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Kheper, a Novel ZFH/δEF1 Family Member, Regulates the Development of the Neuroectoderm of Zebrafish (*Danio rerio*)

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Kheper is a novel member of the ZFH (zinc-finger and homeodomain protein)/δEF1 family in zebrafish. *kheper* transcripts are first detected in the epiblast of the dorsal blastoderm margin at the early gastrula stage and *kheper* is expressed in nearly all the neuroectoderm at later stages. *kheper* expression was expanded in *noggin* RNA-injected embryos and also in *swirl* mutant embryos and was reduced in *bmp4* RNA-injected embryos and *chordino* mutant embryos, suggesting that *kheper* acts downstream of the neural inducers Noggin and Chordino. Overexpression of Kheper elicited ectopic expansion of the neuroectoderm-specific genes *fkd3, hoxa-1,* and *eng3,* and the ectopic expression of *hoxa-1* was not inhibited by BMP4 overexpression. Kheper interacted with the transcriptional corepressors CtBP1 and CtBP2. Overexpression of a Kheper mutant lacking the homeodomain or of a VP16–Kheper fusion protein disturbed the development of the neuroectoderm and head structures. These data underscore the role of Kheper in the development of the neuroectoderm and indicate that Kheper acts as a transcriptional repressor.

Key Words: **zebrafish; ZFH; δEF1; zinc-finger; homeodomain; transcription factor; ectoderm; mesoderm; CtBP; repressor.**

INTRODUCTION

Historical studies revealed the essential roles of the dorsal pore lip (Spemann's organizer) of amphibia in the establishment of the dorsal axis, which includes the neuroectoderm and the dorsal mesoderm. Molecular identification of the inductive signals from the organizer delineated that the organizer proteins Chordin, Noggin, and Follistatin were involved in the induction of the dorsal mesoderm and the neuroectoderm in *Xenopus* (reviewed in Sasai and De Robertis, 1997). These organizer proteins antagonize the signals of BMP2, BMP4, and BMP7, which have strong ventralizing properties. Biochemical studies revealed that these organizer proteins directly bound BMPs and inhibited their functions (Piccolo *et al.*, 1996; Zimmerman *et al.*, 1996). Inhibition of the BMP signals by the organizer proteins or a dominant negative form of BMP4 receptor leads to the ectopic expression of neural genes (Lamb *et al.*, 1993; Sasai *et al.*, 1995; Hawley *et al.*, 1995). In zebrafish, mutations in the *bmp2b/swirl* and *bmp7/snailhouse* genes lead to the dorsalization of the embryos and the expansion of neuroectoderm (Kishimoto *et al.*, 1997; Mullins *et al.*, 1996; Dick *et al.*, 2000). A mutation in the *smad5/somitabun* gene, whose product is involved in the cytoplasmic signal transduction of BMPs, also elicits the dorsalization and expansion of the neuroectoderm (Mullins *et al.*, 1996; Hild *et al.*, 1999). In contrast, mutation in the *chordino* gene leads to the ventralization and reduction of the neuroectoderm (Hammerschmidt *et al.*, 1996). Mutations in the *tolloid/mini fin* gene, whose product inhibits the functions of Chordino by proteolytic cleavages, expand the neuroectoderm in the ectoderm (Mullins *et al.*, 1996; Blader *et al.*, 1997; Connors *et al.*, 1999). Together with the

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overexpression studies of Noggin, Chordin, Follistatin, and BMP4, these data have established a concept that the balance of BMP and anti-BMP determines the fate of the ectoderm during gastrulation and inhibition of the BMP signals is essential for the formation of the neuroectoderm.

Although inhibition of the BMP signal in the ectoderm induces the neuroectoderm, it remains elusive how it neuralizes the ectoderm, in particular, what genes act downstream of it and transmit the signals for neural development. It was reported that the expression of *Xenopus Zic-related-1* (*Zic-r1*), *zic3*, *Sox2*, and *SoxD* was induced by the inhibition of the BMP signals and they were implicated in the specification of the neuroectoderm (Mizuseki *et al.*, 1998a,b; Nakata *et al.*, 1997). Ectopic expression of *Zic-r1* or *SoxD* causes the ectopic formation of the neuroectoderm *in vivo* and initiates neural and neuronal differentiation in animal caps (Mizuseki *et al.*, 1998a,b). Expression of a dominant negative SoxD inhibits the formation of anterior neural tissue (Mizuseki *et al.*, 1998b). A dominant negative Sox2 blocks neural differentiation (Kishi *et al.*, 2000). These reports suggest that these genes act downstream of the BMP-antagonistic neural inducers and they control the neuroectoderm development. However, there should be other factors which link the signals from Spemann's organizer to neural and neuronal differentiation.

The *zfh* family encodes a transcription factor containing both the homeodomains and the zinc-finger DNA binding motifs (Fortini *et al.*, 1991; Lai *et al.*, 1991). Vertebrate homologues include chicken δEF1 (Funahashi *et al.*, 1993), mouse δEF1 (Sekido *et al.*, 1996), human Nil-2-a/AREB6 (Williams *et al.*, 1991; Watanabe *et al.*, 1993) and ZEB (Genetta *et al.*, 1994), mouse MEB1 (Genetta and Kadesch, 1996), hamster BZP (Franklin *et al.*, 1994), and rat Zfhep (Cabanillas and Darling, 1996). They contain highly conserved amino acid sequences. Chicken *δEF1* is first expressed after the gastrulation period in the mesodermal tissues and later in the central nervous system (CNS; Funahashi *et al.*, 1993). Mouse *δEF1* is expressed in a pattern similar to that of chicken and required for skeleton patterning, but not for neural tissues in mutant mice (Takagi *et al.*, 1998). The *Drosophila* homologue ZFH-1 is required for the development of mesodermally derived tissues, but not for the CNS (Broihier *et al.*, 1998; Lai *et al.*, 1993). They are mainly involved in the development of the mesodermal tissues.

We had tried to isolate a gene that was essential for the development of the CNS by RT-PCR under low stringency with several sets of primers. Then we isolated a novel member of the *zfh* gene family, *kheper*, in zebrafish. *kheper* was expressed in nearly all the neuroectoderm and its expression was regulated by the balance of BMP and anti-BMP signals. Overexpression of Kheper expanded the neuroectoderm. Expression of dominant negative forms of Kheper reduced the neuroectoderm and inhibited head formation. Kheper interacted with the transcriptional corepressors CtBP1 and CtBP2 and acted as a repressor. These results provide the identification of a transcriptional repressor that acts in neuroectoderm development.

