THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 3, pp. 1582–1589, January 18, 2013

Hypoxia-inducible Factor-1 (HIF-1)-independent Hypoxia Response of the Small Heat Shock Protein *hsp-16.1* Gene Regulated by Chromatin-remodeling Factors in the Nematode *Caenorhabditis elegans*[*] [S]

Received for publication, July 16, 2012, and in revised form, December 8, 2012 Published, JBC Papers in Press, December 10, 2012, DOI 10.1074/jbc.M112.401554

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Background: To characterize an unknown molecular basis of the *hif-1*-independent hypoxia response, we used the nematode *hsp-16.1* gene as a model.
Results: HMG-1.2, together with chromatin-remodeling factors and calcium ions, is involved in the hypoxia response of *hsp-16.1*.
Conclusion: Chromatin modification is important for the hypoxia response of *hsp-16.1* in an HIF-1-independent manner.
Significance: We report an alternative regulatory pathway for the HIF-1-independent hypoxia response.

Oxygen deprivation is accompanied by the coordinated expression of numerous hypoxia-responsive genes, many of which are controlled by hypoxia-inducible factor-1 (HIF-1). However, the cellular response to hypoxia is not likely to be mediated by HIF-1 alone, and little is known about HIF-1-independent hypoxia responses. To better establish the molecular mechanisms of HIF-1-independent hypoxia responses, we sought to characterize the molecular basis of the hypoxia response of the *hsp-16.1* gene in the nematode *Caenorhabditis elegans*; this gene has been shown to be induced by hypoxia independently of *hif-1*. Using affinity purification followed by LC-MS/MS, we identified HMG-1.2 as a protein that binds to a specific promoter region under hypoxic conditions. By systematic prediction followed by validation of these interactions through RNAi, we identified the chromatin modifiers *isw-1* and *hda-1*, histone H4, and NURF-1 chromatin-remodeling factors as new components of the *hif-1*-independent hypoxia response. These data suggest that the modulation of nucleosome positioning at the *hsp-16.1* promoter may be important for the hypoxia response. In addition, we found that calcineurin acts independently of *hif-1* to modulate the cellular response to hypoxia and that calcium ions are necessary for the induction of *hsp-16.1* under hypoxic conditions.

The cellular response to low oxygen levels is critical for organism survival. In normal development and in many diseased states such as cardiovascular disease and cancers involving solid tumors, cells must cope with the challenge of oxygen deprivation. Dynamic changes in the expression of specific hypoxia-responsive genes underlie the cell's metabolic adaptation to low oxygen conditions. Hypoxia-inducible factor (HIF)[3] is known to be a master regulator of this response, and the roles of HIF in the cellular response to changes in the environmental oxygen concentration have been extensively studied (1–4). HIF is a heterodimeric protein composed of the HIF-1α and HIF-1β subunits. Under hypoxic conditions, HIF-1α stabilizes and subsequently binds to the promoters of target genes, thereby activating their transcription (5, 6). *hif-1* is the *Caenorhabditis elegans* ortholog of HIF-1α, and it functions not only in the adaptive response to hypoxia but also in other biological processes, including stress responses, behavioral responses, neuronal development, and aging (7–12).

HIF-1 has been shown to be a transcription factor that is critical for the hypoxia response in animals under various conditions (13, 14); however, it has become obvious that HIF-1 is not the only regulator of this response. Furthermore, the existence of HIF-1-independent mechanisms mediating the hypoxia response has been reported. For example, c-Myc, NF-κB, and AP-1 seem to regulate hypoxia-responsive genes through an HIF-1-independent mechanism in different types of cancer (15). Nonetheless, only a few attempts have been made to elucidate the regulatory mechanism governing HIF-1-independent adaptations to low oxygen levels. In this study, using *C. elegans* as a model organism, we sought to identify an alternative hypoxia response pathway that is not dependent on HIF-1 function. The *C. elegans* homolog of the HIF-1α subunit and, importantly, most of the core signaling pathways in the hypoxia response are conserved through evolution (7, 16). In addition, feeding RNAi provides an easy way to systematically inactivate genes in *C. elegans*. In a study on the hypoxia-induced alteration of gene expression in *C. elegans hif-1* mutants,

* This work was supported in part by the World Class University Program and the Research Center for Functional Cellulomics.
[S] This article contains supplemental "Experimental Procedures," Figs. S1–S7, Table S1, and additional references.
[1] Supported by Korea Student Aid Foundation (KOSAF) Grant S2-2009-000-01151-1 from the Korean Government.
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[3] The abbreviations used are: HIF, hypoxia-inducible factor; MNase, micrococcal nuclease.

it was reported that HIF-1-independent pathways are involved in the adaptation to hypoxia (17). Our previous study also revealed that the response of small heat shock protein *hsp-16* genes (*hsp-16.1* and *hsp-16.2*) to hypoxic conditions is HIF-1-independent and occurs via *cis*-acting DNA sequences (CAC(A/T)CT), hereafter referred to as "block I," in the promoter region of these genes (18).

To establish the HIF-1-independent hypoxia response pathway, we dissected the molecular mechanism that mediates the hypoxia-inducible transcription of the *C. elegans hsp-16.1* gene. We identified a potential factor that is involved in the HIF-1-independent hypoxia response of *hsp-16.1* using affinity chromatography purification followed by LC-MS/MS. To further understand the mechanism, we employed an interactome network approach, which reveals direct and indirect interactions based on yeast, worm, fly, and human orthology (19, 20). Thereafter, we experimentally validated whether these genes are involved in the hypoxia response of *hsp-16.1* expression. Our results indicate that chromatin-remodeling complex proteins are involved in the modulation of *hsp-16.1* expression under hypoxic conditions. We also show that this hypoxia-inducible transcriptional regulation is mediated by calcium signaling. We report the existence of an alternative, HIF-1-independent mechanism by which cells adapt to hypoxic conditions.

