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Abstract

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The transcription factors ATF-1 and CREB-1 bind constitutively to the hypoxia-inducible factor-1 (HIF-1)
DNA recognition site

Ivica Kvetikova, Roland H. Wenger*, Hugo H. Marti and Max Gasemann

Physiologisches Institut, Universität Zürich-IRchel, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland

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ABSTRACT

The hypoxia-inducible factor-1 (HIF-1) was first described as a DNA binding activity that specifically recognizes an 8 bp motif known to be essential for hypoxia-inducible erythropoietin gene transcription. Subsequently HIF-1 activity has also been found in cell lines which do not express erythropoietin, suggesting that HIF-1 is part of a widespread oxygen sensing mechanism. In electrophoretic mobility shift assays HIF-1 DNA binding activity is only detectable in nuclear extracts of cells cultivated in a low oxygen atmosphere. In addition to HIF-1, a constitutive DNA binding activity also specifically binds the HIF-1 probe. Here we report that CRE and AP1 oligonucleotides efficiently competed for binding of the HIF-1 probe to this constitutive factor, whereas HIF-1 activity itself remained unaffected. Monoclonal antibodies raised against the CRE binding factors ATF-1 and CREB-1 supershifted the constitutive factor, whereas Jun and Fos family members, which constitute the AP-1 factor, were immunologically undetectable. Recombinant ATF-1 and CREB-1 proteins bound HIF1 probes either as homodimers or as heterodimers, indicating a new binding specificity for ATF-1/CREB-1. Finally, reporter gene assays in HeLa cells treated with either a cAMP analogue or a phosphor ester suggest that the PKA, but not the PKC signalling pathway is involved in oxygen sensing.

INTRODUCTION

Oxygen tension was discovered as a regulator of mammalian gene expression when it became evident that the increase in blood erythropoietin (EPO) observed in anaemic individuals was due to transcriptional activation of the EPO gene (1). EPO is secreted primarily by the developing liver and adult kidneys in response to hypoxia and leads to enhanced erythropoiesis by inhibiting apoptosis of erythroid precursor cells (reviewed in 1, 2). The resulting augmented erythrocyte number allows the organism to adapt to reduced oxygen availability by increasing oxygen transport capacity. Other hypoxia-inducible genes have since been reported, including vascular endothelial growth factor (VEGF; 3, 4) and glycolytic enzymes (5–8). Using human hepatoma cell lines (9) and several mouse models (10, 11) a hypoxia-inducible enhancer sequence has been localized in the 3′ flanking region of the EPO gene (12–15). Electrophoretic mobility shift assays (EMSAs) revealed the presence of hypoxia-inducible factor-I (HIF-1), which binds to a short DNA motif corresponding to bases 3458–3465 (numbering according to 16) within this enhancer sequence (17, 18). Interestingly, HIF-1 DNA binding activity, originally identified in EPO-producing human hepatoma cells after hypoxic exposure, was also found in nuclear extracts from a wide variety of non-EPO-producing cell lines, indicating that hypoxic induction of HIF-1 is a general phenomenon in most if not all mammalian cells (19, 20). This widespread capacity for oxygen sensing followed by oxygen-dependent gene expression has been further confirmed by introducing reporter genes linked to the oxygen-dependent EPO enhancer into different cell lines: increased reporter gene expression after hypoxic exposure was observed in all transfected cells (19, 21). Functional binding sites for HIF-1 have also been identified in the 5′ flanking region of the VEGF gene (3, 4) and in the genes encoding the ubiquitous glycolytic enzymes aldolase A (ALDA), phosphoglycerate kinase 1 (PGK1), enolase 1 (ENO1), lactate dehydrogenase A (LDHA) and phosphofructokinase L (PFKL) (6, 7). Analogously to the EPO enhancer, oligonucleotides derived from these genes led to induced DNA binding patterns in EMSAs using either purified HIF-1 protein or nuclear extracts from a variety of hypoxically treated cells. These oligonucleotides mediated oxygen-dependent reporter gene expression in transient expression experiments (3, 4, 6, 7), suggesting that HIF-1 activity is not restricted to EPO gene regulation, but modulates expression of several other genes as well.

