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Hoxa5 overexpression correlates with IGFBP1 upregulation and postnatal dwarfism: evidence for an interaction between Hoxa5 and Forkhead box transcription factors

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SUMMARY

Transgenic mice expressing the homeobox gene *Hoxa5* under the control of *Hoxb2* regulatory elements present a growth arrest during weeks two and three of postnatal development, resulting in proportionate dwarfism. These mice present a liver phenotype illustrated by a 12-fold increase in liver insulin-like growth factor binding protein 1 (IGFBP1) mRNA and a 50% decrease in liver insulin-like growth factor 1 (IGF1) mRNA correlated with a 50% decrease in circulating IGF1. We show that the *Hoxa5* transgene is expressed in the liver of these mice, leading to an overexpression of total (endogenous plus transgene) Hoxa5 mRNA in this tissue. We have used several cell lines to investigate a possible physiological interaction of Hoxa5 with the main regulator of IGFBP1 promoter activity, the Forkhead box transcription factor FKHR. In HepG2 cells, Hoxa5 has little effect by itself but inhibits the FKHR-dependent activation of the IGFBP1 promoter. In HuF cells, Hoxa5 cooperates with FKHR to dramatically enhance IGFBP1 promoter activity. This context-dependent physiological interaction probably corresponds to the existence of a direct interaction between Hoxa5 and FKHR and FoxA2/HNF3β, as demonstrated by pull-down experiments achieved either in vitro or after cellular co-expression. In conclusion, we propose that the impaired growth observed in this transgenic line relates to a liver phenotype best explained by a direct interaction between Hoxa5 and liver-specific Forkhead box transcription factors, in particular FKHR but also Foxa2/HNF3β. Because Hoxa5 and homeogenes of the same paralog group are normally expressed in the liver, the present results raise the possibility that homeoproteins, in addition to their established role during early development, regulate systemic physiological functions.

Key words: Hoxa5, Liver, Postnatal growth, Winged-helix/Forkhead box, Foxa2, HNF3β, FKHR, IGFBP1, IGF1, Mouse

INTRODUCTION

Three main endocrine systems play key roles in postnatal growth: thyroid hormones (Gauthier et al., 1999; Hsu and Brent, 1998) the GH-IGF1 system (Lupu et al., 2001) and insulin (Brogiolo et al., 2001; Tamemoto et al., 1994). To unravel the functions of these endocrine signals in growth regulation, mouse genetic approaches have recently been developed. For example, disruption of the *T3Rα* thyroid hormone receptor (*Thra* – Mouse Genome Informatics) results in hypothyroidism and dwarfism (Fraichard et al., 1997). Mutations in the GH receptor gene or in genes regulating GH cell lineage, or GH synthesis/secretion (i.e. *Pit1*, *Prop1* and *Ghrhr*) impair postnatal growth and lead to dwarfism (Andersen et al., 1995; Camper et al., 1990; Chandrashekar et al., 1999; Li et al., 1990; Lin et al., 1993; Sornson et al., 1996; Zhou et al., 1997). *Igf1* or IGF1 receptor disrupted mice also have impaired embryonic and postnatal growth, demonstrating that IGFs are involved in this physiological function (Baker et al., 1993; Liu and LeRoith, 1999).

According to the somatomedin hypothesis, liver-derived IGF1 is the main mediator of GH functions (Liu and LeRoith, 1999; Mathews et al., 1986). In line with this hypothesis, GH stimulates the liver production of circulating IGF1 (Mathews

et al., 1986; Roberts et al., 1986) and injection of IGF1 into GH-deficient animals restores body growth (Guler et al., 1988). However, in addition to the systemic activity of liver-derived IGF1, GH may have direct effects on target tissues either without IGF1 mediation, or by controlling local IGF1 synthesis at the level of several organs (D'Ercole et al., 1984; Lindahl et al., 1987). The view, that liver-derived circulating IGF1 is not required in postnatal body growth was recently advocated by reports documenting normal growth in transgenic mice in which IGF1 production was specifically disrupted in the liver (Sjogren et al., 1999; Yakar et al., 1999). However, although these mouse models confirm that the liver is a major contributor to circulating IGF1, a lack of function of liver IGF1 in normal postnatal growth is difficult to establish because of compensatory mechanisms.

Complex mechanisms control IGF1 bio-availability that involve the regulation of its synthesis and secretion by all producing organs, including the liver, and at least six circulating IGF-binding proteins (IGFBP1-IGFBP6). IGFBPs prolong the half-life of IGFs and play an important role in controlling their availability and effects on target tissues. For example, IGFBP1, which is mainly produced by hepatocytes, inhibits IGF-dependent cellular growth and differentiation in vitro (Lee et al., 1997), as well as in vivo, as transgenic mice over-expressing IGFBP1 show postnatal growth retardation (Gay et al., 1997; Rajkumar et al., 1995; Schneider et al., 2000).

Transcriptional regulators of the homeobox and Forkhead box/winged helix (Fox) families expressed in the liver play central functions in growth control. Mice that lack HNF1β (*Tcf2*$^{-/-}$) develop a laron type dwarfism (Lee et al., 1998). Postnatal inactivation of the Foxa2/HNF3β transcription factor does not impair hepatocyte function or overall animal physiology – including growth – (Sund et al., 2000), but its overexpression in the liver leads to a striking growth deficit that has been attributed to the upregulation of *Igfbp1* (Rausa et al., 2000). Other Forkhead box transcription factor are thought to play a major role in *Igfbp1* expression, especially in response to hormonal and nutritional status, including FKHR (FOXO1a) and FKHR1 (Foxo1), which are (respectively) the liver-expressed human and mouse homologs of C. elegans *DAF-16* (Biggs and Cavenee, 2001; Guo et al., 1999).

Hoxa5-disrupted mice present posterior homeotic transformations of the cervico-thoracic region, respiratory tract and intestinal maturation defects, but no apparent neural phenotype (Jeannotte et al., 1993; Aubin et al., 1997; Aubin et al., 1999). To verify the possible functions of *Hoxa5* in the developing nervous system, we produced transgenic mice overexpressing Hoxa5 under Hoxb2 regulatory elements. These mice do not show any obvious neural phenotype, even in rhombomeres 3 and 5, where the transgene is ectopically expressed. However, they show an impaired postnatal growth correlated with a 12-fold upregulation of *Igfbp1* and a two-fold downregulation of *Igf1* in the liver. Accordingly, transgenic and endogenous *Hoxa5* genes are expressed in the liver at postnatal stages (this study) (Mizuta et al., 1996). Therefore, in addition to their function in endoderm development (Beck et al., 2000; Grapin-Botton and Melton, 2000), Hox genes may play a physiological role in mature endoderm derivatives (James and Kazenwadel, 1991). It is also proposed that growth regulation by Hoxa5 is due to its interaction with Forkhead box transcription factors, in particular FKHR and HNF3β/Foxa2, two essential regulators of *Igfbp1* and *IgfI* expression, respectively (Guo et al., 1999; Nolten et al., 1996; Yeagley et al., 2001).

