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A role for insulator elements in the regulation of gene expression response to hypoxia

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ABSTRACT

Hypoxia inducible factor (HIF) up-regulates the transcription of a few hundred genes required for the adaptation to hypoxia. This restricted set of targets is in sharp contrast with the widespread distribution of the HIF binding motif throughout the genome. Here, we investigated the transcriptional response of *GYS1* and *RUVBL2* genes to hypoxia to understand the mechanisms that restrict HIF activity toward specific genes. GYS1 and RUVBL2 genes are encoded by opposite DNA strands and separated by a short intergenic region (~1 kb) that contains a functional hypoxia response element equidistant to both genes. However, hypoxia induced the expression of GYS1 gene only. Analysis of the transcriptional response of chimeric constructs derived from the intergenic region revealed an inhibitory sequence whose deletion allowed RUVBL2 induction by HIF. Enhancer blocking assays, performed in cell culture and transgenic zebrafish, confirmed the existence of an insulator element within this inhibitory region that could explain the differential regulation of *GYS1* and *RUVBL2* by hypoxia. Hence, in this model, the selective response to HIF is achieved with the aid of insulator elements. This is the first report suggesting a role for insulators in the regulation of differential gene expression in response to environmental signals.

INTRODUCTION

A large number of biochemical reactions require oxygen as a substrate and metazoa metabolism is largely dependent on oxidative phosphorylation. At the cellular level, the unbalance between oxygen demand and supply (hypoxia) results in the activation of a specific gene expression program aimed at increasing oxygen delivery and reducing its consumption through metabolic reprogramming. This transcriptional response is mostly mediated by an evolutionarily conserved family of transcription factors termed hypoxia inducible factors (HIFs), that belong to the basic helix-loop-helix superfamily (1). HIFs are heterodimers of a constitutive beta subunit (HIFβ also known as ARNT), that partners with several factors and an alpha subunit (HIFα), whose stability (2) and transcriptional activity (3) is regulated by oxygen. Under hypoxia, HIFα subunits avoid degradation, bind to the constitutively expressed beta subunits and the heterodimers translocate to the nucleus where they bind to the RCGTG motif within the regulatory regions of target genes to promote their transcription (4–6). Several works have identified individual HIF targets that, taken together, account for the metabolic adaptation and induction of angiogenesis observed under hypoxia (7). To gain insight into the full range of cellular adaptations to hypoxia, several groups recently attempted the global identification of HIF-targets (5,6,8–11). Interestingly, all these works coincide in that only a few hundred, out of all the genes containing RCGTG motifs, are regulated by hypoxia. Thus, as it is the case for other transcription factors (12), HIF binds only to a small proportion of the

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potential binding sites (5,6,8–11). The basis for this selectivity is incompletely understood, but several mechanisms have been proposed. Among them, the cooperation with other transcription factors, have been well characterized in some instances (13,14). In the case of HIF, requirement of functional HNF-4 (15), AP-1 (16), GATA-2 (16) or ETS (17,18) sites for proper hypoxic induction of selected targets have been described. In agreement with these single locus studies, global analysis of HIF binding sites by means of experimental (5) and computational methods (11) showed the existence of overrepresented transcription factor binding sites (TFBS) in close proximity to the hypoxia response element (HRE) that might account for factors cooperating with HIF. However, the experimental characterization of the role of these TFBS in the regulation of HIF targets by hypoxia is yet to be determined. Thus, the cooperation between HIF and other factors could contribute to the target selectivity, but it is yet unclear to what extent this mechanism explains the observed pattern of targets.

A further mechanism that could dictate the target selectivity is the accessibility to the TFBS. Histone modifications alter the structure of chromatin and hence the availability of the underlying nucleotide sequence for the binding of transcription factors (19). In addition, DNA methylation can preclude the binding of specific transcription factors (20–22). In this regard, a recent study addressed the cell-type specificity in response to hypoxia and concluded that only those loci that were transcriptionally active under basal (normoxic) conditions were permissive to HIF-regulation (8). However, although these results explain most of the intercellular variation in the hypoxic transcriptome, it is clear that an additional layer of regulation is required, as only a small fraction of all the active genes under basal conditions were induced by hypoxia in any of the cell lines studied.

Finally, insulators are included among the regulatory mechanisms employed by eukaryotes to ensure specific patterns of gene expression and as such, they could be involved in the selection of genes to be activated by HIF in response to hypoxia. Insulators are defined as DNA elements that partition chromatin into independent transcriptional domains, thereby contributing, in combination with additional epigenetic mechanisms, to the tight control of gene expression (23) and to the nuclear structure and dynamic organization (24). These elements have been functionally described, according to their ability to block the spread of heterochromatin (barrier function) into adjacent loci and to prevent the promiscuous interaction of distal enhancers with proximal promoters, when placed in between (enhancer blocking function) (25). The role of insulators in the determination of linage-specific patterns of gene expression is well characterized, as illustrated in the chicken β-globin locus (26). However, the participation of these elements in the target discrimination by acutely activated transcription factors, in response to environmental factors, has not been reported.

Here, we investigate the mechanism that restricts HIF activity toward specific genes by the study of a locus that we consider paradigmatic, the *GYS1*/*RUVBL2* genomic region from the human genome. The *GYS1* gene was recently described as a novel hypoxia-inducible gene and a functional HRE was identified upstream its promoter (27). Interestingly, very close to *GYS1*, but encoded by the opposite DNA strand, is located the *RUVBL2* gene. In spite of the location of the HRE between both genes, we found that only *GYS1*, but not *RUVBL2*, was induced in response to hypoxia. The lack of RUVBL2 response to hypoxia was not due to epigenetic silencing of its promoter as it showed a substantial transcriptional activity and the level of RUVBL2 mRNA was comparable with that of GYS1. Instead, the analysis of different reporter constructs derived from the intergenic GYS1/RUVBL2 sequence, revealed an inhibitory region, located between the HRE and the RUVBL2 minimal promoter, that prevented the induction of RUVBL2 by HIF. Removal of this region allowed the up-regulation of the RUVBL2 promoter upon HRE activation. This result hinted the existence of an enhancer blocking element within the inhibitory region that prevented the interaction between the HRE and the RUVBL2 promoter. By means of specific enhancer blocking assays (EBA), performed in cell culture and using heterologous constructs in transgenic zebrafish, we confirmed the existence of an insulator element within this locus that could explain the differential regulation of GYS1 and RUVBL2 by hypoxia. Altogether, our results suggest that HIF selectivity is achieved, at least in this locus, by an insulator element that prevents the activity of the HRE/HIF complex on the *RUVBL2* promoter.

