

# Genome-wide Association of Hypoxia-inducible Factor (HIF)-1 $\alpha$ and HIF-2 $\alpha$ DNA Binding with Expression Profiling of Hypoxia-inducible Transcripts<sup>\*[5]</sup>

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Hypoxia-inducible factor (HIF) controls an extensive range of adaptive responses to hypoxia. To better understand this transcriptional cascade we performed genome-wide chromatin immunoprecipitation using antibodies to two major HIF- $\alpha$  subunits, and correlated the results with genome-wide transcript profiling. Within a tiled promoter array we identified 546 and 143 sequences that bound, respectively, to HIF-1 $\alpha$  or HIF-2 $\alpha$  at high stringency. Analysis of these sequences confirmed an identical core binding motif for HIF-1 $\alpha$  and HIF-2 $\alpha$  (RCGTG) but demonstrated that binding to this motif was highly selective, with binding enriched at distinct regions both upstream and downstream of the transcriptional start. Comparison of HIF-promoter binding data with bidirectional HIF-dependent changes in transcript expression indicated that whereas a substantial proportion of positive responses (>20% across all significantly regulated genes) are direct, HIF-dependent gene suppression is almost entirely indirect. Comparison of HIF-1 $\alpha$ - versus HIF-2 $\alpha$ -binding sites revealed that whereas some loci bound HIF-1 $\alpha$  in isolation, many bound both isoforms with similar affinity. Despite high-affinity binding to multiple promoters, HIF-2 $\alpha$  contributed to few, if any, of the transcriptional responses to acute hypoxia at these loci. Given emerging evidence for biologically distinct functions of HIF-1 $\alpha$  versus HIF-2 $\alpha$  understanding the mechanisms restricting HIF-2 $\alpha$  activity will be of interest.

Cells respond to changes in environmental oxygen levels through the coordinated regulation of the expression of a large number of genes with key functions in processes as diverse as proliferation, differentiation, apoptosis, energy metabolism, and growth factor production that are important in physiological and pathophysiological processes spanning embryonic development, adaptation to altitude, wound healing, inflammation, ischemic vascular disease, and cancer (1–3). Central to many of these responses is the transcription factor hypoxia-inducible factor (HIF),<sup>2</sup> which is regulated by oxygen through enzymatic

post-translational hydroxylation of the  $\alpha$ -subunit (4, 5), which in turn regulates its stability and its interaction with coactivators (5–7).

Analyses of HIF-DNA interactions at ~50 gene loci have defined a core hypoxia response element (HRE), RCGTG that binds HIF (8). However, genome-wide transcript analyses using microarrays have indicated that a much larger number of genes respond to HIF signaling, with significant positive and negative responses extending across several hundreds of transcripts (9–18). Furthermore, recent studies indicate that HIF may affect gene expression profiles indirectly through diverse mechanisms (19–25), raising questions as to the extent of direct versus indirect effects of HIF.

Further complexity is generated by the existence of multiple HIF isoforms, with the best understood being HIF-1 $\alpha$  and HIF-2 $\alpha$ . These have similar domain architectures and mechanisms of regulation, and both bind to HREs and efficiently activate HRE-linked reporter genes (26, 27). However, they generate different developmental phenotypes upon inactivation (28–31). Although, differential expression may contribute to these differences, recent studies have demonstrated that each isoform appears to have distinct transcriptional targets (11, 32–35). Mechanisms of HIF- $\alpha$  transcriptional selectivity are poorly understood and chromatin immunoprecipitation studies at a limited number of loci have not shown selective binding of the two HIF- $\alpha$  isoforms (12, 36, 37). However, to date, such studies have examined only a small set of gene loci and it remains unclear how HIF- $\alpha$  binding correlates with functional effects on gene expression across the genome.

To address this we have undertaken a genome-wide analysis of HIF- $\alpha$  DNA binding in MCF-7 cells using chromatin immunoprecipitation. Here we report on the distribution of HIF-1 $\alpha$ - and HIF-2 $\alpha$ -binding sites across more than 25,500 human gene promoters, and on the correlation of HIF- $\alpha$  binding with functional responses to HIF-1 $\alpha$  and HIF-2 $\alpha$  across the genome (9). The work provides an estimate of the scale of direct versus indirect effects of HIF on early changes in gene expression in response to HIF activation. Despite a large degree of overlap in binding of the two HIF- $\alpha$  isoforms there were striking differences in gene regulation with HIF-2 $\alpha$  contributing very little to the overall HIF response.

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[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Tables S1–S4 and Figs. S1–S4.

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<sup>2</sup> The abbreviations used are: HIF, hypoxia-inducible factor; HRE, hypoxia response element; DMOG, dimethylxalylglycine; ChIP, chromatin immunoprecipitation; MA, moving average; siRNA, small interfering RNA.

## Genome-wide Analysis of HIF DNA Binding

### EXPERIMENTAL PROCEDURES

**Cell Culture**—MCF7 breast cancer cells were grown in Dulbecco's modified Eagle's medium, 2 mM L-glutamine, and 10% fetal bovine serum (Sigma). Subconfluent cell cultures were exposed to 2 mM dimethylxalylglycine (DMOG) (Frontier Scientific) for 16 h prior to harvest.

**Chromatin Immunoprecipitation**—Three independent chromatin immunoprecipitation assays were performed using the Upstate protocol (Millipore). Cells were sonicated in 30-s pulses for a total of 3 min (Sonics & Materials, VCX 500). Chromatin was immunoprecipitated using rabbit polyclonal antisera to HIF-1 $\alpha$  (PM14) and HIF-2 $\alpha$  (PM9) (36, 38). These antibodies have previously been shown to perform well in chromatin immunoprecipitation assays and to be highly specific for HIF-1 $\alpha$  or HIF-2 $\alpha$  (36). Preimmune serum was used as a negative control.

**Chromatin immunoprecipitation (ChIP)-ChIP Analysis Using Affymetrix Human Promoter 1.0R Microarray**—Immunoprecipitated chromatin was amplified using the Sigma Whole Genome Amplification kit according to the manufacturer's instructions. After each stage of amplification, preservation of enrichment of the PHD3 enhancer (36) was demonstrated by quantitative-PCR. 7.5 mg of amplified chromatin was then digested with 150 milliunits of DNase I (Invitrogen) for 35 min at 37 °C, to generate a fragment size of ~70 bp, labeled, and hybridized to Affymetrix Human promoter 1.0R arrays, and detected according to the Affymetrix user manual.

