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The zebrafish buttonhead-like factor Bts1 is an early regulator of *pax2.1* expression during mid-hindbrain development

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SUMMARY

Little is known about the factors that control the specification of the mid-hindbrain domain (MHD) within the vertebrate embryonic neural plate. Because the head-trunk junction of the *Drosophila* embryo and the MHD have patterning similarities, we have searched for vertebrate genes related to the *Drosophila* head gap gene *buttonhead* (*btd*), which in the fly specifies the head-trunk junction. We report here the identification of a zebrafish gene which, like *btd*, encodes a zinc-finger transcriptional activator of the Sp-1 family (hence its name, *bts1* for *btd/Sp-related-1*) and shows a restricted expression in the head. During zebrafish gastrulation, *bts1* is transcribed in the posterior epiblast including the presumptive MHD, and precedes in this area the expression of other MHD markers such as *her5*, *pax2.1* and *wnt1*. Ectopic expression of *bts1* combined to knock-down experiments demonstrate that Bts1 is both necessary and sufficient for the induction of *pax2.1* within the anterior neural plate, but is not involved in regulating *her5*, *wnt1* or *fgf8* expression. Our results confirm that early MHD development involves several genetic cascades that independently lead to the induction of MHD markers, and identify Bts1 as a crucial upstream component of the pathway selectively leading to *pax2.1* induction. In addition, they imply that flies and vertebrates, to control the development of a boundary embryonic region, have probably co-opted a similar strategy: the restriction to this territory of the expression of a Btd/Sp-like factor.

Key words: Zebrafish, Mid-hindbrain, *bts1*, *buttonhead*, Sp factors, *pax2.1*

INTRODUCTION

Neural patterning in vertebrates responds to a combination of planar and vertical inductive signals that progressively subdivide the neural plate into forebrain, midbrain, hindbrain and spinal cord along the anteroposterior axis (Lumsden and Krumlauf, 1996; Appel, 2000). It is a major challenge to understand how this information is encoded at the molecular level, and how the signals are integrated and refined during development to permit the formation of an organized neural plate.

Within the embryonic neural plate, the mid-hindbrain domain (MHD), which comprises the midbrain vesicle and hindbrain rhombomere1 (rh1), follows an interesting mode of patterning. Indeed, a small population of cells located at the junction between midbrain and rh1 ('mid-hindbrain junction' or 'isthmus') was identified as a source of inductive signals controlling the development of the entire MHD (Martinez et al., 1991; Marin and Puelles, 1994; Martinez et al., 1995; Wurst and Bally-Cuif, 2001). From early somitogenesis stages, the secreted factors Wnt1 and Fgf8 are expressed at the isthmus and are involved in cross-regulatory loops with MHD markers of the *engrailed* and *pax2/5/8* families (Wilkinson et al., 1987; McMahon et al., 1992; Crossley and Martin, 1995; Lun and Brand, 1998; Reifers et al., 1998). These regulatory cascades allow for MHD maintenance at somitogenesis stages. Thus, within the MHD, early signalling events are relayed on-site by the isthmus to maintain MHD specification and achieve short-range patterning. It is of great interest to understand in depth the mechanisms and factors which sustain this mode of patterning.

Accordingly, unravelling the processes of mid-hindbrain

specification remains a major issue. To this aim, the expression of MHD markers was analysed in response to different embryonic manipulations or in mutant contexts in several vertebrates. In the mouse and chick, isthmic organizer formation responds to the confrontation of anterior (*Otx2* positive) and posterior (*Gbx2* positive) identities within the neural plate (Broccoli et al., 2000; Katahira et al., 2000; Millet et al., 2000). However, the expression of *Otx2* and *Gbx2* themselves are probably only involved in the refinement of *Fgf8* and *Wnt1* expression rather than in their induction, as *Fgf8* and *Wnt1* are still expressed in $Otx2^{-/-}$ and $Gbx2^{-/-}$ mutants (Acampora et al., 1998; Wassarmann et al., 1997). Recent ablation experiments in the mouse also pointed to a role of the axial mesoderm in the regulation of *Fgf8* expression (Camus et al., 2000). Finally, explant cultures in the mouse and *Xenopus*, and transplantations in the zebrafish showed that *engrailed* genes and *pax2.1* expression could be locally induced within the neural plate by non-neural tissues (Hemmati-Brivanlou et al., 1990; Ang and Rossant, 1993; Miyagawa et al., 1996). Thus, MHD specification probably integrates planar and vertical signals, but the factors involved remain unknown.

We were interested in directly identifying factors regulating the initiation of expression of the early mid-hindbrain markers. In the zebrafish embryo, the earliest known mid-hindbrain-specific marker is the gene *her5* (Müller et al., 1996), expressed in the presumptive MHD from mid-gastrulation onwards (70% epiboly) (Bally-Cuif et al., 2000). Shortly afterwards (80-90% epiboly), *pax2.1* expression (Krauss et al., 1991; Lun and Brand, 1998) is induced in a domain mostly overlapping with that of *her5* (this paper). Finally, at the end of gastrulation (tail bud stage), *wnt1* expression is initiated in the same territory (Molven et al., 1991; Lun and Brand, 1998). Late markers such as *eng* genes (Ekker et al., 1992), *fgf8* (Fürthauer et al., 1997; Reifers et al., 1998) and *pax5/8* (Pfeffer et al., 1998) become expressed in the MHD at early somitogenesis stages only. Analyses of *pax2.1/noi* (*no-isthmus*) zebrafish mutants have demonstrated that the induction of *her5*, *wnt1*, *eng2* and *fgf8* expression is independent of Pax2.1 function, while initiation of *eng3* and *pax5/8* expression requires a functional Pax2.1 protein (Lun and Brand, 1998). Conversely, in the mouse, *Pax2* expression is established independently of Wnt1 (McMahon et al., 1992; Rowitch and McMahon, 1995). The early onset of *her5* expression in the zebrafish suggests that it also does not require Wnt1 function. Taken together, these observations suggest that several initially independent pathways lead separately to the activation of *her5*, *pax2.1*, *wnt1* and *eng2*. The expressions of *eng3* and *pax5/8* are initiated subsequently in a Pax2.1-dependent cascade (see Lun and Brand, 1998).

In the *Drosophila* embryo, *buttonhead* (*btd*) is expressed in and necessary for the development of the antennal, intercalary and mandibular head segments (Wimmer et al., 1993). Recently, re-examination of *btd* expression revealed that it covers two rows of cells in the first trunk parasegment, thus crossing the head-trunk junction (Vincent et al., 1997). *btd* mutant embryos fail to activate the expression of *collier* (*col*) in the last head parasegment and *even-skipped* (*eve*) in the first trunk parasegment and do not form a cephalic furrow, the constriction separating the head from the trunk (Vincent et al., 1997). Thus *btd* is essential to integrate the head and trunk patterning systems and maintain the integrity of the head-trunk junction. Because the MHD also develops in response to the confrontation of anterior and posterior patterning influences, Btd-related factors appeared as good candidate early regulators of mid-hindbrain development in vertebrates, and we initiated a molecular search for zebrafish genes related to *btd*.

btd (Wimmer et al., 1993) encodes a zinc-finger transcription factor of the same family as *Drosophila* and vertebrate Sp factors (Kadonaga et al., 1987; Kingsley and Winoto, 1992; Pieler and Bellefroid, 1994; Supp et al., 1996; Wimmer et al., 1996; Harrison et al., 2000), but has no known vertebrate ortholog at present. We now report the isolation of 11 new zebrafish *btd/Sp1*-related genes (*bts* genes). One of these genes, *bts1*, is transcribed within the presumptive MHD before *her5*, *pax2.1*, *wnt1* and *eng2*. We demonstrate that Bts1 is both necessary and sufficient for the induction of *pax2.1* within the anterior neural plate, but is not involved in regulating *her5*, *wnt1*, *eng2* or *fgf8* expressions. Thus we have identified the earliest known specific regulator of *pax2.1* expression within the embryonic neural plate, and provide further evidence that early specification of the MHD is controlled by several independent genetic cascades. Furthermore, our results imply that flies and vertebrates have likely evolved a similar strategy to cope with the patterning of comparable embryonic regions, by restricting to these regions the expression and function of a Btd/Sp-like factor.

MATERIALS AND METHODS

Fish strains

Embryos were obtained from natural spawning of wild-type (AB), ace^{ti282a} or noi^{tu29a} (Brand et al., 1996) adults; they were raised and staged according to Kimmel et al. (Kimmel et al., 1995).

Cloning of zebrafish buttonhead/Sp-family members

Random-primed cDNA prepared from tail bud-stage wild-type (AB) zebrafish RNA was amplified using degenerate oligonucleotides directed against the first zinc finger of Btd and Sp1-4 proteins (5′ primers Btd-F1 and Btd-F2) and against their third zinc finger (3′ primer Btd-R):

Btd-F1 5′TG(C/T)CA(C/T)AT(C/T)(C/G)(A/C)IGGITG(C/T)G3′; Btd-F2 5′CICA(C/T)(C/T)TI(A/C)GITGGCA(C/T)ACIG3′; and Btd-R 5′TGIGT(C/T)TTI(A/T)(C/T)(A/G)TG(C/T)TTI(C/G)(C/T)IA(A/G)-(A/G)TG(A/G)TC3′. For cloning of cDNAs *1F*, *2F*, *5F*, *g2*, *g5*, nested PCR-amplification was performed: (1) 100 pmol of each primer Btd-F1 and Btd-R, for 1 minute at 94°C, 1 minute at 42°C, 1 minute at 72°C (two cycles), 1 minute 94°C, 1 minute at 48°C and 1 minute at 72°C (28 cycles); (2) 100 pmol each primer Btd-F2 and Btd-R for 1 minute at 94°C, 1 minute at 46°C, minute at 72°C (2 cycles), minute at 94°C, 1 minute at 50°C and 1 minute at 72°C (28 cycles). For cloning of the cDNAs *bts1*, *G2*, *g5.6*, *G5*, *G1* and *G4*, two rounds of PCR were performed with primers Btd-F1 and Btd-R, using 100 pmol of each primer and 1/100 of the first PCR reaction product (following gel extraction) as template for the second round. Amplification cycles were as follows: 1 minute at 94°C, 1 minute at 42°C, 1 minute at 72°C (2 cycles); 1 minute at 94°C, 1 minute at 48°C, 1 minute at 72°C (28 cycles). PCR products of the appropriate size (160-180 bp) were purified by gel electrophoresis, subcloned and sequenced. The fragment encoding the zinc-finger domain of Bts1 was used for high-stringency screening of a somitogenesis stage cDNA library (kindly provided by Dr B. Appel). Positive clones containing the full-length *bts1* cDNA (3kb) were obtained, one of these clones was sequenced (Fig.1); its GenBank Accession Number is AF388363.

