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Biochemical and Biophysical Research Communications 351 (2006) 306–311
BBRC
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Hypoxia-inducible factor-1 mediates the expression of DNA polymerase ι in human tumor cells

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Received 6 October 2006
Available online 16 October 2006

Abstract

Hypoxia generated in tumors has been shown to contribute to mutations and genetic instability. However, the molecular mechanisms remain incompletely defined. Since reactive oxygen species (ROS) are overproduced immediately after reoxygenation of hypoxic cells and generate oxidized guanine, we assumed that the mechanisms might involve translesion DNA polymerases that can bypass oxidized guanine. We report here that hypoxia as well as hypoxia mimetics, desferrioxamine, and $CoCl_2$, enhanced the expression of DNA polymerase ι (pol ι) in human tumor cell lines. Searching the consensus sequence of hypoxia response element to which HIF-1 binds revealed that it locates in the intron 1 of the *pol ι* gene. These results suggest that HIF-1-mediated *pol ι* gene expression may be involved in the generation of translesion mutations during DNA replication after hypoxia followed by reoxygenation, thereby contributing to the accumulation of genetic changes in tumor cells.

Keywords: Hypoxia; HIF-1; Translesion DNA polymerase; DNA polymerase ι; Reactive oxygen species; 8-Oxoguanine

Tumor cells under hypoxic conditions activate many genes including those related to cell survival, glycolysis, and angiogenesis [1,2]. The oxygen sensing mechanisms have been extensively studied and revealed to involve hypoxia-inducible factors (HIFs) as key regulatory transcription factors that are composed of HIF-α subunit and HIF-β/ARNT subunit [1,2]. Under normoxic conditions, the α subunit of HIF-1 (HIF-1α) is hydroxylated at proline-564 and proline-402 residues by specific Fe^{2+}, oxoglutarate, and oxygen-dependent hydroxylases, recognized and ubiquitinated by an E3 ubiquitin ligase complex consisting of the tumor suppressor VHL (von Hippel–Lindau), elongin B and elongin C, and rapidly degraded through the ubiquitin-proteasome pathway while the β subunit of HIF-1 (HIF-1β) is constitutively expressed [3]. Under hypoxic conditions, HIF-1α is stabilized, allowing its nuclear translocation and dimerization with HIF-1β [4]. Chelating or substituting Fe^{2+} with desferrioxamine or $CoCl_2$, respectively, reduces the hydroxylase activity and mimics hypoxia [5,6]. In the nucleus, HIF-1 binds to the hypoxia response element (HRE) of hypoxia-inducible genes and transactivates their transcription. The consensus sequence of HRE has been shown to be 5′-(A/G)CGTG-3′ [7].

It has been shown that exposure of cells to hypoxia results in increased frequencies of point mutations such as C:G → A:T and T:A → G:C transversions [8]. Although the mechanisms underlying hypoxia-induced mutagenesis are not well understood, reactive oxygen species (ROS) are inevitably involved in the mechanisms because ROS are overproduced during reoxygenation and generate

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0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.10.048

highly mutagenic base 8-hydroxyguanine (8-oxo-dG) [9]. The presence of 8-oxo-dG in DNA causes C:G → A:T and T:A → G:C transversions in DNA, since, unless repaired, 8-oxo-dG allows the misincorporation of cytosine and adenine nucleotides opposite the lesion during DNA replication [10].

Recently, several studies suggest the involvement of the deregulation of DNA repair pathways in hypoxia-induced mutagenesis [8,11,12]. Mihaylova et al. reported decreased expression of the DNA mismatch repair gene *Mlh1* under hypoxic conditions [11]. Koshiji et al. demonstrated that HIF-1 induces genetic instability by transcriptionally down-regulating the expression of MutSα which recognizes base mismatches [12]. Hypoxia may cause mutagenesis, at least in part, by hindering repair of ROS-induced DNA damage through down-regulation of DNA mismatch repair enzymes.

To date, several DNA polymerases that are clearly involved in translesion synthesis including Pol η (RAD30), Pol ι (RAD30B), Pol θ, Pol κ, and Rev1 which belong to the Y superfamily have been reported [13]. One of their most distinct features is a high error propensity during DNA synthesis. Among these Y family polymerases, Pol η, Pol ι, and Pol κ can efficiently bypass 8-oxo-dG [14–16]. Pol μ which belongs to the X superfamily has also been reported to possess efficient lesion bypass activities in response to several types of DNA damage including 8-oxo-dG [17].

