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Joint regulation of the MAP1B promoter by HNF3β/Foxa2 and Engrailed is the result of a highly conserved mechanism for direct interaction of homeoproteins and Fox transcription factors

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SUMMARY

The *MAP1B* (*Mtap1b*) promoter presents two evolutionary conserved overlapping homeoproteins and Hepatocyte nuclear factor 3β (HNF3β/Foxa2) cognate binding sites (defining putative homeoprotein/Fox sites, HF1 and HF2). Accordingly, the promoter domain containing HF1 and HF2 is recognized by cerebellum nuclear extracts containing Engrailed and Foxa2 and has regulatory functions in primary cultures of embryonic mes-metencephalic nerve cells. Transfection experiments further demonstrate that Engrailed and Foxa2 interact physiologically in a dose-dependent manner: Foxa2 antagonizes the Engrailed-driven regulation of the *MAP1B* promoter, and vice versa. This led us to investigate if Engrailed and Foxa2 interact directly. Direct interaction was confirmed by pull-down experiments, and the regions participating in this interaction were identified. In Foxa2 the interacting domain is the Forkhead box DNA-binding domain. In Engrailed, two independent interacting domains exist: the homeodomain and a region that includes the Pbx-binding domain. Finally, Foxa2 not only binds Engrailed but also Lim1, Gsc and Hoxa5 homeoproteins and in the four cases Foxa2 binds at least the homeodomain. Based on the involvement of conserved domains in both classes of proteins, it is proposed that the interaction between Forkhead box transcription factors and homeoproteins is a general phenomenon.

Key words: Cerebellum, Mes-metencephalon, Cytoskeleton, Homeodomain, Homeoprotein co-factors, Winged-helix/Forkhead box, Otx2, Goosecoid, Lim1.

INTRODUCTION

Homeoproteins constitute a large family of transcription factors characterized by a highly conserved 60 amino acid-long DNA binding motif, the homeodomain (Gehring et al., 1994), and by specific spatiotemporal expression patterns during development (Krumlauf, 1994; Lumsden and Krumlauf, 1996; Stern and Foley, 1998). Although gain- and loss-of-function experiments have documented the key roles of homeogenes, the molecular mechanisms underlying their biological activity remain unclear. An important problem is the remarkable conservation of the homeodomain, making it difficult to understand how transcriptional specificity can be attained. This is probably why only few direct target genes of distinct homeoproteins have been identified so far (reviewed by Graba et al., 1997; Mannervik, 1999).

A probable explanation for homeoprotein specificity is their association with cofactors. Clearly, homeoproteins have shown associations with numerous proteins, including members of the same homeoprotein family, members of different homeoproteins and non-homeodomain proteins. For example, association of Hox and Engrailed with homeoproteins of the PBC class (*Drosophila* Exd and its vertebrate homologs Pbx1, 2, 3) enhances their DNA-binding specificity and/or affinity (reviewed by Mann and Chan, 1996). In fact, many homeoprotein molecular partners have recently been identified. They belong to several classes of transcription regulators – Groucho (Jimenez et al., 1997), Smad (Germain et al., 2000; Shi et al., 1999), GATA4 (Lee et al., 1998), Nuclear hormone receptor (Budhram-Mahadeo et al., 1998; Kakizawa et al., 1999), bHLH (Poulin et al., 2000), CREB (Edelman et al., 2000), SRF (Carson et al., 2000) and Maf (Kataoka et al., 2001).

The possibility that homeoproteins also interact with the winged-helix/Forkhead box transcription factor HNF3β/Foxa2 (called Foxa2 in the new nomenclature) first came from mice null for Foxa2 and Goosecoid (Gsc) or Lim1. Indeed, the phenotype of these mice suggested that the two latter factors genetically interact with Foxa2 early in development (Filosa et al., 1997; Perea-Gomez et al., 1999). In these two cases, however, direct protein-protein interactions were not investigated. More recently, Foxa2 has been shown to interact directly with homeoprotein Otx2, hence repressing Otx2-directed gene expression ex vivo (Nakano et al., 2000). Another report documents how direct binding of Foxa2 to Pdx1 mediates cooperative interactions of the complex with an

enhancer element of *Pdx1* and regulates *Pdx1* expression in pancreatic β-cells (Marshak et al., 2000). Recently, we demonstrated that Hoxa5 binds Foxa2 and FKHR and that this binding bears important transcriptional and physiological consequences, in particular in the control of body growth (Foucher et al., 2002). Finally FKHR also interacts with Hoxa10 in endometrial cells of the uterus (Kim et al., 2003).

Foxa2 is expressed in different regions of the developing nervous system. In the ventral mesencephalon and cerebellum Foxa2 is co-expressed with Engrailed homeoproteins – En1 and En2, from now on collectively called Engrailed (Dahmane and Ruiz-i-Altaba, 1999; Davis and Joyner, 1988; Hynes et al., 1995; Sasaki and Hogan, 1994) raising the possibility that the two transcription factors interact to regulate common target genes. In a previous report, it was found that the neuronal *Microtubule-associated protein 1B* (*MAP1B/Mtap1b*) promoter is regulated by Engrailed and a region of the promoter, conserved between man and rodent, primarily responsible for this regulation, was identified (Montesinos et al., 2001). In the present work, we show that this promoter fragment contains two conserved overlapping binding sites for Foxa2 and homeoproteins and, when incubated with cerebellum nuclear extracts from the newborn mouse, associates with a protein complex that includes Engrailed and Foxa2. Because of the importance of the interaction between Forkhead box and homeodomain transcription factors we have taken Engrailed and Foxa2 as archetypes of the families, analyzed their molecular and physiological interactions, and identified the domains engaged in their interaction. Interacting regions have been further analyzed for other homeoprotein-Foxa2 interactions, leading to the identification of highly conserved domains for both families of proteins. This strongly suggests that the co-regulation of common targets by Forkhead box transcription factors and homeodomain proteins is a general phenomenon.

MATERIALS AND METHODS

Search for putative Foxa2 binding sites

Computer analysis of potential binding sites for transcription factors within the *MAP1B* promoter sequence was carried out using the matrix search program MatInspector V2 (Quandt et al., 1995). Sequence analysis revealed nine putative Foxa2 binding sites conserved between rat and human.

Plasmids and oligonucleotides

N- and C-terminal deletions of Foxa2, Hoxa5, Lim1 and Gsc were generated by PCR using Pfu polymerase (Promega). T7 promoter was added directly through incorporation into the 5′ oligonucleotide sequence. Owing to low yields of amplification, Hoxa5, Lim1 and Gsc fragments were sub-cloned in pBluescript SKII (Stratagene). The pCMV-Foxa2 expression plasmids [a gift from Dr P. Steenbergh and Dr G. R. Crabtree (Pani et al., 1992)] was used as the PCR-template. GST-En2 (a gift from Dr A. Joliot, ENS, Paris) and GST-Foxa2 fusions were prepared by inserting chick En2 and rat Foxa2 coding sequences into a modified form of pGEX1 (Amersham Pharmacia Biotech). pGEXEn2 was subsequently used to create the Gsc and Lim1 GST fusion proteins. The latter open reading frames were amplified by PCR using Pfu with primers containing appropriate restriction sites in the primers. Truncated constructs of En2 fused to GST, generated by PCR, were a gift from Dr A. Maizel (ENS, Paris). pCLHA-Foxa2 was constructed by first inserting a *Sac*I site in a modified version of pCMV-Foxa2 and then swapping the Foxa2 open reading frame (*Sac*I-*Eco*RI fragment) with Hoxa5 in pCLHAHoxa5. Plasmids containing whole or parts of the *MAP1B* promoter have been described previously (Montesinos et al., 2001). pMAPΔHF1+2-*luc* was constructed by inverse PCR reactions using the ExSite mutagenesis kit (Stratagene).

