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Developmental Biology 269 (2004) 567–579
DEVELOPMENTAL BIOLOGY
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Involvement of *Pax6* and *Otx2* in the forebrain-specific regulation of the vertebrate homeobox gene *ANF/Hesx1*

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Received for publication 29 January 2004, accepted 29 January 2004

Abstract

During early vertebrate development, *ANF* homeobox genes are expressed in the prospective forebrain. Their regulation is essential for correct morphogenesis and function of the prosencephalon. We identified a 1-kb fragment upstream of the chicken *GANF* gene sufficient to drive *lacZ* expression in the endogenous expression domain. Concordant with the high conservation of this sequence in five investigated species, this element is also active in the corresponding expression domain of the zebrafish orthologue. In vivo analysis of two in vitro-identified *Otx2* binding sites in this conserved sequence revealed their necessity for activation of the chicken *ANF* promoter. In addition, we identified a *Pax6*-binding site close to the transcriptional start site that is occupied in vivo by *Pax6* protein. *Pax6* and *GANF* exhibit mutually exclusive expression domains in the anterior embryonic region. Overexpression of *Pax6* in chick embryos inhibited the endogenous *GANF* expression, and in *Pax6*$^{-/-}$ mice the expression domain of the murine *ANF* orthologue *Hesx1* was expanded and sustained, indicating inhibitory effects of *Pax6* on *GANF*. However, a mutation of the *Pax6* site did not abolish reporter activity from an electroporated vector. We conclude that *Otx2* and *Pax6* are key molecules involved in conserved mechanisms of *ANF* gene regulation.

Keywords: Promoter; Transcription; Prosencephalon; DNA binding; Chick; Zebrafish; Mouse

Introduction

The subdivision of the vertebrate prosencephalon into telencephalic, pituitary, optic, and diencephalic territories is reflected by restricted expression patterns of several transcription factors in the developing forebrain (Kobayashi et al., 2002). Among those are the proteins encoded by a subfraction of the paired-like homeobox genes, the anterior neural fold (*ANF*) genes (Hermesz et al., 1996; Kazanskaya et al., 1997). Their common feature is an expression in the rostral region of the embryo during gastrulation and neurulation. The chicken *GANF* gene is initially transcribed in the anterior neural plate of the definitive streak stage embryo (HH4), and then becomes restricted to the anterior neural folds and a small anterior medial region connecting the two folds in a horseshoe-like domain. From the 10-somite stage onwards, expression is only observed in cells of the oral ectoderm and ends with the invagination and differentiation into specialized cells of the pituitary gland (Knoetgen et al., 1999a,b). The murine *ANF* gene, *Hesx1* (*Rpx*), follows the same pattern at gastrula or neurula stages as in the chicken, but exhibits an additional, earlier domain in the anterior visceral endoderm (AVE) of the pregastrula embryo (Dattani et al., 1998; Hermesz et al., 1996; Martinez-Barbera et al., 2000; Thomas and Beddington, 1996). *Hesx1* knockout mice have several defects of midline structures such as optic nerves and the pituitary gland, resembling the human *HESX1* mutant syndrome septo-optic dysplasia (Dattani et al., 1998, Thomas et al., 2001).

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doi:10.1016/j.ydbio.2004.01.044

In this study, we describe the involvement of the homeodomain containing transcription factors *Otx2* and *Pax6* in *ANF* gene regulation. *Otx2* is known for its specific function as a transcriptional regulator during forebrain and midbrain development in vertebrates (Boncinelli and Morgan, 2001). In all examined vertebrate species, it is expressed very early in the endo- and ectoderm, and then becomes restricted to the fore- and midbrain territory. The targeted disruption of the murine *Otx2* gene leads to the loss of anterior neural tissues, emphasizing the central role for *Otx2* during forebrain development (Acampora et al., 1995). *Pax6*, a paired-domain homeobox transcription factor, has important functions during forebrain and eye development (Marquardt et al., 2001; Walther and Gruss, 1991). In the chick embryo, *Pax6* transcripts become first detectable at the early head-fold stage (HH6) in a crescent-shaped expression domain adjacent to the anterior neural plate. With ongoing neurulation, *Pax6* becomes strongly expressed in the forebrain, leaving out the anteriormost part of the telencephalon, the anterior dorsal neural folds, and the ventral midline (Li et al., 1994).

We examined the avian *GANF* promoter using β-galactosidase reporter gene assays. A highly conserved sequence of less than 1 kb was found to be sufficient to drive correct expression in both chick and zebrafish embryos. We identified three *Otx2* and one *Pax6* binding site within this sequence by electrophoretic mobility shift assays (EMSA). The two distal *Otx2*-binding sites proved to be functionally important; however, overexpression of *Otx2* was not enough to activate the *GANF* promoter. Ectopic overexpression of *Pax6* in the dorsal anterior neural folds by electroporation of chicken embryos led to an inhibition of the endogenous *GANF* expression. Conversely, the expression domain of the murine *GANF* orthologue *Hesx1* was expanded in murine *Pax6*$^{-/-}$ embryos. Taken together, these findings indicate key roles for *Otx2* and *Pax6* in the regulation of the *ANF* gene promoter.

Materials and methods

Sequence alignment and analysis

Sequences used for alignment were identified by BLAST searches of DDBJ/EMBL/GenBank (frog) and the Celera database (mouse, rat, and human). Similarities were aligned with the CLUSTAL_W program (Thompson et al., 1994). Searches for potential transcription factor binding sites were performed using the TRANSFAC program (Wingender et al., 2000).

Molecular techniques

GANF clones were isolated from a chicken EMBL SP6/T7 genomic library using *GANF* cDNA (800 bp) as a probe. One clone contained approximately 14 kb of upstream flanking region. Sequence data were submitted to the DDBJ/EMBL/Gene Bank databases under accession number AJ566117. The transcriptional start site was determined by 5′ RACE-PCR (Invitrogen). The translational start codon of the *GANF* gene is part of a *Nco*I restriction site. To clone the flanking region directly into the ATG of the *lac*Z reporter pβgal-BASIC (Clontech), a partial digest of the genomic clone was performed. Secondly, the *lac*Z reporter pβgal-BASIC was modified by PCR to create a *Nco*I site around the ATG with the following primers (pβgal3-1, 5′ -CCACATACAGGCCG-TAGCGGT-3′ and pβgal5-1, 5′ -CCATGGCGTTTACTTT-GACCA-3′ ; AdvantageT cDNA Polymerase Mix, Clontech).

In vitro translated mouse *Pax6* (Kawakami et al., 1997) and chicken *Otx2* proteins were used to perform EMSAs (Bäumer et al., 2003), applying oligonucleotides as described in Briata et al. (1999) (OTS) and Bäumer et al. (2003) (RE2). For control incubations, anti-*Pax6* polyclonal antibody (BABCO), anti-*Pax2* polyclonal antibody (BABCO), and anti-*Otx2* polyclonal antibody were used (Baas et al., 2000). Site-directed mutagenesis was performed with the QuickChange kit (Stratagene) using the oligonucleotides indicated in Figs. 4 and 5.

Chromatin immunoprecipitation (ChIP) assay

Embryonic tissue extracts were prepared from the head or trunk region, respectively, of stage HH10 embryos (28 embryos each, compare Fig. 5). They were collected separately on ice, sonificated (BRANSON SONIFIER, cell disruptor B15: output-control 3, duty-cycle 30, five times 10 pulses with a 1-min break in between each cycle), treated with trypsin for 3 min (37°C), washed two times with ice-cold PBS (2000 rpm, 2 min), and eventually cross-linked with 1% formaldehyde for 10 min (37°C). Chromatin extraction and immunoprecipitation was performed using a ChIP assay kit according to the manufacture's protocol (Upstate Biotechnology). Polyclonal *Pax6* antibodies (BABCO) were used for immunoprecipitation (3 μg antibody per immunoprecipitation). After the reversion of the crosslinks, DNA was recovered with a PCR purification kit (QIAGEN) in 30-μl water, of which 1 μl was used for the PCR reaction. The PCR protocol was as follows: 5 min 94°C (denaturation step), 30 cycles of 1 min: 94°C, 1 min: 60°C, 1 min: 72°C; 7 min 72°C (final extension). For the amplification of the *Pax6* binding site containing promoter fragment (P), the following primer pair was used: 5′ -GGAAATTTCACATACGG-TATCCTTC-3′ and 5′ -GGTGACACTAGGGGGGCCAC-CAA-3′ . For amplification of an upstream region (E) as a negative control (around 5 Kb upstream of *GANF*'s start codon), we used the primer pair: 5′ -CTGTGCTGCAGTCTCCAGG-3′ and 5′ -CAAAAGCCCTTCAGAGTAGTTTGC-3′ (Fig. 5).

