Anoxic Induction of a Sarcoma Virus-Related VL30 Retrotransposon Is Mediated by a *cis*-Acting Element Which Binds Hypoxia-Inducible Factor 1 and an Anoxia-Inducible Factor

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Cells exposed to hypoxia undergo substantial changes in gene expression generally associated with metabolic adaptation and increasing oxygen delivery. In contrast, responses distinct from those elicited by hypoxia are induced in anoxic fibroblasts; this includes activation of a set of VL30 elements. The responses seen in anoxically cultured fibroblasts are expressed physiologically in vivo during the anaerobic phase of wound healing. A fundamental question is whether transcriptional regulatory pathways utilized during anoxia are distinct from those already characterized for hypoxic cells. We report here the isolation of a 14-bp sequence within a VL30 retrotransposon promoter which mediates its anoxia responsiveness. Analyses of the protein complexes binding to this sequence demonstrated the presence of two distinct inducible DNA binding activities. The first is present in both hypoxic and anoxic fibroblasts and is indistinguishable from hypoxia-inducible factor 1. The second activity, which is present only in anoxic fibroblasts, is a previously uncharacterized heterodimeric DNA binding activity that appears to arise via posttranslational modification of an existing complex found in aerobic cells. These results indicate that the strong VL30 transcriptional induction seen with anoxia occurs through a mechanism specific to anoxia.

The abilities of anoxia and hypoxia to impact on many diverse biological systems, such as erythropoiesis (15), wound healing (6, 20), tumor progression (28, 31, 34), and retinal degeneration (27), depend to a large extent upon changes in gene expression. As such, the activity of several different transcription factors is now known to be influenced by low oxygen tensions. In cells which are stressed by oxygen deprivation, NF-κB activity increases as a result of phosphorylation and subsequent degradation of IκBα (23). In other cells, low oxygen tensions induce the transcription of multiple members of the bZIP superfamily (7, 11, 44), result in nuclear accumulation of p53 (17), or induce the activity of hypoxia-inducible factor 1 (HIF-1) (33).

Whereas the aforementioned studies provide multiple examples of changes in the activity of transcriptional regulators in response to hypoxia (0.1 to 1% oxygen), very little work has been done to examine the mechanisms by which complete anaerobiosis (anoxia) affects gene expression. Cellular anoxia represents a biochemical state distinct from hypoxia yet still represents a normal physiological condition present during wound healing (6). Since a response quite different from that seen with hypoxia is induced in anoxic fibroblasts, these cells must possess the ability to activate a different, possibly overlapping set of genes to cope with these different conditions (5). We have investigated whether fibroblasts utilize a distinct pathway to alter gene expression during anoxia by using the transcriptional induction by anoxia of the sarcoma virus-related class of VL30 elements as a model system (4, 13). This system appears to be ideal for identifying anoxia-specific transcriptional regulators, since these VL30s are strongly induced by anoxia but are induced to a far lesser extent in atmospheres

with higher oxygen tensions (0.2 to 2%) (4). Like other components of the anoxic fibroblast response, these VL30s are also induced in vivo during the early anaerobic phase of wound healing (6). Such induction is not stress related, since anoxic fibroblasts in culture neither show a reduction in viability (36) nor induce heat shock proteins (3) during the response. Induction is specific to anoxia; VL30 is induced by neither a variety of respiratory poisons, including those known to induce the heat shock response, nor 2-deoxyglucose, which induces the glucose-regulated proteins (1). The induction of these VL30 elements by anoxia shows biphasic kinetics (5), with low-level (ca. 5-fold) primary induction beginning within 2 h and much higher-level (ca. 25- to 100-fold) secondary induction dependent on new protein synthesis beginning about 6 h into anoxia. Therefore, this system was likely to enable us to elucidate discrete regulatory pathways unique to anoxia.

VL30s represent a group of defective retrotransposons found in the rodent genome with approximately 50 to 100 related but nonidentical copies per cell (19, 22, 43). Although bearing the genomic organization of a retrovirus, VL30s do not appear to encode functional *gag*, *pol*, or *env* genes (9, 25). However, the presence of functional reverse transcriptase primer binding sites and packaging signals allows them to be efficiently pseudotyped by endogenous retroviruses. Such pseudotyped VL30s have been shown to reintegrate into the genome at new sites (9). One class of these elements was initially identified because it was found transduced flanking *ras* in both the Harvey and Kirsten murine sarcoma viruses, which were independently isolated after passaging of the Moloney and Kirsten leukemia viruses in rats (2, 10). Experiments designed to define the sequences necessary for the oncogenic activity of these viruses indicated that the VL30 sequences 3′ of *ras* significantly contribute to their tumorgenic activity in vivo (37, 42). It has been proposed that these VL30 sequences serve to enhance the expression of *ras* (37), although the facts that no other cellular enhancer sequences have been trans-

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duced into the sarcoma viruses and this event independently occurred twice suggest otherwise. Although expressed at low levels in most normal tissues, sarcoma virus-related VL30s are found constitutively expressed at high levels in many rat malignancies and tumor-derived cell lines, in addition to being strongly induced by anoxia in normal rat fibroblasts (2, 5, 8).