MATERIALS AND METHODS

Mice production, genotyping and in situ hybridization

The pB4B2A5m construct (Fig. 1A) was obtained by changing *lacZ* for *Hoxa5myc* in the pB4lacZB2 plasmid (gift from Dr P. Gilardi), containing the *Hoxb4* promoter and *Hoxb2* enhancer regulatory sequences (Sham et al., 1993). A *Sac*I-*Bam*HI (blunted) fragment from pSP9A5m (Chatelin et al., 1996) was inserted into pB4lacZB2 digested by *Nco*I and *Sma*I using a *Nco*I-*Sac*I adapter. The micro-injected fragment was excised from pB4B2A5m by *Xho*I digestion (see Fig. 1).

Transgenic mice were produced as described elsewhere (Hogan et al., 1994) and genotyped by PCR (CR5-CR6 primers, Fig. 1) or Southern blot. Tails or embryo yolk sacs DNA was digested with *Eco*RI, run on agarose, transferred onto nylon membrane (Hybond N+, Amersham) and hybridized with a cDNA probe (*Eco*RI-*Sac*I fragment) derived from *Hoxa5* (Fig. 1) and labeled (α^{32}P-dCTP, Amersham) using a random priming labeling kit (Promega). Transgenic founders were bred and transgene expression was analyzed (by in situ hybridization) in F_1 (from male founders) or F_2 (from female founders) E9.5 embryo. Five independent lines expressing the transgene in rhombomeres 3 and 5 and in the somites at embryonic day 9.5 (E9.5) were selected for breeding.

Digoxigenin-labeled *Hoxa5* probes were prepared from pKHX13A (mouse *Hoxa5*-coding sequence cloned in pBluescript, Stratagene), linearized with *Hin*dIII (antisense probe) or *Xba*I (sense probe) and transcribed with T7 (anti-sense) or T3 (sense) RNA polymerase (Boehringer Mannheim). In situ hybridization was performed in toto on E9.5 mice embryo (Wilkinson, 1992).

lacZ expression, northern blots, radioimmunoassays

lacZ expression was analyzed, using X-Gal staining as previously described (Sham et al., 1993), on various organs of B4B2lacZ mice (mice expressing *lacZ* under the control of the *Hoxb-2* regulatory elements used in B4B2A5m mice) fixed by intracardiac perfusion of 4% paraformaldehyde (PFA).

Total RNA from liver or pituitary was extracted (QIAGEN RNeasy kit). Liver (10 μg) or pituitary (0.5 μg) were separated on denaturing agarose gel, transferred on a nylon membrane, fixed and hybridized with ^{32}P-labeled probes as described above. *Igf1* mRNA was detected with an *Eco*RI-*Pst*I fragment from *Igf1* cDNA (Roberts et al., 1987), and GH mRNA was detected with an oligonucleotide probe (5′-GTC TCT GAG AAG CAG AAA GCA GCC TGG GCA TTC TG-3′ (LeGuellec et al., 1992) labeled with terminal deoxynucleotidyl transferase (Boehringer, Mannheim). Membranes were exposed and analyzed by phosphoimaging (Fuji), stripped in boiling SDS 0.5% and rehybridized with GAPDH (Fort et al., 1985) or ribosomal protein S26 (Vincent et al., 1993) probes for normalization.

In radioimmunoassays (RIA), serum from coagulated blood samples was centrifuged at 1000 ***g*** for 15 minutes and stored at –20°C. IGF1 was measured by non equilibrium RIA (Furlanetto et al., 1977) and GH with a commercial RIA kit (Amersham, France). For T3 and T4 RIA, sodium anilino-naphtalene sulfonate and acidic buffer were used as inhibitors of serum protein binding (Rousset et al., 1984).

RT-PCR, real time PCR and macroarrays

RT-PCR

PolyA+ mRNA were extracted (Ambion Poly-A Pure kit) and treated with 1 U/μg of DNase I (Amp Grade, GibcoBRL). polyA+ RNA (500 ng) were incubated for 1 hour at 45°C with 4 U/μl Superscript II

(GibcoBRL), 10 μg/ml oligo-dT15, 500 μM dNTP, 0.4 U/μl RNasin (Promega) and 10 mM DTT in a final volume of 25 μl. PCR reactions (final volume 25 μl) included 1 μl of cDNA with 10 pmol of each primer, 200 μM dNTP, 1.5 mM $MgCl_2$, 1 U Taq DNA polymerase (Eurobio) in 1×Taq buffer. Amplification conditions were 1 minutes at 92°C, 45 seconds at annealing temperature, 45 seconds at 72°C for 35 cycles; annealing temperature was 55°C for CR5-CR6 and CR7-CR8 couples of primers and 58°C for CR3-CR4. Negative control reactions were obtained in the absence of Superscript. PCR products were analyzed on 2% NuSieve 3:1 agarose gel (FMC).

CR3: 5′-CCA AAT GGC CCG GAC TAC CAG-3′
CR4: 5′-TGC TGC TGA TGT GGG TGC TGC-3′
CR5: 5′-CTG AGA TAA GTT TGT GTT CG-3′
CR6: 5′-TGC ACA TTA GTC ACG ACA AT-3′
CR7: 5′-TGC CGC GGC CAT ACT CAT-3′
CR8: 5′-CCA CTT CAA CCG CTA CCT-3′

Quantitative RT-PCR

Competitor RNA was obtained by inserting a 50 base-pair oligonucleotide in the *Apa*I site of pSP9A5m (between CR6 and CR7) followed by in vitro transcription using SP6 RNA polymerase (riboMAX, Promega). In competition experiments, 470 ng of liver polyA RNA were mixed with serial dilutions of control RNA. CR6 and CR7 primers were used (2.5 μl of cDNA and 3% NuSieve 3:1 agarose gel).

Real time PCR

SYBR green PCR Core Kit (P/N 4304886, Perkin Elmer Applied Biosystems) was used and the experiment performed according to manufacturer's instructions. In brief, 2 μg of total RNA were treated with DNAse I and reverse transcribed in a total volume of 20 μl. Real time PCR was performed, in triplicate, using 1 μl cDNA in a GeneAmp5700 (Applied Biosystems). Primers were:
5′-CGCCACGAGCACCTTGTTCA-3′,
5′-TCCTCTGTCATCTCTGGGCTCTCA-3′ (IGFBP1) and
5′-TGACGTGCCGCCTGGAGAAAC-3′,
5′-CCGGCATCGAAGGTGGAAGAGT-3′ (GAPDH).