Vertebrate embryos

Chick embryos (White Leghorn, obtained from Lohmann Tierzucht, Cuxhaven) were cultured on agarose (Chapman et al., 2001) and electroporated using a procedure modified after Endo et al. (2002). The parameters were 5 μg/μl of reporter construct together with 5 μg/μl of GFP, containing 0.025% Fast Green and 5 μg/μl of GFP alone (overexpression experiment), containing 0.025% Fast Green, three rectangular pulses with 7 V, 25-ms duration within 200 ms (Electro Square Porator ECM 830, Btx Inc.). Besides the reporter constructs described in this article, CMV-GFP and CMV-*Pax6* (Bernier et al., 2001) were used for electroporation. Electroporated embryos were incubated for approximately 4 h at 38°C and stained with X-Gal as described in Bäumer et al. (2002). Embryos were staged according to Hamburger and Hamilton (1951).

For microinjection into one- or two-cell zebrafish embryos, reporter constructs were digested with SalI and Asp718I, vector sequences were removed by electrophoresis, the DNA was purified by phenol–chloroform extraction, and diluted to 10 nM in 10 mM HEPES buffer (pH 7.6). Embryos were staged according to Kimmel et al. (1995).

Mouse embryos were derived from intercrosses of *Pax6*$^{+/-}$ heterozygous males and females on a C57BL/6 background (St-Onge et al., 1997). The day of vaginal plug was considered as day 0.5 postconception (pc). Genotyping was performed as previously described by Bäumer et al. (2002).

Whole mount in situ hybridization (WMISH) and immunohistochemistry

WMISH of zebrafish embryos was performed as described by Köprunner et al. (2001). WMISH of chicken embryos was performed as described by Knoetgen et al. (1999a,b), except that the transcription reaction was done using a PCR-amplified template to avoid false-positive staining in electroporated embryos (Reverse primer, 5′ -AACAGCTATGACCATG-3′ , M13–20 Primer (universal), 5′ -GTAAAACGACGGCCAGT-3′). Combined WMISH and whole mount immunohistochemistry was performed according to Streit and Stern (2001) using an anti-*Pax6* polyclonal antibody (BABCO) or an anti-GFP polyclonal antibody (BD Biosciences). The following probes were used for WMISH: *Krox20* (Oxtoby and Jowett, 1993) for zebrafishes, *GANF* (Knoetgen et al., 1999a,b), *Otx2* (Bally-Cuif et al., 1995), and *Pax6* (Li et al., 1994) for chicken, and *Hesx1* (Thomas et al., 1995) for mouse embryos. For paraffin sections (8 μm), stained embryos were dehydrated and embedded in Paraplast Plus (Sherwood medicals). Double WMISH was performed using simultaneously fluorescein- and digoxigenin-labeled RNA probes that were detected consecutively by the alkaline phosphatase substrates Vector red (Vector Laboratories) and NBT-BCIP (Roche). Because Vector red staining is lost during paraffin histology, double-stained embryos were embedded in a gelatin albumen mixture and cut into sections (30 μm) with a Vibratome (Pelco 101).

Results

The upstream regulatory sequences of ANF homeobox genes are highly conserved among different vertebrates

We screened a genomic chicken library with a *GANF* cDNA and isolated one clone that included an approximately 14 kb upstream region and the first two exons of *GANF*. The transcription start of the *GANF* gene was determined 79 bp upstream of the ATG using RACE-PCR. This indicates a very short 5′ UTR and corresponds with the short mRNA length determined for *ANF* homeobox genes by Northern blotting (around 1000 bp; Samakhvalov et al., 1993). A putative TATA box is at positions −37 to −30 (Fig. 1). A comparison of the chicken sequence with the sequences of frog, mouse, rat, and man revealed a highly conserved sequence of approximately 650 bp directly upstream of the transcriptional start (Figs. 1 and 2A). The overall similarities of this region are chicken–frog (87%), chicken–rat (82%), chicken–mouse (84%), chicken–human (80%), and human–mouse (88%).

A 1-kb regulatory element of the GANF upstream region is sufficient to drive reporter gene expression in the forebrain region in chicken and zebrafish

To identify the regulatory elements of the *GANF* gene, we used different reporter constructs that were electroporated into cultivated chicken embryos of definitive streak stage or early headfold stage (HH4–6). Together with a CMV-GFP control vector, each construct was injected between the vitelline membrane and the ectoderm, electroporated and incubated for approximately 6 h after application of the current. The embryos were analyzed at the four to seven somite stage (HH8–HH9). In this set-up, electroporation of the CMV-GFP control vector resulted in a broad, mosaic expression field of embryonic, extraembryonic, neural, and nonneural territories, covering half of a chick embryo at stage HH9 (Figs. 2C′ and D′). Three different constructs of the 5′ genomic sequence of the *GANF* gene (9 kb *lac*Z, 1 kb *lac*Z, and 0.53 kb *lac*Z, Fig. 2A) fused to the β-galactosidase reporter gene were electroporated. The 9 kb ($n = 24$) as well as the 1 kb upstream sequence ($n = 12$) was sufficient to drive high β-galactosidase expression specifically in the prospective forebrain region (Figs. 2C and D). The reporter gene expression was restricted to the endogenous *GANF* expression domain in the neural ectoderm (Figs. 2B, C, C′ , D, and D′). In contrast, electroporation of the 1 kb *lac*Z vector into the posterior part of the embryo, which normally does not express *GANF*, did not lead to any *lac*Z activity ($n = 12$, data not shown). Controls with co-