By studying the promoter of such an anoxia-responsive VL30 element, we have isolated and characterized a 14-bp sequence which is necessary and sufficient for the secondary phase of its anoxic induction. Sequence analysis of this secondary anoxia-responsive element (SARE) showed it to be similar to the consensus HIF-1 binding site (32). We showed that one DNA binding activity induced in hypoxic, anoxic, and cobalt-treated fibroblasts recognizes this SARE and has electrophoretic mobility similar to that of HIF-1 from hypoxic HepG2 cells. Additionally, we demonstrated a second, more prominent SARE binding activity, termed the anoxia-inducible factor (AIF), which is induced only in anoxic fibroblasts. Two-dimensional gel analysis indicated that AIF is a heterodimer composed of 61- and 52-kDa subunits and is likely to arise from posttranslational modification of a heterodimeric SARE binding complex present in aerobic cells.

MATERIALS AND METHODS

Tissue culture. The FRE cell line (normal rat fibroblasts) was cultured in Dulbecco modified Eagle medium supplemented with 10% calf serum (Gibco) (1, 5). The human hepatoma line HepG2 was grown in minimal essential medium supplemented with 10% fetal calf serum (Gibco), 10 mM sodium pyruvate, 2 mM L-glutamine, and 1 mM nonessential amino acids. Aerobic cultures were grown at 37°C in a humidified incubator with a 95% air-5% CO_2 gas mixture. For anoxic experiments, fresh Dulbecco modified Eagle medium was added to subconfluent cultures 1 h prior to their placement in a humidified anaerobic glove box incubator (Forma Scientific) supplied with an 85% N_2-10% H_2-5% CO_2 gas mixture. Palladium catalysts were utilized to scavenge any trace oxygen contamination. A chemical microsensor (Diamond Electro-Tech) and a methylene blue indicator (35) were utilized to ensure that anoxic conditions were present during experiments. The medium of subconfluent cultures exposed to hypoxia was changed 1 h prior to their placement in a humidified GasPak System chamber (BBL Microbiology Systems) which was subsequently flushed extensively with a 94% N_2-5% CO_2-1% O_2 gas mixture before being sealed.

cDNA library construction and screening. A cDNA library was constructed in a lambda GT11 expression vector with RNA isolated from FRE cells exposed to 6 h of anoxia in accordance with the manufacturer's (Amersham) recommendations. Approximately 10^6 plaques were screened by hybridization of nitrocellulose filters to the P47 VL30 probe (13). To isolate clones containing long terminal repeats (LTRs), DNAs from plaques hybridizing to P47 were used in PCRs containing the 5'V31 (5' CTTCTTGGACTGTGCCTCAGG) primer from the 3' end of P47 and either the GT11 forward or reverse primer which flanks the cloning site present in the vector. Clones which generated an amplified product with the expected size (approximately 1.2 kb) for inserts with intact 3' U3 segments were utilized for subsequent analysis by subcloning into pKT, a pUC18-based plasmid with a promoterless chloramphenicol acetyltransferase (CAT) gene.

Transient transfections and CAT assays. Subconfluent FRE cells were transiently transfected (12 μg of DNA per 10-cm-diameter dish) by the calcium phosphate method (18). Twelve hours after transfection, cells were released from the dishes with trypsin and divided equally between two dishes. Prior to harvesting, half of the transfected cells were placed in an anoxic atmosphere as described above. Cells were harvested, and CAT assays were performed as described previously (16). Cotransfection of a β-galactosidase reporter driven by the simian virus 40 promoter was used to monitor transfection efficiencies. Quantitation was performed with a PhosphorImager (Molecular Dynamics) by calculating the percent conversion of [^{14}C]chloramphenicol to the acetylated form.

RNA isolation and analysis. Total cellular RNA was isolated from subconfluent FRE cells grown under the atmospheres indicated in the figures by guanidinium lysis (30). Fifteen micrograms of RNA per lane was glyoxylated and analyzed on 1% agarose gels. Gels were blotted by capillary transfer to Zetabind (Cuno Inc.). Membranes were probed with a randomly primed, ^{32}P-labelled PvuII-NcoI restriction fragment of the CAT gene and then stripped and reprobed with a randomly primed, ^{32}P-labelled cDNA of ribosomal protein L32 (21) as a control for loading variability.

Plasmid construction. Parental plasmid p−203/25 is an AluI restriction fragment of a VL30 LTR modified by addition of BglII linkers and cloned into pKT at a modified SmaI site. p−126/25 and p−85/25 are 5' deletions created by

opening p−203/25 at the KpnI site, digesting it with Bal31, and then cloning it into pKT at the SmaI and SalI sites. p−203/−20 is a 3' deletion of p−203/25 created by PCR with M13 reverse and 3'V31 (5' cgctgcagGTTATATAGAGA AGGCTAGG) primers. The amplified product was restricted and cloned into pKT at the BglII and PstI sites. p−126/−58 is a 3' deletion of p−126/25 generated by PCR with M13 reverse and 3'V32 (5' ggagatctACAATTGGCTCATT CAA) primers. The amplified product was restricted and cloned into the BglII and HindIII sites of p−35AC, a modification of pCAT Basic (Promega) which has the TATA box from the rat albumin gene inserted upstream of the CAT coding sequences. p−104/−41 was generated by PCR with the 5'V32 (5' ggag atctCTCTTTCTGCTTTGTTCTC) and 3'V33 (5' ccagatctGTTTTGGCGTGG TTACATAC) primers and subsequently cloned into the BglII site of p−35AC. Annealing of complementary oligonucleotides with the sense strand sequences 5' TAGCACGTACTCTT and 5' TTGAATGAGCCAATTGTA was used to generate plasmids p−113/−100 and p−75/−58, respectively, by cloning into p−35AC with HindIII and BglII overhangs present in these oligonucleotides. The nucleotide sequence of each plasmid was confirmed by double-stranded dideoxy sequencing with Sequenase (U.S. Biochemical) as recommended by the manufacturer.

