

A novel fibroblast growth factor gene expressed in the developing nervous system is a downstream target of the chimeric homeodomain oncoprotein E2A-Pbx1

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SUMMARY

Pbx1 is a homeodomain transcription factor that has the ability to form heterodimers with homeodomain proteins encoded by the homeotic selector (Hox) gene complexes and increase their DNA-binding affinity and specificity. A current hypothesis proposes that interactions with Pbx1 are necessary for Hox proteins to regulate downstream target genes that in turn control growth, differentiation and morphogenesis during development. In pre B cell leukemias containing the t(1;19) chromosome translocation, Pbx1 is converted into a strong transactivator by fusion to the activation domain of the bHLH transcription factor E2A. The E2A-Pbx1 fusion protein should therefore activate transcription of genes normally regulated by Pbx1.

We have used the subtractive process of representational difference analysis to identify targets of E2A-Pbx1. We show that E2A-Pbx1 can directly activate transcription of a novel member of the fibroblast growth factor family of intercellular signalling molecules, FGF-15. The *FGF-15* gene is expressed in a regionally restricted pattern in the developing nervous system, suggesting that FGF-15 may play an important role in regulating cell division and patterning within specific regions of the embryonic brain, spinal cord and sensory organs.

Key words: fibroblast growth factor, extradenticle, Pbx1, E2A, homeodomain, nervous system

INTRODUCTION

During embryogenesis, the homeotic selector proteins encoded by the mammalian Hox and *Drosophila* HOM-C gene complexes function to control the identity and pattern of body structures along the anterior-posterior (A-P) axis (Lawrence and Morata, 1994; Krumlauf, 1994). Each region along the A-P axis expresses a unique subset of Hox/HOM-C proteins, which act combinatorially to initiate a gene expression program ultimately leading to the formation of appropriate structures at that position. However, despite intensive study, relatively little progress has been made in identifying the direct downstream target genes regulated by homeodomain proteins.

One obstacle to identifying target genes has been that, although homeodomain proteins act with great biological specificity during development, they exhibit a remarkably low degree of DNA-binding specificity *in vitro* (Hayashi and Scott, 1990). Recent studies indicate that one mechanism for increasing DNA-binding specificity is for homeodomain proteins to form heterodimeric complexes with cofactors (Mann and Chan, 1996). One group of such cofactors is the divergent homeodomain proteins encoded by the mammalian *pbx* genes and their *Drosophila* homolog *exd*. Mutations in *exd* cause homeotic transformations but do not affect HOM-C gene

expression (Gonzalez-Crespo and Morata, 1995; Peifer and Wieschaus, 1990; Rauskolb et al., 1995). Instead, the Exd protein modifies and increases the ability of the HOM-C proteins to regulate downstream target genes (Rauskolb and Wieschaus, 1994). Recent studies have shown that the Exd and Pbx proteins function by forming heterodimers with the HOM-C/Hox proteins, thereby increasing their DNA-binding affinity and specificity (Chan et al., 1994; Chang et al., 1995; Knoepfler and Kamps, 1995; Lu et al., 1995; Neuteboom et al., 1995; van Dijk and Murre, 1994). Known examples of Exd/Pbx-dependent gene expression are regulation of the *teashirt*, *wg* and *dpp* genes by HOM-C proteins in the *Drosophila* embryonic midgut, autoregulation of the *HoxB-1* gene in rhombomere 4 of the mammalian hindbrain, and activation of the somatostatin gene in pancreatic islet cells by the orphan homeobox protein STF-1 (Chan et al., 1994; Peers et al., 1995; Popperl et al., 1995; Rauskolb and Wieschaus, 1994).

In *Drosophila*, the targets regulated by Exd include *wg* and *dpp*, members of the Wnt and transforming growth factor- β /bone morphogenetic protein (TGF- β /BMP) families, respectively. These secreted signalling proteins mediate interactions between groups of cells that are necessary for the correct induction and patterning of tissues during embryogenesis. In vertebrates, embryonic patterning is regulated by four large

classes of secreted signalling molecules: the Wnt, hedgehog (HH), TGF- β /BMP and fibroblast growth factor (FGF) families. Thus, by analogy to *Drosophila*, one might expect targets of Pbx to include genes for some of these factors.

Pbx1 was initially identified due to its involvement in the t(1;19) chromosome translocation which occurs in 30% of pre-B acute lymphoblastic leukemias (Kamps et al., 1990; Nourse et al., 1990). This translocation fuses the 5' end of the *E2A* gene (encoding the bHLH transcription factors E12 and E47) on chromosome 19, to the 3' end of the *Pbx1* gene on chromosome 1, resulting in the production of an E2A-Pbx1 fusion protein (Kamps et al., 1991). Normal Pbx1 has no detectable transactivating activity. However, in E2A-Pbx1 the strong transactivation domain of E2A is fused to the Pbx1 homeodomain, thus converting Pbx1 into a potent transactivator (Lu et al., 1994; van Dijk et al., 1993). This has led to the proposal that E2A-Pbx1 transforms cells by constitutively activating the transcription of genes normally regulated by Pbx1.

In this study, we have used subtractive cDNA cloning to identify genes regulated by E2A-Pbx1 in rodent fibroblasts. One of these genes encodes a new, divergent member of the FGF family. The expression pattern of this gene suggests that it may play an important role in early development of the nervous system. These data indicate that Pbx/Exd proteins may direct embryonic patterning in both insects and mammals by regulating the expression of cell-cell signalling molecules.

MATERIALS AND METHODS

Plasmid construction and mutagenesis

Point mutations in the Pbx1 homeodomain were generated by PCR as described previously (Peltenburg and Murre, 1997). The precise deletion of the homeodomain (mutant Δ HHD) was made by overlap extension PCR (Neuteboom et al., 1995). Intact E2A-Pbx1 cDNAs containing the mutations were assembled in vector pSP65 (Promega).

Luciferase reporter constructs were made by PCR amplification of the promoter region of FGF-15 genomic clones using forward primers with flanking *XhoI* sites and backward primers with flanking *HindIII* sites. The amplified fragments were cloned into the *XhoI* and *HindIII* sites of vector pGL3 basic (Promega). Point mutations were introduced into the FGF-15 promoter using the Quickchange site-directed mutagenesis kit (Stratagene, San Diego).

Generation of stable cell lines by retroviral gene transfer

Wild-type and mutant E2A-Pbx1 cDNAs were cloned into the *BglIII* site of retroviral vector pSLXCMV after filling in the 5' overhanging ends with Klenow fragment (McWhirter and Wang, 1993). Helper-free recombinant retroviruses were made by transient transfection of BOSC 23 cells and used to infect NIH3T3 cells at an moi of 1.0 as described by Pear et al. (1993). 2 days later, the cells were selected in media containing G418 for 8 days, at which point they were harvested for analysis.

Representational difference analysis (RDA) of cDNAs

Total RNA was isolated from 3T3 cells using Trizol reagent (GIBCO/BRL). Poly(A)⁺ RNA was purified using oligo(dT)-conjugated magnetic beads (Dynal). Double-stranded cDNAs were synthesized using the Superscript II cDNA synthesis kit according to the manufacturers protocol (GIBCO/BRL). First strand cDNA synthesis was primed with oligo(dT) using 5 μ g of poly(A)⁺ RNA. RDA of cDNAs was performed as described by Hubank and Schatz (1994). The third difference products were isolated on a 2.5% agarose gel and cloned into vector pGEM-T (Promega, Madison, WI).

Immunoblotting

NIH3T3 cells were lysed by boiling in 10 mM Tris-HCl buffer containing 1% SDS and protein concentrations were determined by Bradford assay. An equal amount of protein from each lysate was separated by SDS-polyacrylamide gel electrophoresis (PAGE) (8.5% gel) and electrophoretically transferred to an Immobilon-P membrane (Millipore). E2A-Pbx1 proteins were detected with monoclonal antibody G193-86.5, which recognizes an epitope in the N-terminal domain of human E2A. Immunoblots were developed using the ECL detection system (Amersham).

