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Advances in Brief

Expression of the Hypoxia Marker Carbonic Anhydrase IX Is Critically Dependent on SP1 Activity. Identification of a Novel Type of Hypoxia-responsive Enhancer[1]

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Abstract

In the present study, we further studied mechanisms of transcriptional regulation of the tumor-associated carbonic anhydrase IX (CAIX). We identified PR5 in the *CA9* promoter as another SP1/SP3-binding site. As shown by electromobility shift assays and block-replacement mutagenesis, PR5 is functionally equivalent to the SP1/SP3-binding PR1 identified previously. However, there is a strong requirement for SP1/SP3 activity in the PR1 position, and SP1/SP3 activity from the PR5 position cannot compensate for this. In various cell lines, the expression of endogenous CAIX and activity of *CA9* promoter constructs depend on SP1/SP3 activity as demonstrated by the dose-dependent inhibitory effect of the SP1 inhibitor mithramycin A. The two conditions of the induction of CAIX expression described previously differ in their sensitivity to mithramycin A inhibition; the hypoxia-mimic-induced expression is less sensitive than the cell density (mild hypoxia)-induced expression. Our present study highlights the importance of SP1/SP3 activity for CAIX expression and provides additional evidence for distinct mechanisms responsible for true and mild hypoxia-induced CAIX expression. The presence of a SP1/SP3-binding element in the PR1 position is absolutely required for mild hypoxia-induced activity, and it significantly up-regulates the true hypoxic induction. The SP1/SP3 and hypoxia-response element in the *CA9* promoter thus may represent a novel type of enhancer capable of mounting responses to a wider range of hypoxic conditions.

Introduction

The expression of CAIX[4] was proposed recently as an intrinsic marker of hypoxia, because it correlates with lowered O_2 tension in tumors (1, 2). CAIX (known previously as MN) was identified in a large number of carcinomas but not in the corresponding normal tissues (Ref. 2 and references therein and Ref. 3). Two separate but interdependent mechanisms, activated by lowered O_2 tension, appear to control CAIX expression. The expression of CAIX was shown to be positively regulated by low O_2 tension ($\leq$1%, hereafter termed true hypoxia; Refs. 2 and 4) via the HRE in the *CA9* promoter immediately upstream of the transcription start (4). HIF-1, which binds to HRE, is a heterodimeric transcription factor, consisting of the regulated HIF-1α and constitutive HIF-1β subunits (5). Activity of the α subunit can be increased through stabilization by either true hypoxic conditions (6) or inactivation of the VHL tumor suppressor that targets the HIF-1α subunit for oxygen-dependent proteolysis (7). In renal carcinoma cells defective for VHL, CAIX up-regulation is associated with the loss of regulation by hypoxia, consistent with the critical function of VHL in the regulation of HIF-1 (4).

In addition to true hypoxic conditions, associated with stabilization of HIF-1α, CAIX expression appears to be controlled by a different mechanism in dense cultures. CAIX was found to be tightly regulated by cell density; CAIX is absent in sparse, rapidly proliferating HeLa cells, whereas its synthesis is induced in dense, overcrowded cultures that express this protein in large amounts (3). The mechanism of CAIX induction operating in dense cultures is apparently triggered by an intermediate decrease of O_2 tension (<5% and >1%, hereafter termed mild hypoxia) that is generated by increased O_2 consumption (8). O_2 tension in dense culture is too high for stabilization of HIF-1α but sufficient for activation of a PI3-K-dependent pathway (8). However, we have shown that for induction of *CA9* promoter activity in dense cultures, a minimal level of HIF-1 activity is necessary but not sufficient (8). The existence of additional mechanism(s) controlling CAIX expression is also supported by colocalization of CAIX and hypoxia in tumor sections. Although substantially overlapping with pimonidazole staining in regions of acute hypoxia in tumors (4), CAIX expression extends beyond the hypoxic regions (4, 9). The patterns of CAIX expression in *in vivo* as well as *in vitro* studies are thus consistent with the existence of two separate mechanisms controlling CAIX expression, both dependent on HIF-1α activity and decreased O_2 levels but differing with respect to the amount of HIF-1α required. True hypoxia is defined as the mechanism that is driven by HIF-1 and requires HIF-1α stabilization to increase the total amount of HIF-1. Mild hypoxia, on the other hand, although still requiring some HIF-1 activity, induces additional pathway(s), including the involvement of activated PI3-K (8), but it is not associated with an additional increase in HIF-1α levels. Varying amounts of HIF-1α have been detected even under normoxic conditions in normal tissue (10) or cell lines (11), and this presumably can provide the small level of HIF-1 activity that is necessary for activation of *CA9* under conditions of mild hypoxia.

The *CA9* promoter, identified in the [−173, +31] region, contains the critical regulatory elements for *CA9* transcriptional activation (4, 12). Of the four stimulatory PRs, PR1 was crucial, whereas PR2 enhanced *CA9* transcriptional activity (12). Detailed mutational and supershift analysis identified SP1/SP3 and AP-1 as the critical factors binding PR1 and PR2, respectively (13).