EXPERIMENTAL PROCEDURES

Worm Strains and Culture—The standard protocol for maintaining *C. elegans* strains was used as described previously (21). *C. elegans* strains were obtained from the *Caenorhabditis* Genetics Center (Minneapolis, MN). The N2 strain was used as wild-type worms.

GFP Fusion Constructs and Microinjection—The full-length *hsp-16.1*::*gfp* fusion plasmid was constructed by subcloning the full-length PCR product from the genomic T27E4.8 sequence into pPD95.77 (a gift of A. Fire). To generate the *hmg-1.2*::*gfp* construct, DNA was amplified by PCR. The PCR product included the 2-kb region upstream of the F47D12.4 promoter, and the whole genomic sequence was cloned into pPD95.77. Microinjection was carried out using standard procedures. The pRF4 plasmid containing the dominant mutant *rol-6* gene was used as a marker. The *hsp-16.1*::*gfp* transgene was integrated into the genome by UV irradiation using Spectrolinker XL-1000 (Spectronics, Rochester, NY).

RNAi Assay—The bacterial feeding protocol was used in RNAi experiments as described previously (22, 23). HT115 bacteria carrying L4440, the plasmid of the empty vector pPD129.36, were used as a control. Most RNAi feeding clones were obtained from the Ahringer RNAi library (Geneservice, Cambridge, United Kingdom). Feeding vectors containing Y53F4B.3 were obtained from the Vidal RNAi feeding library (Open Biosystems, Huntsville, AL). For *nurf-1*, a 950-bp PCR fragment of cDNA corresponding to nucleotides 251–1201 of F26H11.2 was subcloned into pPD129.36 (a gift of A. Fire). For H20J04.2, a 900-bp sequence of the third exon was amplified by PCR and subcloned into pPD129.36. In most experiments, we placed L4 stage worms (parents or P0) on a plate seeded with a bacterial strain carrying specific RNAi plasmids. For *hda-1*, which is an essential gene that causes lethality when severely knocked down, we placed synchronized L1 stage worms under RNAi conditions and performed a hypoxia assay 3 days later.

Heat Shock and Hypoxia Assay—The heat shock treatment was carried out at 30 °C for 6 h. For hypoxia treatment, we soaked worms in M9 buffer without rocking as described by Hong *et al.* (18). The worms were allowed to recover on nematode growth medium at 20 °C before analysis.

ChIP—The ChIP experiments were adapted from Oh *et al.* (24) and performed using a ChIP assay kit (Upstate). Mixed-stage HMG-1.2::GFP transgenic worms were grown on NGM-lite plates and then harvested. Control worms were rocked, whereas hypoxia-treated worms were soaked in M9 medium without shaking. The worms were fixed in M9 buffer containing 2% formaldehyde at room temperature for 30 min. The reaction was quenched with 2.5 M glycine and washed three times with M9 buffer. Lysates were prepared as described above. The lysates were precleaned with salmon sperm DNA/protein G-agarose beads and incubated overnight at 4 °C with either anti-GFP antibody or IgG. The precipitates were washed, the cross-links were reversed, and the DNA was eluted. Real-time quantitative PCR was performed using Bio-Rad iQ SYBR Green Supermix in a Bio-Rad iQ5 real-time PCR machine. The following primer sets were used: sense, 5′-AGGTGCAAAGAGACGCAGAT-3′; and antisense, 5′-CTAGAACATTCGAGCTGCTT-3′.

Microscopy and Measurement of Fluorescence Intensity—All images were taken using an Axioplan 2 microscope equipped with an AxioCam HRc camera and AxioVision 4.7 software (all Zeiss). Fluorescence intensity was analyzed using ImageJ software by outlining the second intestinal cells of the worms. The density was normalized to the L4440 control. Either a one-way analysis of variance ($p < 0.05$) or an unpaired t test was employed to find genes that were significantly different between the control and RNAi at each time. All of the data are expressed as the mean ± S.E.

Nucleosome Preparation and Micrococcal Nuclease Assay—Mixed-stage worms were pelleted and frozen in buffer A (250 mM sucrose, 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM EGTA, 0.2 mM PMSF, and 7 mM β-mercaptoethanol) and protease inhibitor set III (Calbiochem). The worms were ground into a fine powder in liquid nitrogen. The resultant worm dust was resuspended in buffer A. Micrococcal nuclease (MNase; Roche Applied Science), resuspended at 300 units/μl in 10 mM Tris-HCl (pH 7.4), 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, and 0.5 mM spermidine, was added to the worm extract. Digestions were performed at 25 °C and stopped by the addition of worm lysis buffer (0.1 M Tris-HCl (pH 8.5), 0.1 M NaCl, 50 mM EDTA, and 1% SDS), and the sample was treated with proteinase K (20 mg/ml) for 45 min at 65 °C. Purified DNA without MNase treatment was digested with BglII (Enzynomics), and PstI, RsaI (Fermentas), or XbaI (Roche Applied Science). The same amount of DNA was loaded onto a 1.4% agarose gel, and the separated DNAs were transferred to a nylon membrane. The 200-nucleotide fragment of DNA close to the BglII site within the *hsp-16.48* gene as indicated in Fig. 3 was ^{32}P-labeled using a random prime labeling kit (Amersham Biosciences) for indirect end-label analysis.