Drosophila stocks and transgenics

To examine the role played by *bts1* in *Drosophila* we used the IT system (immediate and targeted gene expression) developed by Wimmer et al. (Wimmer et al., 1997). In the conditional *btd>AB >bts1* transgene, the *bts1*-coding region is separated from the *btd* promoter by a flp-out cassette containing *lacZ. btd >AB>bts1* was constructed by inserting a 2659 bp *Not*I-*Cla*I fragment containing the entire *bts1*-coding region and 1387 bp 3′UTR into the *btd >AB>btd* plasmid (Wimmer et al., 1997) open at *Not*I, and used to generate transgenic fly lines (Rubin and Spradling, 1982). The stock β_2-*tub-flp*/Y; *btd >AB >bts1* /TM3, *hb-lacZ* was established and crossed with btd^{XG81} /FM7, *ftz-lacZ.* To identify embryos mutant for *btd* and expressing *bts1, lacZ* in situ hybridization was performed. RNA labelling and in situ hybridization were performed as described (Crozatier et al., 1996). RNA probes were prepared from *col*, *eve*, *en* and *lacZ.*

Ectopic expression analyses in the zebrafish (constructs and injections)

For ectopic expression of wild-type *bts1*, *pXT7-bts1Δ3′* was constructed which contains the full-length *bts1*-coding region and 23 nucleotides of *bts1* 3′UTR (*Spe*I fragment from *pBS-bts1*) subcloned into pXT7 (Dominguez et al., 1995). Mutant forms *bts1ΔZnF* and $bts1^{C\text{->}T}$ were constructed with the Stratagene Ex Site™ PCR-based site-directed mutagenesis Kit using the following oligonucleotides:

(1) *bts1ΔZnF*, ONbts1ΔZnF1, 5′-*P*-GATGTGCTGTTTCTTCTTT-CCGGGCTC-3′; ONbts1ΔZnF2, 5′-CAGAACAAGAAGAGC-AAAAGTCACGACAAAAC-3′

(2) $bts1^{C\text{->}T}$, ONbts1$^{C\text{->}T}$-1, 5′-*P*-AGTCCGGACACACAAA-GCGTTTTTCGC-3′; ONbts1$^{C\text{->}T}$-2, 5′-ACTATAAAAGGTTCAT-GAGGAGCGACCATTTG-3′.

This mutation alters 2 Cys in 2 Tyr in the third zinc finger (TGCTGT→TACTAT). In a null allele of Btd, the second Cys of the third zinc finger is replaced by a Tyr (Wimmer et al., 1993). As Bts1 harbors two adjacent Cys in position 6 and 7 of the third zinc finger, both were mutated. *bts1ΔZnF* and $bts1^{C\text{->}T}$ were subcloned into pXT7. Capped mRNAs were synthesized (Ambion mMessage mMachine kits) and verified by in vitro translation. Injections were carried out at 100 ng/μl into one central blastomere of the 16-cell embryo (10 pl), together with *nls-lacZ* RNA (40 ng/μl) as lineage tracer, and the distribution of the injected progeny was verified a posteriori by anti-β-galactosidase immunocytochemistry (Bally-Cuif et al., 2000). After appropriate staining, embryos were embedded in JB4 resin (Polysciences) and sectioned at 2 μm on an ultramicrotome (Fig. 5D,F).

Design and injections of the *bts1* morpholinos

MO^{bts1} (5′TACCGTCGACACCGACACGACTCCT3′) (Gene Tools LLC, Corvalis, OR) was designed to target positions 1-25 of the *bts1* cDNA. A four bp mismatch morpholino (MO^{bts1}Δ4) (5′TACTGTTGACACCGACACAACCCCT3′) was used as control. A morpholino of unrelated sequence (5′CCTCTTACCTCAGTT-ACAATTTATA3′), biotinylated in 3′ and aminated in 5′ to allow for fixation, was used as a lineage tracer when single cell resolution was necessary (Fig. 6C). For detection of the tracer MO (Fig. 6C), embryos were processed first for in situ hybridisation followed by incubation in avidin-biotinylated β-gal complex (Vector, Roche) revealed with X-gal staining. In other cases, *nls-lacZ* RNA was used as tracer (Fig. 6D-H). All MOs were injected at 1-2 mM in H_2O into a central blastomere of 16-cell embryos.

Transplantation experiments

The full-length coding region of mouse *Wnt1* cDNA (van Ooyen and Nusse, 1984) was subcloned into pXT7 and used to generate capped mRNA. *Wnt1* RNA was injected at 10 ng/μl together with *nls-lacZ* RNA (40 ng/μl) at the one-cell stage, and animal pole cells from injected embryos at the sphere stage were homotopically and isochronically transplanted into non-injected recipients.

Inhibition of Fgf signalling by SU5402

Embryos were incubated in 12 μM SU5402 (Calbiochem) in embryo medium from the dome stage until late gastrulation, and then immediately fixed and processed for in situ hybridisation. To control for SU5402 efficiency, embryos similarly treated from the shield stage were verified to develop a phenotype morphologically indistinguishable from *ace* mutants in the MHD area (not shown).

In situ hybridization and immunocytochemistry in the zebrafish

In situ hybridization and immunocytochemistry were carried out according to standard protocols (Thisse et al., 1993; Hauptmann and Gerster, 1994).

RESULTS

Cloning of *buttonhead*-related genes in the zebrafish

We PCR-amplified tail bud stage wild-type zebrafish cDNA using degenerate oligonucleotides directed against the zinc-finger domains of Btd and Sp factors. Eleven partial cDNAs encoding zinc finger domains were obtained (Fig. 1A), each of them from several distinct PCR reactions, suggesting that they correspond to different genes and not to variations due to Taq polymerase errors. All code for triple zinc fingers, 55-85% similar to each other and with the structure Cys_2-His_2 characteristic of Btd and Sp factors (Kadonaga et al., 1987; Kingsley and Winoto, 1992; Wimmer et al., 1993; Pieler and Bellefroid, 1994; Supp et al., 1996; Wimmer et al., 1996; Harrison et al., 2000). They were named *bts* genes (for *btd/Sp*-related). Except in two cases (g5.6 and g5), they are more closely related to the zinc-finger domain of Sp factors (70-94% identity) than to that of Btd (64-80% identity). g5.6 is equally related to Sp and Btd (75% identity), and g5 is more closely related to Btd than to Sp (69% versus 56% identity).

To determine whether one of these factors could be a functional equivalent of Btd at the *Drosophila* head-trunk junction, we examined their expression profiles at the tail bud stage using high-stringency whole-mount in situ hybridization conditions. With the exception of *g5.6* and *G1*, which proved ubiquitously expressed, all other genes tested showed spatially restricted and distinct expression patterns (Fig. 1A), further confirming that they corresponded to different factors. One of them, *bts1*, appeared selectively expressed in the MHD (see Fig. 3), and was therefore selected for further studies. *g5*, the most related in sequence to *btd*, was not expressed in the mid-hindbrain and thus appeared unlikely to be a functional homologue of *btd* in this domain.

High-stringency screening of a zebrafish somitogenesis-stage library with the PCR product of *bts1* produced six positive clones, covering all or part of the same 3 kb cDNA. The longest open reading frame (1102 nucleotides) is preceded by 126 nucleotides of 3′UTR containing a classical Kozak sequence and two in-frame stops upstream of a start methionine (not shown), and predicts a 368 amino acid protein (Fig. 1B). In agreement with these findings, the in vitro translated products of the entire cDNA (containing 1727 nucleotides 5′UTR) and that of its predicted coding region (*bts1Δ3′*, see Materials and Methods) had the same apparent size (40 kDa) (data not shown). The deduced protein Bts1 presents features characteristic of Btd/Sp factors (Pieler and

B.

