In Vivo Analysis of DNA-Protein Interactions on the Human Erythropoietin Enhancer

BEI HU, ELIZABETH WRIGHT,[†] LAURA CAMPBELL, AND K. L. BLANCHARD*

Center for Excellence in Cancer Research, Treatment and Education, Louisiana State University Medical Center, Shreveport, Louisiana 71130

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The erythropoietin (EPO) gene is one of the best examples of a mammalian gene controlled by oxygen tension. The DNA elements responsible for hypoxia-induced transcription consist of a short region of the proximal promoter and a <50-bp 3' enhancer. The elements act cooperatively to increase the transcriptional initiation rate approximately 100-fold in response to low oxygen tension in Hep3B cells. Two distinct types of transactivating proteins have been demonstrated to bind the response elements in the human EPO enhancer in vitro: one shows hypoxia-inducible DNA binding activity, while the other activity binds DNA under normoxic and hypoxic conditions. We have investigated the DNA-protein interactions on the human EPO enhancer in living tissue culture cells that produce EPO in a regulated fashion (Hep3B) and in cells that do not express EPO under any conditions tested (HeLa). We have identified in vivo DNA-protein interactions on the control elements in the human EPO enhancer by ligation-mediated PCR technology. We show that the putative protein binding sites in the EPO enhancer are occupied in vivo under conditions of normoxia, hypoxia, and cobalt exposure in EPO-producing cells. These sites are not occupied in cells that do not produce EPO. We also provide evidence for a conformational change in the topography of the EPO enhancer in response to hypoxia and cobalt exposure.

Erythropoietin (EPO) is a protein hormone produced primarily in the kidneys and liver that controls oxygen delivery to organs by modulating the erythrocyte mass. Transcription is a major control point in the expression of the EPO gene (10). The best-characterized model of regulated EPO expression is the human hepatoma cell line Hep3B (11). These cells produce EPO in response to hypoxia and cobalt exposure by increasing the rate of transcription of the EPO gene (10) and contain cytoplasmic proteins that bind the 3' untranslated region of the EPO mRNA (20). Hep3B cells have served as the testing ground for reporter gene experiments defining the DNA control elements within the human EPO gene and have been a convenient source of nuclear extracts for in vitro DNA-protein interaction studies on the EPO promoter and enhancer. *cis*-acting DNA elements necessary for the hypoxia-driven expression of the EPO gene have been identified and characterized (1, 3, 23); proteins that interact with these DNA elements in vitro have been described (1, 3, 15, 23), and some have been cloned (29).

Accurate tissue-specific regulation of the human EPO gene requires control elements contained in regions of DNA several kilobases upstream of the transcriptional start site (16, 22). Hypoxia-induced transcription of the EPO gene is facilitated by cooperation between a short basal promoter and an enhancer element located downstream of the polyadenylation site in the human gene (3). The minimal promoter and enhancer share striking DNA sequence homology and bind similar protein factors in vitro. The minimal human enhancer is approximately 42 bp in length and contains three elements that contribute to hypoxic sensing: the binding site for hypoxia-inducible factor 1 (HIF-1), a consensus steroid response ele-

ment in the configuration of a direct repeat with a 2-bp gap (DR-2), and a CACA repeat element. Each of these elements has been shown to contribute to the hypoxic regulation of the EPO gene, while none alone is sufficient (1, 3, 15, 19, 23).

In vitro DNA-protein interactions between nuclear proteins and the EPO enhancer have been studied extensively by electrophoretic mobility shift assays and in vitro footprinting techniques. Two major interactions have been identified: a hypoxia-inducible interaction with a site on the upstream portion of the enhancer (2, 23, 24) and a constitutive interaction with the DR-2 element (3, 23). Both types of interactions have been demonstrated with nuclear extracts from cells that produce EPO and from cells that do not produce EPO (3, 28). Recently the HIF-1 complex was purified by electrophoretic mobility shift assays to identify HIF-1 activity, and the HIF-1 α cDNA was cloned by nucleic acid hybridization based on a partial protein sequence obtained by microsequence analysis (29). HIF-1 α is a previously unidentified protein with features of the PAS family of transactivating proteins, including a basic helix-loop-helix DNA binding motif. HIF-1 β is the aryl hydrocarbon receptor nuclear translocator, a protein that shuttles the aryl hydrocarbon receptor to the nucleus in response to ligand-induced activation of the receptor (25, 29).