EGTA and Thapsigargin Treatment—Worms were treated with EGTA under hypoxic and heat shock conditions at different concentrations (0, 100, 200, and 400 mM). Thapsigargin (Sigma) was used at a final concentration of 1.25 μM while rocking for 6 h. As a control experiment, the same amount of dimethyl sulfoxide was used in parallel.

RESULTS

Identification of HMG-1.2 as a Hypoxia-induced Promoter-binding Protein—Previously, we showed that the sequence CAC(A/T)CT (block I) is required for the HIF-1-independent hypoxia response of *hsp-16* genes; we also demonstrated that regulatory proteins bind to block I-containing sequences *in vitro* (18). To identify the regulatory proteins that bind the *hsp-16.1* promoter under hypoxic conditions, we isolated nuclear extracts from worms incubated under hypoxic conditions and applied them to streptavidin affinity columns containing biotin-labeled block I-containing sequences. As a negative control, we used a biotin-labeled probe in which the block I sequences were mutated (supplemental Fig. S1). We analyzed the isolated proteins using MALDI-TOF and quadrupole time-of-flight mass spectrometry (Fig. 1*A* and supplemental Fig. S2). By comparing the results from the block I-containing column with those from the block I-mutated column, we excluded most bound proteins as nonspecific binders, including HMG-1.1. This analysis showed that HMG-1.2 was the predominant protein that was bound specifically to the block I-containing sequence. We then performed ChIP to evaluate whether HMG-1.2 was bound to the block I-containing promoter *in vivo* and whether the binding efficiency of HMG-1.2 was affected by hypoxia. We found that HMG-1.2 binding was enriched by >20-fold under hypoxic conditions compared with normoxic conditions (Fig. 1*B*). Taken together, our results suggest that HMG-1.2 proteins specifically bind to the block I element of the *hsp-16.1* promoter in response to hypoxic conditions.

Role of HMG-1.2 in the Hypoxia Response of hsp-16.1—To investigate the potential roles of HMG-1.2 in the HIF-1-independent response to hypoxia, we performed RNAi to reduce HMG-1.2 activity. L4 larvae were fed dsRNA-producing bacteria. Thereafter, we monitored the expression of *hsp-16.1* fused with GFP (*hsp-16.1::gfp*) in F1 progeny exposed to hypoxic conditions. We also determined whether the expression of *hsp-16.1* was regulated in a hypoxia-specific manner or as a general response to stress. To do so, worms were also incubated under heat shock conditions, as heat shock can induce *hsp-16.1* gene expression (25, 26). Under hypoxic conditions, disruption of *hmg-1.2* activity by RNAi, compared with the empty vector control, led to an ~60% decrease in the level of HSP-16.1::GFP fusion protein. On the other hand, *hmg-1.2* RNAi did not affect the induction of HSP-16.1 in response to heat shock treatment (Fig. 1, *C* and *D*). These observations suggest that induction of *hsp-16.1* by hypoxia, but not by heat shock, specifically required functional HMG-1.2. As a negative control, we observed no significant change in HSP-16.1::GFP expression in response to either stress in the *hmg-1.1* RNAi animals.

We also examined the effect of *hmg-1.2* inactivation by RNAi on the activation of *hsp-16.1* in the *hif-1*(*ia04*) background. We found that *hsp-16.1* was strongly induced in *hif-1*(*ia04*) mutants and that the induction of *hsp-16.1* was decreased by *hmg-1.2* RNAi but not by *hmg-1.1* RNAi in these mutant animals. Importantly, these observations were similar to those in wild-type animals (supplemental Fig. S3, *A* and *B*). Induction of *hsp-16.1* by heat shock was not affected in *hif-1*(*ia04*) mutants. These results are consistent with the notion that the hypoxia response of *hsp-16.1* is not mediated by the *hif-1* pathway.