Macro array analysis

Atlas Mouse 1.2 array (Clontech laboratory, Palo Alto, CA), including 1176 mouse cDNAs, nine mouse housekeeping control cDNAs with reagents for synthesizing radioactive cDNA probes was used in these experiments. RNA from transgenic and control livers were purified using Nucleospin RNAII kit (Clontech). Total RNA (30 μg) was processed according to the instructions of the manufacturer. Results were analyzed with AtlasImage1.5 (Clontech) and normalized by global standardization.

Cell cultures and transfection assays

HepG2 human hepatoma cell line was cultured in F12 Coon's modified medium supplemented with 5% fetal bovine serum. Human fibroblast cells (HuF) were isolated from decidua parietalis dissected from the placental membranes after normal vaginal delivery at term as previously described (Strakova et al., 2000) and grown in RPMI-1640 (Invitrogen).

IGFBP1 promoter activation was analyzed using the BP1.Luc (320 pb fragment 5′ of the RNA cap site) (Guo et al., 1999). HepG2 and HuF cells were transfected using FuGENE 6 (Roche) or Lipofectamine 2000 (Invitrogen), respectively, as follows. Cells grown in 12-well plates were transfected with 1 μg/well of the IGFBP1 promoter-reporter construct with or without 0.5 μg/well pHA-FKHR (HA-FKHR cloned into *Xba*I/*Acc*I sites within pALTER) and/or 0.5 μg/well pCL9A5m (Hoxa5m expressing vector) (Montesinos et al., 2001) expression vectors with the appropriate empty vectors. Twenty-four hours after electroporation, cultured cells were lysed and assayed for luciferase activity (Ausubel et al., 1995). All transfections were carried out in duplicate or triplicate, and experiments were repeated at least three times.

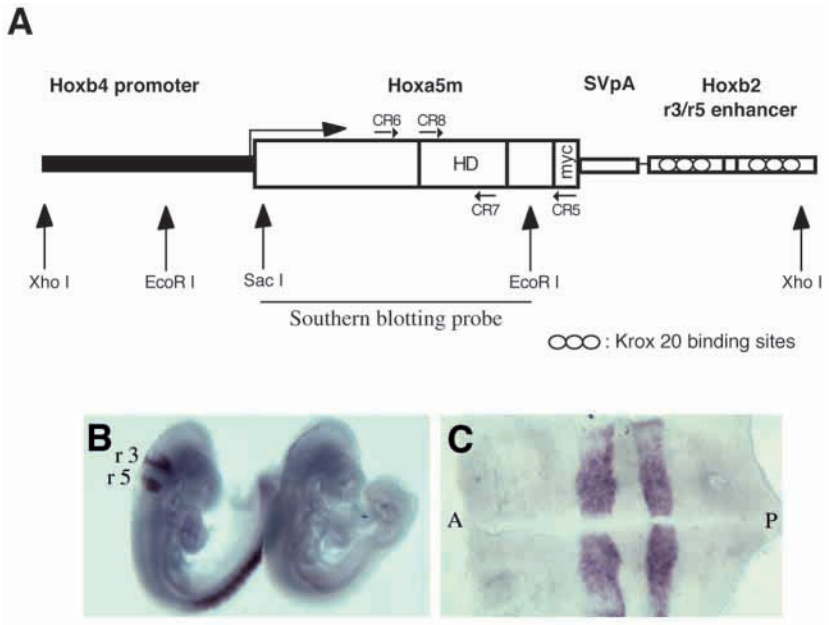

Fig. 1. B4B2A5m construct and expression in E9.5 embryos. (A) Schematic of the B4B2A5m construct. HD, homeodomain; SVpA, SV40 polyadenylation signal; CR5, CR6, CR7 and CR8 are the primers used in PCR and RT-PCR assays (for details see Materials and Methods section). (B,C) *Hoxa5m* transgene expression in E9.5 embryos, visualized by in toto hybridization. (B) Two sibling embryos: one heterozygous transgenic (left) and one control (right). Hoxa5 transgene is observed in r3 and r5, and in the somites. (C) Flat mount of the rhombencephalon of a B4B2A5m mouse, illustrating *Hoxa5m* transgene expression in r3 and r5 (A, anterior; P, posterior).

In vitro and ex vivo protein interactions

GST-Hoxa5 expressing vector (pGA5m) was constructed by inserting in pGEX1 (Pharmacia) the Hoxa5 sequence between GST and Myc tag sequences. GST and GST-Hoxa5 proteins were prepared from *E. coli* strain TG1. Quantities and size-integrity of fusion-proteins were estimated with SYPRO staining (BIORAD) using BSA as a standard. Binding was performed in a final volume of 200 μl of binding buffer 1 (BF1: 20 mM Tris-HCl pH 7, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.01% Nonidet P-40) by incubating 500 ng of glutathione-immobilized fusion proteins with 1 μl of ^{35}S-labeled FKHR, HNF3β or luciferase synthesized by in vitro transcription-translation (rabbit reticulocyte lysates, TNT, Promega) using pHA-FKHR, HNF3β cDNA cloned in pMT21 vector (a gift from Dr A. Ruiz i Altaba) and a luciferase construct (Promega). Glutathione sepharose beads (Pharmacia Biotech, 10 μl) were added to the supernatants, rotated overnight at 4°C, rinsed twice with 1 ml of BF1-200 mM NaCl, once with 1 ml BF1-500 mM NaCl and boiled for 5 minutes in Laemmli buffer before analysis by SDS-PAGE and autoradiography.

For ex vivo protein-protein interaction analyses, pEBG or pEBG-FKHR expressing GST native or fusion proteins in eucaryotic cells (Chen et al., 2002) were co-electroporated with pCL9A5m in COS cells (20 μg of each plasmid per 4,000,000 cells). Twenty-four hours later, cells were scraped in binding buffer 2 (BF2: 400 mM KCl, 20 mM Tris-HCl pH 7.5, 20% glycerol, 5 mM DTT, 1% Triton-X100 plus protease inhibitors), frozen in liquid nitrogen, thawed and

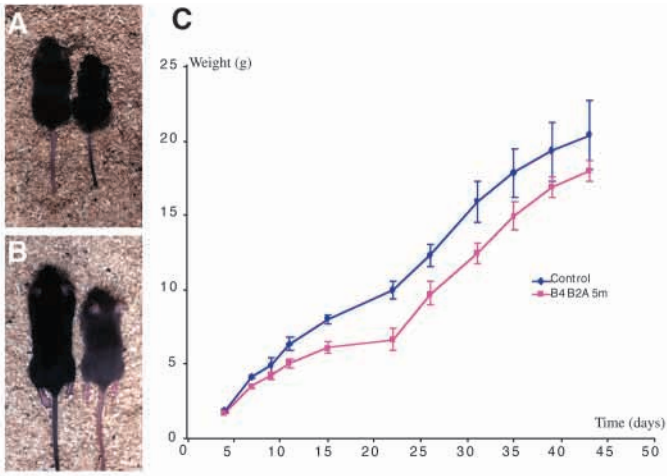

Fig. 2. B4B2A5m mice have an impaired postnatal growth profile. (A,B) Sibling mice of matched sex, one control (left) and one transgenic (right) are shown together at two ages: 2 weeks (A, males) or 5.5 weeks (B, females). (C) Postnatal growth curves comparing the weight of control (n=4) and transgenic (n=7) male mice during the first 7 weeks of postnatal development. The growth rate of transgenic mice is significantly reduced during the second and third postnatal weeks. Growth resumes normally after 3 weeks but the mice remain smaller throughout adulthood.

centrifuged at 4000 ***g*** for 15 minutes at 4°C. Glutathione-sepharose beads were added to the cell extracts and treated as described above. Bound proteins were analyzed by SDS/PAGE and western blot, using either an anti-GST (Amersham Pharmacia biotech) or anti-Myc antibody (9E10 monoclonal antibody) (Evan et al., 1985) to detect GST fusions and Hoxa5m, respectively.