The ChIP-ChIP peak detection tool CisGenome (39–41) was used to define potential protein-binding regions. Three independent HIF-1 $\alpha$  and HIF-2 $\alpha$  chromatin immunoprecipitations were compared with three independent control chromatin immunoprecipitations performed using preimmune sera. Quantile normalization was applied prior to analysis. A moving average (MA) statistic was computed for each probe based on a half-window size of 300 bp or 5 probes. Probes with the MA statistic 4 S.D. away from the global mean were used to define protein-binding regions. Peaks were discarded if they contained less than five probes or were less than 100 base pairs in width. Peaks that were separated by less than 300 base pairs or 5 probes were merged. Peaks that had a left-tail false discovery rate >5% were discounted (39–41). The genomic coordinates of all regions were converted into coordinates based on NCBI build 36 (hg18) and mapped to the nearest transcriptional start site.

**Independent Confirmation of Chromatin Enrichment by Real-time Quantitative-PCR**—Real-time quantitative-PCR for DNA quantification employed SYBR Green gene expression assays on a StepOne thermocycler (Applied Biosystems). Normalization was to  $\beta$ -actin DNA and fold-enrichment at each locus was calculated using the  $\Delta C_T$  method. Primer sequences are given in supplemental materials Table S1.

**Protein Abundance Analysis by Immunoblotting**—Gal-tagged HIF-1 $\alpha$  and HIF-2 $\alpha$  were expressed by the rabbit reticulocyte lysate *in vitro* transcription and translation system (Promega). Relative amounts of each isoform were compared by immunoblot using anti-Gal. These signals were then used to calibrate the abundance of endogenous proteins using the

HIF-1 $\alpha$  and HIF-2 $\alpha$  antibodies. Primary antibodies used were mouse anti-HIF-1 $\alpha$  (BD Transduction Laboratories), mouse anti-HIF-2 $\alpha$  (26), and mouse anti-Gal (Santa Cruz Biotechnology).

**Expression Array**—The microarray analysis of gene expression in response to 16 h of 2 mM DMOG or 1% hypoxia in the presence or absence of siRNA-mediated suppression of HIF-1 $\alpha$  or HIF-2 $\alpha$  has been previously described (9). To correct for multiple testing, we used an arbitrary false discovery rate cutoff of 5% ( $q$  value <0.05) to identify probe sets that are significantly up- or down-regulated between two treatments.

### RESULTS

ChIP analyses from three independent experiments identified 546 HIF-1 $\alpha$ -binding regions (length 128–1738 bp) and 143 HIF-2 $\alpha$ -binding regions (length 142–1866 bp) that met stringent criteria of an MA score more than 4 mean  $\pm$  S.D., a width of 100 bp, or 5 probes or more and a false discovery rate of <5%. Binding regions were annotated using the nearest gene locus, as identified by the shortest distance to a transcriptional start site. The 546 HIF-1 $\alpha$  binding sequences mapped to 394 different gene loci and the 143 HIF-2 binding sequences mapped to 134 different gene loci. The results for the top 25 sequences (as ranked by statistical significance) are given in Tables 1 and 2 and a complete list of all binding regions identified in HIF-1 $\alpha$  and HIF-2 $\alpha$  chromatin immunoprecipitations is provided in supplemental materials Tables S2 and S3. Immunoprecipitation of all of these top 25 sequences for both HIF-1 $\alpha$  and HIF-2 $\alpha$  was confirmed by quantitative-PCR. Genes at the immunoprecipitated loci included both known and previously unknown HIF-target genes. When stratified by gene ontology using the DAVID Bioinformatics Resources (david.abcc.ncifcr.gov) (42, 43) a trend toward a higher proportion of binding to HIF-1 *versus* HIF-2 was observed in genes encoding glycolytic enzymes and oxidoreductase enzymes, as compared with genes encoding molecules involved in angiogenic and hematopoietic pathways. However, the numbers in some functional groups were small, and overall differences were not statistically significant (Fig. 1).

**Relationship of Immunoprecipitated DNA Sequences to the HRE Consensus Sequence**—Previous studies have identified HIF binding sequences at ~70 loci, proposed to encode direct HIF transcriptional targets, defining a consensus core HIF-binding motif, RCGTG (8).

As a first step in analyzing these sequences we sought to determine what proportion of the captured DNA sequences contained the RCGTG core motif. Using an MA score of >4 to define the boundaries of immunoprecipitated sequences, we found that 235 (43%) HIF-1-binding sequences and 92 (66%) of the HIF-2-binding sequences contained an RCGTG consensus, many containing multiple motifs; as expected, in each case, this frequency was very much higher than in a control set of sequences taken from the promoter regions of a randomly selected set of genes ( $p < 10^{-9}$ ). We also observed an increased frequency of the RCGTG motif in the 300 bp (the limit of resolution derived from the DNA fragmentation procedure) flanking each identified sequence ( $p < 10^{-3}$ ), indicating that many loci are associated with RCGTG motifs just outside the defined sequence, most probably reflecting the use of a very stringent

TABLE 1

Anti-HIF-1 $\alpha$  chromatin immunoprecipitation (top 25 gene loci)

The table gives the chromosomal coordinates of each anti-HIF-1 $\alpha$  immunoprecipitated locus meeting criteria of an MA Z score of  $>4$ , a width of  $>100$  bp or  $>5$  probes, and a false discovery rate of  $<5\%$ , together with the maximum Z score for anti-HIF-1 $\alpha$  and anti-HIF-2 $\alpha$  at that gene locus. The presence or absence, and position of any HRE core motif RCGTG within these sequences is given. The table also specifies whether the nearest gene to the immunoprecipitated locus was significantly regulated by exposure of cells to 1% hypoxia or 2 mM DMOG or by transfection of siRNAs directed against HIF-1 $\alpha$ , HIF-2 $\alpha$ , or both siRNAs in the Affymetrix expression arrays. Note that at a number of loci several sequences were immunoprecipitated that shared proximity to a common transcriptional start site. These loci are listed in order with the most strongly enriched site indicated in bold type and others in normal type.

