OUT, a Novel Basic Helix-Loop-Helix Transcription Factor with an Id-like Inhibitory Activity*

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Transcription factors belonging to the basic helix-loop-helix (bHLH) family are involved in various cell differentiation processes. We report the isolation and functional characterization of a novel bHLH factor, termed OUT. OUT, structurally related to capsulin/epi-cardin/Pod-1 and ABF-1/musculin/MyoR, is expressed mainly in the adult mouse reproductive organs, such as the ovary, uterus, and testis, and is barely detectable in tissues of developing embryos. Physical association of OUT with the E protein was predicted from the primary structure of OUT and confirmed by co-immunoprecipitation. However, unlike other bHLH factors, this novel protein failed to bind E-box or N-box DNA sequences and inhibited DNA binding of homo- and heterodimers consisting of E12 and MyoD in gel mobility shift assays. In luciferase assays, OUT inhibited the induction of E-box-dependent transactivation by MyoD-E12 heterodimers. Deletion studies identified the domain responsible for the inhibitory action of OUT in its bHLH and C-terminal regions. Moreover, terminal differentiation of C2C12 myoblasts was inhibited by exogenous introduction of OUT. These inhibitory functions of OUT closely resemble those of the helix-loop-helix inhibitor Id proteins. Based on these findings, we propose that this novel protein functions as a negative regulator of bHLH factors through the formation of a functionally inactive heterodimeric complex.

Transcription factors with a basic helix-loop-helix (bHLH) motif have been demonstrated to play critical roles in cell fate determination and differentiation in a variety of tissues of both vertebrates and invertebrates (1, 2). Examples include myogenic bHLH factors such as MyoD and myogenin in skeletal muscle development (1–4), SCL/TAL1 in hematopoiesis (5, 6), and neuronal factors such as Mash1 and neurogenin in neurogenesis (7–11). The bHLH motif consists of a short region rich in basic amino acids and two amphipathic helices separated by an intervening loop region (12). The bHLH proteins form homo- or heterodimers through the helix-loop-helix (HLH) domains, enabling the basic regions to form a bipartite DNA-binding motif that recognizes so-called E-box sequences, CANNTG, commonly found in the promoter or enhancer regions of numerous developmentally regulated genes (12).

Typically, tissues-specific class B bHLH factors, such as MyoD and neurogenin, dimerize with ubiquitously expressed class A bHLH factors and promote cell fate determination and differentiation into specific lineages (12). Class A bHLH factors are exemplified by so-called E proteins, such as E2A gene products E12 and E47.

Cell differentiation is a complex and well-organized process in which cells respond to stimuli from the environment by carrying out a genetic program. It has been shown that bHLH factors directly or indirectly regulate expression in a gene activation network. The best studied system is skeletal muscle development. Four myogenic bHLH factors, MyoD, Myf-5, myogenin, and MRF4, participate in the development of mammalian skeletal muscles (1, 2). Although all of them can induce skeletal muscle differentiation in a wide variety of non-muscle cell types (13–17), expression analyses (14, 18) and gene-targeting experiments indicate differences in their positions in the genetic network for myogenesis (3, 4, 19–25). MyoD and Myf-5 play redundant roles in establishing myoblast identity of mesodermal progenitors (19–21). Subsequently, myogenin promotes differentiation of myoblasts to myotubes and their maturation (3, 4, 23). MRF4 functions during the differentiation process of myoblasts, together with MyoD, as well as in the terminal stage (25). Combinatorial orchestration of growth factors and other transcription factors such as MEF2 is also involved in this gene activation network, leading ultimately to muscle development (1, 26, 27). Similar cascades of bHLH factors have been also demonstrated in neurogenesis (9).

In addition to these genetic networks of “positive” bHLH factors, “negative” HLH or bHLH factors enable the proper execution of the cell differentiation control through functional modulation of bHLH factors (11, 28). The Id proteins, inhibitors of DNA binding/differentiation, are negative regulators of bHLH factors (28, 29). They possess HLH domains and heterodimerize with bHLH factors, but, due to a lack of the basic region, the resultant heterodimers have no DNA binding activity. As a consequence, cell differentiation is inhibited. Four Id proteins, Id1–Id4, are expressed in a wide range of embryonic tissues and are believed to be involved in the expansion of...
immature cell populations (28–32). The HES proteins are repressive bHLH factors mainly expressed in the developing nervous system (11, 33, 34). A homodimer of HES binds to the E-box-related N-box sequence and actively represses transcription by recruiting a co-repressor through the WRPW domain present in the C terminus (11, 35–37). HES, like Id, also sequesters bHLH factors (34). ABE-13/83/ musclein (39)/MyoR (40), which is expressed in activated B lymphocytes and muscle precursors, binds to the E-box sequence but does not activate transcription. Instead, it represses E-box-mediated transactivation by competing for binding sites with positive bHLH factors and through a transcriptional repressive domain. Mist1 (41), Twist (42), and Stra13 (43) are also repressive bHLH factors with multiple inhibitory mechanisms. Among them, Stra13 is an exception, because it possesses no DNA binding activity. The importance of the negative regulation of bHLH factors in cell differentiation has been emphasized by loss-of-function mutants in Drosophila and mice. For example, Drosophila mutants defective in orthologues of Id and HES, ene (44) and hairy (45), respectively, show developmental defects in the formation of sensory hairs. Mice lacking Id2 show loss of lymph node and Peyer’s patch development and a defect in development of natural killer cells (46). HES-1-deficient mice demonstrate a defect in neural tube closure and microphthalmia due to premature differentiation of neurons (47, 48). Moreover, inactivation of twist results in defective dorso-ventral patterning due to disturbed gastrulation in Drosophila (49, 50) and defects in cranial neural tube closure and mesodermal derivatives in mice (51).