MATERIALS AND METHODS

Cell culture and reagents

Human cervical-carcinoma cells (HeLa) and Human embryonic kidney 293 (HEK293) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and 100 U/ml penicillin and 100 mg/ml streptomycin and cultured at 37°C and 5% CO_2 in a humidified incubator.

For hypoxia treatments, cells were grown at 37°C in sealed chambers (Billups–Rothenberg) flushed with a 1% O_2, 5% CO_2, 94% N_2 gas mixture or in a Whitley hypoxystation (don Whitley Scientific, UK) set at 1% oxygen concentration.

Dimethyloxalylglycine (DMOG, Frontier Scientific, CA, USA) was added to the indicated cultures at a 500 μM final concentration. For the analysis of RUVBL2 and GYS1 expression, cDNA obtained from the following cell lines were also used: HepG2, HepaC1, NIH/3T3; N2a, HEK293 and A549.

Analysis of gene profiling datasets

The expression profiles corresponding to the indicated datasets and series were downloaded from Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) (28) database. In all the cases, untreated normoxic cells were used as reference. For each data set we calculated the mean of probe values in the biological replicates. All probes mapping to the locus of interest, except those with null values, were included in the analysis. Then, for each probe, the effect of hypoxia was

calculated as the logarithm of the ratio of the means of treated and control samples. Finally, individual log-ratio values were normalized by subtraction of the mean of all the log-ratios across the data set and division by their standard deviation. In the case multiple probes mapping to a given gene locus, the average of the log ratio was calculated. Information regarding GEO and probes ID can be found in the Supplementary Table S1.

RNA extraction and quantitative PCR

Total RNA was extracted and purified with the RNeasy Mini Kit (Qiagen). One microgram of RNA from each sample was reverse-transcribed to cDNA (Improm-II reverse transcriptase; Promega) and 1µl of cDNA samples were used as template for amplification reactions carried out with the LC Fast Start DNA master SYBR Green I kit (Roche Applied Science), following the manufacturer's instructions. PCR amplifications were carried out in a Light Cycler System (Roche Applied Science), and data were analyzed with LightCycler software 3 version 3.5.28 (Idaho Technology Inc.). For each sample, duplicate determinations were made, and the gene expression determined by the ΔΔCt method using β-actin as reference gene. The primers used in this study are shown in the Supplementary Table S2.

Plasmid construction

Human genomic DNA extracted from HeLa cells was used as template for PCR amplification of *GYS1* and *RUVBL2* promoter regions. For reporter assays, PCR products were first cloned into pCR2.1-TOPO (invitrogen) and subsequently subcloned into the KpnI/XhoI restriction sites of pGL3-Basic.

For the *in vitro* EBA, the putative insulators were cloned in the plasmid pELuc (29) and assayed their activity by transfection of the resulting constructs into HEK293 cells. This vector carries a CMV enhancer and a minimal promoter controlling the firefly luciferase report gene cassette with the polyadenylation site from SV40. All inserts were cloned into the XhoI site, between the enhancer and the CMV promoter (IN-position), and into SmaI, upstream of the enhancer (OUT-position). The insertion in SmaI site is a control for the assay that tests the potential silencer/repressor effects of our insert. For the *in vivo* EBA the putative insulator elements were cloned in the vector 48RCar (30) and injected resulting constructs into zebrafish embryos to assess their effect on transcription. In this plasmid the GFP gene is carried in the vector under the control of an actin promoter, that directs expression in heart and muscle. The insulator is cloned in the KpnI site, between the actin promoter and the Enhancer 48, which targets the expression to the central nervous system (31).

The identity of all constructs was verified by sequencing. All primer sequences are available in the Supplementary Table S1.

Reporter assays

Reporter assays were performed using the HeLa. Cells were seeded on six-well plates (3×10^5 cells/well) 6 h prior to transfection. A 9 µg DNA mixture containing 3 µg of the indicated reporter construct or empty plasmid and 0.6 µg of a plasmid encoding for renilla (sea pansy) luciferase under the control of a null promoter (Promega, Madison, WI, USA.) was used for transfection using the calcium phosphate method. On completing 16 h after transfection, cells where washed, replated in 24-well plates and incubated in normoxia, in the presence of DMOG or under hypoxia for additional 16 h. After treatments, the cells were lysed and the firefly and renilla luciferase activities of the lysate were determined using a dual-luciferase system (Promega, Madison, WI, USA). The firefly luciferase activity was normalized to that of renilla luciferase.

In vitro EBA

Cells were transiently transfected using Lipofectamine 2000 (invitrogen) and OPTI-MEM® medium (Invitrogen) according to the supplier's instructions. Briefly, 1.8×10^5 HEK293 cells were seeded the day before transfection in 24-well plates. For each well, 0.66 µg of the linearized reporter vector was transfected together with 0.14µg of pCMV-lacZ control plasmid (for normalization purposes). The pELuc-derived constructs were linearized prior to transfection, to avoid bidirectional enhancer activity, using the restiction enzyme Asp-718. This site is located 3′ downstream from the polyadenylation signal. The pCMV-lacZ plasmid was also linearized using ScaI. The cells were incubated 24 h with the transfection mixture and were thereafter lysed with Reporter Lysis Buffer (Promega).

The luciferase activity was determinad using the Luciferase Assay Reagent (Promega) according to the manufacturer's specifications in a microplate luminometer (Orion, Berthold Detection Systems). The sample luciferase activity was corrected by the β-galactosidase activity in and the number of molecules (picomol) of the transfected plasmid construct (according to each plasmid size). Finally, activities were normalized as a fraction of the mean luciferase values obtained for the empty (pEluc) plasmid.

Transgenesis and *in vivo* EBA

Transgenesis and *in vivo* EBA were performed as described (30,31). For zebrafish transgenesis, the Tol2 transposon/transposase method of transgenesis (32) was used with minor modifications. In total, 1 nl was injected in the cell of one-cell stage embryos containing 50 ng/µl of transposase mRNA, 40 ng/µl of phenol/chloroform purified DNA and 0.05% phenol red.

Statistical analysis of data

Statistical analysis of the experimental data was performed with the R software package [(33), http://www.R-project.org/]. The statistical tests applied to each data set are indicated in the figure legends. We adopted the following code to indicate the magnitude of *P*-values throughout the manuscript figures as: $***P = [0, 0.001]$; $**P = [0.001, 0.01]$ and $*P = [0.01, 0.05]$.

