Expression cloning of maternally expressed genes of the ascidian Ciona savignyi demonstrated that the overexpression of syndecan, a member of a multigene family of integral membrane heparan sulfate proteoglycans, resulted in a disturbance of cell adhesion and morphogenesis. The Ciona syndecan gene was expressed both maternally and zygotically. The maternal transcript was distributed evenly in fertilized eggs and early embryos up to the 32-cell stage without any special localization and then became barely detectable in the 64-cell and gastrula stages. The zygotic transcription became evident during neurulation, mainly in cells of epidermis, the central nervous system, and mesenchyme. Embryos with syndecan overexpression via RNA injection cleaved as did normal embryos, but showed loose blastomere adhesion after the 32-cell stage. Gastrulation occurred, but the closure of the blastopore was markedly delayed, resulting in larvae without normal morphology. About half of the syndecan-overexpressing embryos hatched, and differentiation of epidermis, endoderm, muscle, and notochord was evident. However, the formation of pigment cells of the sensory organs was markedly disturbed. These results indicate that an appropriate level of syndecan expression is required for normal cell adhesion and morphogenesis of the ascidian embryo.

Key Words: expression cloning; maternally expressed genes; ascidian; syndecan; cell adhesion; morphogenesis.

INTRODUCTION

During ascidian embryogenesis, several types of embryonic cells are specified autonomously via mechanisms dependent on prelocalized egg cytoplasmic factors or determinants (reviewed by Satoh, 1994). Recent studies have provided convincing evidence for determinants responsible for differentiation of muscle, epidermis, and endoderm, factors for the establishment of the embryonic anteroposterior axis, and for initiation of gastrulation (reviewed by Nishida, 1997; Satoh, 1999). Therefore, the molecular identification of localized maternal factors, the elucidation of the machinery responsible for the localization, and the exploration of the mode of action of the localized factors are key research subjects for the elucidation of the pattern formation of ascidian embryos.

Some of the candidate molecules for the determinants have already been partially characterized. For example, using the two closely-related species Molgula oculata and M. occulta, which produce tailed (urodele) larvae and tailless (anural) larvae, respectively, Swalla et al. (1993) isolated two genes that are maternally expressed only in early embryos of the species with tailed larvae. One of the genes, Manx, appeared to encode a transcriptional factor, and its zygotic expression is likely to be involved in the formation of the tail structure (Swalla and Jeffery, 1996). Analysis of the cDNA of maternally expressed genes in Halocynthia roretzi demonstrated that maternal transcripts of HrWnt-5 (Sasakura et al., 1998a) and a gene for serine/threonine kinase (Sasakura et al., 1998b) are localized in the posterior region of the early embryo.

In our previous studies, to isolate maternal genes with developmental functions, we developed an experimental system consisting of egg fragmentation and fusion of the fragments (Marikawa et al., 1994). Namely, centrifugation of unfertilized eggs of the ascidian Ciona savignyi yielded four types of fragments: a large nucleated red fragment and
small enucleated black, clear, and brown fragments. When fertilized, only red fragments cleave and develop into so-called permanent blastulae in which only epidermal cell differentiation is evident. However, when red fragments are fused with black fragments and the fusion products are fertilized, nearly all of the fusion products develop muscle and endoderm cells, and sometimes morphologically normal tadpole larvae are formed (Marikawa et al., 1994). Clear and brown fragments have no such abilities. Therefore, maternal factors for these processes appear to be preferentially separated into black fragments. In addition, UV-irradiation experiments of black fragments suggested that maternal factors for these processes appear to be preferentially separated into black fragments. In addition, UV-irradiation experiments of black fragments suggested that maternal mRNAs are associated with the activities of these factors (Marikawa et al., 1995).

By differential screening of cDNA libraries of red and black fragments, we isolated and characterized a novel maternal gene named posterior end mark (pem), whose transcript is initially concentrated in the posterior–vegetal cytoplasm of the fertilized egg and later in the posterior end of developing embryos (Yoshida et al., 1996). Overexpression of pem by microinjection of synthetic capped mRNA into fertilized eggs resulted in development of larvae with deficiencies of the anteriormost adhesive organ, dorsal brain, and sensory pigment cells, suggesting that pem may be involved in pattern formation of the embryo (Yoshida et al., 1996, 1997). Further experiments involving treatment of the egg with LiCl suggested that pem function involves the signaling pathways of the wnt/β-catenin cascade (Yoshida et al., 1998). In addition, pem-2, pem-4, pem-5, and pem-6 (Satou and Sato, 1997) were isolated from a cDNA library of C. savignyi maternal mRNAs with gastrula mRNAs subtracted. Further isolation from the subtractive library yielded cDNA clones for three maternally expressed genes (CsEndo 1–3) with mRNA localized in the so-called endoplasm (Imai et al., 1999).