Recombinant protein production

GST-En2, GST-Foxa2, GST-Hoxa5, GST-Gsc, GST-Lim1 and GST proteins were produced in *E. coli* BL21 strain and purified on glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech), according to manufacturer's instructions. En2ΔSP, a deleted version of En2 (amino acids 1 to 9 followed by amino acids 186-289), was produced from expression plasmid pTrc9mEn2ΔSP (Montesinos et al., 2001) and purified on Hitrap heparin-Sepharose columns (Amersham Pharmacia Biotech). Protein concentration was determined by a modified Bradford assay (Bio-Rad) using bovine serum albumin (BSA) as a standard. All samples were verified by SDS-PAGE.

^{35}S-labeled En2, Hoxa5, Gsc, Lim1 and Krox20 were produced using the TNT Quick Coupled Transcription/Translation system or TNT T7 Quick for PCR DNA (Promega), using pKEn2, pKHX13A (Chatelin et al., 1996), pKSGsc (a gift from Dr M. Schaeffer, Karlsruhe), pKSLim1 (a gift from Dr S.-L. Ang, Strasbourg), and pETKrox20 (a gift from Dr P. Charnay, Paris) as templates. ^{35}S-labeled fragments of these proteins were produced in the same way, using PCR-amplified DNA fragments obtained as described above.

Pull-down interaction assay

Binding assays were performed in a final volume of 200 μl of binding buffer (BF1: 20 mM Tris HCl pH 7, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.01% Nonidet P-40) by incubating 100 ng of glutathione-immobilized fusion proteins with 1 μl of ^{35}S-labeled Foxa2, Engrailed, Hoxa5, Gsc or Lim1 variants. Beads were rinsed with 3 ml of BF1-100 mM NaCl and 1 ml of BF1-500 mM NaCl, boiled for 5 minutes before analysis by SDS-PAGE and autoradiography. In the case of GST-En2 mutants, 1 μg of each mutant was used.

Nuclear extracts and electromobility shift assays (EMSA)

Nuclear extracts from mouse neonatal (P0) cerebellum and posterior mesencephalon were prepared as described previously (Beckmann et al., 1997). Dissected tissues were homogenized in 20 mM Hepes pH 7.9, 100 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 0.7% Nonidet P-40, 0.5 mM dithiothreitol, 10% (wt/vol) glycerol and protease inhibitor cocktail Complete 1× (Roche Diagnostics). After centrifugation (10 minutes, 2,000 ***g***) and washes in the same buffer, pellets were resuspended in 20 mM Hepes pH 7.9, 0.5 M KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 25% (wt/vol) glycerol and Complete 1x and then incubated for 30 minutes at 4°C on a rocker. Nuclear debris were removed by centrifugation at 15,000 ***g*** for 30 minutes at 4°C. Protein concentration was determined as for recombinant proteins.

DNA fragments (C, D, E) and oligonucleotides (HF1 upper strand: 5′-GCATATTAAGAAAAGAAATCTGTATC-3′ and HF2 upper strand: 5′-GTATCTAGCATAATATGTCTGCC-3′) were end-labeled by filling with Klenow-fragment polymerase and [α-^{32}P]dCTP (Amersham). Binding reactions were performed in a final volume of 20 μl (15 mM Hepes pH 8.0, 0.5 mM dithiothreitol, 6.25 mM $MgCl_2$, 12.5% glycerol, 1 μg of salmon sperm DNA and 10 μg BSA). Salt concentrations and/or glycerol varied: 30 mM NaCl (Fig. 2A), 80 mM KCl, 25% glycerol (Fig. 2B), 90 mM NaCl (Fig. 4A,B). After incubation on ice for 30 minutes with 0.5 ng of each probe, DNA-protein complexes were analyzed on 4% polyacrylamide gels (acrylamide:bisacrylamide, 60:1) in 0.25× TBE buffer and 2.5% glycerol. For supershift experiments, probes were first incubated with 1 μg of nuclear extracts for 30 minutes on ice, and then for an

additional 30 minutes with 0.8 μl Foxa2 monoclonal antibody (clone 4C7, DSHB, Iowa City). In some control experiments, another unrelated monoclonal antibody [9E10, anti-myc (Evan et al., 1985)] was also used. For oligonucleotide binding, no BSA was included and only 100 ng of salmon sperm DNA were used. In some experiments, En2ΔSP was pre-incubated with GST-Foxa2 for 20 minutes before probe addition for a further 30 minutes. When indicated, En2ΔSP was pre-incubated with the probes (20 minutes) before addition of GST-Foxa2 (30 minutes). Gels were pre-run at 4°C for 45 minutes at 130 V and run at 4°C for 1.5 hours at 240 V, dried and subjected to autoradiography.

Cell transfection assays

N2A cells (Yusta et al., 1988) were grown in MSS-10% fetal calf serum (Rousselet et al., 1988). Plasmid pMAP-*luc* containing the promoter region of rat *MAP1B* (1.7 kb DNA fragment, position –1626 to +60) has been described by Montesinos et al. (Montesinos et al., 2001).

The pCLHA-Foxa2 and a CMV promoter-driven myc-tagged Chick En2 plasmid [pCL9mEn2 (Mainguy et al., 2000)] were electroporated using a Bio-Rad Gene Pulser II apparatus and 4-mm gap cuvettes (170 V and 950 μF in 250 μl culture medium). One million cells were transfected with 2 μg of reporter plasmid and the indicated amounts of expression plasmid, plus appropriate empty vector to keep total DNA constant (12 μg). Transfection rate and protein nuclear localization were determined by immunocytochemistry with anti-myc 9E10 (Evan et al., 1985) and anti-HA (3F10, Roche Diagnostics) antibodies. Cell viability after transfection was checked using the MTT method (Liu et al., 1997).

Neuronal primary cultures were prepared as described previously (Montesinos et al., 2001). Mes-metencephalic regions from 13.5 d.p.c. mouse embryos were dissected and dissociated, and cells were plated at a density of 250,000 cells/well in 24-well dishes. Transfections of neurons with 1 μg of reporter plasmid were performed using the LipofectAMINE 2000 Reagent (Invitrogen) following the manufacturer's instructions.

Luciferase activity was measured 24 hours after transfection (Le Roux et al., 1995) in a Lumat luminometer (Berthold). Results presented are the mean of three independent experiments.

Quantitative RT-PCR

Total RNA from N2A and primary cultures of mes-metencephalic E13.5 mouse nerve cells was isolated using the RNeasy kit and DNase-treated on column with the Rnase-Free DNase Set (Qiagen). First-strand cDNA was synthesized from 450 ng of total RNA, using the Superscript II (Invitrogen) reverse transcriptase and oligo(dT) following the supplier's protocol. Genomic DNA contamination was systematically checked in samples without reverse transcriptase.