Southern and northern blotting

cDNA representations were separated on 2.5% agarose gels and denatured. Poly(A)⁺ RNAs were separated on formaldehyde-agarose gels. The cDNAs or RNAs were transferred to nylon membranes and immobilized by UV crosslinking. Double-stranded DNA probes were labelled with [³²P]dCTP using Klenow fragment and random primers. Hybridizations were done at 42°C in 5 \times SSPE buffer, 50% formamide, 10% dextran sulfate, 1 \times Denhardt's reagent, 0.15% sodium dodecyl sulfate (SDS) and 100 μ g per ml denatured salmon sperm DNA. Washes were done at 60°C in 0.2 \times SSPE buffer, 0.1% SDS.

PCR cloning and analysis of the FGF-15 cDNA

A full-length FGF-15 cDNA was isolated by the rapid amplification of cDNA ends (RACE) method (Frohman et al., 1988). 3' RACE was performed using the forward primer TCTACGGCTGGGGCAAGAT-TAC (corresponding to nucleotides 275-296 of the cDNA) and two rounds of 5' RACE were performed using two nested backward primers: CAGTCCATTTCCTCCCTGAAGG (nucleotides 530-551) and TCCTCCGAGTAGCGAATCAG (nucleotides 502-521). To isolate clones containing the entire FGF-15 coding region RT-PCR was performed using primers flanking the open reading frame of the cDNA. RT-PCR primer sequences are indicated in Fig. 3A. Clones from three independent PCR reactions were sequenced to ensure that no mutations had been introduced during amplification.

Isolation of genomic clones

A 1530 bp fragment of the FGF-15 cDNA (nucleotides 275-1805) was used as a probe to isolate recombinant phages containing genomic DNA from a mouse 129 library (Stratagene, San Diego). Restriction fragments containing the FGF-15 exons were identified by Southern blotting and subcloned into pBluescript. The promoter region and introns were analysed by sequencing and restriction mapping of these subclones.

Transactivation assays

NIH3T3 cells grown in 6 cm dishes were cotransfected with 1 μ g of reporter plasmid, 4 μ g of E2A-Pbx1b -expression plasmid or empty pSLXCMV vector, and 5 μ g of pCMV- β Gal plasmid using the lipofectamine method (GIBCO/BRL). A20 cells (10⁷) were cotransfected with the same plasmids using the DEAE-dextran method (Van Dijk et al., 1993). The cells were harvested 48 hours after transfection. Luciferase activity was measured using the Promega luciferase assay system and β -galactosidase activity was measured using the Galacto-Light Plus chemiluminescent detection system (Tropix).

In vitro translation, preparation of nuclear extracts and electrophoretic mobility shift assays (EMSAs)

Proteins were synthesized using the SP6 TNT rabbit reticulocyte lysate-coupled transcription/translation system (Promega, Madison, WI). Control reactions using [³⁵S]methionine were performed and analysed on a 10% SDS gel to verify that the proteins were produced in similar quantities. Nuclear extracts were prepared according to the protocol of Dignam et al. (1983). EMSAs were performed as described (Neuteboom et al., 1995), except for the following changes in EMSAs using the FGF-15 probes: 10⁵ cts/minute of probe was

added to the reaction instead of 3×10^4 , 0.5 μg of poly(dIC) was added to the reaction instead of 1.0 and electrophoresis was done at 4°C instead of room temperature. The ^{32}P end-labeled probes were 5'-GTCAATTAATGCGCATCAATCAATTCG-3' (probe A) to detect E2A-Pbx1:Hox cooperative binding and 5'-GTCAATTAATGATCAATCAATTCG-3' (probe B) to detect E2A-Pbx1:En-2 cooperative binding. The sequences of the wild-type and mutant FGF-15 promoter probes are indicated in Fig. 6A.

In situ hybridization

An 806 bp fragment corresponding to nucleotides 42 to 848 of the FGF-15 cDNA was used to generate riboprobes for in situ hybridization. Radioactive and non-radioactive in situs were performed as described by Goulding et al. (1993).

RESULTS

Identification of potential downstream targets of E2A-Pbx1

To identify genes that are upregulated by E2A-Pbx1, NIH3T3 cells expressing either empty vector or vector containing the E2A-Pbx1b cDNA were generated by infection with replication-defective retroviruses. Expression of the E2A-Pbx1b transgene was verified by western blotting with an E2A-specific monoclonal antibody (Fig. 1A). Double-stranded cDNAs were prepared from the two cell populations, and representational difference analysis (RDA), a process of subtractive hybridization coupled to PCR amplification which eliminates cDNAs present in both populations leaving only the differences (Hubank and Schatz, 1994), was performed on the two cDNAs to isolate differentially expressed transcripts (Fig. 1B). When the vector cDNA representation was used as driver and the E2A-Pbx1 cDNA representation was used as tester in the RDA, the difference product after the third round of subtraction consisted of three major fragments visible on an ethidium bromide-stained agarose gel (Fig. 1B, DP3). The reciprocal RDA in which the E2A-Pbx1 representation was used as driver and the vector representation was used as tester did not yield any difference products (data not shown). The three DP3 fragments were cloned into a plasmid vector and several clones from each fragment were sequenced. The sequences were compared to the NCBI nonredundant database using the BLAST algorithm (Altschul et al., 1990). The 436 bp fragment was identical to the mouse proglucagon gene at both the nucleotide and peptide level. The 312 bp fragment contained an open reading frame that was 30% identical at the peptide level to several members of the FGF family. The 260 bp band consisted of two comigrating fragments that had no significant similarity to anything in the database but were later found to be derived from the 3' untranslated region of the FGF-related cDNA hereafter referred to as FGF-15.

To verify that the FGF-15 and proglucagon fragments were true difference products, they were labeled with ^{32}P and used to probe Southern blots of the cDNA representations (Fig. 1C) and northern blots of the poly(A)⁺ RNA used to construct the original cDNAs (Fig. 2D). As expected, both genes were detectable only in the representation and mRNA derived from the E2A-Pbx1b-expressing cells (Figs

1C, 2D, lanes 1,2; data not shown). The FGF-15 probe detected a single mRNA transcript of approximately 2.2 kb (Fig. 2D).

The Pbx1 homeodomain is required for induction of FGF-15 by E2A-Pbx1

Previous studies identified several residues in the Pbx1 homeodomain required for cooperative DNA binding with either Hox or En-2 (Fig. 2A). These mutations were introduced into E2A-Pbx1b and analysed by EMSA. The results showed that they behaved the same as the original Pbx1 mutants. One mutation, R2Q, located immediately N-terminal of helix 1 abrogated cooperative DNA binding with Hox proteins but did not interfere with En-2 cooperativity (Fig. 2B, lanes 6,11; Peltenburg and Murre, 1997). Mutation of the valine and glutamine residues (V42E/Q44P) in helix 3 or deletion of the entire homeodomain (ΔHD) abolished cooperative DNA binding with both Hox and En-2 proteins (Fig. 2B, lanes 7,12; unpublished observations). Substitution of a glycine in helix 3 by a glutamine (G50Q) did not affect cooperative DNA binding with either Hox or En-2 proteins (Fig. 2B, lanes 8,13; Peltenburg and Murre, 1997).