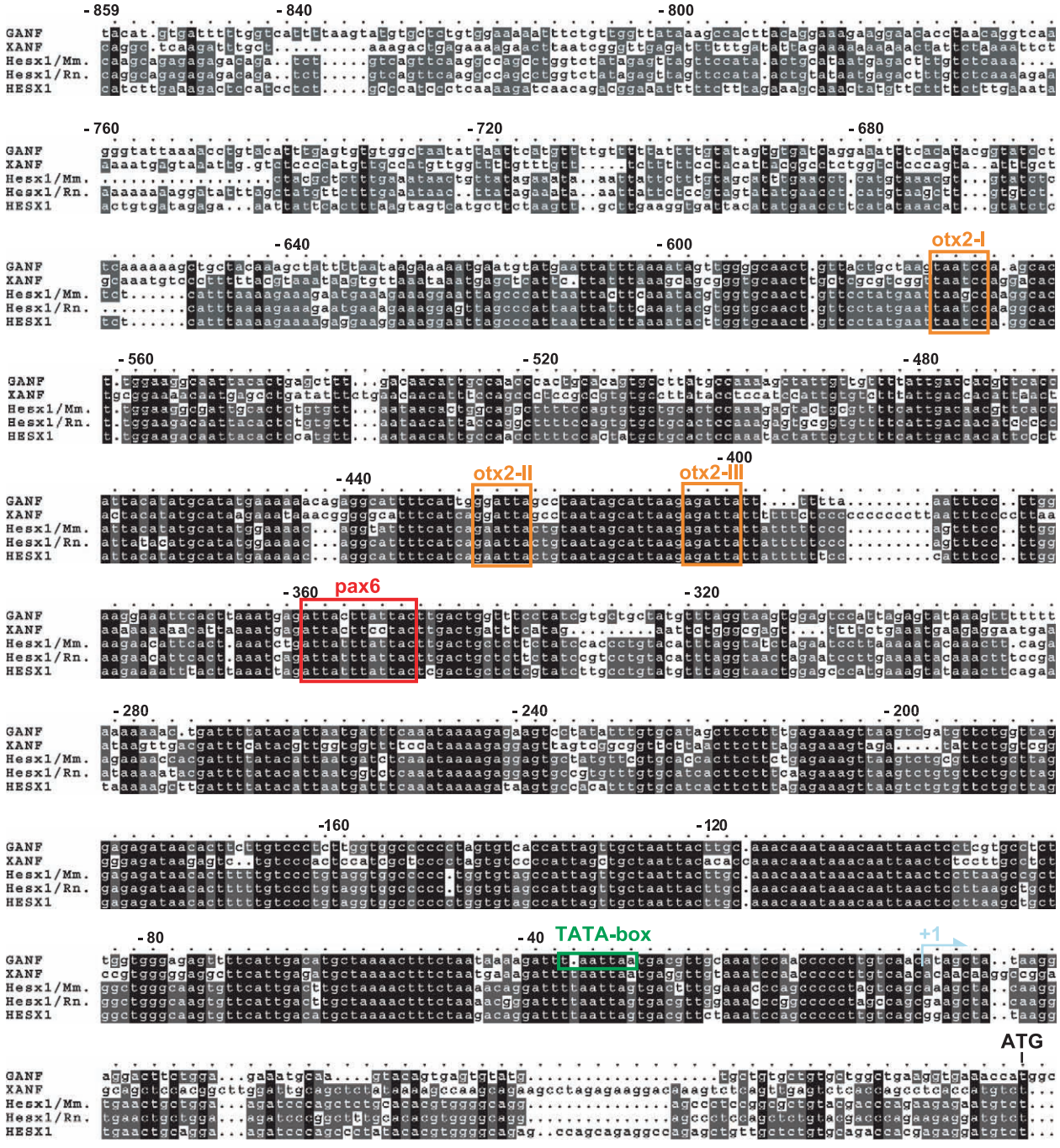

Fig. 1. Conserved cis-regulatory sequence of *ANF/Hesx1* in chicken (*GANF*), frog (*XANF*), mice (*Hesx1/Mm.*), rat (*Hesx1/Rn.*), and human (*HESX1*). Identical bases in all species are underlined in black, identical bases in three or four species are marked with grey. The numbering follows the base pairs of the chicken sequence, counting negatively from the mapped transcription start of *GANF*. The core binding motives of the investigated *Otx2* and *Pax6* binding sites, the transcription start of the *GANF* gene, and the putative TATA box are indicated.

electroporated CMV-GFP proved successful transfections, with a broad, mosaic expression field in the posterior part of the embryo.

Injection of the 9 kb *lacZ* constructs into zebrafish embryos resulted in a restricted forebrain expression domain (n = 96, Fig. 2F) corresponding to the expression domain of the zebrafish *ANF* orthologue and reminiscent of the pattern seen in electroporated chicken embryos (Fig. 2E). In all embryos into which the 0.53 kb *lacZ* reporter construct was injected, expression was found in the anterior part, but the number of positive cells appeared to be decreased compared to embryos injected with the 1 kb *lacZ* construct (Fig. 2G; n = 16). Conversely, a deletion construct lacking the region directly upstream of