MATERIALS AND METHODS

Isolation of kheper cDNA and Plasmid Constructions

A fragment of the *kheper* cDNA was obtained from 1-day-old zebrafish embryos by RT-PCR (Access RT-PCR System; Promega) with chicken *gicerin*-specific primers (Taira *et al.*, 1994). One of the resulting PCR fragments (474 bp) was used as the probe for the further screening of a zebrafish cDNA library in λgt10 (kindly provided by H. Okamoto). Twenty positive clones were isolated and 2 of them, P7-20 and P7-11, were cloned into the pBluescript SK(+) vector. The N-terminal end of the Kheper coding region was amplified by 5′ rapid amplification of cDNA ends (the library was kindly provided by S. Yamashita). Combining them, 5372 bp of sequence was determined and submitted to DDBJ, EMBL, and GenBank as *kheper* cDNA (Accession No. AB016799). The full-length coding region was subcloned into the pCS2+ expression vector (Turner and Weintraub, 1994). Green fluorescent protein (GFP)–Kheper, a fused molecule of GFP and Kheper, was produced by the ligation of *GFP* at the 5′ end of *kheper*. For the dominant negative type of Kheper, dHD, most of the homeodomain was deleted at the *Tth*111I (1603) and *Bgl*II (1768) sites of the cDNA. VP16AD-HD was produced by subcloning the Kheper homeodomain into the CS2+NLS VP16AD vector (kindly provided by M. Itoh), which was constructed with the VP16 activation domain and CS2+NLS vector (Sadowski *et al.*, 1988; Turner and Weintraub, 1994).

RNA Injection

All the constructs of *kheper* in the pCS2+ vector were linearized with *Bss*HII and transcribed *in vitro* with SP6 RNA polymerase in the presence of m7G (5′)ppp(5′)G (New England BioLabs) to produce capped transcripts. pCS2+ *noggin* (mouse cDNA; Y. Yamanaka, M. Hibi, and T. Hirano, unpublished data) was linearized with *Not*I and zebrafish *bmp4* in pSP64T (kindly provided by N. Ueno) was linearized with *Bam*HI, and they were transcribed as in the case of *kheper*. Embryos were injected in the blastomeres at the one-cell stage. Five hundred picoliters of the RNA solution was injected at the concentration of 0.001 to 1.0 mg/ml, using an air pressure injector and glass capillaries.

Whole-Mount in Situ Hybridization

Whole-mount *in situ* hybridization was performed as previously described (Hammerschmidt *et al.*, 1996). BM Purple AP substrate or NBT/BCIP stock solution (Boehringer Mannheim) was used for the alkaline phosphatase substrate. Clone P7-20 was used as the probe for *kheper*. *hoxa-1*, *no tail* (*ntl*), *myoD*, and *eng3* probes were PCR products (Sagerstrom *et al.*, 1996). *eve1* was provided by J. S. Joly. *fgf8* was provided by M. Furthauer. *fkd3* and *fkd6* were provided by Y. Kishimoto. *otx2* and *pax2.1/noi* were provided by H. Takeda. For sections, embryos were embedded in Technovit 8100 (Kluzer, Germany) after fixation with 4% paraformaldehyde/PBS. The mutant embryos *chordino*^{tm84} and *swirl*^{ta72} were supplied by H. Takeda, Y. Kishimoto, and M. Kobayashi.

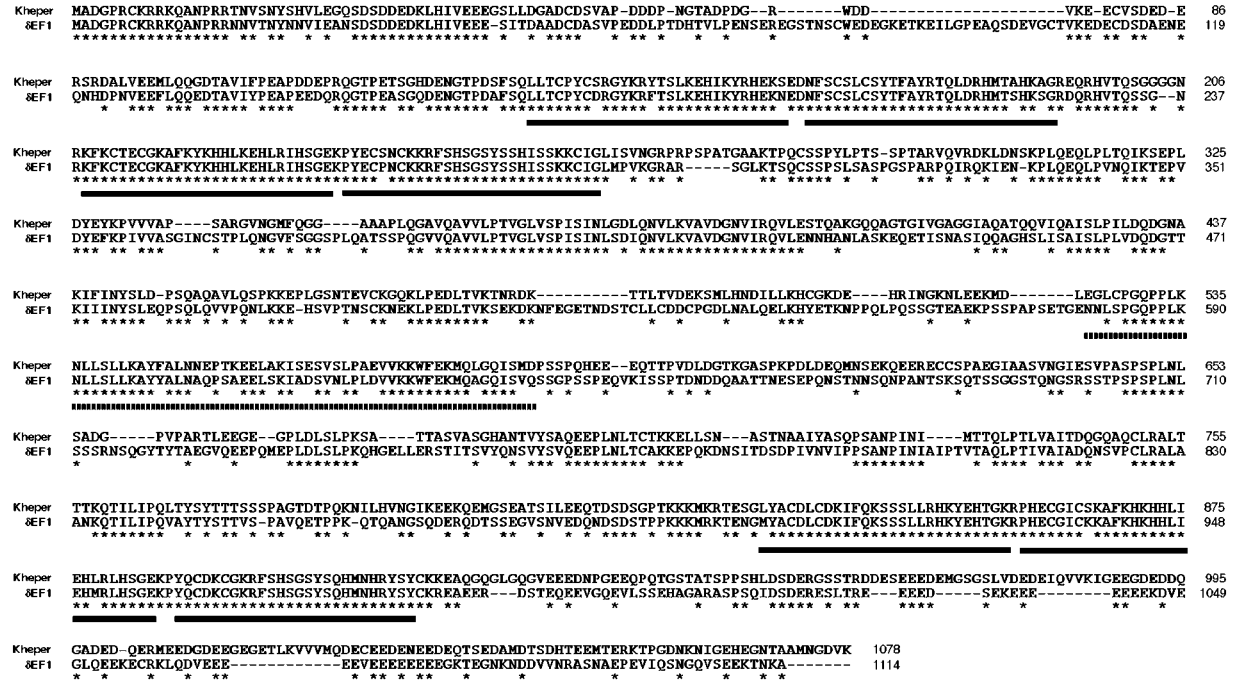


FIG. 1. Alignment of amino acid sequences of Kheper and chicken δEF1, using ClustalW. Identical residues between the two sequences are indicated with asterisks. The solid lines and the dotted line represent the zinc-finger motifs and the homeodomain of δEF1, respectively (Funahashi *et al.*, 1993). The Kheper sequence has been registered in DDBJ, EMBL, and GenBank under Accession No. AB016799.

Fish Maintenance

Zebrafish (*Danio rerio*) were purchased from a pet shop in Osaka. Adult fishes were maintained at 28.5°C and in a 14 h light/10 h dark cycle. Embryos from the zebrafish spawn were collected at 10 min after light on. Embryos were cultured at 28.5°C in embryonic medium. The embryonic stages were determined by the postfertilization hour and by microscopic observation, referring to descriptions in "The Zebrafish Book."

