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Evidence for differential and redundant function of the Sox genes *Dichaete* and *SoxN* during CNS development in *Drosophila*

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SUMMARY

Group B Sox-domain proteins encompass a class of conserved DNA-binding proteins expressed from the earliest stages of metazoan CNS development. In all higher organisms studied to date, related Group B Sox proteins are co-expressed in the developing CNS; in vertebrates there are three (*Sox1*, *Sox2* and *Sox3*) and in *Drosophila* there are two (*SoxNeuro* and *Dichaete*). It has been suggested there may be a degree of functional redundancy in Sox function during CNS development. We describe the CNS phenotype of a null mutation in the *Drosophila SoxNeuro* gene and provide the first direct evidence for both redundant and differential Sox function during CNS development in *Drosophila*. In the lateral neuroectoderm, where *SoxNeuro* is uniquely expressed, *SoxNeuro* mutants show a loss or reduction of *achaete* expression as well as a loss of many correctly specified lateral neuroblasts. By contrast, in the medial neuroectoderm, where the expression of *SoxNeuro* and *Dichaete* overlaps, the phenotypes of both single mutants are mild. In accordance with an at least partially redundant function in that region, *SoxNeuro/Dichaete* double mutant embryos show a severe neural hypoplasia throughout the central nervous system, as well as a dramatic loss of *achaete* expressing proneural clusters and medially derived neuroblasts. However, the finding that *Dichaete* and *SoxN* exhibit opposite effects on *achaete* expression within the intermediate neuroectoderm demonstrates that each protein also has region-specific unique functions during early CNS development in the *Drosophila* embryo.

Key words: *Drosophila, SoxNeuro, Dichaete, Sox,* Neurogenesis, CNS

INTRODUCTION

Some of the earliest molecular events in neural determination have been conserved during metazoan evolution. In most higher eukaryotes, some region of the primitive ectoderm becomes competent to adopt a neural rather than an epidermal fate early in development (Arendt and Nübler-Jung, 1999). In vertebrates, competent neuroectoderm is specified by the antagonistic activity of the neural inducers Chordin and Noggin on the epidermal-promoting factor BMP4. In *Drosophila*, the Chordin homologue Short gastrulation (Sog) promotes neuroectoderm formation by antagonising the BMP4 homologue Decapentaplegic (Dpp) (De Robertis and Sasi, 1996). After a population of ectodermal cells becomes competent to adopt the neural fate, a specific cell within a group of equivalent cells becomes committed to the neural fate and some of the molecular mechanisms involved in this process are also conserved.

In *Drosophila*, the expression of proneural genes, primarily the bHLH transcription factors encoded by the *Achaete Scute Complex* (*AS-C*) (*ac*, *sc* and *l'sc*), render a cluster of ectodermal cells competent to adopt a neural fate. The activity of the Notch-Delta (N-Dl) signalling pathway then results in the elevation of *AS-C* expression in a single cell within a proneural cluster and the subsequent specification of this cell as a neuroblast (Campos-Ortega, 1993). Neuroblasts (NBs) are the stem cells that give rise to the diversity of neuronal and glial cell types within the *Drosophila* CNS (Goodman and Doe, 1993). NBs acquire unique identity, and are thus directed down a specific pathway to produce a particular neuronal lineage, by virtue of the location of individual proneural clusters with respect to the AP and DV axes of the embryo (Udolph et al., 1995). Along the AP axis, neuroblasts acquire specific fates through the activity of segment polarity genes (Bhat, 1999). Along the DV axis, at least in the case of those neuroblasts that segregate early in development, identity is controlled by the combined action of the Epidermal growth factor receptor (Egfr), and a set of homeobox-containing transcriptional regulators encoded by the *ventral nerve cord defective* (*vnd*), *intermediate neuroblasts defective* (*ind*) and *muscle segment homeobox* (*msh*) genes (Skeath, 1999). In this way, the combined activity of an orthogonal array of gene expression along two embryonic axes divides the neuroectoderm into a Cartesian grid system that can specify a set of different neural identities (Skeath, 1999). In mammals, genes encoding bHLH transcription factors and members of the N-Dl signalling

pathway are involved in consolidating neural identity (Lewis, 1996; Lee, 1997; Chitnis et al., 1995). Similarly, the system that specifies neural identity along the DV axis in vertebrates involves homologues of the *Drosophila vnd*, *ind* and *msh* genes that, as with the fly, are expressed in restricted DV domains during neural specification (Davidson, 1995; D'Alessio and Frasch, 1996; Weiss et al., 1998). It is not yet clear precisely how the acquisition of neural competence and the subsequent specification of defined neural fates are molecularly linked and whether there are conserved regulatory pathways that are involved in this process. Members of the Sox family of transcription factors represent one potential set of conserved pan-neural markers, that could regulate early neural specification events.

The Sox gene encompasses a group of transcriptional regulators, related by an HMG1-type DNA-binding domain, to the mammalian testis-determining factor SRY (Gubbay et al., 1990; Pevny and Lovell-Badge, 1997). The Sox family is restricted to metazoans and within the animal kingdom; the family is large and diverse. Many members of the *Sox* gene family have dynamic tissue-specific expression patterns during embryogenesis, suggesting that they may play a variety of roles during development (Wenger, 1999). On the basis of sequence similarity, both in the DNA-binding domain and in other, group-specific conserved motifs, Sox proteins have been divided into at least seven subgroups (A-G) (Bowles et al., 2000). Group B Sox are most closely related to SRY, sharing over 85% sequence identity between their DNA-binding domains and recognising virtually identical DNA sequences (Harley et al., 1994; Collignon, 1996). In flies, frogs, chicks and mammals, group B *Sox* genes are expressed in the neuroectoderm from the earliest stages of neurogenesis (Collignon et al., 1996; Uwanogho et al., 1995; Rex et al., 1997; Russell et al., 1996; Nambu and Nambu, 1996; Wood and Episkopou, 1999; Cremazy et al., 2000). In these animals, related group B genes are co-expressed in the neuroectoderm, leading to the idea that they may function redundantly or influence each other's activity. In mice and chicks, three group B genes (*Sox1*, *Sox2* and *Sox3*) are widely co-expressed in the neuroectoderm and neural tube; in *Drosophila*, only two group B genes, *Dichaete* and *SoxNeuro* (*SoxN*), are expressed early in the CNS.

