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Otx2 and *HNF3β* genetically interact in anterior patterning

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ABSTRACT Patterning the developing nervous system in the mouse has been proposed to depend on two separate sources of signals, the anterior visceral endoderm (AVE) and the node or organizer. Mutation of the winged-helix gene *HNF3β* leads to loss of the node and its derivatives, while mutation of the homeobox gene *Otx2* results in loss of head structures, apparently at least partially because of defects in the AVE. To investigate the potential genetic interactions between the two signaling centers, we crossed *Otx2*+/- and *HNF3β*+/- mice and found that very few *Otx2*+/-;*HNF3β* +/- double heterozygous mutants survived to weaning. Normal Mendelian ratios of genotypes were observed during gestation, but more than half the double heterozygotes displayed a severe anterior patterning phenotype that would be incompatible with postnatal survival. The phenotype was characterized by varying degrees of holoprosencephaly, cyclopia with proboscis-like structures, and anterior forebrain truncations. Regional marker analysis revealed that ventral forebrain structures of *Otx2+/-;HNF3β +/-* mutant embryos were most severely affected. *Shh* expression was completely absent in the anterior region of *Otx2+/-;HNF3β +/-* embryos, suggesting that *Otx2* and *HNF3β* genetically interact, directly or indirectly, to regulate *Shh* expression in the anterior midline. In addition, the forebrain truncations suggest an involvement of both genes in anterior patterning, through their overlapping expression domains in either the AVE and/or the prechordal mesoderm.

KEY WORDS: *head organizer, Otx, HNF3β, Sonic hedgehog, forebrain patterning, holoprosencephaly.*

Introduction

The establishment of the basic body plan of the vertebrate embryo begins at gastrulation. It involves generation of the three germ layers, ectoderm, endoderm and mesoderm, and the elaboration of the three major body axes- anterior-posterior (A-P), dorsal-ventral (D-V) and left-right (L-R). Much of our current understanding of the mechanisms of establishing the body axes is based on the concept of the 'organizer', first proposed by Spemann and Mangold in the 1920s (reviewed (Harland, 1997)). They showed that a small piece of tissue from the dorsal lip of the blastopore in the amphibian gastrula could induce a whole new body axis, when transplanted ventrally. Transplanted tissue gave rise mostly to notochord in the new axis, while host ventral mesoderm was respecified to somites, and ectoderm was transformed into a new neural tube, with appropriate A-P pattern. No other part of the embryo had such a dramatic effect, suggesting that the organizer is a dominant source of signals that can both induce and pattern new neural tissue and respecify existing mesoderm populations. Transplant studies have identified a region of the embryo with similar properties in Zebrafish (the shield)(Shih and Fraser, 1996), the chick (Hensen's node)(Storey *et al.*, 1992; Waddington, 1933) and the mouse (the node)(Beddington, 1994; Tam and Steiner, 1999), suggesting that the organizer is a conserved element in vertebrate embryonic patterning.

As well as inducing correct D-V patterning in the developing mesoderm and inducing and providing A-P pattern information to the forming neural tube, the organizer also gives rise to the notochord, which is a critical source of signals for D-V patterning of the spinal cord and somites(Brand-Saberi *et al.*, 1993; Johnson *et al.*, 1994; Yamada *et al.*, 1991). Finally, a number of studies have implicated asymmetric sources of signals around Hensen's node as the initial event leading to later L-R asymmetry of the viscera (reviewed (Harvey, 1998)). Recent research has identified molecular events upstream of the formation of the organizer, as well as many candidate signaling components for the various activities of the organizer and its derivatives. However, at the same time, it has become apparent that the classic organizer is not the only source of patterning signals at gastrulation and that the situation is more complex than it first appeared.

In particular, there has been revived interest in the concept of a head organizer region physically distinct from the trunk organizer.

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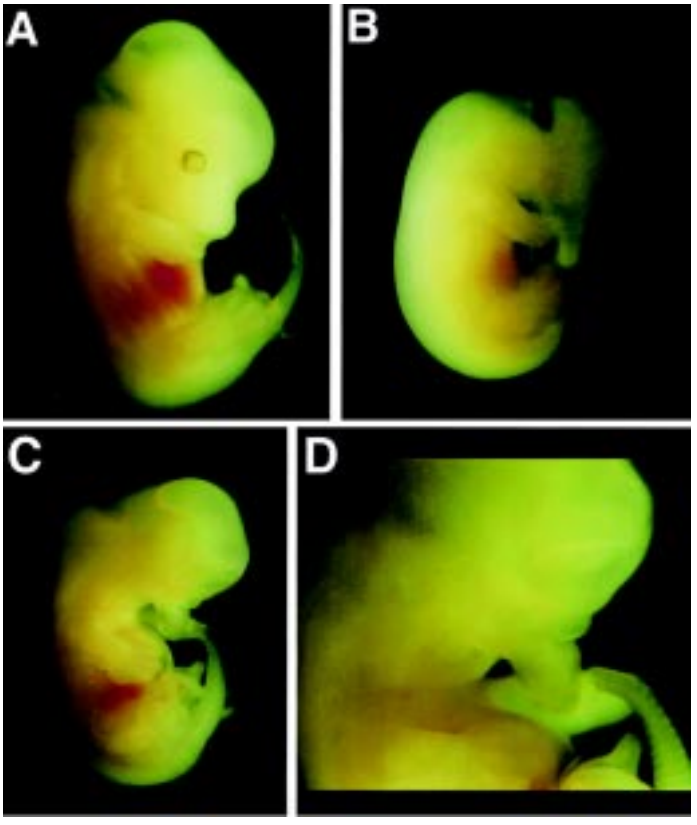

Fig. 1. Phenotype of *Otx2+/-;HNF3β +/-* embryos at E12.5. (A) *Lateral view of normal E12.5 embryo.* **(B,C,D)** *lateral views of Otx2+/-;HNF3β+/- mutants, demonstrating closely spaced or fused eyes with proboscis.*