To determine whether the induction of the *FGF-15* gene required the E2A-Pbx1 homeodomain or interaction with Hox- or Engrailed-like proteins, the mutants were expressed in NIH3T3 cells by retroviral infection. Immunoblotting verified that the proteins were expressed at similar levels (Fig. 2C). The ability of the mutants to induce FGF-15 expression was assayed by northern blotting (Fig. 2D). As expected, the G50Q mutant behaved the same as wild-type E2A-Pbx1 (Fig. 2D, lane 5). Mutants ΔHD and V42E/Q44P were unable to induce FGF-15 expression, indicating that DNA-binding is essential (Fig. 2D, lanes 3,4). Mutant R2Q was still able to induce FGF-15 expression (Fig. 2D, lane 6) but not proglucagon expression (data not shown) indicating that induction of these two genes may involve two distinct binding partners that interact with E2A-Pbx1 via different mechanisms.

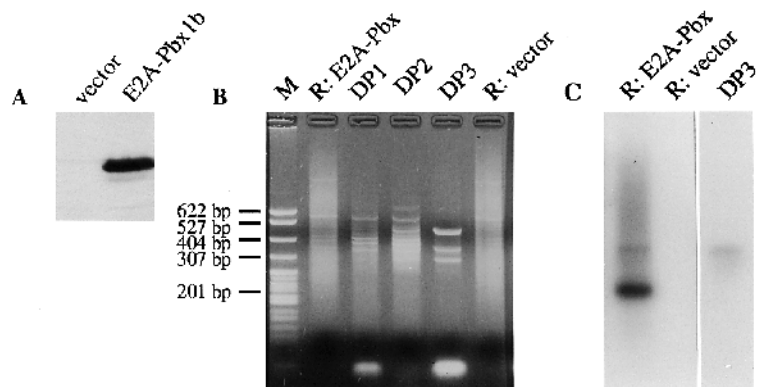


Fig. 1. Identification of genes induced by E2A-Pbx1b in NIH3T3 cells by RDA. (A) Immunoblot of total protein from cells infected with empty retroviral vector or vector containing E2A-Pbx1b cDNA. The blot was probed with a monoclonal antibody for human E2A, G193-86. (B) Ethidium bromide-stained 2.5% agarose gel of cDNA representations (R) and difference products (DP1, DP2 and DP3) from the RDA screen. (C) Southern blot of the cDNA representations (R) and third difference products (DP3) probed with the 312 bp *DpnII* fragment of FGF-15.

Molecular cloning and analysis of the FGF-15 cDNA

A full-length FGF-15 cDNA was isolated from E2A-Pbx1b-expressing 3T3 cells using the rapid amplification of cDNA ends (RACE) method. This strategy yielded two overlapping clones containing a total of 1805 bp (Fig. 3). The sequence contained a 654 bp open reading frame, which would encode a 218 amino acid protein (Fig. 3). To obtain a clone containing the entire coding region, RT-PCR was performed using two gene-specific primers flanking the open reading frame (Fig. 3).

Most members of the FGF family share a conserved genomic organization consisting of three exons and two introns (Smallwood et al., 1996). To determine if the FGF-15 locus also had this organization, the FGF-15 cDNA was used to screen a mouse genomic library. Three overlapping recombinant λ phage clones were isolated. Comparison of the genomic and mRNA sequences showed that two introns of 243 bp and ~2500 bp are present in FGF-15 at precisely the same positions as in the other FGF genes (Fig. 3).

The deduced amino acid sequence of FGF-15 was compared to the sequences of the other mammalian FGFs (Fig. 4). All FGFs share a conserved central region of approximately 120 amino acids which in FGF-1 and FGF-2 consists of 12 antiparallel beta strands that form a structure with threefold internal symmetry called a beta-trefoil (Zhu et al., 1991). Nine of these beta strands contain a highly conserved hydrophobic core residue. Eight of these hydrophobic residues are present in FGF-15 (Fig. 4A). Although the conserved phenylalanine residue in strand 12 is absent in FGF-15, there is a phenylalanine at an adjacent position. Within the conserved region, FGF-15 is between 15 and 30% identical to the other family members, with the greatest similarity to FGF-7 and FGF-1 and the least similarity to FGF-8 (Fig. 4A). Like FGF-1, FGF-3, FGF-5, FGF-7 and FGF-9, FGF-15 contains a variable insert region between beta strands 9 and 10 (Fig. 4A). Of the known FGF family members, FGF-15 is the most divergent (Fig. 4B). One striking feature of the FGF-15 sequence is the unusual pattern of cysteine residues: a serine is substituted for the conserved cysteine in beta strand 1, and nonconservative substitutions of cysteines for consensus residues occur in strands 2, 3 and 6, and in the loop between strands 8 and 9 (Fig. 4A). Other major differences from the consensus are the replacement of two highly conserved glycines in beta strand 5 with charged amino acids and a short deletion in the loop between strands 11 and 12 (Fig. 4A).

The majority of FGFs, with the exception of FGF-1 and FGF-2 and the four FGF-homologous factors (FHF), contain signal peptides and are released from cells via the

normal secretory pathway. The hydrophathy profile of the FGF-15 sequence indicates that it also has a signal peptide and should therefore be secreted (Fig. 3). In vitro transcription and translation of the FGF-15 cDNA resulted in the synthesis of a 25×10^3 M_r protein as predicted by the conceptual translation (data not shown). When an epitope(FLAG)-tagged FGF-15 protein was expressed in NIH3T3 or COS cells, most of the FGF-15-Flag protein was detected in the medium, indicating that it is efficiently secreted and freely diffusible (data not shown).

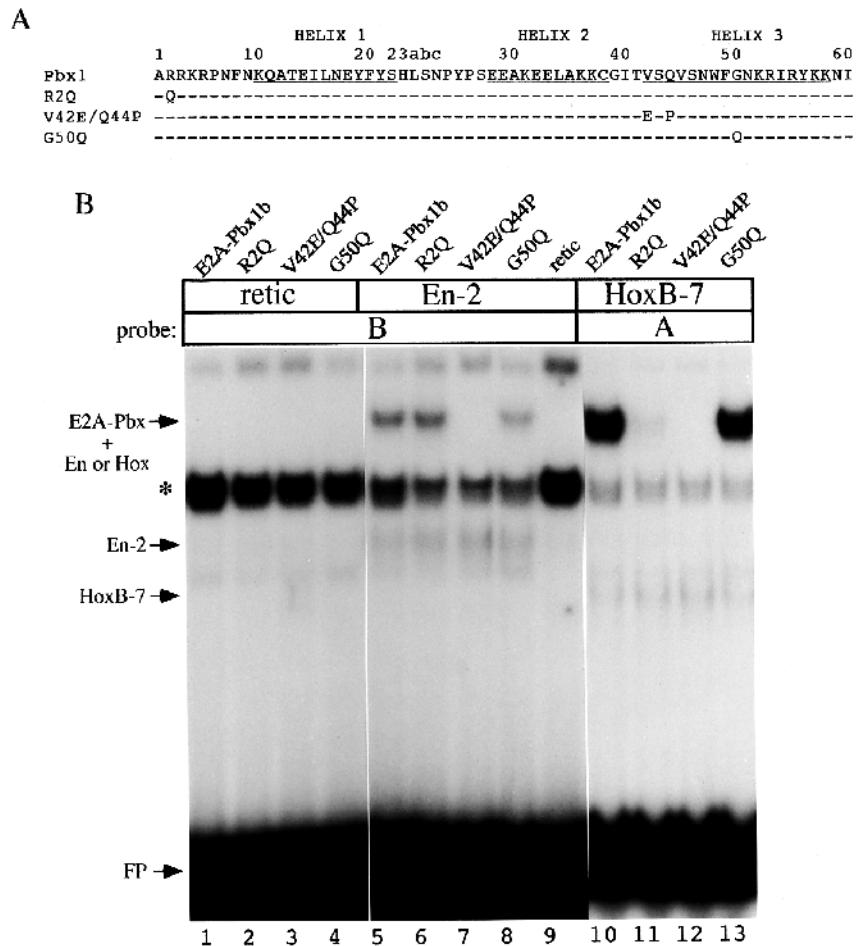
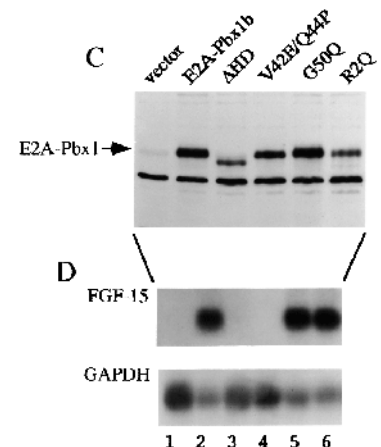


