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Negative Autoregulation of *Mash1* Expression in CNS Development

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***Mash1*, a neural-specific bHLH transcription factor, is essential for the formation of multiple CNS and PNS neural lineages. Transcription from the *Mash1* locus is elevated in mice null for *Mash1*, suggesting that MASH1 normally acts to repress its own transcription. This activity is contrary to the positive autoregulation of other proneural bHLH proteins. To investigate the mechanisms involved in this process, sequences flanking the *Mash1* gene were tested for the ability to mediate negative autoregulation. A *Mash1/lacZ* transgene containing 36 kb of *cis*-regulatory sequence exhibits an increase in *lacZ* expression in the *Mash1* mutant background, which phenocopies the observation of transcriptional autoregulation at the endogenous *Mash1* locus. Using *Mash1/lacZ* lines with progressively less *cis*-acting sequence, autoregulatory responsive elements were demonstrated to colocalize with a previously characterized 1.2-kb CNS enhancer. Mutations of E-box sites within this enhancer did not result in an apparent loss of autoregulation, suggesting that MASH1 does not directly repress its own transcription. Interestingly, these mutations did not indicate any underlying positive auto- or cross-regulation of *Mash1*. Furthermore, the loss of autoregulation in the *Mash1* mutant background is reminiscent of a loss of lateral inhibitory signaling. However, mutations in HES consensus sites, the likely purveyors of Notch-mediated lateral inhibition, do not support a role for these sites in negative autoregulation. We hypothesize that MASH1 normally inhibits its own expression indirectly, possibly through a HES-mediated repression of positive regulators or through novel HES binding sites.** © 2000 Academic Press

Key Words: **bHLH; transcription factor; lateral inhibition; HES; transgenic mice; gene regulation.**

INTRODUCTION

Members of the basic helix-loop-helix (bHLH) family of transcription factors have been demonstrated to play essential roles in the differentiation of hematopoietic, muscle, and neural lineages (Arnold and Winter, 1998; Lee, 1997). The proneural *achaete-scute* complex (AS-C) and *atonal* genes in *Drosophila* have been studied extensively, along with their homologs in *Xenopus*, chicken, and mouse (Jan and Jan, 1993; Kageyama *et al.*, 1995; Lee, 1997). A neural-specific subclass of these bHLHs, including *Mash1*, *Math1*, *Neurogenin1* (*Ngn1*), and *Neurogenin2* (*Ngn2*), is expressed in populations of proliferating and early differentiating neural precursors in complementary domains of the developing vertebrate nervous system (Lee, 1997). Loss-of-function studies of these genes demonstrated their essential roles in the formation of subsets of neurons in the CNS and PNS (Ben-Arie *et al.*, 1997; Bermingham *et al.*, 1999; Fode *et al.*, 1998; Guillemot *et al.*, 1993; Ma *et al.*, 1998). For example, embryos mutant for *Mash1* (mammalian achaete-scute homolog-1) exhibit loss of cells in multiple neural lineages, including olfactory and autonomic systems, neuroendocrine cells in the thyroid and lung, noradrenergic cells in the locus coeruleus, bipolar cells in the retina, and neurons in the ventral telencephalon (Borges *et al.*, 1997; Casarosa *et al.*, 1999; Guillemot *et al.*, 1993; Hirsch *et al.*, 1998; Horton *et al.*, 1999; Lanigan *et al.*, 1998; Lo *et al.*, 1998; Tomita *et al.*, 1996; Torii *et al.*, 1999; Tuttle *et al.*, 1999).

As evidenced by the diverse array of neurons lost in the *Mash1* mutant, *Mash1* is expressed in a complex temporal and spatial pattern of the developing mouse nervous system. Little is known about the direct upstream regulators of *Mash1* expression; however, transgenic studies have identified *cis*-regulatory elements spanning >36 kb that are important for expression in the CNS and PNS (Verma-Kurvari *et al.*, 1996). Further delineation of these sequences led to the identification of a 1.2-kb CNS enhancer located 7 kb 5′ of the coding region, and the information contained within this relatively small region supports the correct

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temporal and spatial expression of reporter transgenes in several domains of the endogenous *Mash1* CNS pattern (Verma-Kurvari *et al.,* 1998). Mutations and deletions within the 1.2-kb region demonstrated the presence of multiple enhancer and repressor elements.

By comparison, the AS-C locus in *Drosophila* has >100 kb of associated regulatory sequence and contains numerous discrete enhancers that control expression in specific proneural clusters (Modolell, 1997). A handful of upstream regulators of *achaete* and *scute* have been identified including *pannier* (Ramain *et al.,* 1993), *ventral nervous system defective* (Skeath and Carroll, 1994), *hairy* (Ohsako *et al.,* 1994; van Doren *et al.,* 1994), *araucan* and *caupolican* (Gomez-Skarmeta *et al.,* 1996), *U-shaped* (Cubadda *et al.,* 1997), and the *enhancer of split* (Heitzler *et al.,* 1996; Nakao and Campos-Ortega, 1996) genes. Only some of these proteins have been demonstrated to directly bind *achaete* sequence. Additionally, *achaete* and *scute* have been shown to directly auto- and cross-regulate each other's expression, although the relevance of cross-activation *in vivo* has been questioned (Ruiz-Gomez and Ghysen, 1993). Positive autoregulation has been proposed to be important for emergence of the sensory mother cell (SMC) from a proneural cluster, and accumulation of high levels of the proneural bHLH protein is the first signal of commitment of the SMC (van Doren *et al.,* 1992). Direct positive autoregulation of expression of another *Drosophila* proneural gene, *atonal,* and one of its mammalian homologs, *Math1,* has been demonstrated by the loss of enhancer/promoter transgene expression in mutant backgrounds (Helms *et al.,* 2000; Sun *et al.,* 1998). These experiments clearly reveal a large component of positive autoregulation in the control of neural bHLH expression.

In addition to the direct positive autoregulation of neural bHLH factor expression, a role for indirect inhibition of proneural gene expression mediated by the *Notch* pathway has also been described. This pathway is also important in choosing the SMC from equipotent cells within the proneural cluster (Artavanis-Tsakonas *et al.,* 1999; Hassan and Vaessin, 1996). *Notch* mediates repression of *achaete* and *scute* expression in the cells neighboring the presumptive SMC, a process dependent on the *enhancer of split* (*E/spl*) genes as well as the activity of the proneural genes themselves (Heitzler *et al.,* 1996). A cell that is expressing high levels of *achaete* or *scute* overcomes this repression and subsequently delivers more inhibition to a neighboring cell by direct activation of *Delta* (Kunisch *et al.,* 1994). DELTA stimulation of the NOTCH receptor on adjacent cells results in an E/SPL-dependent downregulation of AS-C (Heitzler *et al.,* 1996).