RESULTS

Differential regulation of *GYS1* and *RUVBL2* by hypoxia

The muscle glycogen synthase gene, *GYS1*, is regulated by HIF as part of the cellular metabolic reprogramming required for the adaptation to hypoxia (27). The regulation of human *GYS1* by hypoxia is mediated by a functional RCGTG element located 255 bp upstream its transcription start site (TSS) (27). Very close to *GYS1*, but encoded by the opposite DNA strand, is located the *RUVBL2* gene (Figure 1A). The relative position, intergenic distance and orientation of these two genes are conserved across mammals (data not shown). The TSS of *RUVBL2* is located at 288 bp of the HRE driving *GYS1* expression in response to hypoxia (location of the HRE is shown by a black box in the 'blat' track, Figure 1A), raising the possibility of a coordinated regulation of these two genes by HIF. In fact, the intergenic region between *GYS1* and *RUVBL2* can be considered a bidirectional promoter (Figure 1A, 'Elnitski bidirectional promoters' prediction track). To study this possibility, we analyzed publicly available gene expression profiles of cells exposed to hypoxia and found that, whereas *GYS1* mRNA levels were induced by hypoxia in most of the profiles, the expression of *RUVBL2* remained constant or was even repressed under low oxygen tension (Figure 1B). To confirm these results we exposed myotubes to hypoxia and determined its effect on *GYS1* and *RUVBL2* mRNA levels. As shown in Figure 1C, *GYS1* mRNA level significantly increased in response to hypoxia, in agreement with published results (27), but the treatment did not induce *RUVBL2* mRNA. In order to rule out a cell-type specific effect, we determined the effect of hypoxia on the expression of *GYS1* and *RUVBL2* in a variety of cell types (Figure 1D). These analyses confirmed that, whereas *GYS1* expression was increased by hypoxia in virtually all cell lines studied, *RUVBL2* remained largely unaffected. In agreement with this conclusion, a meta-analysis of gene expression profile experiments suggested that, unlike *GYS1*, *RUVBL2* is not significantly modulated by hypoxia (10). These results indicated that hypoxia specifically affects *GYS1*, but not *RUVBL2* transcription. To confirm this possibility, we investigated the effect of hypoxia on a set of reporter constructs derived from this locus. As shown in Figure 1E, a reporter construct containing the region flanking *GYS1* gene and including the HRE (region cG spanning residues +84 to −429 relative to *GYS1* TSS, Figure 1A), was robustly induced by hypoxia and the hypoxia mimetic DMOG in HeLa cells, as expected from the presence of the evolutionarily conserved block containing the HRE within this cG region. In contrast, the analogous region upstream of *RUVBL2* (cR, spanning −396 to +12 relative to *RUVBL2* TSS) did not respond to HIF activation in spite of including the same HRE-containing region (Figure 1E). Importantly, this same result was obtained when the whole intergenic region, maintaining the original genomic structure, was used to drive luciferase expression from the GYS1 (cRcG) or the RUVBL2 (cGcR) promoters (Figure 1E).

Collectively, these results indicate that the HRE located between *GYS1* and *RUVBL2* genes selectively drives the transcription of the former in response to hypoxia.

The lack of *RUVBL2* induction by hypoxia is not due to gene silencing

The accessibility of promoter regions is one that the mechanisms that restricts the activity of a TF toward specific genes. This can be achieved by methylation of promoter regions and/or by altering chromatin compactness through histone modification. In fact, it has been recently found that basal promoter activity determines cell type-specific HIF transcription (8). Thus, we investigated whether a lack of *RUVBL2* transcriptional activity could explain the observed selectivity of hypoxia within this locus. As shown in Figure 2A, the normoxic levels of *RUVBL2* and *GYS1* mRNAs were of similar magnitude, at least for the set of cell lines included in our study. Moreover, the differential effect of hypoxia (Figure 1D) was observed even for cell lines, such as HeLa or A549, in which the relative basal level of *RUVBL2* mRNA was much higher than that of *GYS1*. On the other hand, the genomic region adjacent to the *RUVBL2* gene (cR) showed a strong basal promoter activity when assayed in HeLa cells (Figure 2B). In fact, the promoter activity of the cR region was significantly higher than that of the cG region (Figure 2B). In agreement with our results, published data of genome-wide RNA polymerase II binding shows a similar signal in the *GYS1* and *RUVBL2* promoter regions in a wide range of cell types (Supplementary Figure S1).

Altogether, these results indicate that *RUVBL2* and *GYS1* are transcribed under normoxia to a similar extent and thus, we discarded *RUVBL2* promoter accessibility as a potential explanation for the differential regulation observed under hypoxia.

An inhibitory region prevents *RUVBL2* induction by the HRE

To gain insight into the molecular mechanism responsible for the differential regulation of *RUVBL2* and *GYS1* by hypoxia, we generated a set of reporter constructs containing different deletions and rearrangements of the genomic region between *GYS1* and *RUVBL2* genes (Figure 3A and diagrams on the left of Figure 3B). Based on the evolutionary conservation (Phas Cons elements, see Figure 1A), we differentiated five blocks within this region (Figures 1A and 3): proximal *GYS1* (pG), upstream *GYS1* (uG), HRE-containing block (HRE), upstream *RUVBL2* (uR) and proximal *RUVBL2* (pR). As shown in Figure 3, analysis of the transcriptional activity of these reporter constructs in HeLa cells showed that neither pG nor pR proximal regions responded to HIF activation. However, combination of these proximal regions with the HRE block, regardless of the orientation of the latter, resulted in constructs (HREF_pG, HRER_pG, HREF_pR and HRER_pR) that were robustly induced by the hypoxia mimetic DMOG. The induction of these constructs was of similar magnitude to that observed for the complete *GYS1* construct (cG), suggesting that upstream regions