HIF-1	Gene Annotation	Gene name	RefSEQ number	Regulated in expression array	Chromosome	Start	End	Strand	Max Z-score		RCGTG
									HIF-1	HIF-2	
1	ASMT	Acetylserotonin <i>o</i> -methyltransferase	NM_004043	No	chrX	1,842,483	1,844,214	+	17.9	21.9	Yes
2	PLS3	Plastin 3 (t isoform)	NM_005032	No	chrX	114,898,432	114,899,202	+	15.8	17.4	Yes
3	GPI	Glucose-phosphate isomerase	NM_000175	No	chr19	39,541,340	3,9542,126	+	15.7	9.6	Yes
					chr19	39,540,468	39,540,783	+	7.4		No
4	DARS	Aspartyl-tRNA synthetase	NM_001349	Yes	chr2	136,458,510	136,459,327	-	15.5	9.8	Yes
5	SNAPC1	Small nuclear RNA activating complex, polypeptide 1, 43 kDa	NM_003082	No	chr14	61,291,513	61,291,955	+	13.9	6.0	No
6	SEC61G	Sec61 $\gamma$ -subunit	NM_001012456	No	chr7	54,794,557	54,794,993	-	13.4	12.2	Yes
7	GTF2IRD2B	Gtf2i repeat domain containing 2b	NM_001003795	No	chr7	74,146,820	74,147,110	+	12.7	7.1	No
					chr7	74,145,407	74,145,716	+	6.1		No
					chr7	74,147,633	74,147,933	+	5.4		No
8	SAP30	Sin3a-associated protein, 30 kDa	NM_003864	Yes	chr4	174,527,153	174,528,353	+	12.6	9.3	Yes
9	ZMYND8	Protein kinase C-binding protein 1	NM_012408	Yes	chr20	45,422,559	45,423,749	-	12.1	15.2	Yes
					chr20	45,421,122	45,421,975	-	9.3		Yes
					chr20	45,416,738	45,416,961	-	5.4		No
					chr20	45,324,234	45,324,808	-	5.3		Yes
					chr20	45,382,266	45,382,561	-	5.2		No
					chr20	45,381,728	45,381,912	-	5.0		No
					chr20	45,322,893	45,323,036	-	4.8		No
					chr20	45,380,596	45,380,865	-	4.7		No
10	RSBN1	Round spermatid basic protein 1	NM_018364	Yes	chr1	114,156,633	114,157,374	-	11.9	6.0	Yes
					chr1	114,154,609	114,155,069	-	5.4		Yes
					chr1	114,154,093	114,154,264	-	5.1		No
					chr8	26,297,111	26,297,957	+	11.9	7.9	Yes
11	BNIP3L	Bcl2/adenovirus e1b 19-kDa interacting protein 3-like	NM_004331	Yes	chr7	143,028,228	143,029,042	+	11.8	14.0	Yes
12	FAM139A	Hypothetical protein flj40722	NM_173678	Yes	chr7	73,904,992	73,905,289	-	11.6		Yes
13	GTF2IRD2	Gtf2i repeat domain containing 2	NM_173537	No	chr7	73,906,359	73,906,641	-	6.5		No
14	C3ORF28	Chromosome 3 open reading frame 28	NM_014367	Yes	chr3	123,584,923	123,585,595	+	11.6	4.9	No
					chr3	123,586,518	123,586,736	+	4.7		No
					chr3	123,585,892	123,586,061	+	4.6		No
15	STC2	Stanniocalcin 2	NM_003714	Yes	chr5	172,688,209	172,689,013	-	11.2	8.8	Yes
					chr5	172,689,765	172,690,086	-	6.0		Yes
16	NARF	Nuclear prelamina a recognition factor	NM_001038618	Yes	chr17	78,008,958	78,009,355	+	10.9		No
17	TF	Transferrin	NM_001063	No	chr3	134,944,094	134,944,856	+	10.7	13.6	Yes
18	HK2	Hexokinase 2	NM_000189	Yes	chr2	74,914,233	74,914,441	+	10.5	5.6	Yes
					chr2	74,912,970	74,913,723	+	6.9		No
19	INH1A	Inhibin, $\alpha$	NM_002191	Yes	chr2	220,149,206	220,150,032	+	10.4	9.1	Yes
					chr2	220,150,797	220,151,070	+	5.2		No
20	PCF11	Kiaa0824 protein	NM_015885	No	chr11	82,544,764	82,545,226	+	10.3	8.1	Yes
21	C9ORF30	Chromosome 9 open reading frame 30	NM_080655	No	chr9	102,228,622	10,228,900	+	10.3	9.7	Yes
					chr9	102,231,225	102,231,536	+	5.9		Yes
22	CBWD3	Cobw domain containing 3	NM_201453	No	chr9	70,046,581	70,047,378	+	10.2	5.1	Yes
23	RAD51L1	Rad51-like 1 ( <i>Saccharomyces cerevisiae</i> )	NM_133510	Yes	chr14	67,356,360	67,357,875	+	9.8	8.2	Yes
24	KIAA0195	Kiaa0195	NM_014738	No	chr17	70,960,840	70,961,202	+	9.7	11.6	Yes
25	S100P	S100 calcium-binding protein p	NM_005980	Yes	chr4	6,721,070	6,721,680	+	9.7	9.4	Yes

boundary definition. When these flanking sequences were included, the number of sequences that contain an RCGTG motif rose to 382 (70%) for HIF-1 $\alpha$  and 121 (85%) for HIF-2 $\alpha$ . Thus, whereas the large majority of HIF-binding sequences contain an RCGTG motif, a significant minority apparently do not. Whether these sequences are captured through higher order DNA/protein interactions with HIF bound at HREs, or represent other modes of DNA binding by HIF proteins is unclear.

Overall, however, within the promoter array, only a small proportion ( $<1\%$ ) of DNA sequences containing the core RCGTG motif bound HIF-1 $\alpha$  or HIF-2 $\alpha$ . We next sought to define features of the DNA sequence that might be associated with immunoprecipitation either by anti-HIF-1 $\alpha$  or anti-HIF-2 $\alpha$  antibodies. First, we examined the sequences in the immediate vicinity of the RCGTG motifs. All immunoprecipitated RCGTG motifs were extended by 15 base pairs in both directions. However, analysis did not reveal any significant

overrepresentation of particular bases beyond the core 5-base pair motif for either HIF-1- or HIF-2-binding sites (supplemental materials Fig. S1).

Second, we tested the predictive value of conservation in determining HIF-binding to the consensus motif. Altogether 82 of the 464 RCGTG consensus motifs that bound HIF-1 $\alpha$  and 38 of 188 that bound HIF-2 $\alpha$  were within the top 10% most conserved regions of the genome (as defined by PhastCons score). Although this proportion was significantly greater than would be expected by chance alone ( $p < 0.005$ ), when the same analysis was performed on a set of control sequences from promoters that did not bind HIF, comparable results were obtained, suggesting that, within promoter regions, whether a putative HRE lies within a region of apparent conservation is not a good guide to its ability to bind HIF.