In this study, we identified a novel bHLH factor, OUT, using PCR with degenerate primers. OUT is expressed mainly in the adult mouse reproductive organs and is barely detectable in the developing mouse embryo. In gel shift and oligonucleotide selection assays, OUT failed to bind DNA. In the presence of OUT, E12 and MyoD were prevented from homo- and heterodimer formation and failed to induce E-box-mediated transactivation. By deletion analyses, the bHLH and C-terminal domain(s) were identified as important domains for the inhibitory action of the OUT protein. Furthermore, introduction of OUT in C2C12 myoblasts hampered their terminal differentiation. These functional characteristics indicated that OUT possesses an inhibitory activity similar to that of Id.

**EXPERIMENTAL PROCEDURES**

**RNA Purification**—Total RNA was extracted from organs of ICR mice using the acid guanidinium thiocyanate/phenol/chloroform extraction procedure (52). Poly(A)^+ RNA was isolated using oligo(dT) latex (Oligotex^®^dT30(SUPER), Takara, Otsu, Japan) according to the manufacturer’s recommendations.

**cDNA Cloning and 5’-RACE**—The putative bHLH domain of a novel HLF factor was obtained by the reverse transcription-PCR (RT-PCR) method using the following two degenerate primers: MESO-S CCAA-AC/gaTTGAG(A/C/G/T)GCCACAAGCTACATTGCCACCTC and TCATTTGTTACCAAAAGC-TGGAGA (corresponding to cDNA nt 460–483 and 709–732, respectively). The coding region of OUT together with 20 nucleotides of 5’-untranslated sequence was amplified by PCR with pBS-mOUT as a template. The resultant PCR product containing a BamHI site at the 5’ end was subcloned into XcmI-digested pKRX. After confirming the sequence, the BamHI-EcoRI fragment was subcloned into the BamHI-EcoRI sites of pcMV to generate pcMV-OUT. Other expression vectors of human E47 (pCMV/SV2-E47), of murine E47, a gift from Yoichi Kageyama, Kyoto University, Japan, mouse E12 (gift from Eiji Hara), mouse Id2 (pRCM-ID2) (57), mouse MyoD (pCMV-MyoD; a gift from Eiji Hara), mouse myogenin (pBS-myogenin; a gift from Shosei Yoshida, Kyoto University), mouse Mash2 (pBS-Mash2; a gift from François Guillemot, IGBMC, Strasbourg, France), and mouse TAL2 (pMTAL2) (56) were constructed by subcloning the cDNA from each plasmid into pcMV with appropriate restriction enzymes, generating pcMV-E47, pcMV-E12, pcMV-Id2, pCMV-MyoD, pCMV-myogenin, pCMV-Mash2, and pCMV-TAL2, respectively.

For co-immunoprecipitation assays, expression vectors tagged with 6 repeats of Myc epitope (EQKLISEEDLNE) were constructed using pcMV-6Myc(N), which was generated with the BamHI-XbaI fragment from pcBS2 + MT (a gift from Kunihiro Tsushida, Tokushima University) containing the 6Myc epitope sequence inserted into a BamHI-XbaI-treated pcMV vector. The PCR-amplified OUT coding region (corresponding to OUT cDNA nt 103–729) bearing a KpnI site at the 5’ end and a BamH I site at the 3’ end was ligated in-frame into Kpn1-BamHI-treated pcMV-6Myc(N), generating pcMV-OUT(6Myc). For the expression of the bHLH-deleted mutant of OUT, two PCR-amplified fragments corresponding to OUT cDNA nt 103–321 and nt 493–729 carrying KpnI and SphI/BamHI sites, respectively, were ligated together into Kpn1-BamHI-treated pcMV-6Myc(N), generating pcMV-OUT△bHLH/6Myc. pcMV-Id2/6Myc and pcMV-Id2△HHLH/6Myc (corresponding to Id-2 cDNA nt 62–178 and nt 305–478) were similarly constructed.

For CASTing assays, the OUT expression vector tagged with the FLAG epitope (MYDDDDDDDD) was generated by subcloning the coding

**Northern Blot and RT-PCR Analyses**—Twenty µg of poly(A)^+ RNA of adult mouse organs was separated by electrophoresis on a 1.0% agarose-formaldehyde gel, transferred onto filters, and cross-linked in a UV chamber. A radioactive DNA probe for OUT was prepared by random-primed labeling of a 1.4-kb PstI-XbaI fragment of the OUT cDNA (nt 398–1791). Hybridization and washing were performed under high stringency conditions as described previously (56). The full-length glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was adopted as a probe for an internal loading control. X-ray films were exposed with an intensifying screen at −80°C for 72 h for OUT and 12 h for GAPDH.

For RT-PCR analyses, 5 µg of total RNA from various adult mouse organs was reverse-transcribed with random hexamers (Takara) in a total volume of 20 µl using a manufacturer’s protocol. One µl of the product was subjected to PCR amplification using the following two primers: GCCACAAGCTACATTGCCACCTC and TCATTTGTTACCAAAAGCTGGAGA (corresponding to cDNA nt 460–483 and 709–732, respectively). For an internal control, two primers corresponding to the β-actin gene were utilized.

In *Situ* Hybridization—In *situ* hybridization was performed with paraffin-embedded sections of the uterus and ovary at 7 d.p.c. essentially as described previously (56). 35S-Labeled antisense and sense riboprobes were prepared by *in vitro* transcription with suitable RNA polymerases following linearization of pcMV-OUT (see below) with appropriate restriction enzymes. The probe spanned nt 103–729 of the OUT cDNA. The samples were hybridized and washed at high stringency and autoradiographed with the emulsion of NTB2 (Eastman Kodak Co.).