In *Xenopus*, the axes induced by ectopic expression of organizer-associated gene products, like goosecoid(Cho *et al.*, 1991) and noggin(Smith and Harland, 1992), lack head structures, whereas a novel secreted molecule, Cerberus,(Bouwmeester *et al.*, 1996) produced by the deep endoderm associated with the dorsal lip of the blastopore, induces heads but no tails. In mouse, mutations in the genes *Lim1* (Shawlot and Behringer, 1995) and *Otx2*(Acampora *et al.*, 1997; Ang *et al.*, 1995; Matsuo *et al.*, 1995), expressed in the anterior of the embryo, cause loss of head structures anterior of the hindbrain, without major effect on the node and notochord. These observations, along with early expression patterns of a number of genes, led Beddington and colleagues (Beddington and Robertson, 1999; Thomas and Beddington, 1996) to propose that, in the mouse, there is a very early source of anterior patterning signals associated with the anterior region of the VE (AVE). This region is established before the primitive streak, independent of the node. A number of genes, including a mouse gene related to Cerberus, *Cer1*(Belo *et al.*, 1997; Biben *et al.*, 1998; Pearce, Penny and Rossant, 1999), are expressed in this AVE region, which would then be equivalent to the deep endoderm in frog. Support for the importance of the AVE in anterior patterning include Beddington's experiments showing that some anterior abnormalities occur when the AVE is scraped away(Thomas and Beddington, 1996) and experiments which showed that rabbit visceral endoderm could induce anterior neural structures in chick embryos(Thomas and Beddington, 1996). In addition, the anterior defects in *Otx2* mutant embryos can be partially rescued in chimeras when the visceral endoderm is wild-type(Rhinn *et al.*, 1998), or when the related *Otx1* gene is expressed in the visceral endoderm(Acampora *et al.*, 1998). There is, however, no direct evidence that the AVE alone can act to induce anterior neural structures in the mouse. Tam and colleagues have shown that the AVE fails to induce anterior neural tissue when grafted laterally in the egg cylinder and will only do so when more posterior tissue is included in the graft(Tam and Steiner, 1999). Similarly, we have shown that the AVE alone fails to induce anterior neural markers in naïve epiblast in *in vitro* explant-recombination experiments(Klingensmith *et al.*, 1999), but will do so in combination with mesoderm(Ang and Rossant, 1993).

A functional interdependence between the AVE and the signals of the node in patterning the head region is suggested by recent experiments showing that embryos lacking the two node-derived signaling molecules, noggin and chordin, but with an apparently normal AVE, show defects in both anterior notochord development and anterior truncations of the nervous system(Bachiller *et al.*, 2000). It has been difficult to determine the effect of ablation of the organizer on anterior patterning because of the ability of the organizer to regenerate(Davidson *et al.*, 1999; Psychoyos and Stern, 1996; Yuan and Schoenwolf, 1999). However, mutation of the winged helix gene, *HNF3β* , leads to complete absence of all node and midline structures, including the later notochord, floorplate and gut(Ang and Rossant, 1994; Weinstein *et al.*, 1994).Such embryos do develop a nervous system which shows relatively normal expression of A-P markers, although the domains of expression of the most anterior are reduced(Klingensmith *et al.*, 1999). However, since *HNF3β* embryos are severely compromised by other defects brought about by lack of notochord and gut structures, the exact nature of anterior patterning is not clear in these embryos. *HNF3β* is also expressed broadly in the VE, including the AVE, and tetraploid aggregates with wild-type VE and mutant epiblast show improved anterior patterning, consistent with a role for the AVE(Dufort *et al.*, 1998). Again, however, the embryos do not proceed far enough to determine if anterior patterning is correctly maintained in the presence of a normal AVE but in the absence of the node.

We have been taking various experimental approaches to investigate the possible interactions among the AVE, the node and other signaling centers in the mouse embryo. One approach is genetic. Mice heterozygous for the *HNF3β* mutation are viable but show an incompletely penetrant phenotype affecting development

TABLE 1

FREQUENCY OF GENOTYPES RESULTING FROM *OTX2*$^{+/-}$ AND *HNF3β*$^{+/-}$ INTERCROSSES AT WEANING STAGE

Genotype	Number of mice	Frequency (%)	Expected frequency (%)
(OO;HH)	69	39%	30%
(Oo;HH)	54	30%	26%
(OO;Hh)	48	27%	24%
(Oo;Hh)	8	4%	20%

O: *Otx2+*
o: *Otx2-*
H: *HNF3β+*
h: *HNF3β-*

x^2-16.7
d.f.=3,
p<0.001.

Note: expected frequency of double heterozygotes calculated from combining observed reduction in frequency of single heterozygotes compared with wild-type controls, assuming effects are additive, not synergistic.

TABLE 2

PHENOTYPES RESULTING FROM *OTX2*+/- AND *HNF3β*+/- INTERCROSSES AT E12.5

Genotype	*Otx2*	+/+	+/-	+/+	+/-
	HNF3β	+/+	+/+	+/-	+/-
Number of embryos		26(25%)	29(27%)	26(25%)	25(24%)
Phenotype					
Cyclopia, reduced separation or partial fused eyes and proboscis		-	-	-	10
Anterior abnormality with severe growth retardation		-	-	-	2
Gastroschisis with smaller head		-	-	-	2
No nose and cyclopia		-	1	-	-
Exencephaly		-	1	1	-

of the lower jaw(Ang and Rossant, 1994). This heterozygous phenotype suggests that *HNF3β* is present in limiting amounts. In such a situation, exacerbation of the *HNF3β* heterozygous phenotype might be expected in mutant mice doubly heterozygous for the *HNF3β* mutation and for mutations in genetically interacting loci. We have sought candidate interacting loci by crossing *HNF3β* heterozygotes with mutations in other early acting patterning genes, in an effort to establish the genetic hierarchies of early D-V and A-P patterning in the mouse. Here, we describe a genetic interaction between *HNF3β* and *Otx2*, a gene involved in anterior patterning. Double heterozygous embryos showed varying degrees of holoprosencephaly, and cyclopia with proboscis-like structures, as well as anterior truncations of varying degrees. *Shh* expression was severely reduced in the anterior midline of double heterozygotes, suggesting that *Otx2* and *HNF3β* act together, directly or indirectly, to regulate both D-V and A-P patterning in the anterior of the embryo.

