FIGURES AND TABLES

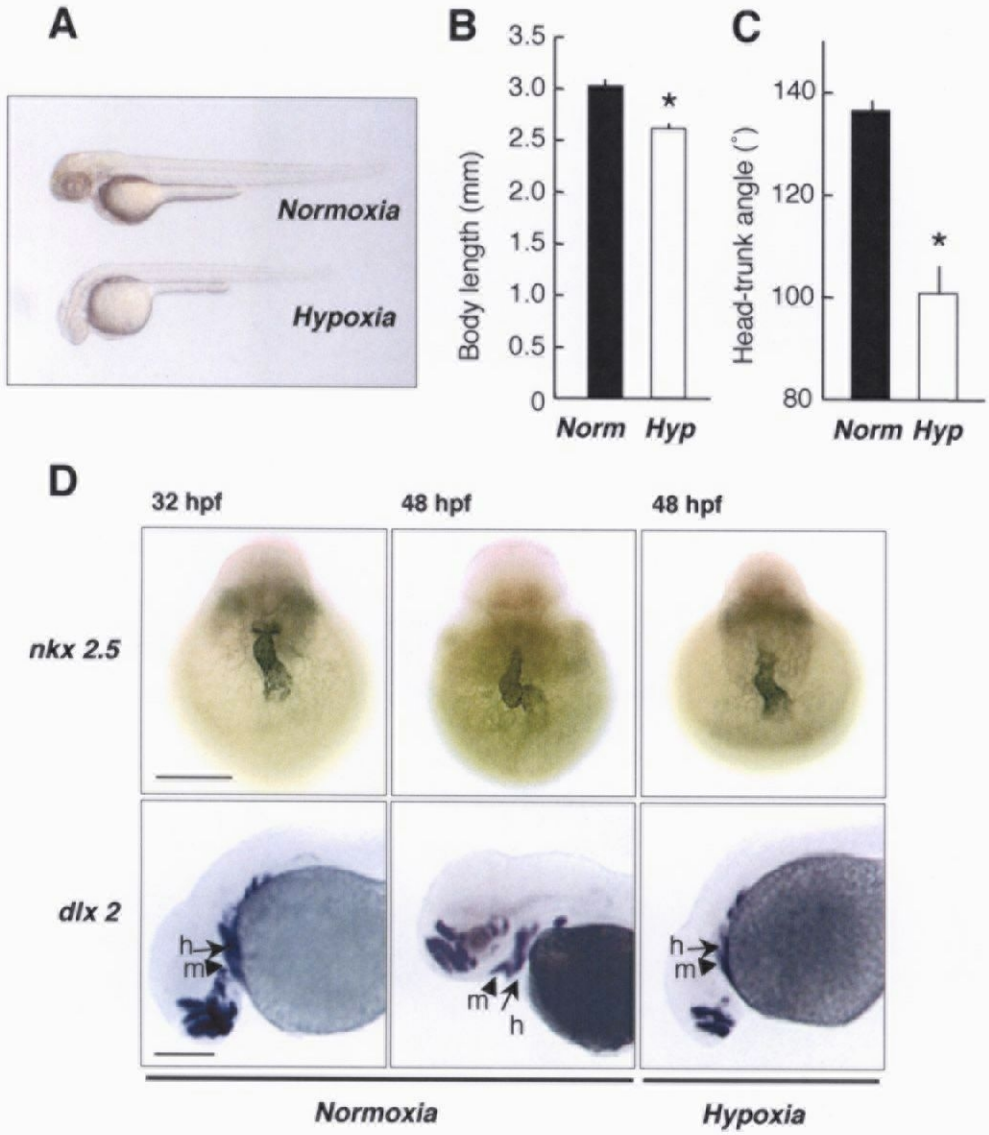


Fig. 1-1. Hypoxia causes growth retardation and developmental delay. (**A**) Morphology of wild-type zebrafish embryos at 48 hours post fertilization (hpf) after 24 h of normoxia or hypoxia treatment. (**B, C**) Effects of hypoxia on embryonic growth and development. 24 hpf embryos were transferred to water in normal (*Norm*, filled bars) or hypoxic water (*Hyp*, open bars). After 24 h, the total body length and head trunk angle were measured. Values are means ± S.E. (n = 28-36). *, $P < 0.05$. (**D**) Effects of hypoxia treatment on heart and head skeleton morphogenesis. Embryos raised in water with normal oxygen (normoxia) were fixed at 32 and 48 hpf. A subset of embryos was subjected to 24 h hypoxia exposure beginning at 24 hpf. Whole mount *in situ* hybridization was performed using a cardiac marker, *nkx 2.5* (upper panels) and a pharyngeal marker, *dlx 2* (lower panels). h, hyroid arch (arrows); m, mandibular arch (arrow heads). Scale bar = 200 μm.

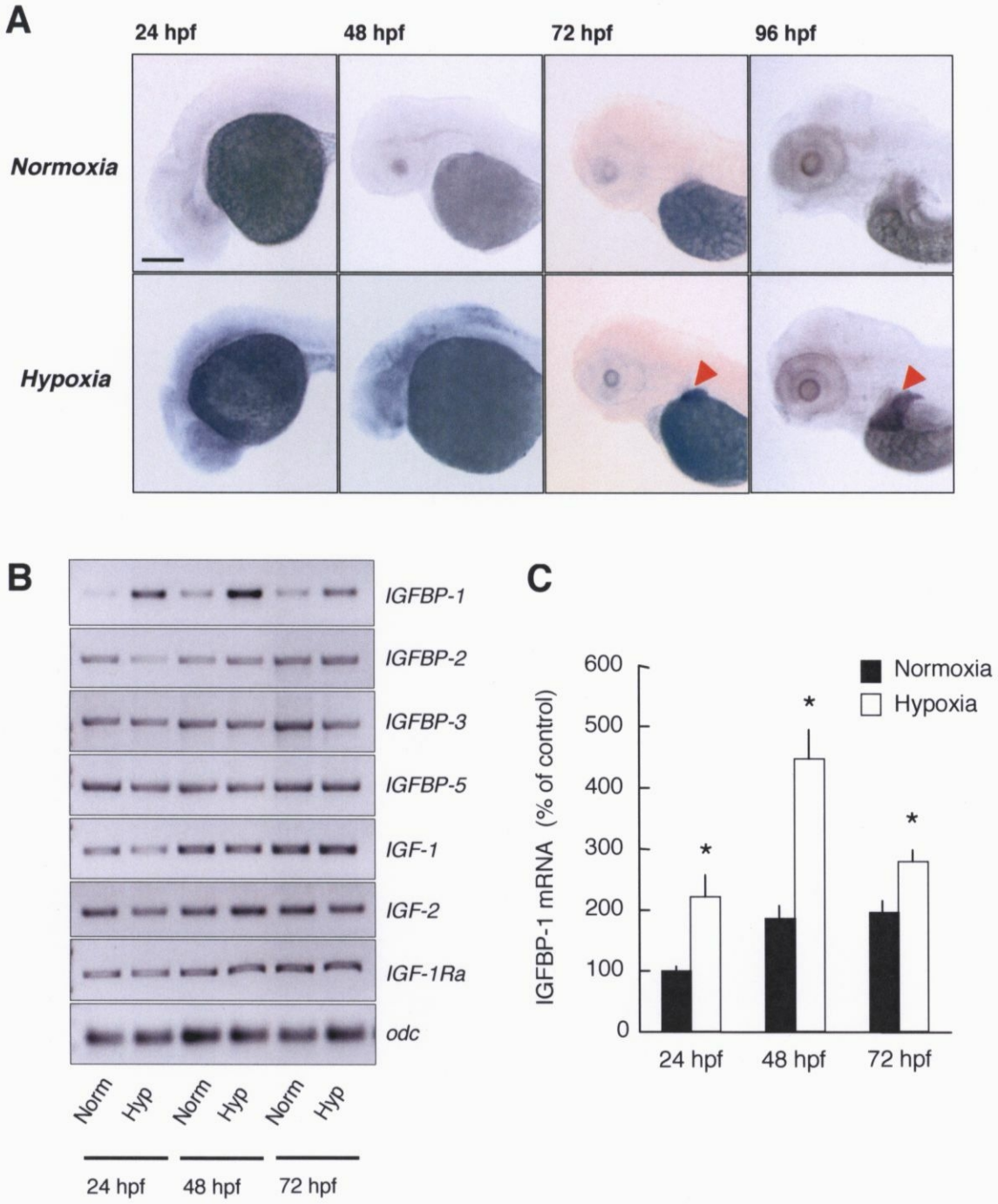


Fig. 1-2. Hypoxia induces IGFBP-1 expression. (**A**) Hypoxia treatment increased IGFBP-1 mRNA expression in early embryonic stages (24 and 48 hpf) and in the liver (indicated by arrow heads) in advanced stages (72 and 96 hpf). Zebrafish embryos at various stages were transferred to normal (upper panels) or hypoxic water (lower panels). After 24 h, the embryos were fixed and subjected to whole mount *in situ* hybridization analysis. Scale bar = 200 μm. (**B**) RT-PCR analysis of IGFBP-1, -2, -3, -5, IGF-1, IGF-2, IGF-1Ra, and odc in embryos of the indicated stages. Norm, normoxia; Hyp, hypoxia. (**C**) Relative IGFBP-1 mRNA levels normalized by the odc mRNA level. Filled bars represent the normoxia group, open bars represent the hypoxia group. Values are mean ± S.E. (n = 6). *, $P < 0.05$ compared with the corresponding normoxia group.

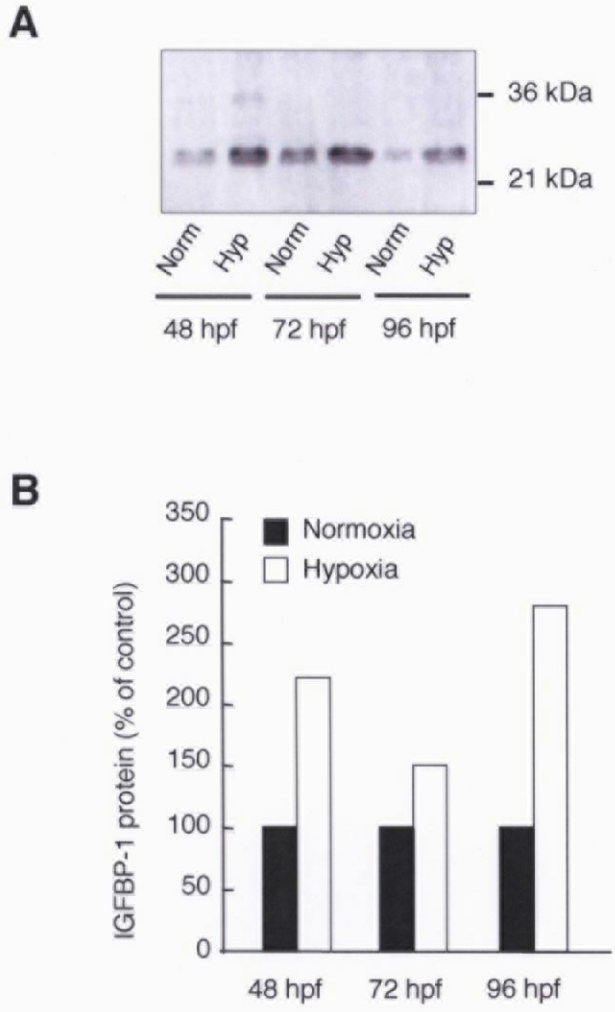
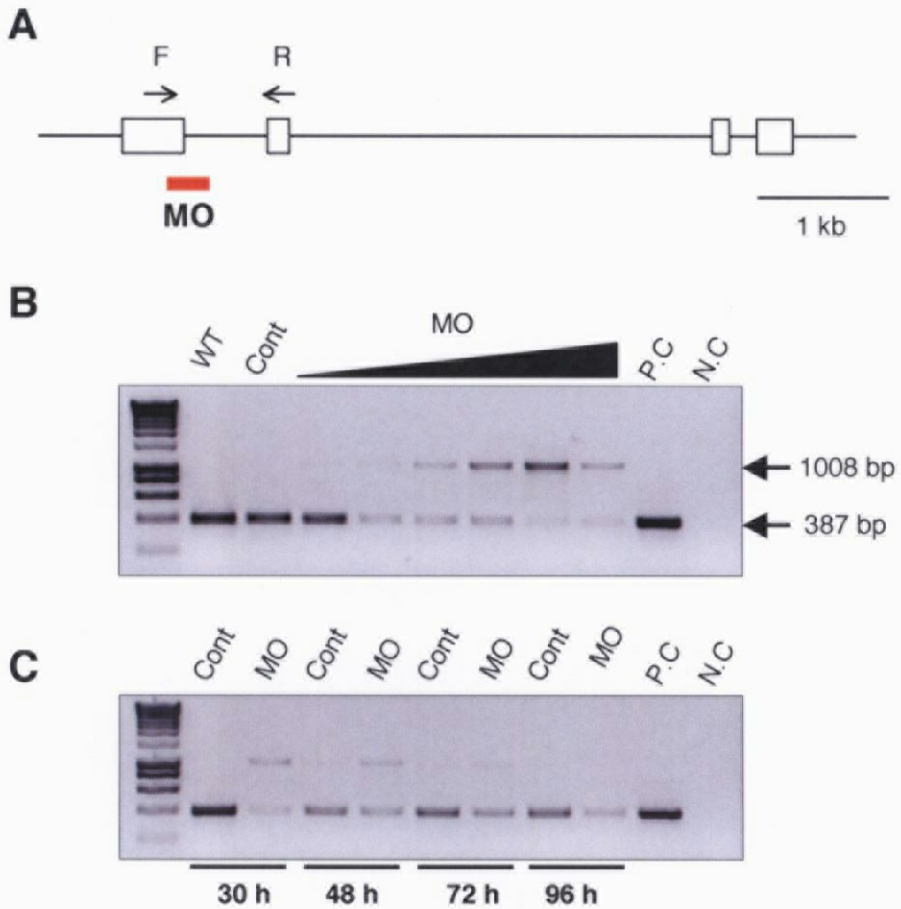