**Plasmid Constructions**—For protein expression, a cytomegalovirus promoter-driven vector, pcMV (a gift from Eiji Hara, Paterson Institute for Cancer Research, Manchester, UK), was used. The coding region of OUT together with 20 nucleotides of 5’-untranslated sequence was amplified by PCR with pBS-mOUT as a template. The resultant PCR product containing a BamHI site at the 5’ end was subcloned into XcmI-digested pKRX. After confirming the sequence, the BamHI-EcoRI fragment was subcloned into the BamHI-EcoRI sites of pcMV to generate pcMV-OUT. Other expression vectors of human E47 (pCMV/SV2-E47), of murine E47, a gift from Yoichio Kageyama, Kyoto University, Japan, mouse E12 (a gift from Eiji Hara), mouse Id2 (pRCM-ID2) (57), mouse MyoD (pCMV-MyoD; a gift from Eiji Hara), mouse myogenin (pBS-myogenin; a gift from Shosei Yoshida, Kyoto University), mouse Mash2 (pBS-Mash2; a gift from François Guillemot, IGBMC, Strasbourg, France), and mouse TAL2 (pmTAL2) (56) were constructed by subcloning the cDNA from each plasmid into pcMV with appropriate restriction enzymes, generating pcMV-E47, pcMV-E12, pcMV-Id2, pCMV-MyoD, pCMV-myogenin, pCMV-Mash2, and pCMV-TAL2, respectively.

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region of OUT (nt 103–732) downstream of the FLAG epitope sequence in pCMV-FLAG-2 vector (Sigma). For use in *in vitro* translation experiment, the FLAG-OUT fragment was transferred to pBluescript using the ScaI-BamHI site, generating pBS-FLAG/OUT. Similarly, pBS-FLAG/MyoD was generated from pCMV-FLAG-2 MyoD (a gift from Shinya Yoshida) digested with EcoRI and XhoI. To generate deletion mutants of OUT, fragments corresponding to amino acid sequences indicated in Fig. 7A were obtained by PCR. The sense and antisense primers contained KpnI and XbaI sites in their 5′ ends, respectively. For deletion of the N-terminal portion, the sequence spanning nt 283–306 was included in respective sense primers to equalize the translation efficiency. For deletion of the C-terminal stop codon was included in antisense primers. Amplified fragments were digested with KpnI and XbaI and inserted into the KpnI-XbaI site of the pCMV vector.

For GAL4 binding assays, various PCR-amplified OUT fragments corresponding to the amino acid sequences indicated in Fig. 7B were ligated in-frame downstream of the GALA DNA-binding domain (DBD), amino acids 1–147 in pEF-GAL4-DBD (a GAL4 DBD expression vector driven by the human elongation factor 1 promoter) (58) using appropriate restriction sites. As positive controls, we utilized pEF-BOS bsr/ GAL4 KRAZI and pEF-BOS bs/ GAL4 KOXI (59).

The authenticity of plasmids constructed by PCR was verified by sequencing.

**Cell Cultures and DNA Transfections**—NIH3T3 fibroblast and C2C12 myoblast cell lines were purchased from American Tissue Culture Collection and provided by Shosie Yoshida (Kyoto University), respectively. They were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) plus 100 units/ml penicillin and 100 mg/ml streptomycin. Dishes coated with type I collagen (IWAKI GLASS, Japan) were used to culture C2C12 cells. For the transient transfections, NIH3T3 and C2C12 cells were plated at densities of 5 × 10^5 cells/25-mm well and 5 × 10^6 cells/35-mm well, respectively, in DMEM supplemented with 10% FCS 24 h before transfection. Transfections were performed by the lipofection method using TransIT-LT1™ (Pan Vera Corp.) according to the manufacturer's instructions. The total amount of DNA added to cells was adjusted to 1.2 μg/25-mm well and 2.0 μg/35-mm well by addition of appropriate empty vector.

**Electrophoretic Mobility Shift Assays**—Electrophoretic mobility shift assays (EMSA) were performed essentially as described previously (60). Oligonucleotides containing an E-box from 52E (61), MCK-R (62), MLCA (62), MLCB (62), CE-2 (63), HEN1 consensus sequence (64), TNi E-box (41), EF1 (65), 6701 (66), or RIP3E (67) and an oligonucleotide containing an N-box from the HES-1 promoter (34) were annealed and end-labeled with [γ-32P]dCTP using the Klenow fragment of Escherichia coli DNA polymerase I. The core sequences within these oligonucleotides were CAGCGT (52E), CACCTG (MCK-R, MLCA and MLCB), CAGCTG (MLCB, HEN1 consensus sequence, CE-2 and TNi E-box), CAGATG (EF1 and 6701), CATCTG (RIPE3), and CACGAG/ MLCA (62), MLCB (62), MLCC (62), CE-2 (63), HEN1 consensus sequence, HES-1 promoter). For the competition assays, we designed a mutant of each N-box in which each core sequence CANNTG was converted to ACNNGT. For the mutant N-box, CAGCGG and CCAAG were replaced by CCGCGG and CCGGG, respectively. The oligonucleotides were 22–26 mers. In *in vitro* transcriptions containing the 5′-7mmGppG cap (New England BioLabs Inc.) were prepared from the linearized plasmid templates using appropriate RNA polymerase. Transcripts were then translated into proteins in *vitro* using a rabbit reticulocyte lysate system (Promega) according to the manufacturer's instructions.

Each protein involved in the DNA-protein complex was identified in supershift assays using anti-E12 antibody (Santa Cruz Biotechnology) and anti-MyoD antibody (PharMingen). The sequence specificity was evaluated by supershift assays using anti-E12 antibody (Santa Cruz Biotechnology) and anti-MyoD antibody (PharMingen). The sequence specificity was evaluated by supershift assays using anti-E12 antibody (Santa Cruz Biotechnology) and anti-MyoD antibody (PharMingen). The sequence specificity was evaluated by supershift assays using anti-E12 antibody (Santa Cruz Biotechnology) and anti-MyoD antibody (PharMingen). The sequence specificity was evaluated by supershift assays using anti-E12 antibody (Santa Cruz Biotechnology) and anti-MyoD antibody (PharMingen). The sequence specificity was evaluated by supershift assays using anti-E12 antibody (Santa Cruz Biotechnology) and anti-MyoD antibody (PharMingen).