Fig. 2. The E2A-Pbx1 homeodomain is required for induction of FGF-15 expression. (A) Amino acid sequences of wild-type and mutant Pbx1 homeodomains used in this study. (B) EMSAs showing cooperative DNA binding of in vitro translated E2A-Pbx1 proteins with Hox-B7 (lanes 5-9) or En-2 (lanes 10-13). Retic (lanes 1-4, 9) indicates unprogrammed reticulocyte lysate, the asterisk indicates a non-specific complex and FP indicates free probe. (C) Immunoblot showing expression of wild-type and mutant E2A-Pbx1 proteins in 3T3 cells infected with recombinant retroviruses. (D) Northern blot of poly(A)⁺ RNA isolated from NIH3T3 cells expressing E2A-Pbx1 proteins. The same blot was sequentially probed with the FGF-15 cDNA and the GAPDH cDNA as a loading control.



Several of the secreted FGFs are oncogenes capable of transforming 3T3 cells. Therefore, the ability of FGF-15 to transform 3T3 cells was measured by focus formation and soft agar colony formation assays. Neither wild-type FGF-15 nor FGF-15-Flag had any detectable oncogenic activity (data not shown). Thus, the ability of E2A-Pbx1 to transform 3T3 cells cannot be due simply to the induction of FGF-15.

E2A-Pbx1 binds directly to a site in the proximal FGF-15 promoter which is required for E2A-Pbx1-dependent transactivation

To determine whether the *FGF-15* gene was a direct target of E2A-Pbx1, 677 base pairs of genomic DNA upstream of the 5' end of the cDNA were sequenced (Fig. 5A). RNAse protection experiments confirmed that the 5' end of the cDNA lies within a few nucleotides of the transcriptional start site (data not shown). A single consensus TATA box was present 31 bp upstream of the cDNA start site (Fig. 5A). Nucleotides -412 to +22 of the FGF-15 promoter were inserted in front of a luciferase reporter gene. Transfection of this reporter into 3T3 cells in the presence or absence of E2A-Pbx1 revealed that E2A-Pbx1 induced transcription from the promoter approximately 20-fold (Fig. 5B, -412/+22). For comparison, a reporter containing 5 copies of an optimal Pbx:Hox-binding site was induced 30-fold by E2A-Pbx1 (Fig. 5B, 5xPbx) and a reporter containing an SV40 enhancer/promoter was induced only two-fold by E2A-Pbx1 (Fig. 5B, +control). Unlike the optimal Pbx:Hox-binding site, which can be transactivated by E2A-Pbx1 in several different cell types, the FGF-15 promoter was not transactivated by E2A-Pbx1 in the pre-B cell line A20 (Fig. 5B). This suggests that induction of the *FGF-15* gene by E2A-Pbx1 requires a cofactor present in 3T3 cells but not in B cells. To map the minimal region necessary for induction by E2A-Pbx1, a set of reporters containing nested deletions in the FGF-15 promoter was constructed. Deletion from the 5' end to nucleotide -204 had no effect on transactivation by E2A-Pbx1 (Fig. 5B, -204/+22) and further deletion to -148 decreased transactivation only slightly (Fig. 5B, -148/+22). In contrast, deletion to -113 or -105 almost completely eliminated transactivation

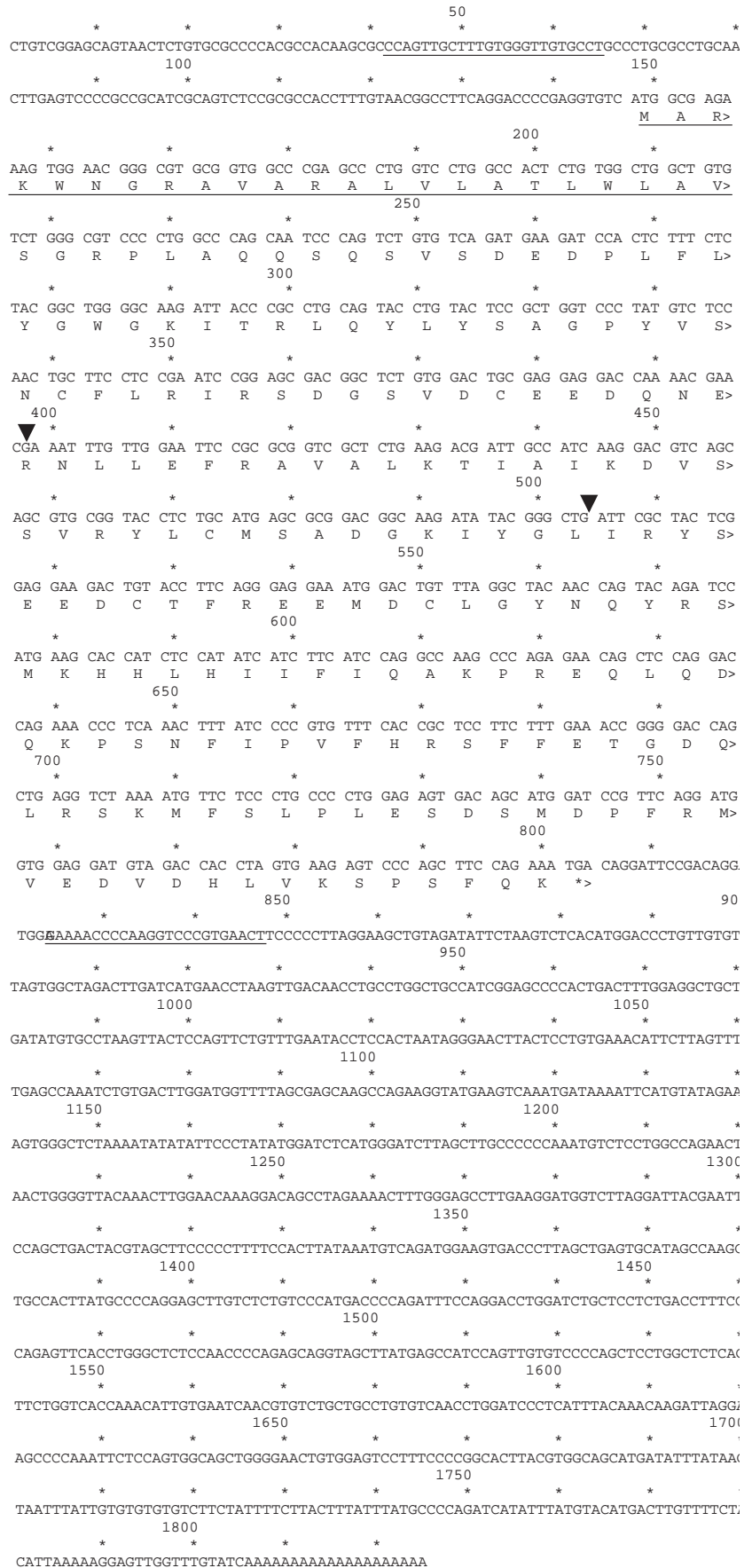


Fig. 3. Nucleotide sequence and conceptual translation of the FGF-15 cDNA. The putative signal peptide and the locations of primers used for RT-PCR cloning are underlined. The locations of exon-intron boundaries are indicated by arrowheads. The GenBank accession number for the FGF-15 cDNA is AF007268.

with the deletion analysis, shows that the TGAT and/or ATTA sites are necessary but not sufficient for transactivation.