Fig. 1-3. Hypoxia induces IGFBP-1 protein levels. (**A**) Western ligand blotting analysis. Wild-type embryos at various indicated stages were subjected to hypoxia or normoxia for 24 h. The levels of IGFBP-1 protein were determined by Western ligand blotting using DIG-labeled IGF-I. See text in detail. (**B**) Densitometric analysis result of A. Filled bars represent the normoxia group, open bars represent the hypoxia group.



D

WT

ACA	GCA	CCA	GAT	CAT	CCG	TGT	TCG	TGA	CCG	GGC
T	A	P	D	H	P	E	S	H	N	G

MO-knockdown

ACA	GCA	CCA	G*GT*	*ACA*	*GGT*	*TTA*	*TCA*	*GTA*	*TAT*	*TAG*
T	A	P	*G*	*T*	*G*	*L*	*S*	*V*	*Y*	*

Fig. 1-4. Splice site targeting MO knocks down functional IGFBP-1. (**A**) Structure of the zebrafish IGFBP-1 gene. Exons are shown as boxes and introns shown as lines. Scale bar is 1 kb. Splice donor site targeted by the IGFBP-1 MO is underlined. The primer set used for RT-PCR is shown as arrows. F, forward primer; R, reverse primer. (**B**) Dose-dependent effect. 0.5, 1, 2, 4, 8, or 16 ng MO was injected to embryos at the 1-2 cell stages. WT, wild-type; Cont, control MO; P.C, positive control of PCR; N.C, negative control of PCR. The specific primer set amplified 387 bp spliced form of IGFBP-1, whereas MO knockdown caused an aberrant splice form (1008 bp). (**C**) Time-course effect. MO at dose of 2 ng was injected to embryos at the 1-2 cell stages and the knockdown effect was analyzed by RT-PCR at 30, 48, 72, and 96 h after the injection. (**D**) Sequence analysis of the IGFBP-1 cDNAs isolated from the wild-type and MO injected embryos. The sequence of the aberrantly spliced form is indicated as red letters. Note that the aberrant splicing form results in a coding frame shift and a premature stop codon.

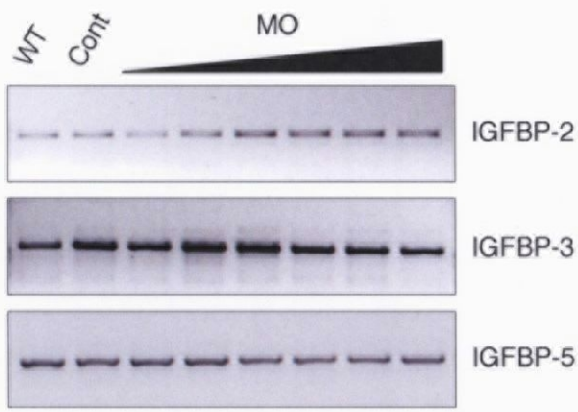


Fig. 1-5. Knockdown of functional IGFBP-1 does not change the expression levels and splicing of other IGFBPs. RT-PCR was performed using specific primer sets for zebrafish IGFBP-2, -3, and –5 using RNA isolated from wild-type, control MO and IGFBP-1 MO1 (0.5, 1, 2, 4, 8 and 16 ng) injected embryo as described in *Materials and Methods*.

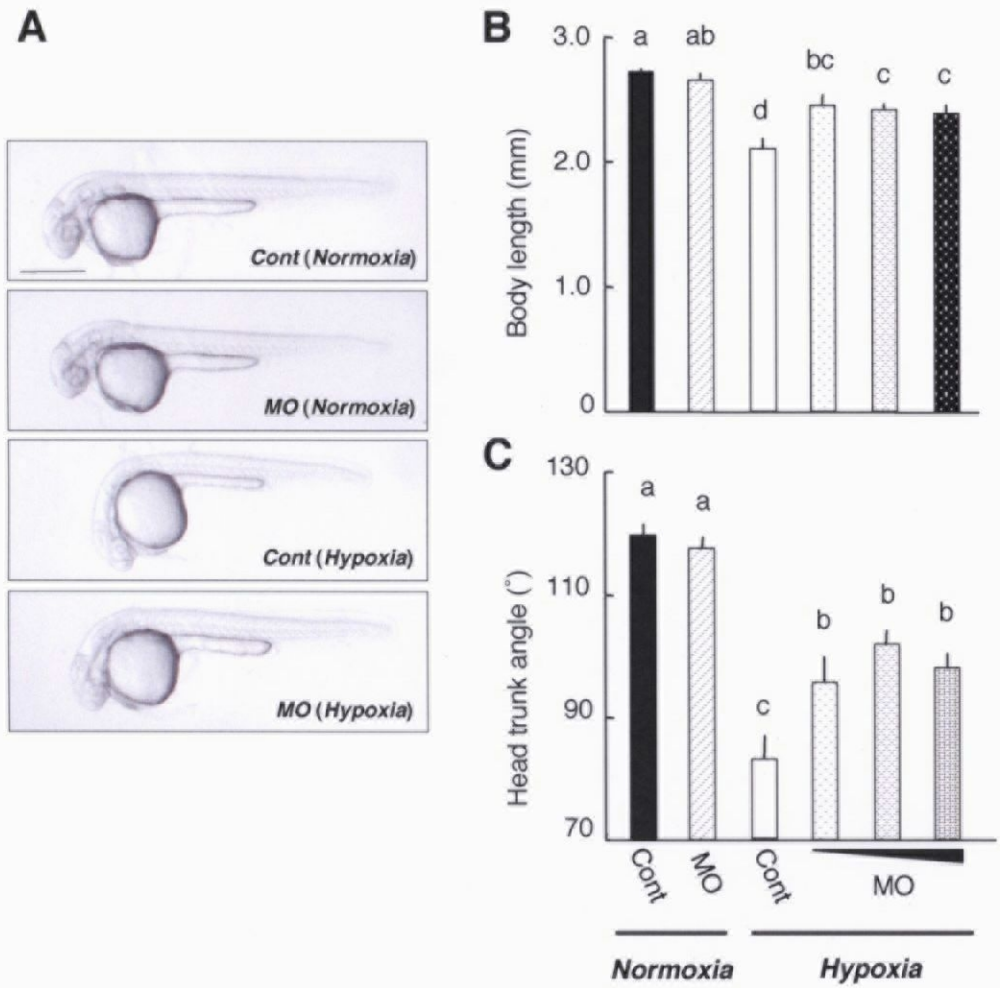


Fig. 1-6. Knockdown of IGFBP-1 partially abrogates hypoxia-caused growth retardation and developmental delay. (**A**) Morphology of control MO (Cont) or IGFBP-I MO (MO) injected embryos at 36 hpf. After raised to12 hpf in normoxic water, the injected embryos were transferred to water with normal (normoxia) or hypoxic water (hypoxia) for 24 h. Scale bar = 500 μm. (**B, C**) Knockdown of IGFBP-1 attenuated hypoxia-induced growth and developmental retardations but had no effect under normoxia. Embryos, injected with 2 ng control MO (Cont) or 1, 2 or 4 ng IGFBP-I MO (MO), were raised to 12 hpf and then transferred to normal (normoxia) or hypoxic water (hypoxia). 24 h later, their body lengths (B) and head-trunk angle (C) were measured. Values are means ± S.E. (n = 20-30). Groups with common letters are not significantly different from each other ($P < 0.05$).

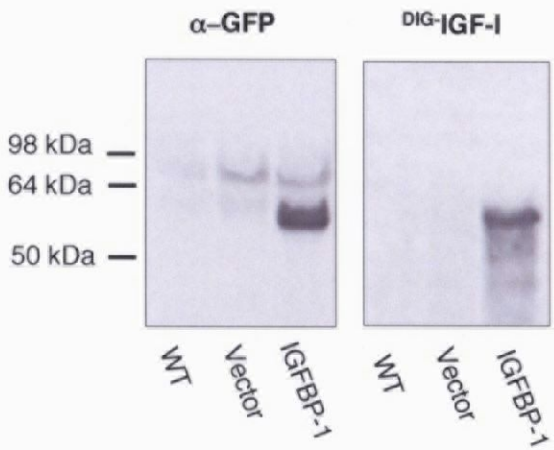


Fig. 1-7. Expression of a functional IGFBP-1:GFP fusion protein. Embryos were injected with the IGFBP-1:EGFP construct (IGFBP-1) or empty vector (vector). At 36 hpf, the embryos were subjected to immunoblot using anti-GFP under reduced condition (left) and Western ligand blot analysis using DIG-IGF-1 under nonreduced condition (right). WT, wild-type embryos; vector, empty-vector-injected embryos; IGFBP-1, embryos injected with the IGFBP-1:EGFP construct.