Third, we analyzed the distribution of the captured sequences and associated RCGTG motifs in relation to the transcriptional start site of the closest gene. Clearly the design

TABLE 2

Anti-HIF-2 $\alpha$  chromatin immunoprecipitation (top 25 gene loci)

The table gives the chromosomal coordinates of each anti-HIF-2 $\alpha$  immunoprecipitated locus meeting criteria of an MA Z-score of  $>4$ , a width of  $>100$  bp or  $>5$  probes, and a false discovery rate of  $<5\%$ , together with the maximum Z-score for anti-HIF-1 $\alpha$  and anti-HIF-2 $\alpha$  at that gene locus. The presence or absence, and position of any HRE core motif RCGTG within these sequences is given. The table also specifies whether the nearest gene to the immunoprecipitated locus was significantly regulated by exposure of cells to 1% hypoxia or 2 mM DMOG or by transfection of siRNAs directed against HIF-1 $\alpha$ , HIF-2 $\alpha$ , or both siRNAs in the Affymetrix expression arrays. Note that at a number of loci several sequences were immunoprecipitated that shared proximity to a common transcriptional start site. These loci are listed in order with the most strongly enriched site indicated in bold type and others in normal type.

HIF-2	Gene Annotation	Gene name	RefSeq number	Regulated in array	Chromosome	Start	End	Strand	Max Z-score		RCGTG
									HIF-2	HIF-1	
1	ASMT	Acetylserotonin <i>o</i> -methyltransferase	NM_004043	No	chrX	<b>1,842,483</b>	<b>1,844,249</b>	+	21.9	17.9	Yes
2	PLS3	Plastin 3 (t isoform)	NM_005032	No	chrX	114,898,432	114,899,202	+	17.4	15.8	Yes
3	ZMYND8	Protein kinase C-binding protein 1	NM_012408	Yes	chr20	<b>45,422,559</b>	<b>45,423,470</b>	-	15.2	12.1	Yes
					chr20	45,421,078	45,421,975	-	12.5		Yes
4	FAM139A	Hypothetical protein flj40722	NM_173678	Yes	chr7	<b>143,028,228</b>	<b>143,029,153</b>	+	14.0	11.8	Yes
5	TF	Transferrin	NM_001063	No	chr3	<b>134,944,026</b>	<b>134,944,856</b>	+	13.6	10.7	Yes
6	DHX35	Deah (Asp-Glu-Ala-His) box polypeptide 35	NM_021931	No	chr20	<b>37,022,195</b>	<b>37,022,900</b>	+	12.7	5.6	No
7	SEC61G	Sec61 $\gamma$ -subunit	NM_001012456	No	chr7	<b>54,794,671</b>	<b>54,794,993</b>	-	12.2	13.4	No
8	HIG2	Hypoxia-inducible protein 2	NM_013332	Yes	chr7	<b>127,882,554</b>	<b>127,883,114</b>	+	12.1	9.5	Yes
9	CITED2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxyl-terminal domain, 2	NM_006079	Yes	chr6	<b>139,738,163</b>	<b>139,739,480</b>	-	12.1	8.1	Yes
10	RAPGEF1	Rap guanine nucleotide exchange factor (gef) 1	NM_005312	No	chr9	<b>133,609,690</b>	<b>133,610,065</b>	-	12.0	6.2	Yes
11	CHD1L	Chromodomain helicase DNA-binding protein 1-like	NM_004284	No	chr1	<b>145,181,416</b>	<b>145,181,919</b>	+	11.9	7.5	Yes
12	UPK1B	Uroplakin 1b	NM_006952	No	chr3	<b>120,386,306</b>	<b>120,387,057</b>	+	11.7	8.6	Yes
13	KIAA0195	Kiaa0195	NM_014738	No	chr17	<b>70,960,840</b>	<b>70,961,202</b>	+	11.6	9.7	Yes
14	BACE2	$\beta$ -Site app-cleaving enzyme 2	NM_138992	Yes	chr21	<b>41,458,829</b>	<b>41,460,026</b>	+	11.4	8.7	Yes
15	C1ORF161	Chromosome 1 open reading frame 161	NM_152367	No	chr1	<b>116,455,368</b>	<b>116,456,452</b>	+	11.2	6.9	Yes
16	GPRC5A	G protein-coupled receptor, family c, group 5, member a	NM_003979	Yes	chr12	<b>12,916,200</b>	<b>12,917,123</b>	+	11.2	6.1	Yes
17	UBC	Ubiquitin c	NM_021009	No	chr12	<b>123,966,902</b>	<b>123,967,854</b>	-	11.2	7.4	No
18	S100A4	S100 calcium-binding protein a4 (calcium protein, calvasculin, metastasin, murine placental homolog)	NM_002961	Yes	chr1	<b>151,784,245</b>	<b>151,784,831</b>	-	11.1	4.7	Yes
19	FLJ16641	Flj16641 protein	NM_001004316	No	chr3	<b>158,017,688</b>	<b>158,018,251</b>	+	10.8	9.3	Yes
					chr3	158,012,829	158,013,289	+	8.9		Yes
					chr3	158,027,265	158,027,560	+	7.4		Yes
20	ARRDC3	Arrestin domain containing 3	NM_020801	No	chr5	<b>90,612,390</b>	<b>90,613,364</b>	-	10.6	9.4	Yes
21	CYP27C1	Flj16008 protein	NM_001001665	No	chr2	<b>127,700,812</b>	<b>127,701,131</b>	-	10.5	8.3	Yes
22	DARS	Aspartyl-tRNA synthetase	NM_001349	Yes	chr2	<b>136,458,799</b>	<b>136,459,291</b>	-	9.8	15.5	Yes
23	PLAC8	Placenta-specific 8	NM_016619	Yes	chr4	<b>84,253,030</b>	<b>84,253,558</b>	-	9.8	4.8	Yes
24	C9ORF30	Chromosome 9 open reading frame 30	NM_080655	No	chr9	<b>102,228,622</b>	<b>102,228,871</b>	+	9.7	10.3	Yes
25	FLJ39743	Hypothetical protein flj39743	NM_182562	No	chr15	<b>96,875,764</b>	<b>96,876,378</b>	-	9.7	5.9	Yes