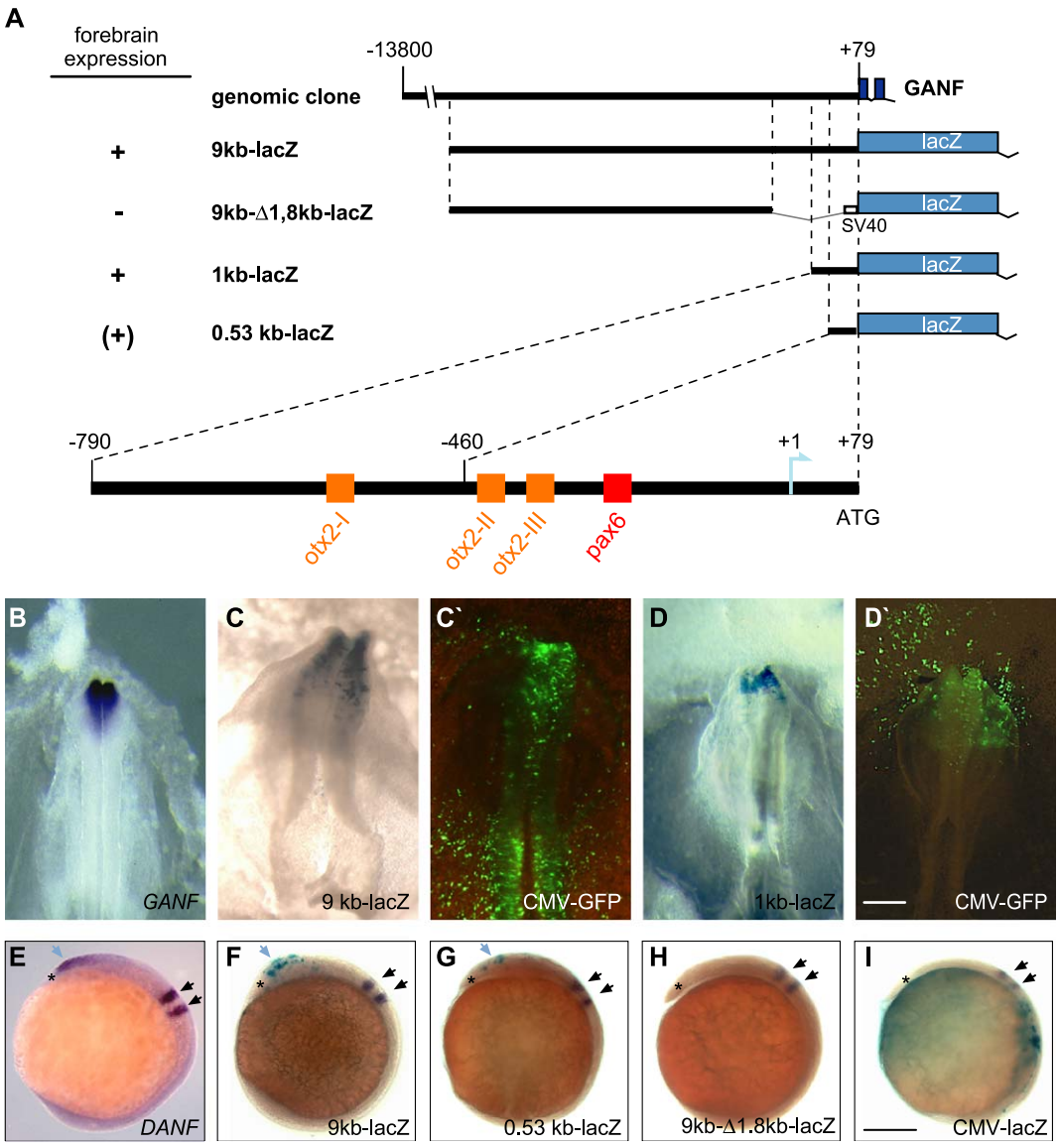

Fig. 2. Reporter constructs in chicken and in zebrafish embryos show the necessity and the sufficiency of the 1000-bp fragment directly upstream of the *GANF* start codon for expression in the endogenous domain. (A) Overview of the cloned reporter constructs. SV40, minimal basal promoter of the SV40 virus, *lacZ*, β-galactosidase. (B–D) The 9 kb *lacZ* and the 1 kb *lacZ* reporter construct in chicken embryos. (B) WMISH for *GANF* in a chicken embryo of the seven somite stage (HH9) shows expression in the anterior neural folds. (C) *lacZ* staining of an electroporated embryo with the 9 kb *lacZ* construct resembles the staining of the endogenous *GANF* expression domain shown in B. (C′) Co-electroporated CMV-GFP exhibits ubiquitous expression in the same embryo compared to the restricted *lacZ* staining in C. (D) *lacZ* staining of an electroporated embryo with the 1 kb *lacZ* construct resembles staining of the endogenous *GANF* expression domain shown in B. (D′) Co-electroporated CMV-GFP exhibits ubiquitous expression in the same embryo compared to the restricted *lacZ* staining in D. The scale bar in D′ corresponds to 500 μm. (E–H) Analysis of chicken reporter constructs in zebrafish embryos. (E) WMISH on a wild-type embryo with *DANF* and *Krox20* to show the endogenous *DANF* expression. (F) Embryo injected with the 9 kb *lacZ* reporter construct exhibits X-gal staining in the prospective forebrain covering the endogenous *DANF* expression. (G) Embryo injected with a construct missing the conserved region does not show *lacZ* staining. (H) Embryo injected with the 0.53 kb *lacZ* reporter construct, where the −700 to −460 bp of the conserved region are depleted and the otx2-I binding site is missing, shows reduced anterior X-gal staining. (I) Injection of CMV-*lacZ* exhibits overall expression in the embryo proper and in the yolk membrane. All shown zebrafish embryos were whole mount in situ hybridized with *Krox20* (two black arrows) after the X-gal staining, indicating rhombomeres 3 and 5 of the hindbrain. Note the so-called polster as the anteriormost structure (*). All embryos are approximately at the tailbud stage (12 hpf). The scale bar in I corresponds to 250 μm.

the *GANF* ATG did not drive β-galactosidase expression (Fig. 2H; $n = 14$). Control injection of CMV *lacZ* resulted in a ubiquitous β-galactosidase expression pattern (Fig. 2I; $n = 28$).

In conclusion, we found that a 1-kb element upstream of the chicken *GANF* gene is sufficient to drive forebrain-specific reporter gene expression in chick and zebrafish embryos.

The activation of the GANF promoter is dependent on Otx2 binding sequences

In chick embryos, *Otx2* transcripts are detectable already before the formation of the primitive streak and are prominent in the anterior neural plate of the definitive streak stage (Fig. 3A). Only at the late streak or early head process stage (HH4+/HH5) *GANF* starts to be expressed within the much wider *Otx2* domain (Fig. 3B). This pattern remains so with ongoing development up to the 10-somite stage (Figs. 3B and C). Within the 650-bp-long conserved sequence of the *GANF* promoter, we identified three *Otx2* binding sites (Figs. 1 and 4A; "otx2-I", "otx2-II", and "otx2-III"). Bandshift assays revealed the specific binding of these sequences by chicken *Otx2* (Fig. 4A; lanes 4, 9, and 14). The specificity of the binding was controlled by incubation with anti-*Otx2* antibody, which impaired the protein–DNA complex formation (Fig. 4A; lanes 5, 10, and 15). We did not observe a supershift presumably due to blocking of the DNA binding motive of *Otx2* by the polyclonal antibody. The complex formation was not impaired by the anti-*Pax2* antibody (Fig. 4A; lanes 6, 11, and 16). Binding of *Otx2* was completely inhibited by mutating the otx2-II oligonucleotide and severely reduced by mutating the otx2-I and otx2-III oligonucleotides (Fig. 4A; lanes 8, 13, and 18).

To test the importance of the *Otx2* binding sites in vivo, we mutated the otx2-I and otx2-II bindings sites within the context of the 1-kb *GANF* promoter construct (Fig. 4B; see Materials and methods). These mutations abolished completely (n = 7/10) or reduced severely β-galactosidase expression (n = 3/10; Figs. 4D and E; 1 kb Δotx2 *lac*Z), while the electroporation efficiencies were at a similar levels (Figs. 4C–E).

Effects of ectopic *Otx2* expression were analyzed after the electroporation of an *Otx2* expression vector by a CMV promoter (CMV-*Otx2*). We neither observe a change of

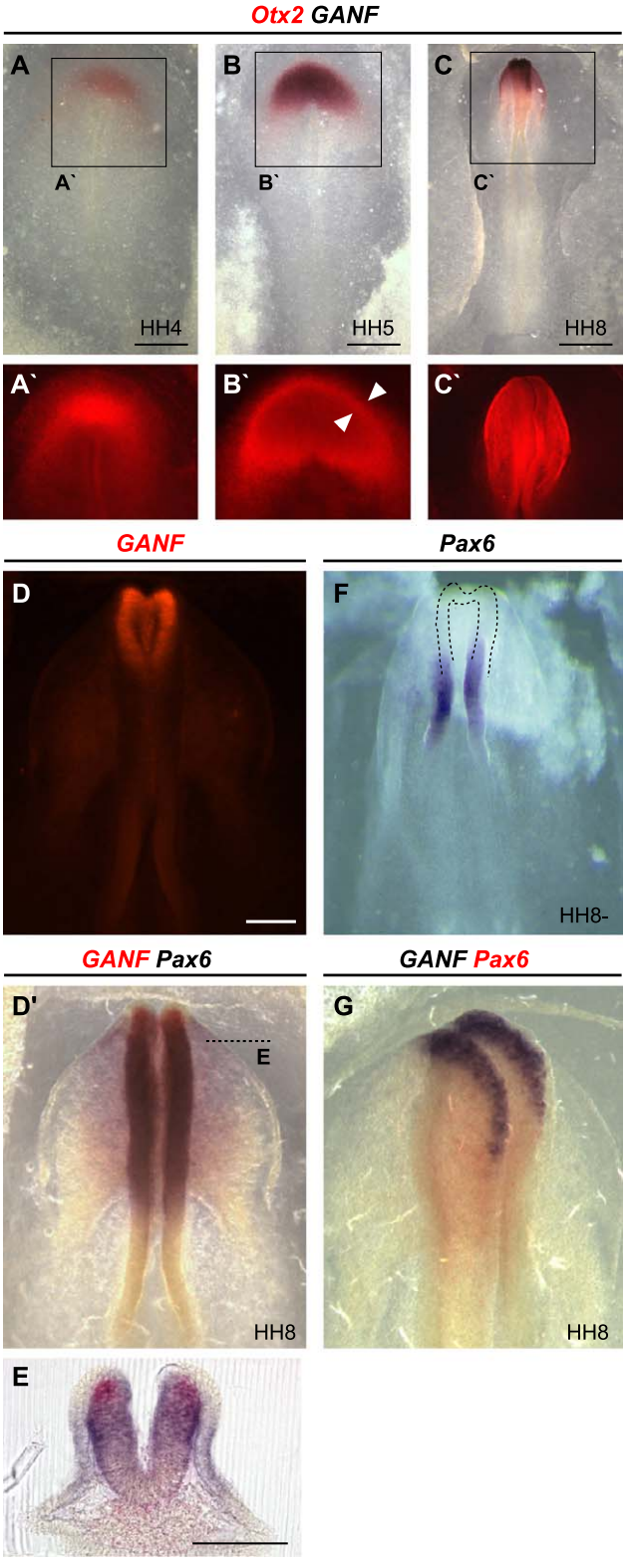

Fig. 3. Relationship of the expression domains of *Otx2* and *Pax6* with *GANF*, respectively. (A–C) Double whole mount in situ hybridization (DWMISH) for *Otx2* and *GANF*. (A and A′) DWMISH of a chicken embryo at the definitive streak stage (HH4). The *Otx2* expression (red) marks the area of the anterior neural plate, no *GANF* expression can be detected. (B and B′) DWMISH of a chicken embryo at the early head process stage (HH5). *GANF* expression (blue) can be detected for the first time within the *Otx2* domain. Note the red fluorescence surrounding the *GANF* domain in B′ labeled with two white arrowheads. (C and C′) DWMISH of a chicken embryo at the four somite stage (HH8). *GANF* (blue) and *Otx2* (red) are co-expressed in the anterior neural folds. The scale bars in A and B correspond to 270 μm, the scale bar in C to 500 μm. (D–G) WMISH and DWMISH of *Pax6* and *GANF* at the four somite stage (HH8). (D, D′, and E) *Pax6* transcripts are stained blue and *GANF* transcripts are detected by red staining. *Pax6* and *GANF* expression domains are adjacent, but exclude each other. *GANF* transcripts are found in the dorsal neural folds of the telencephalon as seen in the cross-section (E) and by fluorescence microscopy (D). (F) WMISH with a *Pax6* probe on a three somite stage embryo (HH8–). *Pax6* transcripts are detectable in the posterior neural folds of the head region. The anterior portion of the headfolds, where at this stage *GANF* is being expressed (compare to D′ and Fig. 2B), remains free of *Pax6*. (G) DWMISH of a chicken embryo at the four somite stage (HH8) with *Pax6* (red) and *GANF* (blue) probes. This lateral view highlights the *GANF* expression in the dorsal neural folds. The scale bar in D corresponds to 230 μm, the scale bar in E represents 70 μm.