Real-time PCR was performed in a LigthCycler apparatus (Roche), using the LightCycler-FastStart DNA Master SYBR Green I kit (Roche). Diluted samples of cDNA derived from 2, 5, 10 or 20 ng of total RNA were used as template. Oligonucleotides used to amplify mouse *En1*, *En2*, *Foxa2* and *GAPDH* sequences were: mEn1-fw: 5′-TGTGTTTCCTTGTGTGTCTGC; mEn1-rv: 5′-GTCTCCAGAAAAGGAAGGGG; mEn2-fw: 5′-AACAAGCGGGCCAAAATCAAGAA; mEn2-rv: 5′-CGCCCTGCTCGCCCTACTC; mFoxa2-fw: 5′-CACAGCCACCACCACCATCAG; mFoxa2-rv: 5′-GCATCCAGGCTCGCTTTGTTC; GAPDH-fw: 5′-TGACGTGCCGCCTGGAGAAAC; GAPDH-rv: 5′-CCGGCATCGAAGGTGGAAGAGT. The PCR program consisted in an initial step of 8 minutes at 95°C for polymerase activation, and 40 cycles as follows: 15 seconds at 95°C; 5 seconds at 60°C; 15 seconds at 72°C. Melting analysis of PCR products was performed to verify the specificity of the amplification reaction. Amplification efficiencies of targets in the conditions described were close to 100%.

RESULTS

The *MAP1B* promoter contains conserved Foxa2 binding sequences

In a previous study, the entire *MAP1B* promoter was cut into several fragments (Fig. 1) (Montesinos et al., 2001). One of them (hereafter called fragment C), a region of high homology between rat and human, binds Engrailed and is essential for in vivo regulation of *MAP1B* promoter activity by this transcription factor (Montesinos et al., 2001). The *MAP1B* promoter also shows nine conserved putative Foxa2 binding

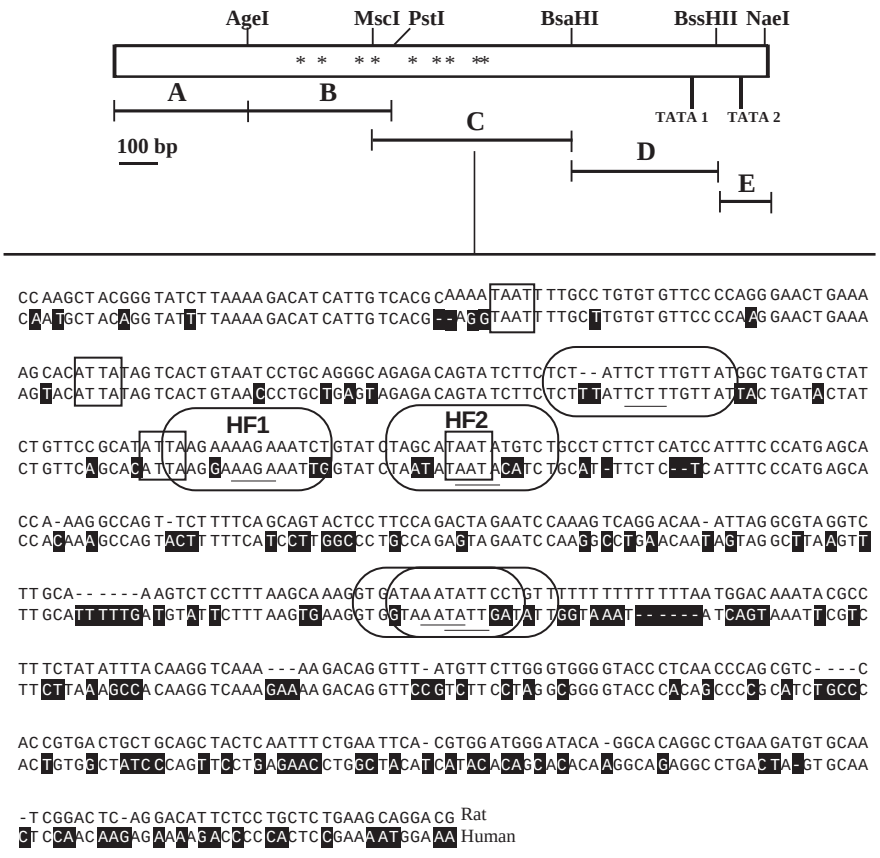


Fig. 1. Conserved Foxa2 consensus binding sites in the *MAP1B* promoter. Schematic representation of the *MAP1B* promoter highlighting potential Foxa2 binding sites (asterisks) on previously characterized A, B, C, D and E fragments (modified from Montesinos et al., 2001). Fragment C rat (top) and human (bottom) sequences have been aligned. Four ATTA (square boxes) and five Foxa2 consensus binding sites (oval boxes, core underlined) are conserved between rodent and human. Note that, in two regions, the putative homeoprotein and Foxa2 sites overlap (HF1 and HF2). Residues that differ between rat and human sequences are shaded in black.

sites, of which five are in fragment C, which also contains all four conserved putative homeoprotein binding sites (Fig. 1). To test whether Foxa2 binds this fragment, electromobility shift assays (EMSA) were performed. As shown in Fig. 2A, GST-Foxa2 binds fragment C, GST shows no binding activity and GST-Foxa2 does not bind fragment E (used as a control) alone (not shown) or in combination with fragment C (Fig. 2A).

The ability of Foxa2 to bind *MAP1B* promoter fragments C, D or E (the two latter taken as control) was tested with nuclear extracts from P0 cerebella that express both Foxa2 and Engrailed. As shown in Fig. 2B, probes C, but not D or E, are retarded. Supershift experiments using the 4C7 anti-Foxa2 monoclonal antibody demonstrate that Foxa2, and Engrailed (Montesinos et al., 2001), are present in the complex formed with fragment C but not with fragment D. Taken together, these results establish that Foxa2 binding sites exist in the most conserved region (fragment C) of the *MAP1B* promoter.

A short conserved region of the MAP1B promoter containing two homeoprotein/Forkhead box binding sites has a regulatory function in mes-metencephalic neurons

Interestingly, within fragment C, two regions contain closely associated Foxa2 and homeoprotein cognate binding sites. The first Foxa2 binding site is fused to an ATTA/TAAT site at its 5′ extremity (defined as HF1, for homeoprotein/Fox 1) and the second contains an internal TAAT/ATTA sequence, defined as HF2 (Figs 1, 4). The functional properties and physiological relevance of these potential targets of both Foxa2 and the homeoproteins were further investigated. To that end, we prepared a truncated version of the *pMAP1B* promoter (pMAPΔHF1+2-*luc*), in which a 43 bp fragment including the HF1 and HF2 sites was excised. Expression of the wild-type and deleted promoters fused to a luciferase reporter was then analyzed in transfected primary cultures of mes-metencephalic E13 mouse neurons expressing both Engrailed and Foxa2 (Fig. 3B). As shown in Fig. 3A, the expression of the deleted *MAP1B* promoter (pMAPΔHF1+2-*luc*) is threefold that of the wild-type promoter, demonstrating that the 43 bp fragment including sites HF1 and HF2 has a regulatory function in mes-metencephalic neurons.