EMSA was performed to determine if E2A-Pbx1 could bind directly to synthetic oligonucleotides containing the wild-type or mutant **TGATGCAATTA** sequence (Fig. 5C). When in vitro translated E2A-Pbx1 was added to the wild-type probe, no protein:DNA complexes were detected (Fig. 5C, lane 4). E2A-Pbx1 was also incapable of binding as a heterodimer with in vitro translated HoxB-7 or En-2 (data not shown). However, when 3T3 nuclear extract was added in addition to E2A-Pbx1, a complex was formed which could be supershifted by antibodies to E2A or to Pbx1 (Fig. 5C, lanes 11-16). Formation of the complex was competed by the addition of cold wild-type probe but not by the addition of cold mutant probe (Fig. 5C, lanes 12-13). Thus, E2A-Pbx1 can bind specifically to the wild-type sequence but this binding requires a factor(s) present in 3T3 nuclear extract. The mobility of the complex is consistent with E2A-Pbx1 binding as a heterodimer with an endogenous protein present in 3T3 cells. The same amount of E2A-Pbx1-containing complex was formed when nuclear extract from 3T3 cells expressing E2A-Pbx1 was added to the probe instead of in vitro translated E2A-Pbx1 (Fig. 5C, lanes 17-22). In vitro translated E2A-Pbx1 did not form a complex when nuclear extracts from the pre-B cell lines A20 or 697 were

used (data not shown). This is consistent with our finding that E2A-Pbx1 is incapable of inducing FGF-15 transcription in pre-B cell lines (Fig. 5B; data not shown). Surprisingly, no complex was detected when in vitro translated Pbx1 was added to the probe in the presence of 3T3 nuclear extract (Fig. 5C,

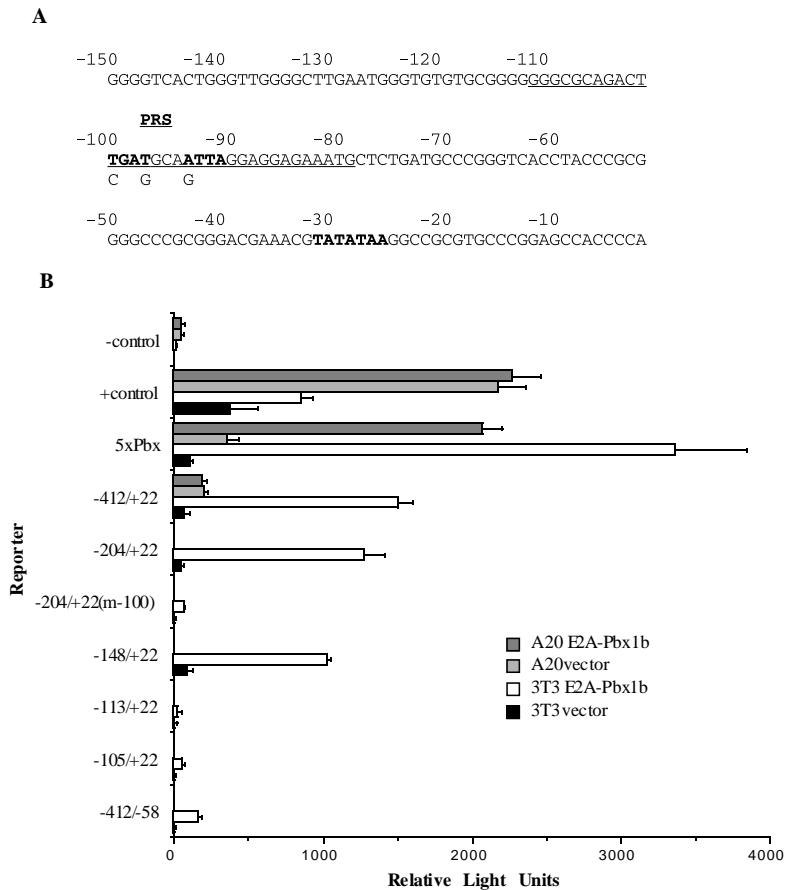
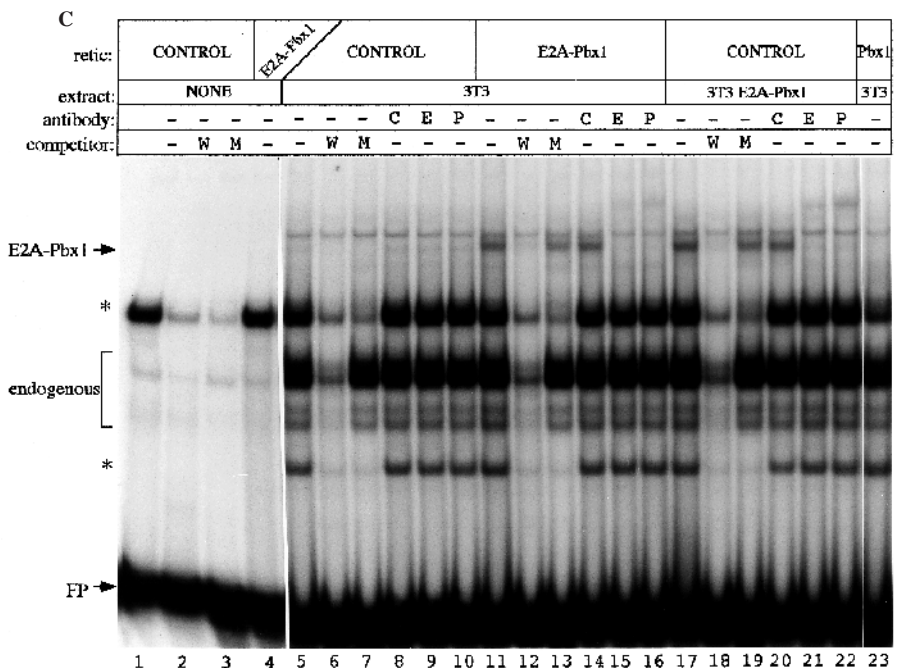


Fig. 5. Transactivation of the FGF-15 promoter by E2A-Pbx1. (A) Sequence of the minimal region of the FGF-15 promoter required for induction by E2A-Pbx1. The TATA box and the E2A-Pbx1 recognition site (PRS) are indicated in bold type. The mutations introduced into the PRS are indicated below the sequence. The 34 base pair region used as a probe for EMSAs is underlined. (B) Relative levels of transcription from luciferase reporter constructs in the presence or absence of E2A-Pbx1b. All determinations are the mean values from three independent transfections and were corrected for differences in transfection efficiency using a CMV-βGal reporter as an internal control. (C) EMSAs showing direct binding of E2A-Pbx1 to the PRS from the FGF-15 promoter. Retic indicates reticulocyte lysate programmed with luciferase (CONTROL), E2A-Pbx1, or Pbx1. Extract indicates nuclear extract (10 μg). Antibodies used were monoclonal anti-KLH (C), anti-E2A (E) or anti-Pbx1 (P). Competitors used were a 200-fold molar excess of wild-type FGF-15 probe (W) or mutant FGF-15 probe (M). Non-specific complexes are indicated by asterisks.



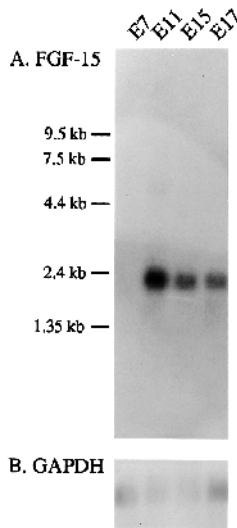


Fig. 6. Northern blot analysis of FGF-15 expression during embryonic development of the mouse.

lane 23). This suggests that the normal DNA-binding activity of Pbx1 has become dysregulated or modified in E2A-Pbx1. Taken together, the data indicate that the *FGF-15* gene is a direct target of E2A-Pbx1.