Hypoxically induced binding of HIF-1 requires phosphorylation and de novo transcription and translation (17, 18). UV cross-linking studies (19) and biochemical purification (22) have revealed that HIF-1 binds DNA mainly as a heterodimeric complex composed of two subunits termed HIF-1α (Mr ~120 kDa) and HIF-1β (Mr ~91–94 kDa), both directly contacting DNA (22). The recent cloning of the HIF-1α and β subunits revealed that both genes belong to the basic helix–loop–helix (bHLH) family of transcription factors (23). While HIF-1α is a novel member of this family with greatest homology to the Drosophila gene SIM, HIF-1β is identical to the aryl hydrocarbon receptor nuclear translocator (ARNT) gene (23). All of these

* To whom correspondence should be addressed
factors share a common region of homology to the bHLH domain termed PAS (PER, AhR/ARNT, SIM). Following binding of aryl hydrocarbon ligands such as dioxin to the aryl hydrocarbon receptor (AhR), ARNT heterodimerizes with AhR to form a functionally active transcription factor activating genes involved in xenobiotic detoxification.

Apart from the induced HIF-1 binding activity, EMSAs using the EPO-derived HIF-1 binding oligonucleotide 5'-GCCCTACGTGCTGCTCTCA-3' show a prominent, specific constitutive band and a weak non-specific band (6, 17, 19, 22-24). Methylation interference studies revealed that HIF-1 and the constitutive activity bind an overlapping DNA sequence, but have distinct methylation interference patterns (18). In contrast, the methylation interference pattern of the non-specific activity was indistinguishable from that of the free probe (18). UV cross-linking experiments with the constitutive probe revealed a Mr of ~30-40 kDa (19), suggesting that the constitutive activity is neither identical to the HIF-1α nor to the HIF-1β/ARNT subunits. In this work we present evidence that activating transcription factor-1 (ATF-1) and cAMP-responsive element binding-1 (CREB-1) are the major constitutive nucleotide binding to the HIF-1 DNA recognition site.

### MATERIALS AND METHODS

#### CELL CULTURE AND HYPOXIC INDUCTION

The murine fibroblast cell line L929 (American Type Culture Collection [ATCC] CCL-1 NCTC clone 929) was a kind gift of V.O’Donnell. L929 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, high glucose; Gibco-BRL) supplemented with 10% heat-inactivated fetal calf serum (Boehringer-Mannheim), 100 U/ml penicillin, 100 mg/ml streptomycin, 1x minimal essential medium non-essential amino acids, 2 mM L-glutamine and 1 mM sodium pyruvate (all Gibco-BRL) in a humidified atmosphere containing 5% CO2 at 37°C. Oxygen tensions in the incubator (Forma Scientific model 3319) were either 140% (20% O2/v, normoxia) or 7% H2 (1% O2/v, hypoxia). Cells at a density of 2 x 10^5 cells/cm² were subjected to hypoxic induction for 4 h.

The human epithelioid carcinoma cell line HeLaS3 (ATCC CCL-22), a subline of HeLa adapted to growth in suspension, was cultured in Ham's F-12 medium (Gibco-BRL) supplemented as described above. Hypoxic induction was achieved by incubation of HeLaS3 cells at a density of 1 x 10^7 cells/ml in a IL 237 tonometer (Instrumentation Laboratory) under continuous stirring for 4 h at 37°C using gas mixtures of either 20% O2, 5% CO2 and 75% N2 (normoxia) or 1% O2, 5% CO2 and 94% N2 (hypoxia) at a flow rate of 500 ml/min.

#### NUCLEAR EXTRACT PREPARATION

Nuclear extracts were prepared according to Semenza and Wang (17). Briefly, 1 x 10^6 cells were washed twice with ice-cold phosphate-buffered saline (PBS), harvested in 5 ml PBS and centrifuged at 400 g for 5 min at 4°C. The cell pellet was washed with 4 packed cell volumes of buffer A containing 10 mM Tris–HCl, pH 7.8, 1.5 mM MgCl2, 10 mM KCl and left on ice for 10 min. Subsequently the cells were lysed by 10 strokes of a Dounce homogenizer using a type B pestle and the nuclei were pelleted at 4500 g for 5 min at 4°C, resuspended in 2 packed cell volumes of buffer C (420 mM KCl, 20 mM Tris–HCl, pH 7.8, 1.5 mM MgCl2, 20% glycerol) and incubated at 4°C for 30 min with gentle agitation. Immediately before use buffers A and C were supplemented with 0.5 mM dithiothreitol (DTT), 0.4 mM phenylmethylsulfonyl fluoride, 2 µg/ml each of leupeptin, pepstatin and aprotinin and 1 mM Na3VO4 (all from Sigma). The nuclear extract was centrifuged at 10 000 g for 30 min at 4°C and the supernatant (0.5 ml) dialyzed twice against 500 ml buffer D (20 mM Tris–HCl, pH 7.8, 100 mM KCl, 0.2 mM EDTA, 20% glycerol) for 4 h at 4°C each. The dialyzed was centrifuged at 10 000 g for 10 min at 4°C, aliquoted, frozen in liquid N2 and stored at –80°C. Protein concentrations were determined using the Bradford protein assay (BioRad) with bovine serum albumin as standard.

#### ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

The sequences of the oligonucleotides used for EMSA are shown in Figure 1. API oligonucleotides were purchased from Promega and the CRE oligonucleotide was from Santa Cruz Biotechnology. Other oligonucleotides were synthesized by Microsynth (Balchach, Switzerland). All oligonucleotides were gel purified on 10% polyacrylamide gels prior to 5'-end-labeling of the sense strand with [γ-32P]ATP (NEN-DuPont) using T4 polynucleotide kinase (Fermentas). Unincorporated nucleotides...
were removed by gel filtration over Bio-Gel P60 (fine) columns (BioRad). Labeled sense strands were annealed to a 2-fold molar excess of unlabeled antisense strands. DNA-protein binding reactions were carried out for 20 min at 4°C in a total volume of 20 µl containing 4–5 µg nuclear extract, 0.1–0.3 µg sonicated denatured calf thymus DNA (Sigma) and 1 × 10⁴ c.p.m. oligonucleotide probe in 10 mM Tris–HCl, pH 7.5, 50 mM KCl, 50 mM NaCl, 1 mM MgCl₂, 1 mM EDTA, 5 mM DTT, 5% glycerol and run on 4% non-denaturing polyacrylamide gels. Electrophoresis was performed at 200 V in TBE buffer (89 mM Tris, 89 mM boric acid, 5 mM EDTA) at 4°C and dried gels were autoradiographed.

For competition experiments a 1- to 500-fold molar excess of unlabeled annealed oligonucleotides were added to the binding reaction mixtures prior to addition of the labeled probes. For supershift analysis 1 µg antibody was added to the completed EMSA reaction mixtures and incubated for 2 h at 4°C prior to loading. All antibodies were obtained from Santa Cruz Biotechnology: D (anti-c-Jun, anti-Jun B and anti-Jun D) rabbit polyclonal IgG; K-25 (anti-c-Fos, anti-Fos B, anti-Fra-1 and anti-Fra-2) rabbit polyclonal IgG; C41-5.1 (anti-ATF-1) mouse monoclonal antibody (mAb) IgG; 25C10G (anti-ATF-1), cross-reactive with CREB-1 and CRED-1) mouse mAb IgG; 24H4B (anti-CREB-1) mouse mAb IgG. A polyhistidine-tagged recombinant ATF-1 protein corresponding to amino acids 39–371 (34 kDa), purified by Ni²⁺ affinity column chromatography to >95% homogeneity, and a truncated 10–14 kDa recombinant CREB-1 (>95% pure), corresponding to amino acids 254–327 (DNA binding and dimerization domains), were both purchased from Santa Cruz Biotechnology. Recombinant proteins were pre-incubated for 5 min at 37°C with 5 µg bovine serum albumin and calf thymus DNA in binding buffer as described above. After adding labeled oligonucleotide probes the reaction mixtures were incubated for 5 min at 37°C and 10 min at 4°C and analyzed by EMSA.

**Transient transfections and reporter gene assays**

Luciferase reporter gene constructs were obtained by inserting the oligonucleotides HIF1 and HIF1mt (Fig. 1b) into the BamHI site 3’ to the luciferase gene present in the pGL3Promoter plasmid (Promega), which contains a heterologous SV40 promoter. Copy number and orientation of the oligonucleotides were determined by single-stranded DNA sequencing using RVprimer4 (Promega). HeLa cells (1 × 10⁷ in 350 µl medium without fetal calf serum) were transiently transfected with 25 µg DNA. Following electroporation at 250 V and 960 µF (Gene Pulser, BioRad) the cells were split in eight 10 cm² tissue culture dishes (Falcon), 1 mM 8-bromo-cAMP (8Br-cAMP; Sigma) or 1 µM phorbol-12,13-dibutyrate (PDBu; Sigma) was added where indicated and incubation was performed for 32 h at 20% or 1% O₂. After washing twice with PBS the cells were lysed in reporter lysis buffer (Promega) and luciferase activity was determined according to the manufacturer’s instructions (Promega). Luminescence was measured in a Biocounter M1500 luminometer (Lumac).

