (*open bars*) or together with the asHNF-3 expression plasmid (*filled bars*). The position of the 5′ end is indicated and all promoter fragments terminate at position +89. The putative HNF-3 target sequences are indicated as *filled boxes* marking sites I (–396 to –407), II (–597 to –608) and III (–951 to –940). The *bars* represent relative luciferase activity of the various constructs either in the absence (*filled bars*) or presence (*open bars*) of added HNF-3. The maximum activity obtained with the construct pGL2-576 was arbitrarily set to 100%. The values are means ± SEM (n = 4)

expression whilst allowing for correct activation in the context of developmental cascades, and thus facilitate the emergence of the fully functional adult phenotype.

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