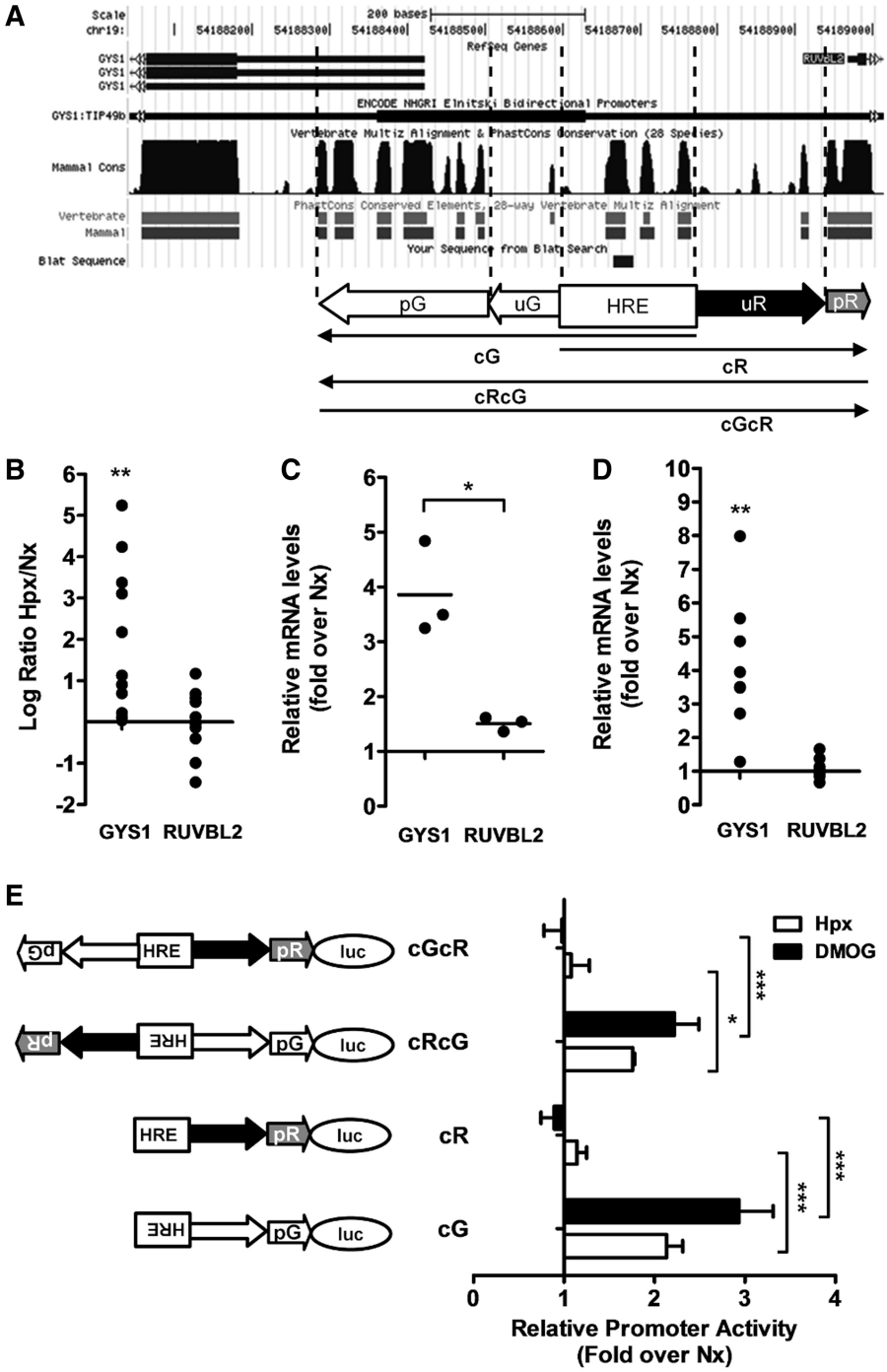

Figure 1. Differential regulation of *GYS1* and *RUVBL2* by hypoxia. (**A**) Schematic diagram depicting the human (hg18 assembly) genomic region containing the intergenic region between *GYS1* and *RUVBL2* and its sequence conservation across mammals [adapted from UCSC genomic browser, http://genome.ucsc.edu/(53)]. The boxes below the diagram represent the different blocks identified within this region according to their evolutionarily conservation and solid lines indicate the regions cloned to generate reporter constructs, cG, cR and cG+cR. (**B**) Effect of hypoxia or the hypoxia-mimetic deferoxamine (GSE5579) on *GYS1* and *RUVBL2* expression extracted from gene expression profiles of human breast carcinoma cell line MCF7 (GSE3188), mouse embryo fibroblast (GSE3196), human B lymphocyte P493-6 cells (GSE4086), human monocyte-derived macrophages (GDS2036), human lymphatic endothelial cells (GSE5579), human aortic smooth muscle cells (GSE4725), human colon adenocarcinoma cell line HT29 cells (GSE9234), mouse hepatocytes (GDS1648), human embryonic kidney cell line HEK293 (GSE2020), human astrocytes (GSE3045) and human epithelial cervical cancer cell line HeLa (GSE3051) exposed to hypoxia. Asterisks indicate that the set of data values was significantly different (one sample *t*-test, $t = 3.6988$, df = 11, $P = 0.003509$) from the value of zero (no induction). (**C**) c2c12 myoblast were exposed to normoxia or hypoxia for 12–24 h and *GYS1* and *RUVBL2* expression was determined by quantitative PCR from total RNA samples. Data were calculated relative to β-actin and expressed as fold change relative to normoxia. Data shown are the results of three independent experiments and their mean (bar). The relative induction of both mRNAs was significantly different (*t*-test, $t = 4.9995$, df = 2, $P = 0.03776$). (**D**) A variety of cell lines (HepG2 and HepaC1, mouse hepatocarcinoma cell lines; primary mouse hepatocytes; NIH3T3, mouse fibroblast cell line; HeLa; N2a, mouse neuroblastoma cell line; HEK293; A549, human lung adenocarcinoma cell line) were exposed to normoxia or hypoxia and the levels of *GYS1* and *RUVBL2* mRNA determined and represented as indicated in C. Asterisks indicate that the set of data values was significantly different (one sample *t*-test, $t = 4.4522$,

(continued)

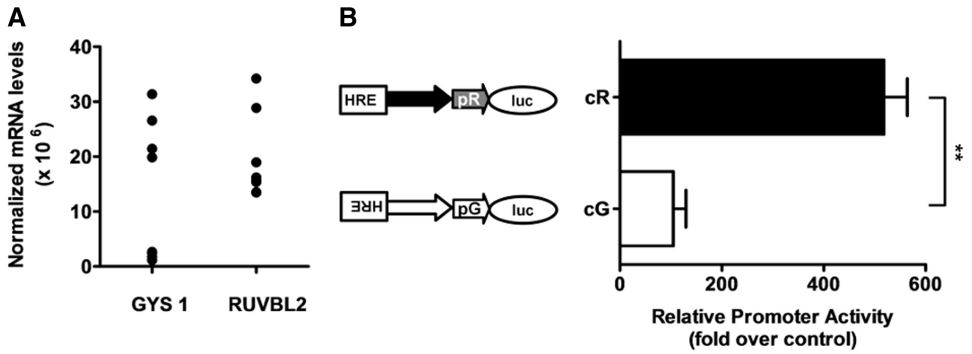

Figure 2. *RUVBL2* is transcribed under normoxia. (**A**) The basal (normoxic) expression of *GYS1* and *RUVBL2* was determined by quantitative PCR for the cell lines indicated in Figure 1D. The graph represents the normalized mRNA levels for each gene across the different cell lines. The difference between both groups was not statistically significant (paired *t*-test, $t = -0.9365$, df = 7, $P = 0.3802$) (**B**) HeLa cells were transfected with a reporter construct containing the indicated region upstream a firefly luciferase gene or the corresponding empty plasmid lacking insert (pGL3basic). The graph represents the corrected luciferase activity values obtained for each plasmid as fold over the activity contained in cells transfected with empty vector. Bars represent the average of values obtained in three independent experiments and errors bars their standard deviation. The difference between the means of both groups was statistically significant (paired *t*-test, $t = -10.2002$, df = 2, $P = 0.009475$).