**RESULTS**

Constitutive and hypoxia-inducible factors bind to the HIF-1 DNA recognition site

An alignment of all DNA sequence motifs known so far to be bound by HIF-1 revealed the tentative consensus binding site BACGTSSSK, with the core ACGT being conserved in all genes (Fig. 1a). In an attempt to investigate the complex pattern of proteins binding to the HIF-1 sequence we analyzed nuclear extracts derived from subconfluent cultures of normoxic (20% O₂) or hypoxic (4 h at 1% O₂) mouse L929 fibroblast cells by EMSA. As shown in Figure 2 and as reported previously (17), the 18 bp HIF1 oligonucleotide probe (Fig. 1b) was bound by a hypoxia-inducible, a constitutive and a non-specific factor (nomenclature according to 17). Homologous competition
A computer-assisted search in the tfsites.dat transcription factor database of the GCG program package (25) was performed using the 18 bp HIF-1 sequence (Fig. 1b) as query. Highest homology was found to the 8 bp consensus activator protein-1 (AP-1) motif (26) with one mismatch at position 4, where a thymidine was replaced by an adenosine. As shown in Figure 1b, the first 2 bases of the AP-1 site overlap the HIF-1 consensus sequence. Increasing amounts of unlabeled API oligonucleotides added to L292 nuclear extracts specifically competed for binding of HIF1 oligonucleotide to the constitutive factor, whereas the hypoxically induced HIF-1 binding activity remained unaffected (Fig. 2b). Specificity of AP-1 binding was demonstrated using a mutant API oligonucleotide (AP1mt, see Fig. 1b and product datasheet, Promega) which competed only marginally with wild-type API oligonucleotides for binding to the constitutive factor (Fig. 2c). A weak residual competition, however, might be attributed to some residual AP-1 factor binding to the APImt oligonucleotide, as found by EMSA using labeled APImt oligonucleotides (not shown). Interestingly, gel migration of shifted protein–API oligonucleotide complexes was comparable with the migration of constitutive factor bound to the HIF1 oligonucleotide (Fig. 2b), indicating similar molecular weights for both proteins. These results raised the question of whether the constitutive factor is composed of Jun and Fos family members known to constitute the AP-1 factor as homodimers or heterodimers.

**Jun and Fos family members do not bind to the HIF-1 DNA recognition site**

Commercially available polyclonal antibodies raised against Jun and Fos family members were used to determine whether one or several member(s) of these transcription factor families are involved in constitutive binding to the HIF-1 recognition site. Gel supershift studies were performed using either anti-Jun antibodies to cross-react with c-Jun, JunB and JunD or anti-Fos antibodies reacting with c-Fos, FosB, Fra-1 and Fra-2 (see Materials and Methods). As shown in Figure 3a, no supershifts were detected with either anti-Jun or anti-Fos antibodies. In contrast, when the API oligonucleotide probe was used, binding of these antibodies resulted in strong supershifts, indicating that L929 cells expressed Jun and Fos family members and that these factors bound to the API oligonucleotide probe independent of whether the cells were grown under hypoxia or normoxia. These observations imply that Jun and Fos family members do not contribute to the constitutive HIF1 oligonucleotide binding activity.

To test the hypothesis that the single mismatch (A→T) of the AP-1 consensus sequence located within the HIF1 oligonucleotide (Fig. 1b) was responsible for the lack of binding of Jun and Fos proteins to this site we restored the wild-type AP-1 motif in oligonucleotides HA20 and HA18 (Fig. 1b). Surprisingly, despite the presence of a perfect AP-1 consensus sequence in the HA20 and HA18 oligonucleotides, the intensity of the constitutive band shown in Figure 3b and c did not increase to the level observed with the API oligonucleotide (Fig. 3a). Furthermore, using the HA20 oligonucleotide probe anti-Jun antibodies only marginally shifted the DNA–protein complex, as seen in normoxic extracts, whereas anti-Fos antibodies did not result in supershifts at all (Fig. 3b). A mutant probe that lacked 2 bp at its 5′-end (HA18,
Fig. 1b) was not bound by HIF-1, while the constitutive binding activity was still present (Fig. 3c). Interestingly, using the HA18 oligonucleotide probe both anti-Jun and anti-Fos antibodies supershifted the constitutive band, irrespective of whether normoxic or hypoxic (i.e. HIF-1 activity-containing) nuclear extracts were used (Fig. 3c). These data demonstrate that a point mutation restoring the wild-type AP-1 site is not sufficient for Jun and Fos binding. However, a double mutation restoring the AP-1 site and preventing HIF-1 binding (HA18, Fig. 1b) allowed replacement of the constitutive factor by Jun and Fos family members. On the other hand, a mutant version of the HIF1 oligonucleotide abolishing the homology to the AP-1 consensus sequence (H3'mt, Fig. 1b) did not influence the constitutive binding activity (Fig. 3d) and addition of anti-Jun and anti-Fos antibodies did not result in supershifts. In summary, these results suggest that the HIF-1 recognition sequence, which precedes (and partially overlaps) the putative homology to the AP-1 site, directs a factor other than Jun and Fos family members to this site. However, wild-type AP1 oligonucleotides specifically and efficiently competed with the HIF1 probe for binding to this factor.