RESULTS

Postnatal phenotype of heterozygous B4B2A5m mice: transient growth arrest and dwarfism

The regulatory elements used to drive the ectopic expression of Myc-tagged *Hoxa5* (*Hoxa5m*) in transgenic mice (thereafter called B4B2A5m mice) contain the *Hoxb4* promoter and an enhancer from *Hoxb2* (Fig. 1A). They drive *lacZ* expression in rhombomeres 3 and 5 (r3 and r5), as well as in the somites of 9.5 day-old (E9.5) embryos (Sham et al., 1993). Accordingly, *Hoxa5m* is expressed in the latter regions at E9.5 (Fig. 1B,C).

Heterozygous B4B2A5m mice obtained by mating heterozygous transgenic males with wild-type females show no obvious abnormality at birth. However, compared with their sex-matched wild-type siblings, they are smaller after a few weeks of postnatal development (Fig. 2A,B). The growth of transgenic mice of both sexes, normal during the first postnatal week, progressively slows down until the end of the third week (when maximum weight difference is observed) and then resumes normally (Fig. 2C). In many cases, transient arrests of growth and even weight losses were observed between the second and third weeks. No full recovery was ever obtained, the weight of the transgenic animals remaining about 20% inferior to that of wild-type siblings, even after 3 months.

Growth deficiency was observed in all lines expressing the transgene, and is therefore not due to insertion. This phenotype cannot be attributed to nursing mothers as heterozygous males

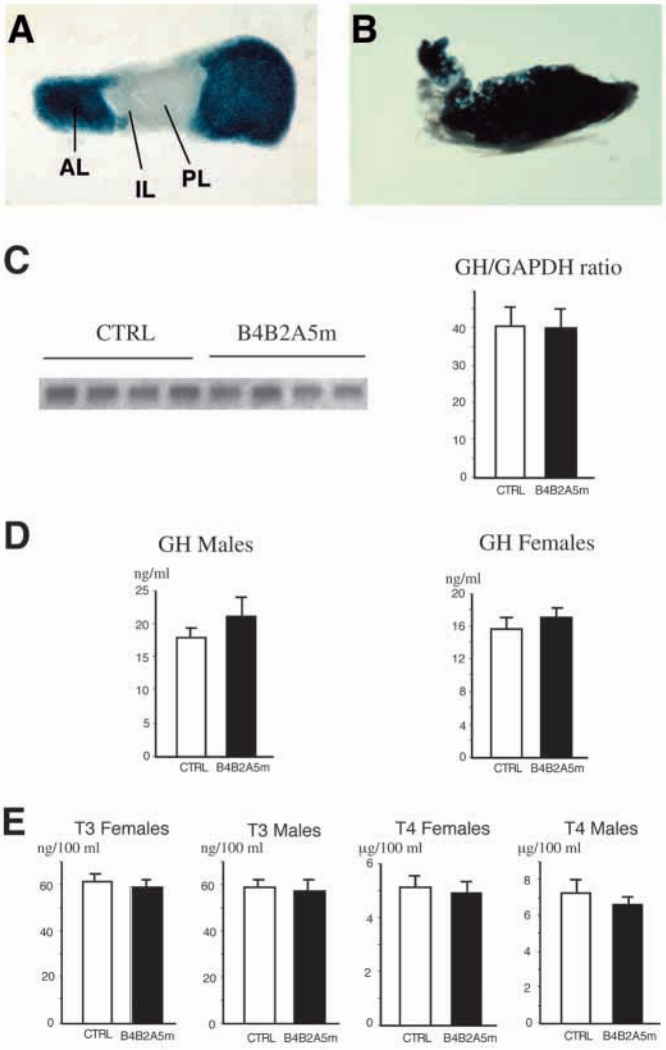

Fig. 3. B4B2A5m mice dwarfism is GH- and T3/T4- independent. (A,B) X-Gal detection of *lacZ* expression in pituitary (200 µm section, A) and whole thyroid gland (B) of adult B4B2lacZ mice. *lacZ* is strongly expressed in many cells of the pituitary anterior lobe (AL), but less so in scattered cells of the intermediate (IL) and posterior lobe (PL). (C) Northern blot analysis of GH mRNA expression in control (CTRL) and transgenic B4B2A5m male mice. No significant difference in GH mRNA concentration is observed between control (n=4) and transgenic mice (n=4). GH mRNA was normalized using the S26 probe (see Materials and Methods). (D) RIA measurements of circulating GH in control (CTRL males, n=8; CTRL females, n=4) and transgenic B4B2A5m (TG males, n=8; TG females n=5) mice. No significant difference could be seen in GH concentration between transgenic and control mice. (E) RIA measurement of total T3 and T4 thyroid hormones in control and transgenic mice of both sexes (n=6 for all groups). Transgenic mice present no alteration in levels of circulating thyroid hormones. All experiments shown in this figure were performed at 3.5 weeks of age.

were crossed with wild-type females. Mating heterozygous males with heterozygous females led to an elevated mortality during the first postnatal weeks and crossing transgenic male survivors with wild-type females revealed that these males were heterozygous. This was tested and observed in three independent transgenic lines. It strongly suggests that