Coimmunoprecipitation and Western Blot Analysis

Zebrafish CtBP1 (Accession No. AB032415) and CtBP2 (Accession No. AB032416) cDNA were isolated by a yeast two-hybrid screening with zebrafish MyT1 as a bait (unpublished result). Zebrafish CtBP1 and CtBP2 had 75 and 84% identity with human CtBP1 and CtBP2, respectively (data not shown). The truncated Kheper (amino acids 336–803) and CtBP were tagged with Myc and GFP, respectively. Forty-eight hours after transfection, COS7 cells were lysed in the lysis buffer (20 mM Hepes, pH 8.0, 12 mM KCl, 2 mM EDTA pH 8.0, 1 mM DTT, 1 mM PMSF, 1% NP-40) and centrifuged. The lysates were immunoprecipitated with anti-Myc monoclonal antibody (Oncogene Products) overnight at 4°C and washed five times with lysis buffer, then 2×Dye (0.12 M Tris–HCl, pH 6.8, 0.12 M DTT, 11.9% sucrose, 4% SDS, and 0.06% BPB) was added and the samples were boiled. The samples were loaded onto a 14% polyacrylamide gel and transferred to the nitrocellulose membrane filter. The membrane filter was serially incubated with anti-GFP polyclonal antibody (Molecular Probes) and anti-rabbit IgG–horseradish peroxidase secondary antibody. After several washes, the membrane was developed by the chemiluminescence method (Amersham Pharmacia Biotech).

RESULTS

Isolation of kheper cDNA

The *kheper* cDNA fragment (5372 bp) encodes a 1078-amino-acid protein that contains seven zinc-finger motifs and a homeodomain (Fig. 1; Kheper is the name of an ancient Egyptian god signifying creation or the scarab beetle). The deduced amino acid sequence of Kheper displayed the strongest sequence similarity to chicken δEF1 through the search of the database. Kheper exhibited 89–100 and 72% identities at the amino acid level with δEF1 within the zinc fingers (amino acids 136–163, 166–192, 208–234, 236–263, 830–856, 858–884, and 887–912) and the homeodomain (amino acids 525–585), respectively. But outside of these domains Kheper displayed less homology to δEF1, and they exhibited 38% overall identity. In addition, Kheper showed 38% identity with mouse δEF1, 40% identity with AREB6, and 39% identity with BZP, although chicken δEF1 displayed 74% identity with mouse δEF1, 80% identity with AREB6, and 73% identity with BZP (data not shown). These data show that Kheper has obviously lower identity than others and suggest that zebrafish Kheper is a novel member of δEF1/ZFH family.

Expression of kheper during Embryogenesis

Northern blotting showed that *kheper* transcripts were detected from early gastrula stage (6 h postfertilization (hpf), the shield stage) and continued to be expressed throughout development (Fig. 2A). Whole-mount *in situ* hybridization revealed that *kheper* was expressed around the embryonic shield at the shield stage (Fig. 2B). Sagittal section further showed that the *kheper* transcripts were detected only in the epiblast and not in the involuting hypoblast of the dorsal blastoderm margin (Fig. 2C). As the gastrulation proceeded, *kheper* was expressed in the epiblast in a dorsoventral gradient with a peak in the dorsal side (Figs. 2D and 2E). Although *kheper* was not expressed in the hypoblast at the shield stage, it was expressed in the hypoblast only in the marginal region after the early gastrula period (Fig. 2F). In the segmentation period, *kheper* was expressed in the neural plate (Figs. 2G–2L). In addition, *kheper* expression in the paraxial mesoderm was detected after the tail bud stage (Figs. 2H and 2J). Double staining of *fkd6*, the neural crest marker, with *kheper* showed that the *kheper* expression domain overlapped with that of *fkd6* (Fig. 2M). This is consistent with the fact that neural crest precursors are intermingled with Rohon–Beard cells in the lateral neural plate (Cornell and Eisen, 2000). Furthermore, the *HuC*-expressing trigeminal placodes was also located in the *kheper* expression domain (Fig. 2N; Kim *et al.*, 1996). Collectively, *kheper* expression defines the neuroectoderm.

kheper Expression Is Regulated by BMP and BMP Antagonists

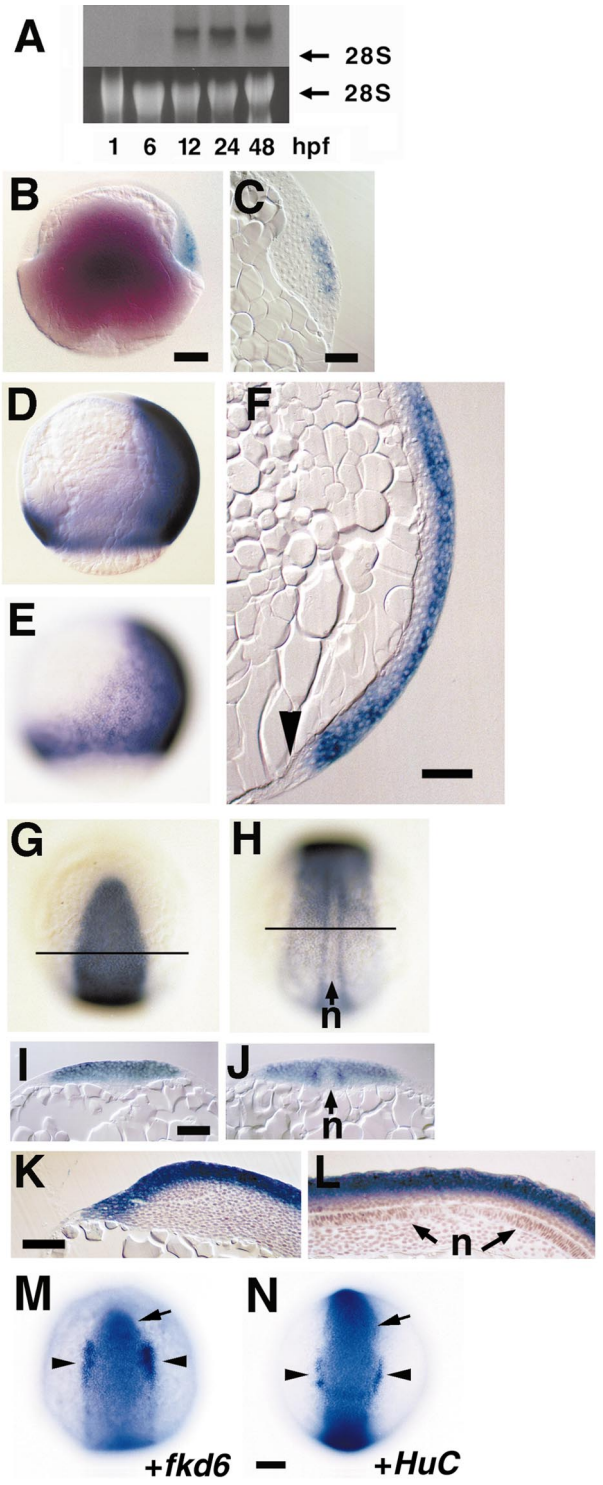
kheper expression in the neuroectoderm suggests that the expression is regulated by the neural inducers, such as Noggin and Chordino. We examined *kheper* expression in the dorsalized and ventralized zebrafish embryos. It was expanded to the ventral side at the midgastrula stage (Fig. 3B) and the transcripts were detected throughout the ectoderm at the segmentation stage (Fig. 3D) in the *noggin* RNA-injected dorsalized embryos. The LiCl treatment of the embryos before the midblastula transition, which dorsalized the zebrafish embryos and elicited expansion of the organizer (Stachel *et al.*, 1993), also expanded the expression of *kheper* (data not shown). In the *swirl*^{ta72} mutant embryos, *kheper* expression was expanded as in the *noggin* RNA-injected embryos (Figs. 3E and 3F, comparing to Figs. 3I and 3C, respectively). In contrast, *kheper* expression was strongly reduced in the zebrafish *chordino*^{tm84} mutant embryos, which lacked the function of the *Xenopus* Chordin orthologue (Hammerschmidt *et al.*, 1996), in particular in the lateral ectoderm (Fig. 3H). Similarly, *kheper* expression was strongly reduced in the *bmp4* RNA-injected embryos (Fig. 3J). Collectively the expression of *kheper* was upregulated in the embryos dorsalized by inhibition of BMP signals and downregulated in the embryos ventralized with a high BMP activity. The expression pattern correlated with the region of the neuroectoderm, suggesting that *kheper* was downstream of the BMP-antagonistic neural inducers in the ectoderm. Consistent with this, the expression domains of *bmp2b/swirl* and *kheper* were almost mutually exclusive in the ectoderm (Figs. 3K and 3L).