We hypothesized that translesion DNA polymerases that can bypass 8-oxo-dG might be involved in the generation of mutations after hypoxia/reoxygenation. We report here that hypoxia enhances the expression of *pol ι* gene through HIF-1 interaction with the consensus HRE site in the intron 1 of the gene.

Materials and methods

Cells and cell culture. Human cervical carcinoma HeLa cells, hepatocarcinoma HepG2 cells, mammary carcinoma MCF-7 and MDA-MB-231 cells, lung adenocarcinoma A549 cells, fibrosarcoma HT1080 cells, colon carcinoma LS174T cells, and glioma U87MG cells were cultured at 37 °C in a humidified atmosphere with 21% O_2/5% CO_2 (normoxia) or 1% O_2/5% CO_2 (hypoxia).

Detection of ROS generation. ROS generation was detected with 2′,7′-dichlorofluorescin diacetate (DCFH-DA) (Molecular Probe, Inc., Eugene, OR) as described previously [18].

Immunostaining of 8-oxo-dG. HeLa cells on glass coverslips were cultured in 21% O_2, or 1% O_2 for 24 h, or 1% O_2 for 24 h followed by 21% O_2 for 30 min. Staining for 8-oxo-dG with anti-8-oxo-dG antibody 1F7 (Trevigen, Inc., Gaithersburg, MD) was performed according to the manufacturer's instructions with some modifications. Briefly, the cells fixed with 70% ethanol at −20 °C were treated with RNase (100 μg/ml) in 10 mM Tris–HCl, pH 7.5, and 1 mM EDTA, and 0.4 M NaCl for 1 h at 37 °C. DNA was denatured with 4 N HCl for 7 min at room temperature. After neutralization, the cells were incubated with 10% fetal bovine serum, and then incubated with anti-8-oxo-dG antibody at 4 °C overnight followed by TRITC-conjugated goat anti-mouse IgG. The nuclei were stained with DAPI (1 μg/ml).

SDS–PAGE and Western blotting. Total cell lysates were prepared by directly solubilizing cells in SDS sample buffer. Nuclear extracts were prepared by using Nuclear Extract Kit (Active Motif, Carlsbad, CA). Proteins were resolved by SDS–PAGE and transferred to nitrocellulose membrane. The membrane was incubated with mouse anti-HIF-1α (Novus Biologicals, Littleton, NO), goat anti-pol ι antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or rabbit anti-E2F-1 antibody (Santa Cruz Biotechnology) followed by incubation with appropriate horseradish peroxidase-conjugated secondary antibody. Proteins were detected using ECL Western blotting detection reagents (Amersham Biosciences, Buckinghamshire, UK).

Semi-quantitative RT-PCR. One microgram of total RNA, which was extracted with guanidinium thiocyanate, was reverse transcribed into cDNA, and the resulting cDNA was used for amplification of target cDNAs using *rTaq* DNA polymerase (TOYOBO, Osaka). The sense and antisense oligonucleotide primers used for PCR were: 5′-GCTGTG CTGGAGTGGCTTCT-3′ and 5′-GCCAGAGCGTGAAGTAGTTG-3′ for *pol ι*, 5′-GCCATGCCAGGATTTATTGCTA-3′ and 5′-CTCCTTTG TTGGTGTTTCCT-3′ for *pol κ*, 5′-ACCCAGGCAACTACCCAAAAC-3′ and 5′-GGGCTCAGTTCCTGTACTTTG-3′ for *pol η*, 5′-AGCCTG-TACCTGTGGAGTGC-3′ and 5′-CCAGGCGGGTAGGGGACTCA-3′ for *pol μ*, 5′-ATGCCTCAACCGTGGACAAT-3′ and 5′-CTTGCT CTCGATGTGCTGC-3′ for *Mlh1*, 5′-GCAGAATCATCACGAA GTGG-3′ and 5′-GCATGGTGATGTTGGACTCC-3′ for *VEGF*, and 5′-TGACGGGGTGACCCACACTGTGCCCATCTA-3′ and 5′-CTAGAA GCATTTGCGGTGGACGATGGAGGG-3′ for *β-actin*, respectively. The PCR conditions were: 95 °C for 2 min, and then 30 cycles with 95 °C for 10 s, 59 °C for 10 s, 72 °C for 1 min for *pol ι*, *pol η*, and *VEGF*, 30 cycles with 95 °C for 5 s, 61 °C for 10 s, 72 °C for 2 min for *pol κ*, 30 cycles with 95 °C for 5 s, 65 °C for 10 s, 72 °C for 1.5 min for *pol μ*, 30 cycles with 95 °C for 5 s, 59 °C for 10 s, 72 °C for 1 min for *Mlh1* or 25 cycles with 95 °C for 5 s, 59 °C for 10 s, 72 °C for 1 min for *β-actin*, and 72 °C for 5 min.