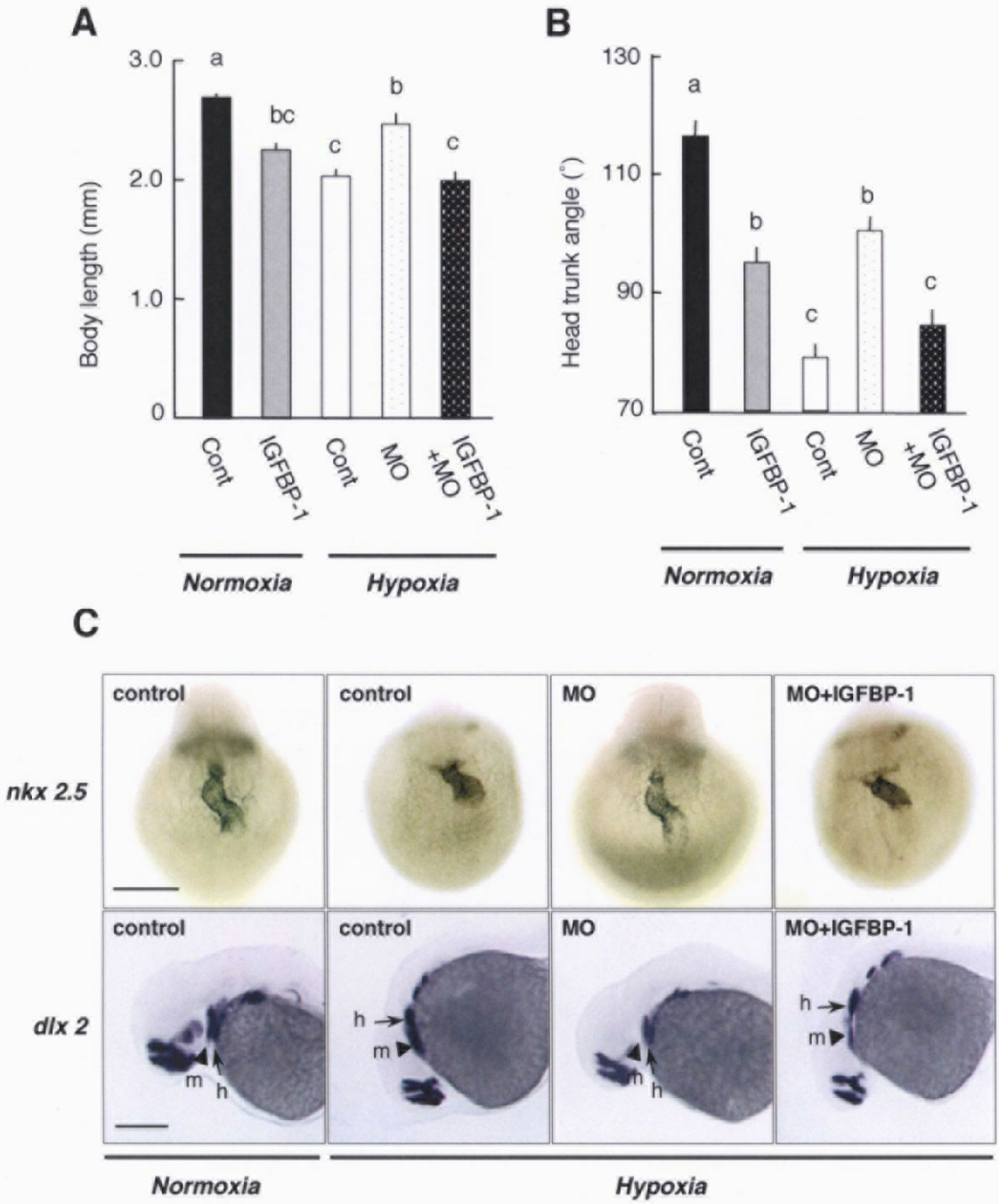


Fig. 1-8. IGFBP-1 inhibits embryonic growth and development by inhibiting IGF actions. (**A**, **B**) Effect of IGFBP-1 overexpression under normoxia and hypoxia. Embryos injected with control MO (Cont), IGFBP-1 MO1 (MO), or IGFBP-1 MO plus IGFBP-1-EGFP overexpression vector at 12 hpf were transferred to normal (normoxia) or hypoxic water (hypoxia). The body length (**A**) and HTA (**B**) were measured 24 h later. Values are expressed as means $\pm$ S.E. (n = 20-34). Groups with common letters are not significantly different from each other ($P < 0.05$). (**C**) MO knockdown abrogates and IGFBP-1 overexpression restores hypoxia-induced delay in head skeleton and heart morphogenesis. Embryos injected with control MO, IGFBP-1 MO, or IGFBP-1 MO plus IGFBP-1:EGFP overexpression vector were transferred to normal or hypoxic water for 24 h and subjected to whole-mount *in situ* hybridization with *nkx 2.5* (upper) and *dlx 2* (lower). h, hyroid arch (arrows); m, mandibular arch (arrow heads). Scale bar= 200 μm.

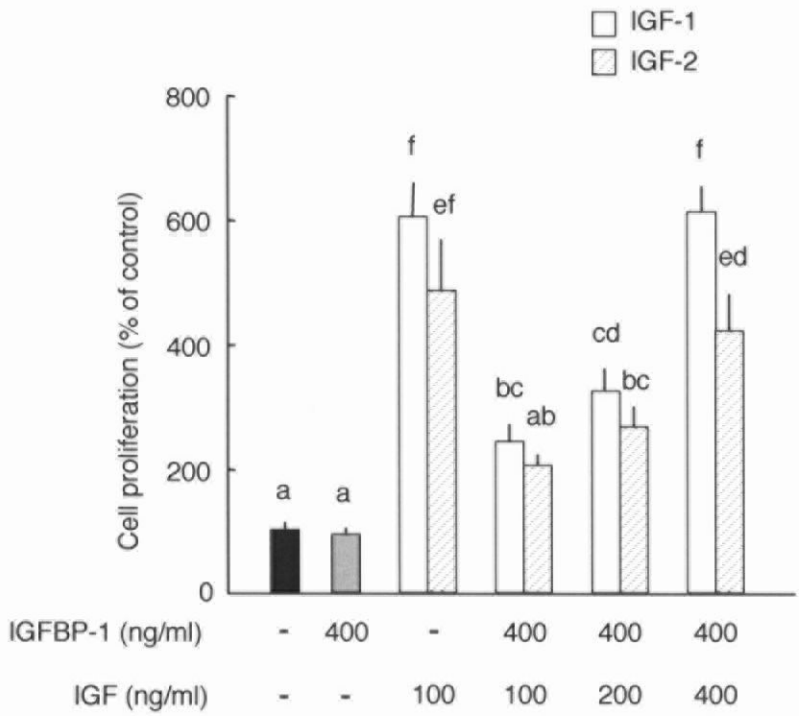


Fig. 1-9. IGFBP-1 inhibits IGF-stimulated zebrafish embryonic cell proliferation. Serum-starved, confluent cells were exposed to IGFBP-1 (400 ng/ml) with or without various concentrations of IGF-1 (open bars) or IGF-2 (shadow bars) in the presence of BrdUrd (20 μM) for 24 h. BrdUrd-labeled cells were detected by immunostaining using anti-BrdU. Values are expressed as means ± S.E. of two independent experiments, each of which was performed in duplicates. Groups with the same letters (a, b, c, d, e, or f) are not significantly different from each other ($P < 0.05$).

Table 1-1. Primer sequences used for RT-PCR

Target gene	Direction	Nucleotide sequence
IGFBP-1	Forward	5'-CTT CTG AAC TTC TTC TGG GTG G-3'
	Reverse	5'-CCC GTT ATG AGA CTC CGG ATG AT-3'
IGFBP-2	Forward	5'-GGA CAC GAT GTT GTC CTA TGT G-3'
	Reverse	5'-GGA TCC ATC TCT TGA CCG TCA A-3'
IGFBP-3	Forward	5'-TAA GTG TGC GCG TGT TCA GC-3'
	Reverse	5'-GAG TCC ATG CCT CAG CAT AC-3'
IGFBP-5	Forward	5'-ATG GCT CTT CTT GTG CTG GGT A-3'
	Reverse	5'-CTC ACT CGT TGT TGT TGT TGC TG-3'
IGF-1	Forward	5'-CTA GCG GTC ATT TCT TCC AG-3'
	Reverse	5'-ACA GGC GCA CAA TAC ATC TC-3'
IGF-2	Forward	5'-ACA GCC ACA AGC ATC ACT CA-3'
	Reverse	5'-ACA GCT CCG AAA GCA GCA TT-3'
IGF-1Ra	Forward	5'-CAA CAG CAC ACT AGT GTC G-3'
	Reverse	5'-GAT GAC AGC TAC GAT CAC GA-3'
odc	Forward	5'-GGA TGT CCT GAA GAA GCA CCT-3'
	Reverse	5'-CCC ACT GAC TGC ACG ATC TGG-3'

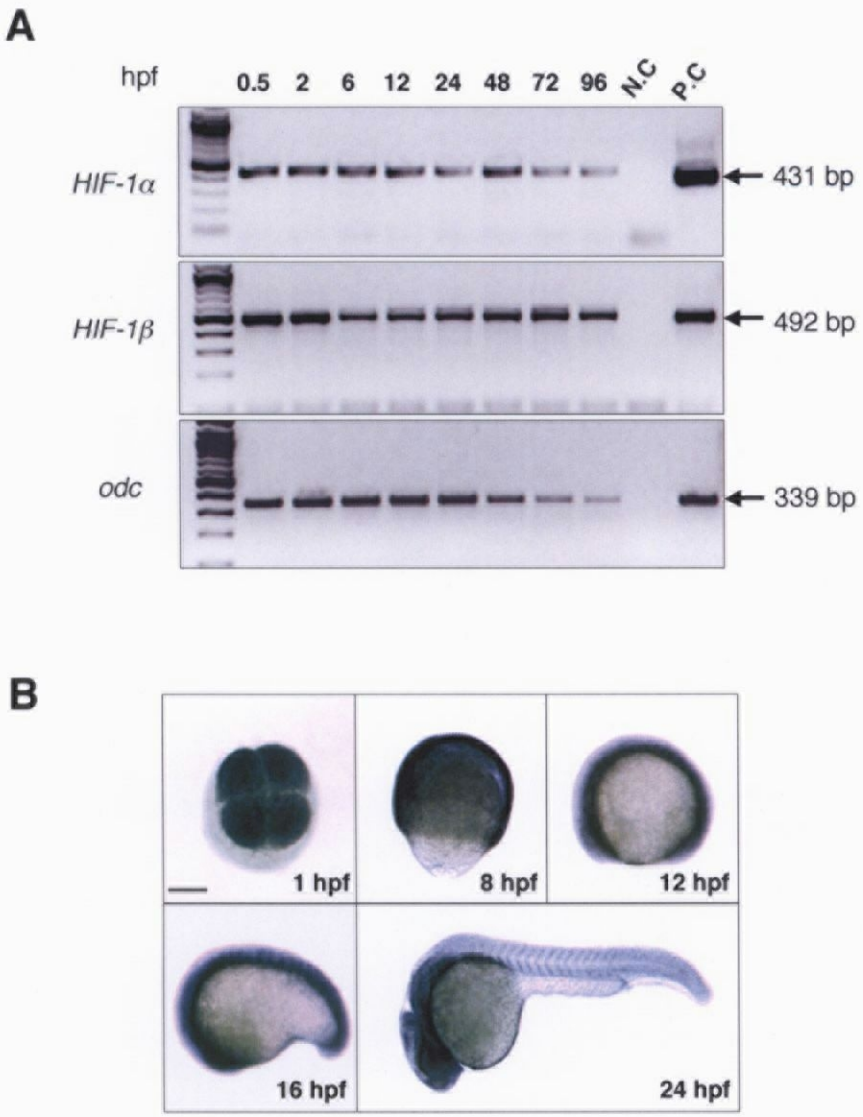


Fig. 2-1. The components of HIF-1 pathway is expressed in early embryogenesis. (**A**) RT-PCR analysis of HIF-1α and HIF-1β mRNA expression in zebrafish embryos of the indicated stages. hpf, hour post fertilization; N.C., negative control; P.C., positive control; and odc, ornithine decarboxylase. (**B**) Whole mount *in situ* hybridization analysis of zebrafish HIF-1α mRNA in embryos of the indicated stages. Scale bar = 200 μm.