(uG and uR) are not required for the activity of the HRE-containing block. These data ruled out a collaboration between HIF and other transcription factors binding to pG or uG as a potential explanation for the differential induction of *GYS1* and *RUVBL2* by hypoxia. On the other hand, comparison of response of the cR and HREF_pR constructs, hinted the existence of an inhibitory region within the uR region as removal of this region significantly increased the response to HIF activation. In agreement, the up-regulation of the HREF_pG construct was blunted by the inclusion of the upstream region of *RUVBL2* between the HRE and proximal *GYS1* block (Figure 3, HREF_pG and HREF_uR_pG).

In summary, these results suggest that the genomic region from −202 to −30, relative to the *RUVBL2* TSS, (uR) prevents the induction of *RUVBL2* by HIF.

The *GYS1*/*RUVBL2* intergenic region contains an enhancer blocking element

Insulators are DNA elements that can prevent the promiscuous effect of enhancers or silencers, restricting their interactions with promoters. Our results suggested that the uR region prevents the interaction of the HRE over the *RUVBL2* promoter, a function that is compatible with the enhancer blocking type activity commonly associated to insulators. To test this possibility, we studied the ability of different sequences, derived from the *RUVBL2*/*GYS1* intergenic region, to interfere with the activity of a heterologous enhancer/promoter pair in a standard EBA, in HEK293 cells (29). Each of the genomic fragments under study was cloned either, between the enhancer and promoter (IN position) or upstream and the enhancer (OUT position) (Figure 4A). As a positive control, we also included the boxes II/III (E II/III) derived from the classical chicken 5′HS4 β-globin insulator element (34), known to bind the nuclear factor CTCF and responsible for the enhancer blocking effect of the 5′HS4 element (35). The enhancer blocking activity of these sequences, represented as fold reduction of the activity of a vector lacking insert, is represented in Figure 4B. In agreement with previous reports (35), the E II/III boxes, but not a mutant form, interfered with the activity of the CMV enhancer when inserted into the XhoI site (Figure 4B, white bars). Enhancer blockers work only in the 'IN' configuration as demonstrated by the lack of activity of the EII/III constructs in the 'OUT' configuration (Figure 4B, black bars). Significantly, all the constructs containing the uR in the 'IN' configuration, except 1R, showed a significant reduction in the enhancer activity (Figure 4B, white bars corresponding to constructs 1F, 2F, 2R, 3F and 3R). Importantly, these same regions had no significant inhibitory effect when cloned in the 'OUT' configuration (Figure 4B, black bars corresponding to constructs 1F, 2F, 2R, 3F and 3R). In contrast to these results, neither the HRE nor uG regions were able to suppress the activity of the CMV enhancer (Figure 4B, constructs 4F, 4R, 5F, 5R, 6F, 6R and Supplementary Figure S2).

The EBA results strongly support the existence of an enhancer blocking element within the genomic region from −202 to −30 relative to the *RUVBL2* TSS (Figure 4B, constructs 3F and 3R). Thus, we decided to functionally validate the presence of an insulator in this region *in vivo* by means of an independent assay using

Figure 1. Continued
df = 7, $P = 0.002964$) from the value of one (no induction). (**E**) HeLa cells were transiently transfected with reporter constructs containing the indicated region (cG, cR, cGcR or cRcG, see Figure 1A) upstream a firefly luciferase gene and exposed to normoxia, hypoxia or the hypoxia mimetic DMOG for 12 h. The graphs represent the corrected luciferase activity values of each construct in cells exposed to hypoxia/DMOG and represented as fold change over the activity obtained in normoxic cells. Bars represent the average of values obtained in three independent experiments and error bars their standard deviation. Statistically significant differences between pairs of constructs are indicated by asterisks (one-way ANOVA, $F_{9,20} = 36.704$, $P = 1.6 \times 10^{-10}$, followed by Tukey's multiple comparison test).