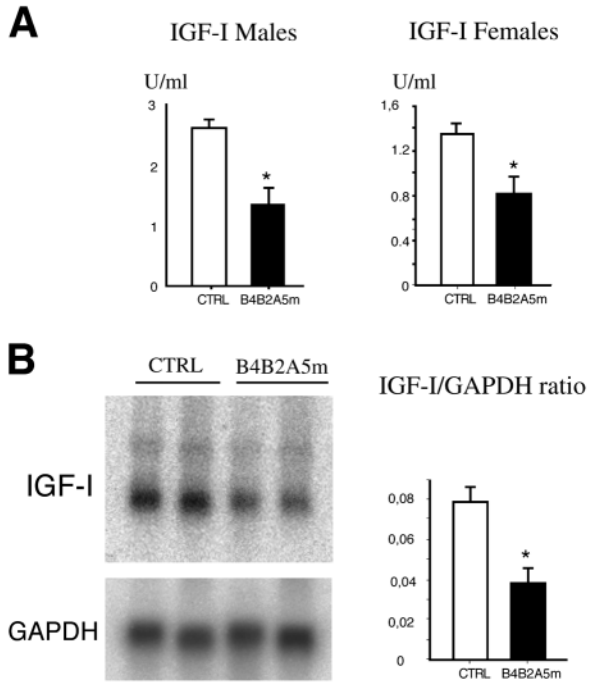

Fig. 4. Decreased liver IGF1 expression and circulating IGF1. (A) Levels of circulating IGF1 in control (CTRL males *n*=8; CTRL females *n*=4) and in B4B2A5m mice (TG males *n*=8; TG females *n*=5). Circulating IGF1 is significantly decreased in transgenic mice of both sexes (male: $P<0.0032$; female: $P<0.018$). (B) Northern blot analysis of liver IGF1 mRNA expression. IGF1 mRNA signal (two transgenic B4B2A5m and two control mice) was normalized with a GAPDH probe. The histogram shows that, compared with control, IGF1 mRNA levels in transgenic animals are halved ($P<0.05$).

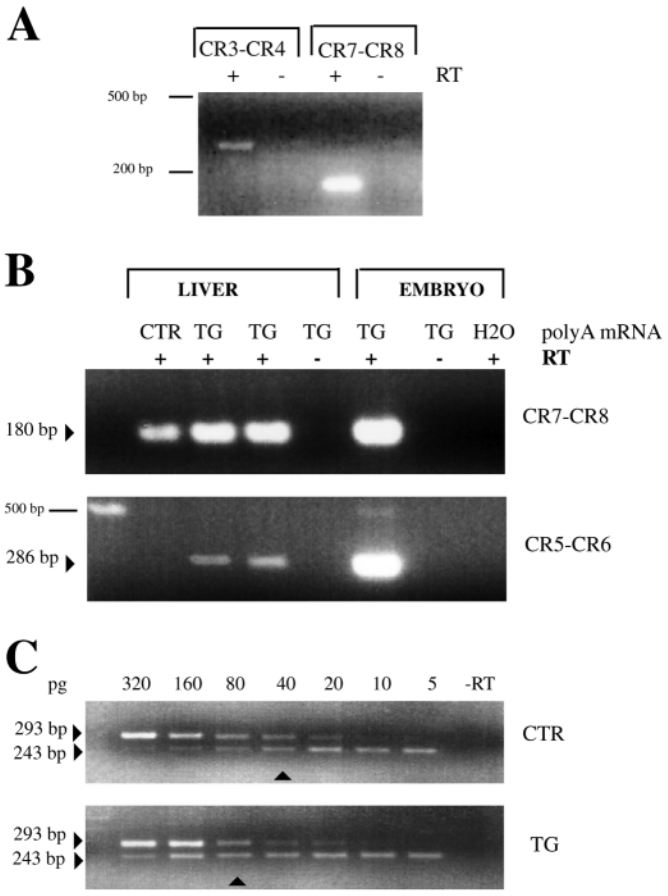

Fig. 5. Expression of *Hoxa5* in HepG2 cells and in liver of control and transgenic mice. (A) Total RNA from HepG2 cells was reverse-transcribed and PCR-amplified using two different couples of primers CR3-CR4 (378 bp) and CR7-CR8 (180 bp) specific for Hoxa5 and recognizing both human and mouse transcripts. (B) PolyA+ RNAs from liver of control (CTR) or transgenic (TG) mice (3.5 weeks), and from E9.5 transgenic embryos (positive control for expression of *Hoxa5m*) were reverse-transcribed and PCR-amplified using three different couples of primers. CR7-CR8 primers amplify a 180 bp fragment, demonstrating the presence of endogenous *Hoxa5* mRNA in control liver and of endogenous *Hoxa5* plus transgene-encoded *Hoxa5m* mRNAs in transgenic livers and embryos. The *Hoxa5m* transgene-specific CR5-CR6 primers amplify a 286 bp fragment only in transgenic livers and embryo. No amplification is obtained in absence of reverse transcriptase (RT–). First lane, molecular mass markers; last lane, PCR performed in the same conditions on H_2O instead of cDNA samples. (C) Liver polyA+ RNAs from control and transgenic mice (1-month-old) were analyzed by quantitative RT-PCR. Equivalence of signal intensity between liver Hoxa5 mRNAs (lower band) and the added standard synthetic RNA (upper band) is obtained at 40 pg for control mice (CTR, arrowhead), and 80 pg for transgenic mice (TG, arrowhead), demonstrating a twofold increase in Hoxa5 mRNA content in transgenic animals.

homozygous B4B2A5m phenotype is lethal and that there is a dose effect of Hoxa5m expression.

Hormonal status of B4B2A5m mice: decreased expression of hepatic IGF1

As shown by the β-galactosidase (β-gal) staining of Fig. 3AB (B4B2lacZ mice), the B4B2 regulatory elements regulating *Hoxa5m* expression in B4B2A5 mice are active in the pituitary and thyroid glands. The levels of GH and thyroid hormones in B4B2A5m mice were therefore investigated. Western blots and immunohistochemistry (data not shown) or northern blots (Fig. 3C) failed to demonstrate any significant difference in the pituitary contents of GH protein and mRNA between transgenic and wild-type animals. RIA measurements of circulating GH performed in 24-day-old mice (when maximum differences of weight are observed) showed no modification of GH concentration in transgenic offspring of both sexes (Fig. 3D). Concerning the pituitary-thyroid hormone axis, no differences were found in the concentration of circulating total T3 and T4 thyroid hormones between transgenic and wild-type mice of either sex (Fig. 3E).

The expression of liver GH receptor and circulating IGF1, two downstream targets of GH, were also evaluated. GH receptor expression, estimated by western blot, is not modified in the liver of B4B2A5 mice (data not shown). However, at 24 days, circulating IGF1 is significantly decreased (about twofold) in transgenic male and female mice (Fig. 4A). This twofold reduction in circulating IGF1 correlates with a 50% decrease in liver IGF1 mRNA (Fig. 4B), indicating that the decrease in circulating IGF1 originates in a downregulation of *Igf1* expression in the liver. This effect is less pronounced in 6-week-old mice (transgenic females: 3.67±0.88 U, *n*=3; control females: 4.33±0.6 U, *n*=3).