Foxa2 physiologically interacts with Engrailed in regulating the *MAP1B* promoter ex vivo

We then analyzed the functional interaction between Foxa2 and Engrailed in the context of N2A neuroblastoma cells which, in contrast with mes-metencephalic neurons, express no, or very little, Engrailed and Foxa2 (Fig. 3B), thus allowing a better control of the expression levels of the two transcription factors. N2A cells were electroporated with a luciferase reporter linked to *MAP1B* promoter, alone or together with various amounts of Foxa2- and/or Engrailed-expressing plasmids. At a high dose (9 μg of plasmid), Engrailed activates the *MAP1B* promoter (Fig. 3C). Interestingly, whereas a low dose of Foxa2 (1 μg of plasmid) has no effect by itself on the promoter, it is sufficient to significantly decrease the Engrailed-dependent activation of

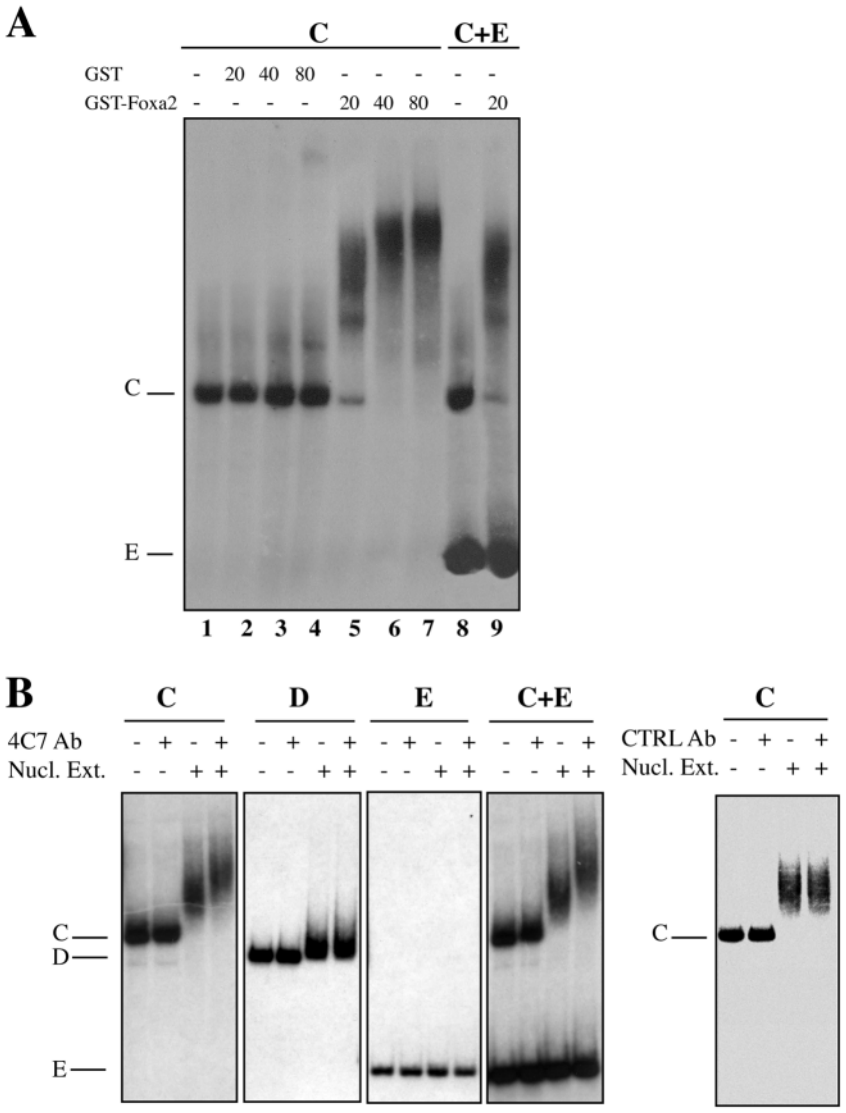
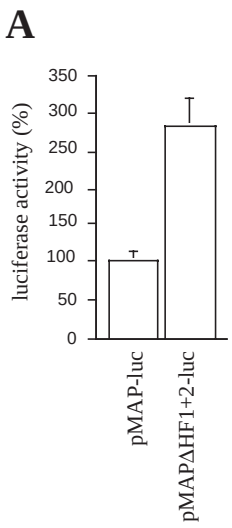


Fig. 2. Binding of Foxa2 to fragment C of *MAP1B* promoter. **(**A) Purified GST (lanes 2-4; 20, 40 or 80 ng) or GST-Foxa2 fusion proteins (lanes 5-7; 20, 40 or 80 ng; lane 9: 20 ng) were incubated with radioactive *MAP1B* promoter fragment C (lanes 1-7) or fragments C plus E (lanes 8-9). Migration of the free probe is indicated on the left. GST-Foxa2 binds to fragment C, but not E, in a dose-dependent manner; GST alone shows no binding activity. (B) Binding of 1 μg nuclear extracts from P0 mice cerebellum to fragments C, D, E and C + E. Migration of free probes is indicated on the left. Both fragments C and D are retarded by the cerebellar nuclear extracts. The complex bound to fragment C contains Foxa2, as demonstrated by the supershift induced by the addition of the anti-Foxa2 antibody (4C7 Ab). No supershift is observed when the 4C7 antibody is used in the absence of cerebellum nuclear extracts. No supershift is observed when another – unrelated – monoclonal antibody (anti-myc, 9E10) is used instead of the 4C7 antibody (CTRL Ab, right panel).



B

	mRNA level (%)	
	E13 mes-metencephalic neurons	N2A cells
En1	32	nd
En2	4	<0.001
Foxa2	0.4	nd
GAPDH	100	93

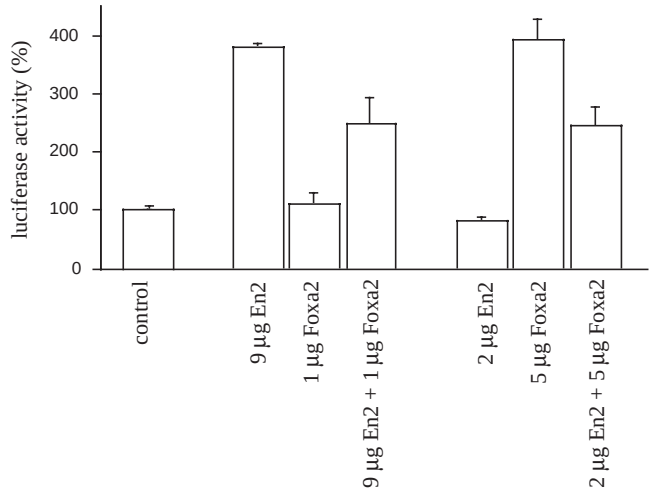


Fig. 3. Ex vivo regulation of the *MAP1B* promoter by Foxa2 and Engrailed. (A) Primary cultures of mouse mes-metencephalic neurons were transfected with the *MAP1B* promoter fused to a luciferase reporter (pMAP-*luc*), or with a modified version of this promoter, in which a 43 bp fragment containing both the HF1 and HF2 sites was deleted (pMAPΔHF1+2-*luc*). The expression of the deleted promoter is about threefold that of the full length promoter, indicating that the 43 bp fragment including HF1 and HF2 has regulatory functions. The data are the results of pooling three independent experiments. (B) Expression levels of *En1*, *En2* and *Foxa2* mRNAs in E13 mes-metencephalic neurons or N2A cells, as determined by real-time RT-PCR. PCR was performed on cDNA derived from 2 ng of RNA (see Materials and Methods). Results are expressed as a percentage of GAPDH mRNA expression level (100% being the level of GAPDH mRNA expression in mes-metencephalic neurons). Significant amounts of En1, En2 and Foxa2 mRNAs were detected in mes-metencephalic cultures. Foxa2 mRNA low abundance is probably due to the fact that *Foxa2* is expressed only by cells located in the ventral mes-metencephalon (the *engrailed* gene being, by contrast, expressed by most cells of the mes-metencephalon). In N2A cells, En2 mRNA level is extremely low and En1 and Foxa2 mRNAs are not detected (nd). (C) N2A cells were co-transfected with various concentrations of *Foxa2*- or *En2*-expressing plasmids, alone or in combination, together with the *MAP1B*-luciferase reporter plasmid (pMAP-*luc*). Control cells were transfected with empty pCL9m plasmid. Transfection of high concentration of En2-expressing plasmid activates the promoter. Transfection of low concentration of Foxa2-expressing plasmid has no effect by itself but partially antagonizes the En2-dependent activation of the *MAP1B* promoter. In a similar way, activation of *MAP1B* promoter by high concentration of *Foxa2* plasmid is partially antagonized by a low dose of En2 plasmid. The data are the results of pooling three independent experiments.