Construction of plasmids. The plasmid pcDNA3.1/HIF-1α^{DN} expressing dominant-negative HIF-1α was prepared essentially as described previously [19]. The plasmid expressing constitutively active HIF-1α, pcDNA3.1/HIF-1α$^{P402A/P564A}$, was constructed by introducing mutations into pcDNA3.1/HIF-1α that change both proline-402 and proline-564 of HIF-1α to alanine by using QuikChange® Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA). A luciferase reporter plasmid harboring the regulatory region (from −1346 to +418) of human *pol ι* gene, where +1 represents the transcription start site, was constructed as follows. First, a DNA fragment was amplified by PCR using genomic DNA, the 5′-primer carrying *Kpn*I site at the 5′ end, 5′-GGTACCCCTCCCTTCTGTCTG TGGA-3′, and the 3′ primer carrying *Sac*I site at the 5′ end, 5′-GAG CTCCTCGGCGTCTTCCTCGTCG-3′, and *Ex Taq*® DNA polymerase (TaKaRa Bio, Shiga). The resulting PCR product was subcloned into pGEM-T Easy vector (Promega Corp., Madison, WI), generating a plasmid pGEM/pol ι. After digesting with *Kpn*I and *Sac*I, the insert was ligated into the *Kpn*I/*Sac*I-cut pGL2-basic (Promega), generating a pGL2/pol ι reporter plasmid. Introduction of a mutation into the core sequence of putative HREs of *pol ι* (named HRE1 (from −400 to −396, 5′-GCGTG-3′), HRE2 (from −176 to −172, 5′-ACGTG-3′), HRE3 (from −149 to −145, 5′-GCGTG-3′), and HRE4 (from +330 to +334, 5′-ACGTG-3′)) that changes the sequence 5′-(A/G)CGTG-3′ to 5′-(A/G)AAAG-3′ was done by using QuikChange® Site Directed Mutagenesis Kit and pGEM/pol ι as a template. The identity of all of the cloned fragments was verified by nucleotide sequence analysis.

Luciferase reporter assays. Transient transfection of the luciferase reporter constructs into HepG2 cells and luciferase reporter assays were carried out as described previously [18]. One day after transfection, the cells were exposed to hypoxia (1% O_2) for 18 h, and luciferase activities in cell extracts were measured.

Electrophoretic mobility shift assay (EMSA). The nuclear proteins for EMSA were prepared from HeLa cells cultured in 21% O_2 or 1% O_2 for 8 h as described previously [20]. The HRE4-specific double-stranded oligonucleotide probe (wtHRE4) or its mutant form (mutHRE4) was prepared by annealing the sense 5′-ACTACAAAT**ACGTG**TCGAGGGT-3′ and the antisense 5′-ACCCTCGA**CACGT**ATTTGTAGT-3′ oligonucleotides (from +321 to +341) (containing putative HRE (marked in bold type)) or the sense 5′-ACTACAAATAAAAGTCGAGGGT-3′ and the

antisense 5′-ACCCTCGACTTTTATTTGTAGT-3′ oligonucleotides (containing mutated HRE (underlined)), respectively. Ten micrograms of nuclear proteins, ^{32}P-labeled double-stranded probe, 0.4 μg of calf thymus DNA, and binding buffer (10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 5% glycerol, and 5 mM DTT) were mixed in a total volume of 20 μl. In competition assays, 40-fold molar excess amount of unlabeled competitors was included in the reaction mixture. The mixture was incubated at room temperature for 30 min and then loaded on a 4% polyacrylamide gel in TBE buffer (89 mM Tris base, 89 mM boric acid, and 5 mM EDTA). Supershift assay was performed using 1 μg of mouse monoclonal anti-HIF-1α antibody (clone H1alpha67, Novus Biologicals) or rabbit polyclonal anti-HIF-2α antibody (Novus Biologicals).