1 MAAVAVLRNDTLQAFLQDRQPNSSPENSKHSPLALLAATCNRIGHHHGSTPTDFIQVPYDTTLGSPSRIFHPWSNYANHGSTLSSNPSFGLSSKSHLQ
SSYASHHELPLTPPADPTYPMISLRLRCCHVNASLQSTCPPTYVPAVTYAAPAPIPPAMPSFVPEHSELVHQQQRQLSPNPGEDIPWWSLQQGNPVAHSV
HPHRFPIQRGLVLGHTDFAQYQTQIAALLHTKSPLATARRCRRCRCPNCQSSSSSDEPGKKKQHI**CHIPGCGKVYGKTSHLKAHLRWH**SGERPFV**CNWLF**
CGKSFTRSDELQRHGRTHTGEKRFV**CPDCCKRFMRSDHLAKHVKTH**QNKKSKSHDKTSDYVKREDLRPRV* 368

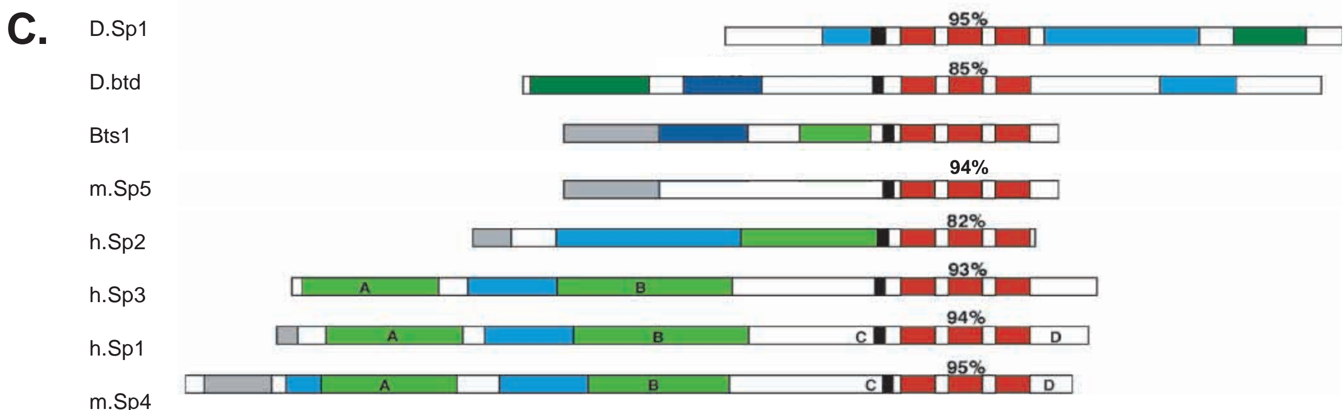

Fig. 1. Structure of the Bts1 protein. (A) Zinc finger domains of the 11 zebrafish Btd/Sp-family members (Bts proteins) isolated, aligned with the corresponding domains of *Drosophila* Btd (Wimmer et al., 1993) and mouse Sp1 (Kadonaga et al., 1987). Positions of the primers used in the degenerate PCR reaction are indicated (arrows). Each zinc finger has the structure $3x(C_2H_2)$ (red boxes highlight Cys and His residues) and is preceded by a 'Btd box' (boxed in black for Btd, mouse Sp1 and Bts1, not indicated for others). The Cys doublet mutated in the negative control-construct Bts1$^{C\text{->}T}$ (see Fig. 5) is boxed in blue. The expression profile of each *bts* gene at the tail bud stage is summarized in the right column. jct, junction; gl, gland; ND, not determined; NT, neural tube; olf, olfactory placodes; TB, tail bud; ves, vesicle. (B) Sequence of the Bts1 protein. The zinc-finger domains are in red and the Btd-box is boxed in black. S/T and Q-rich, potential transcriptional activation domains are, respectively, in green and blue. The N-terminal domain resembling that of Sp1, Sp2, Sp4 and Sp5 is underlined. (C) Structural alignment of Bts1 and other Btd/Sp proteins (Kadonaga et al., 1987; Hagen et al., 1992; Wimmer et al., 1993; Wimmer et al., 1996; Supp et al., 1996; Harrison et al., 2000). Percentages of similarity between Bts1 and other proteins are given for the zinc finger/Btd box (red/black). Q-rich domains are blue (the Q domain of Bts1 only resembles that of Btd (dark blue) but does not align with others (light blue)). S/T-rich domains are green and the N-terminal domain grey. The transcriptional activation domains identified in Sp1, Sp3 and Sp4 are labelled A-D.

Bellefroid, 1994), such as the triple zinc-finger domain (showing highest homology to those of Sp1, Sp3, Sp4 and the recently isolated Sp5) preceded by an arginine-rich 'Btd box' (Fig. 1B,C), a motif implicated in some cases of transcriptional activation by Sp1 (Athanikar et al., 1997). Outside the zinc fingers and Btd box, recognizable motifs include serine/threonine and glutamine-rich regions in the N-terminal half of Bts1. Such domains have been identified in Btd and Sp factors, and were in most instances shown to mediate transcriptional activation (Courey and Tjian, 1998; Kadonaga et al., 1998). The 43 N-terminal amino acids of Bts1 also show significant similarity to the N termini of Sp1, Sp2, Sp4 and Sp5. Outside these domains, similarity with other Sp-like factors is low. Highest homology is found with Sp5 (52% overall identity) but does not reflect an ungapped alignment (see Fig. 1C). *bts1* was mapped in radiation hybrid panels to linkage group 9, 0.10 cM from marker fb18h07, close to the *hoxd* locus (not shown).

In conclusion, *bts1* shows higher overall sequence similarity with Sp factors than with Btd, but its restricted expression in the mid-hindbrain area at the end of gastrulation, is strongly reminiscent of the local expression of *btd* at the head-trunk junction.

Bts1 binds canonical GC boxes and can act as a transcriptional activator in vivo

The sequence of the zinc-finger domain of Bts1 predicts, in analogy to Sp factors, a DNA recognition sequence of the GC box class (Dynan and Tjian, 1983; Gidoni et al., 1984; Gidoni et al., 1985). To investigate the DNA-binding characteristics of Bts1, in vitro transcribed and translated (rabbit reticulocyte lysate) *bts1* protein product was tested in electromobility shift

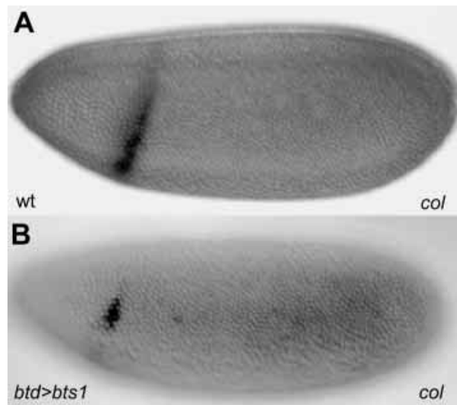

Fig. 2. Bts1 is a transcriptional activator in vivo. Expression of *collier* (*col*) revealed by in situ hybridization at the head-trunk junction of the *Drosophila* blastoderm in wild-type embryos (A) and in *btd* mutant embryos carrying one copy of *bts1* under control of *btd* regulatory elements (B). *btd* mutants show no expression of *col* (not shown). *bts1* can partially rescue *col* expression in *btd* mutants, in a correct spatiotemporal manner.

assay with the zinc-finger binding site of the mouse Pax5 enhancer (Pax5 ZN) (Pfeffer et al., 2000). Bts1 was found to specifically bind to Pax5 ZN but was unable to bind a mutated version of Pax5 ZN in which the zinc-finger binding site has been destroyed (Pfeffer et al., 2000). Thus Bts1 is capable of binding GC boxes in vitro.

Sp factors are highly divergent outside the zinc-finger domain and can act as transcriptional activators or repressors (Majello et al., 1994; Birnbaum et al., 1995; Hagen et al., 1995; Kennett et al., 1997; Kwon et al., 1999; Turner and Crossley, 1999), probably following their interaction with different molecular partners. To determine whether Bts1 behaved as an activator or as a repressor of transcription, we tested whether it could substitute for Btd function in *Drosophila*. Indeed Btd was shown to be a transcriptional activator of the downstream gene *col* (Crozatier et al., 1996), which is necessary for the development of the intercalary and mandibular segments of the head (Crozatier et al., 1999). Transgenic flies were constructed which carry the coding sequence of *bts1* under the control of the *btd* enhancer (Wimmer et al., 1997) (*btd>bts1* flies) and were introduced into a *btd* background. At the blastoderm stage, *btd* embryos completely fail to express *col* (not shown, see Crozatier et al., 1996). We observed that Bts1 was sufficient to partially rescue the expression of *col* in *btd* embryos (Fig. 2B), in a correct spatiotemporal manner along the anteroposterior axis (although in a reduced number of cells, even with two copies of *btd>bts1*; not shown) (compare with Fig. 2A). Thus, at least in this cellular context, Bts1 acts as an activator of transcription.

The similar expression profiles of *bts1* and *btd* at gastrulation, at the junction between anterior and posterior embryonic patterning systems, suggested equivalent developmental functions. However Bts1 and Btd are highly divergent outside the zinc-finger domain, questioning their possible interaction with homologous molecular partners. In addition to *col*, *btd* mutants also fail to express *eve* stripe 1 (Vincent et al., 1997) and *engrailed* (*en*) in the head (Wimmer et al., 1993). Later they lack antennary, intercalary and mandibular head segments. We observed that neither *eve*(1) and *en* expression nor larval head structures was rescued in *btd>bts1* transgenics (not shown). Thus, our results suggest that the correct spatiotemporal activation of *col* mainly requires the zinc-finger domain of Btd, whereas the enforcement/maintenance of *col* expression, as well as the expression of *eve*(1), *en* and the subsequent development of head segmental derivatives would require stronger activity or additional, non-zinc-finger protein modules that are not present in Bts1.

bts1 expression matches the presumptive mid-hindbrain area from mid-gastrulation stages

The spatiotemporal expression of *bts1* at early developmental stages in the zebrafish was determined by whole-mount in situ hybridization. *bts1* transcripts are first detected at 30% epiboly, in the most marginal cells of the blastoderm and in the yolk syncitial layer, excluding the dorsal embryonic side (Fig. 3A,A′). Expression is maintained in epiblastic cells at the margin during gastrulation, with a broader anteroposterior extent as epiboly progresses (Fig. 3B-E). In addition, a restricted number of cells of the dorsal hypoblast, lining the presumptive prechordal plate and anterior notochord, express *bts1* (Fig. 3C-D′). From 70% epiboly, the anterior limit of *bts1* expression in the dorsolateral epiblast is clearly delimited (Fig. 3D-F, arrows), and lies within the presumptive MHD (see below and Fig. 4). At the end of gastrulation, *bts1* transcription in epiblast cells becomes restricted to the MHD and tail bud. It remains prominent in the MHD until at least 24 hours (Fig. 3F-J and not shown). Additional sites of expression arising during late somitogenesis are the otic vesicles, the somites, and restricted nuclei of the diencephalon (Fig. 3I,J).