Although well characterised in terms of expression, in vivo functional studies of *Sox* genes in early CNS development are less well established. In the mouse, *Sox1* null mutants survive until adulthood, where some role in CNS function is suggested by a spontaneous seizure phenotype. However, the fact that homozygous mutants survive without significant defects in CNS development suggests that any major role in early CNS development is dispensable (Nishiguchi et al., 1998). In mice, *Sox2* mutants are reported to die prior to implantation (Collignon et al., 1996) therefore the role of *Sox2* in CNS development has not been described. *Sox3* mutations have not been reported. Direct evidence for the involvement of *Sox* genes in CNS development comes from in vitro stem cell studies, where it was shown that the *Sox1* gene can induce neural fate in competent ectodermal cells (Pevny et al., 1998). Furthermore, a *Sox2*-βGeo insertion construct has been used to select neural precursors from stem cell populations, suggesting that *Sox2* is a marker for early neural fate (Li et al., 1998). In *Xenopus*, the *SoxD* gene is first expressed in the prospective neuroectoderm and then later throughout the neural plate. Injection of *SoxD* mRNA into early embryos can induce ectopic neural tissue and injection of a dominant negative form of *SoxD* leads to loss of neural tissue, establishing a role for this *Sox* gene in *Xenopus* neuralisation (Mizuseki et al., 1998a). Additionally, the *Xenopus Sox2* gene, which acts downstream of *SoxD*, appears to be required for establishing neural competence in neuroectodermal cells (Mizuseki et al., 1998b).

Mutations in the *Drosophila* gene *Dichaete* have specific defects in the specification or differentiation of glial lineages in the midline of the CNS, a structure in which *Dichaete* is a uniquely expressed Sox gene (Sanchez-Soriano and Russell, 1998; Ma et al., 2000). Outside of the midline, in the ventral neuroectoderm where *Dichaete* and *SoxN* are co-expressed (Cremazy et al., 2000), neural phenotypes are relatively weak (N. Sanchez-Soriano, PhD thesis, University of Cambridge, 1999). Recently, Zhao and Skeath (Zhao and Skeath, 2002) have shown that *Dichaete* is involved in the specification of cell fate in the neuroectoderm and in NB formation via interactions with the homeodomain-encoding genes *vnd* and *ind*. Mutations in the other *Drosophila* group B gene, *SoxN*, have not previously been described. We report the identification of a null mutation in *SoxN* and show that, as is reported for *Dichaete*, it is involved in early events in neural cell fate specification. By eliminating *Dichaete* and *SoxN* function simultaneously, we present the first description of the effects of eliminating all group B Sox function in the early CNS of an animal. The severe neural hypoplasia observed in the double mutants together with the reduction of *ac* expression suggests that both genes act on the level of the neuroectoderm. In addition, loss of specific NB lineages in *SoxN* mutant embryos suggests that *SoxN* also has a later role in NB formation. Our observations on *ac* regulation support the idea that group B Sox genes can act redundantly but also antagonistically in early specification events.

MATERIALS AND METHODS

Drosophila stocks

Drosophila stocks were maintained on standard yeasted cornmeal-agar food at 25°C; the wild-type stock used was Oregon R. Mutant nomenclature follows FlyBase conventions (FlyBase, 2002). The following stocks were used: D^{r72} (Sanchez-Soriano and Russell, 1998), *Df(2L)N22-5* (Wustmann et al., 1989), $SoxN^{U6-35}$ (this study), balanced using $Cyo^{wg-lacZ}$, $TM3^{hb-lacZ}$ or $TM6B^{AbdA-lacZ}$ (FlyBase, 2002); GAL4 line *Kr*GAL4 (FBti0002365) (Castelli-Gair et al., 1994); UAS line UAS*SoxN* (this study). Mutant embryos were identified by staining with anti-β-Galactosidase antibody and fluorescently conjugated secondary antibody to detect the balancer chromosomes.

Molecular biology

The coding region for *SoxN* (sequence Accession Number, AJ252124) was amplified by PCR from two differently balanced heterozygous stocks and sequenced on both strands using an ABI Prism kit and automatic sequencer at the Department of Genetics sequencing facility. UAS*SoxNeuro* was generated by inserting bases 1 to 1966 of the *SoxNeuro* cDNA (which encompasses the entire coding region) into the *Eco*RI and *Not*I sites of pUAST (Brand and Perrimon, 1993). The construct was injected into *y w* embryos using standard techniques (Karess, 1985).

Antibody staining

Embryo staging was according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1985). Antibody staining was carried out essentially as described (Patel, 1994). Primary antibodies were detected with AP-conjugated secondaries (Vector labs), biotin-conjugated secondaries (Vector labs) and the ABC elite kit (Vectastain) or with fluorescent secondaries (Jackson Immunoresearch). The following primary antibodies were used at the indicated dilutions: rabbit anti-Dichaete 1/2000 (Sanchez-Soriano and Russell, 1998), rabbit anti-β-Galactosidase 1/2000 (Cappel), mouse anti-Achaete 1/3, mouse anti-BP102 1/100, mouse anti-Fasciclin II 1D4 1/4, mouse anti-Engrailed 4D9 1/10, mouse anti-Eve 3C10 (Developmental Studies Hybridoma Bank, Iowa), rabbit anti-Hunchback 1/1000 (gift of M. Gonzalez-Gaitan), rat anti-ems 1/1000 (U. Walldorf) and rabbit anti-Eagle 1/500 (Dittrich et al., 1997).

RESULTS

Identification of a *SoxN* mutation

We screened a large collection of chemically induced *Drosophila* mutations, isolated on the basis of abnormal CNS phenotypes (Hummel et al., 1999), for lines missing specific neuroblast lineages. One line (U6-35) was identified in which virtually all thoracic and abdominal *eagle* (*eg*)- and *empty spiracles* (*ems*)-expressing neurones and glia were missing from the CNS of homozygous embryos (e.g. NB lineages 2-4, 3-3, 7-3 and 6-4 missing in over 99% of hemisegments, Fig. 1A,B; NB lineages 3-3, 3-5 and 4-4 missing in over 95% of hemisegments, Fig, 1C,D). We localised the mutation genetically by recombination and deficiency mapping and found that it was uncovered by *Df(2L)N22-5*, a deletion encompassing the 29F region (Lindsley and Zimm, 1992). We had previously recovered a Sox-domain containing gene in this region in the course of a molecular screen for new *Drosophila Sox* genes that was subsequently found to be identical to *SoxNeuro* (*SoxN*) (Cremazy et al., 2000). As *SoxN* is known to be expressed early in CNS development, and the related gene *Dichaete* had previously been shown to have specific CNS phenotypes, we considered *SoxN* to be a candidate for the gene mutated in the U6-35 line. We sequenced the *SoxN* gene from the U6-35 stock and found that it carried a C-T transition that changes a glutamine at position 133 of the protein to a stop codon. This premature stop occurs before the DNA-binding domain and is expected to eliminate the function of the gene. In support of this, we find that the phenotype of U6-35 homozygotes is identical to that observed in U6-35/*Df(2L)N22-5* embryos (data not shown). Therefore, U6-35 represents a null mutation in the *SoxN* gene and shall be hereafter referred to as $SoxN^{U6–35}$.