A variety of evidence, including expression patterns and gain-of-function studies in *Xenopus,* suggests that the non-cell-autonomous *Notch/Delta* signaling pathway is involved in vertebrate neurogenesis as well (Lewis, 1996). The role of this inhibitory pathway in vertebrates has not been determined, but one hypothesis is that it plays a role in the timing of differentiation of neural progenitor cells. In mouse, *Mash1* expression is altered in mutants of homologs of the lateral inhibition pathway, including *Notch1, RBP-Jk* (*Suppressor of Hairless* homolog), and *Hes-1* (*Hairy E/spl* homolog-1) (de la Pompa *et al.,* 1997; Ishibashi *et al.,* 1995). Specifically, in *Hes-1* mutant embryos, *Mash1* is prematurely expressed and upregulated in some domains, and it is hypothesized that *Hes-1* negatively regulates *Mash1* and neural differentiation.

Given the importance of autoregulation demonstrated for control of *achaete* and *scute* expression in *Drosophila,* we hypothesized that autoregulatory mechanisms would play a role in controlling *Mash1* expression as well. Recently we demonstrated that transcription from the *Mash1* locus is upregulated in the *Mash1* mutant (Horton *et al.,* 1999). To investigate the mechanism of *Mash1* autoregulation in this context, we have introduced *Mash1/lacZ* transgenes containing different amounts of *Mash1* flanking sequence into *Mash1* mutant mice. Expression of these transgenes in MASH1-deficient mice was dramatically upregulated, phenocopying the results seen at the endogenous locus. Mutations made at E-box and *hairy/E(spl)* (HES) consensus binding sites suggest that the mechanism of *Mash1* negative autoregulation is indirect, possibly through a HES-mediated repression of upstream activators or through novel HES sites.

MATERIALS AND METHODS

Mash1/lacZ Transgene Construction

Figure 1 diagrams all transgenic constructs used in this study. The *Mash1/lacZ* lines J1A, Tg1, Tg5, and Tg14 were described previously (Verma-Kurvari *et al.,* 1996, 1998). Constructs Tg5Δ*Hes* and Tg5Δ*hairy* contain the same *Mash1* regulatory elements as Tg5 with mutations in transcription factor consensus binding sites. Briefly, Tg5 contains a 3.3-kb distal CNS enhancer fragment fused to 0.9 kb of proximal *Mash1* regulatory sequence containing the *Mash1* basal promoter elements and the 5′ UTR, the *lacZ* coding region, and 2.6 kb *Mash1* 3′ sequence. For Tg5Δ*Hes,* a PCR-based strategy was used to generate mutations in the *hairy* and class C *Hes-1* consensus sites (Chen *et al.,* 1997; van Doren *et al.,* 1994) (GCACGCGCCGGGCGCACGCA → CTATGATCCGGCGTGGTACC) found 5′ of the transcription start site (−243 to −225). For construct Tg5Δ*hairy,* a PCR-based strategy was used to generate mutations in tandem *hairy* consensus sites (CACGCGAGCGCCACGCG → CGGTACGATAGATGTAC) at +286 to +302 in the 5′ UTR. Tg14ΔEbox contains the more restricted 1.2-kb CNS enhancer on the heterologous *hsp68* promoter *lacZ* reporter (Verma-Kurvari *et al.,* 1998) with the four E boxes mutated (CAGTTG → TCTAGA, CAGCTG → CCGCGG, CATGTG → CTGCAG, and CAGGTG → TGTTCA). Mutations were generated stepwise by sequential PCR, and the full-length product was cloned into *hsp68lacZpA* (Kothary *et al.,* 1989). All constructs were sequenced to confirm mutations, and fragments for injection were separated from vector sequences and prepared as described previously (Verma-Kurvari *et al.,* 1996).

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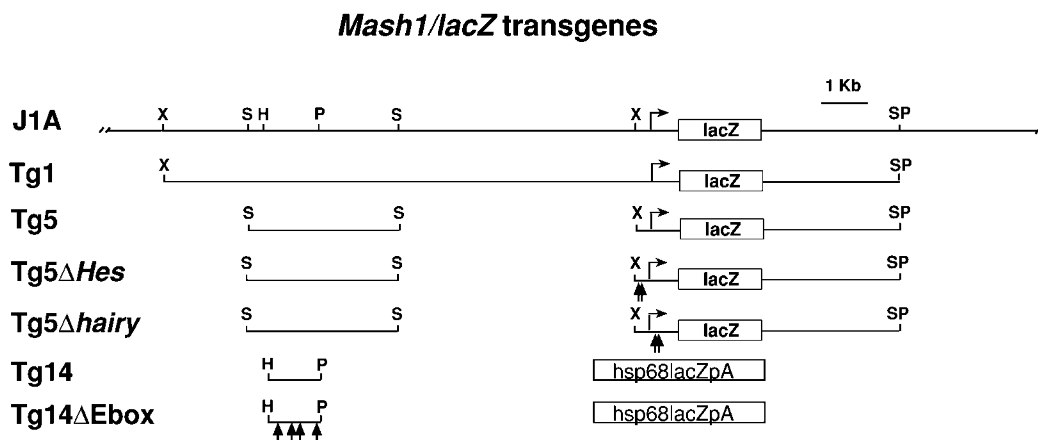

FIG. 1. *Mash1/lacZ* transgenes used to test *cis*-regulatory elements. Transgenes J1A, Tg1, Tg5, Tg5Δ*Hes,* and Tg5Δ*hairy* utilize the basal promoter elements and polyadenylation signal from *Mash1,* including the 5′ and 3′ UTRs, to drive *lacZ* expression. *Mash1* sequence is shown as black lines. J1A (~36 kb total) contains 13 kb 5′ and 23 kb 3′ *Mash1* flanking sequence driving *lacZ* expression. Tg1 (~14 kb total) contains 11 kb 5′ of *lacZ* and 2.6 kb 3′, which includes *Mash1* 3′ UTR and an intron. Tg5 (~7 kb total) contains a 3.3-kb fragment found 6 kb upstream of the coding region fused to a 0.9-kb fragment containing the *Mash1* basal promoter and 5′ UTR, plus the 2.6-kb 3′ flanking sequence. Tg5Δ*Hes* and Tg5Δ*hairy* contain the same sequences as Tg5 but with HES consensus sites mutated, denoted by arrows. Tg14 is the 1.2-kb *Mash1* CNS enhancer found 7.2 kb upstream of the coding region fused to *hsp68lacZpA* (*hsp68* basal transcription elements and an SV40 polyadenylation signal cassette) (Kothary *et al.,* 1989). Tg14ΔEbox contains the same sequences as Tg14 but with four mutated E boxes, denoted by arrows. The bent arrows indicate the transcription start. Relevant restriction sites are shown: H, *Hpa*I; P, *Pst*I; S, *Sac*I; SP, *Sph*I; X, *Xba*I.