Pattern of FGF-15 mRNA expression during mouse embryogenesis

To determine if the *FGF-15* gene was expressed during embryonic development, a cDNA probe was hybridized to a northern blot of poly(A)⁺ RNA isolated from mouse embryos at several stages (Fig. 6). A single 2.2 kb transcript was present at embryonic days 11, 15 and 17, and no expression was detectable at embryonic day 7 (Fig. 6).

To obtain insights into the potential roles of FGF-15 during

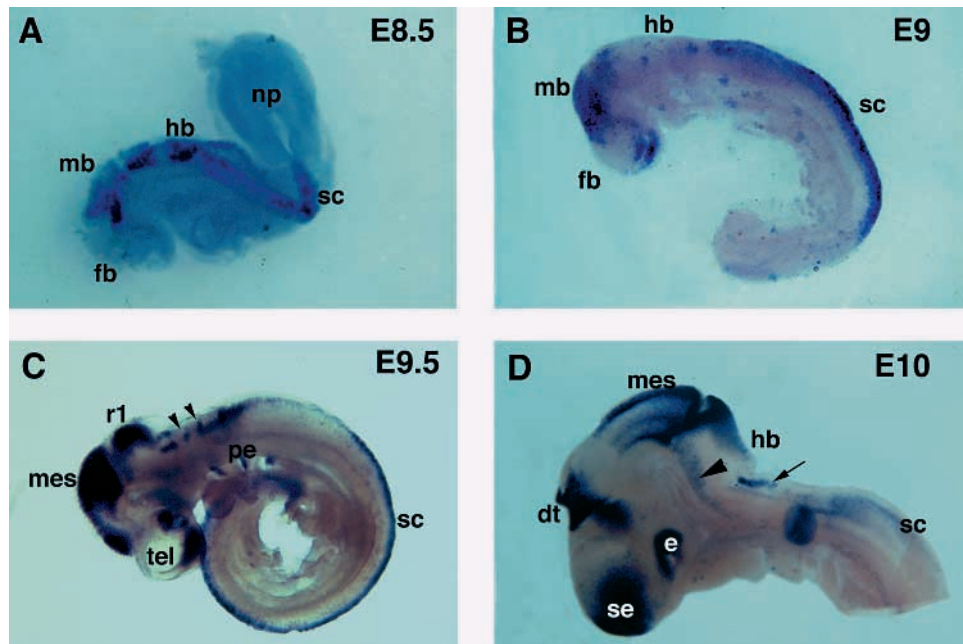
embryogenesis, FGF-15 expression was examined in early and mid-gestation stage mouse embryos by in situ hybridization (Figs 7-9). As expected, FGF-15 transcripts were not detected in E7 embryos. FGF-15 expression first appeared between E7.5 and E8 in the neuroectoderm (data not shown). The predominant site of FGF-15 expression throughout development was the nervous system, with the exception of the pharyngeal pouches where FGF-15 was expressed transiently from E9 to E10 (Fig. 7C,D), and the tail bud where FGF-15 expression first appeared at E9.5 (data not shown). Within the nervous system, the FGF-15 expression pattern was highly dynamic, particularly in the midbrain and hindbrain where the pattern changed substantially between E9 and E14.

From E8.5 to E9, FGF-15 was expressed in a domain that encompasses the presumptive midbrain and diencephalon (Fig. 7A,B). By E9.5, FGF-15 expression in this region had resolved into two domains. In the diencephalon at E9.5 to E10, strong expression was seen in the dorsal thalamus with little or no expression in the ventral thalamus or epithalamus (Figs 7C,D, 8A). Later at E12, expression was seen in both the dorsal and ventral thalamus (Fig. 9B). In the mesencephalon from E9.5 to E12, FGF-15 was expressed in a gradient with high levels caudally, lower levels rostrally, with a sharp boundary of expression at the border between the mesencephalon and diencephalon (Figs 7C,D, 8A). Little or no expression was seen in the isthmus at the caudal end of the midbrain (Figs 7C, 8A). Between E12 and E14, the gradient of FGF-15 expression became reversed, with rostral cells in the superior colliculus now expressing the highest levels of FGF-15 (Fig. 9J).

Beginning at E9, two bilaterally located domains of FGF-15 expression appeared in the telencephalon adjacent to the developing olfactory placodes (Fig. 7B). This region corresponds to the septal region from which the olfactory bulbs will develop and coincides with the first appearance of the olfactory placodes in the mouse. At E9.5, FGF-15 was expressed in both the septal area of the telencephalon and the overlying olfactory placodes, suggesting that FGF-15 may play a role in the early

Fig. 7. FGF-15 expression from E8.5 to E10 of mouse embryogenesis analysed by whole-mount in situ hybridization.

(A) E8.5 embryo showing expression in the midbrain, hindbrain and spinal cord. (B) E9 embryo showing expression in the forebrain and presumptive olfactory placodes (arrow). (C) FGF-15 expression at E9.5. Note expression in the pharyngeal endoderm and at the borders between rhombomeres (arrowheads). (D) FGF-15 expression at E10. Arrowhead indicates expression in the ventral hindbrain and arrow indicates expression in a dorsal stripe of cells in r3. Abbreviations: e, eye; dt, dorsal thalamus; fb, forebrain; hb, hindbrain; mb, midbrain; mes, mesencephalon; np, neural plate; pe, pharyngeal endoderm; r1, rhombomere 1; sc, spinal cord; se, septum; tel, telencephalon.



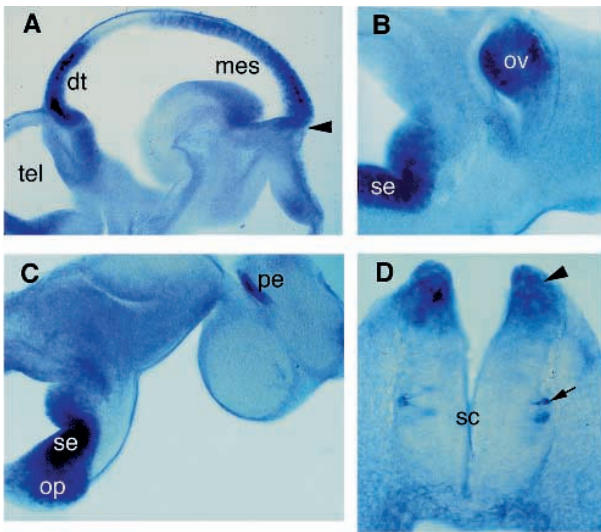


Fig. 8. Vibratome sections of E9.5 embryos after whole-mount in situ hybridization. (A) Midsagittal section showing expression in the dorsal thalamus and mesencephalon. Arrowhead marks the midbrain-hindbrain boundary. (B) Expression in the septum and optic vesicle. (C) Expression in the septum and olfactory placode. (D) Cross section through the spinal cord showing the dorsal domain of expression (arrowhead) and FGF-15-positive cells near the boundary of the alar and basal plates (arrow). Abbreviations: op, olfactory placode; ov, optic vesicle; otherwise as indicated in Fig. 8 legend.

induction of olfactory placode epithelium (Figs 7C, 8C). At E12 and E14, FGF-15 continued to be strongly expressed in the septal region and in differentiating neurons within the olfactory epithelium (Fig. 9B,H).

Beginning at E9.5, expression of FGF-15 was seen in the optic cup where it was restricted to the inner cell layer (Figs 7C,D, 8B). This is the cell layer that gives rise to the neuroretina. FGF-15 continued to be expressed at high levels in the neuroblast layer but not in the ganglion cell layer of the retina until at least E16, suggesting that FGF-15 is expressed in undifferentiated cells of the neuroretina throughout its development (data not shown).