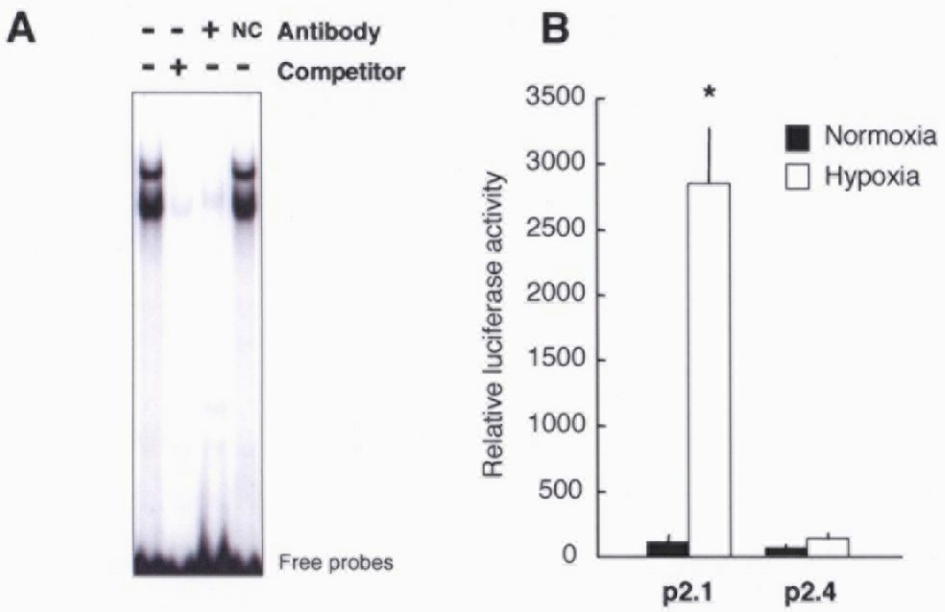


Fig. 2-2. Zebrafish HIF-1α is functional to drive the known target genes. (**A**) Zebrafish HIF-1α specifically binds to a probe containing the functional HRE of the zebrafish IGFBP-1 promoter. EMSA was conducted using nuclear extracts prepared from HepG2 cells transfected with a zebrafish HA:HIF-1α expression plasmid. Addition of excess amount (100 fold) of unlabeled probe (competitor) or HA antibody, but not mouse IgG (NC), abolished the binding. (**B**) Zebrafish HIF-1α activates the promoter activity of a known HIF-1 target gene, human enolase in a HRE-dependent manner. HepG2 cells were transfected with a zebrafish HIF-1α expression plasmid (open bar) or the empty vector (filled bar) together with a human enolase promoter reporter construct (p2.1), or a human enolase promoter reporter construct with the HRE mutated (p2.4). Forty-eight hours after transfection, luciferase activity was measured as described in *Materials and Methods*. Values are means ± S.E. (n = 3-4). *, $P < 0.01$.

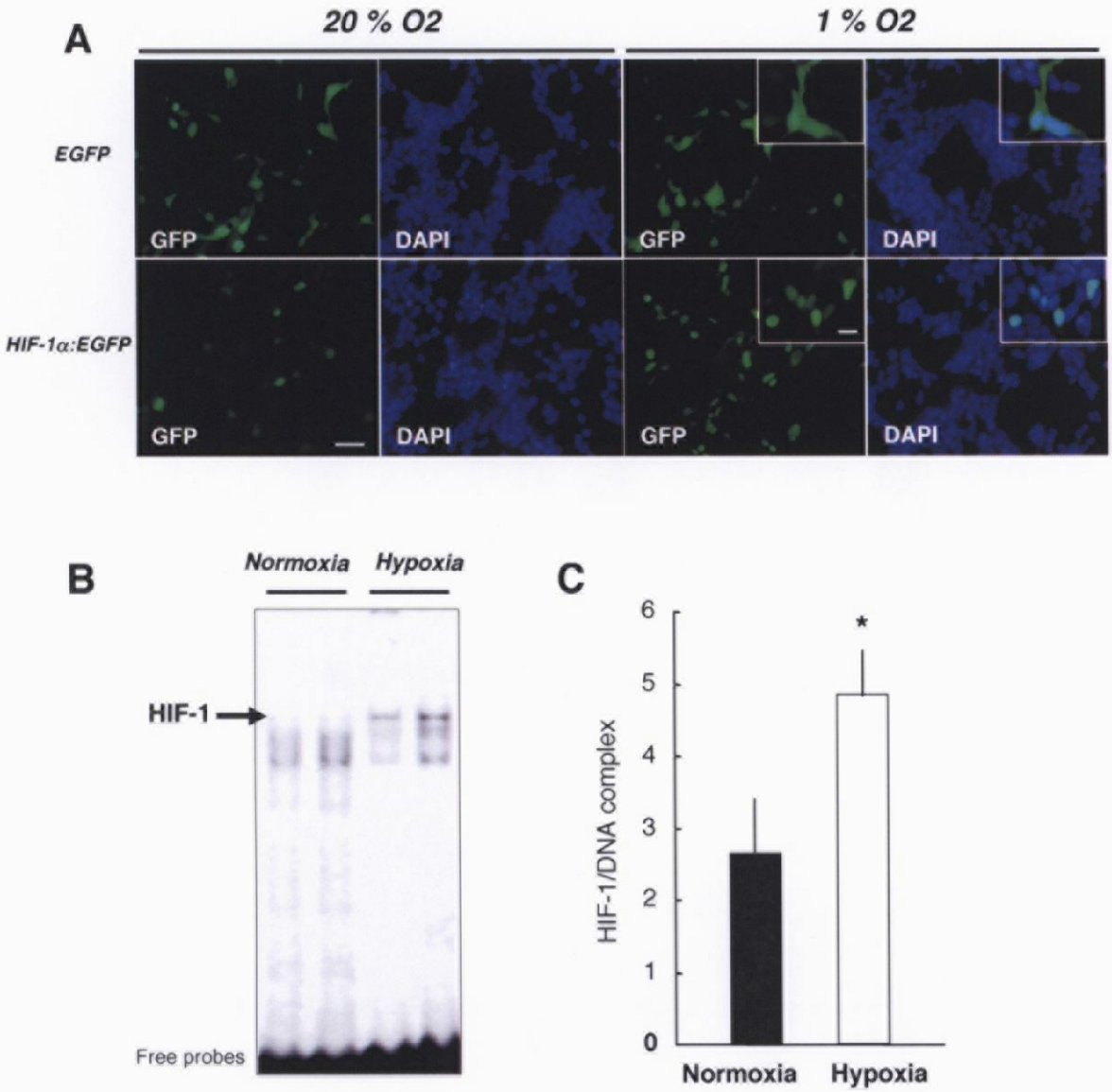


Fig. 2-3. Zebrafish HIF-1α is induced by hypoxia. (**A**) Zebrafish HIF-1α, visualized by EGFP tag, is localized in the nucleus and induced by hypoxia. HEK293 cells were transfected with the constructs containing EGFP only (upper panels) or zebrafish HIF-1α:EGFP fusion protein (lower panels). After 24 hours of the transfection, the cells were transferred to normoxia (20% O_2) or hypoxia (1% O_2) for 24 hours, fixed and stained by DAPI. Scale bar = 50 μm. Inset; high magnification of GFP or overlayed images of GFP and DAPI staining. Scale bar = 5 μm. Note that HIF-1α:EGFP fusion protein is localized in the nucleus. (**B**) Hypoxia induces the HIF-1/DNA complex in zebrafish embryos *in vivo*. EMSA was performed using nuclear extracts prepared from zebrafish embryos kept in normoxic or hypoxic water. (**C**) Densitometric analysis result of (B). Filled bar represents the normoxia group, and open bar represents the hypoxia group. Values are means ± S.E. (n = 6). *, $P < 0.05$.

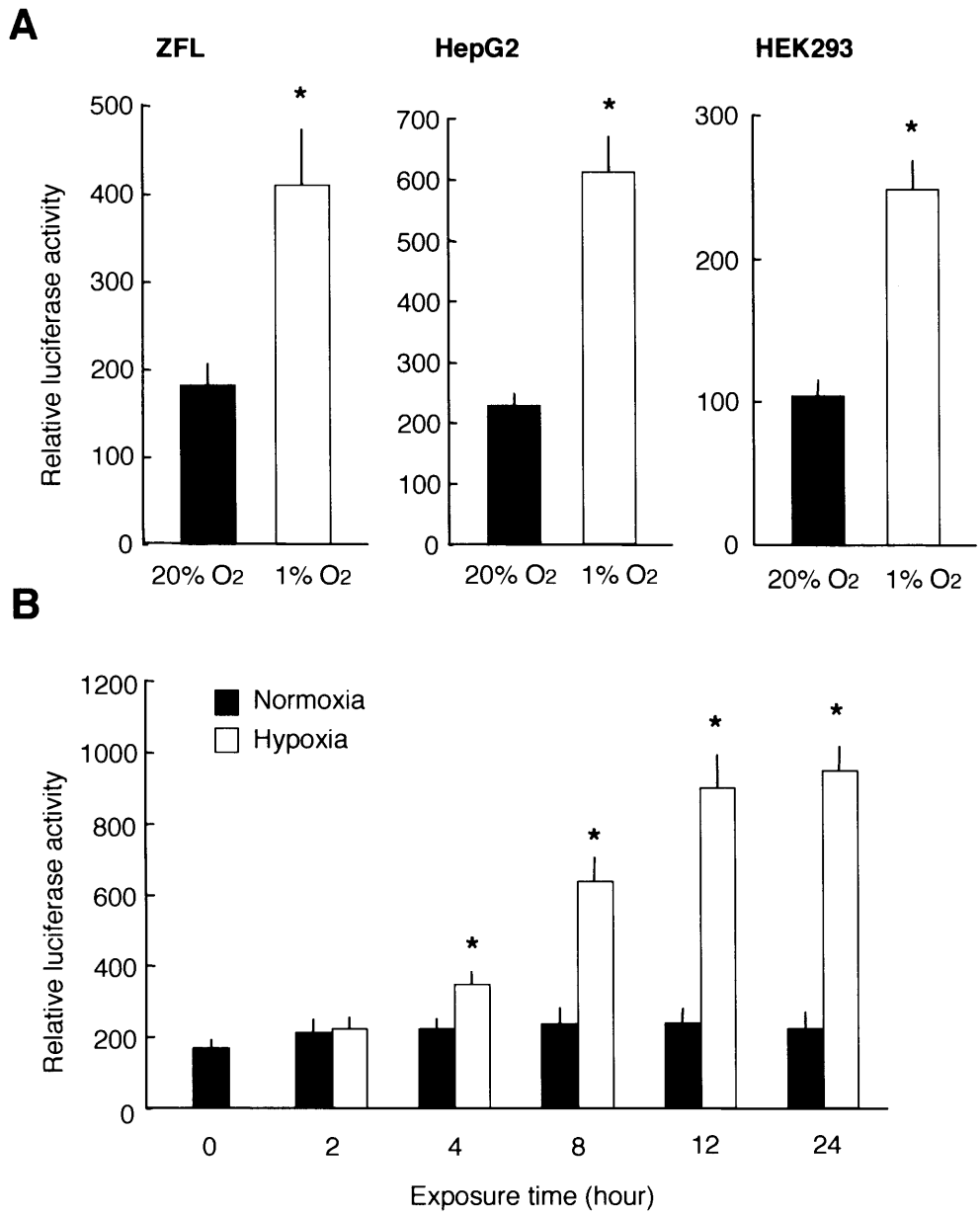


Fig. 2-4. Hypoxia increases IGFBP-1 gene transcription *in vitro*. (**A**) *In vitro* analysis of the hypoxia responsiveness of the cloned zebrafish IGFBP-1 promoter in cultured zebrafish liver cell line (ZF4, left), HepG2 (middle), and HEK293 (right) cells. Cells were transfected with the p2025Luc construct. Twenty four hours after the transfection, they were switched to hypoxia (1% O_2) or kept in normoxia (20% O_2). After another 24 hours, the cells were lysed and the luciferase activity was measured as described in *Materials and Methods*. The transfection efficiency was normalized by measuring *Renilla* luciferase activity. Values are means $\pm$ S.E. (n = 3). *, $P < 0.05$. (B) Time-course effect. HepG2 cells transfected with p2025Luc were exposed to hypoxia (1% O_2) for 2, 4, 8, 12, and 24 hours (empty bars). Cells kept in 20% O_2 were used as controls (filled bars). Values are means + S.E. (n = 3). *, $P < 0.05$.