Chromatin immunoprecipitation (ChIP) assay. HeLa cells cultured in 21% O_2 or 1% O_2 for 8 h were fixed with 1% formaldehyde for 10 min at room temperature. Preparation of chromatin solution was performed essentially as described previously [21]. The chromatin solution was incubated with 5 μg of mouse monoclonal anti-HIF-1α antibody (clone H1alpha67, Novus Biologicals) at 4 °C for 15 h. Normal mouse IgG served as a control. Immunoprecipitation, washing, and elution of immune complexes were carried out with Protein A agarose beads (Upstate, Lake Placid, NY) according to the manufacturer's protocols. After reversing cross-links, the DNA was recovered by phenol:chloroform extraction and precipitated by ethanol. The association of HIF-1α with HRE4 was examined by hot-start PCR using *GoTaq* DNA polymerase (Promega). The sense and the antisense primers used were 5′-GCTGCCTCCCTCTGCCTT-3′ (from +236 to +253) and 5′-GGTTCTGAGCCATCCCTTC-3′ (from +506 to +524), respectively.

Results

To examine whether exposure of cells to hypoxia followed by reoxygenation causes ROS overproduction, HeLa cells were cultured under hypoxic conditions for 24 h and then reoxygenated for 30 min. ROS production was detected with DCFH-DA. The results showed that some of the cells produced ROS under both normoxic and hypoxic conditions (see Supplementary data, S1a). However, at 30 min after reoxygenation, many cells were found to produce a large amount of ROS (S1a). This ROS production was transient and attenuated to the normoxic level within 2 h after reoxygenation (data not shown). Then we examined the formation of 8-oxo-dG by immunostaining the cells with anti-8-oxo-dG antibody. As expected, a larger amount of 8-oxo-dG was formed in the reoxygenated cells than in normoxic and hypoxic cells (S1b).

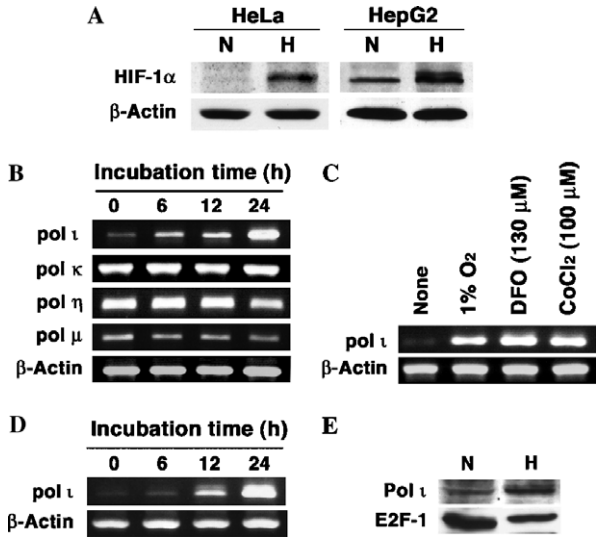

Fig. 1. Effects of hypoxia on the expressions of various error-prone DNA polymerase genes. (A) HIF-1α expression. HeLa and HepG2 cells were exposed to 1% O_2 for 6 h. Total cell lysates were subjected to immunoblot analysis. (B) Expression of mRNAs for various translesion DNA polymerases in HeLa cells exposed to 1% O_2 for the indicated times. Total RNA was subjected to RT-PCR. (C) Expression of *pol ι* mRNA in HeLa cells exposed to 1% O_2 or treated with desferrioxamine (DFO) or $CoCl_2$ for 24 h. (D) Expression of *pol ι* mRNA in HepG2 cells exposed to 1% O_2 for the indicated times. (E) Pol ι protein expression in HepG2 cells exposed to 1% O_2 for 24 h. Nuclear extracts were subjected to immunoblot analysis. E2F-1 was used as a control.