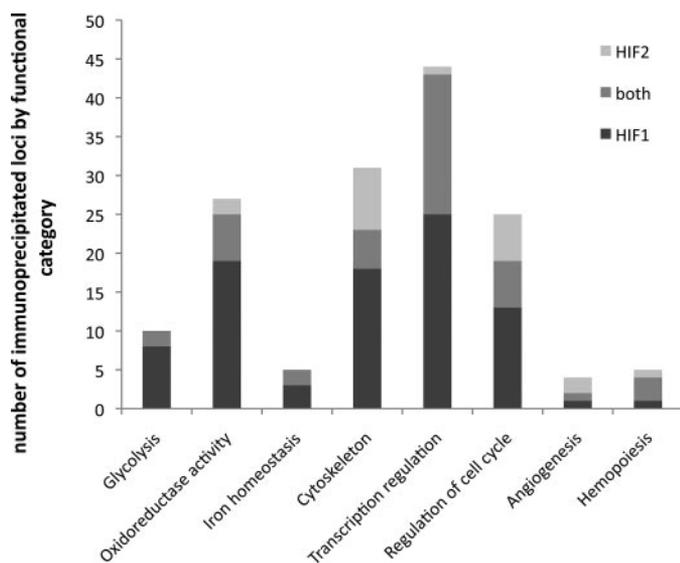


FIGURE 1. **Functional classification of HIF-binding gene loci.** The highest stringency (MA score  $>4$ ) HIF-1 $\alpha$  and HIF-2 $\alpha$  immunoprecipitating gene loci were stratified by gene ontology using the DAVID Bioinformatics Resource. The number of immunoprecipitating gene loci in each functional category is displayed.

of the array (intended to represent sequences from  $-7.5$  to  $+2.5$  kb of the transcription start site) places limits on this analysis, although a proportion of the immunoprecipitated sequences lay outside these regions, most likely representing

changes in gene annotation between that on which the array was designed and the (latest) annotation that we used for locus assignment. Several interesting features were observed irrespective of whether the precise position of the peak of enrichment profile or the associated HRE was used in the analysis. The distribution of immunoprecipitated sequences was closely similar for both HIF-1 $\alpha$  and HIF-2 $\alpha$  with the greatest density of HIF-binding sites being observed within 2 kb of the transcriptional start. This region comprised two distinct frequency peaks. One is a sharp peak centered 500 bp upstream of the transcriptional start site, similar to that observed for other transcription factors (44). The other is a broader peak centered 1000 bp downstream of the transcriptional start site. This distribution contrasted markedly with the distribution of all RCGTG motifs at the same set of promoters, which revealed a single peak centered at the transcriptional start site (Fig. 2).

Finally, to search for overrepresented transcription factor-binding sites that might bind factors cooperating with HIF we identified HIF-binding regions falling within 1 kb of the gene transcriptional start sites (any transcript from the gene). We then extracted the regions 1 kb up- and downstream of the transcriptional start site, omitting known exons (all annotation was taken from the ENSEMBL genome data base (release 51) (45)). We searched these regions using the vertebrate matrices of the TRANSFAC data base (release 2008.4, 621 matrices) (46), using the transcription factor-binding sites perl modules and a

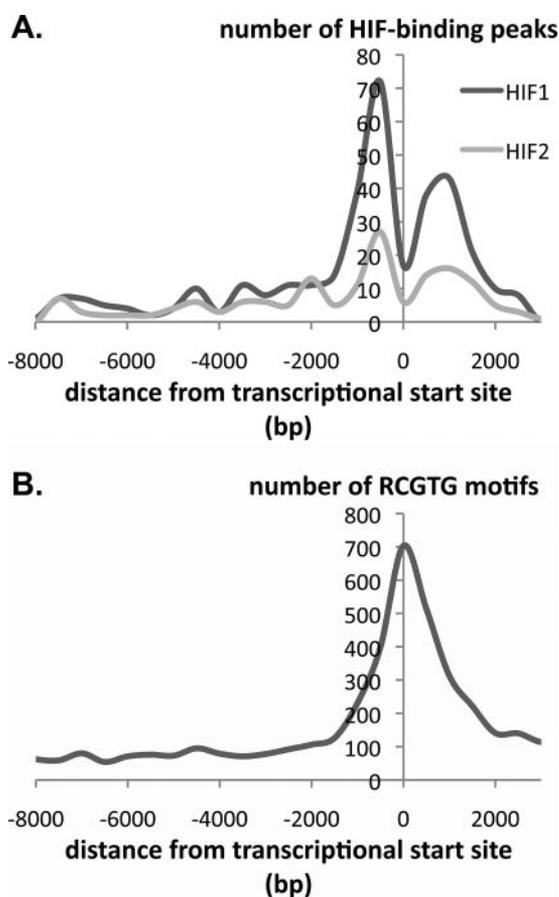


FIGURE 2. **Distribution of HIF-1 $\alpha$ - and HIF-2 $\alpha$ -binding sites.** *A*, distribution of high stringency (MA score  $>4$ ) HIF-1 $\alpha$ - and HIF-2 $\alpha$ -binding sites referred to the nearest transcriptional start site. Number of HIF-binding peaks expressed per 500-bp bin is shown. *B*, frequency distribution of RCGTG motifs at gene loci that bind HIF-1 $\alpha$  or HIF-2 $\alpha$ . The number of RCGTG motifs expressed per 500-bp bin is shown.

90% scoring threshold cut off, recording, for each factor, and each potential target gene, the number of matches per 1000 non-exonic bases. We compared these numbers to the corresponding numbers for all non-HIF binding gene loci in the genome using a Wilcoxon test implemented in the R software package, using a threshold of  $p < 8 \times 10^{-5}$  ( $p < 0.05$  with Bonferroni correction for multiple comparisons).

For all matched HIF regions (combining the HIF-1 $\alpha$  and HIF-2 $\alpha$  sets), six matrices were identified as overrepresented in the HIF-binding loci (Fig. 5). Three are HIF-binding motifs, further validating the currently held HRE consensus; the others being binding motifs for nuclear respiratory factor-1 (47), Myc intron factor (MIF-1) (48), and E2F transcription factors (49). Given the strict definition of statistical significance, it is also likely that a number of other overrepresented matrices are also valid. Indeed, a number of other E2F matrices, as well as those for an array of previously described HIF-interacting transcriptional cofactors, such as STAT, ETS, and MYC (8), were enriched, but failed to reach the statistical threshold (supplemental materials Table S4). When subgroups of HIF-1 $\alpha$  and HIF-2 $\alpha$  interacting gene loci were analyzed no significant difference was identified between the two sets of genes, although the numbers in this subgroup analysis were smaller.