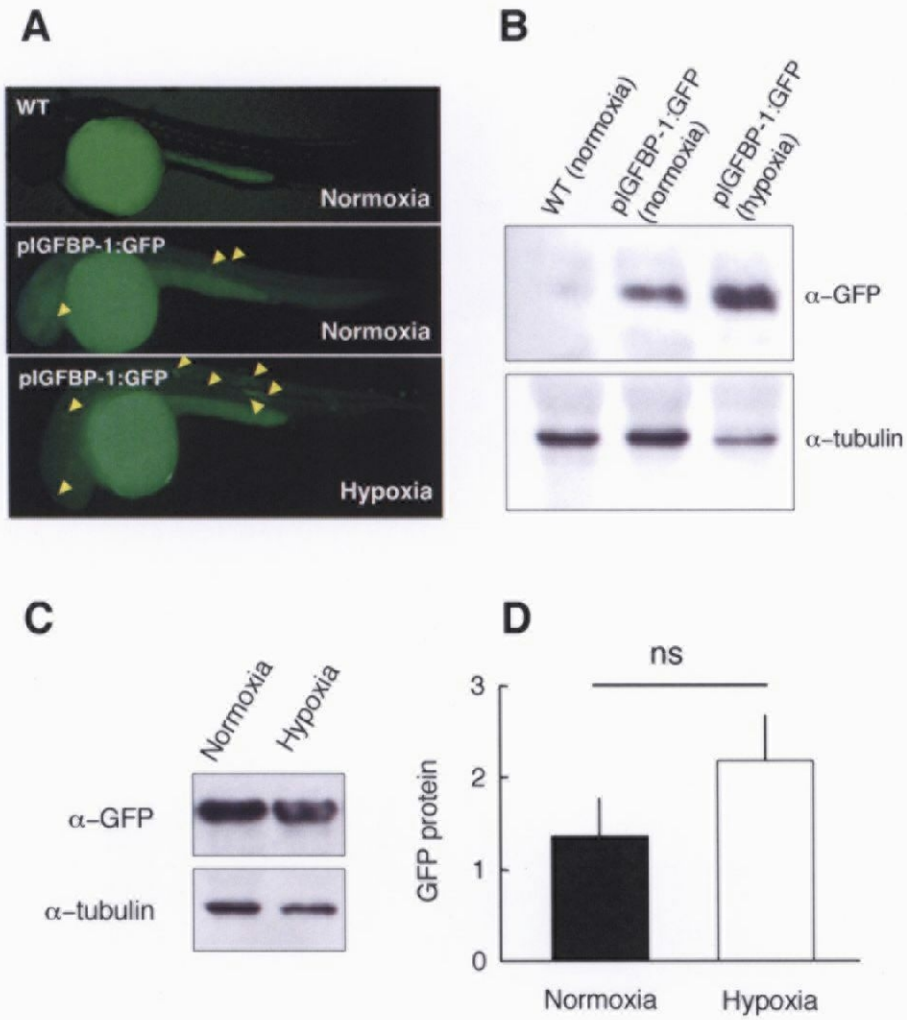


Fig. 2-5. Hypoxia increases IGFBP-1 gene transcription *in vivo*. (**A**) Embryos at 1-2 cell stage were microinjected with linearized pIGFBP-1:GFP DNA (p2025). The injected embryos were raised to 12 hpf and transferred to normoxic or hypoxic water. Twenty-four hours later, GFP expressing cells were observed under a fluorescent microscope. The upper panel is a wild type control embryo kept in normoxic water. The middle and lower panels show a representative pIGFBP-1:GFP injected embryo kept in normoxic or hypoxic water, respectively. Note the increase in visible GFP expressing cells (indicated by arrow heads) in response to hypoxia. (**B**) Pooled embryos were subjected to Western blotting analysis using a GFP antibody. The expression of tubulin protein was also measured using a tubulin antibody. (**C**) Negative control of *in vivo* promoter analysis. Embryos were injected with a CMV:GFP reporter construct, and transferred to normoxic or hypoxic water. Pooled embryos were subjected to Western blotting analysis using a GFP antibody or tublin antibody. (**D**) Densitometric analysis result of (C). Filled bar represents the normoxia group, and open bar represents the hypoxia group. Values are means $\pm$ S.E. (n = 3). ns, not significant.

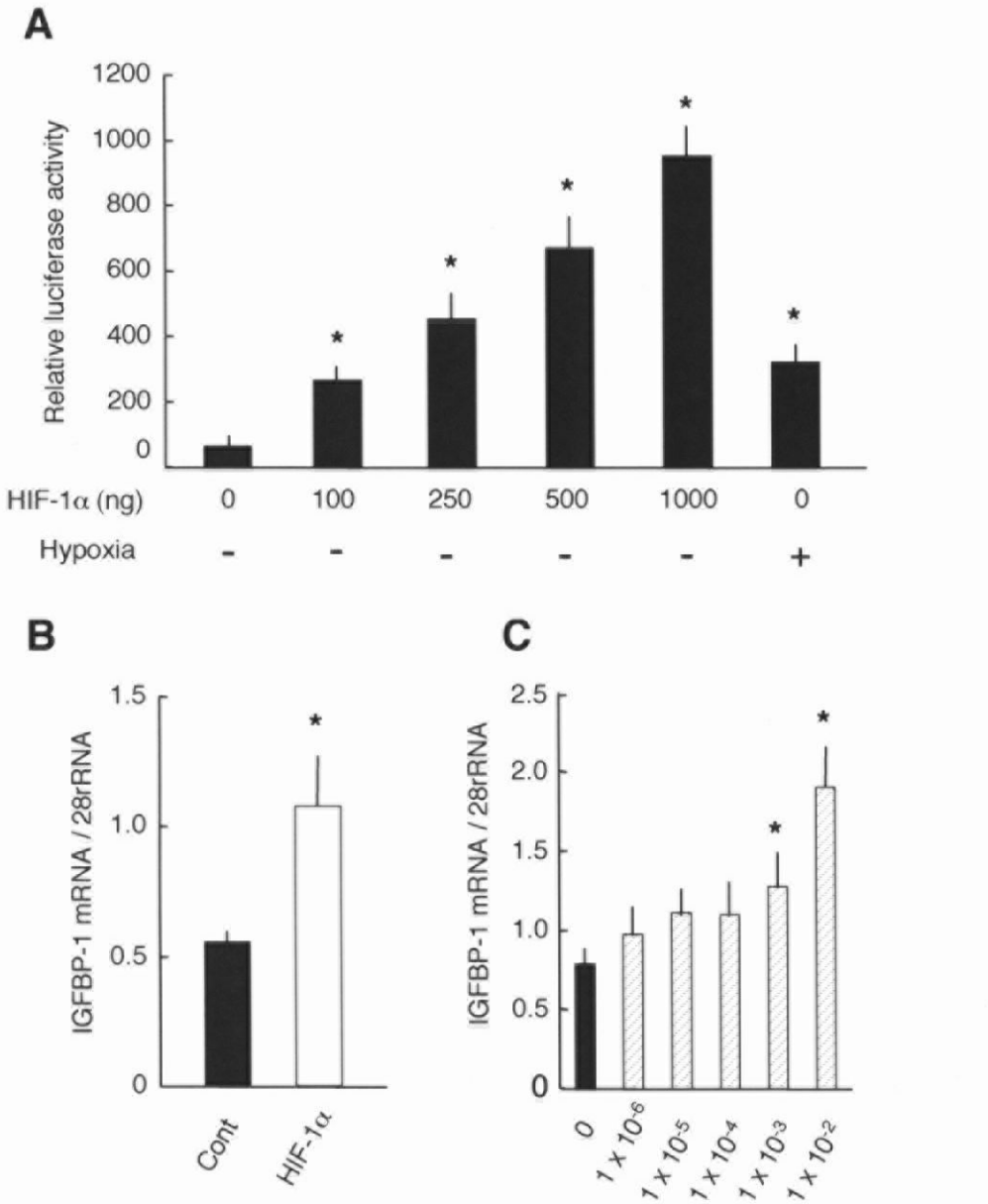


Fig. 2-6. Overexpression of zebrafish HIF-1α increases IGFBP-1 promoter activity *in vitro* and *in vivo*. (**A**) pCMV-HIF-1α at the concentration indicated was transfected into HepG2 cells with a zebrafish IGFBP-1 promoter construct (p1128Luc). The difference in pCMV-HIF-1α DNA was compensated using empty vector DNA. After 48 hours of transfection, luciferase activity was measured. Transfection efficiency was normalized by *Renilla* luciferase activity. Values are means ± S.E. (n = 3). *, $P < 0.05$. (**B**) Injection of zebrafish HIF-1α mRNA into zebrafish embryos increases IGFBP-1 gene expression *in vivo*. Zebrafish HIF-1α mRNA (1 ng/μl) was microinjected into zebrafish embryos at 1-2 cell stage. Twelve-hours later, embryos were collected for RNA extraction. IGFBP-1 mRNA levels were measured by semi-quantitative RT-PCR. Values are means ± S.E. (n = 5). *, $P < 0.05$. (**C**) $CoCl_2$, a hypoxia mimic, increases IGFBP-1 mRNA expression in zebrafish embryos *in vivo*. 24 hpf embryos were treated with $CoCl_2$ for 24 hours at the indicated concentrations. At the end of the treatment, total RNA was extracted and subjected to Northern blotting analysis. IGFBP-1 mRNA levels were normalized by 28S rRNA levels. Values are means ± S.E. (n = 3). *, $P < 0.05$ compared with control.

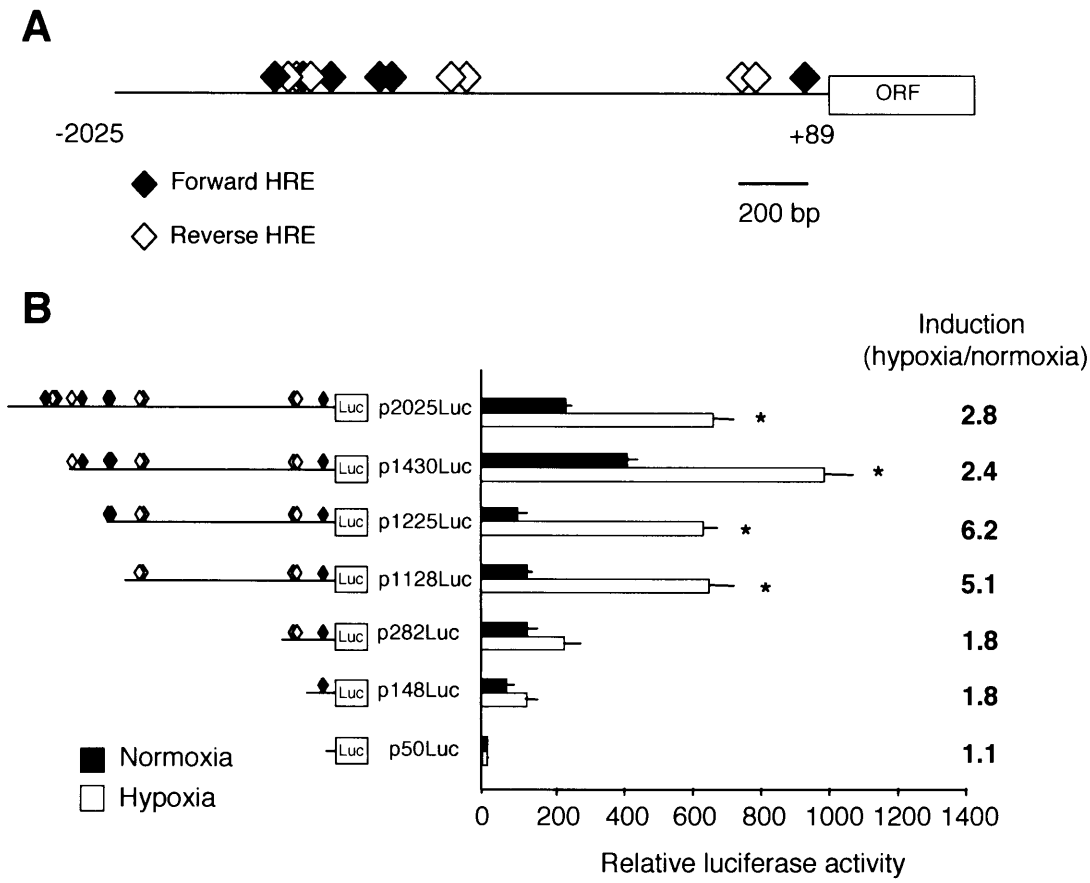


Fig. 2-7. The zebrafish IGFBP-1 promoter contains 13 HREs but only the -1090/-1086 HRE is required for the hypoxia or HIF-1 response. (**A**) Location and direction of the 13 canonical HRE sites identified in the zebrafish IGFBP-1 promoter region. The HREs in forward orientation are indicated as filled diamonds and reverse HREs as open diamonds. (**B**) Deletion analysis of the zebrafish IGFBP-1 promoter. HepG2 cells were transfected with a series of luciferase reporter constructs containing the indicated portion of the IGFBP-1 promoter region. Twenty-four hours after transfection, cells were kept in 20% O_2 (filled bar) or transferred to 1% O_2 (open bar) for 24 hours. The transfection efficiency was normalized by measuring *Renilla* luciferase activity. Values are means $\pm$ S.E. (n = 3). *, $P < 0.05$ compared with normoxia.