Phenotypes of kheper RNA-Injected Embryos

To understand the function of Kheper, we injected synthetic RNA of *kheper* or *GFP-kheper* into the one-cell stage embryos. Overexpression of both Kheper and GFP–Kheper exhibited similar effects on the development of the em-

FIG. 2. The expression of the *kheper* gene. (A) Northern blot analysis. Total RNA was extracted from the embryos at 1, 6, 12, 24, and 48 hpf. Twenty micrograms each of total RNA was loaded. The cDNA of P7-20 was used as the probe (see Materials and Methods). The bottom shows ethidium bromide staining as the loading control. (B–N) Whole-mount *in situ* hybridization. (B) The embryo at the shield stage is oriented with the dorsal side to the right and animal pole to the top. (C) A magnification of the embryonic shield in sagittal section. (D) The 80% epiboly stage. The orientation is same as in B. (E) Surface view of the embryo in D. (F) Sagittal section through the axial hypoblast at the 80% epiboly stage. The dorsal region is magnified. Arrowhead indicates the margin of the blastoderm. (G) Animal pole view of the three-somite stage embryo with the ventral side to the top. (H) Dorsal view of the three-somite stage embryo with the anterior side to the top. (I, J) Transverse sections of the three-somite stage embryo at the positions indicated by the lines in G and H, respectively. (K, L) Sagittal sections of the brain and the trunk at 18 hpf with anterior side to the left. The nuclei are stained with hematoxylin. (M) The three-somite stage embryo hybridized with both *kheper* and *fkd6* probes. The arrowheads and the arrow indicate the expression of *fkd6* in the neural crest and the expression of *kheper,* respectively. The orientation is same as in G. (N) The six-somite stage embryo hybridized with both *kheper* and *HuC* probes. The arrowheads and the arrow indicate the expression of *HuC* in the trigeminal placodes and the expression of *kheper,* respectively. The orientation is the same as in H. Abbreviation: n, notochord. Scale bar, 100 μm (B, N), 50 μm (C, F, I, K). B, D, E, G, and H are at the same magnification.

FIG. 3. Expression of *kheper* in the dorsalized or ventralized embryos. Lateral views of 70% epiboly stage embryos uninjected (A) and dorsalized by injection with 50 pg of mouse *noggin* RNA (B). Lateral views of three-somite stage embryos uninjected (C) and injected with mouse *noggin* RNA (D). Lateral views of *swirl*^{ta72} mutant embryos at the 80% epiboly stage (E) and the three-somite stage (F). Dorsal views of 80% epiboly stage wild-type embryo (G) and *chordino*^{tm84} mutant embryo (H). The expression of *kheper* was decreased in the lateral region. Lateral views of 80% epiboly stage uninjected embryo (I) and ventralized embryo by injection with 50 pg of *bmp4* RNA (J). Animal pole views of the expression of *bmp2b/swirl* (K) and *kheper* (L) at the 80% epiboly stage in wild-type embryos. A–F, I, and J are oriented with the dorsal side to the right and animal pole to the top. K and L are oriented with the dorsal side to the right. Scale bar, 200 μm.



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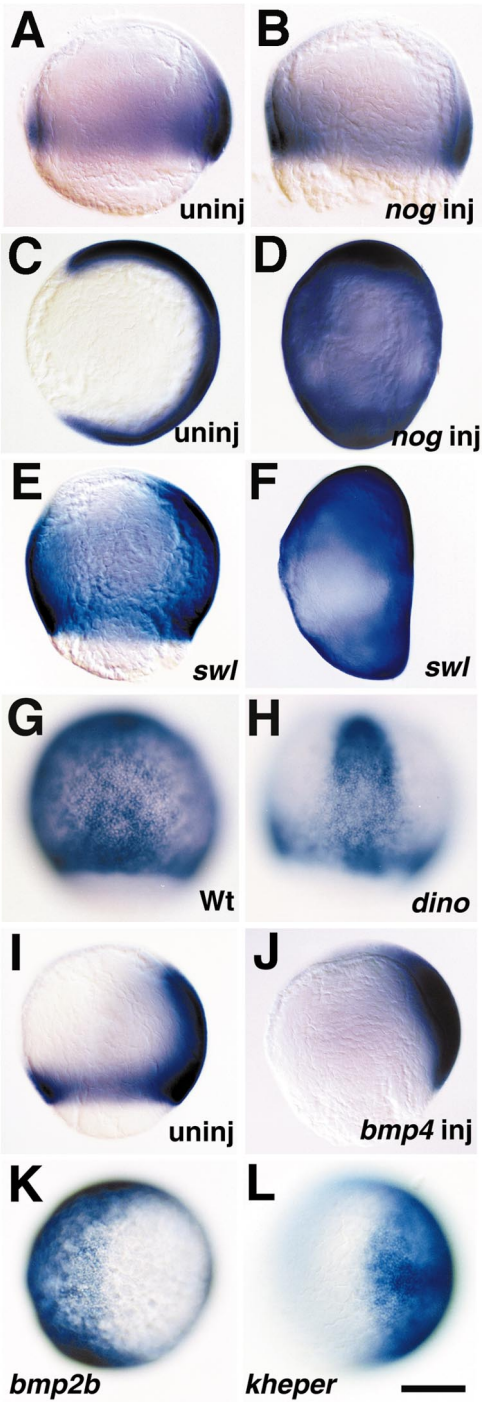

