Hypoxia is a growth inhibitory stress associated with multiple disease states. We find that hypoxic stress actively regulates transcription not only by activation of specific genes but also by selective repression. We reconstructed this bimodal response to hypoxia in vitro and determined a mechanism for hypoxia-mediated repression of transcription. Hypoxic cell extracts are competent for transcript elongation, but cannot assemble a functional preinitiation complex (PIC) at a subset of promoters. PIC assembly and RNA polymerase II C-terminal domain (CTD) phosphorylation were blocked by hypoxic induction and core promoter binding of negative cofactor 2 protein (NC2α/β, Dr1/DrAP1). Immunodepletion of NC2β/Dr1 protein complexes rescued hypoxic-repressed transcription without alteration of normoxic transcription. Physiological regulation of NC2 activity may represent an active means of conserving energy in response to hypoxic stress.

Solid tumors generally have a poor vascular supply, which results in areas of decreased perfusion and hypoxia (1). The hypoxic microenvironment may increase tumor aggressiveness (2), presumably through specific induction of transcription factors such as hypoxia-inducible factor 1 (HIF1) (3) and alterations in gene expression (3). Under normoxic conditions the HIF1α subunit is hydroxylated at proline 564, promoting high affinity interaction with the von Hippel-Lindau (VHL) tumor suppressor protein and ubiquitin-mediated degradation (5, 6). Upon exposure to low oxygen tension, HIF1α is not hydroxylated, is stabilized, and interacts with HIF1β. The HIF1 heterodimer binds to hypoxia-responsive elements and activates the promoters of HIF-responsive genes (7).

In addition to HIF-mediated gene activation, cells under reduced oxygen also limit non-essential cellular processes in order to survive (2). Thus, cells under stress may channel their energy into productive gene expression, whereas non-productive genes and processes are turned off. Gene-specific transcription inhibition can be induced by regulated repressors of transcription (reviewed in Refs. 8 and 9). Certain types of these repressors modify chromatin structure (10), whereas others inhibit transcription through core promoter and general transcription factor interactions (11) (reviewed in Ref. 12). Among this latter group of transcriptional repressors is negative cofactor 2 (NC2α/β, Dr1/DrAP1), which blocks transcription by association with DNA-bound TFID and inhibits PIC assembly in vitro.

NC2α and NC2β are essential genes, and their gene products regulate ~17% of all Saccharomyces cerevisiae genes either positively or negatively (13). In S. cerevisiae, NC2 is required for specific TATA-containing gene repression under conditions of reduced nitrogen availability (14). Recent work surveying Drosophila promoter elements and studies with purified proteins reveal that NC2 represses transcription from TATA element-containing promoters but activates promoters that rely on selective transcription repression. A limited survey of cellular stress conditions reveals that hypoxia may be unique in exploiting this fundamental mechanism of gene regulation.

**EXPERIMENTAL PROCEDURES**

**Cells and Treatments**—Hepatoma cells (Hepa 1–6, American Type Culture Collection) and primary, normal human fibroblasts were plated overnight in glass dishes in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum at 10,000 cells/cm², the media was changed, and the cells were transferred into a hypoxic chamber (Bactron 2, Sheldon Labs) for the indicated times. These conditions have been established in order to maintain stable pH and glucose over the course of the experiment. Cell viability, at the end of all treatments, was judged by trypan blue exclusion and found to be >70% even under severe, long term hypoxic conditions. Cells extracts were prepared as described (18). The drug treatments 150 µM CoCl₂ (in H₂O, Sigma), 0.5 µg ml⁻¹ doxorubicin (in H₂O, Sigma), and 50 µM ALLN (in Me₂SO, Calbiochem) were added to fresh media at the indicated times. Ionizing radiation was delivered from a ¹³⁷Cs source at 3.5 gray/min.