Results

Morphological analysis of Otx2 *and* HNF3β *double heterozygous mutant embryos*

To investigate the possible interaction between *Otx2* and *HNF3β*, *Otx2* heterozygous mice were crossed with *HNF3β* heterozygotes and offspring were genotyped three weeks after birth. Southern blot analysis revealed that the number of double heterozygous weanlings was significantly reduced below the number expected based on the additive effects predicted from the observed losses due to heterozygosity at the individual loci (Table 1). Incompletely penetrant heterozygous defects have been reported previously for both *HNF3β* (Ang and Rossant, 1994; Weinstein *et al*., 1994) and *Otx2*(Matsuo *et al*., 1995). Among the surviving double heterozygotes, two died after eight months and two died after eleven months. All of these had incisor overgrowth and jaw defects, typical of *HNF3β* heterozygotes. The remaining four appeared normal and healthy, although one of them had overgrowth of the incisors. Several double heterozygous pups died on the day of birth and showed severe mandible abnormalities. When litters were dissected at E18.5, some double heterozygous embryos had already begun to be resorbed (data not shown). Taken together, these observations suggest that the doubly heterozygous condition leads to a variably penetrant lethal phenotype.

We then dissected and genotyped litters at E12.5 and E9.5. Genotyping results from E12.5 and E9.5 yolk sacs identified the different genotypes at roughly the expected Mendelian frequencies (Table 2, Table 3). However, about 56% of double heterozygous mutants displayed an obvious phenotype at E12.5, which was characterized by cyclopia or partial cyclopic eyes, and proboscis and other defects (Table 2, Fig. 1 and data not shown). At E9.5, this phenotype was already apparent and varied in its severity (Fig. 2). Some double heterozygotes showed a clear phenotype, with a dramatic reduction in the size of the forebrain. In the most extreme cases, the forebrain was lost (Fig. 2B). However, in most mutants, the forebrain was present but reduced in size (Fig. 2 C,D). In those embryos with an obviously reduced forebrain, the floor and roof of the neural tube were almost in contact with each other at the diencephalic and mesencephalic junction (Fig. 2C). Although the overall size of most *Otx2+/-;HNF3β +/-* mutant embryos was smaller than wild-type or heterozygous embryos, the posterior part of each embryo appeared normal.

Histological analysis of the Otx2+/-;HNF3β *+/- phenotype*

To characterize the *Otx2+/-;HNF3β +/-* phenotype in more detail, E12.5 and E9.5 double heterozygous embryos showing a mutant phenotype were sectioned for histological analysis (Fig. 3, 6 and data not shown). In wild-type or singly heterozygous embryos there are two telencephalic vesicles (future lateral ventricles Fig. 3 A,B). However, in *Otx2+/-;HNF3β +/-* mutants these two vesicles were fused into a single telencephalic vesicle (Fig. 3 D,E). The size of the telencephalon and diencephalon was also reduced (Fig. 3 D,E). The more striking phenotype was a fused single eye (cyclopia) and a proboscis-like structure (Fig. 3 F,H). In those *Otx2+/-;HNF3β +/-* mutants which showed the cyclopia phenotype, the optic vesicles were fused at midline and the lateral optic stalks were absent (data not shown). Some *Otx2+/-;HNF3β +/-* mutants had two smaller eyes close to the midline(data not shown). In all cases, whether single or double, the structure of the eyes appeared morphologically normal. The lens, neural layer of the retina and corneal ectoderm were formed normally (Fig. 3 F,H). There were complex craniofacial abnormalities, including proboscis-like structures and reduced branchial arch derivatives in later *Otx2+/-;HNF3β +/-* embryos (Fig. 3H). Despite these severe anterior abnormalities, the rest of the body axis, including spinal cord, notochord, heart and other organ systems, appeared normal (Fig. 6 M,N). These results suggest that defects in *Otx2+/-;HNF3β +/-* mutants were confined to anterior structures.

Comparison of Otx2 *and* HNF3β *expression in E7.5-E9.5 mouse embryos*

To investigate further how *Otx2* and *HNF3β* could be interacting in the anterior regions of the embryo, we compared the expression of *Otx2* RNA and HNF3β protein in E7.5-E9.5 mouse embryos by

TABLE 3

FREQUENCY OF GENOTYPES RESULTING FROM *OTX2* AND *HNF3β* INTERCROSSES AT E9.5

Genotype	Number of embryos	Frequency	Phenotypes	
			Anterior abnormality	Open neural tube
(OO;HH)	77	27	-	-
(Oo;HH)	70	24	-	4
(OO;Hh)	74	26	-	-
(Oo;Hh)	68	24	28	-

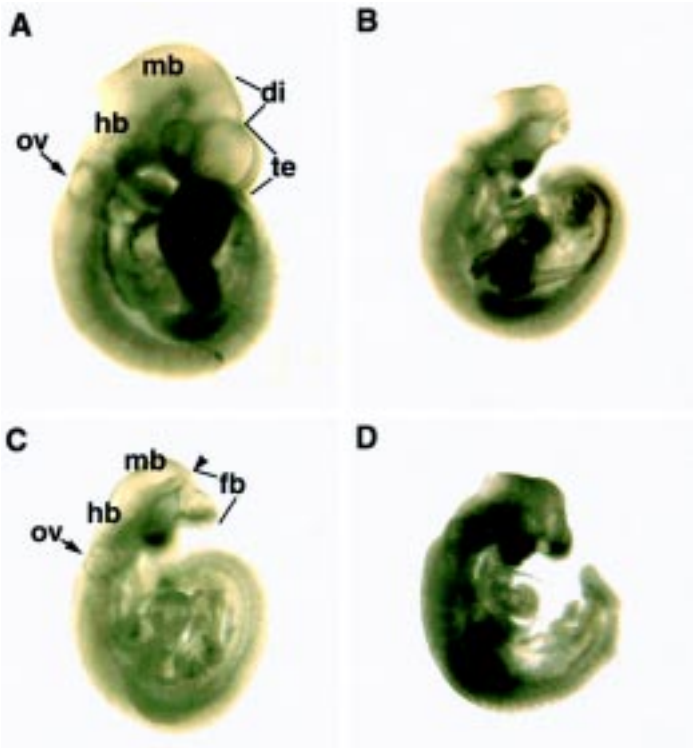

Fig. 2. Phenotype of *Otx2+/-;HNF3β +/-* embryos at E9.5. *Lateral views of* **(A)** *wild type embryo and* **(B,C,D)** *Otx2+/-;HNF3β +/- embryos.* **(B)** *Severely affected Otx2+/-;HNF3β +/- embryo showing forebrain deletion.* **(C, D)** *Less severely affected Otx2+/-;HNF3β +/- embryos display forebrain defects. Note that the floor and roof of the neural tube of the mutants are in contact with each other at the caudal diencephalic and mesencephalic junction (arrowhead). Abbreviations: fb, forebrain; te, telencephalon; di, diencephalon; mb, midbrain; hb, hindbrain; ov, otic vesicle.*