SARE mutagenesis. An oligonucleotide with the sequence 5' CTAAGCTT**T AGCACGTACTCTT**CCAGATCTGG was made so that the internal SARE sequence (in boldface) incorporated 10% random degeneracy during the synthesis. The oligonucleotide was incubated in 50 mM Tris (pH 7.5)–10 mM $MgCl_2$–1 mM dithiothreitol–50 μg of bovine serum albumin per ml at 20°C for 1 h to promote self-annealing at the complementary 3' end. The oligonucleotides were made completely double stranded by addition of 2 mM deoxynucleoside triphosphates and 5 U of Klenow. After additional incubation at 20°C for 2 h, the oligonucleotides were extracted with phenol, precipitated with ethanol, and restricted with HindIII and BglII. The products were then cloned into p−35AC and sequenced.

Mobility shift assays. Nuclear extracts were prepared as previously described (24) with the addition of 0.1% Nonidet P-40 to buffer A and the addition of aprotinin (2 μg/ml), leupeptin (1 μg/ml), pepstatin (1 μg/ml), 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate to buffers A and C. Mobility shift assays were performed in the presence or absence of 0.05% Nonidet P-40 as previously described for the detection of HIF-1 (33, 41). The sequences of the sense strands of the double-stranded oligonucleotides utilized as probes and competitors were as follows: VL30 SARE, 5' CTAGCACGTA CTCTTTCT; MS107 (mutant SARE), 5' TAGCACTTACTCTTTCTG; mouse EPO–HIF-1 site, 5' GCCCTACGTGCTGCCTCGCATGGC; consensus AP-2 site, 5' GATCGAACTGACCGCCCGCGGCCCGT.

Two-dimensional gel analysis. UV cross-linking of proteins to the ^{32}P-labelled SARE oligonucleotide was performed by utilizing the following modifications of a previously described technique (40). A probe (3.6 × 10^5 cpm) was incubated with 8 μg of nuclear extracts. After UV cross-linking and a first-dimension electrophoretic mobility shift assay (EMSA), a second UV irridation was performed on the gel, and then individual lanes of the gel were excised, treated as already described, and then overlaid on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel and electrophoresed in the second dimension.

RESULTS

Characterization of an anoxia-responsive promoter element. To help define regulatory mechanisms responsible for anoxia-induced gene expression and to better understand the biology of sarcoma virus-associated VL30s, we studied the transcriptional activation of these VL30s in anoxic rat fibroblasts. By constructing and screening a cDNA library from anoxic rat fibroblast cell line FRE mRNA, we were able to isolate and clone anoxia-inducible VL30s. Since the genomic structure of these VL30s is similar to that of retroviruses, the U3 portion of the 3' LTR can be found intact at the 3' end of the VL30 mRNA. We chose this strategy rather than screening of a genomic library because there are roughly 100 copies of VL30 found in the rat genome. It is unlikely that all of these are anoxia inducible, since VL30s have previously been shown to be highly heterogeneous (22).

Screening of our anoxic fibroblast library yielded several U3-bearing VL30 clones. These were subcloned into CAT reporter constructs and tested for anoxia responsiveness. Figure 1A shows the results obtained with one of these clones, p−203/25. This clone is composed mostly of U3 sequences and terminates just 5' of the polyadenylation signal in the R region. The results of the CAT assay presented in Fig. 1A show that these VL30 sequences conferred anoxia inducibility on the CAT reporter beginning after 8 h of continuous anoxia and
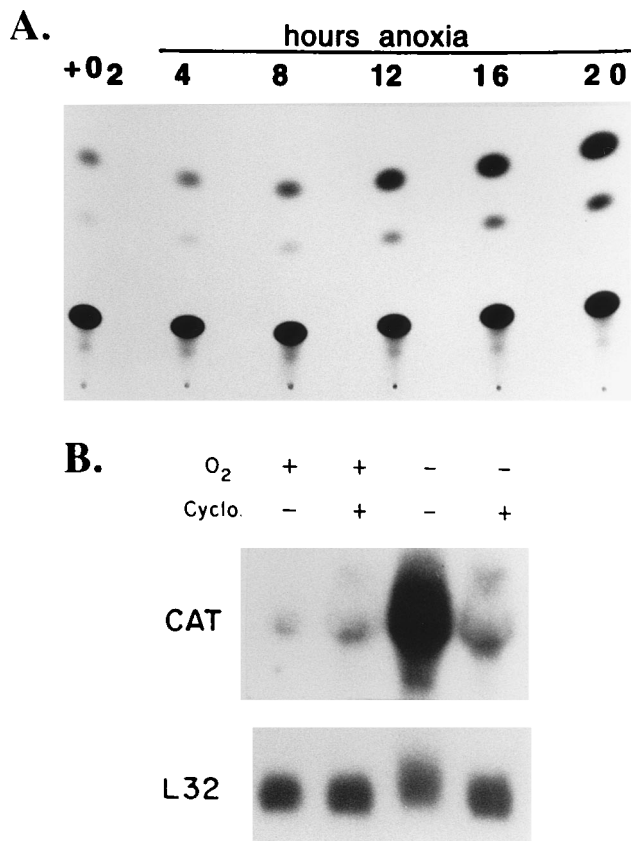
FIG. 1. Identification of an anoxia-responsive rat VL30 promoter element. (A) FRE cells were transiently transfected with the p−203/25 CAT reporter and then grown aerobically or exposed to anoxia for the times indicated prior to harvesting and performance of CAT assays. (B) FRE cells were transiently transfected with the p−203/25 reporter. At 14 h prior to isolation of RNA, half of the transfected cells were placed in an anoxic atmosphere in the presence or absence of cycloheximide (Cyclo.; 10 µg/ml), as indicated above the lanes. A Northern blot was probed with a fragment of the CAT gene and then stripped and reprobed for the ribosomal protein L32 gene as a control for loading variances.
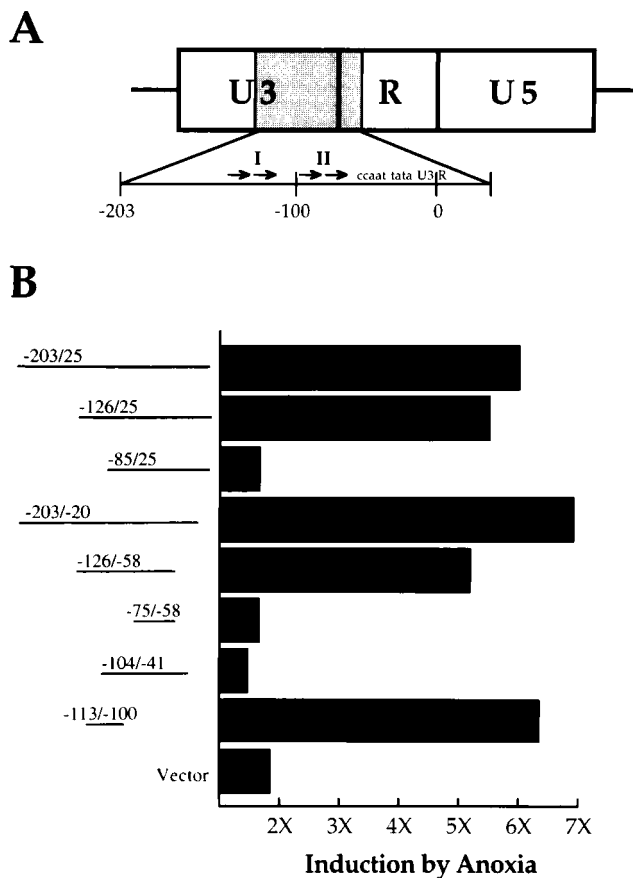