Analysis of Transgenic Mice

Transgenic embryos (Tg5Δ*Hes,* Tg5Δ*hairy,* and Tg14ΔEbox) were generated as described previously (Verma-Kurvari *et al.,* 1996) and were analyzed as founder embryos at E11.5. Previously characterized J1A, Tg1, Tg5, and Tg14 (Verma-Kurvari *et al.,* 1996) transgenic lines were crossed with *Mash1* −/+ mice (Guillemot *et al.,* 1993). Heterozygous intercrosses of *Mash1* +/−;*Mash1/lacZ* mice were used to obtain *Mash1* homozygous, heterozygous, and wild-type embryos expressing *Mash1/lacZ* transgenes. Southern blot analysis was used to determine genotypes of *Bam*HI-digested tail or yolk sac DNA. The *lacZ* probe, a 2.5-kb *Pvu*II fragment from pn*lacZ*F, hybridizes to a 3.1-kb fragment in the *Mash1/lacZ* transgene. The *neo* probe, a 0.6-kb *Xba*I/*Pst*I fragment from pGK-*neo,* hybridizes to a 3.9-kb band from the *Mash1* mutant allele. The *Mash1* probe, a 0.6-kb *Sac*I/*Bam*HI fragment from pM1B3, hybridizes to a 3.1-kb band of wild-type *Mash1.* For β-galactosidase (βgal) staining, embryos were dissected from the uterus in cold PBS at embryonic day 11.5 (E11.5) and fixed in 4% paraformaldehyde (pH 7.2) for 30 min at room temperature. βgal staining was done at 35°C for 12–16 h in 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 5 mM $MgCl_2$ in PBS. Whole-mount embryos were cleared for imaging by dehydration in a series of 12-h incubations in PBS, 70% ethanol, 95% ethanol, and 100% ethanol, followed by 1 h in xylene and 30 min in methyl salicylate. Other whole-mount stained embryos were embedded in 3.5% agar/8% sucrose and Vibratome sectioned at 200 μm.

In Situ Hybridization

Mash1 heterozygous crosses were used to obtain *Mash1* homozygous, heterozygous, and wild-type embryos. E11.5 embryos were harvested in RNase-free solutions and fixed 12 h in 4% paraformaldehyde at 4°C on a rotating platform. Embryos were rinsed in cold PBS and sunk in 30% sucrose in PBS overnight at 4°C, embedded in OCT mounting medium (Tissue Tek), and frozen by gradual immersion in liquid N_2. Cryoprotected embryos were sectioned at 30 μm, and *in situ* hybridization was performed using digoxigenin-labeled probes as described previously (Birren *et al.,* 1993). The antisense *Mash1* and *neo* riboprobes were synthesized from plasmids Nj1-19, containing the rat *Mash1* coding region plus 300 bp of 5′ UTR, and pXP*neo,* containing 630 bp of the *neomycin* coding region. An α-digoxigenin alkaline phosphatase-coupled antibody was used to visualize staining. *Neo in situs* were performed on heterozygous and homozygous mutant sections in parallel, ensuring reaction time and conditions were equal.

RESULTS

Mash1 Negatively Autoregulates through 36 kb of Sequence Flanking the Coding Region

Recently it was shown that transcription from the *Mash1* locus is elevated in MASH1-deficient mice, demonstrating a role for MASH1 in inhibiting its own expression (Horton *et al.,* 1999). In this paper, we address the mechanism of *Mash1* autoregulation by investigating the *cis*-acting sequences required for this phenomenon. A transgenic mouse line containing 36 kb of *Mash1* regulatory sequence directs *lacZ* expression specifically to *Mash1* expression domains in the CNS and PNS (Verma-Kurvari *et al.,* 1996). To determine if sequences responding to the negative autoregulation are within this 36 kb, we compared the activity

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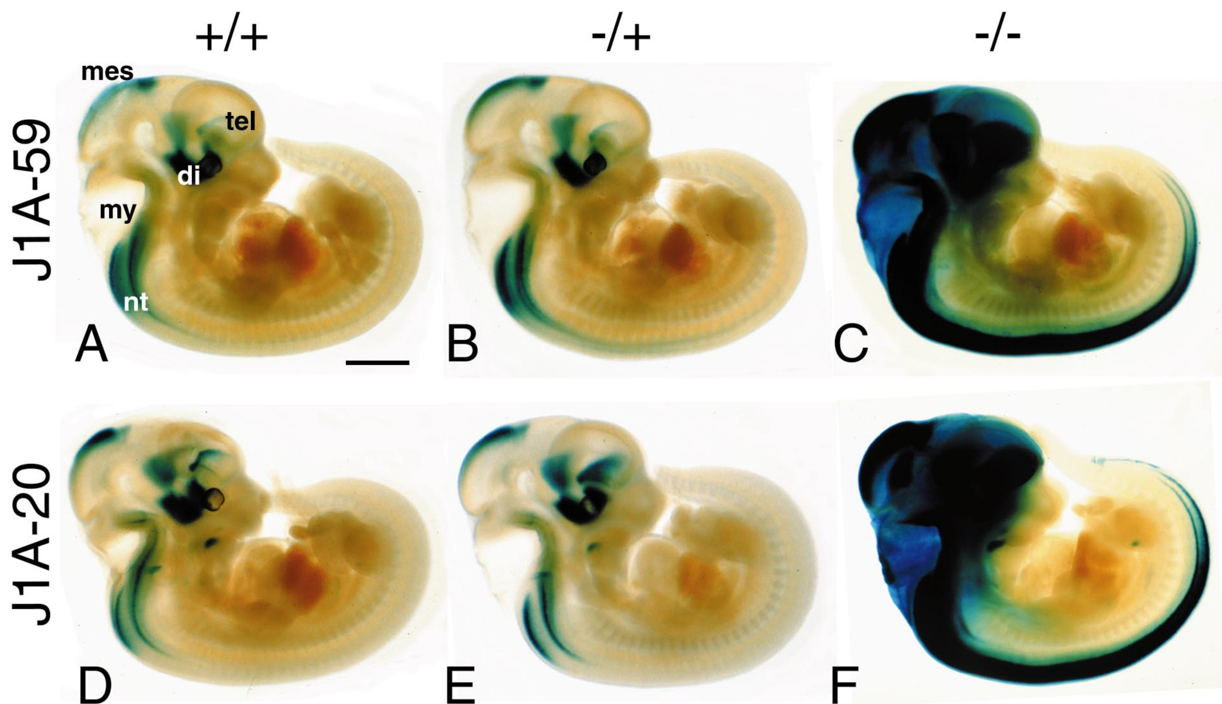