**Luciferase Assays**—As reporter plasmids, we utilized pE7-α-luc (70) for E-box-mediated luciferase assays and tk-GALpl3-LUC or tk-LUC (58) for GALA binding assays. The CMV promoter-driven sea-pansy luciferase plasmid, pRL-CMV (Promega), was used as an internal control to normalize firefly luciferase activity. NIH3T3 fibroblasts plated at 1.2 × 10^5 cells/35-mm well were transiently co-transfected with each expression vector together with 70 fmol of reporter plasmid and 7 fmol of pRL-CMV per 25-mm well. Before transfection, the total amount of DNA per well was adjusted to 1.2 μg by addition of the pCMV empty vector or pEF BOS empty vector. After 48 h of incubation in DMEM with 10% FCS, the cells were lysed, and the luciferase activities were measured using the Dual-Luciferase20™ reporter assay system (Promega) according to the manufacturer's instructions with a Lumat LB 9507 (EG & G Berthold) luminometer. The firefly luciferase activity was corrected by the CMV promoter-driven sea-pansy luciferase activity.

**Differentiation of C2C12 Myoblasts**—The myoblast differentiation assays were performed as described previously (71). C2C12 myoblasts (clinical isolate) above were transiently transfected with 0.5 pmol of each expression vector together with 0.25 pmol of pNLS/lacZ per 35-mm well. The plasmid of pNLS/lacZ (a gift from Nobutake Akiyama, Kyoto University) encodes *E. coli* β-galactosidase with a nuclear localization signal (NLS). The total amount of DNA added to C2C12 cells was adjusted to 2.0 μg by addition of empty pCMV vector. After induction of differentiation in DMEM with 2% horse serum for 96 h, cells were fixed in 10% formaldehyde and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) and then with anti-troponin T (TnT) antibody (Sigma). Differentiation was evaluated by counting the number of TnT-positive cells relative to that of β-galactosidase-positive cells.

**Nucleotide Sequence Accession Number**—The nucleotide sequence of OUT was deposited in the GenBank™ data base with the accession number AF142405.

**RESULTS**—Isolation of a Novel bHLH Factor, OUT—In an effort to identify novel bHLH factors, we performed RT-PCR analyses...
using the total RNA of the mammary gland of pregnant mice at 14 d.p.c. as the source. The bHLH transcription factors comprise a very large family, and the amino acid sequences of the bHLH regions are not highly conserved. We therefore designed several degenerate primer sets targeting the conserved sequences within various bHLH subfamilies. By using primers MESO-S and MESO-AS, which were designed based on the sequences of the mesodermally expressed bHLH proteins paraxis (53) and scleraxis (54), we obtained PCR products with an appropriate size comparable to that of the bHLH region. Sequencing of these products revealed a novel bHLH sequence consisting of 144 nucleotides including the primer sequences at both ends.

Since a preliminary Northern blot analysis using this fragment as a probe revealed a transcript in the adult mouse ovary, we next constructed a mouse ovary cDNA library and screened it with the 144-bp fragment as a probe. Two positive clones were identified among approximately 1 \( \times 10^6 \) independent phage clones. Restriction enzyme and sequence analyses indicated that these clones were overlapping. The longer clone, bearing a 4.1-kb cDNA insert, was used for further analyses.

Nucleotide sequence analysis revealed that this clone contained a 4100-bp cDNA with a single open reading frame of 630 bp (Fig. 1). The novelty of the gene was confirmed by homology searches against databases. Two possible initiation codons were found, and both of them closely matched the Kozak consensus sequence (72). As shown below, the size of the cDNA insert was approximately 100 bp shorter than that of the transcript detected by Northern blot analysis. To obtain information about the 5'-terminal region of the mRNA, we performed 5'-RACE using two specific primers designed to hybridize near the 5'-end of the cDNA and isolated fragments containing an additional 108-bp sequence (4 and 2 clones contained 107- and 108-bp inserts, respectively). This region contained 4 termination codons in the same reading frame (data not shown), demonstrating that translation is not initiated at a site upstream of those noted above. The cDNA sequence thus predicted 2 species of proteins consisting of 210 and 200 amino acids with calculated molecular masses of 22.9 and 21.9 kDa, respectively, depending on the translation initiation site (Fig. 1).

In vitro translated products of this gene migrated on SDS-PAGE with apparent molecular weights consistent with the calculated masses of the two proteins (Fig. 4, lanes 7–10 in the lower panel). Whether these isoforms have functional differences remains to be determined.

We designated this novel factor as OUT, on the basis of the main organs that express this gene, the ovary, uterus, and testis, as shown below.

OUT Is Related to Mesodermal bHLH Factors—Data base searching and motif analysis identified a bHLH motif spanning 56 amino acid residues in the middle of the OUT protein (amino acid residues 75 to 130) (Fig. 1), which closely conformed to the...
Fig. 2. Amino acid sequence alignment of the bHLH regions of OUT and related bHLH factors. The conserved amino acids are shown as white letters on black. The percent identity of each protein with OUT within the bHLH motif is shown on the right. The ranges of the basic, helix1, loop, and helix2 are indicated by arrows above. One proline residue and three arginine residues in the basic region of OUT are indicated by closed and open triangles, respectively. The sequence sources of the related bHLH factors are capsulin (74, 75), ABF-1 (38), paraxis (53), scleraxis (54), dHAND, eHAND (79, 80), myogenin (14), neurogenin (7), Mist1 (78), Twist (88), Stra13 (43), HES-1 (89), and HES-2 (90). The consensus sequence of the bHLH region was derived from Murre et al. (73). ψ in the consensus sequence indicates hydrophobic amino acids. Below the consensus sequence, negative HLH factors Id1-Id4 (28) are aligned.