the promoter. At a higher dose (5 µg of plasmid), Foxa2 activates the *MAP1B* promoter and this activation is partially antagonized by the co-expression of low levels of Engrailed (2 µg of plasmid) which have no significant effect per se (Fig. 3C).

Taken together these results suggest that Foxa2 and Engrailed physically interact and/or compete for overlapping DNA target sequences. The HF1 and HF2 sites described above have regulatory functions in mes-metencephalic neurons, and therefore the binding ability of Foxa2 and Engrailed to HF1 and HF2 sequences, alone or together, was investigated.

Foxa2 inhibits the binding of Engrailed to HF1 and HF2 sequences

The binding of GST-Foxa2 and/or En2ΔSP (a shorter version of Engrailed, see Materials and Methods) to synthetic oligonucleotides containing HF1 shows that En2ΔSP binds HF1 and forms either one or two retarded bands depending on its concentration (Fig. 4A, left panel). GST-Foxa2 binding to HF1 is weak and is only visualized after long exposure times (Fig. 4A, middle panel). Fig. 4A (right panel) illustrates that GST-Foxa2 displaces En2ΔSP from HF1 in a dose-dependent manner (compare lane 3 with lanes 8 and 9). This decrease in En2ΔSP binding is concomitant with a slight increase in GST-Foxa2 binding (compare lane 9 with lane 7), suggesting that En2ΔSP favors the binding of Foxa2 to HF1.

En2ΔSP also binds HF2, generating one shifted band (Fig. 4B lanes 2-3), but GST-Foxa2 binding could not be demonstrated (Fig. 4B, lanes 4 and 5), even after long exposures. Despite its apparent absence of binding to HF2, GST-Foxa2 inhibited that of En2ΔSP even when En2ΔSP was pre-incubated with HF2 for 20 minutes before GST-Foxa2 addition (Fig. 4B, compare lane 2 with lanes 6 and 7, and lane 3 with lanes 8 and 9). This latter experiment strongly suggests that GST-Foxa2 binds En2ΔSP and that, as a result, the En2ΔSP protein detaches from HF2. This prompted us to search for direct physical interactions between Engrailed and Foxa2.

En2 directly binds Foxa2: identification of interacting domains in En2 and Foxa2

GST-En2 was incubated with radioactive Foxa2 generated by in vitro transcription and translation. As shown in Fig. 5A, ^{35}S-labeled Foxa2 binds GST-En2. This interaction is specific since it is not observed between ^{35}S-labeled Foxa2 and GST alone (Fig. 5A) or ^{35}S-labeled luciferase and GST-En2 (not shown). To identify the domains of Foxa2 that interact with Engrailed, ^{35}S-labeled truncated versions of Foxa2 (Fig. 5B) were generated and incubated with GST-En2. Oligonucleotide primers were chosen to specifically delete Foxa2-characterized domains CRI to CRIV (Wang et al., 2000) (Fig. 5B). The pull-