FIG. 2. Upregulation of the J1A *Mash1/lacZ* transgene in the *Mash1* mutant background. Embryos were whole mount stained for βgal activity at E11.5, ensuring equal conditions and staining time. Two independent *Mash1/lacZ* lines containing the J1A transgene (Fig. 1) J1A-59 (A–C) and J1A-20 (D–F) are shown in the *Mash1* wild-type (A, D), heterozygous (B, E), and homozygous (C, F) backgrounds. In both lines, J1A transgene expression is dramatically upregulated in the absence of *MASH1* function in domains normally expressing *Mash1.* di, diencephalon; mes, mesencephalon; my, myelencephalon; nt, neural tube; tel, telencephalon. Scale bar, 0.9 mm.

of the transgene in the *Mash1* mutant and wild-type backgrounds. Mice carrying both the *Mash1/lacZ* transgene (Fig. 1, J1A) and the *Mash1* mutant allele were intercrossed to generate *Mash1* null and wild-type embryos that express *lacZ* under the control of *Mash1* regulatory sequence. Embryos from these crosses were stained for βgal activity at E11.5, a time of high level *Mash1* expression in multiple regions of the CNS and PNS. Consistent with the observation of negative autoregulation at the endogenous *Mash1* locus, a dramatic increase in *Mash1/lacZ* transgene expression was observed in the *Mash1* mutant background compared to heterozygous or wild-type littermates (Fig. 2). Three independently derived transgenic mouse lines carrying the 36-kb *Mash1/lacZ* transgene were analyzed in the *Mash1* mutant background (Fig. 2, J1A-20, J1A-59, and data not shown). In all cases, transgene expression was dramatically increased in the absence of MASH1 function relative to transgene expression the in wild-type background (compare Figs. 2A and 2D with 2C and 2F). As in the wild-type background, the staining in the mutant is still restricted to regions within the spinal neural tube, myelencephalon, mesencephalon, diencephalon, and telencephalon, corresponding to endogenous *Mash1* expression domains (Verma-Kurvari *et al.,* 1996).

The increase in transcription from the *Mash1/lacZ* transgene seen in the mutant background revealed expression in regions of endogenous *Mash1* expression that had not been previously detected with the J1A transgene. These regions include the olfactory epithelium, the ventricular zone of the ventral telencephalon, and the caudal neural tube. Vibratome sections of whole-mount stained embryos more clearly illustrate the differential detection of expression in these domains (Fig. 3). Though endogenous *Mash1* is expressed in neural precursors of the olfactory epithelium (OE), expression of βgal in this area in J1A transgenic mice is not detected in embryos with functional MASH1 (Verma-Kurvari *et al.,* 1996). Parasagittal sections of *Mash1* mutant embryos reveal J1A transgene expression in the OE (Fig. 3B, arrow). Although olfactory neurons do not develop in the *Mash1* mutant (Guillemot *et al.,* 1993), the βgal expression detected in the OE at E11.5 shown here likely reflects transcriptional activity of the locus before these cells die. Consistent with this interpretation, it has been shown that *Mash1*-dependent olfactory progenitors do not die in the mutant until E12.5 (Cau *et al.,* 1997). In contrast, expression of the transgene in the developing sympathetic neurons is absent at E11.5 in the *Mash1* mutant, likely reflecting a difference in timing of formation of the olfactory versus sympathetic systems (compare Figs. 3A and 3B, arrowheads). One conclusion from these data is that the

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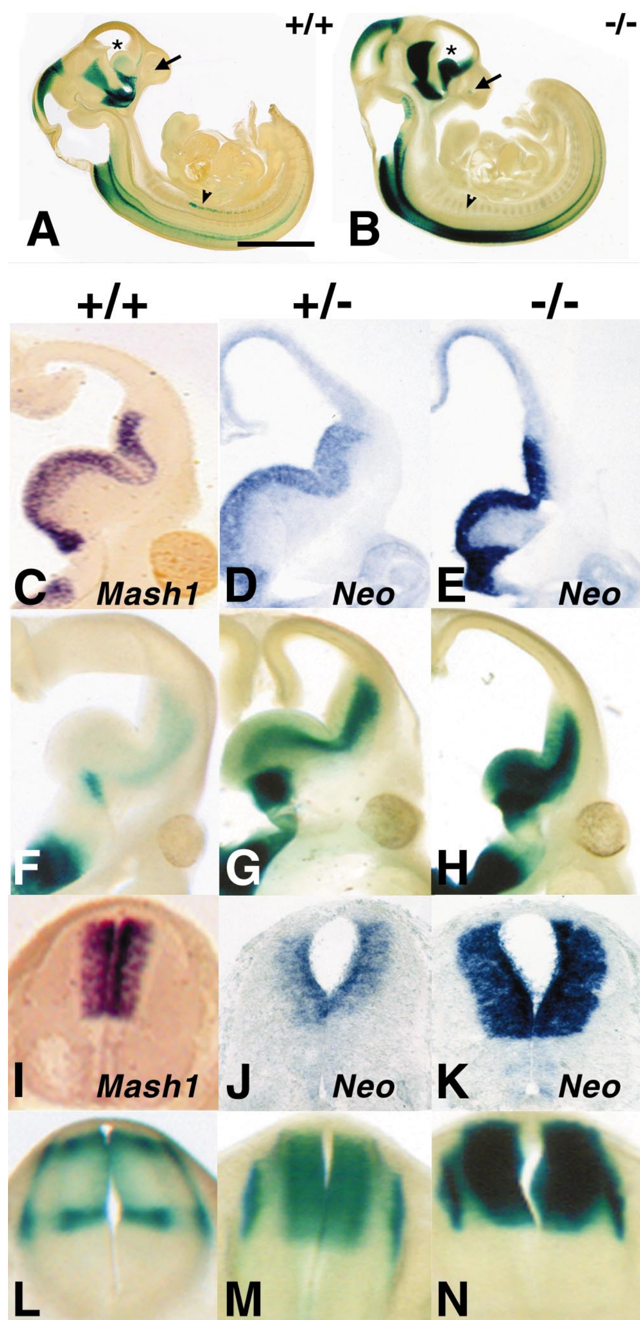