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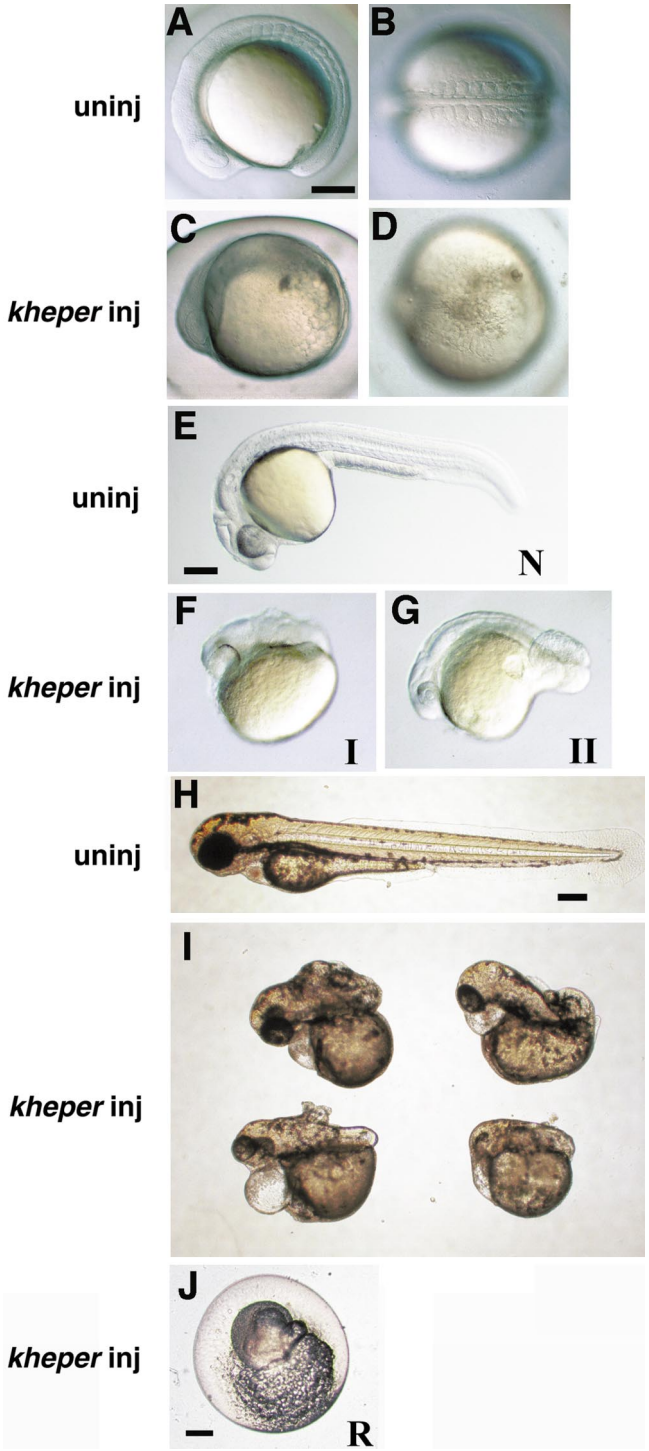


FIG. 4. Phenotypes of the embryos injected with *GFP-kheper* RNA. Fifty picograms of *GFP-kheper* RNA was injected. Embryos are oriented with the anterior side to the left (A–I). The uninjected embryo at 14 hpf, the lateral view (A) and the dorsal view (B). The injected embryo at 14 hpf, the lateral view (C) and the dorsal view (D). At 24 hpf, the lateral views of the uninjected embryo (E) and the injected embryos (F, G). At 60 hpf, the lateral views of the uninjected embryo (H) and the injected embryos (I). The ruptured embryo after injection (J). We designated the phenotype in E as "type N," F as "type I," G as "type II," and J as "type R." Scale bar, 200 μm. A–D, E–G, and H–I are at the same magnifications, respectively.

bryos. These embryos displayed loss or malformation of trunk and tail structures (Figs. 4C, 4D, 4F, 4G, and 4I), as observed in certain dorsalized mutants (Mullins *et al.*, 1996). We could classify the injected embryos into categories by the phenotypes at 24 hpf. "Type I" embryos completely lacked tails (Fig. 4F), "type II" embryos had shortened and curled tails (Fig. 4G), and "type N" embryos were normal embryos at least morphologically (Fig. 4E). In the severely affected embryos (termed "type R"; Fig. 4J), the yolk materials were spilled out during gastrulation and the embryos did not further develop, as observed in the severe dorsalized mutant (Mullins *et al.*, 1996). This is likely due to the strong hydrostatic pressure. Injection of as much as 0.5 pg of *kheper* RNA or 5 pg of *GFP-kheper* generated abnormal phenotypes. Higher amounts of injected *kheper* or *GFP-kheper* RNA increased severity of the phenotypes (from type N to types I and R; Table 1). Since the embryos injected with *GFP-kheper* RNA showed the same phenotypes as those injected with *kheper* RNA, we used *GFP-kheper* for further experiments to monitor the expression of the protein. The results indicate that misexpression of Kheper perturbed development and generated phenotypes similar to those of the dorsalized embryos at some levels.

Misexpression of Kheper Neuralizes the Ectoderm but Does Not Dorsalize the Mesoderm

We examined the expressions of neuroectodermal and mesodermal markers in the Kheper-overexpressing embryos. Expressions of the neuroectodermal genes *fkd3* (panneuroectodermal marker posterior to the prospective telencephalon), *eng3* (marker for posterior midbrain and anterior hindbrain), *fgf8* (marker for mid–hindbrain boundary), and *hoxa-1* (posterior neural maker, expressed in hindbrain and spinal cord) were expanded laterally and ventrally in the embryos injected with 50 pg of *GFP-kheper* RNA, compared to the uninjected control embryos (Figs. 5A–5H; Odenthal and Nusslein Volhard, 1998; Ekker *et al.*, 1992; Reifers *et al.*, 1998; Alexandre *et al.*, 1996). The expression of the anterior neural marker *otx2* was not strongly affected in these embryos (data not shown). In contrast to the neuroectodermal markers, the expression of the ventral marker *eve1* was prominently reduced (Fig. 5J; Joly *et al.*, 1993). These results indicate that overexpression of Kheper dorsalized and neuralized the ectoderm.

The expression of *no tail* (*ntl*) in the axial mesoderm was not affected in the *GFP-kheper* RNA-injected embryos (Fig. 5L). The expression of *myoD* in the paraxial and adaxial mesoderm was not expanded in these embryos, although

TABLE 1
The Effects of *kheper* RNA Injection

Type of RNA injected	Amount of RNA injected (pg)	Phenotype (%)				
		R	I	II	N	*n*
GFP	500	0.8	0	0	95.1	366
kheper	0.5	4.6	5.5	11.9	72.5	109
	5	24.6	11.0	26.5	31.1	264
	50	63.8	6.6	5.9	2.6	423
GFP-kheper	5	15.5	0	21.4	50.0	84
	25	18.6	5.2	33.8	34.8	210
	50	57.0	4.1	6.8	2.3	221

the expression domains were slightly aberrant (Fig. 5N). *pax2.1/noi* is expressed in the intermediate mesoderm, which includes pronephros (Majumdar *et al.*, 2000). Its expression was not reduced by the overexpression of *kheper* (Fig. 5P), although the expression domain in the midbrain extended laterally as *eng3* in Fig. 5H (data not shown). These results indicated that the mesoderm was not dorsalized by the overexpression of Kheper. This is quite in contrast to the dorsalized mutant embryos, such as *swirl, snailhouse,* and *somitabun,* which have expanded notochord and somites and reduced *pax2.1/noi* expression (Mullins *et al.*, 1996). The phenotypes caused by the overexpression of Kheper are not simply explained by the inhibition of BMP signals. In fact, the overexpressed Kheper did not reduce the expression of *bmp2b/swirl,* the loss of which dorsalized the embryo by itself as in the *swirl* mutant (Fig. 5R). Kheper is specifically involved in neuralization of the ectoderm.