At a gross level, $SoxN^{U6–35}$ mutant embryos show a severely disrupted CNS. When examined with the global axonal marker BP102 and the more specific marker FasII we observe a substantial reduction in the longitudinal axon tracts (Fig. 1E,H). In 60% of the mutant hemisegments scored there is a complete loss of longitudinal tracts judged by BP102 staining (n=726). In addition, the anterior and posterior commissures are also affected; in 52% of mutant segments the commissures fail to separate and are sometimes absent (2%). With FasII staining we observe a disruption of the regular axonal fasciculation pattern and many cases of axons inappropriately crossing the midline. There appears to be no difference in the phenotype along the anteroposterior axis. The PNS shows no major defects when examined with the PNS-specific 22C10 antibody (data not shown). Thus the defects in $SoxN^{U6–35}$ suggest a failure in the morphogenesis or differentiation of the CNS. As expected, as *SoxN* expression is initiated after cellularisation (Cremazy et al., 2000), we observe no segmentation defects in $SoxN^{U6–35}$ (data not shown). In addition to these phenotypes, we observe defects in spacing in 68% of $SoxN^{U6–35}$ mutant embryos; within the CNS the spacing between two segments in the middle of the embryo, most often A3 and A4, is greatly increased while spacing in the neighbouring segments is reduced (Fig. 2E); in severe cases there are gaps in the neuroectoderm, however, no segments are lost. As we never observe these defects before stage 12, we believe this phenotype is a result of mechanical defects during germband retraction; in support of this, we observe a failure to complete germband retraction in a small number of mutant embryos (less than 5%).

Loss of specific Neuroblast lineages in *SoxN*

In order to examine the defects in the developing CNS associated with $SoxN^{U6–35}$ in greater detail, we stained mutant embryos with markers for specific NBs and/or their progeny (see Materials and Methods). These data are presented in Table 1 and Figs 1, 2 and can be summarised as follows: using Hunchback (Hb) and Even skipped (Eve) (Fig. 2A,D), along with the Eg and Ems staining described above, we observe a striking asymmetry in NB loss in $SoxN^{U6–35}$ mutants. The use of Hb as a marker for all NBs delaminating in SI shows that medial column NBs are less affected (between 4% (MP2) and 38% (NB5-2) missing) than those that form in the intermediate (52% of NB5-3 missing) and lateral columns (between 23% (NB7-4) and 69% (NB 5-6) missing; Fig. 2A,B). This observation is supported by using Eve as a marker for progeny of certain NBs. The CQ neurones (NB 7-1) and aCC/pCC (NB 1-1), which are progeny of NBs that delaminate in the medial column during the SI wave, are relatively unaffected (less than 4% missing). By contrast, the RP2 neurone, which is a progeny of NB 4-2, an intermediate column SII NB, and the cells of the Eve lateral cluster (ELC), which are progeny of the laterally delaminating SIV NB 3-3, are strongly affected (96% and 100% missing, respectively; n=352; Fig. 2C,D). Additionally, the antibody staining against Eg and Ems show that NBs delaminating in the intermediate or lateral columns in SII-SV are often missing (e.g. 6-4, 7-3, 2-4 and 3-3, greater than 90% loss).

Taken together, these data suggest that *SoxN* is required for the correct specification and/or formation of NBs in both the intermediate and lateral columns. It appears that there is much less of a requirement for *SoxN* in the medial column, at least for early delaminating NBs. As *SoxN* and *Dichaete* expression overlaps in the medial neuroectoderm and *Dichaete* mutants also have little effect on early medial NB lineages (Table 1) (Zhao and Skeath, 2002), it is possible that the proteins are able to functionally compensate in this part of the developing CNS. However, the fact that later-born intermediate and lateral NBs are more affected than the S1 NBs delaminating from these regions additionally suggests a stronger requirement for SoxN function in these post S1 NBs and/or their progeny.

Table 1. Neuroblast loss in *Sox* mutant embryos

				% absence (number of hemisegments)		
Wave	Lineage	Column	Marker	$SoxN^{U6\text{-}35}$	D^{r72}	$SoxN^{U6\text{-}35}$; D^{r72}
S1	1-1	Medial	*hb*	12 (172)	-	-
			eve (aCC/pCC)	0 (352)	0 (500)*	15 (220)†
	MP2	Medial	*ac*	4 (108)	0 (132)‡	54 (176)§
			hb	5 (172)	-	-
	5-2	Medial	*hb*	38 (172)	-	-
	7-1	Medial	*ac*	19 (108)	0 (132)‡	54 (176)§
			hb	4 (172)	-	-
			eve (CQ)	3 (352)	0 (500)	15 (220)†
	5-3	Intermediate	*hb*	52 (172)	2 (46)	79 (24)
	2-5	Lateral	*hb*	36 (172)	-	-
	3-5	Lateral	*ac*	64 (108)	0 (132)‡	80 (176)§
			hb	56 (172)	-	-
			ems	96 (308)	-	-
	5-6	Lateral	*hb*	69 (172)	-	-
	7-4	Lateral	*ac*	75 (108)	0 (132)†	80 (176)§
			hb	23 (172)	-	-
S2	4-2	Intermediate	*eve* (RP2)	96 (352)	3 (500)*	94 (176)
S3	6-4	Lateral	*eg*	100 (114)	-	-
S4	2-4	Intermediate/lateral	*Poxn*	92 (110)	-	-
			eg	100 (304)	-	-
	3-3	Intermediate/lateral	*eve* (ELC)	100 (352)	0 (500)*	99 (176)
			eg	100 (304)	-	-
			ems	96 (308)	-	-
	4-4	Intermediate/lateral	*ems*	96 (308)	-	-
S5	7-3	Lateral	*eg*	100 (304)	-	-
			ey	99 (418)	-	-

*In addition to the occasional loss of RP2 in D^{r72}, we observe a duplication of RP2 and an expansion of ELC in 8% and 1% of hemisegments, respectively, as well as a duplication of cells at the position of the aCC/pCC neurons in 16% of hemisegments.

†Owing to the severe defects found in the double mutants resulting from the $SoxN^{U6\text{-}35}$ spacing defects and the D^{r72} segmentation phenotype, we were unable to score the more severely affected abdominal segments in many embryos; hence, the figures here are rather conservative.