The EPO enhancer is a hypoxia-activated switch that acts over greater than 3,000 bp. The DNA protein machinery involved in the switch has been studied extensively in vitro; however, the architecture of the switch in vivo has not been investigated. We provide the first report of in vivo DNA-protein interactions on the human EPO enhancer in Hep3B and HeLa cells.

MATERIALS AND METHODS

Cell culture. Hep3B cells and HeLa cells were obtained from the American Type Culture Collection. Cells were grown in α minimum essential medium (α -MEM) containing 10% iron-enriched calf serum and 100 U of penicillin and streptomycin in an atmosphere of humidified air with 5% carbon dioxide. Hypoxic conditions were generated by nitrogen displacement of oxygen in a triple-gas incubator (Tabai-Espec) to an oxygen tension of 1%. The oxygen tension was

* Corresponding author. Mailing address: Center for Excellence in Cancer Research, Treatment and Education, Louisiana State University Medical Center, 1501 Kings Hwy., Shreveport, LA 71130. Phone: (318) 675-5974. Fax: (318) 675-5944. E-mail: kblanc@lsu.edu.

[†] Present address: Dyess Air Force Base, Abilene, TX 76907.

verified by gas sampling with a clinical blood gas analyzer. Cobalt chloride-treated cells were incubated in complete tissue culture medium containing 100 μ M cobalt chloride.

Genomic DNA preparation. After incubation of the cells under specific conditions, the medium was removed from the culture plates by aspiration. Neat dimethyl sulfate (DMS) was diluted with α -MEM to the appropriate concentrations by serial dilution (final concentrations: 0.5, 0.05, 0.005, and 0.0005%). No more than 2 min after removal of the cells from the hypoxic atmosphere, DMS-containing medium was applied to the monolayers of cells. After 5 min at room temperature the monolayers were washed with three 25-ml aliquots of phosphate-buffered saline containing 5% bovine serum albumin and 100 mM 2-mercaptoethanol (10 min total). Cell lysis was accomplished with sodium dodecyl sulfate, proteins were digested with proteinase K, and genomic DNA was purified by organic extraction and precipitation in ethanol (18). Genomic DNA for in vitro methylation was prepared from tissue culture cells in an identical manner, except that cells were not treated with DMS prior to DNA purification. In vitro-methylated DNA was prepared by treating deproteinized genomic DNA with DMS at final concentrations of 0.2, 0.1, and 0.05% for 5 min at room temperature.

LM-PCR. In vivo- and in vitro-methylated genomic DNA was incubated in 1 M piperidine at 95°C to produce chain lysis at methylated guanosine residues. The cleaved DNA was subjected to ligation-mediated PCR (LM-PCR) using Vent DNA polymerase (8, 18). The cycle number for PCR was 22, and for the labeling reaction, it was 2. The gene-specific primers from the top strand of the enhancer were EPO-1-top (5'-GAC AAG AAC TGA AAC CAC CAA TAT GAC TC-3'), EPO-2-top (5'-TTC TGT TTT CTG GGA ACC TCC AAA TCC CCT G-3'), and EPO-3-top (5'-CCT CCA AAT CCC CTG GCT CTG TCC C-3'), corresponding to bp 3319 to 3396 of the human EPO gene DNA sequence (14). The bottom-strand primers were EPO-1-bot (5'-AGT GAC CAG GGT TGG CAG CTG TTA-3'), EPO-2-bot (5'-CCA AAA CTC CCA AAA TTA CCC GTC C-3'), and EPO-3-bot (5'-GAC CCG TCC TCT GGC CTC CCT CTC-3'), corresponding to nucleotides 3709 to 3639 of the human EPO gene DNA sequence (6). Annealing temperatures for the top-strand and bottom-strand primers were 60, 65, and 67°C for the primer extension reaction, PCR, and the labeling reaction, respectively. The primers (EPO-3-top and EPO-3-bot) in the labeling reactions were made radioactive by incorporating 5' [32 P]orthophosphate from [γ - 32 P]ATP through the forward kinase reaction catalyzed by T4 polynucleotide kinase. After completion of LM-PCR, the samples were applied to 6 or 8% polyacrylamide gels containing 8 M urea, and the DNA fragments were separated by electrophoresis. Phosphorimager plates were exposed to dried gels for 2 to 3 days, and images were obtained by scanning the plates on a Molecular Dynamics PhosphorImager.