FIG. 2. Anoxia inducibility of promoter deletions. (A) Position of the parental p−203/25 clone relative to the entire VL30 LTR. The U3-R boundary has been arbitrarily designated position zero. This clone contains the VL30 TATA box and two distinct tandem repeats of 9 and 11 bases (double arrows). (B) FRE cells were transiently transfected with CAT reporters bearing the indicated promoter deletion. Transfected cultures were divided equally between two dishes by release with trypsin. After allowance of 5 h for the cells to adhere to the culture dishes, half were placed in an anoxic atmosphere for 20 h prior to harvesting. Mean induction is defined as the activity of each construct (expressed as percent conversion) after exposure to anoxia relative to that of the aerobic control. The mean inductions shown are from two to six separate transfections.

reaching sixfold enhancement of CAT activity relative to the aerobic control.

To determine whether the induction mediated by this promoter element required new protein synthesis, we transiently transfected fibroblasts with the p−203/25 reporter and isolated RNA after exposure to anoxia in the presence or absence of cycloheximide. Figure 1B shows a Northern (RNA) blot of this RNA probed with a fragment of the CAT gene. These results show that the VL30 promoter element, in response to anoxia, conferred 28-fold induction of the CAT gene at the mRNA level. Inclusion of cycloheximide in the media of the anoxic cultures reduced this induction to 1.8-fold, demonstrating a requirement for new protein synthesis for this induction. The induction of CAT enzyme activity does not completely reflect the magnitude observed at the level of the CAT mRNA; the message may be less efficiently translated, or the protein may have a diminished half-life in anoxic cells (26). On the basis of the kinetics seen in Fig. 1A and the requirement for new protein synthesis (Fig. 1B), p−203/25 contains the VL30 promoter element(s) necessary for the secondary anoxic response.

Deletion-and-mutation analysis defines the minimal sequence necessary for anoxic induction. We generated a series of deletions to precisely define the boundaries of this SARE.

Figure 2 shows both the position of the parental p−203/25 clone within the VL30 LTR (Fig. 2A) and a summary of the anoxia inducibility of several deletions assayed in CAT reporters (Fig. 2B). Initially, the SARE was narrowed down to a 68-bp region defined by deletion p−126/−58. Within this region, there is a tandem repeat which shows sequence homology to a hypoxic enhancer found in the maize alcohol dehydrogenase promoter (38), as well as a second repeat downstream which contains a half site for the steroid-thyroid hormone receptor family. However, further deletion analysis within this region showed that anoxia inducibility could be conferred by a 14-bp sequence (p−113/−100) found between these two tandem repeats.

Analysis of this 14-bp sequence showed that it contained a seven-of-eight-nucleotide match to the consensus binding site for HIF-1 (32), a transcription factor shown to mediate the hypoxic induction of erythropoietin and the genes for several glycolytic enzymes (12, 32, 33) (Fig. 3A). To determine if this potential HIF-1 binding site is involved in SARE function, we generated a series of random point mutations spanning the entire SARE. These were then cloned into a CAT reporter and

A.