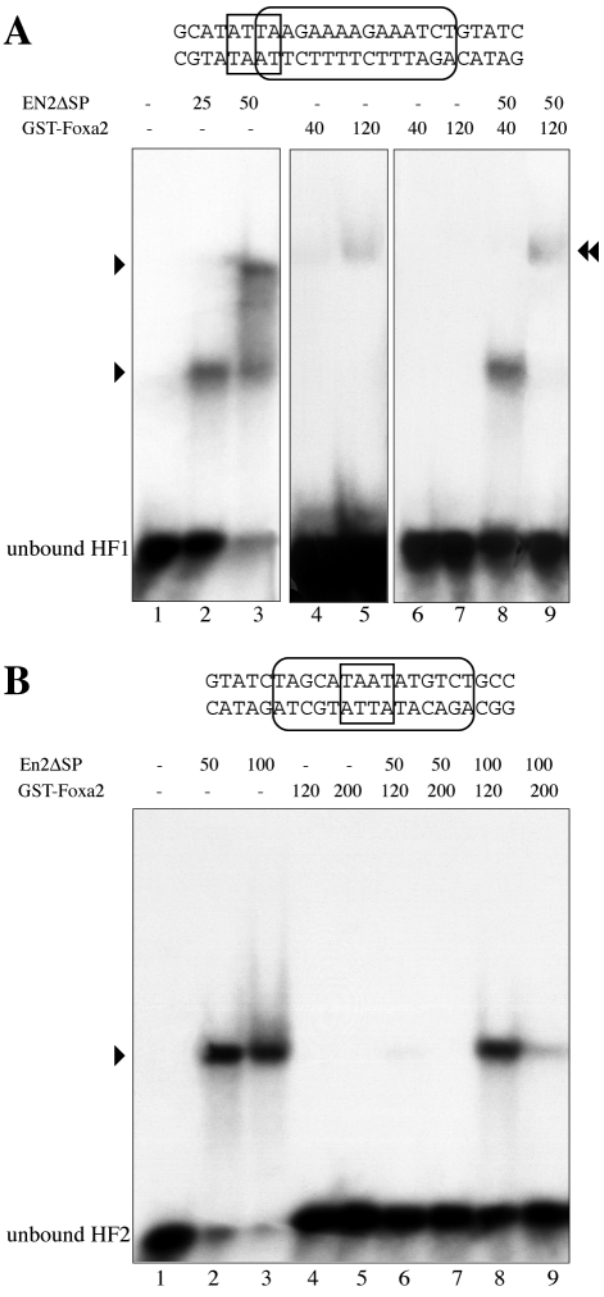


Fig. 4. Foxa2 inhibits En2ΔSP binding to the HF1 and HF2 sites of *MAP1B* promoter. (A) Binding of En2ΔSP and Foxa2 to the HF1 site. The HF1 probe sequence, highlighting the ATTA (square box) and Foxa2 (oval box) sites, is represented above the EMSA panels. Purified En2ΔSP or GST-Foxa2 fusion proteins were incubated independently (lanes 2-7), or in combination (lanes 8-9). Lane 1: free HF1 probe; lanes 2-3: 25 and 50 ng of En2ΔSP, respectively; lanes 4-5 and 6-7 show two different autoradiographic exposures of the same gel area (4-5: long exposure; 6-7: short exposure). Gels were loaded with 40 ng (lanes 4 and 6) or 120 ng (lanes 5 and 7) of GST-Foxa2. An interaction of Foxa2 with HF1 is visible in lane 5 (double arrowhead). At low concentration (lane 2: 25 ng) En2ΔSP gives one retarded band, while at a higher one (lane 3: 50 ng) it generates two retarded bands (arrowheads). Co-incubation of GST-Foxa2 with En2ΔSP leads to an inhibition of En2ΔSP binding (compare lane 3 to lanes 8 and 9). (B) Binding test for En2ΔSP and Foxa2 to the HF2 site. The probe sequence, highlighting the ATTA (square box) and Foxa2 (oval box) sites, is represented above the EMSA panel. Purified En2ΔSP or GST-Foxa2 fusion protein were incubated independently (lane 2: 50, lane 3, 100 ng of En2ΔSP; lane 4: 120, lane 5, 200 ng of GST-Foxa2) or together (lane 6-9) with the HF2 probe. En2ΔSP was preincubated with HF2 for 20 minutes, before adding Foxa2. En2ΔSP binds HF2 (arrowhead), GST-Foxa2 does not bind by itself (lanes 4,5) but dissociates En2ΔSP from HF2 (compare lane 2 with lanes 6-7, and lane 3 with lanes 8-9).

down experiments (see Fig. 5C) demonstrate that the CRI DNA-binding Forkhead domain (amino acids 148-257) is necessary and sufficient for binding to GST-En2.

The same approach was used to identify the domains of En2 (Duboule, 1994) involved in its binding to Foxa2. GST-fused fragments of En2 protein containing the EH1-Groucho binding domain (aa 1-65), the EH2-EH3 Pbx-interacting domain (aa 146-199) and the homeodomain (aa 199-259) were produced (Fig. 6A). As illustrated in Fig. 6B (left panel), fragment 146-199 (containing the Pbx-interacting domain) and the homeodomain bind Foxa2. The 146-199 domain was further sub-divided into fragments 146-167 and 168-199 (the latter containing only the EH2-EH3 Pbx-interacting domain). Fig. 6B (right panel) shows that these two sub-fragments still retain Foxa2, although less efficiently than the entire sequence. It can thus be concluded that the homeodomain and a domain N-terminal to the homeodomain, including the Pbx-interacting domain, bind Foxa2.

Foxa2 also interacts with homeoproteins Hoxa5, Lim1 and Gsc, via conserved domains

The high conservation of the homeodomain and its ability to bind Foxa2 suggests that other homeoproteins may bind Foxa2. To evaluate this possibility, radioactive Hoxa5, Lim1 and Gsc were produced by in vitro transcription/translation and incubated with GST-Foxa2 or GST. This choice was based on the fact that Lim1 and Gsc show genetic interactions with Foxa2 (Filosa et al., 1997; Perea-Gomez et al., 1999) and that Hoxa5 (unlike Lim1 and Gsc but like Engrailed) has a glutamine in position 50 of its homeodomain. Krox20, a zinc-finger transcription factor unrelated to homeoproteins was introduced for control. As shown in Fig. 7A, all three homeoproteins, but not Krox20, bind GST-Foxa2. No binding was seen with GST alone.

To determine which domain of Foxa2 is responsible for its binding to Hoxa5, Gsc and Lim1, its Fox domain (aa 148-257), and the domains located in the N-terminal (aa 1-148) and C-terminal (aa 257-458) were challenged for binding to GST-Hoxa5, GST-Gsc, and GST-Lim1 fusion proteins. Fig. 7B demonstrates that, as for the Foxa2-Engrailed interaction, the only domain retained by all three homeoproteins is the Fox domain. Therefore, Foxa2 binds to all tested homeoproteins through its Fox domain.

Finally, to identify which domains of the homeoproteins Hoxa5, Gsc and Lim1 are involved in the interaction with Foxa2, radioactive fragments of these homeoproteins, including known domains (i.e. the homeodomain and the hexapeptide motif), were synthesized and tested for binding to GST-Foxa2 (not shown). The results of these pull-down experiments are summarized in Fig. 8 which also includes the data reported by Nakano et al. (Nakano et al., 2000) on Foxa2/Otx2 interacting domains. From these mapping

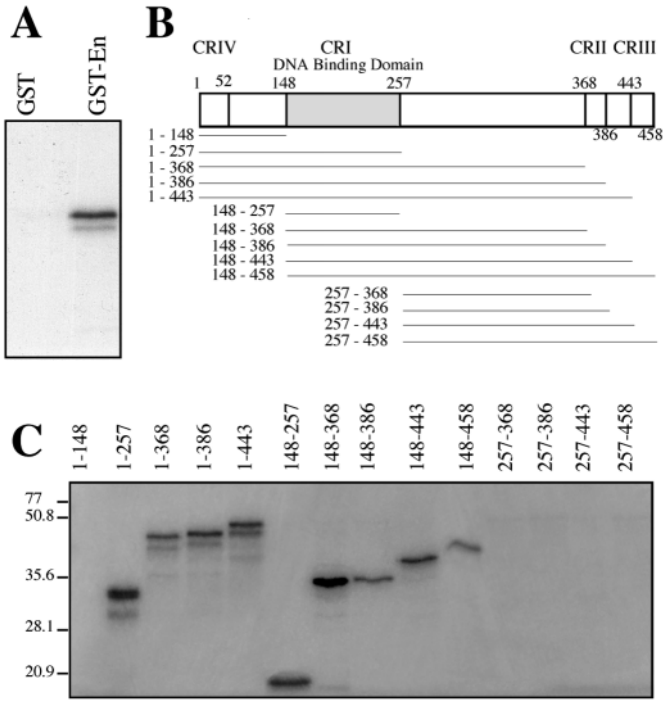


Fig. 5. Foxa2 associates with Engrailed through its winged-helix/Forkhead box domain. (A) ^{35}S-labeled Foxa2 was incubated with bacterially expressed GST or GST-En2 immobilized on glutathione beads, then analysed by SDS-PAGE. Foxa2 binds to GST-En2 but not to GST. (B) Foxa2 constructs used to identify its domain(s) interacting with Engrailed. CRI to CRIV domains of Foxa2 are indicated. Numbers refer to amino acids positions. (C) Each construct was produced and labeled in vitro, and used in a pull-down experiment with GST-En2 immobilized on glutathione beads. Bound proteins were visualized after SDS-PAGE and autoradiography. All constructs containing the CRI domain of Foxa2 bind to Engrailed, and this domain (148-257) is sufficient to mediate this binding. Positions of molecular mass markers (in kDa) are indicated on the left.