**ATF-1 constitutively binds to the HIF-1 DNA recognition site**

Besides Jun and Fos family members, there are other transcription factors known to bind to the AP-1 or closely related motifs, among them members of the ATF/CREB family, which bind to the cAMP-responsive element (CRE). A mAb raised against ATF-1 (C41-5.1; see Materials and Methods) almost completely supershifted the constitutive factor bound to the HIF1 oligonucleotide, as well as the factor bound to the CRE control oligonucleotide (Fig. 4a), indicating that the constitutive factor was immunologically related to ATF-1 and that it was composed of either ATF-1 homodimers or heterodimers with one subunit being ATF-1.

Next we investigated whether the various mutated HIF1 oligonucleotide probes shown in Fig. 1b were still capable of binding ATF-1 by means of supershift experiments in EMSAs using the anti-ATF-1 mAb and L929 nuclear extracts. As shown in Figure 4a, the point mutation restoring the wild-type AP-1 site within the HIF1 oligonucleotide sequence (oligonucleotide HA20) or even a 3 bp mutation that destroyed the homology to the AP-1 site (oligonucleotide H3'mt) did not prevent ATF-1 from binding these oligonucleotides. In contrast, the HA18 oligonucleotide probe, which was identical to HA20 except that the first 2 bases were missing, was not bound by either HIF-1 (see also Fig. 3c) or, because no supershift was obtained with anti-ATF-1 mAb, by ATF-1 homodimers or heterodimers (Fig. 4a). Since the remaining constitutive DNA binding activity to the HA18 oligonucleotide probe was supershifted with anti-Jun and anti-Fos antibodies (Fig. 3c), but not with anti-ATF-1 antibodies (Fig. 4a), it must be concluded that only the combination of both mutations present in HA18, but not the point mutation in HA20 alone (transforming the AP-1 homology to a wild-type AP-1 site), allowed efficient replacement of ATF-1 by Jun and/or Fos proteins.

Irrespective of whether HIF1 or CRE oligonucleotide probes were used, the intensities of the bands supershifted by the anti-ATF-1 mAb were comparable in nuclear extracts derived from either normoxic or hypoxic cells. Since the supershifted constitutive band partially overlapped the hypoxia-inducible HIF1 band (Fig. 4a), we could not conclude whether HIF1 bands were also influenced by the anti-ATF-1 mAb. To circumvent this problem visualization of the constitutive binding activity was prevented by addition of a 100-fold molar excess of unlabeled API (see Fig. 2b) or CRE oligonucleotides prior to incubation with the antibody. As shown in Figure 4b, both competitor oligonucleotides blocked most of the constitutive activity binding the labeled HIF1 probe and the remaining HIF1 band was not shifted by the anti-ATF-1 mAb, suggesting that ATF1 does not participate in HIF1 complex formation. Furthermore, no difference was observed in competition experiments using increasing amounts of either API oligonucleotides (as shown in Fig. 2b) or CRE oligonucleotides (not shown), demonstrating that the constitutive factor had comparable affinities for AP-1 and CRE sites. In agreement with
this, the anti-ATF-1 mAb also supershifted a part of the proteins bound to the AP1 probe (not shown).