Image analysis. Line graphs were created in ImageQuant by bisecting the individual lanes with a 50-pixel line. The line graphs are accurate representations of the raw data: a pixel-by-pixel direct counting of radioactivity in the lanes of the gel. The line graphs were not smoothed, nor were background counts adjusted.

RESULTS

The model. A model of the molecular components of the hypoxia-sensitive switch contained in the human EPO 3' enhancer is presented in Fig. 1. The model is based on previously published in vitro analyses of DNA-protein interactions between fragments of the EPO enhancer and proteins from nuclear extracts or purified fractions of nuclear extracts from Hep3B cells and on functional analyses by transient expression assays (1–3, 15, 23). The human EPO enhancer appears to function as a bi- or tripartite switch consisting of at least two DNA-protein complexes. A strong DNA-protein interaction forms between the DR-2 element (binding site: 5'-TGACCTCTCGACCC-3') and proteins in nuclear extracts prepared from cells incubated under normoxic or hypoxic conditions. Based on sequence similarities between the DR-2 element and cognate steroid response elements (5), the protein moiety is presumed to be a member of the steroid hormone receptor superfamily (3, 7). If the DR-2 binding protein is a steroid hormone superfamily member, hormone binding may modulate transcriptional activation. Differential metabolism of an intracellular hormone as a consequence of hypoxia could produce a hypoxia-dependent ligand that directs graded transcriptional activation. An absence of hormone could eliminate the activation by the receptor or allow the receptor to repress transcription. In this model the protein moiety would be expected to be in contact with the DR-2 element irrespective of cellular oxygen tension, a situation that mimics the interactions

FIG. 1. A model of the EPO enhancer's interactions with transactivating factors based on in vitro experiments. (A) The human EPO enhancer is represented schematically by parallel lines with the binding sites for HIF-1 and the DR-2 binding proteins as a shaded box and a box with diagonal lines, respectively. An open box indicates the position of the CACA repeat sequence. Under normoxic conditions (top), the DR-2 site is occupied by a protein (hatched oval), drawn as a dimer with binding sites for a small chemical ligand. Under normoxic conditions the chemical ligand (triangle) is either not present or in a conformation that is not recognized by the DR-2 binding protein. After exposure to low oxygen tension, two events occur: (i) the HIF-1 complex (cross-hatched ellipse) binds to the HIF-1 element, and (ii) the small chemical ligand is converted to a form (black squares) that can interact with the DR-2 binding protein. The change in shape of the DR-2 binding protein (oval to circle) represents a putative change in the conformation of the protein necessary for transactivation. The combination of HIF-1 element binding and the DR-2 binding proteins is believed to give rise to the transcriptional activation at this site. (B) A *Hae*III DNA fragment containing the human EPO enhancer is presented. This fragment lies approximately 150 bp downstream of the polyadenylation site. The boxes indicate the boundaries of the HIF-1 site, the CACA box, and the DR-2 element (shaded, open, and striped boxes, respectively). bp 42 and 49 (lowercase letters) are nucleotides that were absent from the original genomic sequence. The vertical black bars above and below the sequence are horizontal representations of the expected, idealized G ladders.

between thyroid and retinoid hormone receptors and their DNA response elements (17). Conversely, the DR-2 binding protein could be tethered in the cytoplasm to heat shock proteins, akin to the glucocorticoid receptor, until a hypoxia-dependent hormone binds the receptor and allows it to be translocated to the nucleus to bind its specific DNA element (4, 5) and activate transcription.