FIG. 3. Increase in *neo* expression at the *Mash1* locus and *lacZ* expression from the J1A *Mash1/lacZ* transgene in the forebrain and spinal neural tube of *Mash1* mutant embryos. J1A-59 littermates of different *Mash1* genotypes were whole-mount stained for βgal activity at E11.5 and Vibratome sectioned at 200 μm thickness (A, B, F–H, L–N). Other E11.5 *Mash1* wild-type, heterozygous, or mutant embryos were cryosectioned at 30 μm thickness and hybridized with *Mash1* (C, I) or *neo* (D, E, J, K) riboprobes. Parasagittal sections reveal olfactory expression in the *Mash1* mutant (B) but not wild-type (A) background (arrows). Arrowheads indicate expression in the sympathetic precursors of wild-type (A) embryos that is absent in mutants (B). Transverse sections through the ventral forebrain (F–H) or the spinal neural tube (L–N) show *Mash1/lacZ* expression in the same pattern as endogenous *Mash1* in all genetic backgrounds (C, I), but it is significantly increased in the mutant. J1A transgene activity in the mutant is observed in previously undetectable domains such as the ventricular zone of the ganglionic eminences of the ventral telencephalon (asterisks in A and B and F–H) and the full extent of the dorsal spinal neural tube (L–N). *neo* expression from the neomycin cassette recombined into the endogenous *Mash1* locus is dramatically increased in *Mash1* mutant embryos in the ventral telencephalon (D and E) and spinal neural tube (J and K). The equal reaction times of *neo*-hybridized sections allow direct comparison of the expression levels of mutant (E, K) versus heterozygous (D, J) sections. Scale bar: (A and B) 1.4 mm, (C–H) 475 μm, (I–N) 250 μm.

36-kb *Mash1* flanking sequence contains regulatory information for expression in olfactory epithelium, a fact not previously realized. The upregulation of transgene expression in the mutant background has allowed detection of activity from elements that are too weak to drive expression under normal conditions.

In the CNS, *Mash1* is expressed at high levels in the ventricular (VZ) and subventricular (SVZ) zones of the ventral telencephalon (Fig. 3C). Expression of the *Mash1/lacZ* transgenes was previously not detected in the VZ of the ventral telencephalon even though the adjacent SVZ staining respected the precise dorsoventral borders of the endogenous expression pattern (Verma-Kurvari *et al.,* 1996) (compare Figs. 3C and 3F). Transverse sections through forebrain regions of J1A transgenic embryos demonstrate that in the absence of MASH1, transgene expression is increased and is now detected in the VZ (Figs. 3F–3H). The increased activity of these regulatory sequences mimics the increase in transcriptional activity seen from the *Mash1* locus in the *Mash1* mutant background. Examination of *neo* expression from the mutant allele (Figs. 3D and 3E) demonstrates the increase in expression from the endogenous locus. This upregulation of both the endogenous locus and the J1A transgene is also clearly visualized in transverse sections through spinal neural tube (Figs. 3I–3N). In the wild-type background, the J1A transgene expression reflects the dorsal and ventral borders of endogenous *Mash1* expression but is not detectable throughout the whole dorsal domain (compare Figs. 3I and 3L). However, in the mutant embryos, there is markedly higher βgal activity throughout the appropriate region (Fig. 3N). Again, in the heterozygote, *neo* is expressed appropriately throughout the proper domain (Fig. 3J), and this expression is dramatically induced in the absence of MASH1 function (Fig. 3K). In the spinal neural tube, increased transgene expression was sometimes seen in the heterozygous background (Fig. 3M). Because this increase was not consistently seen in the heterozygous embryos, and it was not detected in the VZ of the ventral telencephalon, we cannot make a case for a heterozygous phenotype. It is clear from the data presented above that

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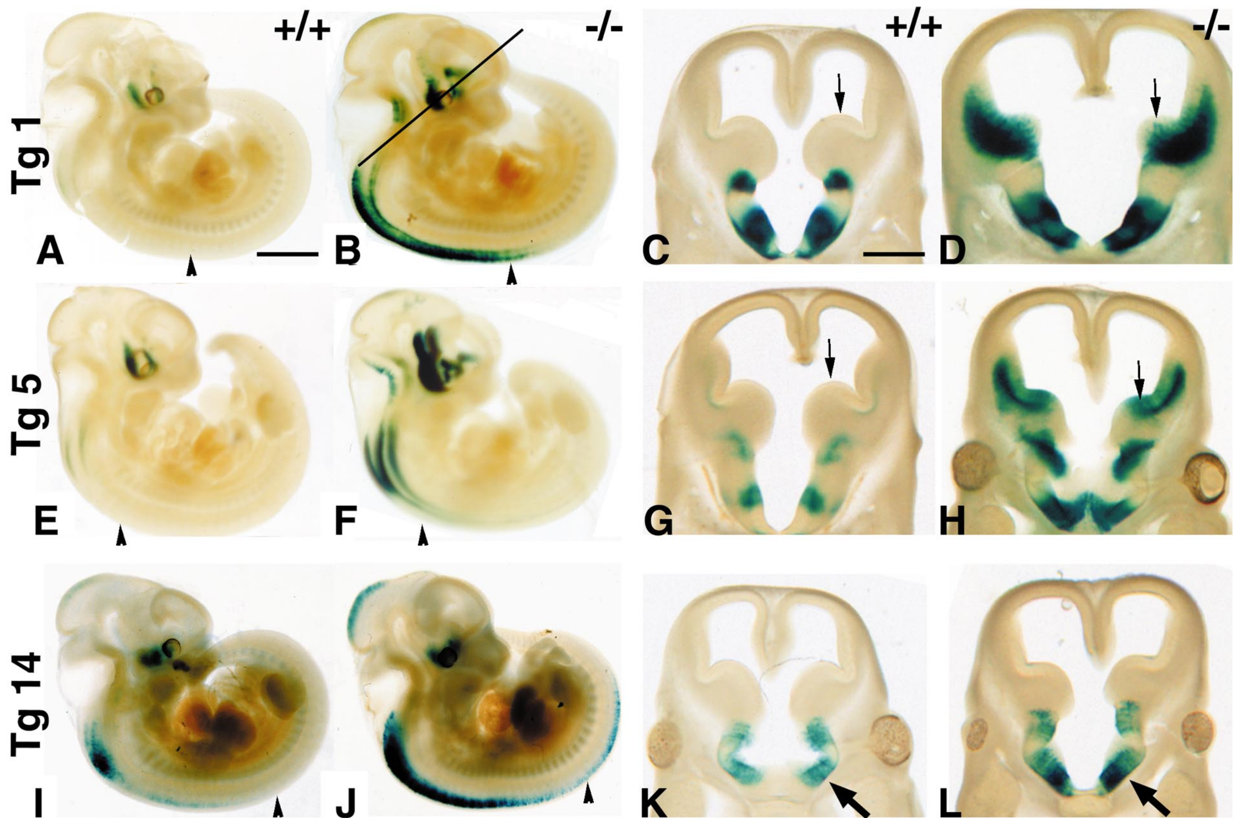