Detection of ATF-1 and CREB-1 binding to the HIF-1 site in nuclear extracts derived from HeLa cells

To confirm the results obtained with the murine fibroblast line L929 we tested additional antibodies raised against ATF-1/CREB-1 family members. We chose to investigate the human epitheloid carcinoma cell line HeLa, which represents a common model for hypoxic HIF-1 induction and has been used for biochemical purification of HIF-1α and HIF-1β/ARNT subunits (22). HeLa cells were induced in a tonometer and nuclear extracts were analyzed by EMSA. As shown in Figure 5a, the previously used mouse anti-ATF-1 mAb C41-5.1 (αATF1), known to be reactive with human, mouse and rat ATF-1 (product datasheet, Santa Cruz Biotechnology), supershifted the constitutive DNA binding activity of HeLa nuclear extracts similarly to that of L929 cells. Two commercially available mAbs, both human-specific, also supershifted the constitutive factor (Fig. 5b); the mAb 2510G, termed α(A/C) in Figure 5b, was raised against human ATF-1 but is cross-reactive with the related factors CREB-1 and CREM-1; the mAb 24H4B (αCREB-1) was raised against human CREB-1 and is not cross-reactive with other members of the ATF/CREB family. However, the extent to which the three mAbs supershifted the constitutive band was clearly different; while the anti-ATF-1 mAb C41-5.1 and the anti-ATF-1/CREB-1/CREM-1 mAb 2510G both resulted in an almost complete supershift, the anti-CREB-1 mAb 24H4B only partially supershifted the constitutive band (Fig. 5b). Since the same pattern was observed with CRE oligonucleotide probes, we conclude that these results reflect the expression levels of the two proteins, suggesting that in HeLa cells ATF-1 represents the main DNA binding activity of the ATF/CREB family. Similar observations have been reported by Hurst et al. (27,28). The stronger band obtained with CRE probes compared with HIF1 probes might be explained by either a higher affinity of ATF-1 for the consensus CRE site or by binding of additional ATF/CREB family members which cannot bind to the HIF-1 sequence. The higher intensity of the constitutive band in hypoxic compared with normoxic HeLa extracts (Fig. 5b) was due to unequal protein loading and was not observed using other HeLa extracts, such as those in Figure 5a. Interestingly, increased bound probe led to decreased non-specific band intensity, suggesting different affinities between the constitutive and non-specific factors for the oligonucleotide probes.

Recombinant ATF-1 and CREB-1 bind to the HIF-1 DNA recognition site

To determine whether ATF-1 and CREB-1 are equally able to bind to the HIF-1 site and, if so, to find out whether homodimers or heterodimers preferentially bind the HIF1 oligonucleotide probe we performed EMSAs using commercially available recombinant ATF-1 (amino acids 39–371) and a truncated form of CREB-1 (amino acids 254–327, corresponding to the DNA binding and dimerization domains), thus allowing easy separation of both proteins on a gel. As shown in Figure 6, at least with recombinant proteins, the HIF1 oligonucleotide probe was bound more strongly by CREB-1 and ATF-1/CREB-1 heterodimers than by ATF-1 alone. A mutation in the HIF-1 motif (oligonucleotide HIF1mt, see Fig. 1b) completely impeded binding of ATF-1 and CREB-1, whereas the two mutations lying 3’ to the HIF-1 site (oligonucleotides HA20 and H3’mt, see Fig. 1b) did not significantly alter the binding pattern. The probe HA18, lacking the two 5’ bases present in HA20 (Fig. 1b), again did not allow ATF-1 binding, as was the case with nuclear extracts (Fig. 4a). In striking contrast, HA18 was still bound to CREB-1, indicating that ATF-1, but not CREB-1, binding is strictly dependent on the presence of these two bases at the 5’-end. On the other hand, the CRE oligonucleotide probe bound to ATF-1 and CREB-1 homodimers with comparable affinities and pre-mixing of ATF-1 and CREB-1 in a 1:1 molar ratio resulted in an almost stoichiometric distribution of homo- and heterodimer binding.

A cAMP agonist enhances hypoxic induction of reporter gene expression

Binding of ATF-1/CREB-1 to the HIF-1 site suggests that the HIF-1 enhancer is cAMP responsive. To test this hypothesis we constructed luciferase plasmids containing a heterologous SV40 promoter and one or three copies of the 18 bp oligonucleotides HIF1 or HIF1mt (Fig. 1b) inserted 3’ of the luciferase gene (Fig. 7). Following transfection into HeLa cells either the PKA activator 8Br-cAMP or the PKC activator PDBu was added and the cells were incubated under normoxia or hypoxia. The resulting luciferase expression was normalized to the untreated normoxic control (Fig. 7). In HeLa cells transfected with a plasmid containing three copies of the HIF1 oligonucleotide hypoxia significantly increased luciferase expression by 6.3-fold (P < 0.01, n = 3, Student’s t-test). The addition of 8Br-cAMP enhanced this.
hypoxic induction by 64% (P < 0.05), whilst PDBu had no effect. 8Br-cAMP also slightly induced normoxic luciferase expression, but this effect was only found to be significant following normalization to the untreated hypoxic values (P < 0.05). Normalization to a co-transfected lacZ plasmid was not practicable, since the β-galactosidase activity itself was induced by 8Br-cAMP (not shown). A luciferase construct containing three copies of the mutant HIF1mt oligonucleotide, as well as constructs containing one copy of either HIF1 or HIF1mt, showed no significant induction by either hypoxia or 8Br-cAMP. A slight inducibility was observed in the pGL3Promoter vector itself, suggesting that the SV40 promoter is weakly hypoxia responsive. Thus one copy of the HIF-1 enhancer sequence seems to be insufficient to markedly enhance hypoxic induction of the pGL3Promoter plasmid.