consensus sequence of the family of bHLH factors and displayed a high percentage of amino acid sequence identity to the sequences of other members within the bHLH region (73) (Fig. 2). Among them, capsulin (74, 75)/epicardin (76)/Pod-1 (77) and ABF-1 (38)/musculin (39)/MyoR (40) are most related to OUT (55.4% identity). Besides these, paraxis (53) (44.6%), Mist1 (78) (44.6%), scleraxis (54) (42.9%), and dHAND and eHAND (79, 80) (42.9%) show a relatively high degree of sequence identity with OUT as shown in Fig. 2. In the basic region of OUT, there are only a few basic amino acids, i.e. three arginine residues, although it preserves the motif of EXXR, which is a determinant of E-box recognition (81, 82). In addition, an arginine residue positioned at the first consensus residue of the bHLH family is replaced by a serine residue in OUT. Of note, the basic region of OUT possesses one proline residue, as do the basic regions of repressive bHLH factors such as HES (33, 34) and Stra13 (43), although the positions of the proline residues are not the same among them. A proline residue is also found in the corresponding region of the Id proteins (29–32). The remainder of the sequence of OUT shows no apparent similarity to any previously described proteins or motifs. The nuclear localization of the OUT protein was verified by using a fusion protein between OUT and the green fluorescent protein (data not shown).

OUT Is Expressed in the Adult Reproductive Organs—To determine the expression pattern of OUT in the adult mouse tissues, Northern blot experiments were performed (Fig. 3A). Twenty µg of poly(A)⁺ RNA from various adult mouse tissues were probed with a 1.4-kb radiolabeled fragment. This probe was designed to contain the 3’-half of the coding region and the following 1.0-kb 3’-untranslated region and to cover one of the putative splicing sites. As shown in Fig. 3A, a single transcript was detected, and its size was estimated to be 4.2 kb. This was about 100 bp longer than the cDNA isolated from the ovary cDNA library. The expression level was highest in the uterus, ovary, and testis, in that order. Faint expression was also noted in the lung, heart, intestine, and spleen.

We also analyzed the OUT expression by RT-PCR using the total RNA from the same set of tissues (Fig. 3B). The primer set was designed to cover one of the putative splicing sites and to give an expected product of 273 bp. Overall, the expression pattern obtained was identical to that seen with Northern blotting. With this method, in addition to the organs in which OUT was detected by Northern blot analysis, faint expression of OUT was detected in virtually all samples analyzed, including the mammary glands from which OUT was initially identified. No apparent fragment of any other size was present.

The reproductive organs are under the influence of hormone action, and the uterus, in particular, shows functional and morphological changes during pregnancy and delivery. To obtain more clues about OUT functions in vivo, we further analyzed the expression in the adult uterus according to the estrus cycle and gestational stages (Fig. 3C). OUT expression in the uterus was higher in the diestrus phase than in the estrus phase and reached a maximum at 7.5 d.p.c., thereafter declining toward the time of delivery. The level of OUT transcripts returned toward the non-pregnant level 4 days after delivery.

To identify the cell types that express OUT in the uterus of the pregnant mouse, we next performed RNA in situ hybridization using an 35S-labeled riboprobe (Fig. 3D). On the sections of the 7.5 d.p.c. uterus hybridized with the antisense OUT riboprobe, OUT expression was detected as double streaks that corresponded to the two layers of myometrium, the inner circular and the outer longitudinal muscle layers (83). On the serial section hybridized with the sense OUT riboprobe, no apparent signal was detected. As compared with control images hybridized with sense riboprobe, a faint signal also appeared to be present in the endometrium.

In contrast to the results in the adult, no detectable signal was observed in the developing embryos by Northern blot analysis using RNA from 7.5, 10.5, 11.5, 14.5, and 18.5 d.p.c. embryos and by whole mount in situ hybridization of 7.5, 8.5, and 9.5 d.p.c. embryos (data not shown). Furthermore, no OUT expression was detected in the uterus before puberty (data not shown).

OUT Does Not Bind DNA but Rather Inhibits DNA Binding of Other bHLH Proteins—Considering the deduced primary structure, the OUT protein was expected to be a transcription factor with a bHLH motif and to possess DNA binding activity specific for the E-box, which is a common target of the bHLH transcription factors (12). To test this, we performed electrophoretic mobility shift assays (EMSA) using 32P-labeled EF1 oligonucleotide bearing the core sequence of CAGATG, one of the well known E-boxes (65). The proteins used were prepared by in vitro transcription of the template cDNA followed by in vitro translation in rabbit reticulocyte lysates. As shown in Fig. 4A, contrary to our expectations, OUT had no binding activity to the EF1 sequence either as homodimers (lane 3) or as heterodimers in the presence of E12 (lane 4), whereas E12 and MyoD homodimers and MyoD-E12 heterodimers exhibited ob-
appropriate amounts of the respective proteins in each lane inhibitory action of OUT using the co-immunoprecipitation method. The results shown above suggested that the inhibitory effect of OUT is similar to that of Id. The main mechanism by which Id inhibits bHLH factors is to quench the activity of the E proteins. We therefore next explored protein-protein interaction between OUT and E12 using the co-immunoprecipitation method. Two kinds of Myc-tagged OUT expression vectors (a full-length and a mutant that lacks the bHLH region) were

![Diagram](image_url)