These studies therefore succeeded in isolation of genes that are maternally expressed in early ascidian embryos. However, the more difficult problem in the characterization of maternally expressed genes is the determination of their function. The function of certain genes, for example pem, may be inferred, because their overexpression by microinjection of synthetic mRNAs results in discrete effects on morphogenesis and differentiation (Yoshida et al., 1996). The function of other genes, such as Manx, can be deduced by treatment of embryos with antisense oligos (Swalla and Jeffery, 1996). However, these techniques do not always work to determine the function of maternally expressed genes. In Xenopus laevis, an expression cloning succeeded in revealing an important role for Xwnt-8 and noggin in organizing the embryo (Smith and Harland, 1991, 1992). Therefore, the present study attempted a similar expression cloning of ascidian embryos. We made a cDNA library of maternally expressed genes of black fragments of C. savignyi and injected fractions of RNA synthesized from cDNAs into fertilized eggs as an assay system. We attempted to identify clones whose injection caused distinct effects on morphogenesis and differentiation. One cDNA clone we obtained showed a marked disturbance of cell adhesion and morphogenesis when injected into fertilized eggs. It became evident that the cDNA corresponds to a gene for an ascidian homologue of syndecan.

**MATERIALS AND METHODS**

**Ascidian Eggs and Embryos**

C. savignyi adults were collected near the Otsuchi Marine Research Center, Ocean Research Institute of the University of Tokyo, Iwate, Japan. They were maintained in aquaria under constant light to induce oocyte maturation. Eggs and sperm were obtained surgically from the gonoduct. After insemination, eggs were reared at about 18°C in Millipore-filtered seawater containing 50 μg/ml streptomycin sulfate.

**Library Construction and Screening**

Production of egg fragments of C. savignyi was described previously (Marikawa et al., 1994). Total RNA was isolated from black fragments by the acid guanidinium thiocyanate–phenol–chloroform method (Chomczynski and Sacchi, 1987). Poly(A)+ RNA was purified using Oligotex beads (Roche Japan, Tokyo, Japan).

Poly(A)+ RNA was converted to double-stranded cDNA which contained an EcoRI site at the 5′-end and an XhoI site at the 3′-end using a cDNA synthesis kit (Stratagene, La Jolla, CA). The cDNAs were ligated to pBS-RN3 (EX) digested with EcoRI and Xhol. pBS-RN3 (EX) is a vector in which the NotI site of pBS-RN3 (Lemaire et al., 1995) has been replaced with XhoI. The cDNAs were electroporated into XL-1 Blue bacteria (Stratagene). The resulting library contained 8 × 10⁵ clones. The library was amplified by conventional methods (Sambrook et al., 1989).

Plasmid DNA was prepared from fractions of the cDNA library by standard methods. For the preparation of templates for in vitro transcription, plasmid DNA was digested with SfiI, followed by proteinase K treatment. RNA was synthesized using a MEGAscript T3 kit (Ambion, Austin, TX). To obtain capped mRNAs, the concentration of GTP was lowered to 1.5 mM and the cap analog 7MeGppG was added at a final concentration of 6 mM. The RNAs were injected into C. savignyi fertilized eggs. A fraction that affects the morphogenesis of embryos was further fractionated. After the fourth sib selection, we obtained a candidate cDNA. The amount of mRNA injected was calculated by the volume and concentration of the RNA solution.

**Sequencing**

Nucleotide sequences were determined for both strands using a BigDye Terminator Cycle Sequencing Ready Reaction kit and ABI Prism 377 DNA sequencer (Perkin-Elmer, Norwalk, CT).