A hypoxia-inducible DNA binding factor, HIF-1, has been demonstrated to interact with the proximal portion of the minimal EPO enhancer (binding site: 5'-TACGTGCT-3') (2, 23). The HIF-1 binding complex is composed of at least two polypeptides, HIF-1 α and HIF-1 β (29). HIF-1 α and HIF-1 β expression rapidly increases over 1 to 2 h in hypoxic HEP3B cells, and hypoxia-induced expression of HIF-1 and EPO mRNAs can be blocked by cycloheximide treatment of cells (25, 27). The phosphorylation state of the HIF-1 proteins plays a role in the regulation of HIF-1 and EPO expression. Treatment of cells with 2-aminopurine, a protein kinase inhibitor,

blocks HIF-1 and EPO induction by hypoxia in HEP3B cells, and treatment of nuclear extracts from HEP3B cells with phosphatase inhibits the DNA binding activity of HIF-1 (26). Whether HIF-1 activity is controlled by the absolute level of protein expression or whether posttranslational modification of HIF-1 complex activates its DNA binding potential and activity, or both, is unclear. Unlike the DR-2 binding protein, the HIF-1 binding complex is believed to bind DNA only under hypoxic conditions.

At a functional level the HIF-1 binding element and the DR-2 site have been shown to be necessary but not sufficient for hypoxia stimulation of transcription by the EPO enhancer (1, 3, 23). Mutations in the CACA repeat region of the enhancer that connects the sites, whether nucleotide substitutions or deletions, also interfere with hypoxia-induced expression (15, 23). This region contains a 10-bp DNA sequence that is also present in the minimal promoter but not present in the murine EPO enhancer (3). No specific DNA-protein interactions have been demonstrated to occur over this region of the enhancer.

General. To analyze DNA-protein interactions in living eukaryotic cells is a technically difficult endeavor. LM-PCR takes advantage of the ability of tight DNA-protein interactions to block the access of chemicals such as DMS to guanosine residues (8, 18). The methylation protection induced by specific DNA-protein interactions is displayed by chemical cleavage of the DNA strands at methylated guanosine residues, primer extension directed by a gene specific primer, ligation of adaptors to the newly created blunt ends, and amplification of the adaptor-accepting, gene-specific, primer-extended DNA fragments. Obtaining reproducible footprinting required optimization of many parameters during all steps of LM-PCR. Cells were grown to no greater than 50% confluence prior to incubation in a 1% oxygen atmosphere. If the cells were allowed to reach higher levels of confluence, a large proportion of the cells appeared to die during the hypoxic growth phase (not shown); furthermore, highly confluent cells grown at 20% oxygen might have areas of local hypoxia that could lead to confusing results. Cells were subjected to hypoxic stress or cobalt exposure for 8 h—a time frame that allows EPO mRNA accumulation but results in no cell death as observed by phase-contrast microscopy and vital staining (not shown). Cobalt chloride exposure was chosen as a stimulus that mimics hypoxia (9) to circumvent the possibility that the hypoxia-associated DNA-protein interactions require constant hypoxia.

Genomic DNA was methylated *in vivo* by incubating monolayers of tissue culture cells with α -MEM containing DMS at concentrations spanning 4 orders of magnitude to optimize the appearance of footprints (21). Methylation at the higher concentrations of DMS led to the production of G ladders indistinguishable from *in vitro*-methylated naked DNA (data not shown). In an attempt to circumvent the inadvertent methylation of protein binding sites uncovered by the general toxicity (for example, extensive protein methylation) of DMS or by cell lysis, quenching agents (bovine serum albumin and 2-mercaptoethanol) were added to the wash buffers applied after DMS treatment.