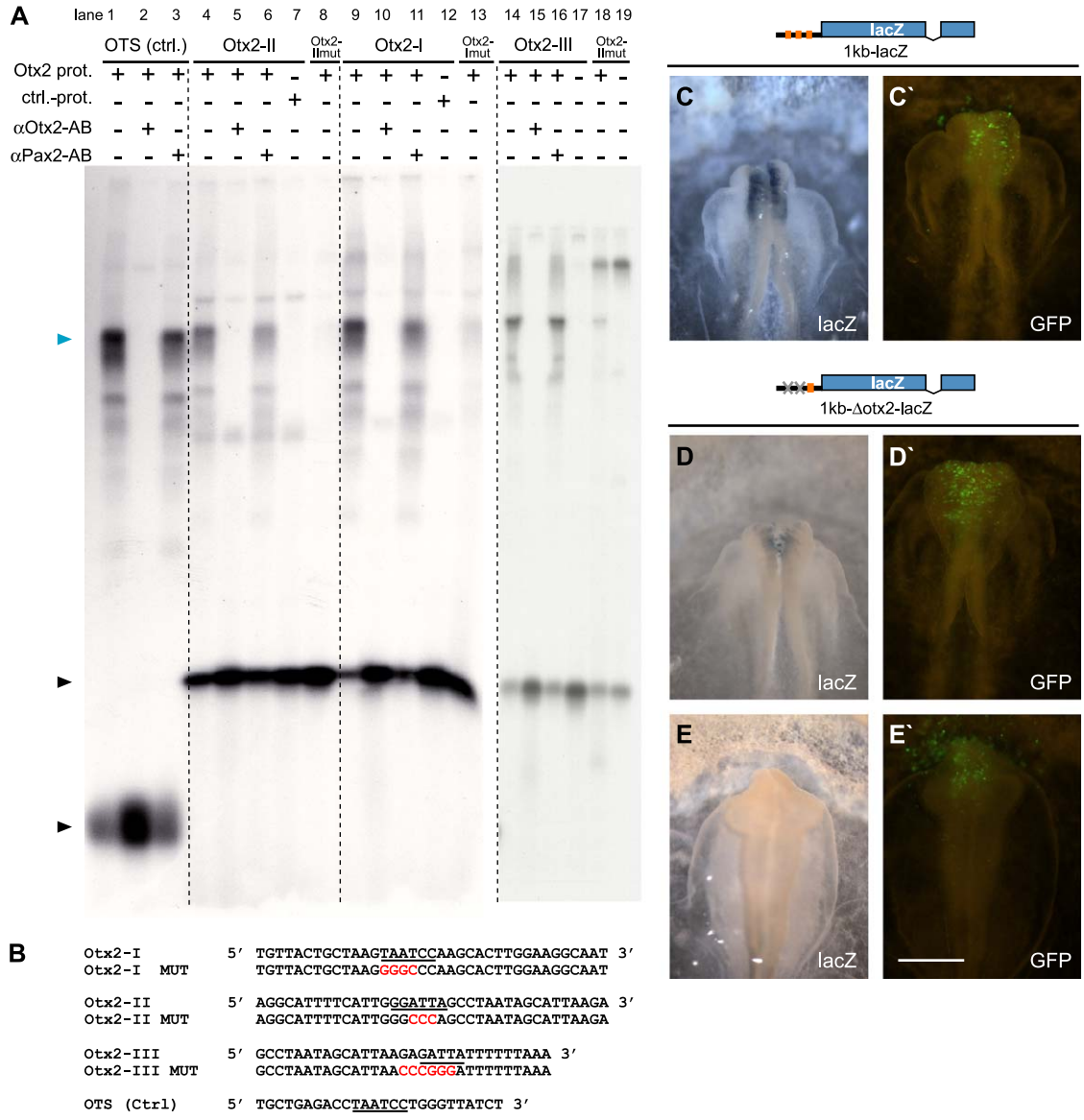

Fig. 4. *Otx2* interacts with three cis-regulatory elements on the *GANF* promoter. (A) Bandshift assay of the three proposed binding sites of *Otx2* in the *GANF* promoter. The positive control nucleotide OTS has previously been shown to bind *Otx2* (Briata et al., 1999). Blue arrowheads exhibit the complex of protein and oligonucleotides; black arrows mark the free oligonucleotides. The difference between the OTS and the other nucleotides is due to different lengths of the oligonucleotides. Clear binding is shown for the proposed binding sites, otx2-I, otx2-II, and otx2-III. The binding is always extinguished by the anti-*Otx2*-antibody, but not by an anti-*Pax2*-antibody. The mutated oligonucleotides show in the otx2-II case a complete and in otx2-I and otx2-III a significantly reduced binding of the protein. (B) Sequence comparison of the nucleotides representing the original, the mutated, and control sequences. (C–E′) Electroporation of chicken embryos with the 1 kb *lac*Z reporter construct (C and C′) and the same construct in which two binding sites are mutated (D–E′). Compared to the *lac*Z staining of the 1 kb *lac*Z, the 1 kb Δotx2 *lac*Z construct shows either a significant reduction of *lac*Z-positive cells or even no *lac*Z expression at all. (C–E) Co-electroporation of CMV-GFP shows comparable ubiquitous transfection (C′–E′). The scale bar in E′ corresponds to 1.3 mm.

intensity nor an enlargement of the endogenous *GANF* expression domain. Co-electroporation of CMV-*Otx2* together with the reporter construct showed no ectopic expression of *lac*Z (data not shown).

In summary, we conclude that *Otx2* can bind specifically at three sites within the *GANF* promoter. Mutation of the two distal sites is sufficient to impair the activity of the *GANF* promoter, and the presence of just the proximal site is not enough for *GANF* transcription.