FIG. 4. *Mash1/lacZ* lines with as little as 1.2 kb of *Mash1* regulatory sequence respond to autoregulation. Tg1 (A–D), Tg5 (E–H), and Tg14 (I–L) transgenic embryos were assayed for βgal activity at E11.5 in the presence or absence of MASH1 function (A, B, E, F, I, and J). Vibratome sections (200 μm) through the forebrain of whole-mount stained embryos in the plane indicated in (B) are shown in (C, D, G, H, K, and L). For each transgene, expression in the mutant background is elevated compared to wild-type littermates. The whole-mount embryo assay illustrates the detection of spinal neural tube expression in more caudal regions of mutant versus wild-type embryos (arrowheads). Vibratome sections through the forebrain reveal upregulated expression in the ventricular zone of the ganglionic eminences (arrows, C, D, G, and H) and diencephalon (arrows, K and L). Scale bar is 1.2 mm for whole-mount embryos and 700 μm for Vibratome sections.

cis-acting sequences responding to the negative autoregulatory mechanisms are contained within the 36-kb sequence present in the J1A transgene. The increase in transcriptional activity observed at both the endogenous locus and the *Mash1/lacZ* transgene occurs in all domains of *Mash1* expression at E11.5, suggesting that the sequences mediating autoregulation seem to influence all elements currently known to direct *Mash1* expression at this stage.

cis-Acting Regulatory Elements Controlling Autoregulation Are Contained within the 1.2-kb Mash1 CNS Enhancer

To localize the important elements for autoregulation within the 36-kb MASH1 flanking sequence, transgenic lines carrying smaller regions of *Mash1* sequence were tested. Transgenes Tg1, Tg5, and Tg14 contain progressively less *Mash1* regulatory sequence, the smallest sequence being the 1.2-kb CNS enhancer in Tg14 (Fig. 1). Stable transgenic lines containing these different *Mash1/lacZ* transgenes were crossed into the *Mash1* mutant background, and E11.5 embryos were assayed for transgene expression (Fig. 4). Although these lines have weaker expression in the wild-type background than the J1A lines, transgene expression in the absence of MASH1 function phenocopies the results observed with J1A transgenes. In each case, there is an obvious upregulation of transgene expression in the mutant background in all expression domains (Fig. 4). Tg1 and Tg5, which contain 13.6 and 6.8 kb of *Mash1* sequence, respectively, are barely detectable in the ventral telencephalon and this expression is restricted to the SVZ. However, as was seen with J1A, the expression of both transgenes dramatically increases in the SVZ and expands appropriately into the VZ in mutant embryos (Figs. 4C, 4D, 4G, and 4H, arrows). Tg14, which contains the 1.2-kb *Mash1* CNS enhancer on the *hsp68lacZpA* reporter, is not detected in the ventral telencephalon of wild-type or mutant embryos (Figs. 4I and 4J). Expression in the dien-

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cephalon, however, is clearly increased in the mutant (Figs. 4K and 4L, arrows). The spinal neural tube expression of all three transgenes is also dramatically increased and is detected more caudally than the wild type (Fig. 4, arrowheads). These data demonstrate that at least some aspects of negative autoregulation are being mediated through sequence within the 1.2-kb CNS enhancer.

Mash1 Does Not Directly Autoregulate through E Boxes in the CNS Enhancer

Basic HLH transcription factors bind class A E-box sites (CANNTG), and many of these consensus sites are present throughout *Mash1* regulatory sequence. It is possible that *Mash1* could directly regulate its own transcription. Direct positive autoregulation has been demonstrated for *achaete* and *scute* proteins, but proneural bHLH proteins have not been shown to repress transcription. However, recent evidence suggests that other class A bHLH proteins may be bifunctional. MyoD can repress transcription of the cyclin B1 promoter, and this repression is dependent upon the expression of MyoD and presence of a pair of MyoD binding sites within the promoter (Chu *et al.,* 1997). To test the involvement of *Mash1* or other bHLH proteins in negative autoregulation, either directly by binding DNA or indirectly by preventing E-box-mediated activation, the four E boxes contained in the CNS enhancer (Tg14) were mutated (Tg14ΔEbox). Mutation of *cis*-acting sites that mediate negative autoregulation would result in an upregulation of transgene expression in the wild-type background. Tg14ΔEbox embryos do not express *lacZ* at significantly higher levels than the intact CNS enhancer (Table 1), arguing against the involvement of these sites, and thus *Mash1* directly, in autoregulation. Interestingly, mutation of these sites had little, if any, detrimental effect on CNS enhancer activity, suggesting that these E boxes are also not essential for any positive regulation of *Mash1.*

Mutation of Conserved HES Consensus Sites Does Not Alter *Mash1/lacZ* Expression

Hairy and the related *Enhancer of Split* [*E(spl)*] bHLH proteins in *Drosophila* can directly bind and repress *achaete* transcription (Ohsako *et al.,* 1994; van Doren *et al.,* 1994). *E(spl)* transcription factors are the downstream mediators of *Notch* signaling in *Drosophila* in sensory bristle formation (Heitzler *et al.,* 1996), and mutations in either *hairy* or *E(spl)* proteins lead to ectopic sensory bristles. In mouse, the *Hes* family has been identified by homology to the *hairy/E(spl)* genes (Akazawa *et al.,* 1992; Ishibashi *et al.,* 1993; Sasai *et al.,* 1992; Takebayashi *et al.,* 1995), and the HES proteins make attractive candidates for mediating the negative autoregulation of *Mash1.* Evidence of a functional conservation of HES regulation of neural bHLH expression in mouse was suggested by a *Hes-1* knockout (Ishibashi *et al.,* 1995). Mutant embryos displayed a disruption in neural differentiation, including upregulation and premature expression of *Mash1* in a subset of its endogenous pattern. In cell transfection assays, *Hes-1* has also been shown to antagonize *Mash1*-dependent transcriptional activation at E-box sites (Sasai *et al.,* 1992). Additionally, expression of *Hes-1* in human small cell lung carcinoma lines resulted in a downregulation of endogenous *Hash1* expression (human homolog of *Mash1*), and this was dependent on its direct binding of a class C *hairy/E(spl)* site in the *Hash1* proximal repressor domain (Chen *et al.,* 1997).