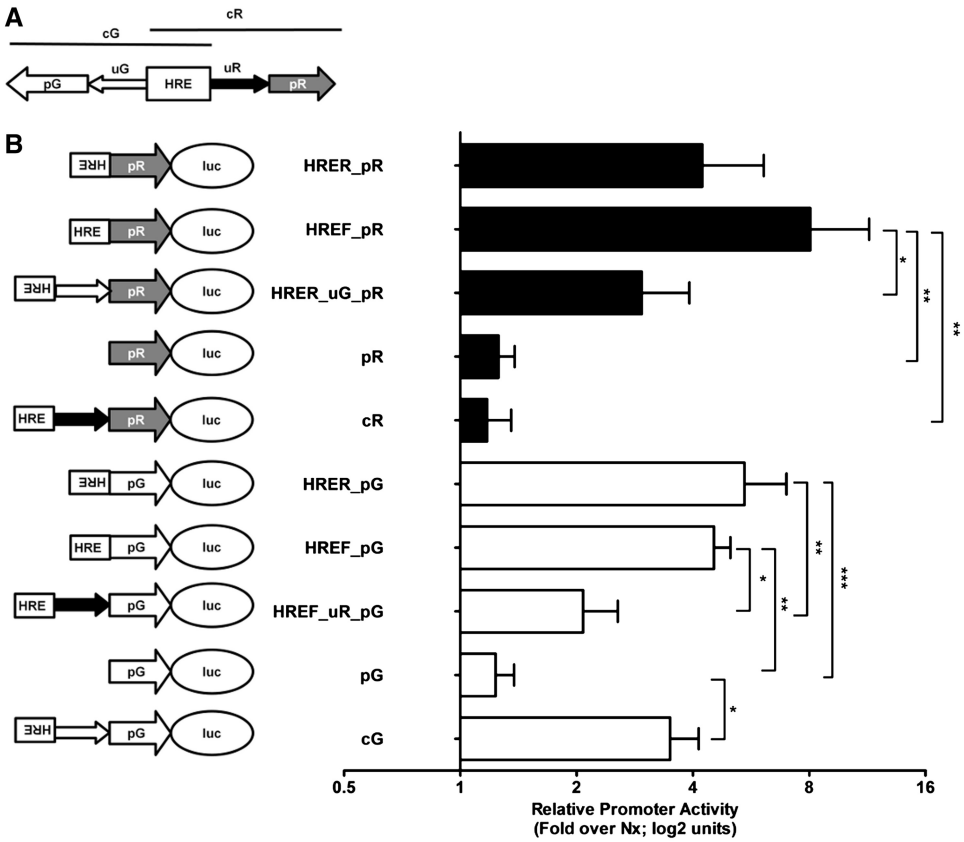

Figure 3. Region uR prevents the increase in transcription mediated by the HRE. (**A**) Schematic diagram of the *GYS1*/*RUVBL2* intergenic showing the different elements included in the reporter constructs. (**B**) HeLa cells were transfected with the indicated constructs and treated with the hypoxia mimetic DMOG or left untreated for 12 h. The graph represents the corrected luciferase activity values of each construct in treated cells represented as fold change over the activity obtained in control (normoxic) cells. Bars represent the average of values obtained in three independent experiments and error bars, their standard deviation. The diagrams on the left are a schematic representation of the genomic elements included in the reporter construct and their orientation relative to the luciferase gene. Statistically significant differences between pairs of constructs within the groups containing pG or pR are indicated by asterisks (one-way ANOVA followed by Tukey's multiple comparison test. pG-containing constructs $F_{4,10} = 13.608$, $P = 0.0004705$; pR-containing constructs $F_{4,10} = 7.518$, $P = 0.004598$).

transgenic zebrafish. To this end, we used a reporter construct in which EGFP expression is under the control of the cardiac actin promoter from *Xenopus laevis* and the Z48 enhancer, which drives transgene expression in the midbrain (Figure 5A) (31). Transgenic zebrafish embryos injected with this construct showed EGFP expression in the heart and in the developing somites (due to the cardiac actin promoter), as well as in the CNS (Figure 5B and C, control), due to the Z48 enhancer, as reported earlier (30,31). Micro-injection of a construct containing the uR region cloned between the enhancer and the promoter, regardless of its orientation, resulted in a strong attenuation of the CNS signal, whereas retaining the EGFP signal in the developing somites (Figure 5C, uR_F and uR_R). Analysis of the somites/CNS EGFP-mediated fluorescence signal ratio in 62 independent transgenic zebrafish lines demonstrated an enhancer blocking activity associated with the uR sequence (Figure 5B, uR_F and uR_R). Importantly, in agreement with the *in vitro* EBA assays (Figure 4), the effect was specific for the uR region as cloning of the uG sequence between the Z48 enhancer and the actin promoter did not interfere with the EGFP expression in the CNS (Figure 5B and C, uG_F and uG_R).

Altogether, these results demonstrate the existence of a powerful enhancer blocking element in the uR region that could explain the differential regulation of *GYS1*/*RUVBL2* by HIF. The activity of this insulator element is observed when assayed in a distant heterologous system (zebrafish) suggesting that its function and potential *trans*-acting factors have been evolutionary conserved in vertebrates.

DISCUSSION

The recognition of short DNA motifs by transcription factors is a key step in the regulation of transcription. However, the low information content of most of the TFBS predicts a promiscuous binding that is in contrast with the observed specificity. This apparent paradox raises the question of how transcription factor selectivity is achieved. Although several mechanisms including chromatin accessibility and TF cooperation, have been shown to restrict the target space for a given TF, a complete explanation is still lacking in most of the cases. In this scenario, HIF transcription factors are not an exception. HIF heterodimers bind to the RCGTG motif

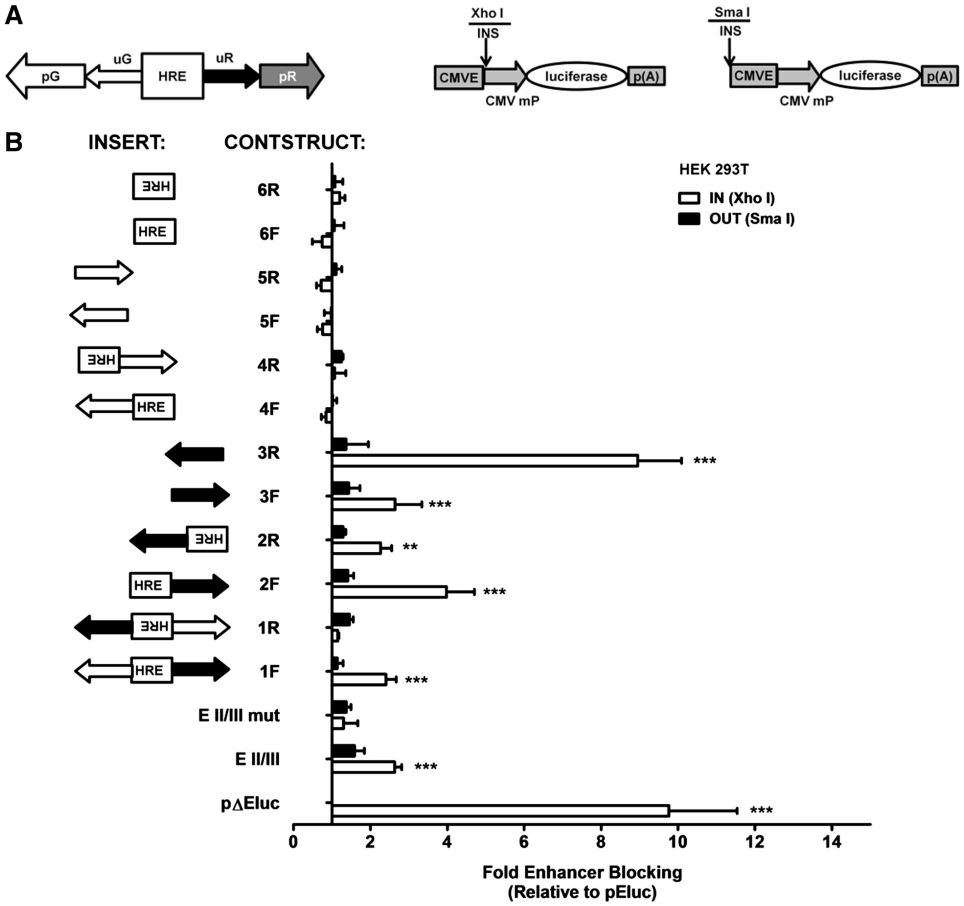

Figure 4. The uR region contains an enhancer blocking element. (**A**) Schematic diagrams of the different elements within the *GYS1*/*RUVBL2* intergenic region (left diagram) and the EBA vector showing the 'IN' (middle diagram) and 'OUT' (right diagram) cloning sites. (**B**) Each of the indicated elements (diagrams on the left under 'INSERT') derived from the *GYS1*/*RUVBL2* intergenic region, or from the β-globin gene (E II/III and a mutated version, E II/III mut), were cloned into the XhoI ('IN' position, white bars) or SmaI ('OUT' position, black bars) sites of the pELuc vector to generate the indicated constructs ('CONST.'). The HEK293 cells were transiently transfected with each one of these constructs, an empty EBA vector lacking insert (pEluc) or an empty EBA vector lacking the enhancer (pΔEluc). On completing 24 h after transfection, the cells were processed to determine the transcriptional activity of the constructs. The figure represents the corrected luciferase activity in each sample and is expressed as fold reduction of the activity observed in cells transfected with the empty vector, pEluc (29). Bars represent the average of values obtained in three independent experiments and errors bars, their standard deviation. Asterisks indicate constructs whose activity was significantly different from that observed for the control, pEluc (one-way ANOVA, $F_{29,237} = 131.10$, $P = 2.2 \times 10^{-16}$, followed by Tukey's multiple comparison test).