To precisely position the domain of *bts1* expression within the presumptive neural plate, we compared its location with known forebrain, MHD or hindbrain markers (Fig. 4). At 75% epiboly, the anterior border of *bts1* expression is located within the posteriormost cell rows of the *otx2*-positive territory, abutting the diencephalic ‘wings’ of *fkh3* expression (Fig. 4A-C). *bts1* expression overlaps the *her5*-positive domain (Fig. 4D), which slightly crosses the *otx2* border (Fig. 4E). At the tail bud stage, *bts1* expression has acquired a posterior limit (see Fig. 3G). It encompasses the *her5*- and *wnt1*-positive domains (Fig. 4G,I), and largely overlaps *pax2.1* expression, albeit with a slight rostral shift (Fig. 4J). All four domains expressing *bts1*, *her5*, *pax2.1* and *wnt1* extend several cell rows posterior to the caudal limit of *otx2* (Fig. 4H). These spatial relationships were maintained at the five-somite stage (Fig. 4M-R).

The anterior ‘wings’ of *fkh3* expression have been fate-mapped to the presumptive diencephalon at the 80% epiboly stage (Varga et al., 1999), and *her5* expression to the presumptive midbrain (with a minor contribution to the anterior hindbrain) at 90% epiboly (Müller et al., 1996). Therefore, at 80% epiboly, *bts1* expression in the neural plate comprises the midbrain and more posterior domains, and it is refined to the midbrain and anterior hindbrain from 90% epiboly onwards. These features make *bts1* the earliest known gene expressed across the entire MHD (see Discussion) and suggest that it might be involved in early mid-hindbrain positioning or patterning.

Bts1 is an early regulator of *pax2.1* expression in the zebrafish MHB

We addressed the function of Bts1 within the zebrafish embryonic neural plate using a combination of gain- and loss-

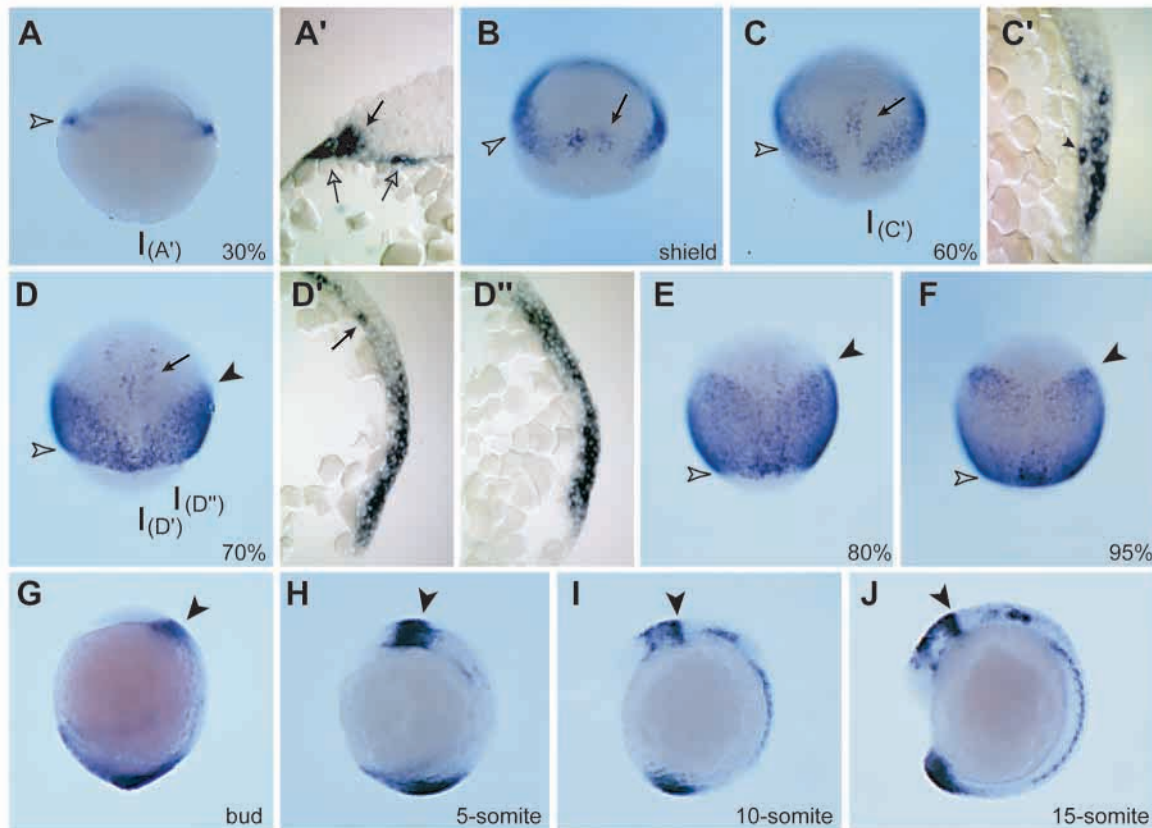

Fig. 3. Expression of *bts1* during gastrulation and early somitogenesis, as revealed by whole-mount in situ hybridization at the stages indicated (% of epiboly). (A-F) Dorsal views, anterior towards the top; (G-J) sagittal views, anterior towards the left. Open arrowheads indicate the blastoderm margin, black arrowheads the mid-hindbrain domain, and small arrows point at hypoblastic expression. (A′,C′,D′,D″) Sagittal sections at the levels indicated, dorsal towards the right, anterior towards the top. *bts1* expression is first detected at 30% epiboly (A,A′) along the ventral and lateral margins of the blastoderm (arrows) and in the yolk syncitial layer (small arrow). During gastrulation (B-F), expression is maintained in the posterior epiblast up to a sharp limit at the mid-hindbrain level, and in hypoblast cells bordering the prechordal plate (small arrow). From the end of gastrulation (F-H), *bts1* expression is confined to the mid-hindbrain level and tail bud and extinguishes from the rest of the epiblast. Additional expression sites during later somitogenesis (I,J) include the otic vesicle, somites and diencephalon.

of-function experiments. To target misexpressions to the neuroectoderm, we injected capped *bts1* mRNA within one central blastomere of the 16-cell blastula. At the 16-cell stage, the four central blastomeres largely contribute to neuroectodermal derivatives (Helde et al., 1994; Wilson et al., 1995). Co-injected *lacZ* RNA served as lineage tracer and we only scored cases where *lacZ*-positive cells were distributed primarily within the neuroectoderm (Fig. 5D,F). Mesodermal markers were unaffected (see *gsc* on Fig. 5E,F; *ntl* and *papc* (data not shown)). Upon misexpression of *bts1*, 50% of embryos injected into regions of the neural plate encompassing the MHD or anterior to it (*n*=72) showed an ectopic expression of *pax2.1* at the tail bud stage (Fig. 5A,B,D-F). By contrast, no induction of *pax2.1* was ever observed in embryos injected only into neural territories posterior to the MHD, or within the epidermis outside the neural plate (*n*=83). Induction of *pax2.1* expression always occurred anterior to the MHD, either in broad patches connected to the MHD (Fig. 5A,D-F) or in scattered cells (Fig. 5B) (at approximately equal frequencies), and in territories showing a high density of injected cells. Within these areas, ectopic *pax2.1* expression appeared restricted to *lacZ*-positive cells (Fig. 5D,F). Notably, no other marker of the early MHD (*otx2*, *her5*, *wnt1*, *eng2*, *pax5*, *pax8*) proved responsive to *bts1* injections (not shown), thus the effect of Bts1 on *pax2.1* expression appeared highly selective. Finally, no patterning defects of the anterior neural plate were observed at somitogenesis or later stages in *bts1*-injected embryos, suggesting that the maintenance of ectopic *pax2.1* expression requires factors other than Bts1 and/or requires the persistence of Bts1 expression. Two mutant versions of *bts1* were constructed as negative controls. *bts1ΔZnF* is deleted in the entire zinc finger-encoding domain of *bts1* and thus should encode a protein incapable of binding DNA. The second mutant form of *bts1*, *bts1*$^{C->T}$, was designed to mimic the *btd* loss-of-function mutation in *Drosophila* (see Materials and Methods). *bts1ΔZnF*- and *bts1*$^{C->T}$-capped RNAs were injected as described for wild-type *bts1* and at similar concentrations; both proved incapable of inducing *pax2.1* expression (100% of cases, *n*=23 and *n*=29, respectively) (Fig. 5C, and data not shown). Taken together, our results indicate that the ectopic expression of Bts1 is sufficient to induce *pax2.1* expression within neural territories anterior to the MHD during gastrulation.