‡In D^{r72} mutant embryos, we frequently observed an expansion of *ac* expression into the intermediate column.

§We were unable to unambiguously identify rows 3 and 7 in double mutant embryos. We have therefore only scored the presence or absence of *ac* in each column.

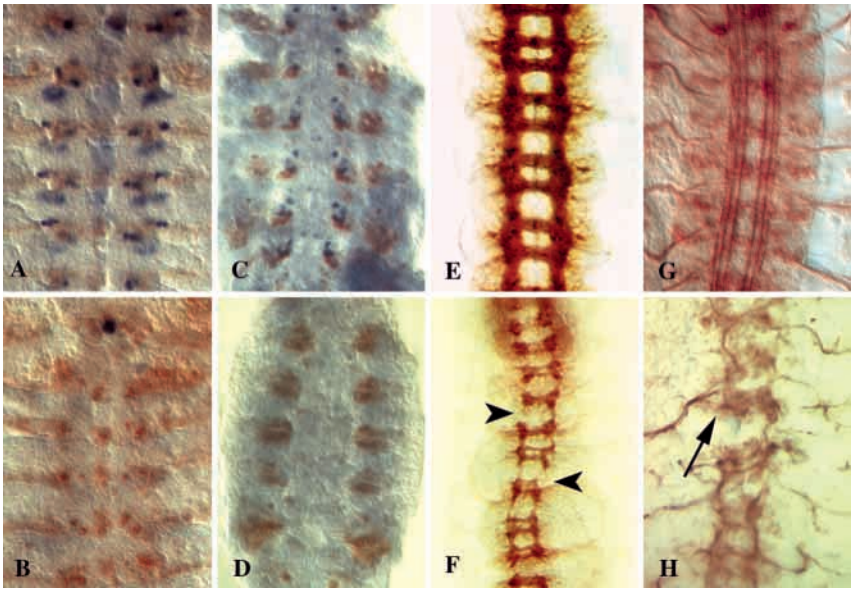

Fig. 1. Nervous system defects in *SoxNeuro* mutants. Flat preparations (A-E,G,H) or a whole-mount (F) of stage 16 (A,B,E-H) and stage 11 (C,D) wild-type (A,C,E,G) and $SoxNeuro^{U6\text{–}35}$ (B,D,F,H) embryos stained with anti-Eagle (blue) and anti-Engrailed (brown) (A,B), anti-Eagle (blue) and anti-Ems (brown) (C,D), monoclonal antibody mAbBP102 (E,F) and anti-Fasciclin II (G,H). (A,B) In wild-type embryos, Eagle staining is observed in progeny of the NB2-4, NB3-3, NB7-3 and thoracic NB6-4 lineages. In $SoxNeuro^{U6\text{–}35}$ embryos, no Eagle staining is seen in these lineages. Eagle expression is still seen in cells in the gnathal midline. (C,D) Ems-expressing progeny of the NB3-5 and NB4-4 and NB3-3 lineages are absent in more than 96% of hemisegments in $SoxNeuro^{U6\text{–}35}$ embryos. Note that in embryos in which one of these cells is observed, we are unable to unambiguously identify which of the three neuroblasts is present. Tracheal Ems expression is still present. (E,F) In $SoxNeuro^{U6\text{–}35}$ embryos, longitudinal BP102 staining is absent in 60% of hemisegments (arrowheads in F); in addition commissures fail to separate correctly in 52% of hemisegments. (G,H) In $SoxNeuro^{U6\text{–}35}$ embryos, the regular axonal fasciculation pattern is disrupted and many axons cross the midline inappropriately (arrow).

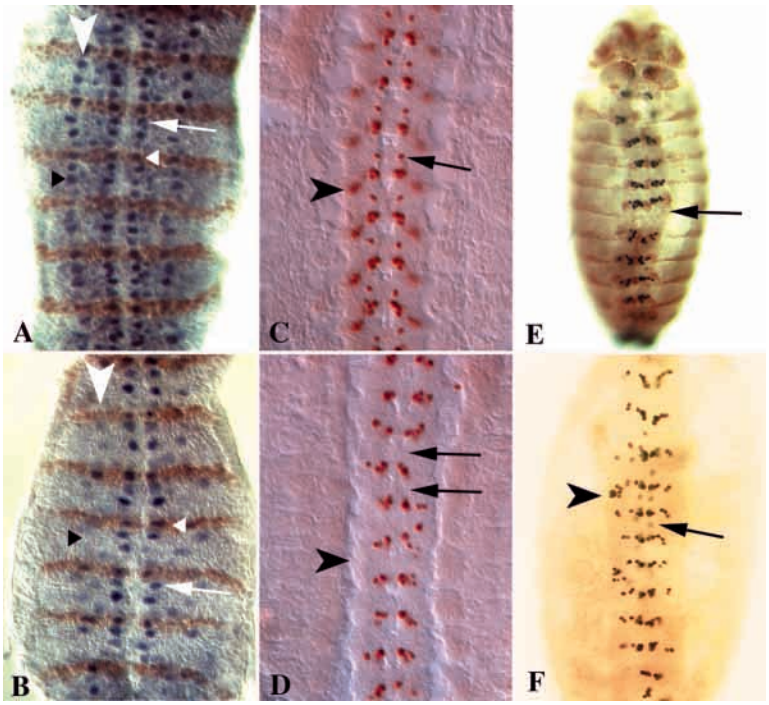

Fig. 2. Lateral neuroblast lineages are absent in *SoxNeuro* mutants. Flat preparations of late stage 8 embryos stained with anti-Hunchback and anti-Engrailed. (A) Wild-type, Hunchback-expressing neuroblasts form an orthogonal array of four neuroblasts per hemisegment in each of the medial and lateral (large white arrowhead) columns, with a single cell in the intermediate column. (B) Lateral column neuroblasts are lost in between 20% and 70% of hemisegments in *SoxNeuro*$^{U6–35}$ mutant embryos (large white arrowhead), for example, NB3-5 (black arrowhead). Medial column neuroblasts are less frequently affected; NB1-1 (white arrow) and NB7-1 (white arrowhead) are present in over 88% of hemisegments. (C,D) Flat preparations of stage 16 embryos stained with anti-Even skipped. (C) Wild-type and (D) *SoxNeuro*$^{U6–35}$. The NB4-2 lineage RP2 motorneurones (arrows) and the NB3-3 lineage lateral cluster (arrowheads) are absent in *SoxNeuro*$^{U6–35}$; however, aCC/pCC and CQ cells are unaffected. (E) Stage 16 whole-mount *SoxNeuro*$^{U6–35}$ embryo stained with anti-Even skipped (black) and anti-Engrailed (brown). The spacing within the CNS is greatly increased between segments A3 and A4 and reduced between A2 and A3; notice that both Eve- and En-expressing neurones are no longer aligned with Engrailed expression in the epidermis (arrow). (F) Stage 16 whole-mount *SoxN*$^{U6–35}$ /*SoxN*$^{U6–35}$; *Kr*GAL4/UAS*SoxN* embryo. Within the domain of *Kr*GAL4 expression, RP2 motorneurones (arrow) and ELC cells (arrowhead) are present in 67% and 33% of hemisegments, respectively.