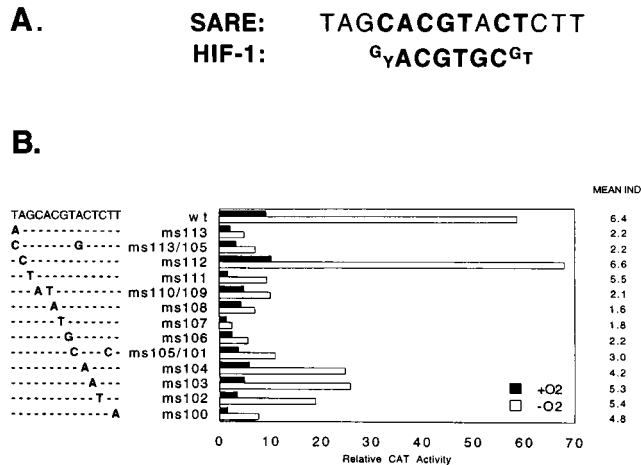SARE: TAGC**ACGT**ACTCTT

HIF-1: ^G_YACGTGC^G_T

B.

FIG. 3. Effects of mutations on the activity of the 14-bp SARE. (A) Comparison of the VL30 SARE sequence to the reported consensus binding sequence for HIF-1 (32). The SARE contains a seven-of-eight-nucleotide match to the consensus HIF-1 site. (B) Randomly degenerate oligonucleotides corresponding to the 14-bp SARE were annealed, cloned into CAT reporters, and then transiently transfected into FRE cells. The position and nucleotide change(s) of each mutant are shown underneath the wild-type (wt) sequence. Transfected cultures were divided equally after release with trypsin. Half of the cells were placed under anoxia for 20 h after the cells had adhered to the culture dishes; cells were then harvested, and CAT assays were performed. CAT activity for each mutant is expressed as mean percent conversion during aerobic culture (solid bars) or anoxic culture (open bars). Mean values were obtained from two to six separate transfections. IND, induction.

FIG. 4. EMSA revealing that an anoxia-inducible DNA binding activity indistinguishable from HIF-1 binds the SARE. (A) Nuclear extracts prepared from aerobic or anoxic (20 h) FRE cells were incubated with radiolabelled oligonucleotides corresponding to the wild-type SARE (lanes 1 to 10) or the mutant (Mut) SARE (MS107) (lanes 11 and 12) in the presence of 0.05% Nonidet P-40. Competition experiments were performed by preincubating the nuclear extracts with a 20-fold (lanes 3, 5, 7, and 9) or 100-fold (lanes 4, 6, 8 and 10) molar excess of the following unlabelled oligonucleotides: wild-type SARE (S), mutant SARE MS107 (MS), the mouse EPO–HIF-1 site (E), or the consensus AP-2 site (AP2). The position of an anoxia-inducible complex is indicated by the arrow, and the free probe is designated FP. (B) Nuclear extracts from FRE and HepG2 cells grown in the atmospheres indicated above the lanes were incubated with radiolabelled oligonucleotides corresponding to the SARE (S) or the mouse EPO–HIF-1 binding site (E). Competition experiments were performed by preincubating the extracts with a 100-fold molar excess of the indicated unlabelled binding site. The position of HIF-1 is indicated by the arrow, and the free probe is designated FP. Comp, competitor.

assayed for anoxia inducibility. The results obtained with these mutants are shown in Fig. 3B. As these data demonstrate, mutations within the core dyad symmetry (ACGT) of this putative HIF-1 binding site reduced the level of anoxia inducibility to that seen with the vector alone. However, four additional mutations outside of the dyad symmetry produced results inconsistent with the hypothesis that the binding of HIF-1 to the VL30 SARE was alone responsible for the anoxic induction. Mutations at the 3′ end of the HIF-1 consensus sequence (see pMS104 and pMS103) resulted in only 33 and 16% reductions, respectively, in anoxia responsiveness. Additionally, SARE constructs with mutations at position −113, which resides outside of the consensus sequence, are not anoxia inducible (see pMS113 and pMS113/105). The failure of mutant pMS113/105 to induce is particularly intriguing given that the A-to-G nucleotide change at position −105 creates a perfect match to the consensus HIF-1 binding site. Taken together, these results suggest that either HIF-1 has a binding site affinity during anoxia that is somewhat different from that found during hypoxia, or else anoxic FRE cells contain a distinct SARE DNA binding activity.