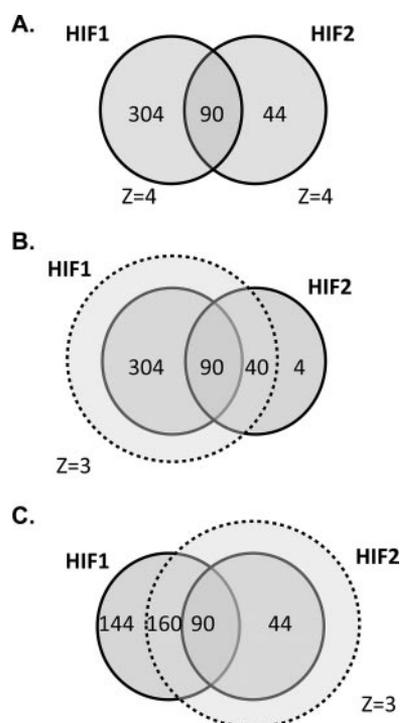


FIGURE 3. **Intersection between HIF-1 $\alpha$ - and HIF-2 $\alpha$ -binding gene loci.** *A*, gene loci that bound either HIF-1 $\alpha$  or HIF-2 $\alpha$  or both isoforms with a Z-score  $>4$ . *B*, defining HIF-1 $\alpha$  capture with a Z-score of 3 encompasses almost all HIF-2 $\alpha$  binding loci. *C*, defining HIF-2 $\alpha$  capture with a Z-score of 3 excludes almost one-third of HIF-1 $\alpha$  binding loci.

*DNA Sequences Immunoprecipitated by Anti-HIF-1 $\alpha$  and Anti-HIF-2 $\alpha$  Overlap*—Overall, there was a substantial overlap between the loci immunoprecipitated with the two HIF isoforms. Thus, of the 394 loci that bound HIF-1 $\alpha$  and the 134 loci that bound HIF-2 $\alpha$ , 90 bound both isoforms. When the stringency of binding for the second isoform was relaxed slightly (MA score  $>3$ ), the overlap increased, with 250/394 HIF-1 $\alpha$  loci binding HIF-2 $\alpha$  and 130/134 HIF-2 $\alpha$  loci binding HIF-1 $\alpha$ . In addition, when both HIF isoforms bind to the same gene locus, they do so at a common site. Thus, of the 90 gene loci that bound both HIF isoforms at the highest level of stringency, all apart from 3 bound HIF-1 $\alpha$  and HIF-2 $\alpha$  at overlapping sites (Fig. 3).

Nevertheless, a substantial number of gene loci apparently bound HIF-1 $\alpha$  in isolation, despite the similar consensus. We hypothesized that this could be due to higher levels of HIF-1 $\alpha$  in the cells, higher affinity of HIF-1 $\alpha$  capture by the immunoprecipitating antibody, a generalized higher affinity binding of HIF-1 $\alpha$  to the DNA across all loci, or specific differences in affinity for the HIF- $\alpha$  isoforms at particular loci despite a similar consensus.

Although we had selected MCF-7 cells for this study on the basis of apparently similar expression of both HIF- $\alpha$  isoforms, we wished to check the relative levels of each isoform using quantitative immunoblotting related to a common standard (supplemental materials Fig. S2). These studies revealed that HIF-2 $\alpha$  levels were at least as high as HIF-1 $\alpha$  (probably in the region of 3–5-fold higher) indicating that the relative abundance of the two HIF isoforms cannot explain the increased capture by anti-HIF-1 $\alpha$ . If the reason for the excess of sites

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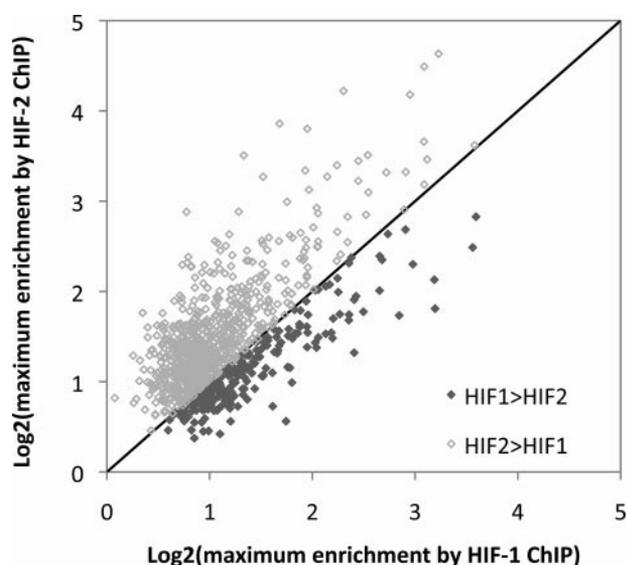


FIGURE 4. **Capture by HIF-1 $\alpha$  and HIF-2 $\alpha$  at common binding sites.** Using a Z-score of 2 for each immunoprecipitation identified 992 genomic regions that bound both HIF-1 $\alpha$  and HIF-2 $\alpha$ . For each of these sequences the maximum enrichment by HIF-2 $\alpha$  immunoprecipitation was plotted against that for the HIF-1 $\alpha$  immunoprecipitation. More points lie above than below the line of identity indicating greater average enrichment by HIF-2 $\alpha$  immunoprecipitation at these common binding loci.

captured by anti-HIF was a greater affinity of the anti-HIF-1 $\alpha$  antibody, or a globally higher affinity of HIF-1 $\alpha$  for the consensus binding motif, then it would be predicted that enrichment with anti-HIF-1 $\alpha$  immunoprecipitation would generally be greater than enrichment by HIF-2 $\alpha$  immunoprecipitation across sequences that were immunoprecipitated by both antibodies. We therefore examined all overlapping HIF-1 $\alpha$ - and HIF-2 $\alpha$ -binding sites for which the MA score was greater than 2. For each of these overlapping binding sites, the maximum fold-enrichment by the two HIF isoforms was compared. In contrast to prediction, across these common sites, HIF-2 $\alpha$  showed a trend to greater maximum enrichment than HIF-1 $\alpha$  (Fig. 4). Therefore, we conclude that excess HIF-1 $\alpha$ -binding sites is not due to better performance of the anti-HIF-1 $\alpha$  antibody or higher affinity of HIF-1 $\alpha$  for its consensus, and is likely to be due to specific cooperative interactions that regulate (positively or negatively) preferential binding of HIF- $\alpha$  isoforms at certain loci. Interestingly, a weak, but significant positive correlation was also seen between the maximum fold-enrichment by each of the two antibodies suggesting that binding of one isoform to a given sequence is not a result of decreased binding of the other isoform.