The primer extension and amplification reactions were optimized by adding hot-start conditions to each step. In some cases, dimethyl sulfoxide was added to the primer extension and amplification steps to enhance the efficiency of DNA melting.

Map. Figure 1B is a map of the human EPO enhancer. The sequence is presented with the sense strand of transcription being collinear with the top strand of the enhancer (placing the polyadenylation site to the left of the enhancer). The sequence

FIG. 2. LM-PCR footprinting of the top strand of the human EPO enhancer in Hep3B cells. Genomic DNA from normoxia-, hypoxia-, and cobalt-treated Hep3B cells was subjected to *in vivo* methylation by DMS. The DNA was treated with piperidine to induce strand cleavage at methylated guanosine residues. Deproteinized genomic DNA from Hep3B cells was methylated *in vitro* as a naked DNA control. After LM-PCR with bottom-strand primers, the fragments were separated by electrophoresis under denaturing conditions. The G ladders read from 5' to 3' from top to bottom. Lane 1 represents naked DNA (methylated *in vitro*, after deproteination); lanes 2, 3, and 4 represent DNA methylated *in vivo* in cells grown under normoxic, hypoxic, or cobaltous conditions, respectively. The black circles indicate guanosine residues that were protected from methylation *in vivo*. The shaded circle indicates a guanosine residue that was hypersensitive to methylation in the cobalt-treated cells only. The horizontal arrows verify at a genomic level the omissions in the original DNA sequence. The boxes represent the regions of the HIF-1 site (shaded), the CACA box (open), and the DR-2 site (striped). The line graphs were created by Imagequant by bisecting the lanes with a 50-pixel line without background subtraction or smoothing. The arrows above line 1 highlight the guanosine residues that differ between *in vitro*- and *in vivo*-methylated DNAs. The arrow above line 3 identifies an artifactual peak created by a hot spot on the electrophoretic gel. T and B indicate the top and bottom, respectively, of the scanned image (also in Fig. 3, 5, and 6).

corresponds to a *Hae*III fragment that contains the entire functional enhancer. The DNA sequence is numbered from 1 to 49, corresponding to bp 3455 to 3502 in the published DNA sequence (14), plus an additional 2 bp (indicated in the map by noncapitalized letters) previously identified as sequencing errors in the original DNA sequence (3). The schematic gel lanes above and below the DNA sequence demonstrate the idealized G ladders that would be produced by 100% cleavage at each guanosine residue for each strand. The regions corresponding to the HIF-1 binding element (bp 4 to 11) and the DR-2 element (bp 29 to 42) are marked with a shaded box and striped box, respectively. The CACA box (bp 16 to 25) is denoted by an open box.

Top-strand footprinting in Hep3B cells. In Fig. 2 the LM-PCR-generated G ladders in the region of the EPO enhancer are presented with corresponding line graphs generated by phosphorimager analysis. The primer set for this series of experiments is EPO-1-bot through EPO-3-bot. Lane 1 contains the baseline control G ladder obtained after *in vitro* methylation of deproteinized DNA from Hep3B cells. Despite titration of DMS concentration, optimization of PCR cycle number, and exhaustive purification of the genomic DNA, the intensity of the bands representing individual guanosine residues was characteristically variable in the enhancer. A similar pattern was obtained with an *in vitro*-methylated subcloned fragment of the human EPO enhancer as the template. Human genomic DNA samples produced even-intensity G ladders in other regions on the same strand and on the complementary strand (data not shown). A similar variation in intensity was observed when the genomic DNA template was isolated from HeLa cells (see Fig. 5). The G ladders in lanes 2, 3, and 4 of