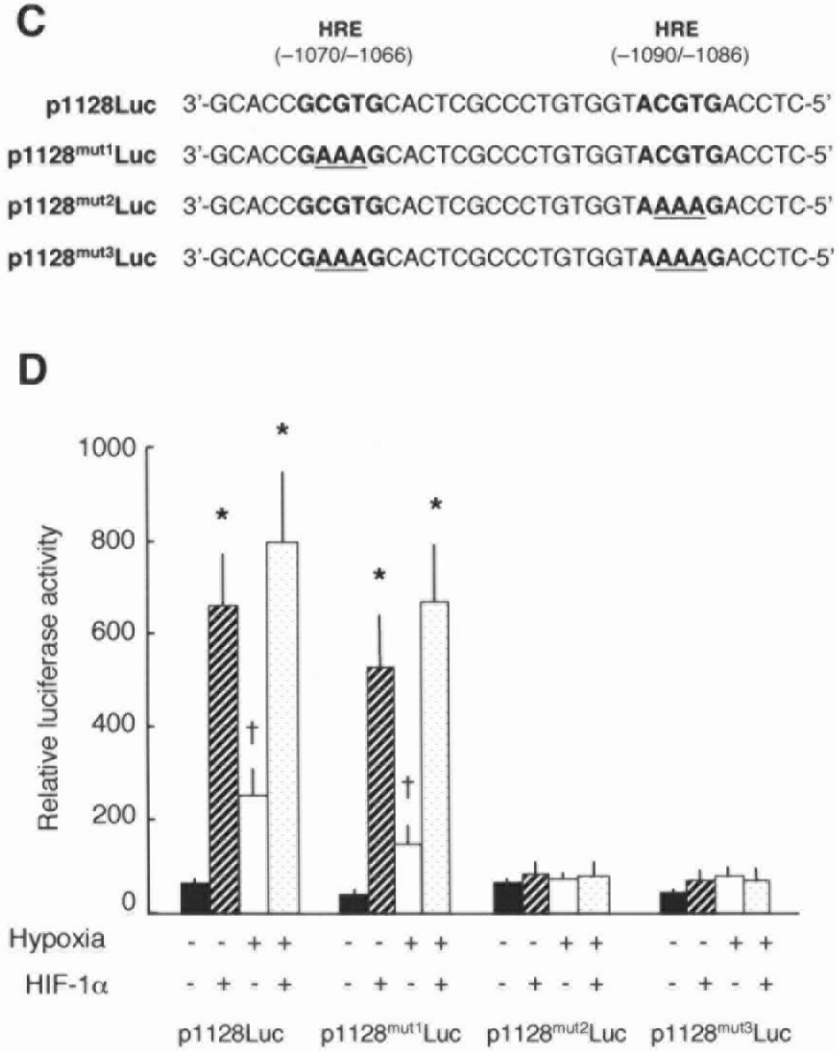


Fig. 2-7 (continued). (**C**) The two HREs and their surrounding sequences in the wild type p1128Luc and various mutants. Because the two HREs are in reverse orientation, the sequences are presented from 3' to 5' direction. The HREs are indicated in bold and mutated sequences are underlined. (**D**) Mutational analysis of the two HREs. HepG2 cells were co-transfected with the constructs indicated in (C) and the zebrafish HIF-1α expressing construct or the empty pcDNA3.1. Twenty-four hours after transfection, cells were kept in 20% O_2 or switched to 1% O_2 (hypoxia) for 24 hours and luciferase activity was measured. Transfection efficiency was normalized by *Renilla* luciferase activity. Values are means ± S.E. (n = 3-4). *, $P < 0.01$ between the HIF-1α and vector-transfected group. †, $P < 0.01$ between the normoxic and hypoxic groups.

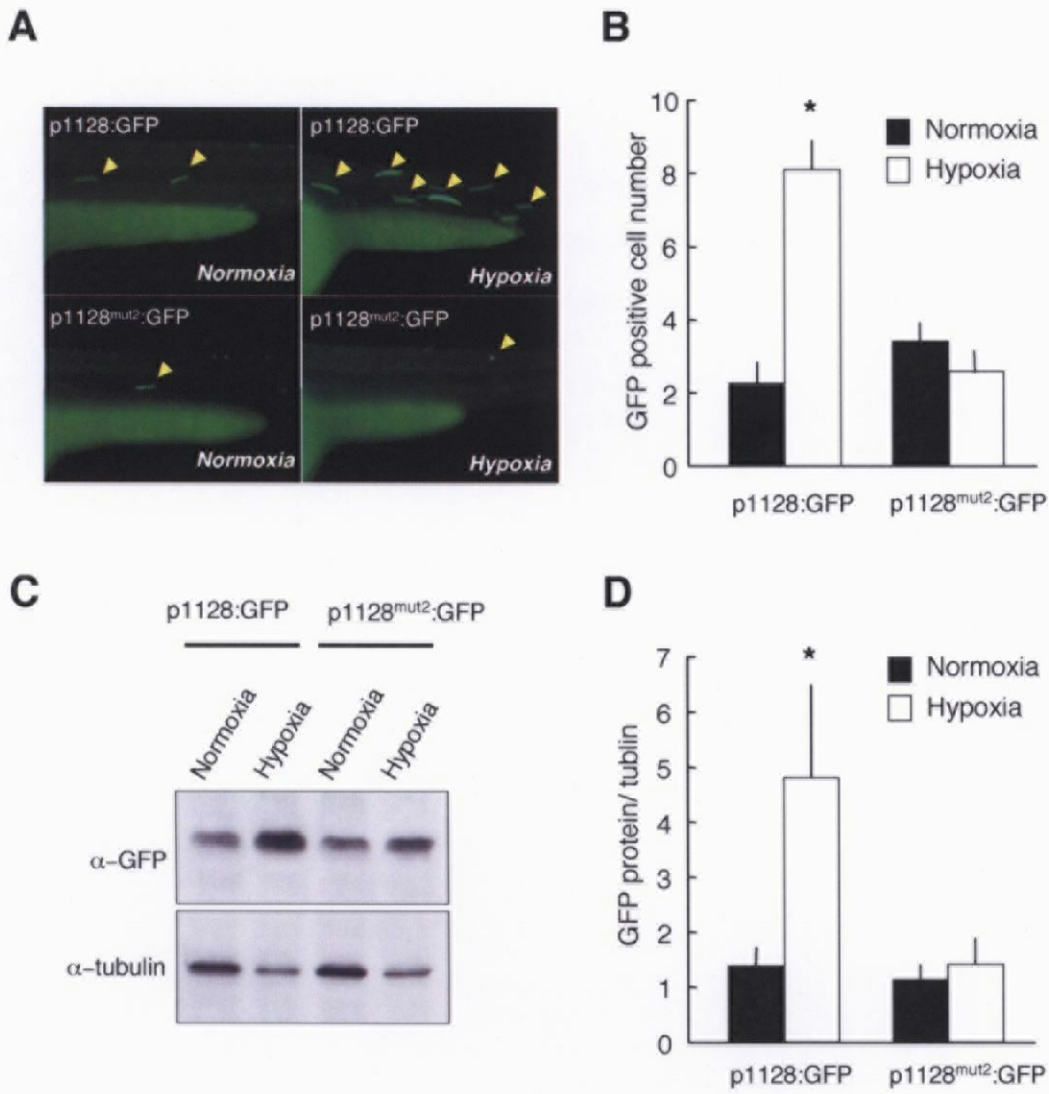


Fig. 2-8. The HRE positioned at -1090/-1086 is required for hypoxia-induced IGFBP-1 gene expression *in vivo*. (**A**) *In vivo* analysis of the involvement of the -1090/-1086 HRE in hypoxia-induced IGFBP-1 gene transcription. Embryos at 1-2 cell stage were microinjected with p1128:GFP or p1128^{mut2}:GFP. The injected embryos were raised to 24 hpf and transferred to normoxic (normoxia) or hypoxic water (hypoxia). Twenty-four hours later, visible GFP expressing cells (arrowed) were counted under a fluorescent microscope. (**B**) Quantification of (A). Filled bars represent the normoxia group, and open bars represent the hypoxia groups. Values are means ± S.E. (n = 69-94). *, $P < 0.01$. (**C**) Western blotting analysis of pooled p1128:GFP or p1128^{mut2}:GFP-injected embryos shown in (A). (**D**) Densitometric analysis of (C). The expression levels of GFP relative to tubulin are shown. Filled bars represent the normoxia groups, and open bars represent the hypoxia groups. Values are means ± S.E. (n = 5). *, $P < 0.05$.

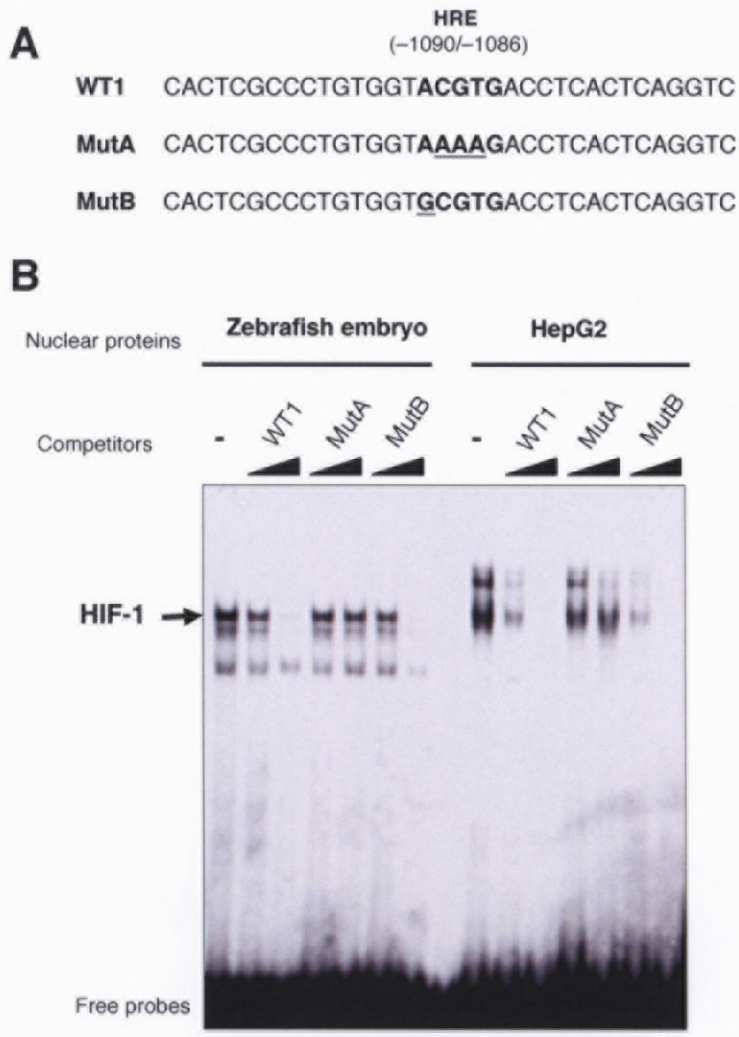


Fig. 2-9. Both ACGTG and GCGTG are functional HRE. (**A**) Sequences of the wild type (WT1) and mutant oligonucleotides used in EMSA. The HRE is indicated in bold and altered sequences are underlined. (**B**) Both forms of HRE (ACGTG and GCGTG) bind HIF-1. [^{32}P]-labeled double-stranded WT1 oligonucleotide was incubated with nuclear extracts prepared from zebrafish embryos or HepG2 cells. For competition, unlabeled WT1, MutA1, or MutB1 were added in 10- and 100-fold molar excess, respectively. The HIF-1/DNA complexes were separated by electrophoresis and visualized by autoradiography.