**Whole-Mount in Situ Hybridization**

To determine syndecan mRNA distribution, RNA probes were prepared with a DIG RNA labeling Kit (Boehringer Mannheim,
FIG. 1. Characterization of an ascidian syndecan. (A) Nucleotide and deduced amino acid sequences of the cDNA clone. The 1058-bp insert includes a single open reading frame that encodes a polypeptide of 209 amino acids. The termination codon is shown by an asterisk. The putative signal peptide is shown by a dotted line, and the transmembrane domain is boxed. (B) Sequence comparison of C. savignyi syndecan with other syndecans. Alignment of the C-terminal regions of C. savignyi syndecan, rat syndecan 1, Xenopus syndecan 1, rat syndecan 2, Xenopus syndecan 2, rat syndecan 3, Xenopus syndecan 3, rat syndecan 4, human syndecan 4, and Drosophila syndecan. Asterisks indicate identity, and dots indicate conservative changes. (C) Molecular phylogenetic tree showing the relationship of C. savignyi syndecan to other known syndecans. C. savignyi syndecan does not show an affinity with any one of the four vertebrate family members, but is located on the base of the branching of the four family members.
Syndecan and Ascidian Morphogenesis

Heidelberg, Germany. Whole-mount in situ hybridization was performed using digoxigenin-labeled antisense probes as described previously (Satou and Satoh, 1997). Control embryos hybridized with a sense probe did not show signals above background. Muscle cell differentiation in syndecan-overexpressed embryos was examined by whole-mount in situ hybridization with probes of a larval muscle-specific actin gene (CsMA1) (Chiba et al., 1998).

Histochemical Staining for Alkaline Phosphatase

The differentiation of endoderm cells in experimental embryos was monitored by the histochemical reaction of alkaline phosphatase.

RESULTS

Expression Cloning of Maternally Expressed Genes with Developmental Functions

To isolate maternally expressed genes with developmentally important functions, we attempted expression cloning. Maternal mRNAs were isolated from black fragments of C. savignyi using the cloning strategy described by Smith and Harland (1992). RNA from black fragments was size fractionated and the active fraction was used to construct a plasmid cDNA library. RNAs were synthesized from pools of plasmids and injected into fertilized eggs. Pools of plasmid DNA that directed the synthesis of functional RNA were sib selected until single clones were isolated. After the fourth sib selection, we obtained one cDNA clone. Northern blot analysis revealed a single transcript of about 1.2 kb in the black fragment (data not shown).

Characterization of an Ascidian Syndecan

Figure 1A shows the nucleotide and deduced amino acid sequences of the cDNA clone. The cDNA sequence was 1058 bp long and had a single open reading frame that predicted a polypeptide of 209 amino acids. Database searches indicated that the polypeptide had a putative signal peptide in the N terminus and a transmembrane domain in the C-terminal half (Fig. 1A). Furthermore, comparison of amino acid sequences of the C-terminal region revealed that the peptide is a C. savignyi homologue of syndecan (Fig. 1B). Syndecan is a major class of integral membrane heparan sulfate proteoglycans (HSPGs), which form a multigene family in vertebrates (reviewed by Bernfield et al., 1992; Rapraeger, 1993). HSPGs play significant roles in cell–cell and cell–matrix interactions, functioning as growth factor co-operators facilitating ligand–receptor interactions and as cell adhesion molecules (Bernfield et al., 1992; Rapraeger, 1993).

Vertebrate syndecans are divided into four subfamilies based on slight differences in their sequences in the transmembrane and cytoplasmic domains (Bernfield et al., 1992) (Fig. 1B). We compared the C. savignyi syndecan sequence to those of the corresponding region of vertebrate syndecans by the neighbor-joining method. The resulting tree revealed that C. savignyi syndecan does not show a particular homology to any one of the four subfamilies but is located at the base of branching of the four subfamilies (Fig. 1C). These results suggest that one original syndecan gene duplicated twice to form the four subfamilies during the evolution of vertebrates, which corresponds to the widely occurring pattern of vertebrate gene duplication (e.g., Holland and Graham, 1995).

Distribution of the Syndecan mRNA in Developing Ascidian Embryos

Results of whole-mount in situ hybridization to determine the spatial expression of the C. savignyi syndecan gene are shown in Fig. 2. Isolation of the cDNA from egg fragments indicated that the gene was expressed maternally. The hybridization signal of the maternal transcript was evenly distributed during early developmental stages from the unfertilized egg (Fig. 2A) to the 32-cell stage. No special localization of the transcript was observed (Fig. 2B). The signal for the maternal transcript, however, gradually disappeared after the 64-cell stage, and at the gastrula stage, the signal became barely detectable (Fig. 2C).