At E8.5, FGF-15 was expressed in two domains in the rostral hindbrain that coincide with rhombomeres 1 and 3, while, in the caudal hindbrain and cervical spinal cord, FGF-15 transcripts were localized within a continuous A-P domain that includes the dorsal neural folds (Fig. 7A). No expression of FGF-15 was seen in the caudal-most regions of the neural plate. Between E8.5 and E9 expression of FGF-15 became downregulated in r3 (Fig. 7B). Then at E9.5 FGF-15 expression reappeared in two lateral stripes of cells in the alar plate of r3, as well as in cells located at the boundaries between rhombomeres (Fig. 7C,D). FGF-15 was strongly expressed in the dorsal region of r1 from E8.5 to E10 (Fig. 7). In the hindbrain at E12 and E14, FGF-15 was expressed in the cerebellum and in dorsally located cells in the fourth ventricle (Fig. 9D,J).

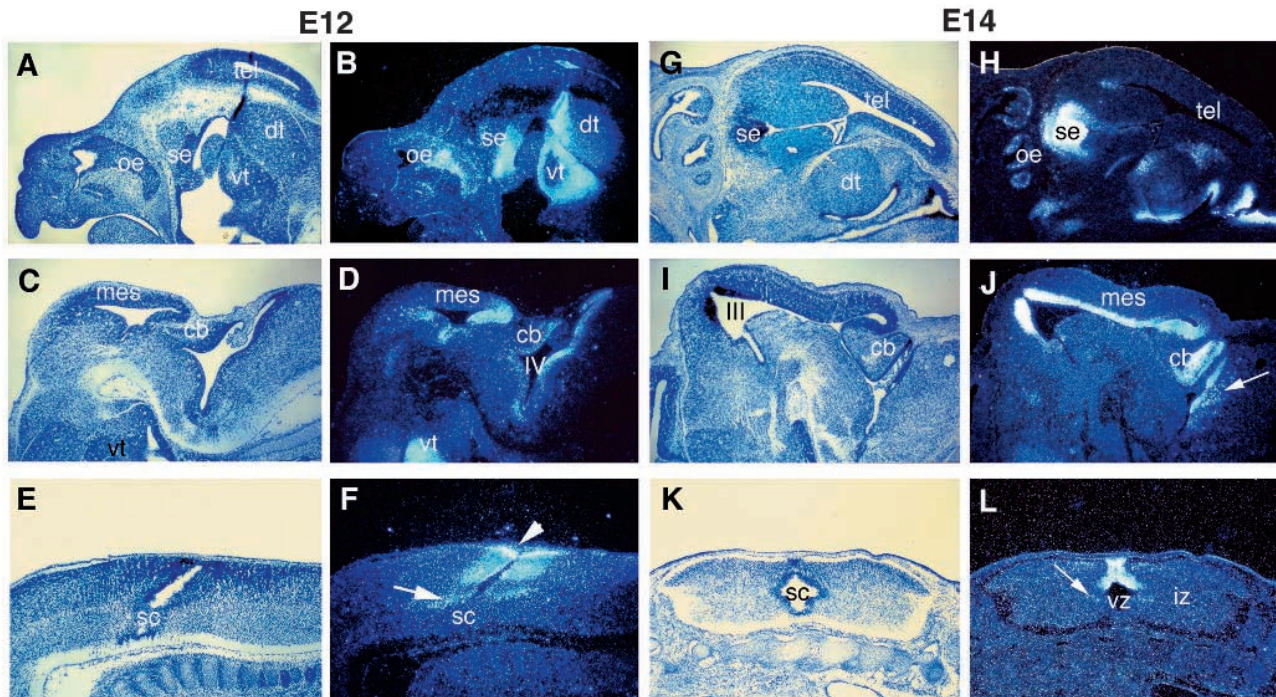


Fig. 9. FGF-15 expression in E12 and E14 embryos analysed by in situ hybridization of frozen sections. Sections were hybridized to an ³⁵S-labelled FGF-15 riboprobe and photographed with bright-field (A,C,E,G,I,K) or dark-field (B,D,F,H,J,L) illumination. (A,B) Parasagittal section showing expression in the E12 forebrain. (C,D) Parasagittal section showing expression in the mesencephalon and hindbrain. (E,F) Transverse section showing expression in the dorsal spinal cord (arrowhead) and in postmitotic cells just ventral to the sulcus limitans (arrow). (G,H) Parasagittal section showing expression in the forebrain at E14. (I,J) Sagittal section showing expression in the mesencephalon and cerebellum at E14. Arrow indicates isolated FGF-15-positive cells in the lateral wall of the fourth ventricle. Note the gradient of expression in the mesencephalon. (K,L) Transverse section showing expression in the spinal cord. The arrow marks postmitotic FGF-15-expressing cells. Abbreviations: cb, cerebellum; iz, intermediate zone; oe, olfactory epithelium; vt, ventral thalamus; vz, ventricular zone; III, third ventricle; IV, fourth ventricle; otherwise as indicated in Figs 8, 9 legends.

From E8.5 to at least E14, FGF-15 was expressed in the dorsal alar plate and roof plate of the spinal cord (Figs 7A-C, 8D, 9F,L). While FGF-15 was expressed in the cervical spinal cord at E9, expression was markedly reduced in this region of the spinal cord by E9.5 and E10 (Fig. 7C,D). In cross sections through the spinal cord at E9.5, two populations of FGF-15 expressing cells were observed: a dorsal population of ventricular cells that includes the roof plate, and 1-2 cells located near the boundary of the alar and basal plate (Fig. 8D). These cells are located close to the pial surface and may represent a population of postmitotic neurons. In E12 embryos, the expression domain in the dorsal spinal cord was expanded compared to E9.5, and transcripts were present throughout the dorsal half of the ventricular zone with a higher level of expression in the roof plate (Fig. 9F). In addition, a population of cells located just ventral to the alar plate-basal plate boundary expressed FGF-15 at E12 and E14 (Fig. 9F,L). From our analysis, it appears that FGF-15 may mark a small population of differentiating interneurons in the spinal cord; however, their identity will require further investigation.

DISCUSSION

Recent studies revealed that the Pbx/Exd proteins can act in concert with other homeodomain proteins to regulate common downstream target genes. Thus far, however, only a small number of potential targets have been identified (Rauskolb and Wieschaus, 1994; Popperl et al., 1995; Peers et al., 1995). As an initial step towards identifying downstream targets of Pbx proteins in mammals, we used the subtractive process of RDA to isolate genes upregulated by the E2A-Pbx1 oncoprotein in NIH3T3 cells. One of these genes encoded a novel FGF, designated FGF-15. We presented evidence for direct activation of FGF-15 transcription by E2A-Pbx1 and described the embryonic expression pattern of the *FGF-15* gene.

Transactivation of the *FGF-15* gene by E2A-Pbx1

Our results indicate that E2A-Pbx1 transactivates the *FGF-15* gene through direct binding of the Pbx1 homeodomain to a site in the promoter in combination with an endogenous cofactor. However, it remains unclear whether this reflects a normal function of Pbx1 or an abnormal function acquired upon fusion of the Pbx1 homeodomain to E2A, since we have been unable to demonstrate binding of normal Pbx1 to the E2A-Pbx1-binding site. One possibility is that DNA binding by Pbx1 is subject to post-translational regulation, whereas E2A-Pbx1 is able to bind DNA constitutively. For example, a recent report showed that Exd can exist in a latent state in the cytoplasm until it becomes activated in response to Wg or Dpp signalling (Mann and Abu-Shaar, 1996). A second possibility is that the E2A portion of E2A-Pbx1 interacts with a factor bound to the FGF-15 promoter and this protein-protein interaction is sufficient to stabilize binding of E2A-Pbx1 to the TGAT half-site in the absence of a Hox partner.