**RNA Analysis and in Vitro Transcription**—Northern blot probes were obtained from the Image Consortium (Incyte) and sequenced prior to use to confirm inserts. Primer extension analyses with lacZ-specific primers (3′-HRE and VEGF), chic β-globin-specific primer, and a chloramphenicol acetyl transferase (CAT)-specific primer (p21) were performed under standard conditions.
FIG. 1. Extracts from hypoxic cells show decreased transcription in vitro. A, AFP DNA was transcribed in extracts made from Hepa 1–6 cells as follows: normoxic cells (lane 1) and cells exposed to ionizing radiation (4 h post 6 or 8 gray, IR1 and IR2, respectively), hypoxia (0.01% for 24 h), CoCl2 (150 μM for 24 h), and a proteasomal inhibitor, ALLN (1 h). Hepa 1–6 extracts were mixed together at the indicated ratios and tested for transcription of AFP as indicated. C, hypoxic repression is dominant in mixed extracts. Control and hypoxic Hepa 1–6 extracts were mixed as indicated (microgram extract protein of each, total microgram protein held constant) and tested for transcription of AFP. All newly synthesized transcripts were quantitated by primer extension. Molecular weight standards (MW) are radiolabeled 4X174 DNA digested with HaeIII (Invitrogen).

Hypoxic Repression Is Widespread but Selective—The general nature of hypoxia-mediated repression was revealed by transcription of three gene templates that share few if any trans-acting regulatory elements upstream of the proximal promoter, i.e. the p21/WAF1 gene (which is p53-activated) (22), AFP (which is repressed by p53) (19), and chick β-globin (which is not regulated by p53) (23) (Fig. 2A). Transcription of each gene was strongly inhibited in hypoxic cell extracts. By contrast, treatment of cells with doxorubicin (0.5 μg ml^-1 for 24 h) robustly induced p53 in these cells (data not shown) but had varied effects, including none (AFP), 2-fold inhibition (β-globin), and induction of several transcripts from aberrant start sites (p21). These data confirmed hypoxia-dependent repression of transcription conferred through a widely conserved regulatory element independently of p53 activation.

Though multiple genes were repressed in hypoxic extracts, this repressive effect is selective. As observed in vivo, the transcription of gene templates containing hypoxia-inducible HIF1 regulatory elements is activated in vitro rather than repressed (Fig. 2B). One of these constructs contains three copies of an HRE from the 3’-untranslated region of the erythropoietin (EPO) gene (24) fused to a heterologous promoter/luciferase reporter (3X HRE). The other hypoxia-activated template is a natural VEGF promoter and upstream regulatory region (from -2275 to +51) plasmid containing a single HRE and a luciferase reporter (VEGF). This construct lacks the identified VEGF RNA stability element (25). The 3X HRE and VEGF constructs are transcriptionally induced when transfected into cells and incubated under hypoxic conditions (data not shown). Each of these DNA templates was transcribed in the same extracts under identical conditions and exhibited a different profile of time- and “dose”-dependent responses to hypoxia. AFP showed hypoxic repression and moderate hypoxia (2% oxygen) yielding moderate repression, whereas severe hypoxia (0.01% oxygen) showed increasing repression with time, and CoCl2 showed no effect. The 3X HRE template showed hypoxic activation, and moderate hypoxia showed induction, whereas severe hypoxia clearly induced expression at 12 h, and by 24 h expression was reduced to control levels; CoCl2 treatment also showed significant induction. In contrast, the VEGF promoter showed induction that required more extreme hypoxic conditions. The natural VEGF HRE-containing, TATA-less promoter transcribed poorly and was slightly acti-
Fig. 2. Hypoxia effects selective transcription repression or activation. A, several tested templates showed hypoxic repression (+); p21, AFP, and β-globin genes were transcribed in Hepa 1–6 extract from control (lanes 2, 5, and 9), hypoxic (lanes 3, 6, and 10), or doxorubicin-treated cells (lanes 4, 7, and 11). Transcription reactions without extract (lane 1) and with HeLa extract (lane 8) were used as controls. B, hypoxia-induced genes showed transcriptional activity in vitro. AFP/tacZ, 3× HRE, and VEGF/luc were transcribed under identical conditions in extracts from cells under control conditions, CoCl2, 24 h under 2% oxygen and 12 and 24 h under 0.01% oxygen treatment. C, Northern blot analysis of total RNA from normal human fibroblasts exposed to the control, hypoxia, or cobalt chloride. Probes are indicated (p53R2, ribonucleotide reductase isofrom 2; Hif1, hypoxia inducible factor 1α), and methylene blue-stained 18S RNA was used as a loading control.