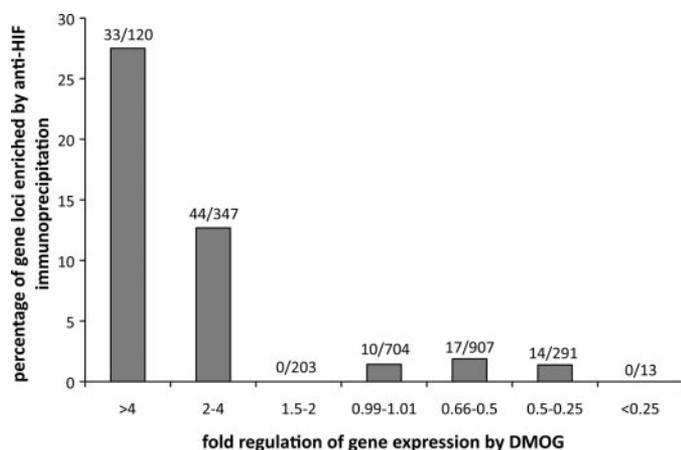
**Relationship of Chromatin Immunoprecipitation to the Functional Effects of the HIF- $\alpha$  Isoforms**—To better understand the functional consequences of promoter binding by HIF-1 $\alpha$  and HIF-2 $\alpha$  we next compared chromatin immunoprecipitation data with functional effects on gene expression across the genome, assayed in the same cell line using 2 mM DMOG or 1% hypoxia with or without siRNA to the HIF- $\alpha$  isoforms (9). Overall, 4% of Affymetrix transcripts were significantly up- or down-regulated by one or another of the stimuli with very strong correlations being observed between up-regulation by DMOG and hypoxia and down-regulation by HIF-1 $\alpha$  siRNA (9). Genes at loci that were immunoprecipitated by anti-HIF-1 $\alpha$

or anti-HIF-2 $\alpha$  antibodies were very significantly more likely to be up-regulated by the hypoxia/HIF-response. Thus 20.8% of the loci immunoprecipitated by anti-HIF-1 $\alpha$  manifest significant up-regulation by at least one of these stimuli *versus* 3.6% of those not immunoprecipitated ( $p < 10^{-10}$ ), whereas for loci immunoprecipitated by anti-HIF-2 $\alpha$  the equivalent figures were 32.8 *versus* 3.7% ( $p < 10^{-10}$ ). Although, as expected, these correlations are highly significant, the proportion of HIF-binding loci associated with HIF-inducible transcripts was still well below 50%. To explore this further we examined other genes in the vicinity of the immunoprecipitated loci. When all genes within 100 kb of the immunoprecipitated sequences were analyzed, only a few additional regulated transcripts were identified. For instance, at HIF-1 $\alpha$  binding loci only 18 further regulated genes were identified in the expression array. Hence, the majority of HIF immunoprecipitating sequences do not regulate expression of a gene within 100 kb of the binding site.

When HIF-1 $\alpha$  and HIF-2 $\alpha$  binding was separately correlated with functional effects of siRNA suppression a very striking difference was observed. Whereas 15.6% of gene loci that were immunoprecipitated by the HIF-1 $\alpha$  antibody were down-regulated by HIF-1 $\alpha$  siRNA as compared with 2.3% of gene loci that were not immunoprecipitated ( $p < 2 \times 10^{-5}$ ), only 1.5% of the gene loci immunoprecipitated by the HIF-2 $\alpha$  antibody were reduced by HIF-2 $\alpha$  siRNA, compared with 0.3% of gene loci that did not immunoprecipitate. Furthermore, when quantitative comparisons were made between maximum fold-enrichment by anti-HIF- $\alpha$  chromatin immunoprecipitation and reduction in gene expression by HIF- $\alpha$  siRNA, a clear correlation was observed for HIF-1 $\alpha$  ( $r = -0.367$ ,  $p < 0.001$ ) but not HIF-2 $\alpha$  ( $r = -0.183$ ,  $p = 0.04$ ) where the gradient of any correlation was close to zero (supplemental materials Fig. S3). Similarly no correlation was found between the ratio of combined HIF-1 and -2 $\alpha$  siRNA to HIF-1 $\alpha$  siRNA (as a measure of HIF-2 $\alpha$  activity) and enrichment by anti-HIF-2 $\alpha$  ChIP, whereas responses to hypoxia ( $p = 0.02$ ), DMOG ( $p = 0.015$ ), combined HIF-1 and -2 $\alpha$  siRNA ( $p < 0.001$ ), and the ratio of combined HIF-1 and -2 $\alpha$  to HIF-2 $\alpha$  siRNA (as a measure of HIF-1 $\alpha$  activity) all correlated with enrichment by HIF-1 $\alpha$  ChIP.

Despite the increased binding of HIF-2 $\alpha$  compared with HIF-1 $\alpha$  at gene loci that bound both isoforms, only one of 90 (TMTC2) was significantly down-regulated by HIF-2 $\alpha$  siRNA compared with 25 that were down-regulated by HIF-1 $\alpha$  siRNA. Furthermore, TMTC2 was down-regulated by both siRNAs.

In addition, when the top 500 HIF-2 $\alpha$  responsive genes (defined as those manifesting the greatest reduction by HIF-2 $\alpha$  siRNA) were compared with the 500 least responsive HIF-2 $\alpha$  genes no differences were observed either in the numbers reaching an MA score of at least 4 or in the average MA score or fold-enrichment by anti-HIF-2 $\alpha$  ChIP. Thus, even the modest effects of HIF-2 $\alpha$  on gene regulation that were observed do not appear to relate to HIF-2 $\alpha$  binding at these loci, suggesting that these effects of HIF may be indirect. This suggests that although HIF-2 $\alpha$  is (on average) binding more strongly than HIF-1 $\alpha$  at these loci, HIF-1 $\alpha$  is largely or even entirely responsible for transcriptional responses over the time course of the experiments.



**FIGURE 5. Relationship between amplitude and direction of regulation and chromatin immunoprecipitation by anti-HIF-1 $\alpha$  or HIF-2 $\alpha$ .** Gene loci were assigned to functional groups according to fold up- or down-regulation by DMOG in the Affymetrix expression array. The proportion of gene loci captured by either of the anti-HIF- $\alpha$  immunoprecipitations is given for each of the functional group.

*Direction of Regulation by Hypoxic Stimuli and HIF Binding*—Finally, to pursue the question of the extent to which hypoxia/DMOG inducible and HIF-dependent changes in gene expression are directly or indirectly dependent on HIF binding at the relevant locus, we categorized genes based on the amplitude and direction of changes in expression in response to these stimuli, and determined the proportion of genes in each category that were associated with sequences binding to HIF (Figs. 5 and supplemental S4). This analysis revealed a striking difference between genes that were up-regulated by hypoxic stimuli and those that were down-regulated by these stimuli. Thus for genes that were significantly up-regulated by DMOG, 28% of all loci bound HIF-1 $\alpha$ , HIF-2 $\alpha$ , or both, whereas for genes that were down-regulated the figure was not significantly different from the 1.4% of genes defined as unregulated (on the basis of fold-regulation by DMOG between 0.99 and 1.01). Grouping of genes by the magnitude of regulation by DMOG revealed that more of the highly up-regulated genes bound HIF (27.5% of those regulated >4-fold by DMOG *versus* 12.7% of those regulated 2–4-fold and 0% of those regulated 1.5–2 fold;  $p < 10^{-12}$ ) (Fig. 5). However, no such association was observed among the down-regulated genes where even the most strongly down-regulated genes were no more likely than unregulated genes to bind HIF. Similar results were obtained whether functional responses were stratified on the basis of the response to hypoxia, or suppression of that response by siRNA directed against HIF- $\alpha$  (supplemental materials Fig. S4).