(4,6,9), which is present in almost every gene in the human genome, yet hypoxia results in the regulation of a few hundred genes only and, accordingly, HIF only binds to a subset of the potential binding sites (5,6,9,10). It has been recently demonstrated that HIF binds preferentially to RCGTG motifs present in the promoters of genes actively transcribed under normoxic conditions (8). Although this restriction results in a large reduction in the number of potential targets, it does not fully explain HIF selectivity as many genes transcribed under normoxia and containing RCGTG motifs are not induced by hypoxia. With the aim of gaining insight into the mechanisms of HIF target selectivity, we investigated the *GYS1*/*RUVBL2* genes as a particularly striking example of differential regulation. Given their close proximity and the location of the functional HRE between both genes, we had expected their coordinated regulation by HIF. However, our results showed that while *GYS1* was induced by hypoxia, *RUVBL2* levels remained unchanged (Figure 1). The basal level of mRNA, RNA polymerase II binding and promoter activity (Figure 2 and Supplementary Figure S1) ruled out the accessibility of chromatin as an explanation for the lack of induction of *RUVBL2* by hypoxia. Detailed analysis of the promoter activity of different fragments derived from the *GYS1*/*RUVBL2* intergenic region revealed the existence of an inhibitory region between the HRE and the *RUVBL2* gene (Figure 3). Finally, specific EBAs performed *in vitro* (Figure 4), as well as *in vivo* (Figure 5) demonstrated the existence of an insulator element that could explain the lack of effect of the HRE over the *RUVBL2* promoter and thus, the differential regulation of *GYS1* and *RUVBL2* genes by hypoxia.

Insulators have been shown to play a key role in the differential patterns of gene expression during development and cell-lineage specification (23). However, to our

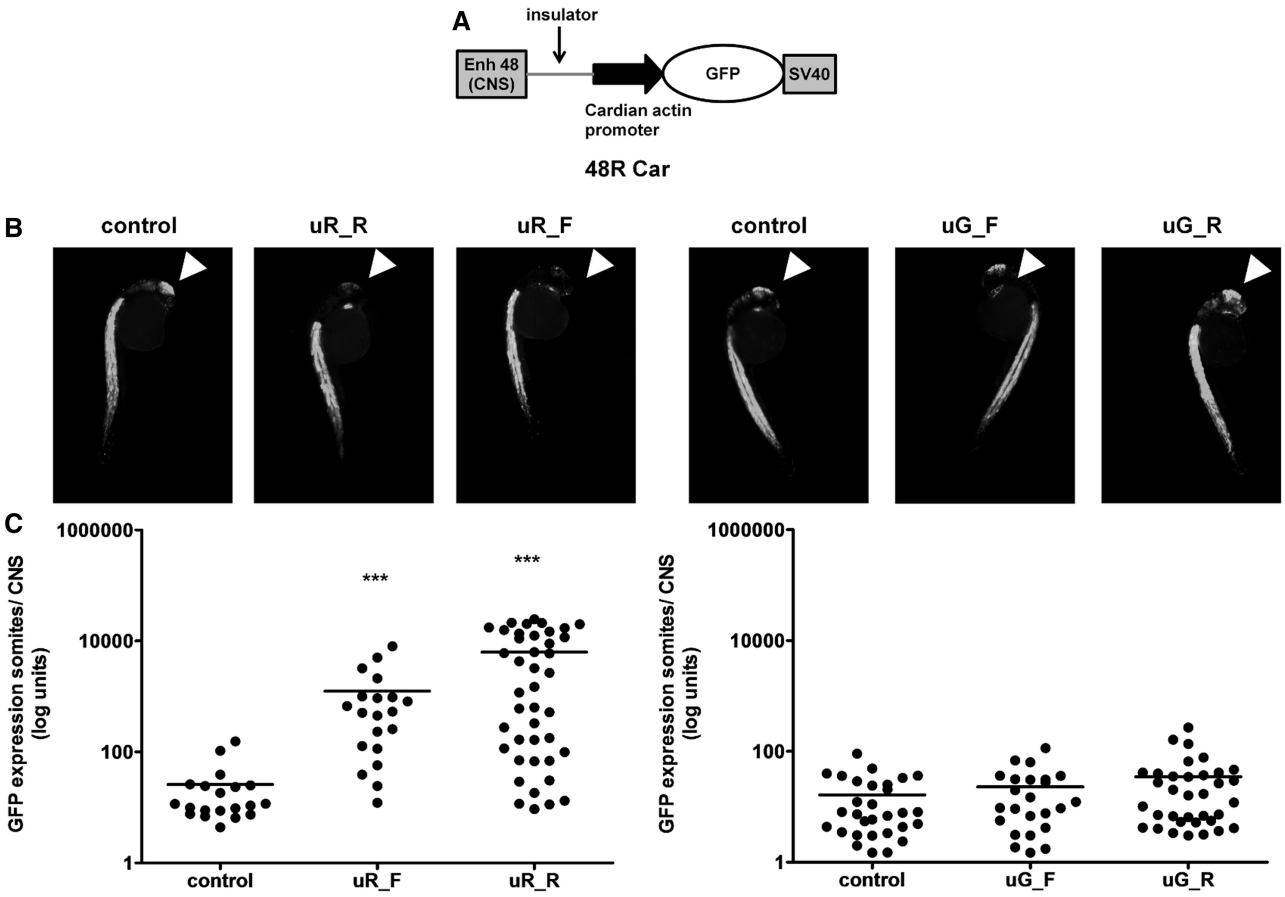

Figure 5. The insulator element derived from *RUVBL2* genomic region blocks enhancer activity *in vivo*. (**A**) Schematic representation of the ZED construct. (**B** and **C**) zebrafish embryos were micro-injected with an empty ZED vector (control), lacking insert between the enhancer and promoter, or constructs containing the *RUVBL2*-derived enhancer blocking element (uR) or the corresponding region upstream the GYS1 gene (uG). Both elements were cloned in the forward (uR_F, uG_F) or reverse (uR_R, uG_R) orientation. After 36 h after micro-injection, the GFP signal in CNS and somites was determined by fluorescence microscopy and quantified with LaserPix (Bio-Rad) image analysis software, as reported before (30). (B) A representative image of each group of animals is shown. Arrowheads indicate the location of the CNS. (C) The graph represents the ratio between the GFP signals in somites and CNS for each of the transgenic fish. Horizontal line represents the median of the ratios for each group of animals. Asterisks indicate set of values that were significantly different from those obtained for the empty construct Kruskal–Wallis, chi-squared = 89.2081, df = 5, $P < 2.2e\text{-}16$; *P*-values for comparisons were calculated by Wilcoxon rank and corrected for multiple comparison using Bonferoni's method.