**DISCUSSION**

The discovery of a nuclear factor (HIF-1) whose DNA binding activity can be induced by low oxygen concentrations provided the first step towards understanding the mechanisms of hypoxia-regulated gene expression. That hypoxic induction is unique to HIF-1 is supported by EMSAs, performed with a variety of probes other than HIF1 and using our L929 nuclear extracts, which do not show any difference in factor binding between hypoxic and normoxic cell culture conditions. The probes contained binding sites for the following factors: AP-1 (Jun and Fos family members, Fig. 2); CRE-binding factors (ATF/CREB family members, Fig. 4); AP-2, AP-3, Sp-1, MTF (not shown); C/EBPβ, NF-κB, STAT-1 and STAT-3 (V.Poli, personal communication).

Characterization of the HIF-1 DNA binding activity in EMSAs revealed the presence of a constitutive activity which, in addition to the hypoxia-inducible factor HIF-1, binds to the HIF1 oligonucleotide probe. Based on the data presented in this work, we found the following evidence that most if not all of this constitutive factor is closely related or identical to the transcription factors ATF-1 and CREB-1: (i) CRE oligonucleotides specifically competed for HIF1 oligonucleotide probe binding to the constitutive factor, whereas the inducible HIF-1 activity remained unaffected; (ii) mAbs raised against ATF-1 and CREB-1 supershifted most of the constitutive factor in nuclear extracts derived from mouse and human cell lines, whereas the HIF-1 shift was not influenced; (iii) purified recombinant ATF-1 and CREB-1 proteins bound HIF1 oligonucleotide probes as homo- or heterodimers.

Transcription factors of the ATF/CREB family were originally defined by their specific binding to a palindromic sequence (TGACGTCA), known as the cAMP-responsive element (CRE), which has been found in many cAMP-inducible cellular genes
and in several viral promoters (reviewed in 29–31). All of these proteins share a common basic DNA binding domain followed by a leucine zipper dimerization domain. Among the increasing number of ATF/CREB family members CREB-1 is the best characterized factor. CREB-1 has been shown to be a target of cAMP-inducible PKA-mediated phosphorylation, which is required for transcriptional activator function. Binding of nuclear factors to the CRE site, however, is usually not induced by PKA-dependent phosphorylation and requires leucine zipper-mediated dimerization. Homodimerization and heterodimerization of CREB-1 with other members of the ATF/CREB family and even with members of the Jun/Fos family have been reported (32). In contrast to CREB-1, the function of ATF-1 is poorly understood. It is known that multiple phosphorylation of ATF-1 leads to a dramatic change in conformation which affects the stability of DNA binding (33). Compared with CREB-1, PKA-dependent phosphorylation of ATF-1 does not seem to be a major inducer of transcriptional activator function (28). ATF-1 is most homologous to CREB-1 and is known to form heterodimers with CREB-1. With regard to the unusual DNA recognition specificity of ATF-1/CREB-1 bound to the HIF-1 site (see below), we cannot rule out the possibility that ATF-1 binds the HIF1 probe as an heterodimer with a yet to be identified factor. Indeed, altered DNA recognition specificity after differential heterodimer formation has been reported for other ATF/CREB family members (30,32).