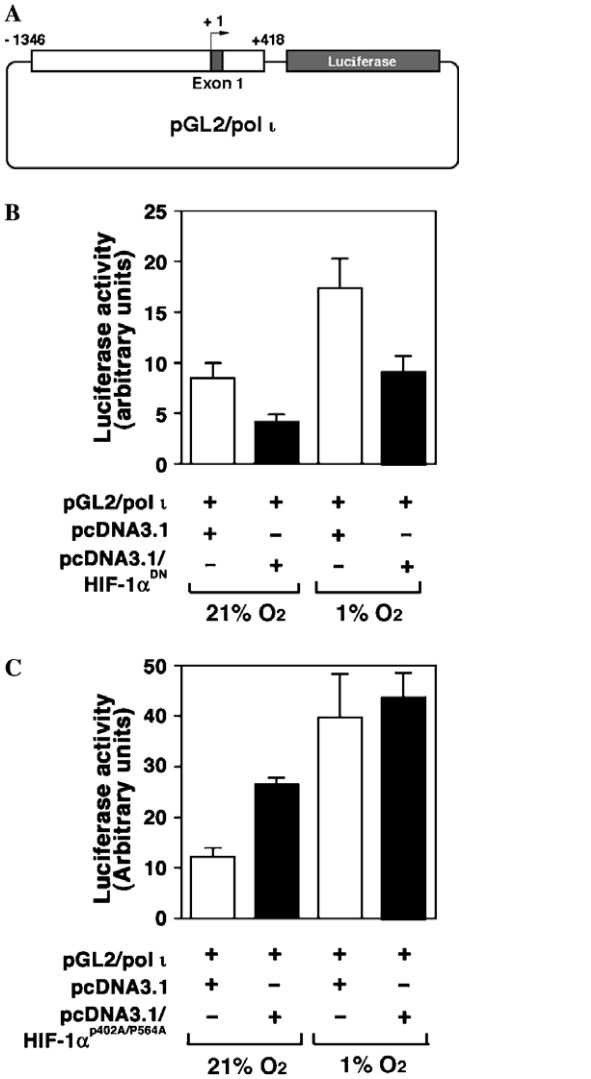

Fig. 2. Responsiveness of the luciferase reporter plasmid harboring *pol ι* regulatory region to hypoxia. (A) Construct of pGL2/pol ι luciferase reporter plasmid. (B) Effects of hypoxia and dominant-negative HIF-1α on luciferase activity. The reporter plasmid pGL2/pol ι was co-transfected with pcDNA3.1 or pcDNA3.1/HIF-1α^{DN} into HepG2 cells. The cells were exposed to 1% O_2 for 18 h. (C) Effects of hypoxia and constitutively active HIF-1α on luciferase activity. The reporter plasmid pGL2/pol ι was co-transfected with pcDNA3.1 or pcDNA3.1/HIF-1α$^{P402A/P564A}$ into HepG2 cells. Bars, SD.

Exposure of HeLa and HepG2 cells to hypoxia for 6 h resulted in the accumulation of HIF-1α (Fig. 1A). We then examined the expressions of translesion DNA polymerases that can bypass 8-oxo-dG in hypoxic cells by semi-quantitative RT-PCR. The results clearly showed that the expression of *pol ι* mRNA, but not of *pol κ*, *pol η*, or *pol μ* mRNA, was significantly increased by hypoxic stress in HeLa and HepG2 cells, depending on the incubation periods (6–24 h) (Fig. 1B and D). The hypoxia mimetics, desferrioxamine and $CoCl_2$, also increased the expression of *pol ι* mRNA (Fig. 1C). Accordingly, the amount of Pol ι protein was elevated in the nuclear extracts of HepG2 cells (Fig. 1E). Furthermore, hypoxia increased the level of *pol ι* mRNA in other cell lines such as A549, HT1080, LS174T, MCF7, MDA-MB-231, and U87MG (S2a). The induction of *pol ι* mRNA by hypoxia was observed in parallel with that of *VEGF* mRNA that is a well-known hypoxia-inducible gene. On the other hand, the decrease in the expression of *Mlh1* mRNA was detectable in both HeLa and HepG2 cells after a 48-h incubation under hypoxic conditions (S2b).