FIG. 3. LM-PCR footprinting of the bottom strand of the human EPO enhancer in Hep3B cells. LM-PCR was performed with top-strand primers as described in the legend to Fig. 2. The template for lane 1 was naked DNA; the templates for lanes 2, 3, and 4 were in vivo-methylated DNA from Hep3B cells exposed to normoxia, hypoxia, or cobalt, respectively. The black circles indicate guanidine residues that were protected from methylation in vivo. Guanidine residues that became hypersensitive to methylation after treatment with hypoxia and cobalt are indicated by open circles (robust sites) or open arrows (less robust sites). The diamond indicates a site within the HIF-1 element that is hypersensitive to methylation under all conditions in vivo. The solid horizontal arrow verifies an omission in the original DNA sequence. The boxes represent the regions of the HIF-1 site (shaded), the CACA box (open), and the DR-2 site (striped). The line graphs were created by Imagequant by bisecting the lanes with a 50-pixel line without background subtraction or smoothing. The arrows above line 1 highlight the guanidine residues that are different between in vitro- and in vivo-methylated DNA.

Fig. 2 were generated from in vivo-methylated DNA isolated from Hep3B cells incubated for 8 h under 20 or 1% oxygen or in 20% oxygen in medium containing 100 μ M cobalt chloride, respectively. Within the minimal enhancer sequence the access of DMS to three guanidine residues (top-strand residues 7, 30, and 38) was reproducibly impeded. A fourth guanidine residue outside of the enhancer sequence was similarly affected. Each of the affected guanidine residues is denoted by a filled circle. G-7 is located within the binding site for HIF-1, while G-30 and G-38 are the 2nd and 10th residues in the DR-2 element, respectively. These guanidine residues were protected from in vivo methylation under each condition tested: normoxia, hypoxia, and cobalt exposure. The shaded circle identifies G-12, a residue that is hyperreactive to DMS only under conditions of cobalt exposure.

Bottom-strand footprinting in Hep3B cells. The bottom-strand G ladders in the region of the human EPO enhancer generated by LM-PCR from Hep3B genomic DNA and the corresponding line graphs are presented in Fig. 3. The primer set for this experiment was EPO-1-top through EPO-3-top. The bottom strand of the enhancer is more G rich than the upper strand and hence provides more topographical information. Four guanidine residues within the minimal enhancer demonstrated reduced in vivo reactivity towards DMS compared to in vitro reactivity towards deproteinated DNA (lane 1 versus lanes 2, 3, and 4): G-45, G-40, G-32, and G-6 (denoted by closed circles). G-6 falls within the HIF-1 binding element, G-40 and G-32 fall within the DR-2 element, and G-45 is immediately downstream of the DR-2 element. These guanidine residues were similarly affected under each condition tested: normoxia, hypoxia, and cobalt exposure. The G ladders from this region also demonstrate that exposure to hypoxia and cobalt induced robust DMS hypersensitivity at

FIG. 4. A composite map of in vivo footprinting in the human EPO enhancer in Hep3B cells. The DMS reactivity of individual guanidine residues within the EPO enhancer as measured in Hep3B cells has been added to the map from Fig. 1B. Black circles indicate guanidine residues that were protected from methylation, the shaded circle indicates a guanidine residue that became hyperreactive during cobalt exposure, the open arrows indicate low-level-hypersensitivity sites during stimulation (hypoxic or cobalt exposure), the open circles indicate robust hypersensitivity sites during stimulation, and the diamond indicates a guanidine residue that was hypersensitive under all in vivo conditions.

positions G-1 and G-2 (open circles) and less reactive hypersensitivity sites at positions G-16 and G-18 (open arrows). G-1 and G-2 showed increased reactivity to DMS under hypoxia and cobalt exposure, while G-16 and G-18 were hyperreactive under all in vivo conditions but more so after hypoxia and cobalt exposure. G-10 (black diamond), a guanidine residue within the HIF-1 binding element, was hypersensitive to DMS methylation after exposure to normoxia, hypoxia, and cobalt. A composite schematic diagram of the in vivo DMS reactivity within the human EPO enhancer in Hep3B cells is presented in Fig. 4.