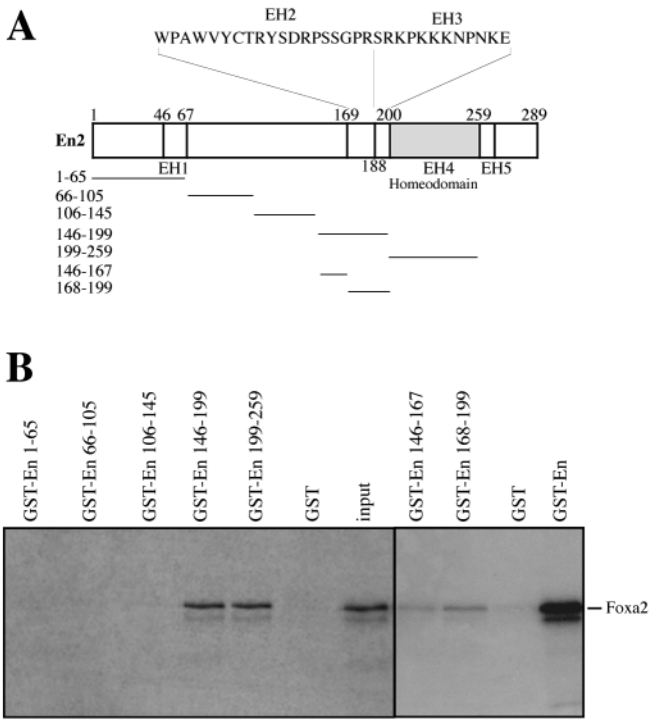


Fig. 6. Identification of the domains of Engrailed interacting with Foxa2. (A) Engrailed truncated constructs produced as GST fusions to identify Foxa2-binding domains within the En2 sequence. The homeodomain (EH4), as well as other previously characterized domains of Engrailed [EH1-5 (Duboule, 1994)] are indicated. Numbers refer to amino acids positions. (B) ^{35}S-labeled Foxa2 was incubated with immobilized GST-fusion proteins. Bound radioactive protein was visualized, following SDS-PAGE, using autoradiography. Left panel illustrates that the homeodomain and the 146-199 domain fully retain Foxa2 (compare with input). Right panel demonstrates that, compared with the full 146-199 domain, the two sub-domains 146-167 and 168-199 bind Foxa2 poorly.

experiments performed on five distinct homoproteins, Engrailed, Hoxa5, Gsc, Lim1 (this paper) and Otx2 (Nakano et al., 2000), it appears that in addition to the homeodomain, which is sufficient for binding to Foxa2, other interacting domains can exist in either the N-terminal (Engrailed, Hoxa5, Gsc) or C-terminal (Otx2) domains of homeoproteins.

DISCUSSION

Regulation of the *MAP1B* promoter by Foxa2

The first indication that Foxa2 may regulate *MAP1B* expression is the presence, in fragments B and C of the *MAP1B* promoter, of several putative consensus Foxa2 binding sites. The fact that fragment C binds Foxa2, either produced in vitro or present in neonatal cerebellum extracts, demonstrates that at least some of these putative Foxa2 binding sites are functional. The presence of these functional Foxa2-binding sites in the *MAP1B* promoter correlates with the fact that Foxa2 activates this promoter in N2A cells. Taken together, these results suggest that Foxa2 regulates *MAP1B* promoter activity and that this regulation involves direct binding and/or indirect mechanisms requiring the inhibition or activation of other transcription factors or co-factors. A Foxa2 site has also been found in the promoter region of *MAP1A* (Nakayama et al., 2001), a gene closely related to *MAP1B*, and derived from a common ancestral gene (Langkopf et al., 1992).

A physical interaction between Foxa2 and Engrailed and its physiological relevance

The existence of interactions between Foxa2 and Engrailed is supported by the following observations. First, in fragment C conserved consensus ATTA/TAAT homeoprotein binding sites are present in the vicinity of Foxa2 binding sites. Second, within nuclear extracts, Engrailed binds the *MAP1B* promoter in a complex that includes Foxa2 (this study) (Montesinos et al., 2001). Third, gel mobility-shift data obtained with HF2 sites are best interpreted in terms of protein-protein interaction. Indeed, although Foxa2 does not show any detectable binding activity to the HF2 site, it displaces En2ΔSP from this DNA sequence. The absence of Foxa2 binding precludes a competition mechanism and therefore favors a model in which Foxa2 binds En2ΔSP, and provokes its dissociation from DNA.

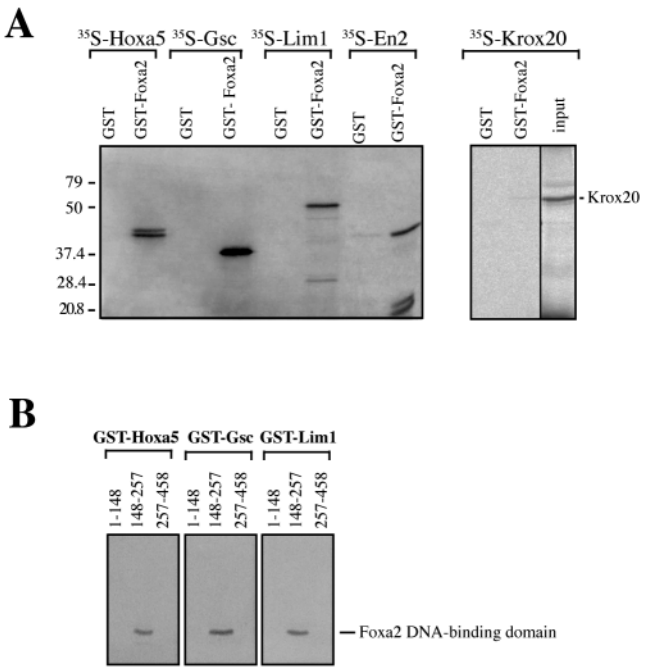


Fig. 7. Binding of Foxa2 to Hoxa5, Lim1 and Gsc homeoproteins through its Forkhead box domain. (A) ^{35}S-labeled homeoproteins Hoxa5, Gsc, Lim1, and Engrailed (En2), as well as ^{35}S-labeled Krox20 were incubated with immobilized GST or GST-Foxa2. All four homeoproteins bind Foxa2 but Krox20 does not. Positions of molecular mass markers (in kDa) are indicated on the left. (B) Mapping of the domain of Foxa2 involved in its interaction with Hoxa5, Gsc and Lim1. ^{35}S-labeled Forkhead box domain (148-257) and its flanking N-terminal (1-148) and C-terminal (257-458) domains were challenged for binding to GST-Hoxa5, GST-Gsc and GST-Lim1. In all three cases, only the Forkhead box domain of Foxa2 binds to the homeoprotein.

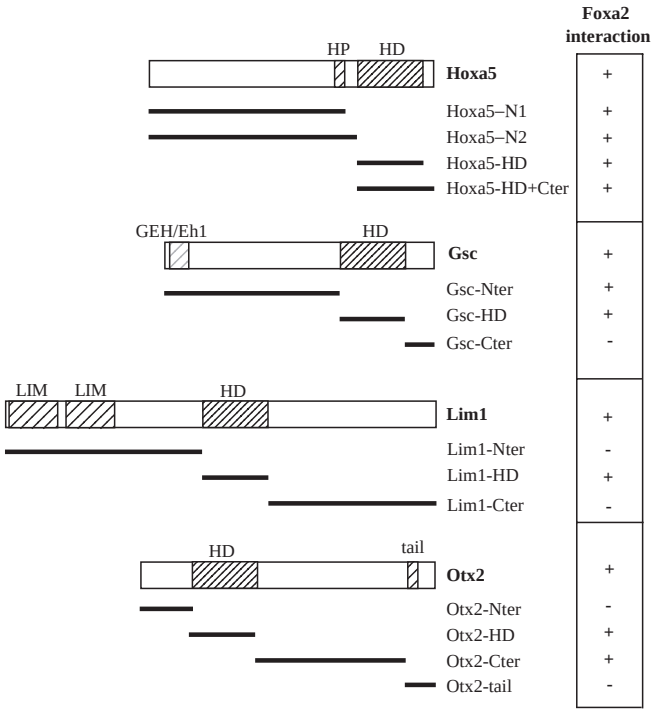


Fig. 8. Homeoprotein domains interacting with Foxa2, as revealed in pull-down assays. Summary of the protein domains within the four homeoproteins studied (other than Engrailed) that bind Foxa2 [Hoxa5, Gsc, Lim1 (this work) and Otx2 (Nakano et al., 2000)]. Radioactive fragments of the homeoproteins were tested for binding to Foxa2 (+ indicates positive binding; – indicates absence of binding). In all cases, the homeodomain (HD) binds Foxa2. Moreover, three homeoproteins out of four have, like Engrailed, one additional interacting domain, located either in their N-terminal domain (Hoxa5, Gsc) or in their C-terminal (Otx2) sequence. HP, Hexapeptide; GEH/Eh1, Gsc-Engrailed homology/Eh1 domain; LIM, LIM domains; Tail, tail domain of Otx2.