Expression of *SoxN* in the developing CNS rescues *U6-35* phenotypes

To unambiguously demonstrate that the phenotype of U6-35 mutant embryos is due to the mutation in *SoxN*, we tried to rescue *SoxN*$^{U6–35}$ phenotypes by driving UAS*SoxN* expression in the developing CNS with *Kr*GAL4 (Castelli-Gair et al., 1994). The *Kr*-Gal4 line expresses Gal4 at high levels in the neuroectoderm within the central domain of the embryo from stage 9 onwards (data not shown). In *SoxN*$^{U6–35}$/*SoxN*$^{U6–35}$; *Kr*GAL4/UAS*SoxN* embryos, we observe a substantial rescue of RP2 neurones and ELC cells (progeny of NB4-2 and NB3-3, respectively; Fig. 2F). Absence of the RP2 neurone is now observed in only 33% of hemisegments, while the ELC cells are absent in 67% of hemisegments compared with 96% and 100% respectively in embryos without UAS*SoxN*. These data indicate that the CNS phenotype of U6-35 embryos results from a specific loss of *SoxNeuro*.

SoxN in the neuroectoderm

The differential loss of NBs and their progeny in the DV axis may result from the failure of neuroectodermal cells to be specified to a neural fate. As *SoxN* is expressed throughout the neuroectoderm prior to neuroblast delamination and *Dichaete* is reported to have effects on proneural gene expression (Zhao and Skeath, 2002), we examined proneural gene expression in *SoxN*$^{U6–35}$ mutants. *ac* is a marker for certain medial and lateral proneural clusters but is not normally expressed in the intermediate column. We observe a striking reduction in the number of lateral column proneural clusters expressing *ac* (Fig. 3A-D). In 70% of these clusters, *ac* expression is no longer detected, compared with 12% of medial column proneural clusters (n=108). The loss of lateral column *ac* expression suggests that *SoxN* functions early in the neuroectoderm to specify proneural clusters correctly. In addition to this, there is an overall reduction in *ac* expression levels in the medial proneural clusters compared with the heterozygous sibling embryos stained in the same reaction (compare Fig. 3E with 3F). This implies that *SoxN* is required more generally in the neuroectoderm to establish the appropriate level of *ac* expression. We also examined the expression of the related proneural gene *l'sc* in the neuroectoderm prior to neuroblast delamination and, in contrast to the results with *ac*, we see no appreciable effect (data not shown). Thus it appears that *SoxN* is selectively required in the neuroectoderm for the regulation of some proneural gene expression. The loss of *ac* expression in lateral proneural clusters partly explains why we see such a dramatic loss of lateral NBs in *SoxN*$^{U6–35}$ mutants. However, all lateral NBs are strongly affected in *SoxN*$^{U6–35}$ embryos, including those which express *l'sc* and not *ac*. Hence, the normal expression of *l'sc* in *SoxN*$^{U6–35}$ mutant embryos argues against a simple linear pathway in which *SoxN* acts only upstream of proneural genes, and suggests a mechanism in which *SoxN* functions both upstream and in parallel to the proneural genes to promote neuroblast formation. This parallel function of *SoxN* is additionally supported by the observation that the severe hypoplasia of *SoxN* mutant embryos resembles the phenotype in *AS-C* mutants, and is more severe than can be accounted for by the effect on *ac* expression, as loss of *ac* alone does not produce severe phenotypes (Jimenez and Campos-Ortega, 1987).

Zhao and Skeath (Zhao and Skeath, 2002) have reported a derepression of *ac* expression in the intermediate column in *Dichaete* mutants and we confirm those observations.

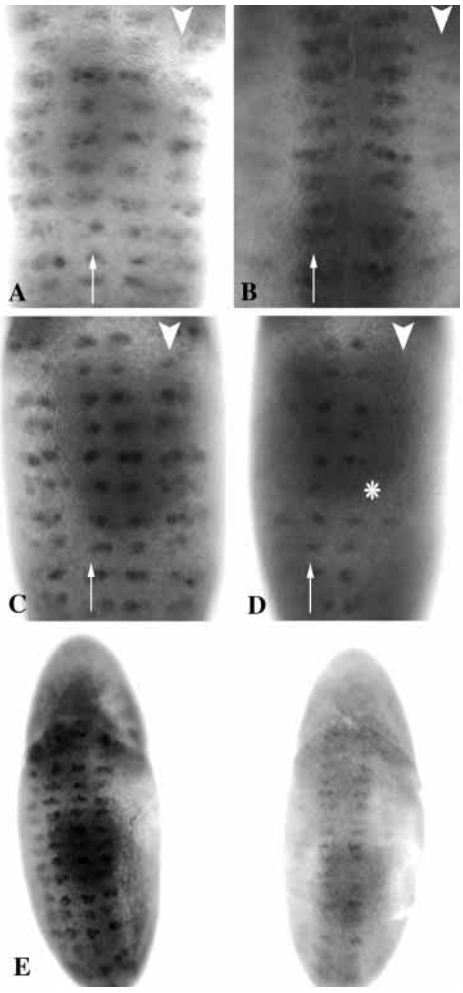

Fig. 3. Lateral Achaete expression is lost in *SoxNeuro* mutant embryos. Late stage 8 (A,B) and stage 9 (C,D) whole-mount views of wild-type (A,C) and *SoxNeuro*$^{U6–35}$ (B,D) embryos stained with anti-Achaete. In wild-type embryos, Achaete is expressed in proneural clusters of five to seven cells that give rise to the medial MP2 and NB7-1 and the lateral NB3-5 and NB7-4 neuroblasts. In *SoxNeuro*$^{U6–35}$ embryos, Achaete protein is undetectable laterally in 70% of rows (white arrowhead); however, Achaete is still observed in 80% of rows medially (white arrow); asterisk in D shows absence of Achaete in one NB7-1 neuroblast. (E) Achaete expression is greatly reduced in both medial and lateral columns in *SoxNeuro*$^{U6–35}$. The *SoxN*$^{U6–35}$/*SoxN*$^{U6–35}$ embryo to the right shows a much lower level of Achaete expression than its *SoxN*$^{U6–35}$/Cyo sibling of the same stage from the same staining reaction.