In the E2A-Pbx1-binding site that we have identified, the Pbx half-site is separated from the nearest potential Hox site by a three base pair spacer (Fig. 6A). In the optimal Pbx:Hox sites defined by binding site selection, separation of the two half-sites by even a single base pair has been shown to disrupt binding of Pbx:Hox heterodimers (Chang et al., 1996). In

agreement with this result, we find that E2A-Pbx:Hox and E2A-Pbx:En-2 complexes do not bind to our site. Moreover, induction of FGF-15 transcription by E2A-Pbx1 is not affected by mutations in the Pbx1 homeodomain that disrupt Pbx:Hox or Pbx:En-2 cooperativity in vitro (Fig. 2; data not shown). Therefore, it is likely that the mechanism of E2A-Pbx1 binding to the FGF-15 promoter is quite different from the mechanism previously described for binding of Pbx:Hox complexes. Characterization of this new mechanism will require isolation of the binding partner for E2A-Pbx1 from 3T3 cells.

We have observed that nuclear extracts from t(1;19)-positive pre-B ALL cell lines do not support binding of E2A-Pbx1 to the FGF-15 promoter nor is transcription from the FGF-15 promoter induced by E2A-Pbx1 in pre-B cell lines. Thus, it is likely that the cofactor required for FGF-15 induction is not expressed in B cells. We have also used RDA to identify several genes that are upregulated in t(1;19) pre-B ALL cell lines (J. R. McWhirter, S. Neuteboom, and C. Murre, manuscript in preparation). Like *FGF-15*, several of these genes belong to families known to be involved in intercellular signalling and pattern formation. Together, these results indicate that E2A-Pbx1 interacts with different tissue-specific cofactors in each cell type, thus causing distinct subsets of target genes to become activated.

FGF-15 expression in the developing brain

To date, fourteen genes encoding FGFs and four genes encoding transmembrane FGF receptors with intrinsic tyrosine kinase activity have been reported (Baird, 1994 and references therein; Smallwood et al., 1996; Yamasaki et al., 1996; Coulier et al., 1997). In various assays, FGFs have been shown to function as mitogens, motogens, angiogenic factors, neurotrophic factors, differentiation factors or oncogenes, and to be involved in several developmental processes including limb formation and mesoderm induction (reviewed by Baird, 1994 and Slack, 1994). FGF signalling also plays important roles in the induction and patterning of neural tissues. In *Xenopus*, FGF-2 can transform anterior neural tissue into neural tissue with a posterior identity (Cox and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995) and FGF signalling is necessary for the correct targeting of retinal axons to the optic tectum (McFarlane et al., 1996). In chicken embryos, implantation of an FGF-8-soaked bead into the diencephalon can induce the formation of an ectopic midbrain (Crossley et al., 1996). FGF-2 can be used as a mitogen to isolate and culture neural stem cells from the embryonic CNS, suggesting that FGFs may also control proliferation of these cells in vivo (Johe et al., 1996).

The expression pattern of FGF-15 suggests that it may perform a specialized role in development of the nervous system: it first appears in the neuroectoderm soon after the start of neurulation and its expression is largely confined to the nervous system throughout development. Only two other FGF genes, *FGF-3* and *FGF-8*, have been shown to be expressed during the early stages of neurulation in the mouse (Crossley and Martin, 1995; Wilkinson et al., 1988). Unlike FGF-15, these two FGFs are also expressed at high levels in the primitive streak during gastrulation as well as in various other non-neuronal tissues. Comparison of their expression patterns in the brain at E8.5-9.5 reveals that the three genes are expressed in non-overlapping, reciprocal regions of the neural plate, suggesting that they each may perform distinct functions

during neural development. For example, FGF-3 expression is restricted to rhombomeres 5 and 6 of the hindbrain, whereas FGF-15 is expressed in rhombomeres 1 and 3, and at the borders between rhombomeres (Wilkinson et al., 1988).

In the mesencephalon-metencephalon (mes-met) region, FGF-8 expression is restricted to the isthmus, the organizing center for the mes-met region (Ang, 1996). In contrast, FGF-15 is absent from the isthmus but is expressed throughout the rest of the mes-met region. FGF-15 continues to be expressed in a highly dynamic pattern in the ventricular zone of this region until at least E14. An ectopic midbrain can be induced in the caudal forebrain of chick embryos either by transplantation of isthmus tissue or by implantation of an FGF-8-soaked bead (Crossley et al., 1996). This suggests the possibility that FGF-8 acts as the primary signal for midbrain induction and FGF-15 acts as a secondary signal to direct subsequent outgrowth and patterning of the midbrain. It will be important to determine if FGF-8 induces FGF-15 expression in these ectopic midbrains and if FGF-15 is required downstream of FGF-8 for midbrain induction.

Studies of forebrain organization based on the expression patterns of homeobox genes and other molecular markers have suggested that the forebrain consists of six transverse segments or prosomeres designated P1 through P6, where P1 is the most caudal and P6 is the most rostral segment (Rubenstein et al., 1994). In the E9.5 forebrain, FGF-8 is expressed in the ventral thalamus (P3), the telencephalic commissural plate (P5) and the ventral midline of the hypothalamus (P5) whereas FGF-15 is expressed in the dorsal thalamus (P2) and the septum (P6) (Crossley and Martin, 1995). The juxtaposition of FGF-8 and FGF-15 expression in adjacent prosomeres suggests that the two signaling molecules might act cooperatively during forebrain development to establish segmental boundaries or identities. Interestingly, complementary expression patterns are also observed in the developing eye: FGF-8 is expressed in the optic stalk and optic nerve, whereas FGF-15 is expressed in the inner cell layer of the optic cup which will give rise to the neuroretina. The early onset of FGF-15 expression in the septum and overlying olfactory placodes, which will give rise to the olfactory bulbs and olfactory epithelia, respectively, suggests that FGF-15 may play a role in the early induction and patterning of these structures.

FGF-15 expression in the spinal cord

FGF-15 is the first FGF found to be expressed in the spinal cord during early neural development. Early dorsoventral patterning in the spinal cord is controlled by signalling from ventrally expressed sonic hedgehog (SHH) and dorsally expressed BMPs (Tanabe and Jessell, 1996). At the neural fold stage, SHH signalling causes expression of the homeodomain-containing transcription factors Pax-3, Pax-7, Msx-1, and Msx-2 to become dorsally restricted (Goulding et al., 1993). Differentiation of cell types in the dorsal spinal cord requires both expression of these homeodomain transcription factors and signalling by BMPs expressed in the epidermal ectoderm and roof plate (Liem et al., 1995). Since FGF-15 expression is not detected in the dorsal spinal cord until the stage of neural tube closure, FGF-15 must act at a later stage in spinal cord development than SHH or BMPs. It is likely that FGF-15 expressing cells in the dorsal neural tube include premigratory neural crest precursors. FGF-15 may therefore play a role in the early

specification of these cells. Nevertheless, FGF-15 expression is retained in the dorsal neural tube following neural crest cell emigration, which suggests additional roles in spinal cord development.

Concluding remarks

We have identified a novel member of the FGF family as a downstream target of E2A-Pbx1. These findings, in combination with previous reports, suggest that downstream targets of Exd/Pbx proteins in both *Drosophila* and mammals include genes for intercellular signalling molecules that contribute to the patterning of body structures during development. This suggests that the regulation of secreted signalling molecules such as Wg, Dpp and FGF-15 may be a common feature of morphogenetic regulation by homeodomain proteins. Further experiments will be necessary to determine whether the *FGF-15* gene is a target of the Pbx proteins during embryogenesis and to determine the function of the *FGF-15* gene product during development.

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