In addition to the maternal transcript, the hybridization...
signal of the zygotic transcript became evident during neurulation (Fig. 2D). At the neurula stage, the signal was found in a broad area involved in forming the central nervous system (CNS). Then at the early tailbud stage, intense signals were detected in at least three regions (Figs. 2E and 2F). First, a signal was evident in the antiormost

**FIG. 4.** Injection of syndecan mRNA affects the formation of the anterior and dorsal structures of tadpole larvae. Syndecan mRNA was microinjected into fertilized eggs, and injected eggs were allowed to develop into tadpole larvae. (A) A control larva derived from an egg injected with lacZ-RN3 mRNA, showing normal larval morphology with lacZ expression. (A') Enlargement of the sensory vesicle showing ocellus (Oc) and otolith (Ot) pigment cells. (B–F) Experimental larva derived from an egg injected with syndecan mRNA, showing deficiencies of the normal morphogenesis. Larvae developed (B) two, (C) one, or (D) three pigment cells. Tk, trunk; Tl, tail of the tailbud embryo. (E) Histochemical detection of alkaline phosphatase showing the differentiation of endoderm in the trunk region of the embryo (arrow). (F) In situ hybridization of a muscle actin mRNA showing muscle-specific gene expression in the embryo (arrows). Scale bar represents 100 μm.
region of the embryo, a part of which forms the adhesive organ of the tadpole larva, which is used by larvae to attach to substrates during metamorphosis. This signal was continuous to the dorsal midline of the embryo (Figs. 2E and 2F). Cells with the signal were the outermost epidermal cells and those of the CNS just beneath the ectoderm. Second, a strong signal was evident at both sides of the neck region between the trunk and the tail of the embryo (Figs. 2E and 2F). This area contains mesenchyme precursor cells. The other region with a distinct signal was the posterior-most region of the tail of the tailbud embryo (Figs. 2E and 2F). Therefore, zygotic expression of the syndecan gene is restricted to cells of the CNS, mesenchyme, and probably tailbud.

Overexpression of Syndecan Affects the Cell Adhesion and Morphogenesis of the Embryo

We injected various amounts of syndecan mRNA into single fertilized eggs to examine the effects of its overexpression. As controls, we injected the same amounts of lacZ mRNA. Injection of 6 pg mRNA did not affect the morphogenesis. However, injection of 15 pg and more mRNA disturbed the morphogenesis in a concentration-dependent manner. Because eggs injected with 60 pg of syndecan mRNA developed into cell masses (data not shown), we describe here results of 30 pg mRNA injection.

As shown in Fig. 3, 30 pg syndecan mRNA injection did not alter the cleavage pattern. The cleavage tempo of experimental embryos (Figs. 3A'–3C') was the same as that of normal embryos (Figs. 3A–3C). However, the adherent activity of blastomeres of experimental embryos was down-regulated (Figs. 3B' and 3C'), so that their blastomeres looked round compared with blastomeres of normal embryos, which showed tight adhesion at the midphase of each division cycle (Figs. 3A–3C). This inclination continued during embryogenesis, so that some embryonic cells detached from the embryo proper (Fig. 3F').

Morphogenetic movements related to gastrulation and neurulation were also affected in syndecan mRNA-injected embryos. As shown in Figs. 3C' and 3D', the gastrulation of experimental embryos initiated at the same developmental time as that of normal embryos. The most apparent effect of syndecan overexpression was retardation of the blastopore closure (Figs. 3D' and 3E'). In Fig. 3D, the normal embryo was at the neural plate stage, during which the blastopore closure was accomplished. In Fig. 3D', however, the experimental embryo still had a large blastopore. The blastopore closure of experimental embryos was not accomplished even when normal embryos reached the early tailbud stage (Fig. 3E'). However, experimental embryos also elongated to some extent along the anteroposterior axis (Fig. 3E'). Partially due to nonclosure of the blastopore, primordial muscle cells located on both sides of the posterior part of the blastopore could not converge in the single tail. Therefore, the posterior part of experimental embryos became bifurcated, resulting in a forked tail (Figs. 3E' and 3F').