**FIG. 3. Expression of the OUT mRNA.** **A,** Northern blot analysis of adult tissues. Twenty µg of poly(A)⁺ RNA derived from various adult mouse tissues were hybridized with a radiolabeled OUT probe (**upper panel**). Mammary glands were from pregnant mice at 14 d.p.c. The size of the OUT cDNA was estimated to be approximately 4.2 kb without any apparent alternative splicing product. The filter was re-probed with the full-length GAPDH cDNA to correct for differences in the amount of RNA loaded (**lower panel**). **B,** RT-PCR analysis. Total RNA from the same set of tissues as in A was subjected to RT-PCR analysis using specific primers within the OUT cDNA. The expected OUT product (273 bp) (**upper panel**) and β-actin product for the internal control are shown (**lower panel**). The order of the tissues in the lanes is the same as in A. **C,** Northern blot analysis of OUT expression in the uterus under different physiological conditions. The diestrus and estrus phases of the estrus cycle are indicated, as well as d.p.c. P1, P4, and P7 are 1, 4, and 7 days postpartum. The filter was re-probed with the full-length GAPDH cDNA as a loading control (**lower panel**). **D,** in situ hybridization analysis. Sections of the uterus at 7.5 d.p.c. were hybridized with 35S-labeled antisense or sense OUT riboprobe. A bright-field image (**upper panel**), a dark-field image hybridized with antisense OUT riboprobe on the same section (**middle panel**), and a dark-field image hybridized with sense OUT riboprobe on a serial section (**lower panel**). Lu, the lumen of the uterus; E, the endometrium; C, the inner circular muscle layer; L, the outer longitudinal muscle layer; P, the perimetrium.

Various binding activities under the same conditions (**lanes 2, 5**, and 6). Moreover, OUT inhibited the DNA binding of E12 and MyoD homodimers (**lanes 4 and 7**) and E12-MyoD heterodimers (**lane 8**). Similar results were obtained with 10 other oligonucleotides containing different E-box sequences that have been reported so far (data not shown, see under “Experimental Procedures”). In addition, there was no evident binding of OUT to an N-box, with which HES proteins, repressive bHLH factors, preferentially interact (data not shown, see under “Experimental Procedures”). Thus, we could not detect any DNA binding activity of OUT, but instead we found that it inhibited the DNA binding of other bHLH factors.

These functional features of OUT are reminiscent of those of the Id proteins, which lack the basic region but possess the HLH region (28, 29). Id proteins form inactive heterodimers with bHLH factors and negatively regulate their function. Therefore, we next investigated the dose dependence of the inhibitory action of OUT using the eE2 oligonucleotide, containing the core sequence of CAGGTG (61), in comparison with the inhibitory action of Id. As shown in Fig. 4B, the DNA binding activities of the E12-MyoD heterodimers and MyoD homodimers were attenuated, in a manner dependent on the dose of OUT protein added in the reaction mixtures (**lanes 2 and 7–10**). Meanwhile, Id2, one of the Id proteins (31), showed an activity similar to but stronger than that of OUT (**lanes 2–6**). Appropriate amounts of the respective proteins in each reaction were confirmed by SDS-PAGE of 35S-labeled proteins as shown in the lower panel of Fig. 4B.

Cyclic amplification and selection of targets, CASTing, was next performed to identify OUT-binding DNA sequences, which might be different from the E-box or the N-box. FLAG epitope-tagged OUT was co-translated with E12 in vitro. For a positive control, FLAG epitope-fused MyoD was used and similarly co-translated with E12. After incubation of in vitro translation product with double-stranded degenerate oligonucleotides, the mixture was precipitated with an anti-FLAG antibody, and the bound DNA was subjected to amplification by PCR. After six rounds of CASTing with the positive control, the bound DNA was detected by gel electrophoresis and subcloned into pBlue-script. Sequence analyses indicated that all of 22 clones examined contained the E-box sequences, CANNTG (data not shown). However, no obvious DNA fragment was obtained from the tagged OUT-E12 complex, supporting the idea that OUT has no DNA binding activity (data not shown).

**OUT Interacts Physically with Class A bHLH Factor E12—** The results shown above suggested that the inhibitory effect of OUT is similar to that of Id. The main mechanism by which Id inhibits bHLH factors is to quench the activity of the E proteins. We therefore next explored protein-protein interaction between OUT and E12 using the co-immunoprecipitation method. Two kinds of Myc-tagged OUT expression vectors (a full-length and a mutant that lacks the bHLH region) were
construted and transfected into COS-7 cells together with pCMV-E12. As controls, Myc-tagged Id2 expression vectors (a full-length and a mutant lacking the HLH region of Id2) were prepared and analyzed. Nuclear extracts were subjected to the immunoprecipitation with anti-Myc antibody, and the precipitates were separated by SDS-PAGE and probed with anti-Myc or anti-E12 antibodies. The results are shown in Fig. 5. As anticipated, E12 was co-immunoprecipitated together with the Myc-tagged Id2 by anti-Myc antibody (Fig. 5, lane 2) but not with the HLH-deleted mutant Id2 (Fig. 5, lane 5). Similarly, an association of OUT with E12 was detected (Fig. 5, lane 8). E12 was not detected in the precipitate of the nuclear extract of cells transfected with the cDNA of Id2 and mutant OUT lacking the bHLH region (Fig. 5, lane 11). The specificity of the immunoprecipitation was confirmed with an isotype-matched nonspecific mouse IgG1 (lanes 3, 6, 9, and 12). These results indicate that the OUT protein forms a complex with the E12 protein by physical interaction through the bHLH domain. The smaller species of OUT products observed in vitro translation (Fig. 4B, lower panel) was barely detectable in the expression system with COS-7 cells.