vated at moderate hypoxia but was activated in severe hypoxia slightly at 12 h, increasing at 24 h, and was not activated by CoCl2. This pattern of hypoxia-induced VEGF expression, including the limited response to CoCl2, matched that observed for endogenous VEGF activation (Fig. 2C and Ref. 26). We determined by Northern blot analysis that hypoxia can result in repression of specific mRNAs in vitro. Fig. 2C shows that, in primary human fibroblasts exposed to severe hypoxia, specific genes show decreased mRNA levels, whereas others are unchanged or activated by hypoxia. The selective repression of certain genes with the activation of others suggests an active process rather than a passive loss of macromolecular synthesis under conditions of reduced energy. This decline of steady-state RNA levels for a variety of genes, combined with in vitro transcription results, supports a role for hypoxia-regulated transcription repression through widely conserved regulatory elements.

Hypoxia-induced Repression Acts at the Core Promoter—Because a diverse set of templates was repressed (Fig 2A), we focused on the promoter region and examined PIC assembly in control and hypoxic cell extracts (Fig. 3A). Under established conditions for in vitro single-round transcription (20), transcription is dependent solely on protein-DNA interactions (PIC assembly) established (in the absence of NTPs) before washing the protein-bound DNA template and adding Sarkosyl detergent, which prevents subsequent protein binding (see Fig. 3A, diagram). Addition of Sarkosyl after PIC assembly (Fig. 3A, lanes 4–8) revealed that hypoxic cell extract is incapable of functional PIC assembly (Fig. 3A, lanes 6 and 8). PICs assembled in hypoxic extract could not elongate even if transcription be rescued by control extract when DNA binding of control extract proteins is precluded (Fig. 3A, lane 6). Control extract rescue of hypoxic transcription does occur in the absence of Sarkosyl (Fig. 3A, lane 3). Importantly, we found that hypoxic extract did not inhibit transcription elongation by PICs preassembled in control extract (Fig. 3A, lane 5). Thus, any inhibitory factors present in the hypoxic cell extract must act during PIC assembly rather than by altering preassembled transcription initiation complexes or inhibiting transcription elongation.

One direct consequence of complete PIC assembly is phosphorylation of the RNA polymerase II C-terminal domain (CTD) before initiation (27, 28). Western blot analyses with specific antibodies raised against the RNA polymerase II amino terminus (N-20), CTD phosphoserine 5 (H14), CTD phosphoserine 2 (H5), and unmodified CTD (C-19) (29) revealed marked differences between the hypoxic cell extract and other control or treated cell extracts (Fig. 3B). All of the cell extract preparations contained similar levels of unphosphorylated RNA polymerase II (IIA form) as revealed by the N-20 and C19 antibodies. However, there was a dramatic absence of RNA polymerase II with its CTD phosphorylated (IIO form) at serine 5, and very low amounts of IIO phosphorylated at serine 2 in the hypoxic cell extract. Comparison of blots probed with phospho-specific antibodies and functional analysis of transcription in vitro (Fig. 1) revealed a correlation between loss of RNA polymerase II CTD phosphorylation and repression of transcription. RNA polymerase II CTD-hypophosphorylation at both serines 5 and 2 in hypoxic extracts is most consistent with inhibition of initiation (30). These findings support a
Hypoxia-induced NC2 Mediates Selective Repression