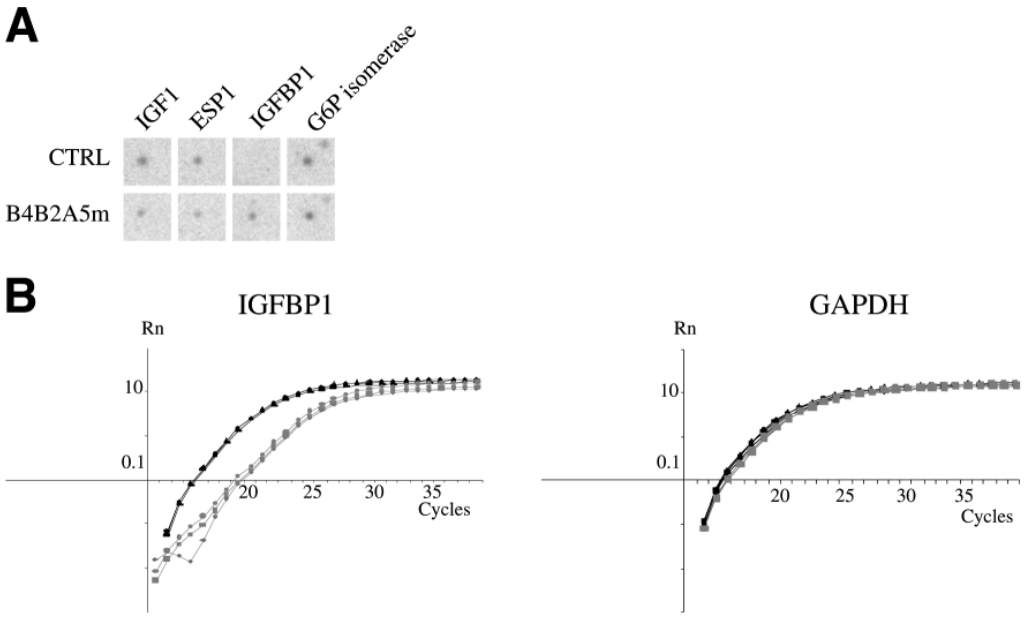

Fig. 6. Macroarray analysis and real time PCR of liver cDNAs from B4B2A5m and control mice. (A) Radioactive spots obtained for IGF1, ESP1, IGFBP1 and glucose-6-phosphate (G6P) isomerase from membranes hybridized with labeled cDNAs obtained from control (CTRL) and B4B2A5m livers. Results are normalized using global standardization with AtlasImage 1.5 software. (B) Graphs representing normalized fluorescence intensity (Rn) as a function of cycle number and showing threshold cycles (C_T) for IGFBP1 and GAPDH transcripts amplicons.

Hoxa5 and Hoxa5m transgene are expressed in the liver of B4B2A5m transgenic mice

Hoxa5 is normally expressed in the rodent liver (Mizuta et al., 1996) and also in the human hepatocyte cell line HepG2 (Fig. 5A). *Hoxa5* expression was thus analyzed by RT-PCR from transgenic and wild-type livers. cDNAs were prepared from polyA+ mRNAs and specific oligonucleotides were used to identify the expression of *Hoxa5m* (oligos CR5 and CR6, see Fig. 1). Oligonucleotides CR7 and CR8, which do not discriminate between the *Hoxa5m* transgene and endogenous *Hoxa5* (Fig. 1A) were also used. Fig. 5B illustrates that *Hoxa5* is expressed in the liver from wild-type and transgenic animals and that only transgenic animals express *Hoxa5m*.

Expression of total (transgene and endogenous) *Hoxa5* was compared between control and B4B2A5m livers by semi-quantitative RT-PCR, using primers allowing the amplification of both endogenous and transgenic *Hoxa5* mRNAs. Fig. 5C illustrates that *Hoxa5* mRNA is overexpressed by twofold in the liver of B4B2A5m animals.

Altered gene expression in the liver of B4B2A5m transgenic mice

Hoxa5 overexpression in the liver of transgenic mice correlates with a downregulation of *Igf1* expression. To identify other modifications in gene expression, a global analysis was performed using macroarrays on mRNA from 24-day-old mice. Only a few mRNAs showed different expression levels between transgenic and control livers (examples in Fig. 6A). The decrease in *Igf1* mRNA in transgenic animals was confirmed (ratio between control and transgenic: R=0.58) and several other mRNAs were slightly downregulated, for example, ESP1 (Enhancer of split 1 related to *Drosophila Groucho* gene, R=0.47) and albumin D box-binding protein (R=0.56). By contrast, prothymosin alpha and clusterin-apoJ mRNAs were upregulated (R=2.26 and 3.45, respectively).

Interestingly, in the context of growth control, *Igfbp1* mRNA is upregulated in transgenic livers. However this upregulation could not be quantified on macroarrays as no signal was detected in control liver (expression decays rapidly after birth) (Gay et al., 1997). Therefore, we used real time RT-PCR assay to quantify *Igfbp1* mRNA in the transgenic B4B2A5m mice and could measure a 12-fold increase of *Igfbp1* mRNA in transgenic compared with control livers (Fig. 6B).

Taken together, these results strongly suggest that the dwarfism of B4B2A5m mice is primarily due to an upregulation of *Igfbp1* and, secondarily, to a downregulation of *Igf1* in transgenic hepatocytes.

Hoxa5 modulates the FKHR-dependent activation of IGFBP1 promoter

A potent activator of *Igfbp1* expression in hepatocytes is the Forkhead box FKHR transcription factor. In its non-phosphorylated form, FKHR activates *Igfbp1* transcription through direct binding to FKHR-specific binding sites (Guo et al., 1999; Rena et al., 1999; Tomizawa et al., 2000). We thus used a cell transfection assay to investigate the ability of Hoxa5 to modulate the *Igfbp1* promoter activity. In the human hepatocyte cell line HepG2, whereas Hoxa5 alone has no significant effect on *Igfbp1* promoter, it reduces its activation by FKHR (Fig. 7A). In primary cultures of human fibroblasts (HuF cells), Hoxa5 and FKHR activate the IGFBP1 promoter when expressed separately, and their co-expression results in a dramatic enhancement of the promoter response (Fig. 7B). Taken together these experiments demonstrate that Hoxa5 and FKHR cooperate at the transcriptional level and that the sense and intensity of this cooperation is context dependent.