**OUT Inhibits Transactivation Induced by bHLH Factors**—To evaluate the effect of OUT on E-box-mediated transactivation, luciferase assays were performed using NIH3T3 cells (Fig. 6). pCMV vectors expressing OUT, E12, MyoD, and Id2 were co-transfected with a reporter plasmid in various combinations indicated in Fig. 6. As anticipated, OUT failed to induce the transactivation over the basal level (lanes 2, 5, and 7). Next, the inhibitory effect of OUT on E12-MyoD-induced transactivation was studied at varying molar ratios and compared with the inhibitory effect of Id2. In the presence of Id2, E12-MyoD-mediated luciferase activity was greatly reduced in a dose-dependent manner (lanes 6 and 8–12). The reduction was about 50% even at the molar ratio of 0.125:1 (lanes 6 and 8) and about 80% at the molar ratio of 1:1 (lanes 6 and 11). OUT showed a similar effect, but the reduction was smaller at the same molar ratio; the reductions were about 50 and 70% at the molar ratios of 1:1 and 2:1, respectively (lanes 6 and 13–17). In addition, luciferase activity could be restored as the molar ratio of E12 and MyoD to OUT increased (data not shown).

To exclude the possibility that the inhibitory effect observed with OUT was the result of the overexpression of exogenous genes in NIH3T3 cells, we overexpressed the neural bHLH TAL2 (56) and placental bHLH Mash2 (84) in the same context, instead of OUT. As shown in Fig. 6A, although a 10–20% reduction was caused by TAL2 at a 1:2 ratio (lane 16), no major inhibition of luciferase expression was caused by either TAL2 or Mash2 in the luciferase expression, excluding the above possibility.

These results demonstrated that the inhibitory effect of OUT on the E-box-mediated transactivation by bHLH factors was in accordance with the effects seen in the EMSA.

**Delineation of the Functional Domain of OUT**—To determine the region responsible for the inhibitory activity of OUT, various deletion mutants were constructed, as indicated in Fig. 7A. Each vector was co-transfected into NIH3T3 cells with a molar equivalent of pCMV-E12, pCMV-MyoD, and pE7-luc, and the luciferase activity was evaluated. Since mutants lacking the bHLH region exhibited no inhibitory activity (Fig. 7A, rows 5, 11, and 12), the bHLH region was suggested to be essential for the function of OUT. However, the bHLH region alone did not reduce the induction of luciferase activity by E12-MyoD heterodimers (Fig. 7A, row 7). Additionally, inclusion of the whole N-terminal portion caused only a marginal inhibition (Fig. 7A, row 6). To investigate the effect of the C-terminal portion of the protein, we subsequently prepared the constructs consisting of the bHLH region and various parts of the C-terminal portion. These mutants showed inhibitory activity proportional to the length of their C-terminal regions (Fig. 7A, rows 7–10). The results suggested that both the bHLH region and the C-terminal portion are essential for the inhibitory function of OUT. From the structural features of bHLH proteins (1, 2), it is most plausible that the bHLH region is the main functional domain that interacts with dimerization partners. On the other hand, the C-terminal portion of the protein is probably required to facilitate dimerization or to stabilize already formed heterodimers, as demonstrated for Id3 (85).

It has been reported that Stra13, an inhibitory bHLH factor, has no DNA binding activity but possesses a repressor domain (43). By using the GAL4 system (58, 59), we next attempted to elucidate whether OUT has a transcriptional repressor domain. The full-length and three portions (N-terminal region, bHLH region, and C-terminal region, indicated in Fig. 7B) of OUT were fused to the GAL4 DNA-binding domain (GAL4 DBD) under the control of the human elongation factor 1α promoter (pEF-BOS). These expression vectors were transfected into NIH3T3 cells with the firefly luciferase reporter plasmid carrying five repeats of GAL4-binding sites upstream of the thymidine kinase (tk) promoter. The reporter produced a
high basal level of transcription activity. This activity was strongly suppressed by co-expression of transcriptional repressors KRAZ1 or KOX1, as reported (59), but not by the parental GAL4 DBD plasmid alone (Fig. 7B, lower panel, lanes 7–10). In this assay system, none of the OUT domains displayed an apparent repressive activity (Fig. 7B left panel, lanes 6–8), although the full-length OUT showed a slight repression (Fig. 7B left panel, lane 5). However, this repression was almost negligible as compared with the repressor activity induced by positive controls (Fig. 7B left panel, lanes 3 and 4). Moreover, similar repression was detected with the other reporter plasmid lacking the GAL4 binding site (Fig. 7B right panel, lane 5), suggesting that the slight repression induced by the full-length OUT protein was due to a nonspecific effect on the transfected...
Fig. 7. Deletion studies and evaluation of the transcriptional repressor activity of OUT. A, to delineate the functional domain of OUT, various deletion mutants of OUT were analyzed in the same E-box-mediated luciferase assay system used in Fig. 6. Schematic representations of deletion mutants are shown on the left. Id2 was used as a positive control. The results are expressed as relative luciferase activity. Error bars, S.E. B, the GAL4 binding assay was performed to identify a transcriptional repressor activity of OUT. Schematic representation of reporter plasmids.
Transcriptional repressors KRAZ1 and KOX1 were utilized for positive controls. The results are expressed as fold repression relative to GAL4 DBD almost all (97.1%) of the co-transfected. When the cells were transfected with myogenin, differentiated into muscles, respectively. To evaluate the specificity of the effect of OUT further, the neural bHLH factor Mash2 (84) was heterodimerized with Id2. As demonstrated by comparison with Id2, OUT is a novel bHLH factor with an inhibitory function similar to that of the Id proteins, although OUT does have the basic region as well as the HLH region.