Fig. 3. Hypoxic repression occurs at the core promoter. A, hypoxic extracts are dysfunctional for PIC formation. Immobilized AFP templates were preincubated with control (C; lanes 1, 2, 4, 5, and 7) or hypoxic extract (H; lanes 3, 6, and 8) without NTPs to allow PIC assembly. Protein bead-DNA complexes were washed, and transcript elongation was initiated with an NTP addition to control (lanes 1, 3, 4, and 6), hypoxic (lanes 2 and 5), or no extract (lanes 7 and 8) and plus (+; lanes 4–8) or minus (−; lanes 1–3) 0.025% Sarkosyl (Sigma). B, RNA polymerase II CTD is hypophosphorylated under hypoxia. Fifty micrograms of total extract protein from control, hypoxia, doxorubicin, CoCl2, ALLN, and ionizing radiation exposed cells alongside immunopurified epitope-tagged RNA polymerase II (Fl-RNA Pol II, lane 1) were immunoblotted sequentially with RNA polymerase II N-terminal antibody N20 and CTD-specific antibodies C19 and phosphoserine H14 and H5 antibodies (Research Diagnostics). C, hypoxic repression overcome by recombinant TPB and immunopurified RNA polymerase II. TBP protein alone (lane 3; 25 ng), increasing amounts of purified FLAG-RNA polymerase II (lanes 4–6; 75, 150, and 300 ng, respectively) alone or a combination of both TBP (25 ng) and FLAG-RNA polymerase II (lanes 7–9; 75, 150, and 300 ng, respectively) were added to hypoxic extract (lanes 2–9). Lane 1 shows equal total protein levels of control normoxic extract transcription. D, addition of high levels of TBP squelches hypoxia-mediated repression to basal transcription levels. TBP (lanes 3–5; 25, 50, and 100 ng) was added to hypoxic extract (lanes 2–5). Transcription levels in the presence of TBP are comparable with basal levels generated by purified FLAG-RNA polymerase II (lanes 6 and 7; ~135 ng) and TFIID (lanes 6 and 7; 0.75 ng) in the presence of factor PC4 (lane 7; 100 ng).

model in which PIC assembly is affected by hypoxia, a dysfunction marked by the lack of RNA polymerase II CTD phosphorylation.

Assembly of a PIC is regulated at numerous levels and involves the interactions of many proteins (recently reviewed in Refs. 8 and 31). We surveyed a number of purified proteins for their potential ability to restore transcription function to the hypoxic extract. These proteins included TFIID, TFIIH, mediator complex, RNA polymerase IIIA, RNA polymerase IIO, immunopurified RNA polymerase II (21), and recombinant TBP (Fig. 3C, and data not shown). Among these factors, only relatively high concentrations of recombinant TBP could overcome hypoxia-mediated transcription repression to any degree (to 15% of the control level; Fig. 3D) when added alone. FLAG-tagged RNA polymerase II (Fl-RNA Pol II; Ref. 21) alone was also unable to enhance hypoxic transcription (Fig. 3C, lanes 4–6). However, the combination of both TBP and purified RNA polymerase II (Fig. 3C, lanes 7–9) reversed hypoxia-mediated repression and increased transcription of hypoxic extract to control activated transcription levels (Fig. 3C, lane 1). From these data, we hypothesized that high levels of recombinant TBP partially squelched the repressive effect of hypoxia to regain a basal level of transcription (compare with transcription driven by purified RNA polymerase II plus PC4 and TFIID Fig. 3D, lane 7). The addition of both FLAG-RNA polymerase II, which is not hyperphosphorylated (Fig. 3B), and TBP could effectively rescue hypoxic transcription to control activated levels. These data suggest that a hypoxia-induced repressor interacted with both transcription factors and/or that the RNA polymerase II preparation contained proteins that augmented the ability of TBP to squelch inhibition and promote activated transcription.