To examine whether HIF mediates the expression of *pol ι* mRNA in hypoxia, we made a pGL2/pol ι luciferase reporter construct harboring *pol ι* gene regulatory region (from −1346 to +418) (Fig. 2A). Transfection of the construct into HepG2 cells followed by exposure to hypoxia resulted in an approximately 2- to 3-fold increase in the luciferase activity (Fig. 2B and C). Co-transfection of pcDNA3.1/HIF-1α^{DN} abolished the increase (Fig. 2B). On the other hand, co-transfection with pcDNA3.1/HIF-1α$^{P402A/P564A}$ increased luciferase activity even under normoxic conditions (Fig. 2C), suggesting the involvement of HIF in the expression of *pol ι* mRNA in hypoxia.

Searching HRE consensus sequence (5′-(A/G)CGTG-3′) within this region showed that there are four putative HREs, three of them locating upstream of the transcription start site, named HRE1, HRE2, and HRE3, and the one locating in the intron 1, named HRE4. To determine which is functional, we generated a series of luciferase reporter plasmids in which three or all of the putative HREs were destroyed by introducing mutations (Fig. 3). Co-transfection of each of them with pcDNA3.1/HIF-1α$^{P402A/P564A}$ into HepG2 cells revealed that the reporter plasmid with intact HRE4 was most responsive while that with intact

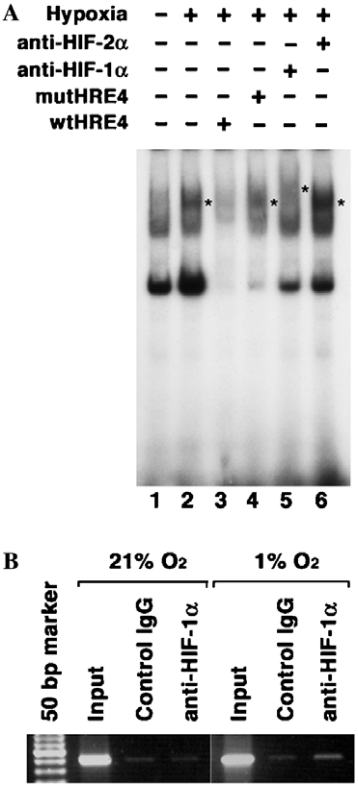

Fig. 4. HIF-1 binds to the HRE located in the intron 1 of the *pol ι* gene. (A) EMSA. Hela cells were cultured under 21% O_2 or 1% O_2 for 8 h. Nuclear extracts were subjected to EMSA using ^{32}P-labeled wtHRE4 as a probe. Asterisks indicate binding activity. (B) ChIP assay. HeLa cells were cultured under 21% O_2 or 1% O_2 for 8 h. ChIP assay was performed with mouse monoclonal anti-HIF-1α antibodies or mouse IgG as a control.

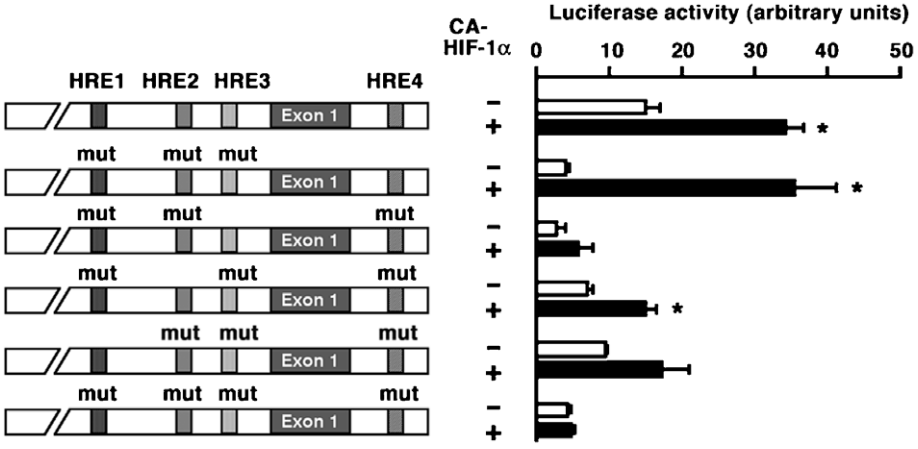

Fig. 3. Analysis of functional HRE in the *pol ι* regulatory region by luciferase reporter assays. Each of pGL2/pol ι plasmids with or without mutated (mut) HREs as indicated was co-transfected with pcDNA3.1 or pcDNA3.1/HIF-1α$^{P402A/P564A}$ (CA-HIF-1α) into HepG2 cells. The cells were exposed to 1% O_2 for 18 h. Bars, SD. *$p < 0.01$.