To confirm it further, we injected *bmp4* RNA with *GFP-kheper* RNA. In the co-injected embryo, the *ntl* expression in the dorsal midline was reduced (Fig. 6D), indicating the ventralization of the mesoderm. Overexpression BMP4 also reduced the *hoxa-1* transcripts in the ectoderm (Fig. 6A), but the coexpression of Kheper elicited the expansion of the *hoxa-1* expression domain (Fig. 6B). These data indicate that Kheper mediates signals for neuralization specifically in the ectoderm.

Kheper Interacts with CtBPs

Recently it has been reported that δEF1 homologues are transcriptional repressors that interact with the corepressor CtBPs (Postigo *et al.*, 1999; Furusawa *et al.*, 1999). CtBP was discovered as a protein that interacted with the PLDLS sequence located in the C-terminal region of E1A (Boyd *et al.*, 1993; Schaeper *et al.*, 1995). The PLDLS sequence is also found in the Kheper sequence, which is conserved with chicken δEF1 (Fig. 1), suggesting that Kheper interacts with zebrafish CtBP. To address this issue, we examined the expression of *CtBP1* and *CtBP2* in zebrafish and the interaction between Kheper and CtBPs. *CtBP1* and *CtBP2* are maternally deposited and ubiquitously expressed until the onset of the gastrulation. During the gastrulation and segmentation periods, *CtBP2* is expressed ubiquitously, whereas *CtBP1* is expressed in the ectoderm, indicating that *CtBPs* and *kheper* are coexpressed in the ectoderm (data not shown).

When the GFP-tagged CtBP1 and Myc-tagged Kheper were coexpressed in COS7 cells, CtBP1 was coimmunoprecipitated with Kheper (Fig. 7), showing the interaction between Kheper and zebrafish CtBP1. Kheper also interacted with CtBP2 (data not shown). As the expressions of *CtBP1* and *CtBP2* were detected in the ectoderm, Kheper may act as a transcriptional repressor with CtBP1 and CtBP2 in the ectoderm.

Kheper Is Required for Neural Development

To further reveal the role of Kheper, we constructed a mutant of Kheper which inhibits the function of endogenous Kheper. The dHD mutant was constructed by the deletion of the homeodomain. The embryos injected with *GFP-dHD* RNA displayed the defect of the head formation with various severities (Figs. 8B and 8C). These phenotypes were classified as "type III" (slightly disturbed head structure; Fig. 8B) and "type IV" (loss of head structure; Fig. 8C) at 24 hpf. Coexpression of the wild-type GFP–Kheper suppressed the phenotypes caused by GFP–dHD expression. Similarly, coexpression of GFP–dHD suppressed the phenotypes caused by the wild-type GFP–Kheper (Table 2), suggesting that GFP–dHD specifically inhibited the activity of Kheper. In the *dHD* RNA-injected embryos, the expression of *otx2* (Mori *et al.*, 1994) and *hoxa-1* was prominently reduced (Figs. 8F and 8G) although the expression of *ntl* was not affected (data not shown). These data indicate that Kheper is required for the formation of the neuroectoderm but not for the dorsalization of the mesoderm. Kheper was suggested to be a transcriptional repressor and we constructed another dominant negative Kheper by the fusion of the Kheper homeodomain and the transactivation domain of VP16 (VP16AD-HD; Sadowski *et al.*, 1988). The embryos injected with the *VP16AD-HD* RNA displayed phenotypes

TABLE 2
The Effects of Dominant Negative *kheper* and *dHD* RNA Injection

Type of RNA injected	Amount of RNA injected (pg)	Phenotype (%)						
		R	I	II	N	III	IV	*n*
GFP-kheper	50	57.0	4.1	6.8	2.3	0	0	221
GFP-kheper/	50/35	30.2	5.5	9.5	12.7	19.8	0.8	126
GFP-dHD	50/70	22.5	2.0	9.9	27.8	12.6	0	151
GFP-dHD	70	1.2	0	0	67.4	16.3	11.6	86

Note. The data for 50 pg of *GFP-kheper* from Table 1 are shown at the top.

similar to those injected with *GFP-dHD* RNA (data not shown) and the expressions of *otx2* and *hoxa-1* were also reduced in these embryos (Figs. 8H and 8I). The results suggest that Kheper acts as a transcriptional repressor for neural development.

DISCUSSION

The ZFH/δEF1 family is an emerging group of transcriptional regulators that have both zinc fingers and the homeodomains. Among the family, the sequence of Kheper exhibits only weak similarities to those of other members outside of the zinc fingers and homeodomain (Fig. 1). Unlike other homologues, Kheper is mainly expressed in the forming neuroectoderm (Fig. 2). These data suggest that Kheper is a novel member of the ZFH/δEF1 family and it exhibits a unique function in the formation of the neuroectoderm.