Drosophila hairy has a preferred binding site (CACGCG) that differs from the class A E-box sites recognized by activator bHLH proteins (van Doren *et al.,* 1994). Other bHLH proteins of the *E(spl)* family, including the mammalian *Hes* homologs, can bind to class B and C noncanonical variant sites, including the N-box (CACNAG), and repress transcription directly (Akazawa *et al.,* 1992; Fisher and Caudy, 1998; Ishibashi *et al.,* 1993; Sasai *et al.,* 1992; Takebayashi *et al.,* 1994). *Mash1* genomic sequence in transgenes J1A, Tg1, and Tg5 contains multiple HES consensus binding sites. To test the hypothesis that direct repression by HES factors is important in negative autoregulation, four sites within the context of Tg5 were mutated. These sites were chosen because of their positions in a region of high conservation located just 5′ of the coding region between mouse and human sequences. One of these sites was reported to be important in HES-mediated repression of *Hash1* in small lung cell carcinoma cells (Chen *et al.,* 1997). Since the sites were clustered in two locations, two sites each were mutated in two *Mash1/lacZ* constructs (Fig. 1, arrows). Tg5Δ*Hes* mutates a *hairy* preferred site

TABLE 1
E-Box and HES Sites Are Not Required for *Mash1* Negative Autoregulation

Mash1/lacZ constructs	No. TgM	CNS	High levels
Tg5	6	4	0
Tg5Δ*hes*	7	2	0
Tg5Δ*hairy*	16	8	1
Tg14	6	5	1
Tg14ΔEbox	8	3	0

Note. Transgenes diagrammed in Fig. 1 were injected into mouse one-cell eggs to generate transgenic embryos. Founder embryos were harvested at E11.5 and assayed in whole mounts for βgal activity. Typical expression levels for the wild-type transgenes Tg5 and Tg14 are depicted in Figs. 4E and 4I. For mutant transgenes, *lacZ* levels were compared to those of the parent transgene. If a *cis*-acting site important for *Mash1* autoregulation were mutated, an upregulation of transgene expression in the wild-type background would be expected. No significant effect on transgene expression was detected from any of the mutant constructs. No. TgM, number of transgenic embryos assayed; CNS, number of embryos expressing *lacZ* in the *Mash1* CNS pattern; High levels, number of embryos exhibiting higher than normal levels of βgal activity.

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(CACGCG) and the adjacent class C *Hes1* site (CACGCA) found immediately 5′ of the transcription start site. Tg5Δ*hairy* mutates tandem *hairy* sites in the 5′ UTR. Deletion of sites involved in negative regulation of *Mash1* should result in higher levels of βgal activity in the *Mash1* domain in transgenic embryos. *Mash1/lacZ* expression in embryos containing either Tg5Δ*Hes* or Tg5Δ*hairy* did not demonstrate significantly higher βgal levels than the original Tg5 (Table 1). We conclude that these *cis*-acting HES consensus sites are not essential for the negative regulation of *Mash1* in transgenic mice.

DISCUSSION

In contrast to the positive autoregulatory loops described for AS-C, ATONAL, MATH1, and nonneural bHLH proteins such as MyoD, MASH1 represses its own expression. This repression does not require bHLH or HES binding sites, suggesting that direct repression by MASH1 or HES factors is unlikely. Thus, regulatory mechanisms not currently understood underlie the negative feedback. HES proteins are the most likely candidates for mediating NOTCH signaling. A reasonable model is one in which MASH1 induces HES proteins, which act to repress the activity of upstream factors whose normal function is to activate or maintain *Mash1* expression. The CNS enhancer contains many elements necessary for *Mash1* expression, and it is likely that surrounding sequence serves to further modulate the activity of these core elements. While our data clearly demonstrate that elements responding to MASH1 autoregulation are contained within the 1.2-kb CNS enhancer, this does not preclude the possibility that surrounding sequence also mediates autoregulation. The observation of an upregulation of *lacZ* in all areas of expression for each construct tested supports this hypothesis.

Autoregulation and bHLH Transcription Factors

Many bHLH transcription factors have been shown to positively regulate their own expression. The autoregulation of MyoD and related proteins (myogenin, Myf5, and MRF4) is thought to be important for maintaining stable expression in myoblasts until appropriately signaled to terminally differentiate. Studies in transfected cells demonstrated that members of the MyoD family can auto- and cross-regulate the others' expression (Braun *et al.,* 1989; Thayer *et al.,* 1989). Direct autoregulation of MyoD seems to be mediated by E boxes in proximal promoter sequences (Zingg *et al.,* 1994). However, distinct *MyoD* and *myogenin* autoregulatory responsive enhancers that contain E boxes have been identified, but these sites are dispensable for autoregulation (Dechesne *et al.,* 1994; Edmondson *et al.,* 1992). Despite the identification of autoregulatory responsive elements in cell culture studies, in transgenic mice, the expression patterns of a *MyoD/lacZ* transgene with deleted E boxes or a *myogenin/lacZ* transgene examined in the *myogenin* mutant background were unperturbed (Cheng *et al.,* 1995; Goldhamer *et al.,* 1995). These data demonstrate that a proportion of the positive autoregulation seen for these myogenic bHLH factors is through indirect mechanisms.