HRE1, HRE2, or HRE3 and that without all of the putative HREs showed weak and no responsiveness, respectively. To obtain evidence that HIF-1 binds to HRE4, we carried out EMSA using wtHRE4 as a DNA probe. The results showed that hypoxia enhanced binding activity (Fig. 4A, lane 2) that was competed with excess wtHRE4 (lane 3), but not with its mutant form mutHRE4 (lane 4). Addition of antibodies directed against HIF-1α, but not HIF-2α, induced a supershift of the binding activity (lanes 5 and 6). Furthermore, ChIP assay demonstrated that HIF-1α bound to the region containing HRE4 (Fig. 4B). From these results, we concluded that HIF-1 indeed interacts with the consensus HRE site (from +330 to +334) in the intron 1 of the *pol ι* gene.

Discussion

In this study, we confirmed that ROS were transiently overproduced in HeLa cells during reoxygenation and indeed caused 8-oxo-dG formation in the cells. We then focused on the expressions of translesion DNA polymerases that can bypass 8-oxo-dG, and found that hypoxia enhanced the expression of *pol ι* mRNA in various tumor cell lines.

Human Pol ι has low processivity and lacks an intrinsic 3′–5′ exonuclease activity, and has the lowest fidelity among so far reported eukaryotic polymerases [22]. Purified Pol ι has been observed to be able to efficiently bypass oxidized guanine and cytosine residues, as well as a variety of uracil lesions [23]. Not only can Pol ι mediate translesion replication in damaged DNA, but it also can misincorporate bases in a template-dependent manner in undamaged DNA [24,25]. Although these data are based on the *in vitro* studies, Yang et al. recently reported that Pol ι is overexpressed in human breast carcinoma cells and, importantly, that the expression level of Pol ι correlates with a significant decrease in DNA replication fidelity [26]. Therefore, up-regulation of Pol ι under hypoxic conditions might contribute to hypoxia/reoxygenation-induced mutagenesis.

Three lines of evidence suggested the involvement of HIF-1 in the mechanisms by which hypoxia induces *pol ι* mRNA expression. First, desferrioxamine and $CoCl_2$ also increased the expression of *pol ι* mRNA. Second, the reporter assays showed that dominant-negative HIF-1α suppressed the hypoxia-enhanced luciferase activity. Third, constitutively active HIF-1α enhanced the luciferase activity under normoxic conditions. Sequence analysis revealed the presence of four putative HREs which could explain the described effect of hypoxia on the induction of *pol ι* mRNA. Introduction of mutations in these HREs revealed that the reporter construct with intact HRE present in the intron 1 showed the maximal response to the co-transfected constitutively active HIF-1α construct, pointing out the major contribution of this HRE. Binding of HIF-1 to the HRE sequence was corroborated by EMSA. A shifted band was detected in hypoxic nuclear extracts, and a supershifted band was also detected after the incubation of the probe with anti-HIF-1α antibody. Since anti-HIF-2α antibody did not induce a supershifted band, HIF-2 is unlikely to bind the HRE. HIF-1 binding to the same sequence was further strengthened by the results of ChIP assay. Altogether, these results demonstrate that *pol ι* is a hypoxia-inducible gene through HIF-1 interaction with the consensus HRE site located at +330 in the intron 1 of the gene.

Recent studies highlight the deregulation of DNA mismatch repair enzymes in hypoxia as a mechanism of hypoxia-induced mutagenesis [11,12]. Loss of DNA mismatch repair renders cells hypersensitive to the mutagenic effect of oxidative stress [27]. In addition to this mechanism, our results may provide another mechanism underlying hypoxia/reoxygenation-induced mutagenesis. Through these mechanisms, hypoxia could lead to the genetic instability in tumor tissues.

Acknowledgments

This work was supported by Grants-in-Aid from the Ministry of Health, Labour, and Welfare for Third Term Comprehensive Control Research for Cancer and from the Ministry of Education, Culture, Sports, Science and Technology for Scientific Research, Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.10.048.

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