double-labeling to examine regions of overlap. In these studies, embryos were first stained for *Otx2* RNA by whole-mount *in situ* hybridization and then for HNF3β protein by whole-mount antibody staining. Further analysis was performed on sections. At E7.5, there were *Otx2* and HNF3β coexpressing cells in the foregut pocket and anterior midline (Fig. 4D). Sections through embryos double-labeled for *Otx2* and HNF3β revealed coexpression of these two genes in prechordal mesoderm (Fig. 4C), anterior ventral neural fold and ventral endomesoderm (Fig. 4E). At E8.5, sections through forebrain, midbrain and hindbrain regions of embryos showed *Otx2* and HNF3β co-expressing cells in the ventral forebrain and midbrain region (Fig. 4 G,H). However, by E9.5, the expression of these two genes only overlapped in the ventral midbrain regions (Fig. 4J). Our double-labeling studies clearly suggest that *Otx2* and *HNF3β* are co-expressed in different midline structures as the embryo develops. Previous analysis has also shown that they are co-expressed in the early AVE(Ang *et al.*, 1993; Rhinn *et al.*, 1998; Sasaki and Hogan, 1993; Simeone *et al.*, 1993).

Anterior abnormalities in Otx2+/-;HNF3β *+/- mutant embryos*

To characterize the anterior abnormality of *Otx2+/-;HNF3β +/-* mutant embryos further, the expression of several genes normally transcribed along the A-P axis of the embryos was examined at E9.5 (Fig. 5). *Otx2, Hoxb1* and *Krox20* were all expressed at the correct level of A-P axis, suggesting that A-P patterning in the neural tube of *Otx2+/-;HNF3β+/-* mutants was not completely disrupted. However, the domain of *Otx2* expression, which normally encompasses the entire forebrain and midbrain area, was considerably reduced. This suggested that there could be specific truncations of the most rostral regions of the neural tube. To assess this, we analyzed *BF1* and *Six3* expression in *Otx2+/-;HNF3β +/-* embryos. BF1, a winged-helix transcription factor, is normally expressed in the telencephalon of brain. In homozygous mutants of *BF1,* the dorsal telencephalon is reduced in size while the ventral telencephalon is almost completely absent (Xuan *et al.*, 1995) suggesting a role for *BF1* in the development of telencephalon, especially ventral telencephalon. The entire expression domain of *BF1* was missing in the most severely affected *Otx2+/-;HNF3β +/-* mutant embryos (Fig. 6B and data not shown), suggesting that both dorsal and ventral telencephalon were reduced or absent. *Six3* is normally expressed in the ventral forebrain and optic vesicles at this stage. In *Otx2+/-;HNF3β +/-*

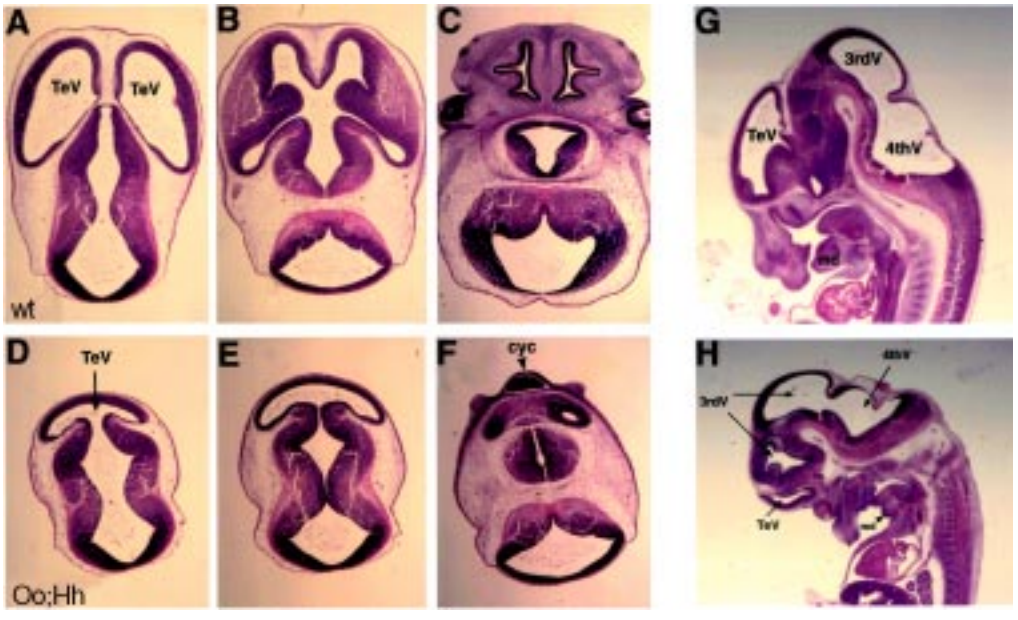

Fig. 3. Histological analysis of *Otx2+/-; HNF3β +/-* embryos at E12.5. *Cross-sections of* **(A,B,C)** *wild-type and* **(D,E,F)** *Otx2+/-;HNF3β+/- mutants.* **(A,B)** *The wild-type embryo has two telencephalic vesicles (future lateral ventricles) while* **(D,E)** *in the double mutant these two lateral ventricles are fused to form a single ventricle.* **(C,F)** *show cross-sections at the level of the eyes.* **(F)** *The Otx2+/-;HNF3β +/- mutant has a single eye (cyclopia).* **(G,H)** *Middle sagittal sections of* **(G)** *wild type and* **(H)** *double heterozygote embryos.* (H) *Note a single eye in the anterior midline and the smaller mandible in the double mutant. Abbreviations: TeV, telencephalic vesicle; cyc, cyclopia; 3rdV, the third ventricle; 4thV, the fourth ventricle.*

embryos, the expression domain of *Six-3* in ventral forebrain was missing but reduced expression was still detectable in the reduced single optic vesicle (Fig. 5 E,F). This analysis suggested that there were anterior truncations in the *Otx2+/-;HNF3β +/-* double heterozygotes, and that the defects were more severe ventrally.