The relative molecular weight \( M_r \) of ATF-1 has been shown to be dependent on its phosphorylation status (33) varying between ~35 and 40 kDa (28). This is in agreement with UV cross-linking experiments of constitutive factor binding to the HIF1 oligonucleotide, which revealed a \( M_r \) of ~30–40 kDa (19). The protein–DNA contact sites of the constitutive factor have been analyzed by methylation interference and were found to be closely related to the HIF-1 contact sites (18). This is not consistent with the result of the computer search, which revealed a putative AP-1/CRE motif located 3' to the HIF-1 site (model a in Fig. 8). Based on the observations presented in this study, we conclude that the ATF-1 binding site does indeed match the HIF-1 DNA recognition site: (i) the mutant oligonucleotide HIF1 Int (see Fig. 1b) was not bound by either HIF-1 or the constitutive factor and HIF1 Int did not compete with the wild-type HIF1 probe for binding to the constitutive factor; (ii) a mutation in the putative AP-1/CRE site located 3' to the HIF-1 motif (probe H3 Int) did not abolish constitutive factor binding; (iii) generation of a perfect homology to the AP-1 consensus located 3' to the HIF-1 motif (probe HA20) only allowed efficient Jun and Fos binding when in addition to this mutation the first two 5' bases were absent (probe HA18), which abolished HIF-1 and constitutive factor (ATF-1, but not CREB-1) binding. Thus both HIF-1a/HIF-1b (ARNT) heterodimers and ATF-1/CREB-1 heterodimers bind to the HIF-1 DNA recognition site in vitro, as shown by EMSAs. A re-inspection of the homology comparison between the CRE motif and the HIF1 oligonucleotide (model a in Fig. 8) revealed another possible match in which the core CRE motif overlaps the core HIF-1 motif ACGT (model b in Fig. 8), which is conserved in all HIF-1 binding sites found so far (Fig. 1a). Despite a reduced overall homology, model b is more consistent with our data than model a. Based on the result obtained with the HA18 probe (Fig. 1b), which efficiently binds CREB-1 homodimers, but only marginally ATF-1 homodimers or ATF-1/CREB-1 heterodimers (Fig. 6), one must conclude that ATF-1 binding is dependent on the presence of the two 5' bases of the HIF1 probe. Thus ATF-1 most probably occupies the 5' half-site when bound to DNA as a heterodimer.

It remains an open question whether HIF-1 and the constitutive factor (ATF-1/CREB-1) bind to the HIF-1 DNA recognition site simultaneously in vitro or whether following hypoxic stimulation ATF-1/CREB-1 is replaced by HIF-1. However, based on methylation interference assays revealing that HIF-1 and the constitutive factor contact the same guanosine residues (18), we favor the model that following hypoxic induction ATF-1/CREB-1 is replaced by HIF-1; even though in vitro ATF-1 and CREB-1 are still capable of binding to the HIF1 probe, which was offered in excess. In this context it is interesting to note that while protein kinase A-dependent phosphorylation of ATF-1 only marginally affects transcriptional activator function (28), phosphorylation-dependent conformational changes of ATF-1 are known to contribute to the instability of the interaction with DNA (33). It is therefore tempting to speculate that such a conformational change could allow replacement of ATF-1 by HIF-1 following hypoxic stimulation.

Our finding that a CRE site overlaps the hypoxia-inducible cis-acting element raised the question whether the cAMP signal transduction pathway is involved in the oxygen sensing mechanism. Functional reporter gene studies using the PKA activator 8Br-cAMP demonstrated that under hypoxic conditions the HIF-1 site was cAMP responsive. This result is in line with our previous report showing that hypoxia induces activity of the glycolytic enzyme LDH and also increases the cAMP content in vascular smooth muscle cells (8). Furthermore, activators of adenylate cyclase induced LDH activity, while inhibitors of PKA blocked hypoxia-inducible LDH up-regulation (8). Since functional HIF-1 cis-regulatory elements have also been localized in the LDH gene (6,7), these results on LDH regulation support the idea that cAMP-dependent phosphorylation of ATF-1 might be a crucial component of HIF-1-mediated hypoxia-inducible gene expression in mammalian cells.

### Table 1

<table>
<thead>
<tr>
<th>Model</th>
<th>HIF-1 consensus</th>
<th>HIF1 probe</th>
<th>AP-1 consensus</th>
<th>CRE consensus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model a</td>
<td>HIF-1 consensus</td>
<td>BACGTSSK</td>
<td>GCCCTACGTGCTGTC-TCA</td>
<td>TGACGTCA</td>
</tr>
<tr>
<td>Model b</td>
<td>HIF-1 consensus</td>
<td>BACGTSSK</td>
<td>GCCCTACGTGCTGTC-TCA</td>
<td>TGACGTCA</td>
</tr>
</tbody>
</table>

**Figure 8.** Two possible homologies between the HIF1 oligonucleotide probe and AP-1 CRE consensus motifs. For AP-1 and HIF-1 consensus sequences see Figure 1. The palindromic consensus CRE sequence is from Lalli and Sassone-Corsi (31). K = G or T; S = C or G; M = A or C; B = C or G or T.
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