Hypoxia Induces Accumulation of a Negative Regulator of PIC Assembly—One of several negative regulators of PIC assembly is NC2 αβ (or Dr1/DrAP1 protein) (12, 32, 33). Additionally, interactions between NC2 and RNA polymerase II, which affect RNA polymerase II CTD phosphorylation, have been reported previously (34). In vitro experimentation supports a model wherein the NC2 protein associates with TBP bound at TATA boxes, which inhibits further assembly of the PIC. We examined this candidate repressor of hypoxic transcription by Western blot analysis with antibodies specific for the NC2 subunits Dr1/NC2β and DrAP1/NC2α. We found that both Dr1/NC2β and DrAP1/NC2α protein levels were elevated in extracts of hypoxia-treated hepatoma cells (Fig. 4A). NC2 is likely regulated post-translationally, as levels of both NC2α and NC2β mRNA remain unchanged with hypoxic treatment (data not shown). In these same extracts, TBP levels are un-
Fig. 4. Hypoxia-induced NC2 inhibits PIC assembly. A, NC2 is induced in hypoxic extracts. Fifty micrograms of control and hypoxic Hepa 1–6 extracts were immunoblotted with both anti-AFP and anti-TBP antibodies (top panel) and sequentially with anti-Dr1 and anti-DrAP1 monoclonal antibodies (bottom two panels). B, control (C, lanes 1–5) and hypoxic (H, lanes 6–9) extracts were supplemented with recombinant NC2β (Dr1) protein (lanes 2–5 and 7–9; 2, 20, 40, and 100 ng, respectively), and transcription was performed. C, PIC assembly is incomplete in hypoxic extracts. Immobilized AFP templates were incubated with control, hypoxia, or 1:1 control/hypoxia mixture without NTPs for 10 min and processed for immunoblotting of PIC-assembled proteins with anti-TBP, TFIIB (both from Santa Cruz Biotechnology), and NC2β polyclonal antibodies. Total extracts were immunoblotted as a control. D, PIC assembly and immunoblotting for the bound RNA Pol IIA and Pol IIO forms were similarly performed for control, hypoxic, or mixed extracts. E, transcription levels in control extracts were unaffected by immunodepletion using nonspecific (NS; CCAAT/enhancer-binding protein) or NC2β antibodies but were severely inhibited by anti-TBP depletion. Hypoxic extracts (H) were reactivated for transcription by depletion with an anti-NC2β but were not reactivated by immunodepletion with nonspecific or TBP antibodies (Ab).

changed, and AFP protein levels are reduced, reflecting the transcription response of AFP under hypoxic conditions. The addition of recombinant Dr1/NC2β to control transcription extracts effected repression in a concentration-dependent manner (Fig. 4B, lanes 1–7) of the control extract to levels observed in hypoxia-incubated cell extracts (lanes 6–10). The ability of the single subunit to repress transcription in vitro has been previously shown with multiple gene templates (reviewed in Ref. 35).

NC2 (Dr1/DrAP1) Induction Blocks PIC Assembly—We assayed for endogenous NC2 activity by comparing the PIC components (TBP, TFIIB, and Dr1/NC2β) present in control and hypoxic cell extracts (Fig. 4C) to those bound to promoter DNA, as described previously (Fig. 4C, diagram, and Ref. 36), under conditions for single-round transcription (Fig. 2A). Similar levels of total TBP (or TFIID) and TFIIB proteins were present in hypoxic and control extracts, but NC2β/Dr1 was increased by hypoxia (Fig. 4C, lanes 4–6). Comparison of soluble extract to DNA-bound PIC proteins (lanes 1–3, long exposure) revealed an inverse relationship between NC2 and TFIIB in the PICs (lanes 2 and 3). Analyses of mixed hypoxic/control (1:1) extracts support a concentration-dependent profile of proteins specifically bound at the PIC (TBP and NC2) versus those excluded from the PIC (TFIIB) rather than effects on protein degradation or modification.