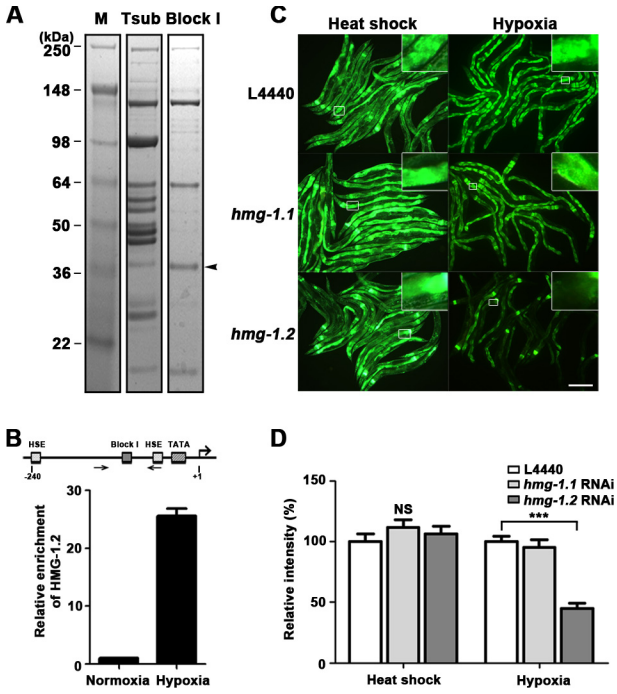

FIGURE 1. **HMG-1.2 is a potential regulator of the *hsp-16.1* hypoxia response.** *A*, identification of block I-binding proteins. Purified proteins were separated on SDS-polyacrylamide gels and strained with Coomassie Blue. The identities of the bands were confirmed by MALDI-TOF and quadrupole time-of-flight mass spectrometry. *First lane*, markers (*M*); *second lane*, eluate bound to T-substituted (*Tsub*) sequence; *third lane*, eluate bound to block I. Molecular mass standards (in kilodaltons) are shown on the left. The *arrowhead* indicates HMG-1.2. *B*, ChIP with anti-GFP antibody and real-time quantitative PCR revealed that HMG-1.2 is directly associated with block I under hypoxic conditions. *HSE*, heat shock response element; *TATA*, TATA box. The *arrows* indicate the primers used for real-time quantitative PCR. *C*, a reduction in *hmg-1.2* function significantly down-regulated the induction of *hsp-16.1* under hypoxia, whereas *hmg-1.1* RNAi did not affect the *hsp-16.1* hypoxia response. Inlets are the second intestine cells that show the most consistent fluorescence intensity within worms. All of the photographs were taken under identical exposure conditions. *Scale bar* = 200 μm. *D*, the relative fluorescence intensity of stress-induced HSP-16.1::GFP was quantified using ImageJ software. Statistical significance was determined using a one-way analysis of variance and Dunnett's multiple comparison test with the hypoxia controls (***, $p < 0.001$). *NS*, not significant. *Error bars* indicate S.E.

Hypoxia-inducible Expression of hsp-16.1 through Chromatin-remodeling Factors and Histone 4—It has been suggested that if two genes are connected in any way, they are likely to participate in a common biological process (27). Therefore, we used functional gene networks to further characterize the mechanism by which *hmg-1.2* mediates the HIF-1-independent hypoxia response. We used tools provided by the WormNet v.2 (19, 20) that can predict the genetic or physical interaction of a gene with a gene of interest, in our case, *hmg-1.2*. WormNet provides functional gene networks by combining different types of data such as co-cited gene associations, mRNA coexpression, protein-protein interaction, physical interaction, and genetic interaction among *C. elegans*, yeast, fly, and human

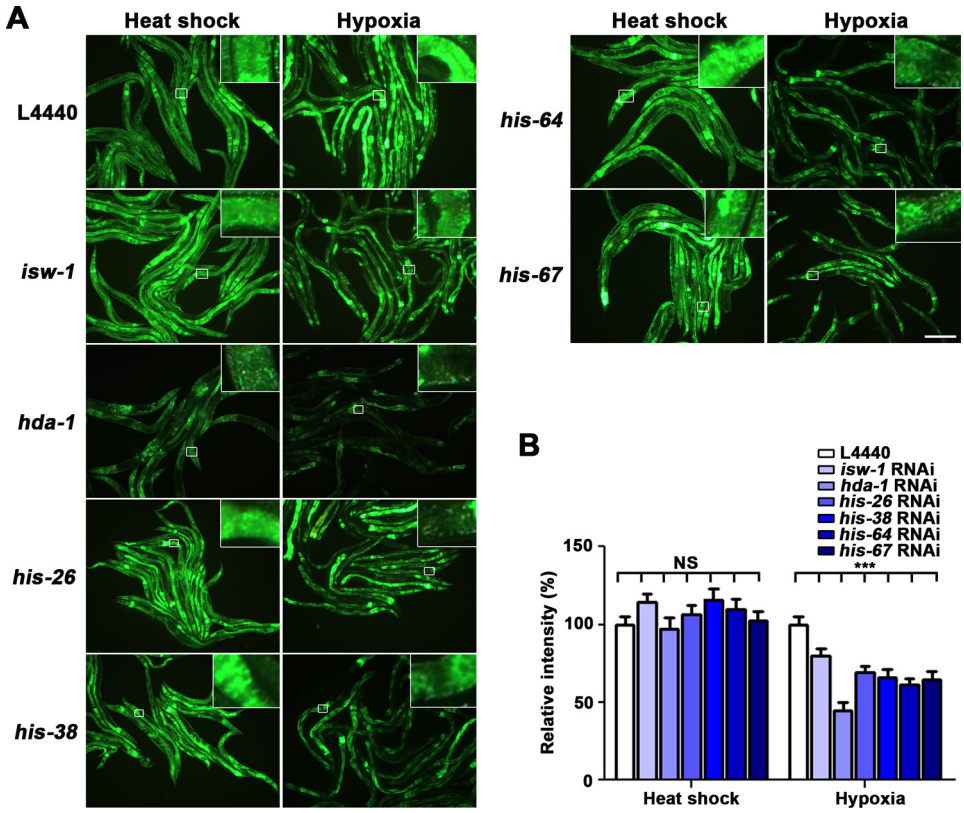

FIGURE 2. **Chromatin-remodeling components histone deacetylase and histone H4 are involved in the *hsp-16.1* hypoxia response.** *A*, a reduction in *isw-1*, *hda-1*, *his-26*, *his-38*, *his-64*, and *his-67* RNAi suppressed the hypoxia up-regulation of *hsp-16.1*. *Scale bar* = 200 μm. *B*, quantification of the relative intensity of HSP-16.1::GFP fluorescence was carried out using ImageJ software. ***, values that differ from the L4440 hypoxia controls at the $p < 0.001$ significance level (Dunnett's test). *NS*, not significant. *Error bars* indicate S.E.