To study D-V patterning effects further, we analyzed expression of two homeobox genes, *Nkx2.1(Lazzaro et al., 1991)* and *Nkx2.2* (Price *et al*., 1992; Shimamura *et al*., 1995) in *Otx2+/-;HNF3β +/-* mutants at E9.5. Both genes are normally expressed in the ventral forebrain (Fig. 5 G,I) but are missing in the anterior of *Otx2+/-;HNF3β +/-* mutants (Fig. 5 H,J). No defects in the expression of *Nkx2.1* and *Nkx2.2* were observed in the more posterior regions of the embryos. These observations suggest that the ventral forebrain is severely affected in double heterozygotes.

Shh *expression in anterior of* Otx2+/-;HNF3β +/- *mutant embryos is severely affected*

Loss of ventral forebrain expression of *Nkx2.1* and *Nkx2.2,* the fused telencephalon, cyclopia and craniofacial defects in *Otx2+/-;HNF3β +/-* mutants was reminiscent of the phenotype of *Shh* homozygous mutants in the anterior region (Chiang *et al*., 1996).Therefore, we analyzed *Otx2+/-;HNF3β +/-* mutant embryos at E9.5 for the expression of *Shh.* At E9.5, *Shh* is expressed in the ventral forebrain, midbrain, notochord, floor plate and gut. Expression of *Shh* was almost completely lost at the anterior end of the *Otx2+/-;HNF3β +/-* embryos (Fig. 6 and data not shown). Sections through these embryos showed that the expression of *Shh* was lost in ventral forebrain and floor plate, although the floor plate was present morphologically. Very weak expression of *Shh* could still be detected in the ventral midbrain (Fig. 6E, arrows) and was detectable in the notochord from the hindbrain back (Fig. 6 G,M). The expression of *Shh* in the rest of the notochord, foregut and hindgut was still present in the normal domains, although the level of the expression was reduced, perhaps due to reduced dosage of *HNF3β* (Fig. 6 M,N and data not shown).

Discussion

Otx2+/-;*HNF3β* +/- doubly heterozygous mutant mice exhibited a variably penetrant lethal phenotype. The major phenotype was characterized by varying degrees of holoprosencephaly, cyclopia and proboscis development, with truncations of the anterior nervous system. The extent of these truncations was very variable and never extended beyond the midbrain/hindbrain boundary, as judged by the persistence of *Otx2* expression. However, expression of *BF1*, a marker of both dorsal and ventral telencephalon (Tao and Lai, 1992)was lost completely in the most severely affected cases, and the expression domain of *Six3*, another anterior marker (Oliver *et al*., 1995)was severely reduced. This suggests that the anterior defects are more than simply loss of ventral pattern elements, but may involve anterior-posterior (A-P) patterning effects as well.

In previous single-mutant studies, incompletely penetrant haploinsufficiency phenotypes had been demonstrated in *HNF3β +/-* or *Otx2* +/- animals (Ang and Rossant, 1994; Matsuo *et al*., 1995; Weinstein *et al*., 1994). About 20% of *HNF3β* heterozygous adults exhibit malocclusion of the jaws and overgrowth of the incisors. This can lead to death, although special care such as providing soft food and cutting the overgrowth of incisors periodically can significantly reduce mortality of these mice. In this study, some *Otx2*+/- heterozygotes exhibited open neural tube defects at E9.5 leading to characteristic exencephaly at E12.5(data not shown). This phenotype contributed to about 5% loss of heterozygotes. Variation in the threshold levels of factors or modifiers which may act synergistically with either *HNF3β* or *Otx2* may contribute to the variable penetrance