Characterization of a HIF-1-like SARE binding activity. We examined the DNA-protein interactions at the SARE with extracts prepared from aerobic and anoxic FRE cells by EMSAs. As Fig. 4A demonstrates, we were able to detect an anoxia-inducible DNA binding activity (lane 2) which specifically recognized the SARE. This activity could be successfully competed for by an excess of unlabelled wild-type SARE (lanes 3 and 4) but not by the noninducible mutant SARE MS107 (lanes 5 and 6) or the unrelated consensus binding site for transcription factor AP-2 (lanes 9 and 10). As might be expected given the inability of MS107 to compete for this complex, this mutant SARE was unable to form this anoxia-inducible complex (lane 12). Additionally, an unlabelled oligonucleotide containing the HIF-1 binding site from the mouse
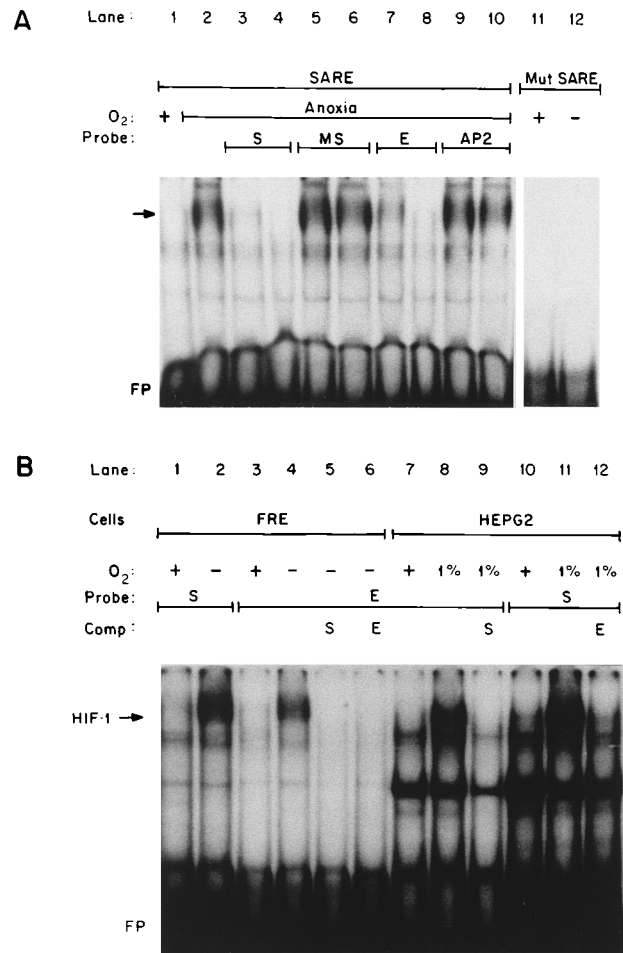
EPO gene also successfully competed for this activity (lanes 7 and 8). This supports the hypothesis that the VL30 SARE binds HIF-1 or a related activity. To further examine this issue, we directly compared the anoxia-inducible SARE binding activity from FRE cells to that of HIF-1 induced in hypoxic (1% oxygen) HepG2 cells (Fig. 4B). Nuclear extracts from both anoxic FRE cells and hypoxic HepG2 cells formed inducible complexes with similar migrations during EMSAs when incubated with either the VL30-SARE or the EPO–HIF-1 oligonucleotide (compare lane 2 with lane 11 and lane 4 with lane 8). In addition, both the SARE and HIF-1 binding sites were able to successfully cross-compete for this DNA binding activity (see lanes 5, 9, and 12). Taken together, these results sup-
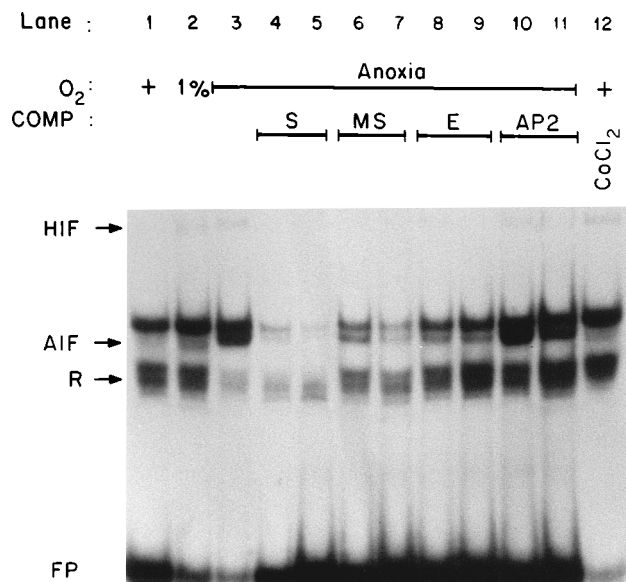
FIG. 5. Detergent-free EMSAs reveal an AIF binding to the SARE. Nuclear extracts from aerobic, hypoxic (20 h), or anoxic (20 h) FRE cells exposed to 75 μM cobaltous chloride (20 h) were used in EMSAs with the SARE probe. Competition was performed with a 20-fold (lanes 4, 6, 8, and 10) or 100-fold (lanes 5, 7, 9, and 11) molar excess of the following unlabelled binding sites: SARE (S), mutant SARE MS107 (MS), the mouse EPO–HIF-1 site (E), or the consensus AP-2 site (AP2). The position of AIF is indicated, and the free probe is designated FP. The position of HIF-1, as determined by a longer exposure of the gel, is also indicated. COMP, competitor.
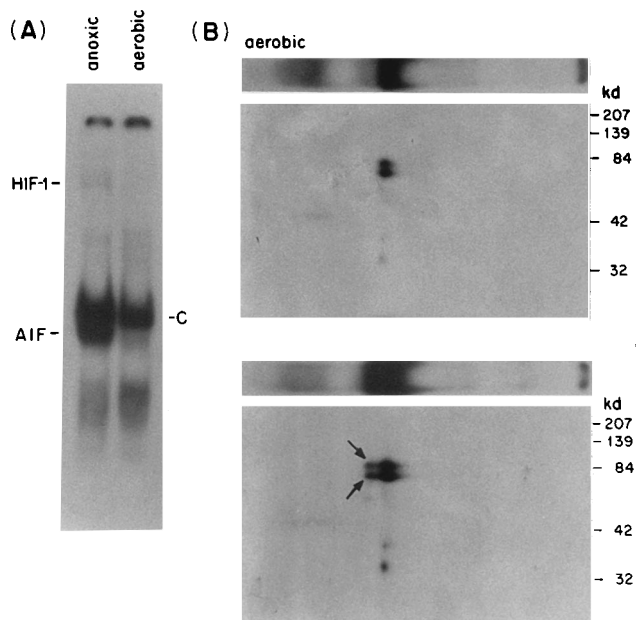

FIG. 6. Two-dimensional gel analysis of SARE binding complexes. (A) First-dimension analysis of SARE binding complexes by detergent-free EMSA. The positions of HIF-1 and AIF are indicated, as is the position of a constitutive DNA binding activity (C). (B) UV cross-linked SARE binding complexes were removed from the EMSA gel by excising the entire lane, and then they were overlaid on an 10% SDS–polyacrylamide gel and subsequently electrophoresed in the second dimension. The positions of molecular size standards are shown for the second dimension, and the original first-dimension EMSA lane is above the SDS-polyacrylamide gel electrophoresis autoradiogram. The two AIF components with approximate molecular masses of 61 and 52 kDa (exclusive of bound DNA) are shown by arrows.

port the hypothesis that HIF-1 is expressed in anoxic fibroblasts and specifically binds the VL30 SARE.