proteins (19, 20, 28). Probability of interactions is scored with a Bayesian network (28). Genes predicted to interact with *hmg-1.2* included a transcription factor (*mab-5*); a nuclear transport factor (28); a protein kinase (*hpk-1*); and several chromatin-associated genes such as a predicted subunit of FACT (facilitates chromatin transcription; *hmg-4*), components of the chromatin-remodeling complex (*psa-4* and *isw-1*), histone H4-coding genes, an H2A.Z histone variant (*htz-1*), and histone deacetylase (*hda-1*) (supplemental Table S1). We then inactivated the candidate genes using RNAi to test whether they were involved in the hypoxia response of *hsp-16.1*. Although we found no effect for some genes, including *mab-5*, *ima-3*, *hpk-1*, *hmg-4*, *psa-4*, and *htz-1*, we did find that the inhibition of *his-26*, *his-38*, *his-64*, *his-67*, *isw-1*, and *hda-1* by RNAi decreased the expression of the *hsp-16.1* gene under hypoxic conditions (Fig. 2, *A* and *B*). Given the sequence similarity between *his-26*, *his-38*, *his-64*, and *his-67* and their similarity to other histone H4 genes (supplemental Fig. S4), it is conceivable that the inactivation of the four histone H4 genes by RNAi may also have resulted in the silencing of other histone H4 genes. ISW-1 is an ortholog of *Drosophila* ISWI and acts as an ATPase in chromatin-remodeling complexes. HDA-1 is a histone deacetylase involved in chromatin modification. Our results thus suggest that chromatin modifiers are involved in the hypoxia response of *hsp-16.1*. Furthermore, the inactivation of *isw-1* and histone H4-coding genes by RNAi in *hif-1*(*ia04*) deletion mutants showed results that were consistent with the HIF-1 independence of *hsp-16.1* in wild-type worms (supplemental Fig. S5, *A* and *B*).

The NURF Chromatin-remodeling Complex May Mediate the hif-1-independent Hypoxia Response of hsp-16.1—There are three chromatin-remodeling complexes that contain the ATPase subunit ISWI: ACF (ATP-dependent chromatin assembly and remodeling factor) (29), CHRAC (chromatin accessibility complex) (30), and NURF (nucleosome-remodeling factor) (31). Genes encoding these components of chromatin-remodeling complexes are well conserved in *C. elegans*: ACF-1 (*flt-1* and *athp-2*), CHRAC-14 (T27A5.8), and CHRAC-16 (Y53F4B.3) (32). In the *Drosophila* NURF complex, ISWI is composed of NURF301, NURF38, and NURF55 (33, 34); these subunits are homologous to *nurf-1*, *pyp-1*, and *rba-1* in *C. elegans*, respectively. We determined whether the *C. elegans* orthologs of the ACF, CHRAC, and NURF proteins were required for the hypoxia response of *hsp-16.1*, similar to *isw-1*. Inactivation of *flt-1*, *athp-2*, T7A5.8, and Y53F4B.3 did not affect the induction of *hsp-16.1* in response to hypoxia (supplemental Fig. S6). Only RNAi inhibition of the gene encoding the NURF301 ortholog failed to fully induce *hsp-16.1* by hypoxia, but not by heat shock (Fig. 3*A*). Furthermore, we found that NURF-1 regulation of *hsp-16.1* was independent of HIF-1 (supplemental Fig. S7, *A* and *B*). Our results indicate that ISW-1 likely functions as a component of the NURF complex to regulate the expression of *hsp-16.1* in response to hypoxia and that this activity is independent of HIF-1.

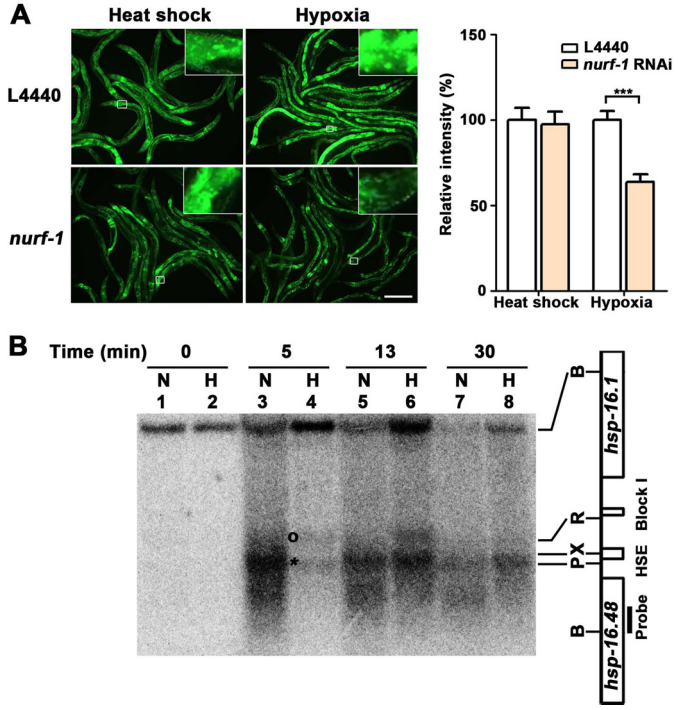

FIGURE 3. **NURF-1 chromatin-remodeling factor mediates the hypoxia response of the *hsp-16.1* gene.** *A*, decreased *nurf-1* function (*Drosophila* NURF301) reduced the activation of *hsp-16.1* by hypoxia. The results for ACF and CHRAC RNAi are presented in supplemental Fig. S7. Quantification of the relative intensity of HSP-16.1::GFP fluorescence was carried out using ImageJ software. Statistical significance was determined using an unpaired *t* test (***, $p < 0.001$). *Error bars* indicate S.E. *Scale bar* = 200 μm. *B*, MNase digestion patterns of the normoxic (*N*) and hypoxic (*H*) chromatins. The DNA of MNase-digested nuclei was digested by BglII and analyzed by Southern blot hybridization using a 200-nucleotide probe made from the region designated in the figure. Chromatin was incubated in the presence of MNase for 0 min (*lanes 1* and *2*), 5 min (*lanes 3* and *4*), 13 min (*lanes 5* and *6*), and 30 min (*lanes 7* and *8*). The *asterisk* indicates the genomic position that is more sensitive to MNase cleavage in normoxia. The *circle* indicates the band with increased sensitivity to MNase cleavage in hypoxia. *B*, BglII; *P*, PstI; *X*, XbaI; *R*, RsaI; *HSE*, heat shock response element.