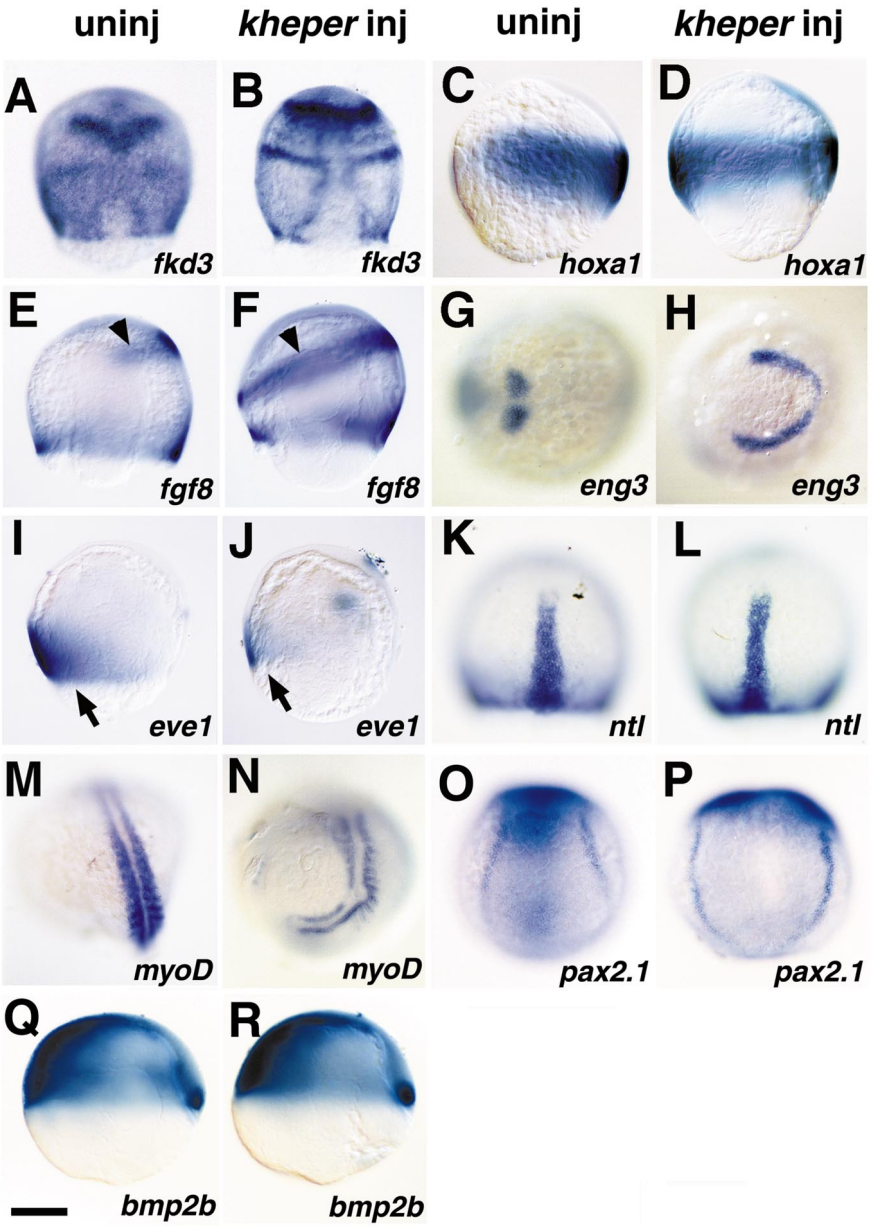
The *kheper* expression in neuroectoderm was expanded and reduced in the embryos expressing Noggin and BMP4, respectively (Fig. 3). Consistent with this, the expression was expanded in the *swirl/bmp2b* mutant embryos and diminished in the *chordino* mutant embryos (Fig. 3). These data suggest that *kheper* expression in the neuroectoderm is regulated by the balance of BMPs and the BMP antagonists Chordino and Noggin1, which are secreted by the dorsal organizer region (Miller-Bertoglio *et al.*, 1997; Furthauer *et al.*, 1999).

Misexpression of Kheper caused dorsalized-like phenotypes, in which the neuroectoderm was expanded. The phenotypes are similar to those observed in the dorsalized mutants *swirl*, *snailhouse*, and *somitabun* in terms of the neuroectodermal expansion (Mullins *et al.*, 1996). However, the Kheper-overexpressing embryos did not display the expansion of dorsal mesoderm (Fig. 5). This is quite in contrast to the dorsalized mutant embryos, which have the dorsal mesoderm expanded (Mullins *et al.*, 1996). Therefore, the expansion of the neuroectoderm in the *kheper* RNA-injected embryos cannot be explained by the inhibition of the BMP signals. Consistently, misexpression of *kheper* did not suppress *bmp2b/swirl* expression (Fig. 5R), and the expansion of prospective posterior neural tissue expressing *hoxa-1* by Kheper overexpression was not inhibited by the overexpression of BMP4 (Fig. 6B). These data indicate that Kheper acts downstream of the BMP antagonist and mediates its signals only in the neuroectoderm and not in the mesoderm.

The effects of misexpression of Kheper and the dominant negative Kheper suggest that Kheper functions in the formation of all the neuroectoderm (Figs. 5 and 8). However, overexpression of Kheper did not expand *otx2* expression (data not shown). Coexpression of Kheper and BMP4 expanded *hoxa-1* expression to the animal pole side (Fig. 6B). These results suggest that Kheper is sufficient to transmit the anti-BMP signals for the formation of posterior neuroectoderm but it requires other downstream targets of the anti-BMP signals to form the anterior neuroectoderm. In

FIG. 5. The effects of microinjection of *GFP-kheper* RNA on tissue-specific gene expression. Uninjected embryos (A, C, E, G, I, K, M, O, Q) and embryos injected with 50 pg of *GFP-kheper* RNA (B, D, F, H, J, L, N, P, R). *fkd3* (A, B), *hoxa-1* (C, D), *fgf8* (E, F), *eve1* (I, J), and *ntl* (K, L) expression at the 75–80% epiboly stage. *eng3* expression at the three-somite stage (G, H). *myoD* expression at 15 hpf (M, N). *pax2.1/noi* expression at the three-somite stage (O, P). *bmp2b/swirl* expression at the 60% epiboly stage (Q, R). Arrowheads in E and F indicate the *fgf8* expression in the anterior hindbrain primordium. Arrows in I and J indicate the expression of *eve1*. (A, B, K, L, M, N, O, P) Dorsal view with the animal pole to the top. (C–F, I–J, Q–R) Lateral view and (G, H) animal pole view with the dorsal side to the right. Scale bar, 200 μm.

FIG. 6. Co-injection of *bmp4* RNA with *GFP-kheper* RNA. *hoxa-1* expression at the 75% epiboly stage in the embryo injected with 100 pg of *bmp4* RNA (A) and in the embryo co-injected with 100 pg of *bmp4* RNA and 50 pg of *GFP-kheper* RNA (B). *ntl* expression at the 75% epiboly stage in the uninjected embryo (C) and the co-injected embryo (D). Arrows indicate *ntl* expression in the dorsal midline. (A, B) Lateral view with the dorsal side to the right. (C, D) Dorsal view with the animal pole to the top. Scale bar, 200 μm.



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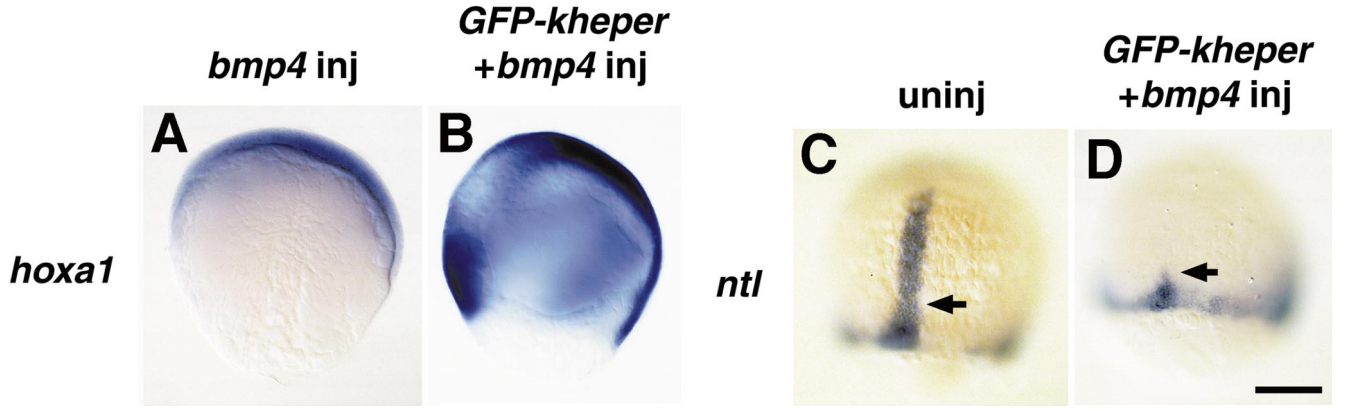