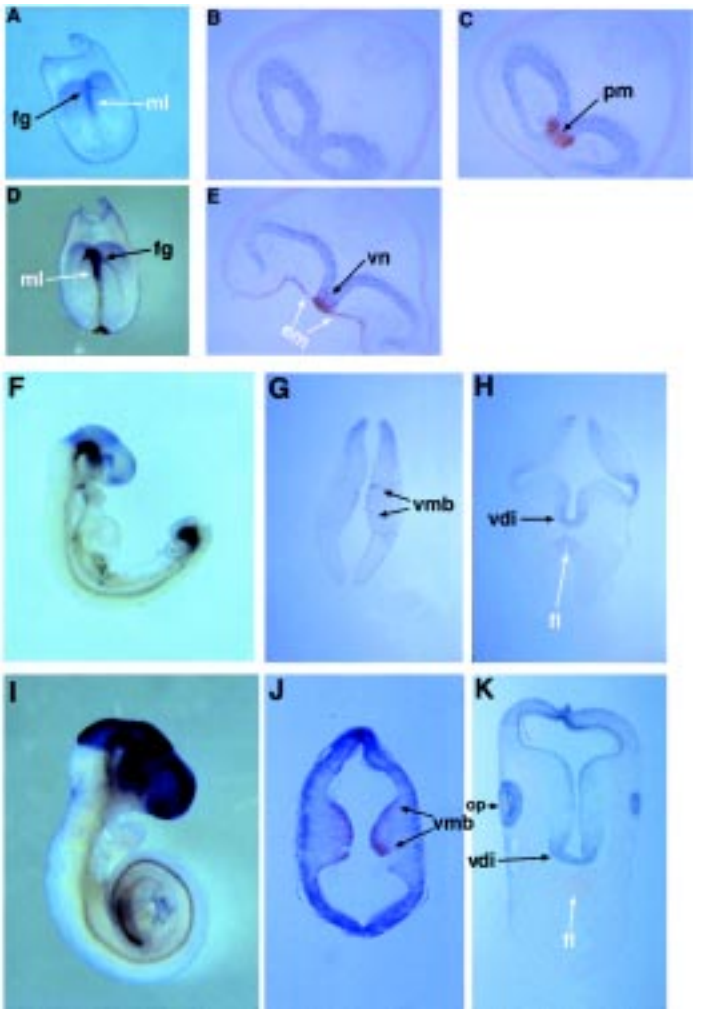

Fig. 4. Comparison of *Otx2* and HNF3β expression in wild type mouse embryos between E7.5 and E9.5. (A,D) *Anteroventral views of E7.5 embryo, stained for Otx2 RNA (purple)* **(A)** *and then stained for HNF3β protein (brown). Co-expressing Otx2 and HNF3β cells were found in the foregut pocket (fg) and anterior midline (ml). (B,C,E) are cross sections through progressive rostral-caudal levels of the anterior head fold region of a double stained embryo, demonstrating the presence of cells coexpressing Otx2 and HNF3β in prechordal mesoderm, pm* **(C)**, *anterior endomesoderm, em* **(E)**, *and anterior ventral neural fold, vn* **(E)**. **(F,I)** *Lateral views of E8.5 and E9.5 embryos, double stained for the expression of Otx2 and HNF3β.* **(G,H)** *Cross sections show cells co-expressing Otx2 (purple) and HNF3β (brown) in the ventral midbrain (vmb) and (vdi) at E8.5.* **(J)** *Cross section shows cells co-expressing Otx2 (purple) and HNF3β (brown) in ventral midbrain (vmb) at E9.5.* **(K)** *Cross section shows expression of Otx2 in the ventral diencephalon and HNF3β in the floor plate (white arrow) at E9.5. At this stage, the two genes are no longer coexpressed in the ventral diencephalon. Abbreviations: em, endomesoderm; fg, foregut pocket; fl, floorplate; ml, midline; op, optic vesicle; pm, prechordal mesoderm; vdi, ventral diencephalon; vmb, ventral midbrain; vn, ventral neural fold.*

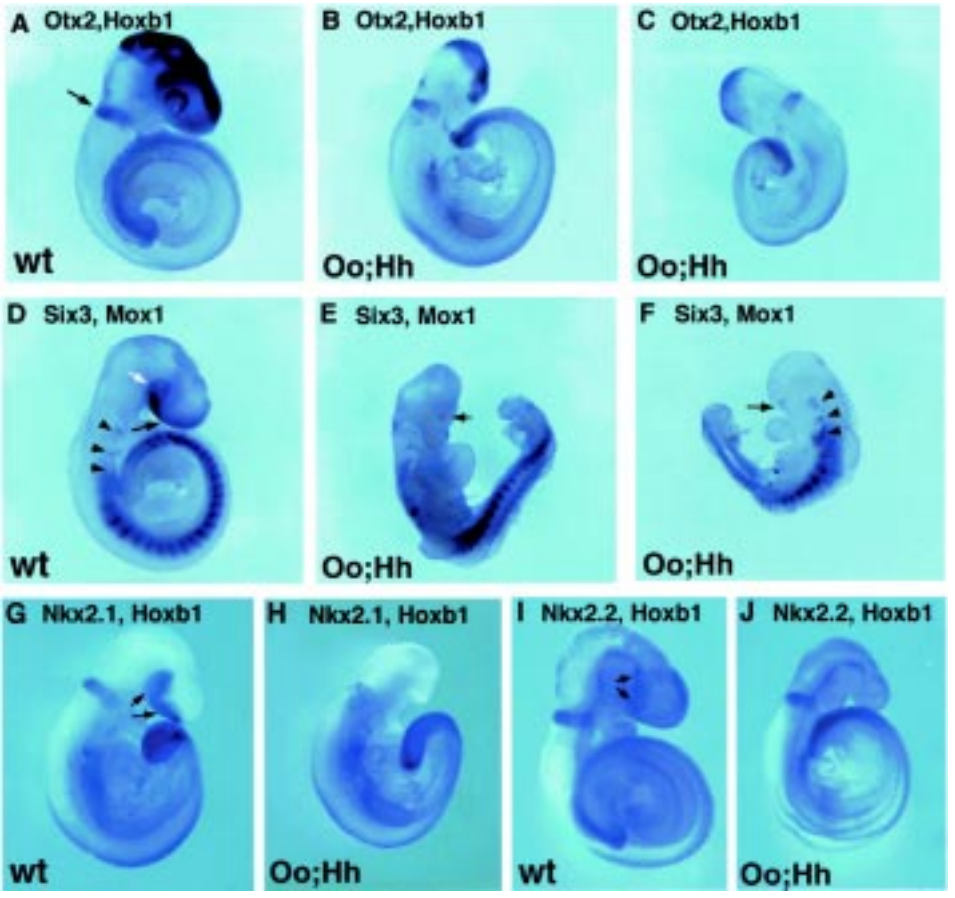

Fig. 5. Expression of *Otx2, Hoxb1, Six3, Mox1, Nkx2.1 and Nkx2.2* in *Otx2+/-;HNF3β+/-* embryos. (A) *Lateral view of wild-type embryo, showing Otx2 expression in forebrain and midbrain and Hoxb1 expression in rhombomere 4 (arrowed) and posterior spinal cord.* **(B,C)** *Otx2+/-;HNF3β +/- embryos showing reduced Otx2 expression domain.* **(D)** *Lateral view of wild-type embryo, showing Six3 expression in the optic vesicle (white arrow) and ventral forebrain (black arrow), and Mox1 expression in second, third, and fourth branchial arches (arrowheads), and somites.* **(E,F)** *Otx2+/-;HNF3β +/- embryos, showing loss of Six3 expression domain in ventral forebrain but resistant weak expression in the reduced single optic vesicle (arrows).* **(G)** *Wild-type expression of Nkx2.1 in the ventral diencephalon and telencephalon (arrows).* **(H)** *In the Otx2+/-;HNF3β +/- mutants, Nkx2.1 expression is missing in the entire ventral forebrain.* **(I)** *Wild-type expression of Nkx2.2 (arrows, expression in the ventral diencephalon).* **(J)** *In the Otx2+/-;HNF3β +/- mutants, Nkx2.2 expression is not detected in the diencephalon region.*

of these haploinsufficiencies. However, the new severe lethal phenotype observed here in double heterozygotes cannot readily be explained by additive effects of the two single low penetrance haploinsufficient phenotypes, but appears to represent a genetic interaction between *HNF3β* and *Otx2*, leading to specific defects in anterior patterning.