Similar inhibitory interactions have been described for En1/Pax6, Ey/Antp, Hox/Maf and CDP/SATB1 (Jinqi et al., 1999; Kataoka et al., 2001; Plaza et al., 1997; Plaza et al., 2001). In this study, the interaction between the two transcription factors results in the displacement of Engrailed from a cognate binding site. This was also observed for Otx2 (Nakano et al., 2000) but is not a general rule since the two partners can show cooperative binding to specific enhancer elements [Pdx1 (Marshak et al., 2000)]. Finally, as will be discussed later, the proteins interact directly and the interaction domains have been identified.

Two main factors indicate a physiological interplay of Engrailed and Foxa2 in regulating *MAP1B* expression. First, deleting the 43 bp fragment encompassing HF1 and HF2 binding sites up-regulates *MAP1B* promoter activity in mesmetencephalic neurons expressing Engrailed and Foxa2. Second, in a cell context devoid of Engrailed or Foxa2 (N2A), dose-dependent gain-of-function experiments demonstrate a regulatory activity of both transcription factors as well as an interaction between the two factors to regulate *MAP1B* promoter activity. In the latter experiment each transcription factor acts as a co-factor for the other one. Separately the two factors activate *MAP1B* at high expression levels and have no effect at low levels but, in co-expression experiments, low levels of either factor down-regulate the activity of the other one. This pattern of regulation suggests at least two possible and non exclusive modes of interaction: binding of the first factor conferring access to the second through conformational changes of the promoter (Fig. 4A) or a direct interaction between the two factors (Fig. 4B). In fact it is well accepted that, depending on the cellular context (i.e. co-expression of co-factors), some transcription factors have opposite functions on a given promoter. For example, Engrailed has opposite regulatory functions on the *polyhomeotic* promoter in *Drosophila*, depending on both Engrailed concentration and Extradenticle expression (Serrano and Maschat, 1998).

Foxa2 and homeoproteins interact through their conserved DNA-binding sequences with various additional interacting domains in homeoproteins

Mapping of the interacting domains identified the Forkhead box binding domain in Foxa2 as the only domain interacting with Engrailed, Hoxa5, Lim1, and Gsc (this study) and Otx2 (Nakano et al., 2000). Similarly, for all homeoproteins tested, the homeodomain alone binds Foxa2. However, and in contrast with Foxa2, four out of these five homeoproteins contained

additional Foxa2-interacting regions: Engrailed, Hoxa5, Gsc (in all three cases in the N-terminal sequence; this study) and Otx2 [in its C-terminal sequence (Nakano et al., 2000)]. In this study a detailed analysis of the interacting domains has been done for Engrailed only and the mapping of the other homeoproteins has been limited to the homeodomain, and its flanking N- and C-terminal regions, at large. In the case of Engrailed, in addition to the homeodomain, a short sequence (amino acids 146-199) overlapping the Pbx-interacting domain also binds Foxa2. This latter domain and the homeodomain bind independently to Foxa2 and the possibility that they interact with different sub-regions of the Forkhead box domain was not investigated. Such an additional non-homeodomain Foxa2 interacting domain was also present in the N-terminal sequences of Hoxa5 and Gsc, but not in Lim1. With the exception of the hexapeptide sequence present in Engrailed and Hoxa5 (see below), no further similarities were found between the Foxa2-binding domains identified outside the homeodomain in Engrailed, Hoxa5, Gsc and Otx2. It is thus possible that, in addition to the homeodomain, different homeoproteins have evolved separate Foxa2-binding regions with regulatory functions.

In this context it is interesting that the fragment 146-199 of Engrailed includes the EH2 (homologous to hexapeptide in Hox proteins) and EH3 domains of Engrailed both implicated in functional interactions with Exd/Pbx homeoproteins (Peltenburg and Murre, 1996). The same observation also stands for Hoxa5, for which the N-terminal sequence containing the hexapeptide sequence binds Foxa2. A possibility, not investigated here, is that both Pbx and Foxa2 bind Engrailed (or Hoxa5) to form a tripartite complex or, alternatively, that Foxa2 and Pbx binding are mutually exclusive. Also intriguing is the fact that Engrailed and Gsc, as well as different Forkhead box proteins – including BF1 and Foxa2 – interact with co-factors of the Groucho/TLE family (Chen and Courey, 2000; Wang et al., 2000; Yao et al., 2001). Since the Groucho/TLE-interacting domains of Engrailed and Foxa2 have been mapped to the EH1 and CRII domains, respectively (two domains not involved in the Foxa2-Engrailed interaction) it is possible that larger complexes involving Groucho/TLE proteins, Forkhead transcription factors and homeoproteins form in vivo.

How general is the interaction between homeoproteins and Forkhead box transcription factors?

The Forkhead box DNA-binding domain and the homeodomain are highly conserved among winged-helix/Forkhead box transcription factors and homeoproteins, respectively. This observation lends weight to the idea that interactions between Fox proteins and homeoproteins could be a general phenomenon. This is supported by the report of direct physical interactions between Foxa2 and Otx2 (Nakano et al., 2000) or Pdx1 (Marshak et al., 2000). In addition, *Foxa2* interacts genetically with *Lim1* (Filosa et al., 1997) and *Gsc* (Perea-Gomez et al., 1999). The former interaction regulates *Sonic hedgehog* expression in the neural tube and the latter participates in the organizer activity of the visceral endoderm. This led us to investigate if (and to demonstrate that) Foxa2 binds Lim1 and Gsc. Homeoproteins that bind Foxa2 therefore presently include Otx2, Pdx1, Hoxa5, Engrailed, Lim1 and Gsc, and the conservation of some of the binding sequences suggests that this is probably a general rule for both classes of partners, the homeoproteins (see above), but also the Forkhead box transcription factors. In line with this hypothesis, we recently showed that such interactions are not limited to Foxa2, but that they also exist for another Forkhead box transcription factor playing a key role in regulating *IGFBP1* (*Igfbp1*) expression, FKHR (Foxo1). Indeed, Hoxa5 physically interacts with FKHR, and this interaction has important physiological consequences in regulating the *IGFBP1* promoter in the liver, a key parameter in postnatal growth (Foucher et al., 2002). A similar physical interaction between FKHR and Hoxa10 was also reported, and it was shown that both transcription factors cooperate on FKHR-binding sites, within the *IGFBP1* promoter, to regulate its cyclic activity in cells of the adult uterus (Kim et al., 2003). Taken together, these data demonstrate that Fox proteins and homeoprotein can interact physically and functionally to regulate many distinct functions, from the earliest events of embryonic development throughout adulthood.

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