Hypoxia Response Occurs through Modification of the Chromatin Environment—Next, we investigated the roles of ISW-1 and NURF-1 during hypoxia. Because NURF has been shown to have nucleosome disruption activity *in vitro* (35, 36) and because the chromatin structure of *hsp-16* genes has been shown to markedly differ upon heat shock treatment (37), we decided to examine whether hypoxia affected the chromatin structure of the *hsp-16.1* promoter *in vivo*. We purified nuclei from control and hypoxia-treated worms and subsequently performed MNase digestion coupled with Southern blot analysis to map nucleosome positioning. Purified nuclei were free from endonucleases, as no degraded bands were observed (Fig. 3*B*, *lanes 1* and *2*). The differences in nucleosome arrays were obvious when nuclei were partially digested by MNase (*lanes 3–6*). To map the relative positions, we digested naked DNA with restricted enzymes (data not shown). An MNase-hypersensitive site (which marks the heat shock response element) was detected under normoxic conditions near the PstI and XbaI sites (*asterisk*, *lanes 3*, *5*, and *7*), and this was attenuated in hypoxia (*lanes 4* and *8*). In contrast, an MNase-sensitive site (which corresponds to the block I region) appeared in hypoxia-stressed nuclei (*circle*, *lanes 4* and *6*). These data suggest that nucleosome remodeling has occurred along the block I region in response to hypoxia and that the block I region is exposed outside of nucleosomes, which in turn results in the transcriptional activation of *hsp-16.1*.

Calcium Signaling May Mediate the Hypoxia Response in an HIF-1-independent Manner—Cells are known to increase intracellular calcium levels as a primary response to hypoxia (38). Furthermore, the binding of calcium to the C-terminal domain of HMGB1, another mammalian homolog of HMG-1.2, is known to modulate the DNA-binding properties HMGB1 (39, 40). Therefore, we investigated whether the disruption of the cellular calcium balance affected the hypoxia response of *hsp-16.1*. We used chemical reagents to manipulate the endoplasmic reticulum release of calcium: thapsigargin, which induces the release of endoplasmic reticulum calcium, and EGTA, which specifically chelates calcium (41). We found that treatment with EGTA decreased the induction of *hsp-16.1* in response to hypoxia in a concentration-dependent manner, whereas heat shock-responsive activation remained unaffected (Fig. 4, *A* and *B*). In contrast, thapsigargin treatment was sufficient to activate *hsp-16.1* at normal oxygen concentrations (Fig. 4*C*). Interestingly, *tax-6*, which encodes the catalytic subunit of calcineurin, was up-regulated in the *hif-1*(*ia04*) mutant animals under hypoxic conditions (17). Calcineurin contains a catalytic subunit, calcineurin A, and a regulatory subunit, calcineurin B, which are encoded by the *C. elegans* genes *tax-6* and *cnb-1*, respectively (41, 51). We found that inactivating *tax-6* or *cnb-1* resulted in failure to induce *hsp-16.1* expression under hypoxia (Fig. 4, *D* and *E*). This phenotype is similar to that of *hif-1*(*ia04*) mutants (supplemental Fig. S7, *A* and *B*). Taken together, our results indicate that changes in cellular calcium levels generate a primary signal that induces the expression of *hsp-16.1* in response to hypoxia.

DISCUSSION

In this study, we sought to better establish the molecular mechanisms of HIF-1-independent hypoxia responses in *C. elegans*. We have shown that chromatin-remodeling factors, including HMG-1.2, are involved in the *hif-1*-independent hypoxia response of *hsp-16.1* expression. In addition, we have shown that calcium is important for induction of *hsp-16.1* transcription in response to hypoxia. Taking all of our data and previous studies together, we propose a mechanism for the HIF-1-independent hypoxia response (Fig. 5). As this regulatory mechanism may not act specifically on *hsp-16.1*, future study will be needed to investigate the extent to which this mechanism is involved in hypoxia-responsive regulation. Because chromatin-remodeling factors are well conserved in evolution, it is conceivable that a similar mechanism of *hif-1*-independent hypoxia response may occur in mammals.