Although the syndecan-overexpressing embryos had abnormal morphology, about half of them hatched. When we examined the differentiation of epidermis, endoderm, and muscle cells in the fork-tailed embryos, differentiation of endoderm (Fig. 4E) and muscle cells (Fig. 4F) was evident. Differentiation of epidermis was ascertained by the occurrence of tunic surrounding the embryo (Fig. 4C).

A marked disturbance induced by syndecan mRNA injection was evident in the formation of sensory pigment cells in the brain vesicle, the anterior–dorsal portion of the ascidian larval CNS (Fig. 4A'). The brain of a Ciona larva contains two sensory organs: the otolith and the ocellus (Fig. 4A'). The otolith or statocyte is an unusual cell which contains a large melanin granule, and the ocellus of the Ciona tadpole is composed of a single cup-shaped pigment cell, some 15–20 sensory cells, and 3 lens cells (Eakin and Kuda, 1971). The pigment cell forms part of the wall of the sensory vesicle and contains many discrete membrane-bound melanin granules. The otolith pigmentation pattern is distinguishable from the ocellus pigmentation pattern (Fig. 4A').

As summarized in Table 1, all of the control embryos injected with 30 pg of lacZ mRNA formed two sensory pigment cells. In contrast, microinjection of 30 pg of syndecan mRNA resulted in disturbance of pigment cell formation. We examined two experimental series of 45 embryos. Among them, 12 embryos (26.7%) failed to develop pigment cells, while 10 embryos (22.2%) formed only one pigment cell (Fig. 4C). Ten embryos (22.2%) formed two pigment cells (Fig. 4B). Furthermore, 9 (20%), 3 (6.7%), and 1 embryo (2.2%) developed three (Fig. 4D), four, and five pigment cells, respectively. It was noticed that most of the pigment cells were of the ocellus type rather than of the otolith type (Figs. 4B–4D).

DISCUSSION

Expression Cloning of Ascidian Maternally Expressed Genes with Developmental Functions

The present study attempted expression cloning of maternally expressed genes with developmental functions in ascidian embryos. One cDNA we obtained corresponds to a gene for an ascidian syndecan. The ascidian syndecan is expressed both maternally and zygotically. Overexpression of syndecan resulted in disturbance of cell adhesion and morphogenesis. Experimental embryos became fork-tailed, and the formation of pigment cells in the brain vesicle was markedly affected; some embryos failed to form the pigment cells while others formed additional pigment cells.

As the source for isolation of maternal mRNA, we selected black fragments of C. savignyi, because the fragments contain maternal factors for differentiation of muscle cells and endoderm cells, for the embryonic axis.
formation, and for normal morphogenesis (Marikawa et al., 1994). In previous experiments exploring the maternal factors in C. savignyi, red fragments were used to monitor their molecular identification (Marikawa et al., 1994). Therefore, the best recipient for injection of fractions of cDNA may be red fragments. However, that was technically infeasible. We also considered lithium-treated eggs as the injection target. Recently, Yoshida et al. (1998) showed that lithium treatment of C. savignyi eggs resulted in the reduction of the larval tail and that this reduction was caused by the fate change of the A4.1-line notochord cells to endoderm cells. In addition, this effect of lithium was rescued by pem mRNA injection. Therefore, it is highly probable that the expression cloning of maternally expressed genes using lithium-treated eggs as target would result in isolation of pem cDNA. Therefore, as the first step of expression cloning, we injected mRNA into normal eggs and obtained syndecan cDNA.