We next determined whether *bts1* expression was also necessary to MHD development and/or *pax2.1* expression (Fig. 6A-D). Antisense ‘morpholino’ oligonucleotides have now

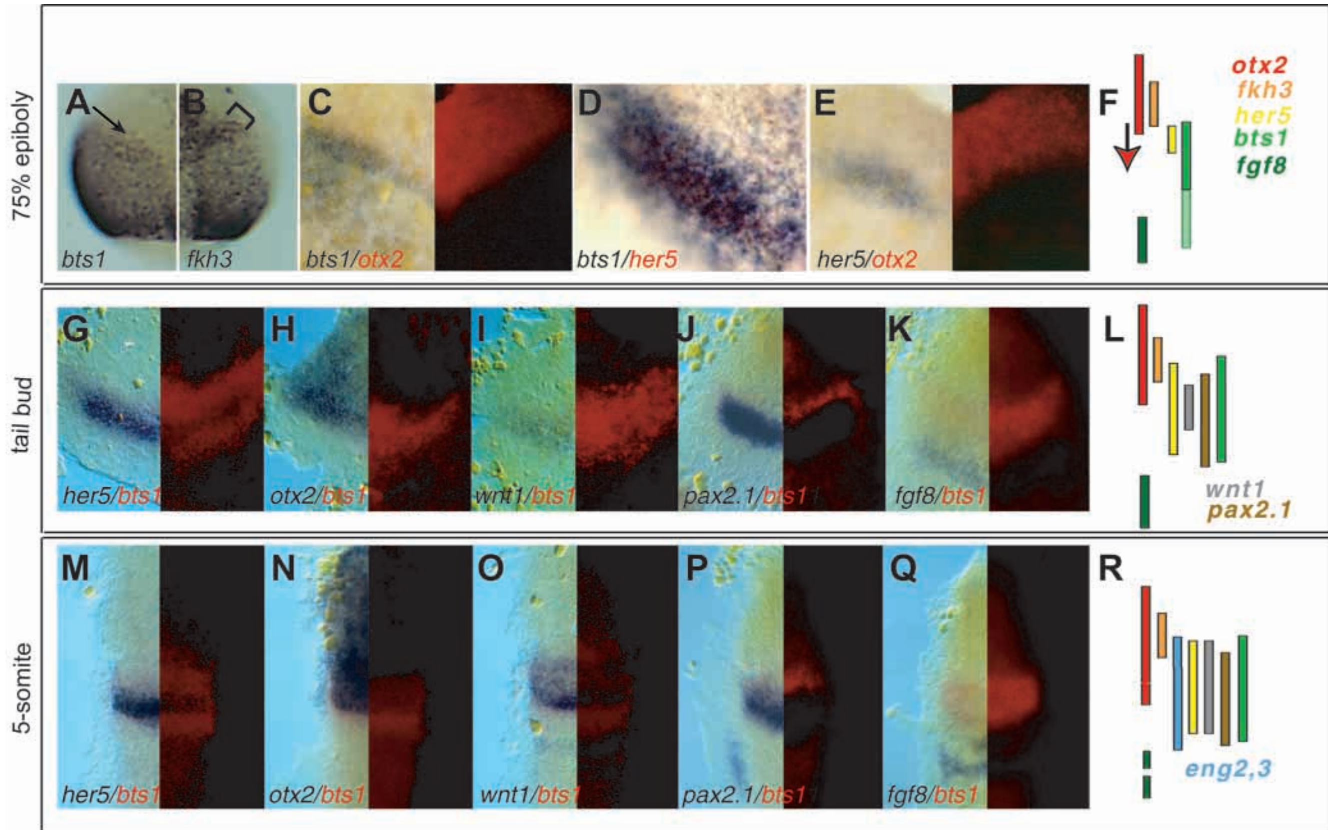

Fig. 4. Comparison of *bts1* expression with other mid-hindbrain markers. Whole-mount in situ hybridization was performed at the 75% epiboly (A-E), tail bud (G-K) and five-somite (M-Q) stages with the probes indicated (colour-coded) (dorsal views, anterior towards the top). (A,B) Single staining for *bts1* and *fkh3*, respectively (whole-mount views of half embryos) (arrow in A indicates anterior limit of *bts1*; bracket in B indicates 'diencephalic wings' of *fkh3* expression). (D) Bright-field view of a flat-mounted MHD, all other panels show a bright field view (left, red and blue labelling) and the contralateral fluorescence view (right, red labelling only) of flat-mounted neural plates. (F,L,R) Corresponding schematics of genes expression profiles (including data not shown) at 75% epiboly, tail bud and five somites, respectively. Note that anteriorly, *bts1* expression never extends to the presumptive diencephalon (compare A with B), and that it crosses the caudal border of *otx2* expression at all stages.

proven to reliably and selectively inhibit RNA translation in many instances in *Xenopus* as well as in the zebrafish embryo (Heasman et al., 2000; Nasevicius and Ekker, 2000; Yang et al., 2001). A morpholino targeting the translation initiation site of *bts1* mRNA was designed (MO^{bts1}) and injected into a central blastomere of the 16-cell zebrafish embryo together with a tracer MO (MO^{ctrl}) (see Material and Methods). At the same concentration, a four base-pair mismatch control MO ($MO^{bts1}\Delta4$) of unrelated sequence had no effect (n=32) (Fig. 6D). In all embryos injected with MO^{bts1} across the MHD (n=23) and observed at the tail bud stage, a strong reduction of *pax2.1* expression was observed (Fig. 6A) (lineage tracing experiments often revealed a unilateral and patchy distribution of the injected cells; accordingly, *pax2.1* expression was most often diminished on only one side of the neural plate). To determine whether *bts1* expression was necessary to induce and/or maintain *pax2.1* expression, we performed a timecourse analysis of the effect of the MO^{bts1}. We observed that *pax2.1* expression was abolished from its onset (90% epiboly) (n=13, Fig. 6B), indicating that *bts1* is necessary for *pax2.1* induction. Some *pax2.1*-expressing cells were always retained. Their varying number and distribution in each embryo (see Fig. 6A,B) suggests that these cells were most likely not or poorly targeted by the injection. Co-detection of *pax2.1* expression and MO^{ctrl} confirmed this hypothesis as cells maintaining *pax2.1* transcripts do not stain for MO^{ctrl} (Fig. 6C). Therefore, Bts1 appears necessary in all MHD cells for *pax2.1* induction.

However, at the concentrations of MO^{bts1} used, *pax2.1* expression was progressively recovered between the five- and ten-somite stages (n=26) (see Fig. 6I,J), and brain development appeared normal at late somitogenesis stages (not shown).

Taken together, our results reveal that *bts1* expression is sufficient to induce ectopic expression of *pax2.1* in the neural plate anterior to the MHD, and is necessary for the induction and early maintenance of *pax2.1* expression in the MHD. Thus endogenous Bts1 may be an early regulator of *pax2.1* expression, a conclusion supported by its expression profile (Fig. 4).

Distinct requirements of mid-hindbrain markers for *bts1* expression

We next examined whether MHD genes other than *pax2.1* require *bts1* for their expression. Upon injection of MO^{bts1} within the embryonic neural plate, the expressions of *her5*, *otx2*, *fgf8*, *wnt1*, *eng2* and *krox20* were never affected (Fig. 6E-H and data not shown). By contrast, expression of *eng3* and

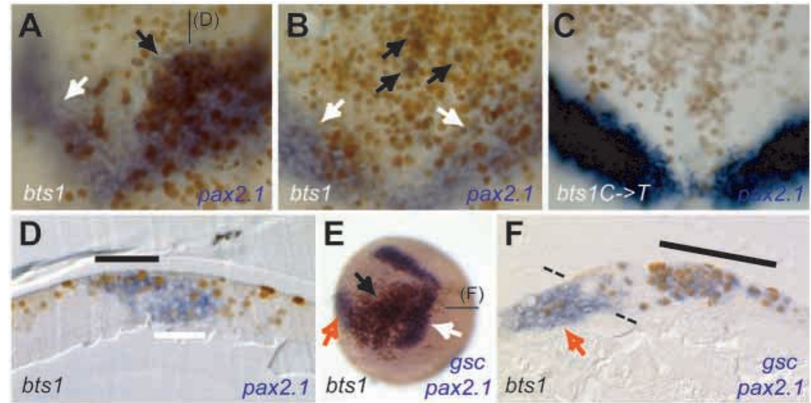

Fig. 5. Bts1 is sufficient to induce *pax2.1* expression in the anterior neural plate. *pax2.1* (A-D) or *pax2.1* and *gsc* (E,F) expression revealed by whole-mount in situ hybridization (blue staining) at the tail bud stage on embryos injected with (A,B,D-F) wild-type *bts1* RNA, or (C) mutant *bts1*$^{C\text{->}T}$ RNAs, as indicated (bottom left of each panel). (A-C) High magnifications of the MHD in flat-mounted embryos, anterior towards the top. (E) A whole-mount view, anterior towards the left. (D,F) Sections of the embryos in A,E (respectively) at the levels indicated, anterior towards the left. The red arrows in E,F point to *gsc* expression, and the broken line in F delimits the anterior mesendoderm/neural plate border. All injections were made into in one central blastomere of the 16-cell embryo, leading to a mosaic distribution of the injected RNA in the presumptive neural plate (see expression of the β-galactosidase tracer (brown nuclei) and in particular D,F). Misexpression of *bts1* induces ectopic *pax2.1* expression (black arrows in A,B,E, black bars in D,F) anterior to the MHD (endogenous *pax2.1* expression is indicated by the white arrowheads or white bars), in broad patches (A,D-F) or in scattered cells (B). Mutant *bts1* RNAs (C, and data not shown) have no effect.