The double heterozygous phenotype seems to have two components. One is a loss of ventral structures, specifically in the anterior, associated with loss of Shh signaling. This can be explained by *Otx2* acting to enhance the *HNF3β* mutant phenotype in a region-specific manner. The severe D-V patterning defects seen in the anterior of *Otx2*+/-;*HNF3β* +/- embryos would not have been predicted since there has been no report of D-V patterning defects associated with *Otx2* mutants. However, we show here that *HNF3β* and *Otx2* do overlap in expression in anterior, *Shh*-expressing cells. At E7.5, *Otx2* and *HNF3β* are co-expressed in the cells of the foregut pocket and anterior midline but not in the posterior of the embryo. At E8.5, the two genes continue to be co-expressed in the *Shh*-expressing cells of ventral forebrain and midbrain. At E9.5, the expression domains of the two genes no longer overlap in the ventral diencephalon but still overlap in ventral midbrain. *Shh* has been shown to be involved in specifying ventral neuron identity in the forebrain and midbrain (Hynes *et al.*, 1995; Kohtz *et al.*, 1998; Wang *et al.*, 1995). In these regions the most likely endogenous source of Shh is the cells of the ventral telencephalon and the loss of these anterior domains of *Shh* expression in the *Otx2*+/-;*HNF3β* +/- double heterozygotes presumably explains the loss of ventral structures.

The region-specific loss of *Shh* expression in the *Otx2*+/-;*HNF3β* +/- embryos and the overlapping expression of these two transcription factors in the anterior *Shh*- expressing domains suggest the possibility that the two genes may interact directly to co-regulate expression of *Shh* in the anterior of the embryo. It has been suggested that *HNF3β* is upstream of *Shh* in several systems (Chang *et al.*, 1997; Echelard *et al.*, 1993; Sasaki and Hogan, 1994). Recent studies on the regulation of the mouse *Shh* gene have defined both HNF 3β-dependent and independent regulatory elements(Chang *et al.*, 1997). Interestingly, in this study it was shown that separate enhancers drive *Shh* expression in the ventral brain and in the floorplate, suggesting region-specific control of *Shh* expression. It is thus possible that *Otx2* may be directly involved with *HNF3β* in regulating *Shh* expression in a region-specific manner. However, both genes could be acting in parallel pathways that converge on the expression of *Shh*. It will be interesting to determine whether the regulatory elements of the *Shh* gene contain region-specific enhancers with both HNF3 and Otx binding sites.

The second component of the compound phenotype is a loss of anterior structures in the head. This suggests that *Otx2* and *HNF3β* may also work together to promote anterior patterning. Recent evidence has suggested that an important source of signals for anterior patterning of the embryo is the anterior visceral endoderm (AVE) of the primitive streak stage embryo (reviewed(Beddington and Robertson, 1999)). *HNF3β* and *Otx2* are both expressed in this region. Chimera studies have suggested that both genes have critical functions in this tissue(Dufort

et al., 1998; Rhinn *et al.*, 1998), since improved anterior development is observed when the AVE is wild-type and the embryo mutant for either gene. *Otx2* and *HNF3β* are also co-expressed in the prechordal mesoderm, which has been shown to play a potential role in A-P patterning of the brain at slightly later stages (Dale *et al.*, 1997; Dale *et al.*, 1999; Foley, Storey and Stern, 1997). Thus the defects observed may result from defects in the AVE, the prechordal mesoderm, or both. Future experiments using chimera and explant-recombination analysis with double mutant tissues will resolve these issues. Whether the anterior defects observed are simply the additive effects of the reduction in activity of the two genes in these regions, or whether the two genes interact more directly, perhaps to regulate the production of anterior signaling molecules, also remains to be seen.

In mouse, the most striking phenotype of *Shh* homozygous mutant embryos is a single fused telencephalic vesicle with a single fused optic vesicle and proboscis-like structure(Chiang *et al.*, 1996). The phenotype demonstrated from *Otx2+/-;HNF3β +/-* mutants is not only reminiscent of *Shh* homozygous mutant mouse embryos but also reminiscent of a variable spectrum in facial defects in patients with *Shh* mutations (Belloni *et al.*, 1996; Roessler *et al.*, 1996). The most severe group of these patients exhibits cyclopia, synophthalmia, proboscis and microcephaly with normal development of the rest of the body, suggesting a role for *Shh* in anterior midline patterning in humans. Interestingly, all the human patients are heterozygous for *Shh* mutations, a situation that gives no detectable phenotype in mice. This suggests that there may be other associated mutations in these individuals that accentuate the heterozygous *Shh* effect. The observation of cyclopia in *Otx2+/-;HNF3β +/-* embryos suggests these genes as possible candidate modifiers, or, indeed, as candidates for some of the other genetic loci associated with holoprosencephaly, that are unlinked to *Shh* (Gurrieri and Muenke, 1996).

The sensitivity of *HNF3β* to gene dosage has also revealed a genetic interaction with another pathway that can lead to cyclopia. It has been reported that embryos heterozygous for both *HNF3β* and the *TGFβ*-related gene, *nodal*, show cyclopia and midline patterning defects(Varlet *et al.*, 1997). In this case, there is minimal overlap in expression between the two genes, showing that there are a number of pathways that seem to converge, through *HNF3β*, on to Shh midline signaling. A search for further mutations that enhance the *HNF3β* heterozygous phenotype may thus lead to identification of novel components of the hierarchy of anterior patterning in the embryo, as well as components involved in human holoprosencephaly syndromes.