Therefore, whereas both *SoxN* and *Dichaete* mutants show loss of neuroblasts, the effect in the neuroectoderm differs: *SoxN* mutants display loss of *ac* expression but *Dichaete* mutants show some *ac* derepression.

Evidence for functional redundancy between group B Sox proteins

SoxN and *Dichaete* are both expressed early in the neuroectoderm. *Dichaete* is restricted to the ventral region, extending from the midline to the position of the intermediate column (Zhao and Skeath, 2002), and *SoxN* is excluded from the midline and extends more dorsally to encompass the entire neuroectoderm (Cremazy et al., 2000). *Dichaete* mutants show strong phenotypes in the midline, where it is uniquely expressed (Sanchez-Soriano and Russell, 1998), and *SoxN* mutants exhibit strong phenotypes in the lateral half of the CNS where it is uniquely expressed. In *Dichaete* mutants, SI medial NBs are not affected (Table 1) (Zhao and Skeath, 2002) but there is a loss of later delaminating SII and SIII NBs from both medial and intermediate columns. *SoxN* and *Dichaete* overlap in the medial and intermediate neuroectodermal columns and in the medial column, *SoxN* phenotypes are weaker than those observed in the lateral columns. These data are consistent with the idea that the genes may be able to compensate functionally in the medial column neuroectoderm. To examine the consequences of removing group B *Sox* function from the early CNS, we constructed a double mutant combination, using null alleles for both *Dichaete* and *SoxN*. We examined the overall structure of the CNS as well as markers for specific NBs and/or progeny in the double mutant embryos.

Staining the double mutants with BP102 reveals a severe disruption in the organisation and structure of the CNS (Fig. 4A,B). We observe a complete loss of longitudinal axons in many segments with frequent gaps in the neuropil. Commissures are often absent, and those that do form are virtually never separated. The phenotype of the double mutants is far more severe than observed with either single mutant and supports the idea that the genes can act redundantly or in related pathways. If this is the case then we expect to see an enhanced effect on medial NBs and their progeny when we remove both *SoxN* and *Dichaete* function compared with each of the single mutants, as this is the region in which they are extensively co-expressed. In line with this expectation we observe that in the SI medial lineages of NB1-1 and NB7-1, identified by *eve* expression, there is a rather severe reduction in the number of aCC/pCC and CQ cells in double mutants (15% loss; however, due to difficulties in reliably scoring the severely affected abdominal segments of the double mutant embryos, we believe this figure to be a conservative measure; Fig. 4C,D) compared with each of the single mutants. Note that these lineages are virtually unaffected in either of the single mutants. Additionally, in the intermediate column, the Hb expressing neuroblast 5-3 is absent at a higher frequency in double mutant embryos than in *SoxN* or *D* mutants (79% compared with 52% and 2%, respectively, Fig. 2A.B, Fig. 4H,I), indicating that *Dichaete* is to some extent able to compensate for a loss of *SoxNeuro* within this lineage. Although it is impossible to determine accurately the identity of the remaining Hb expressing SI NBs in the double mutants, we have counted the total number of cells in thoracic segments and find that, in *SoxN*$^{U6–35}$ homozygotes, 30% of Hb expressing NBs are missing, in *Dichaete* mutants less than 1% are missing, whereas 56% are missing in the double mutants. Taken together, we conclude that in the cases of overall CNS structure as well as medial and intermediate column SI NBs, we see evidence for functional redundancy between related Group B Sox genes.

As described above, the situation in the neuroectoderm appears to be different. In *Dichaete* mutants the proneural gene *ac* is partially derepressed in intermediate column neuroectoderm, there are no major effects on the medial column (Zhao and Skeath, 2002). In *SoxN*$^{U6–35}$ we see a loss of lateral column *ac* expression as well as an overall