LM-PCR of the EPO enhancer in HeLa cells. To investigate the tissue specificity of the in vivo footprints on the human EPO enhancer found in Hep3B cells, HeLa cells were grown in 20% oxygen (Fig. 5, lane 2), 1% oxygen (lane 3), and 20% oxygen–100 μ M cobalt chloride (lane 4). After incubation, the HeLa cells were subjected to in vivo methylation with DMS followed by LM-PCR. Deproteinated HeLa DNA was subjected to in vitro methylation and LM-PCR to develop baseline G ladders (lane 1). As demonstrated in Fig. 5, the top-strand baseline control G ladder developed on in vitro-methylated DNA is essentially identical to the G ladder from Hep3B genomic DNA. In contrast to Hep3B cells, the patterns of the G ladders obtained from in vivo-methylated genomic DNA

FIG. 5. LM-PCR footprinting of the top strand of the human EPO enhancer in HeLa cells. LM-PCR was performed as described in the legend to Fig. 2. Genomic DNA templates were prepared from HeLa cells exposed to normoxia, hypoxia, or cobalt. Primers were identical to those used for LM-PCR for Hep3B cells. The template for lane 1 was naked DNA; the templates for lanes 2, 3, and 4 were in vivo-methylated DNA from HeLa cells exposed to normoxia, hypoxia, or cobalt, respectively. The boxes represent the regions of the HIF-1 site (shaded), the CACA box (open), and the DR-2 site (striped). The circle indicates a guanidine residue that was hypersensitive to methylation after cobalt exposure only. The line graphs were created by Imagequant by bisecting the lanes with a 50-pixel line without background subtraction or smoothing.

FIG. 6. LM-PCR footprinting of the bottom strand of the human EPO enhancer in HeLa cells. LM-PCR was performed as described in the legend to Fig. 3. Genomic DNA templates were prepared from HeLa cells exposed to normoxia, hypoxia, or cobalt. Primers were identical to those used for LM-PCR for Hep3B cells. The templates for lanes 0 and 1 were naked DNA; the templates for lanes 2, 3, and 4 were in vivo-methylated DNA from HeLa cells exposed to normoxia, hypoxia, or cobalt, respectively. The naked-DNA sample was subjected to electrophoresis on two separate denaturing gels, due to electrophoresis artifacts in the higher-molecular-weight region of lane 1. The boxes represent the regions of the HIF-1 site (shaded), the CACA box (open), and the DR-2 site (striped). The line graphs were created by Imagequant by bisecting the lanes with a 50-pixel line without background subtraction or smoothing.

from HeLa cells are basically indistinguishable from that of in vitro-methylated deproteinated DNA. G-12 (shaded circle) in the top strand does appear to have increased reactivity towards DMS in the cobalt-stimulated cells. In Fig. 6 the same HeLa genomic DNAs were subjected to LM-PCR using bottom-strand primers. No footprints were observed on the bottom strand of the enhancer in HeLa cells.

DISCUSSION

The human EPO enhancer is a true enhancer that acts in a position- and orientation-independent fashion (1, 3, 23). As with most enhancers, the EPO enhancer has binding elements for more than one transactivating factor. In vitro analyses of DNA-protein interactions between the EPO enhancer and nuclear proteins and transient expression assays suggest the involvement of the HIF-1 factors and a steroid hormone receptor superfamily member in the regulation of EPO expression. A conservative model of EPO expression requires the production of a new transactivating function during hypoxia by new transcription or posttranslational modification of an existing factor (HIF-1) and the activation of a constitutive transactivating factor (DR-2 binding protein). The involvement of a metabolically modulated hormone is at present conjectural.