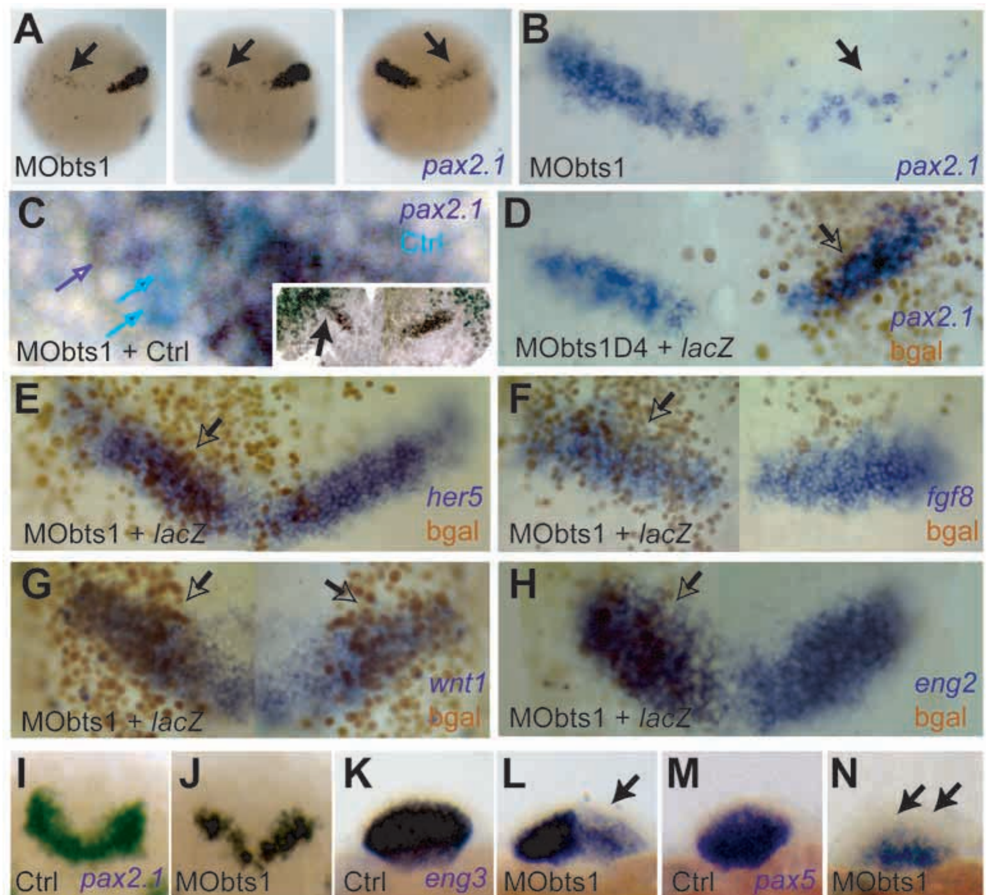

Fig. 6. Bts1 is necessary to the expression of *pax2.1* and its dependent cascade in the MHD. (A-D) *pax2.1* expression revealed by whole-mount in situ hybridisation (purple) at 90% epiboly (B) or tail bud (A,C,D) after injection of MO^{bts1} (A-C) or the mismatch control $MO^{bts1}\Delta4$ (D). All injections were made at the 16-cell stage into one central blastomere. (C) A biotinylated control MO of unrelated sequence co-injected as a tracer (turquoise staining) to monitor the exact distribution of targeted cells (turquoise arrows) compared with *pax2.1*-expressing cells (purple arrows); the area shown is a high magnification of the domain indicated by the black arrow in the inset. (D) *nls-lacZ* RNA used as a tracer to reveal the targeted area (brown staining). (A) Whole-mount views; (B-D) flat-mounts, anterior towards the top; arrows point to injected areas (affected and unaffected expression are indicated by filled and open arrows, respectively). Note that the injection of MO^{bts1}, but not $MO^{bts1}\Delta4$, strongly diminishes the number of *pax2.1*-positive cells from the onset of *pax2.1* expression (B), and that cells maintaining *pax2.1* expression have not been targeted by the injection (C). (E-H) Expression of *her5* (E, tail bud), *fgf8* (F, tail bud), *wnt1* (G, one to two somites) and *eng2* (H, three somites) upon injection of MO^{bts1} (conditions as in D). Note that these expression are unaffected. (I-N) Expression of *pax2.1* (I,J), *eng3* (K,L) and *pax5* (M,N) at the five-somite stage upon injection of control MO or MO^{bts1}, as indicated. (I,J) Dorsal views, anterior towards the top; (K-N) Optical coronal sections, dorsal towards the top. Note that at five somites, the *pax2.1*-dependent markers *eng3* and *pax5* are also affected.

pax5, starting at the three- and five-somite stages, respectively, were transiently inhibited from their onset until approximately the 10-somite stage (Fig. 6K-N). Thus, first, the territories located anterior and posterior to the MHD do not require Bts1 for their early development. Second, at least two initially independent early gene regulatory pathways operate within the MHD: one requires Bts1 and permits the induction of *pax2.1* expression, and the other is independent of Bts1 and leads to the induction of expression of *her5*, *wnt1*, *eng2* and *fgf8*. Whether *pax5* and *eng3* expressions are directly regulated by

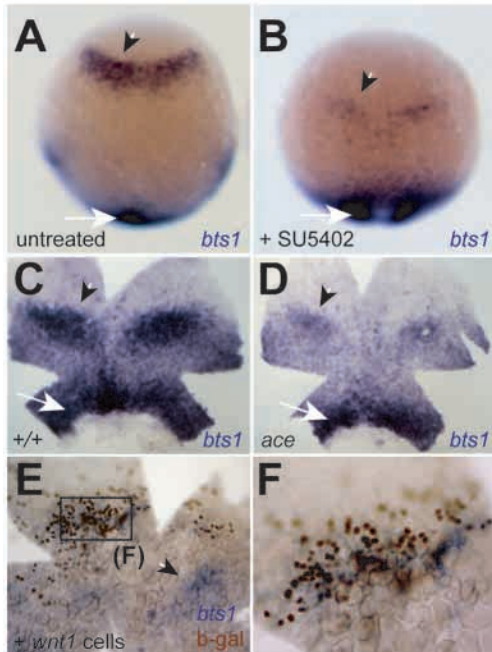

Fig. 7. *bts1* expression at the MHD during gastrulation requires Fgf8 and is activated by Wnt signalling. (A,B) Whole-mount dorsal views of *bts1* expression at the tail bud stage, anterior to the top, without (A) or after (B) treatment with the inhibitor of Fgf signalling SU5402 between the stages dome and tail bud. Note the strong reduction in expression at the mid-hindbrain in B (arrowhead), while expression at the blastoderm margin is not affected (white arrow). (C,D) Flat-mounted views of *bts1* expression in the mid-hindbrain area at the 90% epiboly stage, anterior towards the top, in wild-type (+/+) (C) versus *ace* homozygous mutants (D), as indicated. Mid-hindbrain expression of *bts1* is strongly reduced and maintained only laterally (arrowheads); it remains unperturbed at the blastoderm margin (white arrow). (E,F) *bts1* expression in embryos grafted with *wnt1*-expressing cells within the anterior neural plate. Endogenous *bts1* expression at the MHD is indicated by the arrowhead. Grafted cells were co-injected with *nls-lacZ* RNA and are visualized by anti-β-galactosidase immunocytochemistry (brown nuclei). (F) A high magnification of the grafted area (boxed in E). *bts1* expression is induced around *wnt1*-expressing cells.

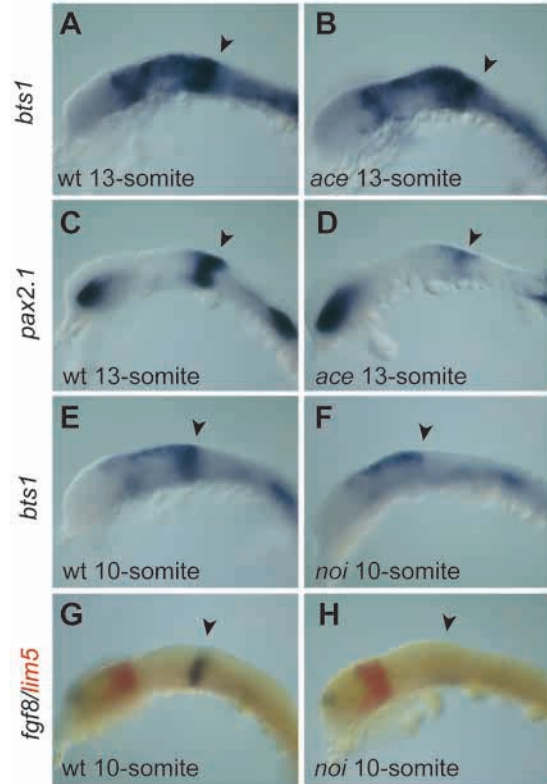

Fig. 8. *bts1* expression during somitogenesis distinguishes Pax2.1 and Fgf8 functions. (A-D) Comparison of *bts1* and *pax2.1* expression in wild-type (left) or *ace* mutant (right) embryos at the 13-somite stage. The MHD is indicated by the arrowhead. *bts1* expression is unperturbed in *ace* (B), when most *pax2.1* expression has already been eliminated (D). (A,B) *ace* embryos identified by their reduced otic vesicles, which also express *bts1* (not visible on the figure). (E-H) Comparison of *bts1* and *fgf8* expression in wild-type (left) or *noi* mutant (right) embryos at the 10-somite stage. *bts1* expression is strongly diminished following the same schedule as other MHD markers (e.g. *fgf8*). *lim5* expression (red) is unperturbed.

Bts1 cannot be immediately concluded from our data, as *pax5* and *eng3* expressions require Pax2.1 at all stages (Lun and Brand, 1998; Pfeffer et al., 1998).

bts1 expression at gastrulation responds to Fgf and Wnt signalling

The crucial role of Bts1 as a selective regulator of *pax2.1* within the neural plate prompted us to investigate the mechanisms regulating its own expression.