We extended the PIC analysis to RNA polymerase II and its phosphorylated forms bound to the DNA versus total protein (Fig. 4D). Again, there were similar levels of unphosphorylated RNA polymerase IIA in both hypoxic and control extracts (N-20 antibody) and sharply reduced hypoxic levels of CTD-phosphorylated RNA polymerase II (H14 antibodies). Parallel analysis of PICs revealed that no RNA polymerase II was bound in hypoxic-assembled PICs. Therefore, in the presence of NC2 (Dr1/DrAP1), TFIIB and RNA polymerase II cannot assemble as part of a functional PIC. Reported roles for NC2 (Dr1/DrAP1) complex function (reviewed in Refs. 12, 35, and 37) in the repression of transcription by blocking entry of TFIIB in PIC assembly is consistent with our results of PIC analysis in hypoxic extracts.

To determine whether NC2 protein complexes were primarily responsible for transcription repression induced by hypoxia, we immunodepleted NC2β protein complexes from hypoxic and control cellular extracts. Hypoxic and control extracts were incubated with antibody-coated beads, the beads were removed, and the depleted extracts were assayed for transcription function (Fig. 4E). Incubation with nonspecific antibody did not alter the transcription properties established for hypoxic and control extracts. Depletion of TBP protein from control extract obliterated transcription and demonstrated the effectiveness of immunodepletion. Incubation with NC2β antibody-coated beads restored transcription capability to hypoxic cell extract and did not alter the ability of control extract to transcribe in vitro (lanes 4 and 5). These data show that either NC2β or an NC2β-associated protein complex is an essential component of hypoxia-mediated transcription repression, the removal of which rescues transcription function.

The NC2 (Dr1/DrAP1) complex could assume multiple roles in both transcription repression and activation due to either post-translational modifications or association with specific protein binding partners. Interpretation of the NC2/TBP/TATAA DNA ternary complex crystal structure suggests that transcriptional activators or co-activators could overcome NC2-mediated inhibition of functional PIC assembly (38). Post-translational modification may be “stressor-” or target site-specific, as phosphorylation of NC2 by casein kinase II inhibits binding to DNA in general and increases the specificity of TBP
interaction (39). Genome-wide expression analyses and chromatin immunoprecipitation of temperature shift-induced NC2 protein in *S. cerevisiae* revealed NC2 association with both positively and negatively regulated promoters in response to the stress of heat shock (13). More recently, a role for NC2 in stabilizing TBP-DNA binding to promote basal transcription and the displacement of NC2 to effect activated transcription has been demonstrated both in *vivo* and in *vitro* for *S. cerevisiae* (40).

A mechanism for a dual role in positive and negative regulation of transcription has been proposed for NC2 (15, 16) (reviewed in Ref. 31). NC2, which represses PIC assembly at TATA-containing promoters but as an activator at different subsets of genes, presents a potential paradigm for mammalian cells in that one induced protein such as NC2 could act as a repressor at many TATA-containing promoters but as an activator at different subsets of genes, e.g. those regulated by HIF1, evoking a timely and energy-efficient response to stress. Potential interaction(s) between HIF-responsive gene promoters and NC2 protein therefore becomes an important question for future investigations.

**Acknowledgments**—We are grateful to M. Czyzyk-Krzeska, T. Oegelshlager, D. Reinberg, K. Ladaroute, and J. Abraham for providing essential materials and to L. Sang and J. Irish for technical support. We thank M. Czyzyk-Krzeska, A. Giaccia, J.A.K. Harmony, D. Reinberg, and A.J. Crowe for helpful discussions.

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Hypoxia Actively Represses Transcription by Inducing Negative Cofactor 2 (Dr1/DrAP1) and Blocking Preinitiation Complex Assembly
Nicholas Denko, Kara Wernke-Dollries, Amber Buescher Johnson, Ester Hammond, Cheng-Ming Chiang and Michelle Craig Barton

J. Biol. Chem. 2003, 278:5744-5749.
doi: 10.1074/jbc.M212534200 originally published online December 10, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M212534200

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