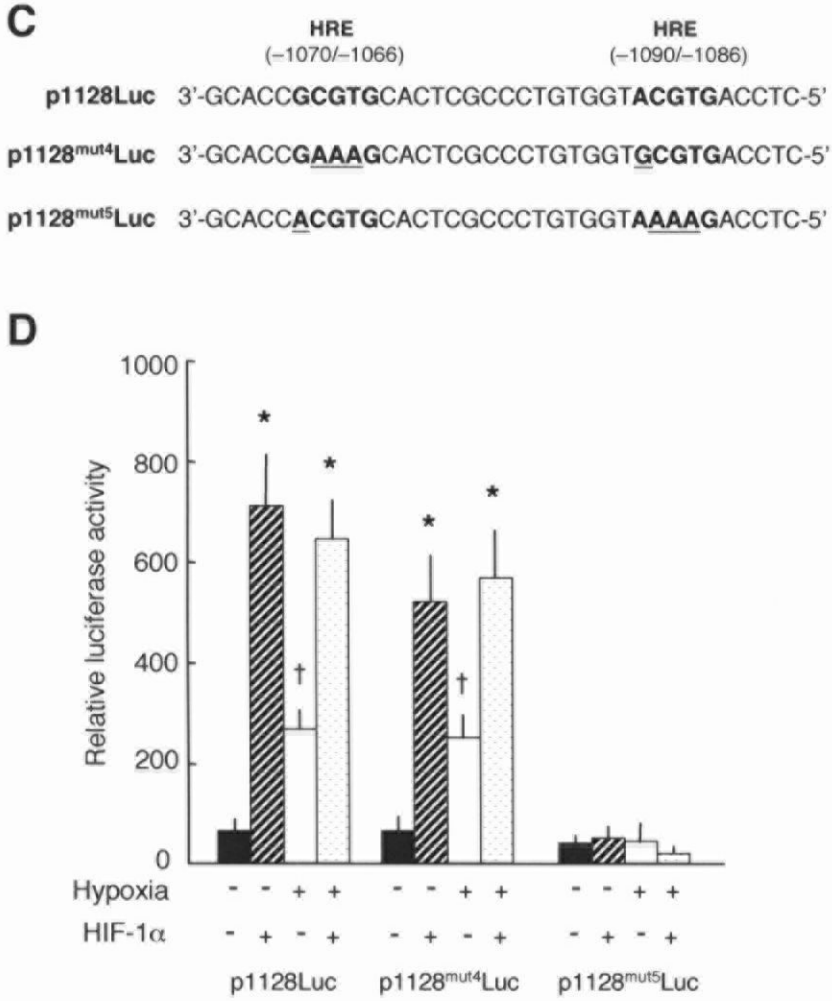


Fig. 2-9 (continued). (**C**) The two distinct HREs and their surrounding sequences in the wild type p1128Luc and two mutants. The HRE sequences are indicated in bold and mutated sequences are underlined. (**D**) HepG2 cells were co-transfected with the zebrafish HIF-1α expressing construct or empty pcDNA3.1 together with individual constructs indicated in (C). See details in the text. Values are means ± S.E. (n = 4-6). *, $P < 0.01$ between the HIF-1α and the empty vector-transfected group. †, $P < 0.01$ between the normoxia and hypoxia groups.

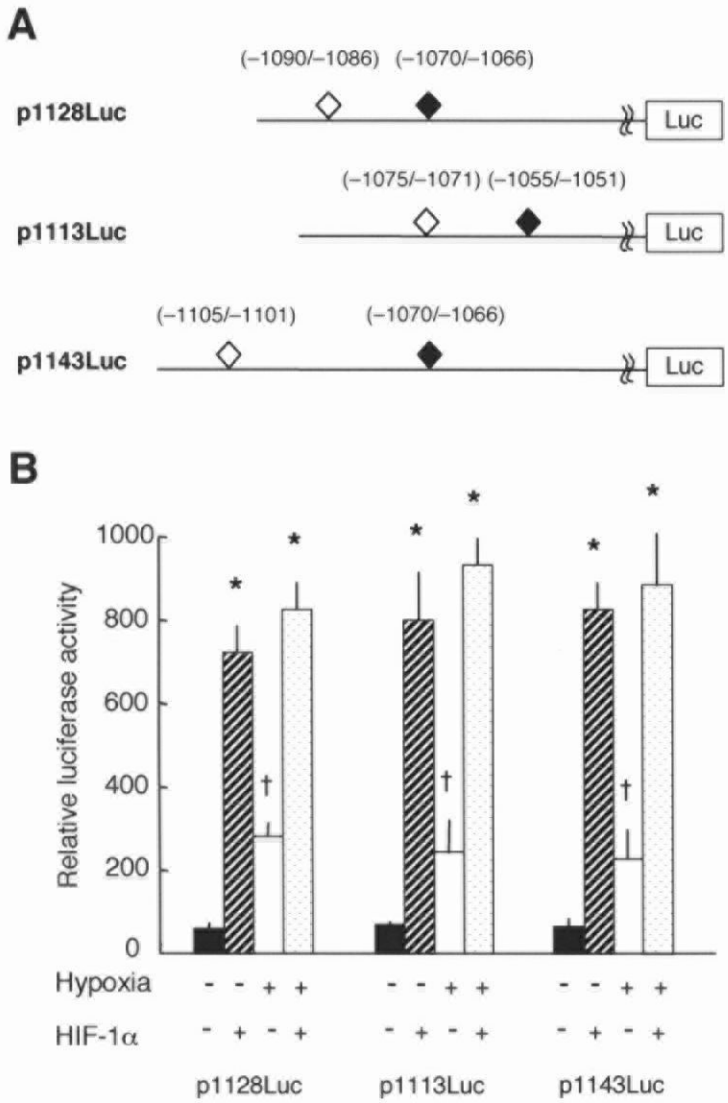


Fig. 2-10. The selective use of the -1090 /-1086 HRE cannot be attributed to its location. (**A**) A diagram showing the locations of the two HREs in p1128Luc (wild type), p1113Luc and p1143Luc, the latter two were generated by deleting or adding 15 bp. The functional HRE is represented by an open diamond and the non-functional HRE by a filled diamond. (**B**) Moving the functional HRE by 15 nt to either direction has no effect on the hypoxia or HIF-1 responsiveness. HepG2 cells were co-transfected with individual reporter constructs indicated in (A) and a zebrafish HIF-1α expression plasmid or the empty vector. See details in the text. Values are means ± S.E. (n = 3). *, $P < 0.05$ between the HIF-1α and the empty vector-transfected groups. †, $P < 0.05$ between the normoxic and hypoxic groups.

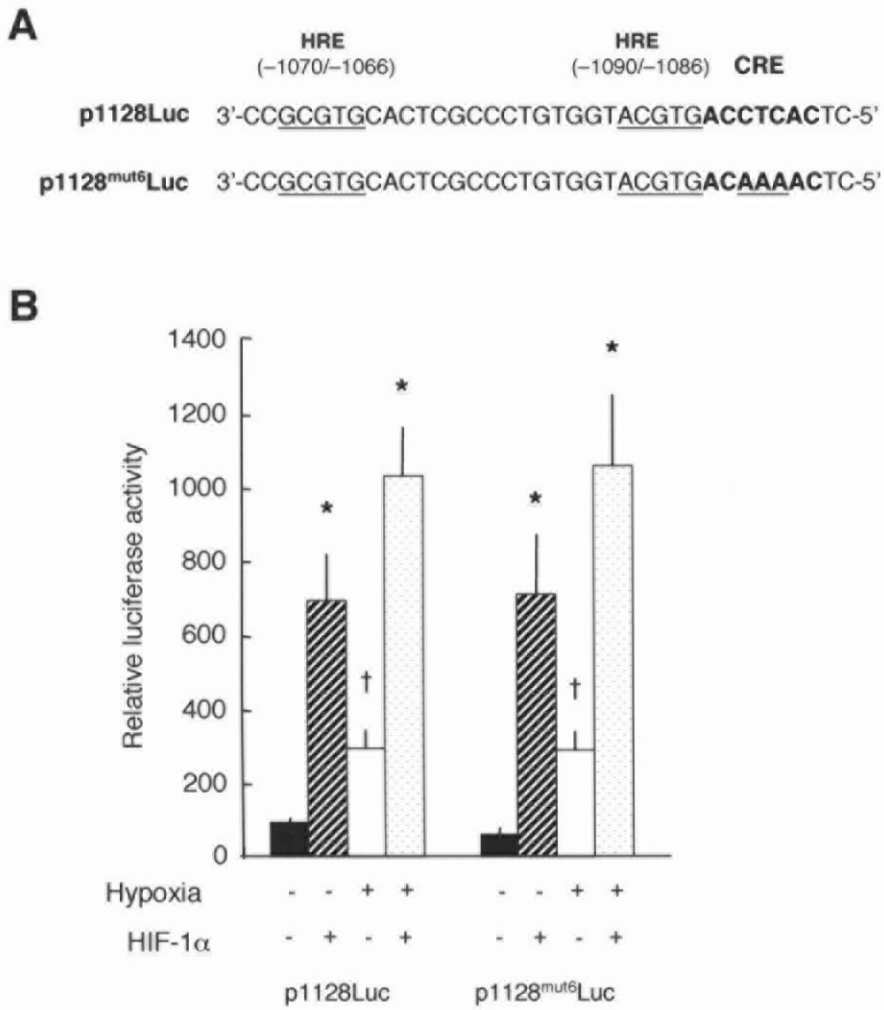


Fig. 2-11. A CRE site is not critical for the hypoxia- and HIF-1 responsiveness of the IGFBP-1 promoter. (**A**) The two HREs and the adjacent CRE sequences in the wild type p1128Luc and p1128Luc^{mut6}. The HREs are underlined and CRE sequence is in bold letters. The mutated sequences are in bold and underlined. (**B**) Mutation of the CRE does not alter the hypoxia or HIF-1 responsiveness of the IGFBP-1 promoter. HepG2 cells were co-transfected with individual reporter constructs indicated in (A) and a zebrafish HIF-1α expression plasmid or the empty vector. See details in the text. Values are means ± S.E. (n = 4). *, $P < 0.05$ between the HIF-1α and the empty vector-transfected groups. †, $P < 0.05$ between the normoxic and hypoxic groups.