HMG-1.2 is a homolog of the human HMGB2 protein and exhibits ~55% identity within the HMG boxes (42). HMGB proteins are non-histone chromatin-binding proteins that are generally thought to have limited sequence-specific DNA recognition ability, preferring to bind to bent DNA and four-way junction DNA (43). Nevertheless, evidence was found in *C. elegans* that HMG-1.2 might also have site-specific sequence recognition abilities, *e.g.* in Wnt signaling in specific develop-

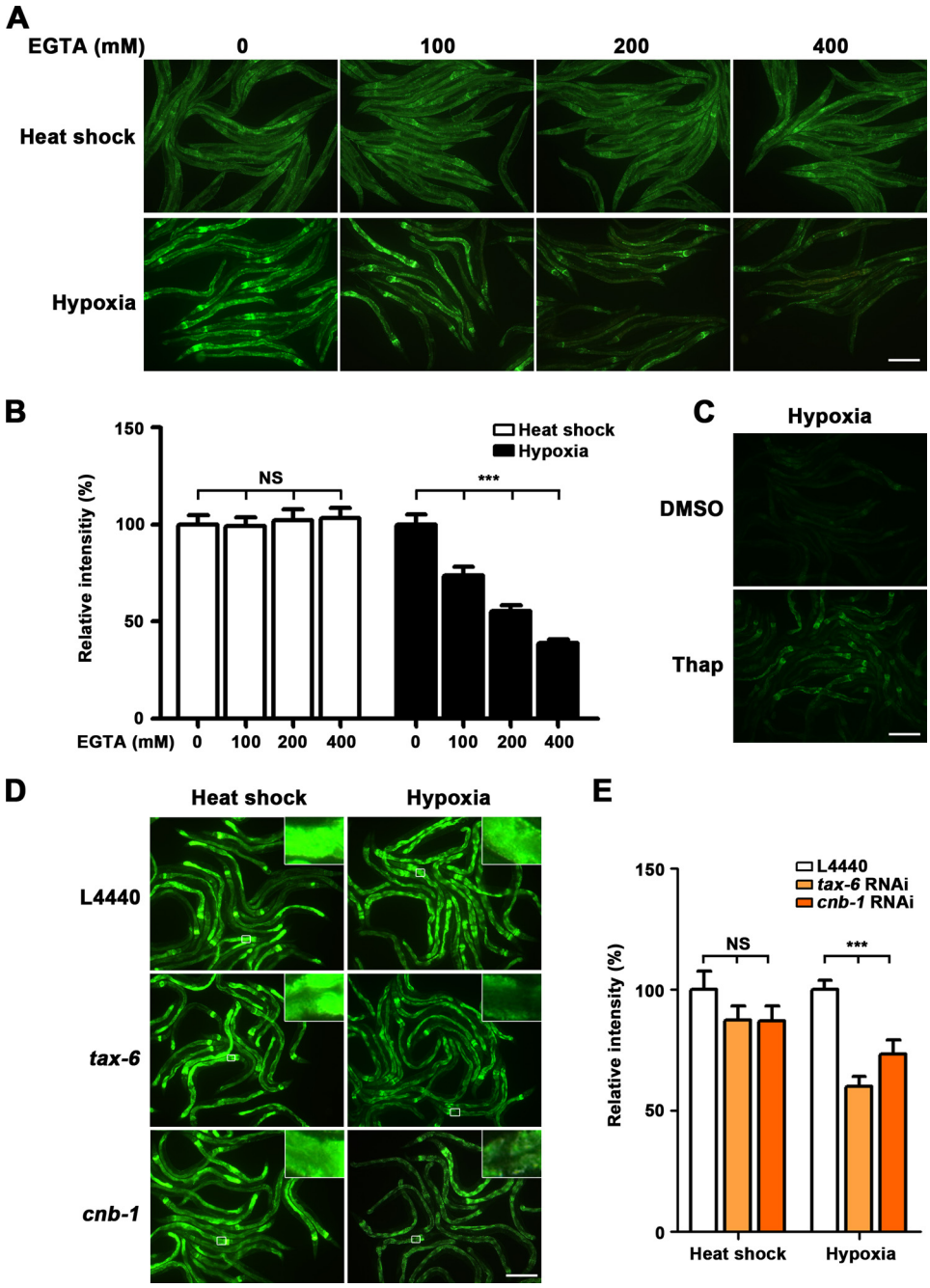

FIGURE 4. **Manipulation of calcium balance affects hypoxia-induced *hsp-16.1* expression.** *A*, treatment with the calcium chelator EGTA at different concentrations (0, 100, 200, and 400 mM). EGTA-driven depletion of calcium decreased the induction of *hsp-16.1* under hypoxia but not with heat shock treatment. However, EGTA did not affect the heat shock-inducible expression of *hsp-16.1*. *B*, quantification of the relative intensity of HSP-16.1::GFP. ***, $p < 0.001$ (Dunnett's test). *NS*, not significant. *C*, thapsigargin (*Thap*)-induced Ca^{2+} release mimicked the effect of hypoxia to activate the expression of *hsp-16.1*. *DMSO*, dimethyl sulfoxide. *D*, inactivation of calcineurin decreased the activation of *hsp-16.1* under hypoxia. *E*, quantification of the relative intensity of HSP-16.1::GFP fluorescence was carried out using ImageJ software. ***, values that differ from the L4440 hypoxia controls at the $p < 0.001$ significance level (Dunnett's test). *Error bars* indicate S.E. *Scale bars* = 200 μm.

mental processes (42). The sequence specificity of HMG-1.2 may allow it to regulate transcription of the *hsp-16.1* gene under oxygen-deprived conditions independently of HIF-1. It would be of a great interest to determine whether genes other than *hsp-16.1* and *hsp-16.2* that contain the block I sequences at their promoter regions also require HMG-1.2 for their response to hypoxia.