Fgf3 and Fgf8 are expressed at the blastoderm margin during gastrulation (Fürthauer et al., 1997; Koshida et al., 1998; Reifers et al., 1998) and the reception of an Fgf signal by marginal cells has been indirectly implicated in the posteriorization of the adjacent neural plate (Koshida et al., 1998). To determine whether *bts1* expression was influenced by Fgfs during gastrulation, we examined its response to SU5402, a general inhibitor of Fgf signalling (Mohammadi et al., 1997). Incubation of embryos in SU5402 from the dome stage onwards lead to a strong reduction of *bts1* expression at the presumptive MHD (Fig. 7A,B). Thus, during gastrulation, *bts1* expression within the neural plate depends on Fgf signalling. By contrast, expression of *bts1* at the blastoderm margin (or later in the tail bud, Fig. 7A,B) remained unaffected by SU5402 treatments. To determine which combination of Fgf3 and Fgf8 might be involved in the early regulation of *bts1* expression in the MHD, we examined *bts1* expression in *acerebellar* (*ace*) mutants, which are solely deficient in Fgf8 function (Reifers et al., 1998). At the 90% epiboly stage, *bts1* expression in the presumptive MHD was severely reduced in 25% of embryos from a cross between two *ace/+* parents (n=63) (Fig. 7C,D). Thus, *bts1* expression in the presumptive MHD at gastrulation probably requires Fgf8 signalling, originating from the hindbrain territory or marginal cells (see Reifers et al., 1998). Whether this signal acts directly within the neural plate or via patterning the embryonic margin cannot be ascertained at this point.

bts1 expression was never totally abolished in the absence of Fgf signalling, however, suggesting that additional factors contribute to regulating its expression. As Wnt molecules are produced both at the embryonic margin (Wnt8) (Kelly et al.,

1995) and at the mid-hindbrain junction (Wnt1, Wnt8b) (Molven et al., 1991; Kelly et al., 1995), we tested whether *bts1* expression was also responsive to Wnt signalling. Capped mRNA encoding the mouse Wnt1 protein (van Ooyen and Nusse, 1984) was injected at the one-cell stage into donor embryos, and five to ten cells taken at the sphere stage from the animal pole of these donors were homotopically transplanted into non-injected recipients. At 80% epiboly, 50% of grafted embryos (*n*=38) had received Wnt1-expressing cells within the neural plate anterior to the MHD, i.e. in a region normally not expressing *bts1*. In 30% of these embryos, *bts1* expression was induced around the grafted cells (Fig. 7E,F). Mouse Wnt1 is likely to have the same activity as zebrafish Wnt1, as embryos injected at the one-cell stage displayed a strong headless phenotype (not shown) characteristic of enhanced zebrafish Wnt signalling (Kim et al., 2000). Thus, ectopic Wnt signalling can positively regulate *bts1* expression within the neural plate, and the expression of endogenous *bts1* might also depend on Wnt factors produced at the embryonic margin and/or within the MHD during gastrulation and somitogenesis. Again, this regulation might or not occur directly within the neural plate.

The maintenance of *bts1* expression is differently affected by Pax2.1 and Fgf8 functions

In agreement with the early onset of *bts1* expression in the prospective MHD area, we found that the initiation of *bts1* expression was not affected in *pax2.1/noi* mutant embryos (Lun and Brand, 1998), and thus was independent of Pax2.1 function (not shown). However, the maintenance of *bts1* expression in the MHD during somitogenesis appeared dependent on *pax2.1/noi*: it was gradually lost from the five-to six-somite stage onwards in *noi* homozygous embryos, and disappeared completely by the 10-somite stage (Fig. 8E,F), following the same schedule as other mid-hindbrain markers (see Fgf8 on Fig. 8G,H; Lun and Brand, 1998). The maintenance of expression of all MHD genes studied to date was shown to be also dependent on Fgf8/ace function, within a similar time frame (between the five- and ten-somite stages), suggesting that Fgf8 and Pax2.1 are involved in a common regulatory loop that controls MHD maintenance (Lun and Brand, 1998; Reifers et al., 1998). Thus, surprisingly, we found that following a transient decrease at gastrulation (Fig. 7) *bts1* expression was not affected in *fgf8/ace* mutant embryos at somitogenesis until late stages. At 13 somites, *bts1* expression was normal (Fig. 8A,B), while the lateral and ventral expression domains of other markers were already absent (see *pax2.1* on Fig. 8C,D; Reifers et al., 1998). *bts1* expression started to decline around the 17-somite stage, and was undetectable at 20 somites (not shown). This downregulation might parallel the loss and/or transformation of mid-hindbrain tissue, which is likely to start around that stage. Thus, while *bts1* maintenance depends on Pax2.1, it appears primarily independent of Fgf8 function, suggesting that exit points exist in the Pax2.1/Fgf8 loop to differentially control the expression of some MHD genes.

DISCUSSION

In this study, we relied on the comparable locations of the

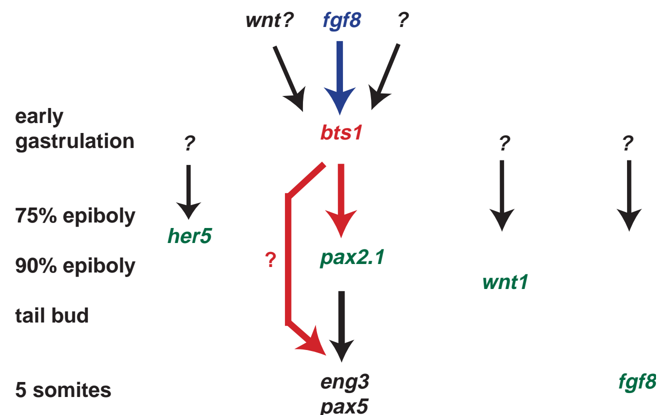

Fig. 9. A model of MHD induction incorporating Bts1 function. Evidence in all vertebrates suggest that the expression of early MHD markers (*her5*, *pax2.1*, *wnt1* and *fgf8*) (green) is established by following independent pathways. Bts1 (red) is a selective inducer of *pax2.1* expression, and its own expression depends on Fgf8 signalling (blue). Other factors regulating *bts1* expression might include Wnt molecules. In turn, Pax2.1 induces *eng3* and *pax5*. Bts1 might also directly regulate the expressions of *eng3* and *pax5* (red arrows).

Drosophila head-trunk junction and of the vertebrate mid-hindbrain within the embryonic body plan to identify candidate regulators of early mid-hindbrain development. In *Drosophila*, Btd is expressed at the head-trunk junction and the zebrafish Btd-related factor Bts1 is an early marker of the MHD. We demonstrate that, in the zebrafish, Bts1 is both necessary and sufficient for the induction of *pax2.1* expression within the anterior neural plate and is expressed at the appropriate time and place during development to exert such a role. We therefore move one step upstream in our understanding of MHD specification by identifying the first known selective and early regulator of *pax2.1* expression (Fig. 9). In addition, our results have important evolutionary implications. They suggest that flies and vertebrates have probably evolved a similar mechanism to cope with the patterning of a hinge region of the embryo, by restricting to these territories the expression of a Btd/Sp factor.

Identification of a large family of *btd-Sp*-related genes in the zebrafish

Our study has revealed the existence of a family of at least eleven zebrafish Bts proteins, related to *Drosophila* Btd and to Sp factors. Stringent in situ hybridisation revealed, for most genes, distinct expression profiles, highly specific of a subset of embryonic structures. Thus, these different Bts factors might take part in a restricted number of non-overlapping developmental processes. Within this family, *Drosophila* Btd and Sp1 and five mammalian Sp factors are known to date. Thus, it is likely that many more members remain to be discovered in mammals. *Drosophila Sp1* and mammalian *Sp1-Sp4* are widely expressed, and Sp1-Sp4 transregulate a multitude of promoters, thereby controlling cellular activities as general as cell cycle progression and growth control (Fridovich-Keil et al., 1991; Kingsley and Winoto, 1992; Hagen et al., 1994; Hagen et al., 1995; Karlseder et al., 1996; Lin et al., 1996; Supp et al., 1996; Zwicker et al., 1996; Jensen et al., 1997) or nuclear architecture (Jongstra et al., 1984;

Philipsen et al., 1993); *Sp5* expression is in contrast very dynamic (Harrison et al., 2000; Treichel et al., 2001). *bts1* is in sequence most closely related to mouse *Sp5*; the two genes also share strong expression in the presumptive midbrain, and a similar map location (*Sp5* lies close to *Hoxd* genes on chromosome 2, a region syntenic to the *hoxd* locus on zebrafish linkage group 9). However, the orthology of *bts1* and *Sp5* is questionable, as outside a few conserved domains, Bts1 and Sp5 sequences are highly divergent (30% deduced amino acid identity). The proline-rich N-terminal half of SP5, proposed to have evolved by domain swapping from BTEB/KLF family members (Treichel et al., 2001), is not identifiable in Bts1. Rather, in Bts1, S/T- and Q-rich domains like in Sp1-4 have been maintained. Further, *bts1* and *Sp5* expressions do not always coincide, and these genes seem to exert different roles during embryogenesis. Indeed the genetic disruption of *Sp5* did not cause brain patterning defects in mouse embryos (Harrison et al., 2000). A definite answer on the possible orthology of *bts1* and *Sp5* will await availability of more sequence information on the zebrafish genome.