OUT shows a high degree of homology to bHLH factors that are expressed in tissues of mesodermal origin. Among them, capsulin (74, 75), also known as epicardin (76) or Pod-1 (77), and ABF-1 (38), also known as musculin (39) or MyoR (40), are the most closely related bHLH factors, with 55.4% identity in the bHLH region at the amino acid level. As the identity between the bHLH regions of capsulin/epicardin/Pod-1 and ABF-1/musculin/MyoR is 96.5%, and these two factors form a subfamily within the bHLH factors. In this context, it is highly probable that OUT belongs to a new subfamily within the bHLH factors. Interestingly, ABF-1/musculin/MyoR has been reported to be a transcriptional repressor (38, 40), whereas many of the bHLH factors induce or enhance expression of their target genes through E-box elements present in the promoter or enhancer regions of downstream genes. ABF-1/musculin/MyoR binds E-boxes as a homodimer or heterodimer with the E protein but fails to induce transcription. Instead, it inhibits the transcriptional activation induced by other bHLH factors. Capsulin/epicardin/Pod-1 also binds DNA but is unable to induce the E-box-mediated transactivation, depending on the situation (74, 75). Thus, OUT is closely related to the repressive bHLH factors not only in structure but also in function. However, the mechanism by which OUT inhibits the functions of bHLH factors is different from those of these repressive bHLH factors. As demonstrated in co-immunoprecipitation experiments, OUT is able to heterodimerize with E12 through the HLH region, but the resultant heterodimeric complexes are functionally inactive, being unable to bind DNA in EMSA. By titrating out E12 and MyoD, OUT inhibits the DNA binding of the E12-MyoD heterodimer. This feature distin-

and deletion mutants of GAL4-OUT fusion proteins are indicated in the upper right corner. Numbers next to the names of the constructs correspond to the numbers of the bars in the plots below. These expression vectors were transfected into NIH3T3 cells together with the ptk-GAL4 × 3-luc reporter plasmid carrying 3 repeats of the GAL4 binding site (left panel) or ptk-luc reporter plasmid lacking a GAL4 binding site (right panel). Transcriptional repressors KRAZ1 and KOX1 were utilized for positive controls. The results are expressed as fold repression relative to GAL4 DBD alone. Error bars, S.E.
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guishes OUT from these repressive bHLH factors.

Although less closely related to OUT, the HES proteins, Mist1, Twist, and Stra13, are also repressive bHLH factors and exert an inhibitory effect at least partly via a mechanism similar to Id (35, 34, 41–43). These factors are functionally related to OUT but display repressive activities, also through alternative mechanisms. HES proteins bind weakly to the E-box sequence, and homodimers of HES prefer the N-box as a binding site (11, 34). In the main mechanism employed by HES proteins, a WRPWM motif in the C terminus recruits a co-repressor, such as Groucho or TLE, resulting in active suppression of the transcription of their downstream genes (11, 35–37). On the other hand, Mist1 and Twist repress the activities of myogenic bHLH factors by occupying specific E-box target sites and through their repressor regions, which are capable of inhibiting activators, in addition to the mechanism of titrating bHLH factors (41, 42). Twist can also inhibit transactivation by MEF2 proteins, which are transcription factors containing the MADS domain, and regulate muscle-specific genes cooperatively with myogenic bHLH factors (42), whereas MEF2 is directly activated by Twist (86). Another repressive bHLH factor is Stra13, which is structurally highly related to HES (43). Although Stra13 can form dimers well with Mash1 and poorly with E proteins, it has no DNA binding activity. It possesses an α-helix-rich domain through which it directs repression of transcription (43). On the other hand, OUT contains no obvious repressor domain and no apparent WRPWM motif, suggesting that OUT belongs to a different category from these repressive bHLH factors in terms of their inhibitory mechanisms.

The DNA binding activities of bHLH transcription factors are determined by amino acid residues that constitute the basic region. Crystallographic analyses of bHLH proteins indicate that the determinants of E-box recognition are the first glutamate and last arginine residues in the ERXR motif of Murre’s consensus sequence (81, 82). The glutamate residue, in particular, contacts cytosine and adenine bases (81, 82). The replacement of this glutamate with other amino acid residues disturbs the DNA binding activity (87). The remaining amino acid residues in the region contribute to the DNA binding of bHLH factors by interacting with the phosphodiester backbone of DNA or by defining the specificity of interactions between the central dinucleotides of the E-box sequences and bHLH factors (81, 82). OUT contains the motif ERXR in the basic region and was expected to be able to bind DNA through E-box sequences. OUT protein, however, failed to bind E-box or N-box sequences. In addition, no obviously bound DNA was recovered from the CASTing assay. What is the molecular basis for the inability of OUT to bind DNA? The one proline and relatively few basic amino acid residues in the basic region may account for this inability. Site-directed mutagenesis of the proline residue, however, indicated that its replacement with an arginine, asparagine, or glycine residue is not sufficient to restore the DNA binding activity of OUT in EMSA (data not shown). Alternatively, OUT may require an as yet unknown bHLH factor to form a functionally active heterodimer for binding to the E-box and for induction of transactivation.

The in vivo function of OUT remains to be determined at present. As OUT mRNA is barely detectable in the developing mouse embryo by Northern blot and whole mount in situ hybridization analyses, OUT appears not to be involved in organogenesis or cell differentiation during development. In the adult, however, OUT is expressed mainly in the reproductive organs, particularly in the uterus and ovary. This expression profile of OUT is distinct and contrasts with those of other bHLH factors reported so far. The other factors show embryonic expression in addition to expression in the adult organs and participate in morphogenesis and organogenesis of the developing embryo. The unique expression pattern of OUT suggests a role of OUT in relation to the reproductive organs under the regulation of sex hormones after sexual maturation, particularly in females. In support of this notion, Northern blot analyses indicate that OUT expression is maximal in early pregnancy and minimal around parturition. OUT expression recovers to non-pregnant levels 4 days after parturition. Additionally, in situ hybridization studies demonstrate that the myometrium is a predominant site of OUT expression. These results suggest that OUT is involved in the regulation or modulation of smooth muscle contraction of the uterus during pregnancy and particularly around the time of delivery. The physiological role of OUT is not clear in the ovary or other organs, including testis, mammary gland, lung, intestine, and pancreas.

The results presented here indicate that OUT has an inhibitory activity similar to those of the Id proteins, the mechanism of which distinguishes OUT from other bHLH factors reported so far. Further characterization will clarify the in vivo function of OUT and our understanding of the mechanisms underlying the functional regulation of the adult reproductive organs by bHLH factors.

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