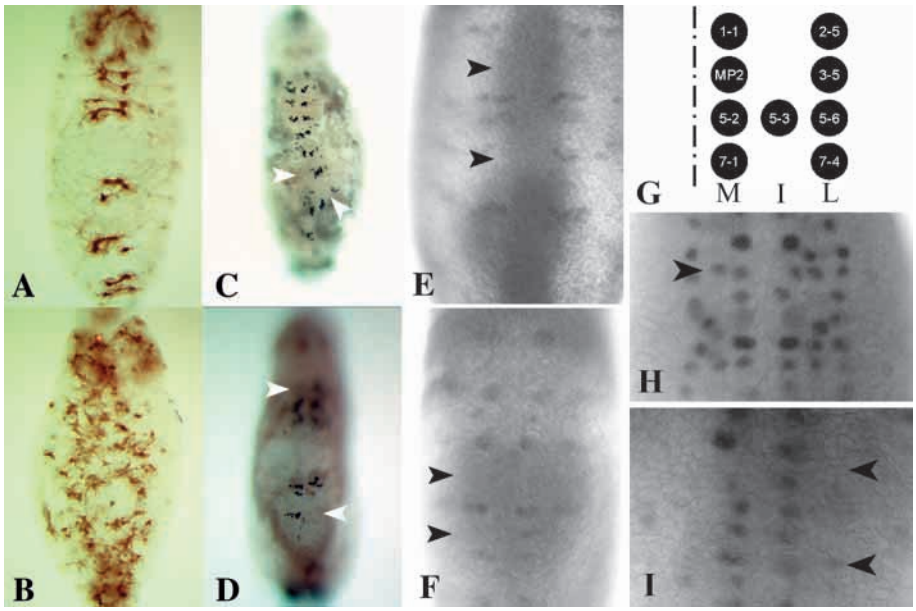

Fig. 4. *SoxNeuro; Dichaete* double mutant embryos are far more severely affected than either single mutant. Whole-mount ventral views of stage 16 (A-D), stage 8 (E) and stage 9 (F) *SoxNeuro*$^{U6–35}$; *D*^{r72} double mutant embryos stained with BP102 (A,B), anti-Eve (C,D), anti-Achaete (E,F) and anti-Hb (H,I). (A,B) Double mutant embryos show a variable severe hypoplasia; longitudinal axons are almost totally absent and there are frequent gaps in the neuropil. (C,D) As in *SoxNeuro*$^{U6–35}$, RP2 and ELC staining is variably absent in double mutant embryos; in addition, we see a loss of aCC/pCC and CQ cells in 15% of hemisegments (arrowheads). The defects in D are much more extreme than can be accounted for by the *Dichaete* segmentation phenotype, suggesting that many segments fail to express Eve in any cells. (E,F) Achaete protein is absent laterally in *SoxNeuro; Dichaete* embryos as in *SoxNeuro*$^{U6–35}$; however, Achaete is now undetectable in 54% of medial clusters (arrowheads); Achaete staining is completely absent in 21% of segments (arrowheads). (G) Diagrammatic representation of S1 neuroblasts at late stage 8. Neuroblasts are arranged in an orthogonal array of four rows and three columns [medial (M), intermediate (I) and lateral (L)]. (H,I) Late stage 8 *D*^{72} (H) and *SoxN*$^{U6–35}$; *D*^{r72} (I) embryos. S1 neuroblasts are barely affected in *Dichaete* mutant embryos [e.g. NB5-3 (arrowhead) is missing in 2% of hemisegments]. As in *SoxN*$^{U6–35}$, lateral NBs are frequently absent in double mutant embryos; in addition, within the intermediate column we observe Hb-expressing cells in only 21% of hemisegments compared with 48% in *SoxN* mutants (arrowheads). In I, we are unable to unambiguously identify these cells as NB 5-3.

reduction in *ac* levels. When we examine the double mutants for *ac* expression, we observe a synergistic and an additive effect (Fig. 4E,F). As with *SoxN*, the overall level of *ac* expression is lower compared with heterozygous siblings and there is a marked loss of lateral column *ac* expression. In addition, the double mutants display the *Dichaete* phenotype as we see ectopic intermediate column expression in some rows (6%). However, in 21% of segments we do not detect any *ac* expression, suggesting that both Sox genes are principally able to positively regulate *ac* expression. Taken together, we conclude that in the neuroectoderm, the elimination of group B Sox expression results in an early failure in neural specification and subsequent loss of neural progenitors.

Both *SoxN* and *Dichaete* are expressed early in the neuroectoderm, *SoxN* expression being initiated slightly before that of *Dichaete*. It is therefore possible that SoxN regulates the expression of *Dichaete* and we examined this possibility by staining *SoxN*$^{U6–35}$ mutant embryos for Dichaete (Fig. 5). We observe a rather unexpected phenotype; in around half of the mutant embryos, Dichaete levels are apparently normal (Fig. 5A,B). However, in the remaining half we noticed that Dichaete levels were reduced, but only in the anterior half of the neuroectoderm, the posterior appeared to be normal (Fig. 5C,D). We know that this is not due to a staining artefact because in the affected embryos Dichaete is expressed normally in the midline all along the AP axis. Thus, it appears that *SoxN* does have an effect on *Dichaete* expression, but that this effect is variable and restricted along the AP axis. In any case we cannot explain the *SoxN* phenotypes by a loss of *Dichaete* expression in the neuroectoderm because we would expect to see ectopic expression of *ac* in *SoxN*$^{U6–35}$ as we do in *Dichaete* and the double mutants.

Therefore, we conclude that in the neuroectoderm the two group B Sox proteins, SoxN and Dichaete, can functionally compensate but that they also have antagonistic functions, particularly within the intermediate neuroectoderm.

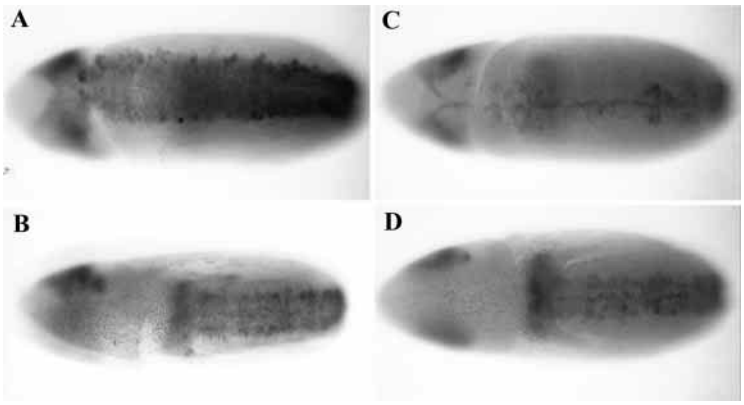

Fig. 5. Dichaete expression is variably reduced in *SoxNeuro*$^{U6–35}$ mutant embryos. Whole-mount ventral (A,C) and dorsal (B,D) views of stage 9 wild-type (A,B) and *SoxNeuro*$^{U6–35}$ (C,D) embryos stained with anti-Dichaete. In half of mutant embryos, Dichaete expression is reduced in the anterior region of the neuroectoderm but appears normal posteriorly. Note that expression of D in the midline and brain is unaffected.

DISCUSSION

In this paper, we provide the first report of completely removing Group B *Sox* gene function from the developing CNS of an animal. As we describe above, in many metazoans the developing CNS is marked by the expression of related *Sox* genes and the idea that the related genes may be functionally redundant has been widely discussed (see Wenger, 1999). Our results support the idea that related group B genes show a degree of functional redundancy because, in double mutants, severe neural phenotypes are observed in regions where the genes are co-expressed and much weaker phenotypes displayed by each of the single mutants.

Redundant Sox function in the CNS

We have presented a number of observations that support the view that group B *Sox* genes, *Dichaete* and *SoxNeuro*, are to some extent functionally redundant in the developing CNS of *Drosophila*. While we observe strong phenotypes in *Dichaete* and *SoxN* single mutant in regions where they are uniquely expressed, *Dichaete* in the midline and *SoxN* in the lateral CNS, we see much weaker phenotypes where the expression domains overlap, especially in the medial neuroectoderm. In double mutants, we find strong phenotypes, apparent at a gross level in the overall structure of the CNS, and also with molecular markers for the neuroectoderm or specific NBs and their progeny. Taken together, these observations support the view that when one group B *Sox* gene is lost the other is able to functionally substitute in a part of the region where they are co-expressed. This is not entirely unexpected as both genes share a virtually identical DNA-binding domain and would be expected to recognise the same DNA sequence. It is interesting to note that, outside of the DNA-binding domains, there is little sequence similarity between *SoxN* and *Dichaete*, suggesting that redundant function is mediated solely through DNA binding.