We provide the first evidence that in cells expressing EPO, the EPO enhancer is occupied by proteins during normoxic, hypoxic, and cobaltous growth conditions. Specifically, we provide compelling evidence that the HIF-1 site is resistant to methylation by DMS under normoxic conditions, as well as during hypoxia and growth in medium containing cobalt chloride. These findings are in direct contradistinction to the in vitro DMS interference analysis reported by Wang and Semenza (27), which demonstrated protein contacts with the HIF-1 site from proteins in nuclear extracts derived from cells exposed to hypoxia but not from those exposed to normoxia. The identities of the proteins that produce the in vivo footprints on the HIF-1 binding element cannot be determined by

our experiments. Wang et al. (25) have presented evidence that the level of expression of the HIF-1 factors rises rapidly after hypoxic stress or exposure to cobalt and that hypoxia-associated phosphorylation of the HIF-1 factors may modulate the activities of these important proteins. The HIF-1 binding site could be occupied by inactive HIF-1 complex during normoxic growth, to be activated by a change in phosphorylation status. Alternatively, a different protein could bind the HIF-1 site during normoxic growth, only to be displaced by HIF-1 factors during the stress of hypoxia or growth in the presence of cobalt. In support of this model, ATF-1 and CREB-1, transactivating proteins that are constitutively present in cell extracts from a variety of cells, have previously been shown to bind in vitro to the general region of the HIF-1 element of the EPO enhancer (13). Local areas of hypoxia could exist on a tissue culture plate, despite incubation under normoxic conditions, if the cells were allowed to reach high levels of confluence. A subpopulation of locally hypoxic cells could express HIF-1 proteins and would taint the normoxic footprinting results. Extreme care was taken to ensure that the tissue culture cells did not reach high levels of confluence and were well dispersed across the tissue culture plates, making unlikely this more prosaic explanation for normoxic HIF-1 element footprints.

The DR-2 element in the human EPO enhancer appears to be occupied at all times, irrespective of the presence of oxygen or cobalt, corroborating the in vitro analyses of DNA-protein interactions between nuclear extracts from Hep3B cells and the EPO enhancer (3). These data are consistent with a model in which a steroid hormone receptor superfamily member binds the DR-2 element at all times and is activated only under appropriate metabolic conditions. The protection from methylation of the first guanosine residue downstream of the DR-2 element is evidence for an extended steroid response element in the EPO enhancer. This guanosine residue is within the boundaries of the in vitro DNase footprint generated with Hep3B nuclear extracts and the human EPO enhancer (3). The functional significance of this extended steroid response element remains to be tested.

DNA footprinting techniques are used to examine conformational changes in fragments of DNA based upon the appearance of sites that are hypersensitive to cleavage by the footprinting agent. Under appropriate conditions the access of footprinting agents to nucleotides is facilitated by protein-induced DNA bending (12). Evidence for a conformational change in the topography of the EPO enhancer induced by hypoxia or cobalt exposure is presented in Fig. 3. The DMS-hyperreactive sites may be the result of a modification of the HIF-1 complex, either by a change in the proteins involved in DNA binding or by a posttranslational event, for example, a change in phosphorylation status. Alternatively, the conformational change in the EPO enhancer could be evidence for a hormone-induced alteration of the protein complex at the DR-2 site.

EPO is produced in a tightly controlled tissue-specific, condition-specific, and developmentally specific manner. The native EPO enhancer is transcriptionally inactive in HeLa cells. Our in vivo footprinting experiments demonstrate that DNA-protein complexes do not form on the EPO enhancer in HeLa cells, cells that do not produce EPO. HeLa cells contain protein factors able to interact with the human EPO enhancer in vitro. The HIF-1 complex proteins were purified from HeLa cells (29), and a DR-2 element binding moiety has been demonstrated in vitro (3). Functionally, the access of transactivating proteins to the EPO enhancer in HeLa cells must be blocked in vivo. The mechanism that renders the enhancer

inaccessible in HeLa cells is unknown. The architecture of the chromatin surrounding the EPO gene could force the enhancer into an inactive configuration in nonexpressing cells, or the binding of regulatory proteins to the enhancer in nonexpressing cells could be blocked by tissue-specific DNA methylation. An essential cofactor or the appropriate machinery to complete a specific posttranslational modification of a transactivating factor could be lacking.

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