Pax6 is involved in the regulation of the GANF promoter

The expression domains of *GANF* and *Pax6* are mutually exclusive in the forebrain region of 4–10 somite embryos (HH8–10); Figs. 3D–G). This led us to investigate the effect of ectopically expressed *Pax6* on the endogenous *GANF* expression. We electroporated a CMV-*Pax6* cDNA expression vector into the anterior region of gastrula or early neurula stage chicken embryos (HH4–HH6). WMISH

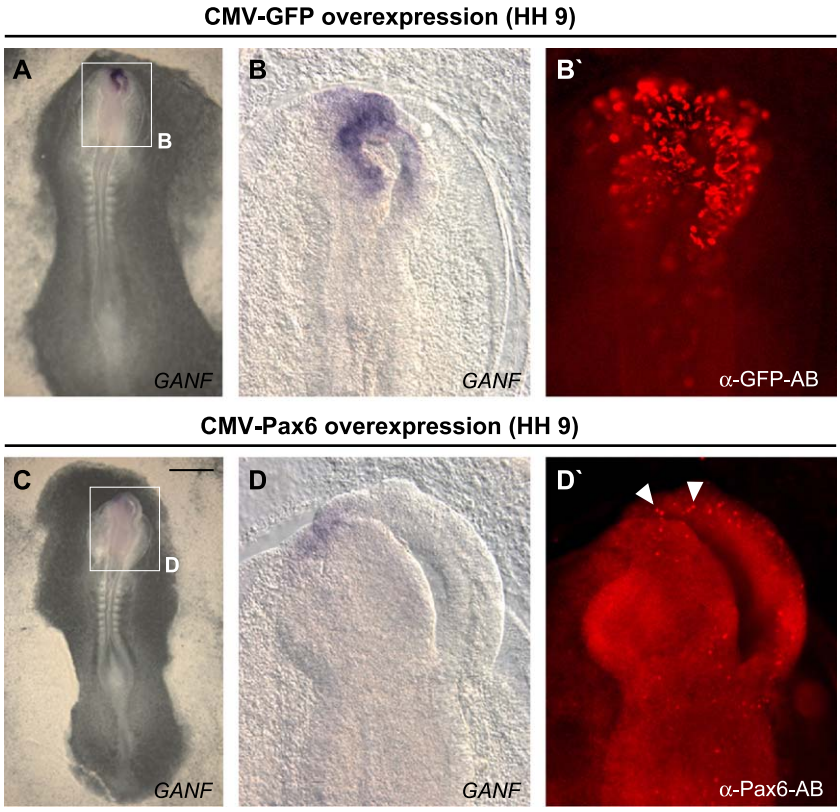

Fig. 5. Downregulation of *GANF* expression by *Pax6* overexpression. (A and B) Overexpression of CMV-GFP shows no alteration of the endogenous *GANF* expression. (C and D) Overexpression of CMV-*Pax6* by electroporation into the endogenous *GANF* expression domain of chicken embryos exhibited a clear reduction of the endogenous *GANF* expression. (D′) Immunostaining of *Pax6* after WMISH exhibits *Pax6*-overexpressing cells within the endogenous *GANF* expression domain. *Pax6*-overexpressing cells are indicated by arrowheads.

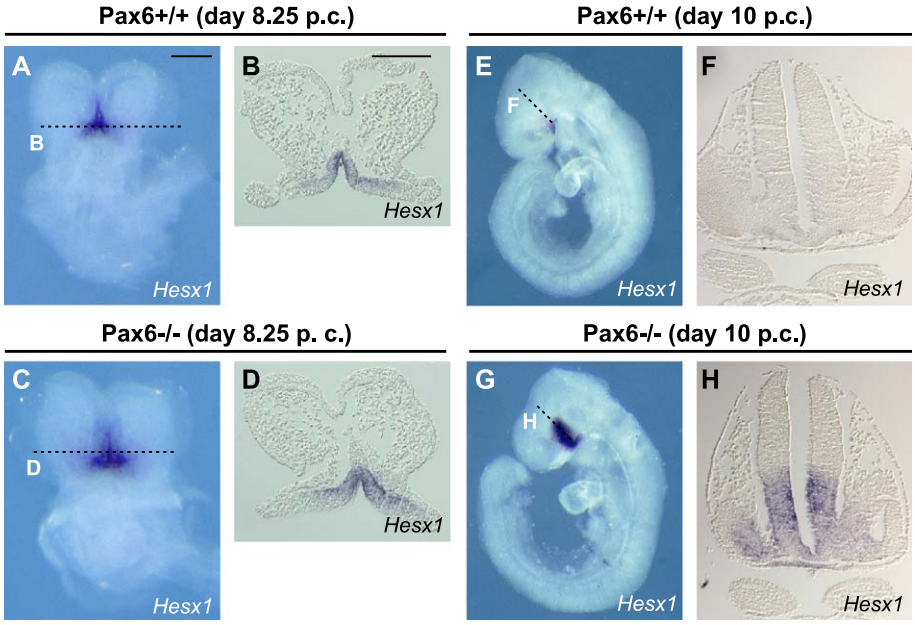

Fig. 6. Analysis of *Hesx1* expression in *Pax6* mutant mice. (A–D) A slight difference can be detected in the expression domain of the murine *GANF* orthologue *Hesx1* in *Pax6*$^{-/-}$ mice of stage d. 8.25 pc. (E–F) An enlargement of the expression domain of *Hesx1* can be detected compared to the wild type in stage day 10 pc. *Hesx1* expression is extended dorsally into the diencephalon and laterally into the optic stalks, where in the wild-type embryo *Pax6* is expressed. The scale bar in A represents 250 μm for A and C; the scale bar in B corresponds to 125 μm in B and D and 200 μm for F and H.

showed a downregulation of endogenous *GANF* expression in the electroporated cells (Figs. 5A–D), thus demonstrating a negative regulation of *GANF* by *Pax6*.

To study the effect of *Pax6* on *ANF* genes further, we studied *Hesx1* expression in murine *Pax6* mutants. In wild-type embryos, the *Hesx1* expression domain develops from an anterior domain on day 7 pc, via the typical, large domain in the anterior neural folds on day 8.5 pc, and to a restricted, weak domain in the ventral medial part of the telencephalon and oral ectoderm on day 10 pc (Figs. 6A,B,E and F). In *Pax6*$^{-/-}$ and *Pax6*$^{+/-}$ mice, *Hesx1* expression developed quite differently (Figs. 6C,D,G,H and data not shown). On day 7 pc, it still resembled the wild-type pattern, whereas on day 8.25 pc, a marginal broadening of the anterior neural fold domain became apparent (Figs. 6C and D). By day 10 pc, a dramatic difference had developed, with *Hesx1* expression expanding laterally and anteriorly into the ventral dienceph-alon and the optic stalks, where in wild-type embryos *Pax6* is