Hoxa5 physically interacts with FKHR and FoxA2/HNF3β

The above data demonstrating that Hoxa5 modulates FKHR transcriptional activity could be explained by a physical interaction between Hoxa5 and FKHR. Indeed, physical

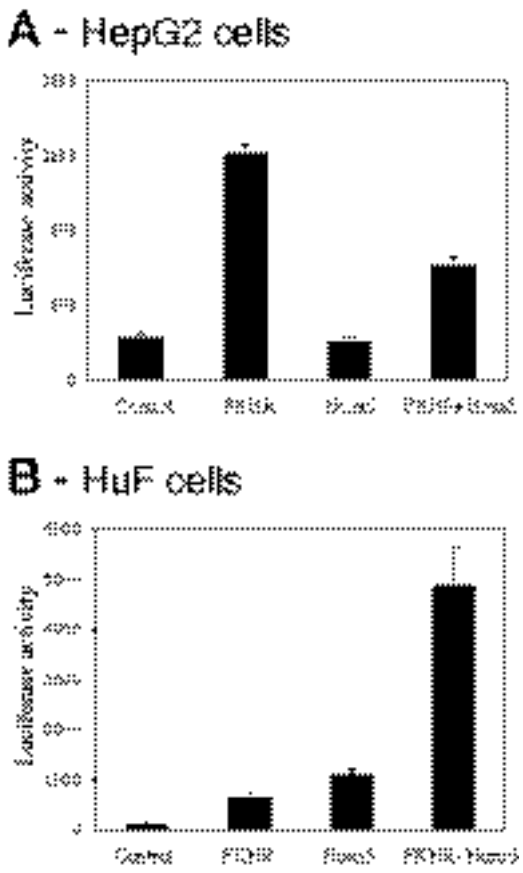

Fig. 7. Regulatory actions of Hoxa5 on the IGFBP1 promoter expression in HepG2 and HuF cells. Cells were transfected with the IGFBP1 promoter linked to luciferase, with/without FKHR and/or Hoxa5. (A) in the HepG2 hepatoma cell line, Hoxa5 alone has no effect, but it inhibits the FKHR-dependent activation of the promoter. (B) In the HuF cells, Hoxa5 activates the IGFBP1 promoter, and it cooperates with FKHR to enhance its activity dramatically. Graphs illustrate the result of one representative experiment; each bar represents the mean and s.e.m. from triplicates.

interactions between Forkhead box transcription factors and homeoproteins have been reported in the case of Otx2 (Nakano et al., 2000), Pdx1 (Marshak et al., 2000) and Oct-4 (Guo et al., 2002). This possibility was tested for both FKHR and FoxA2/HNF3β, using a pull-down assay. Radioactive FKHR and FoxA2/HNF3β were produced in reticulocyte lysates and challenged for binding to GST-Hoxa5 in vitro. As shown in Fig. 8A, the two Forkhead box transcription factors bind to GST-Hoxa5. No binding was observed with GST alone or when FKHR or FoxA2/HNF3β was replaced by luciferase. Finally, to determine whether Hoxa5/FKHR complexes could be retrieved from cells co-expressing these two transcription factors, we performed GST pull-down experiments on extracts from cells transfected with Hoxa5m and GST-FKHR. Fig. 8B shows that Hoxa5 is pulled down only when cells are transfected with GST-FKHR, but not when they are transfected with GST. Altogether, these data demonstrates that Hoxa5 has the ability to interact with FKHR and with FoxA2/HNF3β, and that complexes including Hoxa5 and Forkhead box transcription factors can form within live cells.

DISCUSSION

Dwarfism phenotype in B4B2A5m mice is GH- and T3/T4-independent

In B4B2A5m mice, *Hoxa5m* expression is found in tissues that normally express *Egr2*. This is illustrated by expression in r3 and r5 between embryonic days 8.5 and 10.5 and, at later developmental stages and in the adult, in the pituitary and thyroid. The possibility that the phenotype could be explained by the early and transient *Hoxa5* expression in r3 and r5 was eliminated because no obvious abnormality of the rhombencephalon organization was detected. It was in particular verified that the trajectories of the cranial nerves (visualized by neurofilament staining) were identical between wild-type and B4B2A5m mice (not shown).

Growth retardation starts at the end of the first week of life. This is slightly earlier than in GH-deficient *Snell, Ames* and *lit* mutants, which are indistinguishable from wild-type littermates for the first two weeks (reviewed by Efstratiadis, 1998). The possibility that *Hoxa5m* expression in the pituitary could explain the dwarfism of B4B2A5m mice was explored. The levels of GH mRNA and protein in pituitary extracts and of circulating GH are identical in sibling transgenic and wild-type mice. These data are in sharp contrast with those obtained from other dwarf mice with an impaired GH system (Li et al., 1990; Lin et al., 1993) (reviewed by Voss and Rosenfeld, 1992) suggesting that growth retardation in B4B2A5m mice is GH independent.

Postnatal growth is also regulated by thyroid hormones. Inactivating the thyroid hormone T3Rα receptor gene results in hypothyroidism, reduced levels of T3/T4 and total growth arrest after two weeks of life and death (Fraichard et al., 1997). Phenotypic similarities with B4B2A5m animals pointed towards a pituitary-thyroid axis dysfunction as a possible origin of the dwarfism. However, T3/T4 plasma concentrations are similar in B4B2A5m and wild-type siblings, thus precluding any significant alteration of the pituitary-thyroid axis physiology as a cause B4B2A5m dwarfism.

Postnatal growth defect of B4B2A5m mice probably originates in the liver

The respective 50% decrease and 12-fold increase in liver *Igf1* and *Igfbp1* mRNAs suggest that the origin of B4B2A5m dwarfism is in the liver. It has been shown that overexpressing IGFBP1 in the liver leads to postnatal growth retardation (Gay et al., 1997; Rajkumar et al., 1995). In another transgenic mouse model, it was observed that overexpressing FoxA2/HNF3β in the liver upregulates *Igfbp1* expression and induces growth retardation (Rausa et al., 2000). These phenotypes support the idea that body growth is negatively correlated with IGFBP1 expression and plasma concentration, and that IGFBP1 functions to inhibit IGF1 actions in vivo, most probably by decreasing its bio-availability (Lee et al., 1997).

Recent studies in transgenic mice have shown that postnatal growth was not impaired when targeted disruption of *Igf1* was performed in the liver, even though circulating levels of IGF1 were reduced by 80% (Sjogren et al., 1999; Yakar et al., 1999). However, mice produced by Yakar and colleagues also display an increase in circulating GH and insulin levels, resulting in a decrease in IGFBP1 and IGFBP2 and in normal levels of free IGF1 (Yakar et al., 2001). Thus, B4B2A5m mice growth