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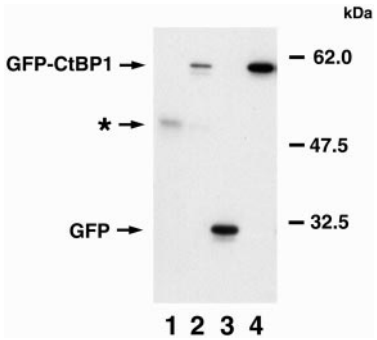

FIG. 7. Kheper binds to zebrafish CtBP1. The expression vectors for GFP (lanes 1 and 3) or GFP-tagged zebrafish CtBP1 (lanes 2 and 4) were introduced with Myc-tagged Kheper (amino acids 336–803) into COS7 cells. After 48 h, cells were lysed, and after immunoprecipitation with anti-Myc antibody, binding to Kheper was detected by Western blot using anti-GFP antibody as described under Materials and Methods (lanes 1 and 2). As input control, the expression of GFP and GFP–CtBP1 protein in the lysate was checked with anti-GFP antibody without immunoprecipitation (lanes 3 and 4). The signal indicated with the asterisk is nonspecific. The weak signal below the major GFP–CtBP1 signal seems to be the degraded GFP–CtBP1 protein. CtBP2 also bound to Kheper (data not shown).

Xenopus, SoxD, which is a target of the anti-BMP signals, is required for the formation of anterior neuroectoderm (Mizuseki *et al.,* 1998b). The zebrafish homologue of SoxD might be a cofactor for Kheper for the anterior neuroectoderm development.

Previously several transcription factors were reported to act downstream of the BMP-antagonistic neural inducers and function in neuroectoderm development. These include *Zic-r1, Zic3, Sox-2,* and *SoxD* (Nakata *et al.,* 1997; Mizuseki *et al.,* 1998a,b). Expressions of *Zic-r1, Zic3,* and *SoxD* in animal cap ectoderm induce the expression of neural markers, indicating that overexpression of these genes is sufficient to neuralize the ectoderm. In contrast, overexpression of Sox-2 alone is not sufficient to induce the expression of neural markers, but can synergistically act with FGF signaling to induce the neuroectoderm (Mizuseki *et al.,* 1998a). When Kheper-overexpressing blastomeres were transplanted to the ventral side of the sibling embryos at the late blastula stage, they did not express the neural marker (data not shown), suggesting that overexpression of Kheper alone was not sufficient for neural induction. However, expression of the dominant negative Kheper inhibited neuroectoderm formation, indicating that Kheper was required for neuroectoderm development. The situation is similar to that of Sox-2, the dominant repressor form of which inhibits neuroectoderm differentiation (Kishi *et al.,* 2000). However, Sox-2 acts as a transcriptional activator, whereas Kheper may act as a transcriptional repressor. Kheper may collaborate with those factors for the development of the neuroectoderm.

δEF1 homologues are reported to be transcriptional suppressors (Postigo *et al.,* 1999; Williams *et al.,* 1991; Franklin *et al.,* 1994). ZEB, ZFH-1, and δEF1 interact with CtBP to

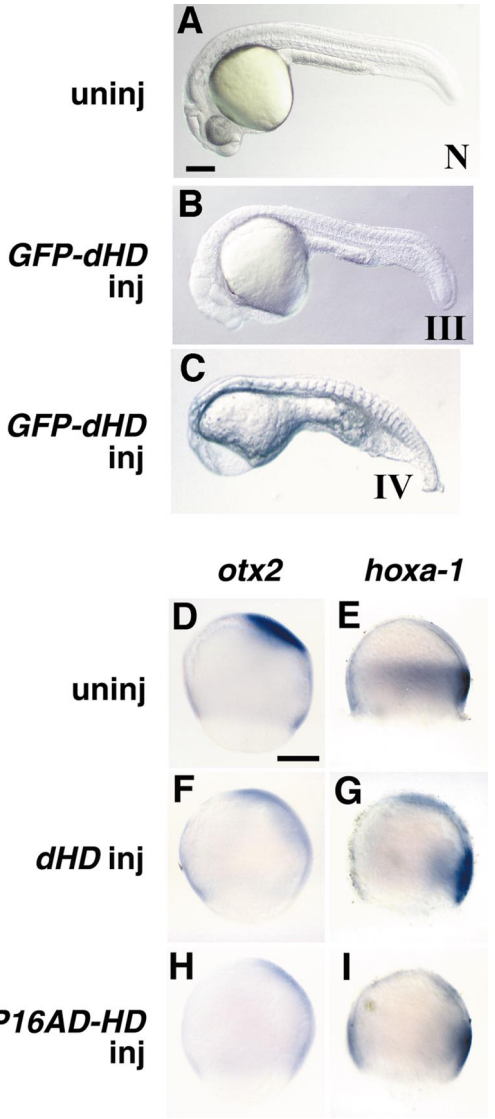

FIG. 8. Effects of the dominant negative type of Kheper, dHD, and VP16AD-HD. dHD was constructed by deletion of the Kheper homeodomain, and VP16AD-HD was constructed with the VP16 activation domain and Kheper homeodomain (see Materials and Methods). 24-hpf embryos uninjected (A) and injected with 170 pg of *GFP-dHD* RNA (B, C). C shows a more severe phenotype than B. We designated the phenotype in B as "type III" and C as "type IV." Whole-mount *in situ* hybridization with *otx2* probe (D, F, H) and *hoxa-1* (E, G, I). 75% epiboly stage embryos uninjected (D, E), injected with 170 pg of *dHD* RNA (F, G), and injected with 240 pg of *VP16AD-HD* RNA (H, I). (A–C) Lateral views with the head to the left. (D–I) Lateral views with the dorsal side to the right. Scale bar, 200 μm.

repress transcription (Postigo *et al.*, 1999; Furusawa *et al.*, 1999). CtBP is the corepressor that interacts with other factors to repress transcription (Sollerbrant *et al.*, 1996; Brannon *et al.*, 1999; Nibu *et al.*, 1998). The consensus amino acid sequence for the interaction of CtBP is PLDLS with some variations, P-X-D/N-L-S/T (Postigo *et al.*, 1999; Turner and Crossley, 1998). Kheper also has consensus and similar sequences and interacted with zebrafish CtBPs, as we expected. In addition, expression of VP16AD-HD inhibited the neuroectoderm development as another dominant negative form. These data indicate that Kheper acts as a transcriptional repressor and its repressor function is required for the neuroectoderm development.

In summary, our data provide the evidence that Kheper is a transcriptional repressor which promotes the neuroectoderm development, acting downstream of the BMP-antagonistic neural inducers.

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