Btd and all Sp factors isolated to date bind GC-rich promoter sequences (GC-box; Dynan and Tjian, 1983; Gidoni et al., 1984; Gidoni et al., 1985), and we have shown that Bts1 was capable of recognizing such a motif with an affinity similar to Sp1. The specificity of action of Sp factors has been proposed to arise from the non DNA-binding modules of the proteins, which may interact with different molecular partners (Courey and Tjian, 1988; Kadonaga et al., 1988; Schöck et al., 1999a; Schöck et al., 1999b). In addition, multiple protein isoforms can derive from a single Sp gene and differ in their capacity to activate or repress transcription in a similar cellular context (Kennett et al., 1997). We have used an in vivo system, the *Drosophila* embryo, to determine the properties of Bts1 as a transcriptional regulator. Our results demonstrate that Bts1 is capable of activating the expression of *col*, an immediate downstream target of Btd, suggesting that Bts1, like Btd, acts as an transcriptional activator. This conclusion is in agreement with our finding that in the zebrafish, the initiation of expression of *pax2.1* rapidly follows *bts1* expression at the MHD and is positively dependent upon Bts1 function.

bts1 expression and specification of the mid-hindbrain territory

The earliest known mid-hindbrain-specific markers of the zebrafish neural plate are expressed after mid-gastrulation (75% epiboly). Before that stage, AP regional markers within the neural plate rather cover broad anterior or posterior territories. Until now, the most extended caudal marker was *hoxa-1*, in the spinal cord and rhombencephalon up to the presumptive location of rhombomere 3 (Koshida et al., 1998). This left a gap of more than 10 cell rows between the *otx2*- and *hoxa1*-positive domains (Koshida et al., 1998; A. T. and L. B.-C., unpublished). At 75% epiboly, *bts1* expression overlaps entirely that of *hoxa1* (not shown), and slightly the caudal limit of *otx2* expression. Thus, *bts1* is the first gene expressed in this intermediate territory, which at 75% epiboly would cover most of the presumptive MHD, as it abuts the presumptive diencephalon identified by *fkh3* expression (Varga et al., 1999). In other vertebrates, the anteriormost posterior marker during gastrulation is the homeobox gene *Gbx2* (Wassarman et al., 1997), which precisely abuts *Otx2* from the end of gastrulation and labels the anterior hindbrain. We found that the rostral limit of *bts1* was at all stages anterior to that of zebrafish *gbx* genes (A. T. and L. B.-C., unpublished).

Our observations further suggest that mid-hindbrain identity is progressively established after mid-gastrulation. Indeed, until late gastrulation, gene expression boundaries in this domain move relative to each other. While newly expressed mid-hindbrain-specific markers align with *bts1*, the caudal limit of *otx2* expression is displaced caudally relative to the *bts1* domain. In the mouse and chick, the caudal border of *Otx2* expression is believed to position the mid-hindbrain junction and to encode midbrain fate. Thus, our expression data suggest that mid- and anterior hindbrain identities are progressively established and refine until late gastrulation. These results are in agreement with the finding that the embryonic margin exerts a posteriorizing activity on hindbrain cells until late gastrulation (Woo and Fraser, 1997; Woo and Fraser, 1998). By contrast, presumptive mid-hindbrain cells transplanted into the prospective forebrain at 55% epiboly are capable of maintaining their fate (Miyagawa et al., 1996).

The factors involved in mid-hindbrain induction remain mostly unknown. In the zebrafish, as in other vertebrates, a combination of vertical and planar signals is likely to operate during gastrulation to specify this territory. The anterior hypoblast of the late zebrafish gastrula has the capacity to induce *pax2.1* expression within the neural plate (Miyagawa et al., 1996). In addition, Fgf signalling received by marginal cells is necessary to posteriorize the neural plate and position the borders of *otx2* and *hoxa1* expressions (Koshida et al., 1998). We extended these findings by showing that the mid-hindbrain component of *bts1* expression at gastrulation is (directly or indirectly) dependent on Fgf8 signalling, originating either from the hindbrain territory or from the embryonic margin (Reifers et al., 1998). However, the role of Fgf8 on *bts1* expression is transient, as *bts1* expression is restored in *ace* mutants from the tail bud stage. Other factors, not affected in *ace*, might relay Fgf8 in its regulation of neural plate patterning at that stage. Given the crucial role of Bts1 in the activation of *pax2.1* expression and of the subsequent Pax2.1-dependent cascade, this rescue of *bts1* expression might explain why early mid-hindbrain development still continues normally in *ace* mutants. Our findings additionally imply that, contrary to previous assumption, early stages of mid-hindbrain development are affected (albeit indirectly) in *ace* mutants. The defects are, however, rapidly compensated for.

Bts1 is an early regulator of *pax2.1* expression and the Pax2.1-dependent molecular cascade

To date, no zebrafish mutants were mapped to the *bts1* locus. We thus addressed Bts1 function by combining gain- and loss-of-function approaches. The specificity of our manipulations is supported by the selective and opposite effects of *bts1* and MO^{bts1} injections on *pax2.1* expression. Taken together, our results identify Bts1 as the first known factor that selectively controls *pax2.1* induction and the immediate Pax2.1-dependent cascade at gastrulation and early somitogenesis, and refine our molecular picture of MHD induction (Fig. 9).

It is most probable that, upon MO^{bts1} injection, enough non-targeted mid-hindbrain cells remained to progressively

reorganize on-site a complete MHD, after the initial perturbations, which explains our transient phenotypes. A requirement for Bts1 at later stages of mid-hindbrain development, such as during the maintenance phase, is suggested by its persistent expression within the mid-hindbrain territory during somitogenesis. Further analyses will be necessary to directly address this issue.

Our lineage tracings in Bts1 misexpression experiments strongly suggest that Bts1 acts primarily within the neural plate. The fact that *pax2.1* induction is not observed in all ectopic *bts1*-expressing cells in the anterior neural plate, however, might indicate an indirect effect and/or that additional factors or a community phenomenon must reinforce Bts1 activity. It will be most interesting to determine whether Bts1 directly binds and transactivates the *pax2.1* promoter.

Finally, we show that Bts1 can only induce *pax2.1* expression in territories anterior to the MHD. These results suggest that Bts1 needs to act in conjunction with spatially restricted molecular partners to induce *pax2.1* expression, and/or needs to be alleviated from the dominant influence of a posterior inhibitor. It will be of interest to determine which local factors are necessary to potentiate or inhibit Bts1 activity.

bts1 expression and the mid-hindbrain maintenance phase

During mid-hindbrain maintenance, expression of the different mid-hindbrain markers become interdependent. In zebrafish *pax2.1/noi*^{tu29a} mutants, all mid-hindbrain markers, including *fgf8*, are completely downregulated between the 5- and 14-somite stages (Lun and Brand, 1998). In *fgf8/ace* mutants, all markers tested, including *pax2.1*, also begin to be affected at a similar stage (Reifers et al., 1998). These results point to a regulatory loop involving Pax2.1 and Fgf8 functions during mid-hindbrain maintenance. However, the mid-hindbrain phenotypes of *noi* and *ace* mutants are clearly different, in particular as regards *bts1* expression. Indeed in *noi* mutants *bts1* expression is affected and completely downregulated within the same time-frame as other mid-hindbrain markers, whereas it remains unperturbed in *ace* until late somitogenesis. The most likely explanation for this finding is that *bts1* expression is only transiently dependent on Pax2.1, requiring Pax2.1 function at early somitogenesis only but not after the five- to ten-somite stage. Enough Pax2.1 activity would be spared in *ace* mutants until that stage to allow for *bts1* maintenance. Thus, our results highlights the existence of mid-hindbrain markers that only transiently require, and then escape, the Pax2.1/Fgf8 regulatory loop (see also Reifers et al., 1998).

Functional characteristics of Bts1 and their evolutionary implications

Our experiments have allowed us to test the starting hypothesis that factors expressed at the *Drosophila* head-trunk and vertebrate mid-hindbrain junctions would be conserved during evolution. This hypothesis was based on previous reports that documented the expression of homologous genes of the *otd/Otx*, *engrailed/En* and *pax2/5/8* families at equivalent AP levels in urochordate, vertebrate and insect embryos (Wada et al., 1998; Wurst and Bally-Cuif, 2001). We found that Bts1 and Btd do share some functional characteristics, as Bts1 could rescue the expression of *col* in a correct spatiotemporal manner in *btd* mutants. We observed that Bts1 was neither capable of rescuing the expression of *eve* and *en* nor the formation of posterior head structures in *btd* mutants. Under similar conditions, Sp1 could partially restore *en* expression and mandibular derivatives (Wimmer et al., 1993; Schöck et al., 1999a; Schöck et al., 1999b). As a chimeric protein composed only of the SP1 zinc finger fused to the activation domain of VP16 also rescues *en* expression (Schöck et al., 1999b), and given the conservation of Bts1 and Sp1 zinc fingers, Bts1 might simply not have sufficient activity to transactivate the *en* promoter. A similar hypothesis might hold true for the failure of both Bts1 and Sp1 to sustain the development of intercalary and antennal segments (Wimmer et al., 1993; Schöck et al., 1999b). Alternatively, in these processes, Btd might need to interact with cofactors incapable of recognizing the divergent non DNA-binding modules of Bts1 and Sp1.

Taken together, our results indicate that Btd and Bts1 share expression and function characteristics in their control of the development of a comparable boundary region of the embryo. *btd* and *bts1* might have diverged from a common ancestor involved in the development of posterior head territories, or might have been co-opted during evolution in the fly and in vertebrates. We favour the second hypothesis, as Bts1 is more related in sequence to the extant subfamily of Sp factors, including *Drosophila* Sp1, than to the Btd subfamily (which comprises zebrafish members such as our clone g5). Our results therefore have interesting evolutionary implications as they strongly suggest that flies and vertebrates, by restricting to the head-trunk or mid-hindbrain junction the expression and functional domain of a Btd/Sp-family member, have independently developed a similar strategy to pattern comparable territories. Whether Bts1 and Btd are part of a conserved molecular cascade awaits further analysis; we note, for example, that *col* has no vertebrate homologue expressed at the mid-hindbrain junction (Garel et al., 1997; Bally-Cuif et al., 1998; Dubois et al., 1998).

Finally, Bts1 might be an interesting tool to approach other evolutionary questions. For example, the existence or the secondary loss of a MHD-like territory in cephalochordates have been questioned, based on the non-expression of *Pax2/5/8* and on the late onset of expression of *en* homologues at this AP level in *Amphioxus* (Holland et al., 1997; Kozmik et al., 1999). *Amphioxus bts1*, as it acts upstream of the 'traditional' MHD maintenance loop that involves Pax and En, might help resolve this issue.

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