## Expression Pattern of the Ascidian Syndecan

The ascidian syndecan was expressed both maternally and zygotically. The maternal transcript was distributed evenly in early embryos, whereas the zygotic transcript was found in presumptive cells of the CNS, mesenchyme, and tailbud. Expression patterns of syndecans have been well characterized during Xenopus embryogenesis (Teel and Yost, 1996). During Xenopus embryogenesis, three syndecan genes, Xsynd-1, Xsynd-2, and Xsynd-3, are expressed in specific manners. Maternal mRNAs of Xsynd-1 and Xsynd-2 are localized to the animal pole in blastulae and are expressed in the ectoderm of gastrulae. In neurulae, Xsynd-1 mRNA is restricted to nonneural ectoderm and Xsynd-2 mRNA is restricted to neural ectoderm. In tailbud embryos, the three syndecans are expressed in adjacent, nonoverlapping patterns. Xsynd-2 is expressed in the heart while Xsynd-1 is expressed in the underlying anterior endoderm. Xsynd-3 is expressed in the hindbrain, midbrain, and forebrain, while Xsynd-2 is expressed in the intervening regions. Interestingly, the combined patterns of Xsynd-1 and Xsynd-2 expression are similar to that of the single ascidian syndecan. This suggests that the gene duplication that occurred during vertebrate evolution may have split the primary expression pattern (and function) of a single ancestral gene into a few discrete patterns (and functions) of its descendant genes.

### Possible Function of Ascidian Syndecan in Embryogenesis

Cell-cell and cell-matrix interactions, mediated by growth factors and cell adhesion molecules, are key events in the earliest steps of animal development. Heparan sulfate proteoglycans play roles in these interactions. They function as growth factor co-operators facilitating ligand-receptor interactions and as cell adhesion molecules. It has been reported that heparan sulfate is required for the activity of several fibroblast growth factor (FGF) family members (Yayon et al., 1991) and interacts with many other peptide factors, including VEGF (Gitay-Goren et al., 1992); HB-EGF (Higashiyama et al., 1993); the cytokines IL-3, GM-CSF, and IFN-γ (Ruoslahti and Yamaguchi, 1991); and members of the hedgehog family of signaling proteins (Lee et al., 1994). In X. laevis, HSPGs are implicated in mesoderm induction, gastrulation, and left–right development. Heparinase treatment of animal cap explants inhibits mesoderm induction by basic FGF, activin, and Xwnt-8 (Sack et al., 1987; Brickman and Gerhart, 1994; Itoh and Sokol, 1994), whereas heparitinase treatment of dorsal marginal zone explants inhibits morphogenetic movements and mesoderm induction (Brickman and Gerhart, 1994; Itoh and Sokol, 1994). In addition, the left–right orientation of the heart and gut in the tadpole is randomized by injection of heparinase into the blastocoel at early gastrula stages (Yost, 1992). Thus, HSPGs are crucial for early Xenopus development. Treatment of C. savignyi eggs and early embryos with heparinase, however, did not affect cell adhesion and morphogenesis of the embryo (data not shown).

A major class of HSPGs is made up of syndecans, a multigene family of integral membrane HSPGs (Bernfield et al., 1992; Rapraeger, 1993). However, the roles of syndecans in the earliest events in development have not been extensively studied, except for Xenopus syndecans. As shown in the present study, overexpression of syndecan resulted in less adhesion of embryonic cells and in abnormal morphogenesis. One of the most apparent effects was on the formation of pigment cells, because this feature is easily detectable. However, it is likely that the effects of overexpression of syndecan are more general but less specific. As shown in Fig. 3, the closure of the blastopore was much delayed, and the neurulation movement of cells located on both sides of the embryo to converge toward the midline to form an organ consisting of a single cluster of cells was severely inhibited. In the posterior region of the embryo, this damage resulted in a forked tail. In the anterior and central parts of the embryo, formation of neuronal cells was inhibited. During normal neurulation, presumptive pigment cells (right a8.25 and left 8.25) converge toward the midline and align properly to form the anterior otolith pigment cell and posterior ocellus pigment cell (Nishida...
and Satoh, 1989). Inhibition of this movement by overexpression of syndecan resulted in abnormal development of pigment cells. The two pigment cells of ascidian embryos constitute the so-called equivalence group (Nishida and Satoh, 1989). In the case of H. roretzi, formation of ocellus pigment cells is a dominant fate so that if one precursor cell is experimentally killed, the remaining cell differentiates into an ocellus pigment cell. Syndecan overexpression induced numerous pigment cells, most of which were ocellus pigment cells. This suggests that ocellus is a dominant fate in C. savignyi embryos, as in the case of H. roretzi. It is easily predictable that an inhibition of syndecan function by treatment with some specific proteases would result in the disturbance of morphogenesis and cell specification. Because the present study revealed that overexpression of syndecan affected the cell adhesion and morphogenesis, it is worth mentioning that an appropriate level, neither too high nor too low, of syndecan expression is required for normal embryogenesis of ascidians.

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