The regulation of neural specific bHLH genes has not been as extensively characterized as their myogenic relatives, possibly due to the lack of model culture systems. Cross-regulation of AS-C genes has been demonstrated, and *cis*-acting elements mediating these interactions have been identified at multiple sites in AS-C regulatory sequence (Culi and Modolell, 1998; Gomez-Skarmeta *et al.,* 1995; Martinez and Modolell, 1991; Martinez *et al.,* 1993; Skeath *et al.,* 1992; van Doren *et al.,* 1992). The relevance of cross-activation in cluster-specific *achaete* or *scute* expression is unclear (Modolell, 1997). However, autoregulation is thought to play a critical role in the normal expression profile of the proneural genes in a fashion mechanistically similar to that of the myogenic bHLHs. In *Drosophila,* although prepattern factors likely set up the initial expression of the AS-C genes in a proneural cluster, positive autoregulation is thought to be necessary for singling out one of these cells to become the neural precursor (Ruiz-Gomez and Ghysen, 1993). For *achaete,* direct positive transcriptional autoregulation has been demonstrated and is dependent in the transgenic fly assay upon E boxes in the *achaete* promoter (Martinez *et al.,* 1993; van Doren *et al.,* 1992). Additionally, positive autoregulation has also been shown for *scute* and the related proneural gene *atonal* by identification of enhancers whose expression is dependent upon presence of the respective proteins in flies (Culi and Modolell, 1998; Sun *et al.,* 1998). The enhancer/promoter fragments that autoregulate via E boxes constitute a substantial component of the endogenous expression patterns, indicating that autoregulation is an important part of the basic expression profile of these bHLH proteins.

In mouse, little is known about the mechanisms regulating expression of the neural bHLH factors. Recently, however, a direct role for MATH1 in positively regulating its expression has been demonstrated (Helms *et al.,* 2000). An E box required for enhancer activity was identified and this E-box site is bound by MATH1/E12 heterodimers *in vitro.* In addition, the expression of the *Math1/lacZ* transgene is lost in embryos null for MATH1. In contrast, these same types of *in vivo* experiments with *Mash1* reveal negative rather than positive autoregulation. In addition, the 1.2-kb CNS enhancer that was previously reported for *Mash1* (Verma-Kurvari *et al.,* 1998) does not require the four E-box sites found in its sequence. These experiments detect no direct or indirect positive autoregulatory mechanism controlling *Mash1* expression. The identification of only negative autoregulation of *Mash1* may represent a divergence of function from its *Drosophila* homologs. Unlike the role of the proneural genes of the *achaete-scute* complex, which involves singling out a neuron from a cluster of competent cells, the role of MASH1 may be to coordinate events

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during neuronal differentiation (Casarosa *et al.,* 1999; Cau *et al.,* 1997; Horton *et al.,* 1999; Sommer *et al.,* 1995; Torii *et al.,* 1999). However, the difference between autoregulation of MASH1 and MATH1 and the *Drosophila* proneural proteins indicates that each bHLH factor may have an individually characteristic capacity for self-regulation, and this may be important for the unique developmental processes of each region that expresses a particular bHLH.

What Is the Mechanism of Negative Autoregulation of Mash1?

The loss of negative autoregulation in the *Mash1* mutant background is observed as an increase in *lacZ* expression from reporter transgenes and, at the endogenous locus, as an increase in *neo* expression. The latter appears to be due to cells expressing higher levels of *neo,* as well as more cells with detectable levels of *neo* (Horton *et al.,* 1999). This phenotype is reminiscent of a loss of lateral inhibitory signaling mediated by the *Notch* pathway. The involvement of the proneural genes in a *Notch/Delta*-mediated lateral inhibition pathway in *Drosophila* neural precursor specification has been well studied. Homologs of *Notch, Delta, hairy/E(spl), Su(H),* and the proneural genes have been postulated to play a similar role in vertebrate neurogenesis. *Mash1* levels are increased in *Notch1, RBPJ-κ*, and *Hes-1* mutants (de la Pompa *et al.,* 1997; Ishibashi *et al.,* 1995), indicating that lateral inhibition probably functions to restrict *Mash1* expression in the developing CNS. Expression of *Mash1* in these mutants was more uniform compared to its normal patchy expression in the wild-type CNS, consistent with a loss in lateral inhibition regulation of *Mash1* expression (de la Pompa *et al.,* 1997). Additionally, the potential downstream effectors of *Mash1* function in lateral inhibition, *Delta1* (*Dll1*), *Delta3* (*Dll3*), and *Hes-5,* are reduced or missing in the CNS of *Mash1* mutant embryos (Casarosa *et al.,* 1999). It is likely that the observed negative autoregulation of *Mash1* is a non-cell-autonomous downregulation of expression at the *Mash1* locus in neighboring cells.

To investigate a mechanism of negative autoregulation by the *Notch* pathway, specific *cis*-acting sites were mutated and assayed for upregulation of *lacZ* in transgenic embryos, a phenotype that would indicate the loss of an important negative element. We investigated a direct role for HES factors, proteins which likely mediate *Notch/Delta* lateral inhibition (de la Pompa *et al.,* 1997; Jarriault *et al.,* 1995, 1998), in regulating *Mash1/lacZ* transgene expression. Mutation of HES consensus binding sites had no effect on transgene expression. It is possible that other HES sites contained in the 36-kb *Mash1* flanking sequence may be responsible for some aspect of *Mash1* negative autoregulation although no data currently available suggest the importance of one site over others. However, since the 1.2-kb CNS enhancer with no recognizable HES consensus binding sites mediates autoregulation, it is possible that if HES proteins are involved, it is not through direct binding of DNA or through binding DNA at a previously characterized site.

This negative result is similar to observations from *scute/lacZ* studies using a sensory mother cell-specific enhancer that suggest that *E(spl)* regulation of *scute* may not be as simple as direct repression (Culi and Modolell, 1998). While *E(spl)* proteins mediate *Notch*-dependent repression of AS-C, and *E(spl)*-m8 binds the N box in the SMC enhancer (along with another nonconsensus binding site), mutation of these sites did not release the enhancer from lateral inhibition. Instead, an NF-κB site was sufficient to restrict expression to one cell of a proneural cluster. It was proposed that *E(spl)* family members may interact with an unidentified protein that binds the NF-κB site to mediate *Notch* signaling. NF-κB family members have been shown to associate with Groucho/TLE proteins, which themselves functionally associate with *Hairy/E(spl)* family members (Fisher and Caudy, 1998). These associations suggest that a mechanism of HES-dependent indirect autoregulation is possible through unidentified sites in the *Mash1* CNS enhancer. Recently, additional HES family members have been identified and are expressed in the developing nervous system, but their functional properties have not been characterized (Kokubo *et al.,* 1999; Leimeister *et al.,* 1999). Experiments to identify *trans* factors that bind to these sequences and link NOTCH signaling to *Mash1* regulation are currently being pursued. In vertebrate CNS development, the *Notch* pathway has been postulated to control the timing and relative proportion of proliferating and differentiating cells (Lewis, 1996). The functional significance of a non-cell-autonomous autoregulation may be to coordinate the timing of *Mash1*-dependent aspects of differentiation during this transition by preventing simultaneous expression of *Mash1* in precursors competent to differentiate.

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