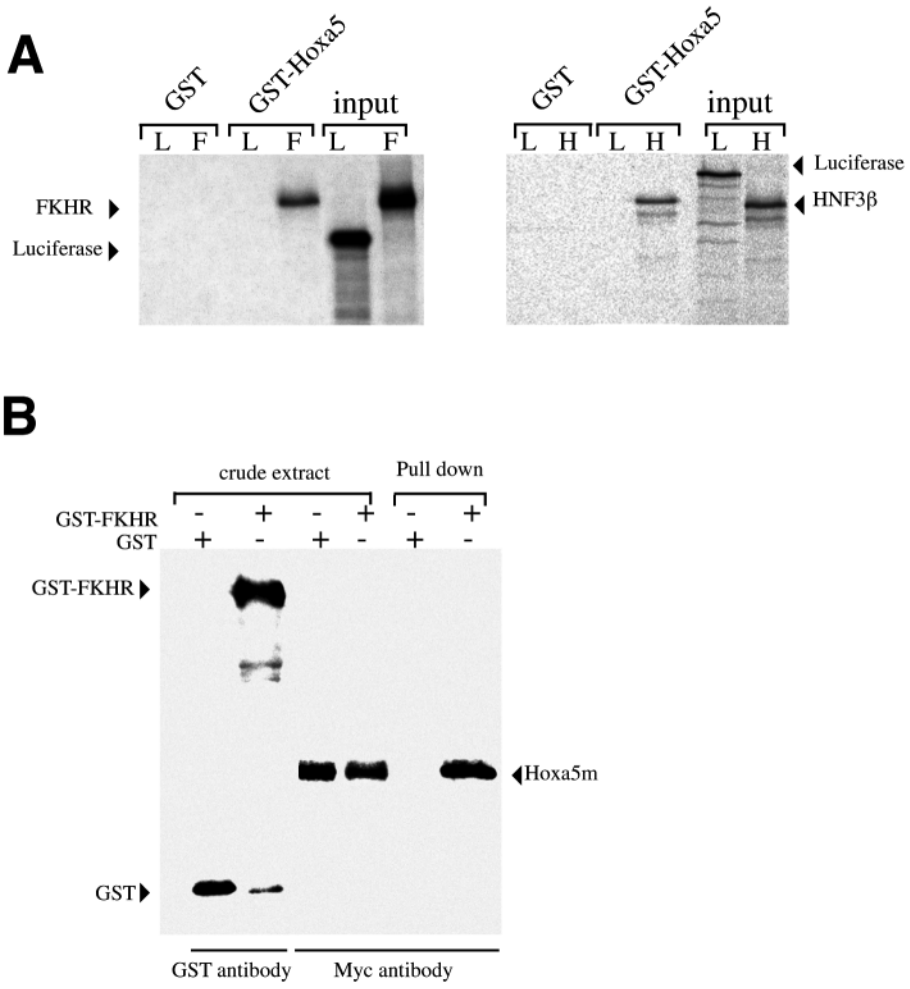

Fig. 8. Hoxa5 directly interacts with FKHR and HNF3β. (A) Binding of ^{35}S-labeled FKHR and HNF3β to GST or GST-Hoxa5 fusion protein. GST or GST-Hoxa5 produced in bacteria were immobilized on glutathione-sepharose beads, which were subsequently loaded with either radioactive luciferase (L), FKHR (F) or HNF3β (H). The last two lanes on each gel (input) show that the same amounts of radioactive luciferase and FKHR or HNF3β were added to the beads. The first four lanes demonstrate that FKHR and HNF3β bind specifically to Hoxa5 (absence of binding on GST alone, and absence of luciferase binding on GST-Hoxa5). (B) Binding of Hoxa5m to GST-FKHR extracted from transfected COS cells. GST or GST-FKHR constructs were co-transfected with Hoxa5m in COS cells, cell extracts were obtained and incubated with Glutathione Sepharose beads. Bound proteins were analyzed by SDS-PAGE and western blot. Whereas Hoxa5m protein is expressed as well in the GST transfected cells as in the GST-FKHR transfected cells, it is pulled down only from cells expressing GST-FKHR, but not from cells expressing GST.

retardation is probably due to the 12-fold increase in *Igfbp1* expression that reduces the bio-available fraction of circulating IGF1, independently of its sites of synthesis in the organism and not, or not only, to the 50% decrease in liver *Igf1* transcription.

Igf1 and *Igfbp1* are expressed in hepatocytes and their up- and downregulation have been observed in the liver. It is therefore tempting to propose that the observed phenotype reflects a cell-autonomous activity of *Hoxa5* or of its paralogs in hepatocytes. Indeed, we confirm that *Hoxa5* is normally expressed in the liver (Mizuta et al., 1996) and in human-derived hepatocyte HepG2 cells (this report). In B4B2A5m mice, *Hoxa5m* transgene expression results in a twofold increase in total *Hoxa5* mRNA levels in the liver. Expression of the transgene in the liver could be driven by low levels of Egr2 or by other transcription factors capable of binding the Hoxb2 enhancer, as is probably the case for somitic expression.

Hoxa5 and Forkhead box transcription factors interact to regulate liver genes playing important roles in postnatal growth

Forkhead box transcription factors are key regulators of several liver genes playing important roles in postnatal growth, including IGF1 and IGFBP1. FoxA2/HNF3β is an activator of the P1 promoter of the IGF1 gene, the most active promoter of this gene in liver (Nolten et al., 1996). FKHR, in its non-phosphorylated form, is a strong activator of *Igfbp1* transcription through direct binding to FKHR-specific binding sites. FKHR transcriptional activity is negatively regulated by insulin, which provokes its phosphorylation at several sites, leading to its exclusion from the nucleus (Brunet et al., 2001; Guo et al., 1999; Rena et al., 1999; Tomizawa et al., 2000).

In absence of a change in FKHR transcription in B4B2A5 mice (assessed by real time PCR, not shown), it was thus tempting to address the possibility that Hoxa5 could either regulate, or interfere with, *Igfbp1* expression. We first addressed this issue in the human HepG2 hepatocyte cell line, using the human *Igfbp1* promoter. Our data indicate that although Hoxa5 by itself is devoid of regulatory activity on this promoter in this cell context, it significantly inhibits its activation by FKHR. This piece of data is at odd with the dramatic overexpression of IGFBP1 observed in the transgenic mice analyzed in the present work. A likely explanation is that the cellular context, i.e. the presence or absence of particular co-factors and/or transcription factors, differs between hepatoma HepG2 cells and the developing liver. This interpretation is comforted by the strong synergy between Hoxa5 and FKHR in another human cellular system (the HuF primary cultures). In any event the two sets of experiments demonstrate without ambiguity that the two transcription factors cooperate to regulate *Igfbp1* promoter expression and that this cooperation is context dependent.

Our results are in line with the observations that Homeoproteins and Forkhead box transcription factors interact to regulate target gene transcription (Filosa et al., 1997; Perea-Gomez et al., 1999). Of particular interest, Pdx1 acts with

FoxA2/HNF3β to regulate its own transcription (Marshak et al., 2000) and Oct-4 almost completely inhibits the FoxD3-dependent activation of the FoxA2/HNF3β promoter (Guo et al., 2002). In a similar way, Engrailed modulates the FoxA2/HNF3β-dependent regulation of the MAP1B promoter, an effect possibly caused by an interaction between both transcription factors on particular homeoprotein-Forkhead box (HF) DNA-binding sites identified within the MAP1B promoter sequence (I. F., A. P. and A. T., unpublished). In all cases, as for the FKHR-Hoxa5 interaction documented in the present work, a physical interaction between the homeoprotein and the Forkhead box transcription factor has been demonstrated.

In conclusion, it can be suggested that, through FKHR and its homologs (i.e. Daf-16 in *C. elegans*), homeoproteins play a role in the regulation of growth and longevity (Lin et al., 1997; Ogg et al., 1997) and that, in addition to their well-established function during development, they have more systemic and physiological roles at late developmental stages and throughout adulthood.

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