A functional gene network enabled us to predict interplays between HMG-1.2 and chromatin-remodeling factors. It is known that HMGB proteins bind to nucleosomes, thereby loosening the wrapped DNA and enhancing its accessibility to chromatin-remodeling complexes (43, 44). HMGB proteins have also been suggested to accelerate the sliding activities of chromatin remodeling and to enhance the binding of chromatin-remodeling factors to nucleosomal DNA (44). In addition, recent genetic evidence has shown that HMG-1.2 is up-regulated by hypoxia and that knockdown of *hmg-1.2*, *isw-1*, and *hda-1* significantly increases sensitivity to hypoxia (45). These

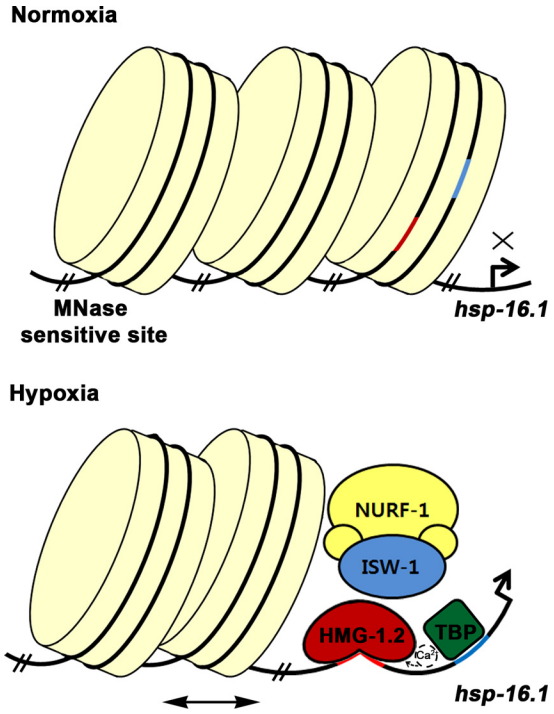

FIGURE 5. **Model of HIF-1-independent hypoxia regulation of *hsp-16.1*.** Under normal oxygen conditions, the block I region (*red line*), surrounded by nucleosomes, may not activate the transcription of *hsp-16.1*. Hypoxia induces the release of calcium from the endoplasmic reticulum and influences HMG-1.2 in a way that HMG-1.2 has a higher affinity for DNA. In concert with HMG-1.2, ISW-1 and the NURF-1 remodeling complex are involved in remodeling the positioning of the nucleosomes on block I. The *horizontal bidirectional arrow* indicates a possible nucleosome sliding. Once HMG-1.2 binds to DNA, it can bend the DNA to allow efficient binding by several elements of the transcription machinery such as TATA-binding protein (*TBP*). The TATA box is indicated by the *blue line*.

studies support a relationship between HMG-1.2 and chromatin modifiers under hypoxic conditions. Moreover, the N terminus of histone H4 is essential for stimulating ISWI ATPase activity and inducing nucleosome sliding (46, 47). This suggests that histone H4 may act to modulate the activity of chromatin remodelers in the hypoxia response. It would be interesting to investigate whether the deacetylation at the N-terminal region of histone H4 proteins by HDA-1 is involved in this modulation.

Our data suggest that ISW-1 may act with NURF chromatin-remodeling factors to modulate the hypoxia response of *hsp-16.1* independently of HIF-1 and that NURF-mediated unwrapping of the block I region by nucleosome movement may contribute to the activation of *hsp-16* under hypoxic conditions. Additionally, *nurf-1* is up-regulated by hypoxia in *hif-1*-deficient animals (17). This implies that NURF-1 is regulated under hypoxia independently of HIF-1. Unfortunately, because the null mutants of *hmg-1.2*, *isw-1*, and *nurf-1* showed severe defects in both development and fertility, we could not determine whether the nucleosome remodeling was abolished in these null mutants *in vivo*. However, ISWI and NURF are known to promote nucleosome sliding at promoters, which leads to the disruption of regularly ordered arrays and thereby activates gene transcription (36, 48). Given this knowledge, we propose that the chromatin-remodeling complex of ISW-1 and NURF-1 catalyzes the nucleosome sliding at the *hsp-16.1* promoter to facilitate transcriptional activation and that transcriptional regulation via nucleosome and chromatin modifications is important for the regulation of the hypoxia response. As knockdown of *pyp-1* and *rba-1* resulted in embryonic lethality, we could not test whether these genes were involved in the hypoxia response.

Our finding that the hypoxia response of *hsp-16.1* is calcium-dependent is consistent with the idea that calcium release is a landmark of early hypoxia response. There are several possible explanations for how *hsp-16.1* expression is regulated by calcium signaling under hypoxic conditions. First, an increase in the intracellular calcium levels increases the affinity of HMG-1.2 for DNA, which is necessary to induce the hypoxia response of *hsp-16.1*. Another possibility is that calcineurin activated by a high cytosolic calcium level may dephosphorylate unidentified substrates, which in turn positively modulate the HIF-1-independent hypoxia response of *hsp-16.1*. It would be of interest to pursue the issue of the calcium action mechanism in hypoxia response.

HIF-1α is overexpressed in common human cancers, in cells in the center of a solid tumor, which experience hypoxia (49). Although HIF-1α has been considered to be a major target for tumor therapy, HIF-1 is not the only regulator of the hypoxia response in cancer cells (50). Our findings propose a novel alternative mechanism that regulates gene induction in hypoxia in an HIF-1-independent manner. Because *C. elegans* cellular adaptations to hypoxia are homologous to those found in mammals, HMG-1.2 and the ISW-1 chromatin factor may be therapeutic targets, in addition to HIF-1, and could block hypoxia-induced responses in a combinatorial manner in cancer cells.

Acknowledgment—We thank the Caenorhabditis Genetics Center for the nematode strains.

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