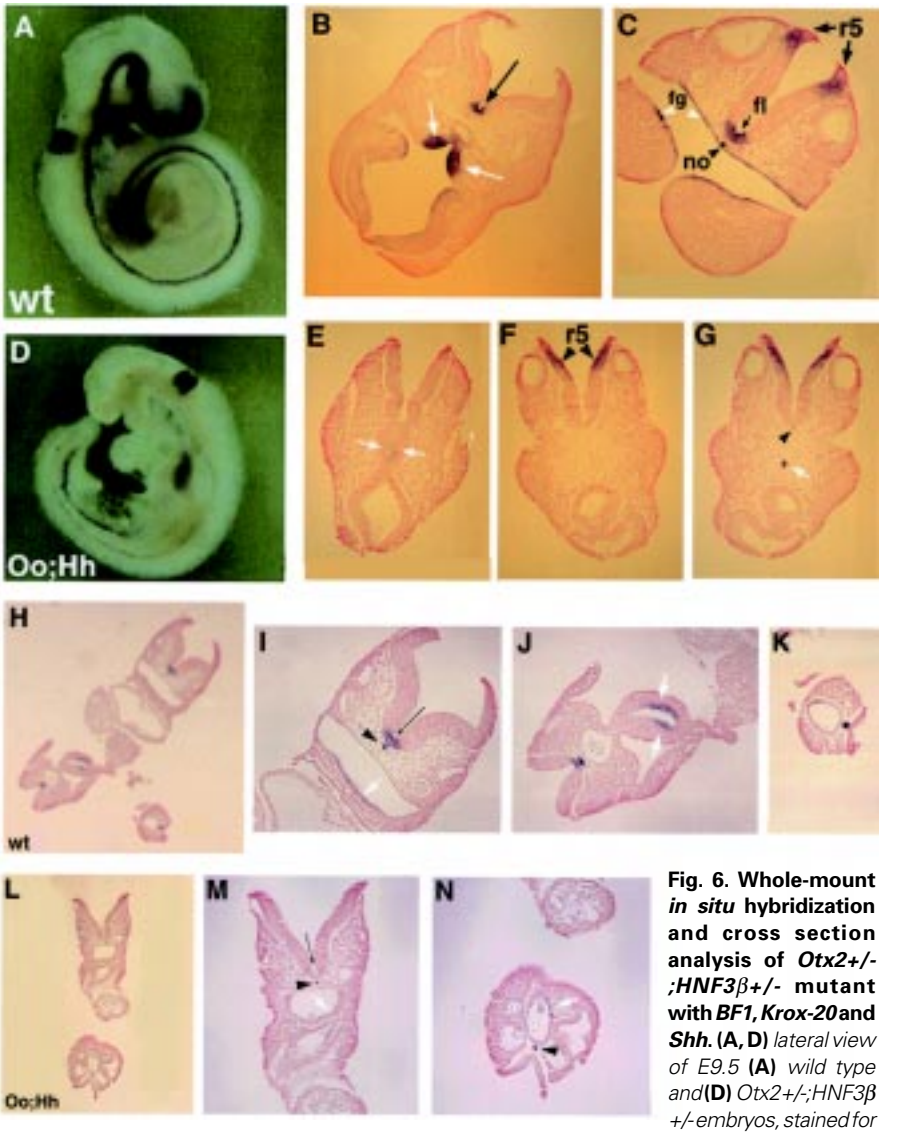

Fig. 6. Whole-mount *in situ* hybridization and cross section analysis of *Otx2+/-;HNF3β+/-* mutant with *BF1, Krox-20* and *Shh*. **(A, D)** *lateral view of E9.5* **(A)** *wild type and* **(D)** *Otx2+/-;HNF3β +/- embryos, stained for BF1, Krox-20 and Shh expression. Severely affected Otx2+/-;HNF3β +/- embryo demonstrates lack of BF1 expression in telencephalon. Shh expression is absent from the anterior ventral midline and much reduced in the posterior region of the embryo.* **(B,C)** *Cross sections of a wild type embryo shows Shh expression in the ventral neural tube, notochord and foregut. Note Krox20 expression in the rhombomere 5.* **(E,F,G)** *cross sections of Otx2+/-;HNF3β +/- embryo, showing loss of Shh expression in anterior ventral neural tube. Note weak expression of Shh in ventral midbrain (E, white arrows), anterior notochord in hindbrain region (G, black arrowhead) and expression in the tip of Rathkes's pouch (G, white arrow). Krox20 is still normally expressed in rhombomere 5.* **(H,I,J,K)** *cross sections of wild type embryo through foregut and hindgut level show Shh expression in the floor plate, notochord, foregut and hindgut.* **(L,M,N)** *cross sections of Otx2+/-;HNF3β +/- embryo show reduced levels but a normal pattern of Shh expression in the notochord, foregut and hindgut. Abbreviations: fg, foregut; fl, floor plate; no, notochord; r5, rhombomere 5; wt, wild type; Oo:Hh, Otx2+/-;HNF3β +/-.*

Materials and Methods

Generation and genotyping of wild-type and mutant mice

Otx2 heterozygous mice on a mixed 129/Sv/CD1 background(Ang *et al.*, 1996) were crossed with *HNF3β* heterozygous mice on the same mixed background (Ang and Rossant, 1994) to generate double heterozygotes. Genotyping of newborn mice and embryos was performed by Southern blot analysis with genomic DNA prepared from biopsies of tails and yolk sacs. Each DNA sample was divided into two aliquots, one of which was analyzed

with an *Otx2* probe and the other with an *HNF3β* probe. Probes used for Southern blot analysis were described (Ang and Rossant, 1994; Ang *et al.*, 1996). Hybridizations were carried out at 42˚C overnight in 50% formamide, 5x Denhardt's, 5x SSC, 1% SDS, 100 μg/ml sheared salmon sperm DNA. The filters were finally washed in 0.2x SSC at 63◦C. The filters were then exposed to phosphor imager screens overnight.

Histology, whole-mount RNA in situ *hybridization and immunocytochemistry*

Midday of the day of the vaginal plug was considered as E0.5 in the timing of embryo collection. Embryos were dissected and staged according to morphological criteria (Kaufman, 1992). Embryos were photographed on a Leitz Wild M10 microscope. For histological analysis, embryos were fixed overnight in 4% paraformaldehyde at 4°C, processed, embedded in wax and sectioned. 5-6 μm sections were dewaxed in xylene, rehydrated through an ethanol series into PBS and stained with hematoxylin and eosin.

Whole-mount RNA *in situ* hybridization was performed as described previously (Conlon and Hermann, 1993). Single-strand RNA probes were labeled with digoxigenin as directed by the manufacturer (Boehringer Mannheim Biochemicals). The probes used for the whole-mount *in situ* hybridization studies were as follows: *Shh* (Echelard *et al.*, 1993), *Otx2* (Ang *et al.*, 1994),*BF1* (Tao and Lai, 1992),*Hoxb1* (Wilkinson *et al.*, 1989), *Six3* (Oliver *et al.*, 1995), *Mox1* (Candia *et al.*, 1992), *Nkx2.1* and *Nkx2.2*(Shimamura *et al.*, 1995). After RNA *in situ* hybridization, embryos were post-fixed in 4% paraformaldehyde at 4°C overnight and then subject to whole-mount antibody staining. Whole-mount antibody staining was performed as described (Davis *et al.*, 1991)using an anti-*HNF3β* antibody at a dilution of 1:1000 (Sasaki and Hogan, 1993). For sectioning of whole-mount stained specimens, embryos were post-fixed in 4% paraformaldehyde at 4°C overnight. Sections were cut at 5-6 μm and some sections were counterstained lightly with eosin, and photographed using a Leitz Orthoplan compound microscope and Nomarski optics.

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