## DISCUSSION

Genome-wide analysis of DNA binding to the HIF transcription factors defined more than 600 sites that bound HIF-1 $\alpha$ , HIF-2 $\alpha$ , or both proteins, although, given the highly stringent criteria we used to define binding sites and the restriction of the tiled array to known or predicted gene promoter regions, the actual number of HIF-binding sites is likely to be substantially greater.

Our results confirmed the core HIF-binding motif, RCGTG (8), and did not define other bases in the immediate vicinity that

were significantly overrepresented at bound regions. Nevertheless, HIF binding to this motif was highly selective, indicating that its epigenetic context must in some way define binding. Less than 1% of such motifs within the tiled array bound HIF, and bound sites showed a highly selective distribution in relation to the transcriptional start site. Unexpectedly, binding sites were found to cluster in two distinct regions  $\sim 0.5$  kb upstream and 1 kb downstream of the start site, presumably representing the operation of other factors that promote HIF binding within these regions, or restrict accessibility or binding of HIF in the immediate vicinity of the start sites. Indeed, such binding patterns have been previously described for several other transcription factors as well as for histone modifications (50, 51). *In silico* searches for transcription factor binding motifs that were overrepresented at HIF binding promoters *versus* equivalent regions at other promoters revealed a number of sites. Interestingly, after the HIF binding consensus itself, the most strongly enriched site was the core binding motif for nuclear respiratory factor-1, a key transcriptional regulator of genes involved in mitochondrial biogenesis and function (47). Whether this reflects specific cooperation in the response to hypoxia, or the operation of physiologically distinct pathways on a group of common genes will require further study.

Pan-genomic comparison of the chromatin immunoprecipitation data with functional responses (9) enabled HIF-binding and transcriptional responses to be compared across the genome. Surprisingly, even though analysis of HIF-binding sequences was restricted to high stringency binding at known or predicted promoter regions, the region of 70% of bound loci were not associated with genes that manifest significant responses to either hypoxia or HIF, within 100 kb of the bound locus. The proportion of HIF-binding loci responding to HIF is comparable with the ratio of Myc binding genes that were differentially modulated in response to Myc (52), where additional transcription factor binding is necessary so that targets are poised to respond (50). Furthermore, although it is also possible that these sites affect gene expression over even longer distances, or are entirely non-functional, the findings also raise the possibility that HIF has as yet unknown effects on genome integrity or function that are mediated by binding at such sites.

Comparison of chromatin immunoprecipitation and functional responses to HIF also allowed the proportion of direct *versus* indirect HIF-dependent responses to be estimated. Across the genome, in the region of 20% of genes reported to demonstrate positive HIF-1 $\alpha$ -dependent responses bound HIF at their promoters. Although we cannot be certain that all these HIF-binding sites are functional, the use of highly stringent criteria to define HIF-binding and limited genome coverage by the promoter array would suggest that 20% is more likely to be a lower estimate of the proportion of positive transcriptional responses that are directly dependent on HIF.

These results are in striking contrast with the analysis of genes manifesting down-regulation of expression in response to HIF. Expression array studies have defined similar numbers of genes that are positively and negatively regulated by HIF. In some cases promoter analyses of negatively regulated genes have defined HIF-binding sites and it has been proposed that displacement of more powerful transcriptional activators, or

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recruitment of corepressors to HIF, accounts for down-regulation of gene expression by HIF (53–55). Our results suggest that these particular mechanisms of HIF-dependent down-regulation of genes are unusual. When promoters of negatively regulated genes were surveyed for HIF binding we did not observe an excess of binding over that in the promoters of genes that were entirely unresponsive to HIF. Thus it appears that for the large majority of genes, HIF-dependent down-regulation of expression is likely to be due to indirect effects “in *trans*,” rather than direct effects of HIF on the promoter. In keeping with this, a number of genes encoding transcriptional repressors have been identified as positively regulated HIF targets in this and other studies (56, 57). In addition, recent studies have established a role of hypoxia and HIF in the regulation of specific microRNAs, notably miR-210, which act to down-regulate gene expression (58, 59).

Comparison of HIF-1 $\alpha$  and HIF-2 $\alpha$  DNA binding revealed that whereas many loci bound both HIF- $\alpha$  isoforms, substantially more bound HIF-1 $\alpha$  than HIF-2 $\alpha$ . Further analysis indicated that this was not due to greater abundance, better antibody capture, or a generally higher affinity of HIF-1 $\alpha$  for DNA. Rather, distinct patterns of binding were observed. At common binding sites there was a tendency to greater enrichment of DNA in the anti-HIF2 $\alpha$  chromatin immunoprecipitations, suggesting that affinity for HIF-2 $\alpha$  was at least as high as for HIF-1 $\alpha$ , whereas at other sites, binding was essentially restricted to HIF-1 $\alpha$ . How this relates to functional transcriptional selectivity is currently unclear.

Recently, studies of selected HIF target genes in mouse embryonic stem cells demonstrated that bound HIF-2 $\alpha$  is transcriptionally inactive at the loci analyzed, possibly due to a titratable repressor (12). Although studies in other cells also reported that HIF-2 $\alpha$  is transcriptionally inactive on specific HIF target genes (60, 61), we had assumed that this phenomenon would be highly locus specific, because analyses conducted have demonstrated a range of HIF-2 $\alpha$ -dependent transcriptional effects under specific circumstances (11, 32, 34, 35). Our genome-wide studies of both chromatin immunoprecipitation and HIF- $\alpha$ -dependent gene expression indicate that this is not the case. Rather, under the conditions of these experiments, HIF-2 $\alpha$  appears to exert little or no direct transcriptional activity at HRE-associated targets across the genome despite high affinity binding to these sites. Whether this is due to interaction of HIF-2 $\alpha$  with other pathways such as Myc (22, 62) or temporal differences between HIF-1 $\alpha$  and HIF-2 $\alpha$  activation (34) remains to be determined. Overall, our results provide a framework for a better understanding of the HIF transcriptional cascade that should be of importance in understanding the biology of hypoxia and opportunities for therapeutic manipulation of the response.

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