knowledge, this is the first example of an enhancer blocking element contributing to the selectivity of a transcription factor acutely induced by environmental factors, such as HIF. Although further work is required to determine whether this is a general mechanism contributing to HIF specificity, our working hypothesis is that this mechanism could be particularly relevant in cases of bidirectional promoters, such as the one described herein, where chromatin accessibility and DNA methylation is likely to be similar across the intergenic region. In support to this possibility, we have found at least one further example of bidirectional promoters differentially regulated by hypoxia, *BCKDHA* and *EXOSC5*. Preliminary results indicate that, in spite of similar basal transcription, *BCKDHA*, but not *EXOSC5*, is induced by hypoxia (Supplementary Figure S3).

Another issue raised by our results relates to the molecular identity of the insulator element located upstream of the *RUVBL2* gene. In vertebrates, several regulatory elements including CTCF binding motifs (36–38), repetitive elements, [such as ALUs (39), SINE B2 (29) and SINE B1 (30)] and scaffold/matrix-attachment regions [S/MARs; (40,41)], have been shown to function as insulators (25,42). Among them, the most widespread and well characterized are CTCF-binding elements (43). We have not found obvious CTCF binding motif within the −202 to −30 region upstream of *RUVBL2*, and published ChIP data shows CTCF binding to the GYS1 TSS region, but not to the region between the HRE and *RUVBL2* gene (Supplementary Figure S4A, CTCF ChIP signal track). On the other hand, there are no repetitive elements within this genomic region (Supplementary Figure S4A, repeats tracks) arguing against the existence of a SINE element that could explain the observed enhancer blocking activity. Finally, although we found no locally high proportion of A/T nucleotides, typically associated with S/MARs elements (41) in the region upstream of *RUVBL2* (Supplementary Figure S4A, GC percent track), computer prediction of S/MAR sites, found a significant signal in the upstream region of

RUVBL2 (Supplementary Figure S4B). Thus, the insulator activity described herein could be mediated by S/MAR elements. However, as this evidence has been obtained through *in silico* approaches, further work is required to identify the minimal region required for the enhancer blocking function, including its molecular identity, underlying mechanism and the associated potential *trans*-acting factors. This is yet another perfect example demonstrating the diversity of mechanisms, most of them not known to date, that cells are using to organize functional insulator elements (25).

The mechanism by which enhancer blockers prevent the activity of upstream enhancers is unclear, although our current understanding is that insulators will probably not be using unique mechanisms but, rather, adaptations of pre-existing ones already in place for the normal regulation of gene expression (23). Several of the proposed models invoke the generation of chromatin loops that segregate enhancer-sensitive and resistant promoters in distinct domains (44,45). In the case described herein, the model is further complicated by the short distance existing between the *cis*-elements involved. A piece of information that could shed light into the mechanism is the intriguing observation that the inclusion of the uG region seems to abolish the enhancer blocking effect of uR in EBA (Figure 4B, compare constructs 1R/1F with 3R/3F). Although we cannot currently explain this behavior, it would suggest that the EBA of uR can be modulated by elements located in its proximity. In this regard, it has been previously found that the EBA of the 'gypsy' element is affected by the number of copies of this element. When two copies, instead of one, are located between the enhancer and promoter, its blocking effect is abolished (46). However, the enhancer blocking effect is restored by the insertion of a third copy (47), depending on the order and distance of the insulator elements, indicating that complex protein–protein interactions are responsible for these unexpected effects and underlying a major role of insulators in whole nuclear genome organization (24,48). Thus, it is possible that the impairment of the EBA of uR by uG can be reverted by other *cis*-elements, present in the native genomic context, but not included in this set of constructs. A further possibility is that the HRE-containing block could enhance the transcription from the minimal CMV promoter. If this is the case, the enhancer blocking element within uR would be located upstream of the HRE element in the construct 1R and would be thus, unable to prevent its action upon the minimal CMV promoter. Regardless of the specific mechanism by which uR exerts its effect, our data clearly demonstrates that it contains an EBA.

Reprogramming of cellular metabolism, in particular glucose metabolism, is central in the cellular adaptation to hypoxia. The hypoxic induction of *GYS1*, encoding for an isoform of glycogen synthase, is part of this reprogramming (27). On the other hand, the existence of an enhancer blocking element between the HRE and the *RUVBL2* promoter raises the question of why *RUVBL2* expression has to be shielded from the HIF-mediated induction. *RUVBL2* gene encodes for Reptin, an AAA+ ATPase that forms part of chromatin remodeling complexes. Interestingly, it has been recently shown that hypoxia leads to reptin methylation and that, upon this post-translational modification, it is able to repress HIF-mediated transcription (49). Thus, it is plausible that *RUVBL2* expression might not be induced by hypoxia so as not to upset the balance between methylated/unmethylated reptin that could lead to premature termination of HIF-mediated transcription. However, this hypothesis does not provide an explanation for the close proximity of these genes. The conservation of the *GYS1*/*RUVBL2* genomic arrangement from opossums to humans suggests a selective pressure to maintain both genes in close proximity. Previous studies have shown that a substantial proportion of mammalian genes is arranged in a divergent head-to-head structure and controlled by bidirectional promoters (50), so that the pair of genes tend to be co-expressed (50,51). The need for co-regulation of the pair of genes under the control of a bidirectional promoter could explain the selective pressure that keeps them in close proximity. However, in the case of *GYS1*/*RUVBL2*, co-regulation of both genes is an unlikely reason for their close proximity as the existence of the insulator element would prevent the action of flanking *cis*-elements on the opposite promoter.

In summary, we have identified an insulator, acting as a functional enhancer blocking element, that explains the differential response of *GYS1* and *RUVBL2* genes to hypoxia. To our knowledge, this is the first report describing a role for this type of genetic elements in dictating the specificity of acutely induced transcription factors in response to environmental, as opposed to developmental, signals. Importantly, the generalization of this model adds to the arsenal of strategies, including chromatin accessibility and combinatorial assembly of TFs, that are employed by eukaryotes to ensure a highly specific gene expression based on an otherwise promiscuous set of *cis*-regulatory elements.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1 and 2 and Supplementary Figures 1–4, Supplementary Reference [54].

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