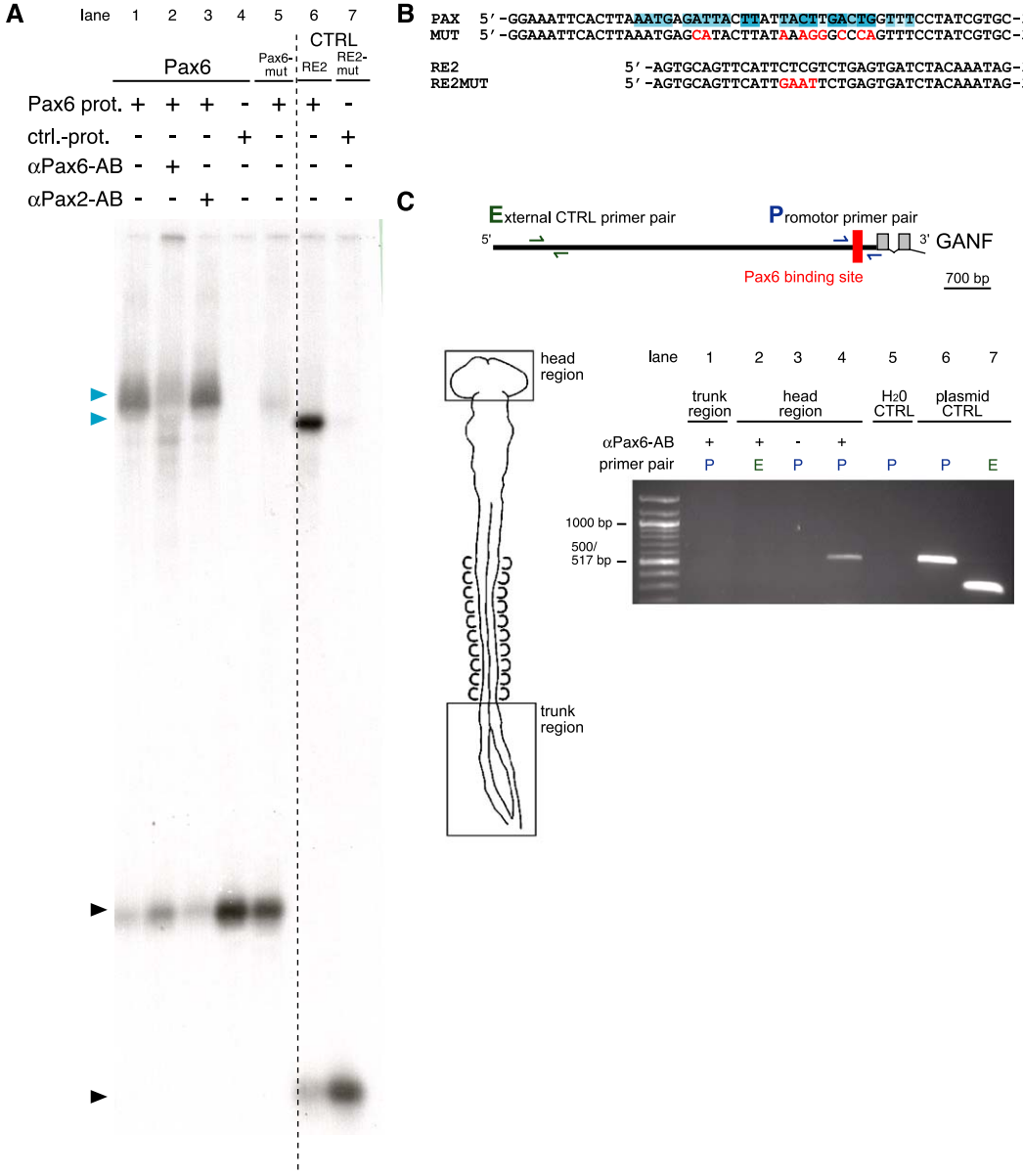

Fig. 7. Interactions of *Pax6* with the *GANF* promoter. Bandshift assay of the proposed binding site of *Pax6* in the *GANF* promoter. (A) Blue arrowheads exhibit the complex of protein and oligonucleotides; black arrows show the free oligonucleotides. As a control, an oligonucleotide was used for which binding has been shown (RE2; see Materials and methods). The difference between the control oligonucleotides and the other is due to different lengths. Clear binding is shown for the proposed binding site. The binding is extinguished by the anti-*Pax6* antibody, but not by an anti-*Pax2* antibody. The protein control consists just of TNT reticulocyte lysate; there is no binding observed. The mutated oligonucleotide shows significantly reduced binding of the protein. (B) Sequence comparison of the nucleotides representing the original and mutated and control sequences. (C) ChIP assay. No binding was detected when the trunk instead of the head region was used, when the PCR amplification was carried out with a different upstream primer pair, and when no antibody was added during the ChIP procedure (lanes 1–3). A positive PCR amplification of an approximately 500-bp-long fragment covering the *Pax6* binding site investigated by EMSA is shown in lane 4.

expressed (Figs. 6G and H; Bäumer et al., 2003; Walther and Gruss, 1991). However, *Hesx1* transcription did not extend all the way into the dorsal *Pax6* expression territories seen in wild-type embryos.

The conserved upstream region of *GANF* includes a *Pax6* binding site (Epstein et al., 1994; Fig. 1, "*PAX6*"; Fig. 7B). In bandshift assays using oligonucleotides representing this binding site, *Pax6* was found to bind specifically (Fig. 7A; lane 1). A competitive anti-*Pax6* antibody inhibited this binding (Fig. 7A; lane 2), while an anti-*Pax2* antibody did not (Fig. 7A; lane 3). In addition, the specificity of this complex formation was confirmed by the impaired binding of *Pax6* to a mutated version of this oligonucleotide (Fig. 7A, lane 5; Fig. 7B). To determine whether the binding site in the *GANF* promoter is indeed occupied by *Pax6* protein, we performed chromatin immunoprecipitation (ChIP) assays using preparations from head or trunk regions of stage HH10 chicken embryos. *Pax6* antibodies precipitated chromatin, in which we could detect a 500-bp fragment from the *GANF* promoter covering the *Pax6* binding site (Fig. 7C, lane 4). In contrast, no binding was detected when trunk instead of head extracts were used, when the PCR amplification was carried out with a different upstream primer pair, and when no antibody was added during the ChIP procedure (Fig. 7C, lanes 1–3). These assays indicate that *Pax6* protein binds in vivo to the *GANF* promoter. We tried to detect the negative influence of *Pax6* on the *ANF* promoter also in our reporter gene assay. However, electroporation of a 1 kb *lac*Z construct, in which the *Pax6* binding site was deleted, did not show any difference in β-galactosidase expression compared to the wild-type construct (data not shown).

Taken together, we found evidence for a direct involvement of *Pax6* on *ANF* gene expression by DNA binding as well as ChIP assays. A repressing function was indicated in ectopic overexpression experiments and in murine mutants. However, we were unable to demonstrate such a repressive effect with a mutated reporter construct.

Discussion

Similarities and differences in the control regions of vertebrate ANF homeobox genes

ANF genes are present in the genome of five vertebrate species, *Xenopus laevis*, *Gallus gallus*, *Mus musculus*, *Rattus norvegicus*, and *Homo sapiens*, but no homolog exists in the genome of *Drosophila melanogaster*. The 650 bp directly upstream region of the vertebrate *ANF* genes is extraordinarily highly conserved. This is in spite of some significant differences in the transcriptional patterns of *ANF* genes observed between mammals on one hand and other vertebrates on the other. The analysis of *ANF* gene promoters may provide the tools to separate conserved and evolved mechanisms.

While the zebrafish promoter is at this point neither sequenced nor functionally studied, it is striking to see that our construct derived from the avian *GANF* promoter is faithfully transcribed also in fish embryos. The promoter of the *X. laevis XANF* gene was previously studied by luciferase reporter assays on dissected embryonic parts (Eroshkin et al., 2002). These authors demonstrated that the presence of the two proximal *Otx2* binding sites otx2-II and otx2-III ("Box 1") was sufficient to generate promoter activity in anterior embryonic fragments, whereas their deletion decreased activity drastically. Their results are consistent with our observation in the chick system, showing the importance of *Otx2* as an enhancing transcription factor for *GANF* and the necessity of two binding sites, that is, otx2-I and otx2-II for the chick, and otx2-II and otx2-III for the frog promoter.

A principal, cell autonomous role of *Otx2* for the expression of the *M. musculus Hesx1* (*Rpx*) gene is evident in *Otx2* knockout mutants (Rhinn et al., 1999). The promoter of the *Hesx1* gene was previously studied using *lac*Z vectors in transgenic mice (Hermesz et al., 2003). In this study, the complete early expression pattern of *Hesx1* in the mouse was only reflected by reporter constructs containing the three *Otx2* and the *Pax6* binding sites studied by us. The shortest reporter construct tested by Hermesz et al. contained just 390 bp upstream of the translation start and does not cover these binding sites. This fragment directed reporter gene activity into the endogenous *Hesx1* expression domains of embryos older than day 8.5 pc, but not in earlier stages (Hermesz et al., 2003). Thus, the results obtained in mice are significantly different from those obtained in frog and chick. It remains to be seen if this difference relates to other differences observed between mammals and other vertebrates with respect to *ANF* genes. In contrast to other vertebrate homologs, mammalian *ANF* genes begin to be transcribed before gastrulation in the anterior visceral endoderm (AVE), and not initially in the anterior neural plate at gastrula stages as in frogs and chicken (Knoetgen et al., 1999a,b). A further peculiarity of mice is the existence of two, temporally controlled *Hesx1* transcripts that differ in the length of their 5′ UTRs (Hermesz et al., 1996). Only the smaller RNA corresponds to the molecule found in *Xenopus* or chick embryos (Samakhvalov et al., 1993; this study).