If the HIF-1 found in anoxic fibroblasts is responsible for the in vivo induction of VL30s, then VL30s should also be induced at 1% oxygen. However, previous results (4) have shown that the magnitude of VL30 induction at 0.1 to 1% oxygen is much less than that seen during anoxia. We tested what affect HIF-1 has on SARE-mediated VL30 expression by exposing fibroblasts transiently transfected with a SARE CAT reporter to cobalt chloride, hypoxia, or anoxia; cobalt has been previously shown to induce HIF-1 DNA binding activity regardless of the state of oxygenation (39, 40). The SARE conferred a weak 2.2-fold induction of CAT activity on normoxic fibroblasts treated with 75 μM $CoCl_2$ and a 2.8-fold induction on untreated fibroblasts cultured under 1% O_2. However, a much stronger 8.8-fold induction was seen in anoxic fibroblasts alone and 6.8-fold induction occurred in anoxic fibroblasts treated with cobalt. These data indicate that induction of HIF-1, whether by hypoxia or by cobalt, is alone not sufficient for the activity of the SARE observed during anoxia. This might reflect the requirement for a new factor which fails to bind under our original EMSA conditions. Alternatively, HIF-1 may undergo anoxia-specific changes in expression or posttranslational modifications.

Characterization of an anoxia-inducible SARE binding activity. To test the hypothesis that there might be additional anoxia-inducible factors which bind the SARE, we performed EMSAs with nuclear extracts from aerobic, hypoxic, anoxic, and cobalt-treated fibroblasts under different, detergent-free conditions that had been used in other studies to detect HIF-1 (33). Under these conditions, we detected similar levels of HIF-1 in hypoxic, anoxic, and cobalt-treated cells, along with an additional second activity strongly induced only in anoxic fibroblasts (Fig. 5). We have termed this activity the AIF. With

extracts from anoxic cells, the abundance of the AIF-SARE complex relative to that of the HIF-SARE complex in these assays was approximately 10:1. In these detergent-free EMSAs, the abilities of unlabelled oligonucleotides to compete for the formation of AIF and HIF complexes with the SARE were nearly identical. The wild-type SARE (lanes 4 and 5) efficiently competed for both of these complexes, whereas the unrelated sequence of the AP-2 binding site (lanes 10 and 11) did not significantly affect HIF or AIF formation. Anoxia-nonresponsive SARE mutant MS107 (lanes 6 and 7) and the mouse EPO–HIF-1 sites were roughly similar in the ability to partially compete for the formation of the two complexes. The identification of a second SARE binding complex induced only in anoxic cells, together with the inability of HIF-1 to fully activate SARE-mediated transcription, strongly suggests that AIF is required for full SARE activity. The formation of an additional complex, R, migrating faster than AIF was generally reduced but varied considerably in reactions containing anoxic cell extracts in these experiments. This aspect is under continued investigation.

To characterize the composition of AIF, we utilized two-dimensional gel electrophoresis. This included a first-dimension EMSA comparing aerobic and anoxic fibroblast extracts in which the bound proteins were UV cross-linked to the ^{32}P-labelled SARE oligonucleotide. After electrophoresis, the complexes were again UV irradiated in situ. Individual EMSA lanes were excised from the gel and overlaid on an SDS-polyacrylamide gel and electrophoresed in the second dimension. Figure 6 shows that AIF is a heterodimer composed of subunits with calculated molecular masses of 61 and 52 kDa when the mass of the oligonucleotide (14.5 kDa) is subtracted.

AIF may arise via posttranslational modification of a SARE binding complex constitutively expressed in aerobic fibroblasts, since these two activities each appear to be composed of subunits of the same sizes. By this model, the requirement for de novo protein synthesis in the anoxic induction of VL30 could be for that of the modifying activity. However, if the two AIF subunits are themselves directly posttranslationally modified, this modification must be of a type which does not visibly alter their mobility on the second-dimension SDS gel. This may exclude phosphorylation or major glycosylation but could accommodate conformational or redox changes. A second possibility is that the major subunits of AIF are themselves unmodified, but an additional subunit becomes synthesized under anoxia and it is the binding of this new subunit which produces the first-dimension mobility shift on EMSAs. Consistent with this, a weak signal of a 33-kDa protein which comigrated with AIF specifically in the anoxic cell extracts was seen (Fig. 6). The weakness of this signal relative to those of the 61- and 52-kDa proteins may reflect inefficient cross-linking to the ^{32}P-labelled oligonucleotide, perhaps because the 33-kDa protein binds one of the two major proteins but does not directly bind the oligonucleotide itself. Since the appearance of AIF does not occur at the expense of the constitutive SARE binding activity, a third possibility is that anoxia induces the de novo synthesis of one or both of the major AIF subunits. Such a hypothesis would require that these AIF subunits be very similar in size to the proteins which make up the constitutive SARE DNA binding activity. All three of these possibilities are currently being examined.