Differential functions of *SoxN* and *Dichaete*

Zhao and Skeath (Zhao and Skeath, 2002) found that *ac* expression was derepressed in the intermediate column neuroectoderm of *Dichaete* mutants; this is not the case with *SoxN*. In the double mutants, we observe the *SoxN* loss of *ac* expression in lateral and medial neuroectoderm but a low frequency of ectopic intermediate column *ac* expression, presumably as a consequence of the loss of *Dichaete*. This suggests that, although the two Sox proteins may be functionally equivalent in the medial neuroectoderm they show antagonistic functions within the intermediate neuroectodermal region. Within this area, *Dichaete* functions as a negative regulator of the *ac* gene, whereas *SoxN* is a positive regulator of *ac*, as judged by the reduction of ectopic *ac* expression seen in the *SoxN/Dichaete* double mutants. Similarly, in the case of late NB formation we observe differences between SoxN and Dichaete. SoxN is required for both early and late forming NBs in the intermediate and lateral columns, Dichaete, however, appears to be required for the formation of some late delaminating NBs in the medial and intermediate columns (e.g. NB6-1, NB4-1 and NB4-2) (Zhao and Skeath, 2002). In some situations where *SoxN* and *Dichaete* expression domains apparently overlap, for example, in the intermediate column, we observe a severe loss of the SI intermediate column NB 5-3 in $SoxN^{U6–35}$, as judged by loss of Hb expression, a phenotype that is enhanced in $SoxN^{U6–35}$; D^{r72}. This suggests that, at least in the case of this early forming NB, *Dichaete* can only partially compensate for a loss of *SoxNeuro* in NBs derived from the intermediate column. This may be explained in part by the observation that *Dichaete* is rapidly downregulated in early NBs as they delaminate (Zhao and Skeath, 2002).

We presume that the differences between the two proteins may well reflect interactions between each Sox protein and a different partner mediated by protein domains outside the highly conserved DNA-binding domain. In accordance with this, Zhao and Skeath suggest that, in the neuroectoderm, *Dichaete* interacts with the product of the *ind* gene to mediate repression of *ac*. As *ind* is specifically expressed within the intermediate neuroectoderm, it is tempting to speculate that this protein might interact specifically with Dichaete to repress *ac* while it does not interact with SoxN in the same way if indeed at all. However, Zhao and Skeath (Zhao and Skeath, 2002) provide evidence for interactions between *Dichaete* and both *ind* and *vnd* in the context of NB specification. As our data suggest that SoxN and Dichaete function is at least redundant within the *vnd*-positive medial row, it is very likely that Vnd interacts with SoxN as well as Dichaete.

The interaction of HMG-domain proteins and homeodomain containing proteins has been recognised for some time, and appears to be a general feature of HMG1-type DNA-binding domains (Kamachi et al., 2000; Dailey and Basilico, 2001). More specific interactions between Sox-domain proteins and homeodomains have been demonstrated in mouse, where Sox2 interacts with Oct4 (Yuan et al., 1995), and in flies, where Dichaete interacts with Vvl (Sanchez-Soriano and Russell, 1998; Ma et al., 2000). Therefore we consider it likely that *SoxN* can interact with the DV patterning protein Vnd, and also with Ind and Msh to regulate expression in the *AS-C*; the observation that *vnd*, *ind* and *msh* transcript levels are unaffected in $SoxN^{U6–35}$ suggests that *SoxN* is likely to act in parallel with rather than upstream of these DV patterning genes (data not shown). Chu et al. (Chu et al., 1998) have shown that the loss of *ac* in *vnd* mutant embryos is insufficient to explain the loss of S1 NBs as restoration of *ac* expression in this background does not rescue the phenotype, which itself is more severe than would be expected if the sole action of *vnd* was through *AS-C*; as in $SoxN^{U6–35}$, expression of *l'sc* is unaffected in lineages which fail to delaminate. The authors suggest an additional proneural activity regulated by *vnd* – we consider it likely that the role of *SoxN* parallel to *AS-C* in the neuroectoderm is to act alongside the DV patterning genes in controlling this activity. However, given that we see an overall reduction in *ac* expression throughout the neuroectoderm in $SoxN^{U6–35}$ it is possible that *SoxN* plays a more general role in regulating *ac*, perhaps acting as a factor for modulating chromatin structure.

Potential Sox target genes are complex

One feature that stands out in the studies of Sox activity in *Drosophila* is the structure of the target genes that have been identified to date. During embryonic segmentation, prior to the establishment of neuroectoderm, Dichaete is required for the correct expression of the primary pair-rule genes *even skipped*, *hairy* and *runt* (Nambu and Nambu, 1996; Russell et al., 1996). In the midline, *slit* is a direct target (Sanchez-Soriano and Russell, 1998; Ma et al., 2000), in the neuroectoderm *ac* is a likely target

and in the hindgut *hedgehog* and *decapentaplegic* (Sanchez-Soriano and Russell, 2000). Each of these genes is characterised by having complex structure and complex regulation. This is most apparent for the pair-rule genes, whose regulatory sequences extend over many kilobases. We notice that where *Dichaete* is the only Sox gene involved in the regulation of these genes (e.g. at cellular blastoderm) we observe a variable phenotype in *Dichaete* mutants, both at the gross morphological level and at the molecular level. We have previously suggested that this reflects an architectural role for Sox proteins in gene regulation, as Sox-domain proteins bind DNA in the minor groove and are capable of modulating chromatin structure (Russell et al., 1996). We note with interest that the regulatory sequences that control the expression of *ac* are also extremely complex (Campuzano et al., 1985) and the *ac* expression phenotype we observe in *SoxN-Dichaete* mutant embryos is also variable. This suggests that at least one of the functions of Sox proteins may be modulating chromatin structure at complex regulatory regions, allowing the integration of many different regulatory inputs. In this view, the loss of Sox function would destabilise gene expression but would not be expected to completely eliminate it.

Conservation of Sox function

As we describe in the introduction, many of the molecular mechanisms that control early events in CNS development have been conserved during evolution. We also describe how the expression of group B Sox genes has similarly been conserved in the early neuroectoderm. Is it possible that Sox genes perform similar roles in neural specification in mammals and flies? The expression of *Sox1* in the mouse suggests that it is expressed in the neuroectoderm as an early response to neural inducing signals and may function to direct cells toward a neural fate (Pevny et al., 1998). This is analogous to the situation we observe in *Drosophila*, where *SoxN* is reported to be regulated by the DV signalling system (Cremazy et al., 2001) and regulates *ac*, a neural fate determining gene. We have previously shown that *Drosophila* and mouse Sox genes are functionally conserved as we have rescued *Dichaete* CNS midline phenotypes with the mouse *Sox2* gene (Sanchez-Soriano and Russell, 1998). Therefore it seems possible that the role of Sox proteins in early CNS development may indeed be conserved, and it will be of considerable interest to ascertain whether or not mouse group B Sox genes can regulate proneural targets such as the mouse *AS-C* homologues.

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