Otx2 as a necessary, but not sufficient, activator of ANF genes

Otx2 is a central protein for head induction and head development. It was shown to be necessary for the regulation of a variety of genes involved in morphogenesis, cell migration, and the acquisition of anterior neural identity (Boncinelli and Morgan, 2001). A direct interaction of *Otx2* proteins with the murine *Hesx1* gene was suggested by the analysis of *Otx2* knockout mutants in which forebrain-specifying genes, including *Hesx1*, failed to be transcribed in the anterior neural plate (Rhinn et al., 1999). In our study,

we present in vitro evidence for three *Otx2* binding sites within the *GANF* promoter. All sites match to the bicoid-like binding motive for which *Otx2* binding with high affinity was previously shown (Briata et al., 1999). The relevance of *Otx2* binding to the described sites on the *GANF* promoter was further confirmed in vivo by the use of a reporter construct with mutated otx2-I and otx2-II sites. In these experiments, the promoter activity was completely abolished or at least severely decreased. A residual *lac*Z activity could possibly be attributed to the remaining otx2-III site or to a residual weak binding activity of the otx2-I site.

Ectopic *Otx2* overexpression did not result in an ectopic activation of the endogenous *GANF* gene, implying that additional factors were necessary for *GANF* activation. The conserved 650-bp element contains, for example, additional putative binding sites for *SOX*-type transcription factors (data not shown). *GANF* expression in the anterior neural plate is turned on at the late definitive streak or early head process stage (HH4+/HH5). At these stages, the rostral definitive endoderm has spread under the anterior ectoderm and the early axial mesendoderm of the head process ingresses through the anterior tip of Hensen's node forming the prechordal plate. Transsection assays, where the anterior part of an intermediate streak embryo was separated from influences of the forming organizer, demonstrated that from the anterior definitive endoderm, instructive signals emanate, which activate *GANF* expression (Chapman et al., 2003). Also, the early axial mesoderm, namely the prechordal plate, is a source of *GANF*-inducing signals (Knoetgen et al., 1999a). However, the molecular nature of these signals is still not known.

In summary, we conclude that *Otx2* activity in the anterior neural ectoderm of chick embryos in combination with factor(s) yet to be identified is necessary to activate *GANF*.

The role of Pax6 in the regulation of ANF/Hesx1 expression

Interactions of *Pax6* with target genes were previously studied for many aspects of cell specification and differentiation. *Pax6* mediates the activation or repression of downstream target genes during the differentiation of retinal progenitor cells (Marquardt et al., 2001). In the spinal cord, an inhibitory activity of *Pax6* was demonstrated: cross-repressive interaction of *Pax6* with *Nkx2.2* positions the ventral progenitor cells (Briscoe et al., 2000; Ericson et al., 1997a). Mutual repression between *Pax6* and *Pax2/En1* was described, and both factors were found in excluding expression domains at the di-mesencephalic boundary (Matsunaga et al., 2000).

The exclusive expression domains of *Pax6* and *GANF* suggested an inhibitory function of *Pax6* on *GANF* expression. An overexpression study of the *Xenopus ANF*-class gene, *XANF-1*, already demonstrated a repressive function of *XANF-1* on *Pax6* (Ermakova et al., 1999). In our experiments, overexpression of *Pax6* in the endogenous *GANF* expression domain led to a clear downregulation of *GANF* transcripts. A strong extension of the *Hesx1* expression was found in the *Pax6*$^{-/-}$ mutant mice in day 10 pc, where *Hesx1* expression was shifted dorsally into the diencephalon and laterally into the optic stalks, areas where *Pax6* is normally expressed. This observation resembles the ventral-to-dorsal shift of *Nkx2.2* expression in the spinal chord of *Pax6* mutant mice (Ericson et al., 1997b). We did not observe any profound morphological alterations in the mutant mice, arguing against indirect effects, such as the deletion of a *Hesx1* expressing morphological structure. The obvious difference of *Hesx1* expression between wild-type and mutant embryos was not detectable in very early stages, but became prominent by day 10 pc. This observation indicates that an extended early *Hesx1* expression domain becomes increasingly restricted in parallel to the broadening of the *Pax6* domain. Such a mechanism would not suggest an extension of *Hesx1* all the way into the *Pax6* domain of *Pax6* mutant mice because the *Hesx1*-activating mechanism may not be altered.

Mutant mice and direct overexpression experiments indicated a repressor role for *Pax6* on *ANF/Hesx1*. Evidence for a direct binding of *Pax6* to an enhancer element, resulting in transcriptional repression, was previously reported as a mechanism for the transcriptional control of the lens fiber gene *βB1*-crystallin (Duncan et al., 1998). In the case of *Pax6* and *GANF*, the electroporation of the 1 kb *lac*Z construct, in which the *Pax6* binding site was deleted, did not, however, exhibit a difference in β-galactosidase expression. Thus, while both the EMSA and the ChIP assay strongly suggest that *Pax6* acts directly on the *GANF*

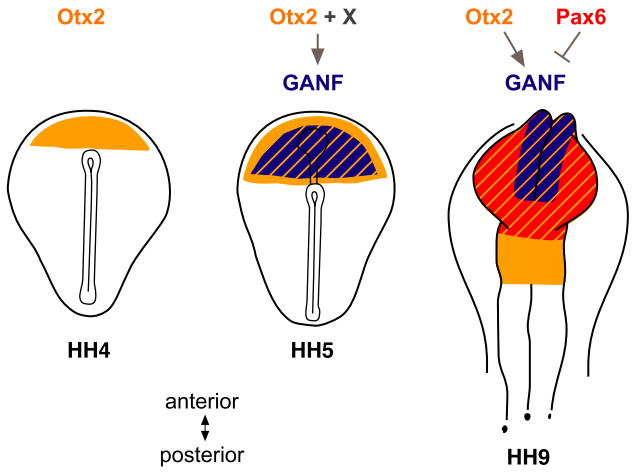

Fig. 8. Schematic drawing of the proposed interactions of *Pax6* and *Otx2* with *GANF* during the regionalization of the forebrain. In the definitive streak stage (HH4), *Otx2* expression marks the anterior neural plate. At the head process stage (HH5), *GANF* is expressed within the *Otx2* domain in the anterior neural ectoderm, dependent on the expression of *Otx2* and a signal from the prechordal plate mesendoderm (X). During later stages, from the headfold to the 10 somite stage (HH6–HH10), *Pax6* controls *GANF* expression negatively, establishing the restricted *GANF* expression domain in the telencephalon and the dorsal neural folds.

promoter, a direct proof of its mechanism of action is still to be found.

Interactions among Pax6, Otx2, and GANF/Hesx1 during forebrain development

The investigated regulation of the *GANF* gene sheds light on the highly dynamic expression domain of *GANF* during forebrain development. Before the activation of *GANF* in the anterior neural plate, *Otx2* provides the base for ectodermal cells to activate *GANF* (Fig. 8, HH4). Signals from the mesendoderm activate *GANF*, with *Otx2* as a key, directly interacting, transcriptional activator (Fig. 8, HH5). *GANF* expression persists in the anterior neural plate until *Pax6* becomes activated in increasingly large areas of the diencephalic neural folds. From then on, *Pax6* restricts the *GANF* expression to the anterior neural folds of the telencephalon and the anterior floorplate (Fig. 8, HH9).

Acknowledgments

We are grateful to Ralf Altschäffel, Wiebke Behrens, Gordon Dowe, Silke Eisenbeiss, Birgit Raschke, and Hartmut Sebesse for excellent technical assistance and would like to thank Christopher Brink, Hendrik Knötgen, Ahmed Mansouri, and Dieter Treichel for helpful advice and discussions, and Peter Gruss for his support. The chicken *Otx2* expression construct was a generous gift of Paola Bovolenta Nicolao. The anti-*Otx2* antibody was generously provided by Flora Vaccarino. The authors were supported by Studienstiftung des deutschen Volkes (D.S.), the Max-Planck-Gesellschaft (N.B., M.K.) and DFG-Grants (L.W., U.T.) and schweizerischer Nationalfonds (J.S.), Volkswagen-Stiftung and DFG-Grants (M.Kö., M.R.-F., and E.R.).

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