DISCUSSION

Low oxygen tensions affect a diverse range of physiological and pathological conditions, ranging from would healing to tumor progression and retinopathy (6, 20, 27, 28, 31, 34). This diversity is reflected in the number of cellular transcription factors affected by various degrees of oxygen deprivation. Whereas very few of the target genes for most hypoxia-induced transcription factors have been identified, HIF-1 has several well-defined targets. First characterized as the transcription factor necessary for hypoxic induction of EPO in hepatoma cells (33), HIF-1 has subsequently been demonstrated to be induced in many hypoxic cells (40). This suggests that it is part of a general regulatory pathway necessary for cellular adaptation to hypoxic conditions. Such a hypothesis is consistent with the recent observation that HIF-1 or a related activity participates in mediating the hypoxic induction of several genes involved in glycolytic metabolism (12, 32).

The transcriptional induction of VL30 retrotransposons in anoxic fibroblasts provides a valuable model system to test the hypothesis that under anoxia cells can alter gene expression via pathways distinct from those utilized during hypoxia. Analysis of this system has identified a 14-bp cis-acting promoter element (SARE) and two inducible DNA binding activities (HIF and AIF). Surprisingly, both factors interact with this one cis-acting element. The SARE bears considerable similarity to the consensus binding site reported for HIF-1 (32) and, as might be expected, forms a complex in hypoxic, anoxic, and cobalt-treated fibroblasts which is indistinguishable from the ubiquitous HIF-1 found in most hypoxic cells. In addition, we have found a second, more abundant SARE DNA binding activity (AIF) which is induced only by anoxia.

The data presented here indicate that both of these factors have the capacity to activate VL30 transcription in response to reduced oxygen tension. However, since anoxia is a much more potent activator of VL30 expression or a CAT reporter cou-

pled to the VL30 SARE, it appears that AIF is also more potent than HIF-1 in activating the VL30 system. Our observation that in detergent-free EMSAs AIF-SARE complexes are 10-fold more abundant than HIF-1–SARE complexes indicates that AIF has a higher affinity for the SARE or that considerably more AIF than HIF-1 is expressed. As cells progress from a normoxic to an anoxic state, they likely pass through a period of hypoxia sufficient for induction of HIF-1. We hypothesize that the initial primary induction of VL30s in response to oxygen deprivation is mediated by HIF-1. As the cells become functionally anoxic, AIF induction occurs and in turn drives the secondary, stronger phase of the anoxic induction. Both of these events appear to be mediated by the same cis-acting element, the 14-bp SARE described here, although the precise binding sites may slightly differ within these sequences.

The anoxia-responsive VL30 elements represent the first mammalian transcriptional regulatory system identified which is capable of discriminating between hypoxia and anoxia. The presence of a cellular transcription factor specifically induced by anoxia may provide a more general mechanism for the activation of a larger set of response genes specific to anoxia. We have previously demonstrated that fibroblasts exposed to anoxia in vitro secrete the same proteases (procathepsins D and L) and endonuclease as they do in vivo during the debridement phase of wound healing (6, 31). This anoxic response must be tightly controlled, since inadvertent activation in tissues made simply hypoxic by processes such as vigorous exercise or temporary ischemia could prove detrimental. The presence of an anoxia-specific transcriptional regulator (AIF) should allow more precise expression of this anoxic response than otherwise could be achieved in the heterogeneous, damaged tissue environment of a wound.

Although HIF-1 and AIF recognize similar sequences, several lines of evidence suggest that AIF is distinct from HIF-1. Whereas HIF-1 binds DNA as a heterodimer composed of 120- and 94-kDa subunits (39, 41), AIF is composed of 61- and 52-kDa subunits. In addition, hypoxic atmospheres (1% oxygen) sufficient for the induction of HIF-1 do not induce AIF. Although active AIF is likely to be generated via posttranslational modification of a complex present in aerobic cells, the possibility exists that the generation of AIF requires de novo synthesis of one or both subunits, as has been proposed for the induction of HIF-1. If this is the case, the anoxia-inducible protein(s) would have to be very similar in size to the subunits which make up the constitutive SARE DNA binding activity.

The nature of the signal transduction pathway utilized during the sensing of anoxia and the subsequent production of AIF remains an enigma. It clearly does not involve a typical stress response (3, 36), nor does it appear to be dependent upon disruption of the electron transport chain (1). The inability of either cobalt or hypoxia to induce AIF also appears to rule out a role for a heme-bearing oxygen sensor, such as that believed to participate in the activation of HIF-1 (14). Several other questions regarding the biology of AIF remain to be answered, in particular, those of the identities of the protein components of AIF and the roles of de novo synthesis versus posttranslational modification in the production of AIF. Since both the SARE and the consensus HIF-1 binding sites have five-of-six-nucleotide matches to an E box, the binding site for members of the basic helix-loop-helix family of transcription factors such as Myc and Max (29), cloning of AIF and HIF-1 may reveal that AIF shares membership in this family with HIF-1 (39). Since the anoxic induction of VL30s occurs only in actively cycling cells (1), AIF expression is likely to be proliferation related.

Identification of a mammalian anoxic response element has interesting implications. This element may prove useful in gene therapy regimens for targeting expression to physiological situations in which functional anaerobiosis exists, such as during wound healing. Deregulation of genes normally expressed during anoxia is often seen in cancer cells regardless of their state of oxygenation (5). Understanding the molecular basis of the mammalian anoxic regulatory pathway and how genes become constitutively activated in malignancy may further lead to unique approaches to diagnosis or therapeutic intervention.

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