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 rhombomere 1 (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<sup>−/−</sup> and Gbx2<sup>−/−</sup> mutants (Acampora et al., 1998; Wassermann 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 transplantsations in the zebrafish showed that engrailed 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 (Molvén 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 Sp factors (Kadonaga et al., 1987; Kingsley and Winoto, 1992; Pieler and Belferfroid, 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<sub>al262</sub> or noi<sub>al262</sub> (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(T/C)CA(C/T)AT(C/T)(G/A)(A/G)GTTTGTC(T/G)G3;

Btd-F2 5'CIC(A/C)(T/C)TTI(A/T)(C/G)TG(T/C)TTI(G/C)T3;

Btd-R 5'TGGT(C/T)TTI(A/T)(C/G)A(G)TG(T/C)TTI(G/C)T3;

Btd-F1 5'CIC(A/C)(T/C)TTI(A/T)(C/G)TG(T/C)TTI(G/C)T3;

Btd-F2 5'CIC(A/C)(T/C)TTI(A/T)(C/G)TG(T/C)TTI(G/C)T3;

Btd-R 5'TGGT(C/T)TTI(A/T)(C/G)A(G)TG(T/C)TTI(G/C)T3;

Btd-F1 5'CIC(A/C)(T/C)TTI(A/T)(C/G)TG(T/C)TTI(G/C)T3;

Btd-R 5'TGGT(C/T)TTI(A/T)(C/G)A(G)TG(T/C)TTI(G/C)T3;

For cloning of cDNAs F1, 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, 1 minute at 72°C (2 cycles), 1 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, 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 *bts>bts* transgene, the *bts*-coding region is separated from the *btd* promoter by a flip-out cassette containing lacZ. *btd >AB>bts* was constructed by inserting a 2659 bp NorI-ClaI fragment containing the entire *bts*-coding region and 1387 bp 5′UTR into the *btd >AB>btd* plasmid (Wimmer et al., 1997) open at NorI, and used to generate transgenic fly lines (Rubin and Spradling, 1982). The stock *bts-tub-flpY; btd >AB>bts1 /TM3, hh-lacZ* was established and crossed with *btdGSGI/FM7, ftc-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 (5′pfl fragment from pBS-bts1) subcloned into pXT7 (Dominguez et al., 1995). Mutant forms *bts1ΔZfN* and *bts1ΔZfY* were constructed with the Stratagene Ex Site™ PCR-based site-directed mutagenesis Kit using the following oligonucleotides:

1. *bts1ΔZfF*: ONbts1ΔZfF1, 5′-GATGTGCTGTCTCTCTTCCGGGCTC-3′; ONbts1ΔZfF2, 5′-CAGAACAAAGAAGGACAAAAATCGACGACAAA-3′

2. *bts1ΔZfY*: ONbts1ΔZfY1, 5′-AGTCCGGGACACAAAAGCCTTTTCCGC-3′; ONbts1ΔZfY2, 5′-AC4AAGAAGGACCAATTG-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 +Tyr in this domain.

**Preliminary screening of transgenic lines**

Restriction and distinct expression patterns (Fig. 1A), further confirmed 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-His 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.

**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-His 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).

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**RESULTS**

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**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 2465 bp NorI-ClaI fragment containing the entire *bts*-coding region and 1387 bp 5′UTR into the *btd >AB>btd* plasmid (Wimmer et al., 1997) open at NorI, and used to generate transgenic fly lines (Rubin and Spradling, 1982). The stock *bts-tub-flpY; btd >AB>bts1 /TM3, hh-lacZ* was established and crossed with *btdGSGI/FM7, ftc-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*.

**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 iso-schizomorphically 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 (Thissie et al., 1993; Hauptmann and Gerster, 1994).

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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
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-
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. bts1DZnF 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, bts1C->T, was designed to mimic the btd loss-of-function mutation in Drosophila (see Materials and Methods). bts1DZnF- and bts1C->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
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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*D4) 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. 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.
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
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 unperturbed 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.,...
In 30% of these embryos, within the neural plate anterior to the MHD, i.e. in a region of grafted embryos (transplanted into non-injected recipients. At 80% epiboly, 50% 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
Philippsen 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 homeotic 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 Ghx2 (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\textsuperscript{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\textsuperscript{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\textsuperscript{nu29a} 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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REFERENCES