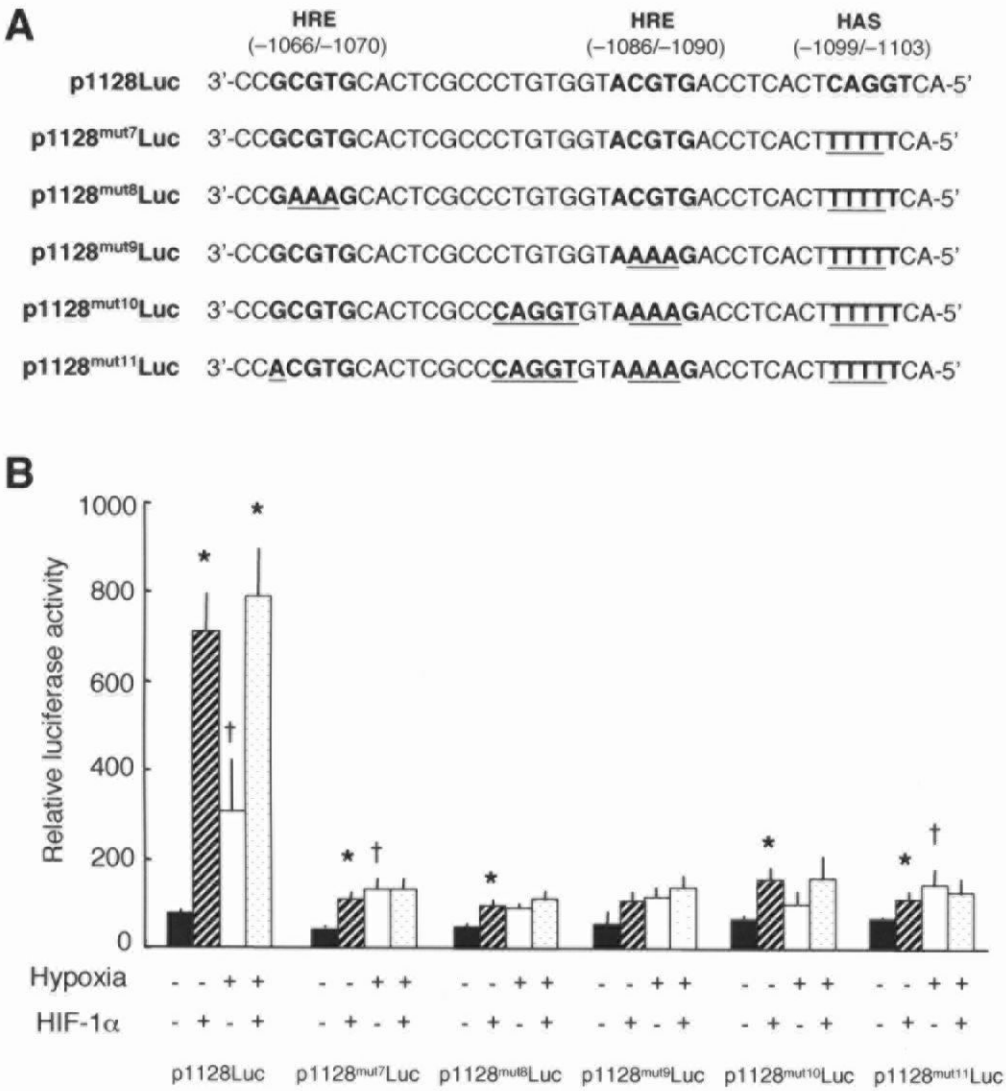


Fig. 2-12. A HAS adjacent to the functional HRE is required, but not sufficient for the hypoxia- and HIF-1 responsiveness of the IGFBP-1 promoter. (**A**) The two HREs and the HAS sequences in the wild type p1128Luc and four mutants. The HREs and HAS are indicated in bold and mutated sequences are underlined. (**B**) Mutational analysis of the HAS. HepG2 cells were co-transfected with individual reporter constructs indicated in (A) and a zebrafish HIF-1α expression plasmid or the empty vector. See details in the text. Values are means ± S.E. (n = 3-6). *, $P < 0.05$ between the HIF-1α and the empty vector-transfected groups. †, $P < 0.05$ between the normoxic and hypoxic groups.

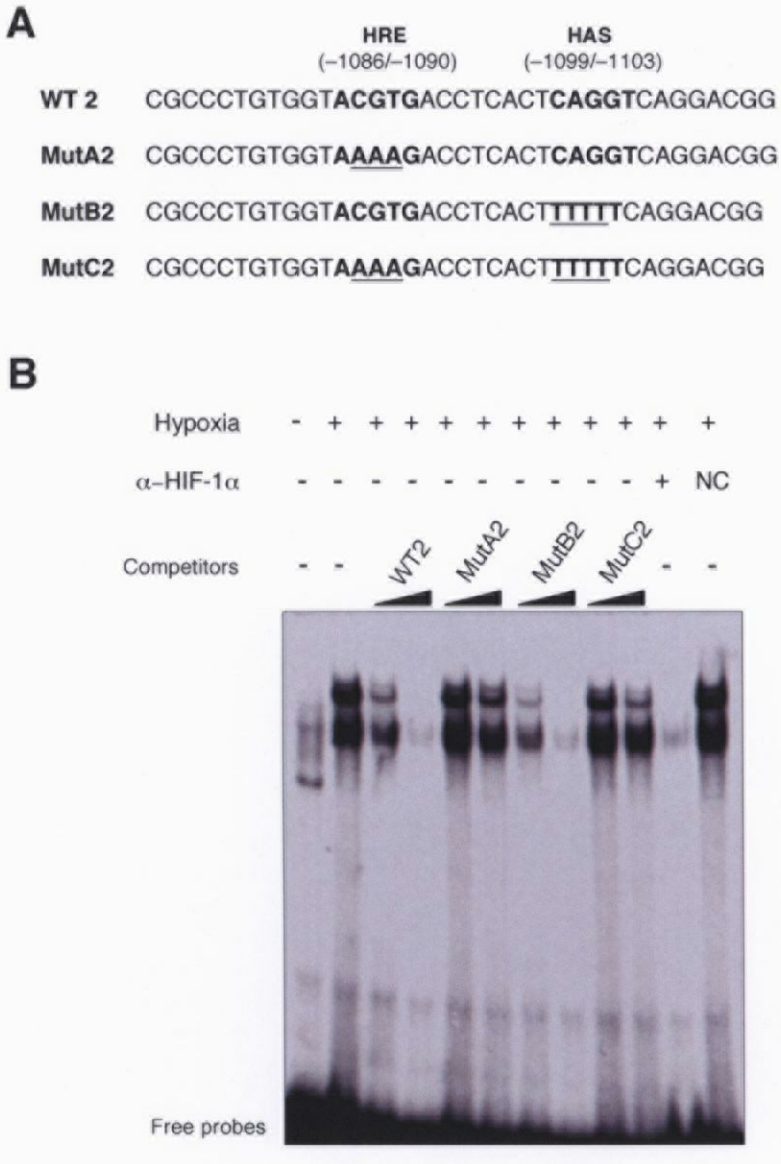


Fig. 2-13. The adjacent HAS dose not affect the HIF-1/HRE binding but is bound to a nuclear protein under normorxic condition. (**A**) Sequences of the oligonucleotide probe (WT2) and various competitors used in EMSA. The HRE and HAS are indicated in bold and mutated sequences are underlined. (**B**) The HAS dose not affect the HIF-1/HRE binding. [^{32}P]-labeled double-stranded WT2 was incubated with nuclear extracts prepared from HepG2 cells kept in 20% or 1% O_2. For competition, unlabeled WT2, MutA2, or MutB2 was added in 10- and 100-fold molar excess, respectively. In the last two lanes, human HIF-1α antibody or mouse IgG (negative control; NC) was added prior to the probe.

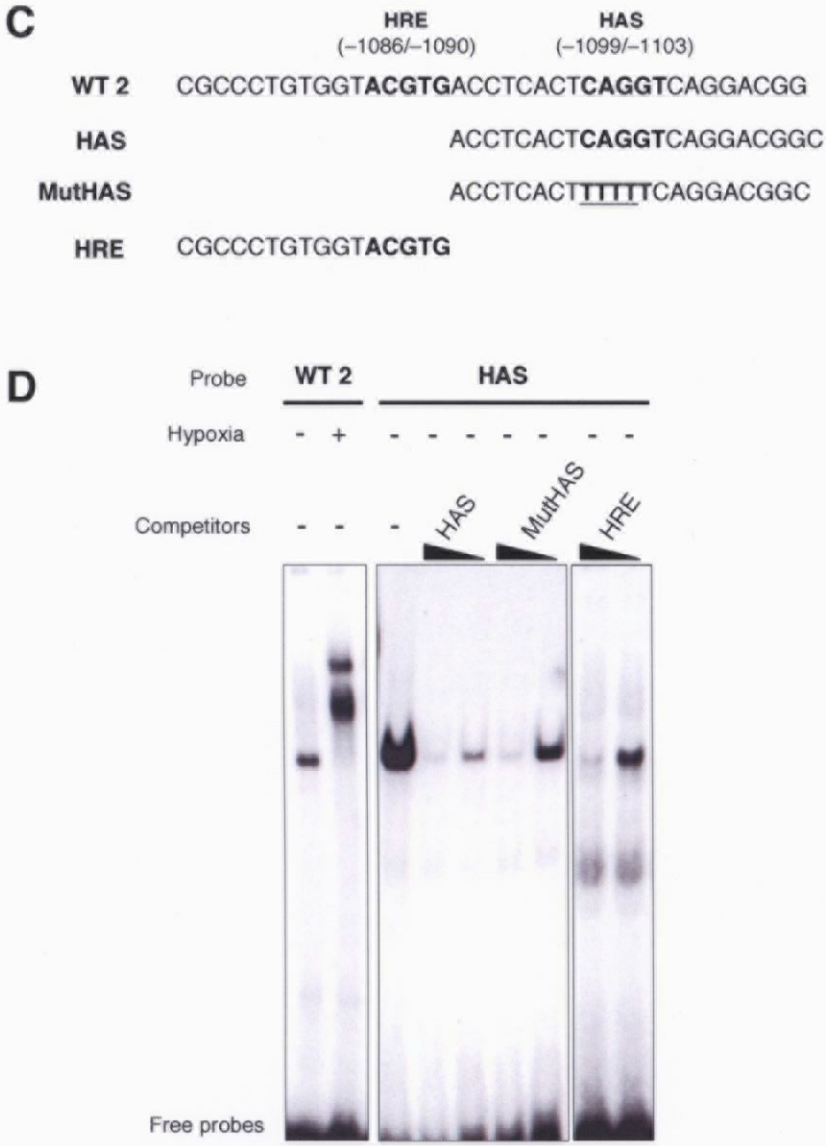


Fig. 2-13 (continued). (**C**) Sequences of the HAS probe and various competitors used. The HRE and HAS are indicated in bold and mutated sequences are underlined. (**D**) [^{32}P]-labeled double-stranded WT2 or HAS was incubated with nuclear extracts prepared from HepG2 cells kept in normoxia or hypoxia. For competition, unlabeled HAS, MuHAS, or HRE were added in 10- and 100-fold molar excess, respectively. The protein/DNA complexes were separated by electrophoresis and visualized by autoradiography.

Table 2-1. The presence of a HAS site adjacent to the functional HRE in known IGFBP-1 promoter regions. HRE is shown in bold, and HAS is underlined.

Genes	HRE
Human IGFBP-1	5'-G<u>CACGG</u>TCTTGGCAGG**ACGTG**CTC-3'
Mouse IGFBP-1	3'-AGG**ACGTG**CATGCAGCC<u>CAGATG</u>G-5'
Rat IGFBP-1	3'-CAC**ACGTG**CTTTCTAGG<u>CACGTC</u>A-5'
Zebrafish IGFBP-1	3'-GGT**ACGTG**ACCTCACT<u>CAGGTC</u>AG-5'
Human VEGF	5'-CAT**ACGTG**GGCTCCAA<u>CAGGTC</u>CT-3'