Although undoubtedly critical for activation under hypoxic conditions (4), it is of note that HRE alone was not sufficient for activation of the *CA9* promoter by mild hypoxia (8). The minimal mild hypoxia-inducible module within the *CA9* promoter consisted of the juxtaposed PR1 and HRE, suggesting a requirement for cooperation between transcription factors binding to these two independent regulatory elements (8). It has been recognized for some time that a single HRE in isolation is not very active in inducing gene expression

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[4] The abbreviations used are: CAIX, carbonic anhydrase IX; AP-1, activator protein; Ab, antibody; CA9, carbonic anhydrase 9; DFO, desferrioxamine mesylate; EMSA, electrophoretic mobility shift assay; HIF-1, hypoxia-inducible factor 1; HRE, hypoxia-response element; MMA, mithramycin A; NE, nuclear extract; PI3-K, phosphatidylinositol 3′-kinase; PR, protected region; RCE, retinoblastoma control element; SP, specificity protein; pRL-CMV, *Renilla* luciferase-cytomegalovirus immediate early enhancer-promoter; VEGF, vascular endothelial growth factor; VHL, von Hippel-Lindau.

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in response to hypoxia (14), and adjacent sequences were identified as being necessary for hypoxic induction (15), *e.g.,* a cyclic AMP response element in the lactate dehydrogenase A promoter (16).

As a part of our ongoing effort to understand functioning of the *CA9* promoter, we characterized PR5 and its role in transcriptional activation. Given the importance established previously of PR1 for *CA9* promoter function (8, 12, 13), we wished to further investigate the role of SP1/SP3 in regulation of true and mild hypoxia-induced CAIX expression. These studies further confirmed the distinction between the two mechanisms on the basis of their different dependency on SP1/SP3 activity. In addition, we define a novel, minimal true, and mild hypoxia-inducible enhancer element consisting of SP1/SP3- and HIF-1-binding elements.

Materials and Methods

Sequences are written in the 5′-3′ direction, and numbers in brackets indicate each position relative to the *CA9* transcription start. Kits, enzymes, antibodies, and reagents were used according to the manufacturers' recommendations.

Plasmid Constructions. All promoter fragments were cloned in pGL2 basic vector (Promega). The [−173, +31], [−135, +31], [−46, +14], [−36, +14], and [−25, +14] constructs contain the corresponding *CA9* fragments. The [−173, +31] (PR1mut) and [−46, +14] (PR1mut) constructs contain the following mutations (bold, underlined) in PR1 ([−46, −24]): AGGCTTGCTCCT**AA**CCCACCCAG. The [−46, +14] (HREmut) construct contains the following mutations in HRE ([−17, +5]): TTTCCAATGC**TTT**TACAGCCCG. The [−135, +31] (PR1→PR5) construct has PR1 replaced with PR5 and was prepared by block-replacement mutagenesis as described previously (17). The SP1HRE construct is a derivative of the [−46, +14] construct with the SP1-binding GC-box sequence TCCGATCGGGGCGGGGCGAGC in place of PR1. Deletions and mutations in all promoter fragments were verified by sequencing.

Cell Lines and Culture. Human cervical carcinoma HeLa and fibrosarcoma HT 1080 cell lines were grown in DMEM (BioWhittaker), supplemented with 10% FCS (Life Technologies, Inc.), 1.10^2 units/ml penicillin (Sigma), 1.10^2 μg/ml streptomycin (ICN), and 125 ng/ml amphotericin B (Sigma). All cell lines were regularly tested for microbial contamination (18) and uniformly negative. The effects of the SP1 inhibitor MMA (Sigma) on endogenous CAIX expression were tested on the cells that had been seeded at 10,000/cm^2 and grown for 3 days. For cell density-dependent CAIX induction, these cells were plated at 160,000/cm^2 and incubated in the presence of the indicated MMA concentrations for 24 h. Hypoxic CAIX induction was mimicked with DFO (Sigma). The cells were plated at 40,000/cm^2 and pretreated with various concentrations of MMA for 30 min, followed by the addition of 100 μM DFO and incubation for 24 h.

Western Blot Analysis. Western blot analysis was performed as described previously (8).

Transient Transfection Assay. Cells were transfected with the *CA9* pGL2 basic construct (expressing firefly luciferase) and pRL-CMV (Promega; expressing *Renilla* luciferase) as described previously (8). After 7-h exposure to the transfection mixture, the cells were rinsed with PBS, trypsinized, and plated at 20,000 and 160,000/cm^2 in the absence/presence of 500 nM MMA. For hypoxia-mimic experiments, the cells were plated at 40,000/cm^2 in the absence/presence of 500 nM MMA for 30 min, followed by the addition of 100 μM DFO. The transfected cells were harvested at 42-h post-transfection. Alternatively, the transfected cells were exposed to 0.5% O_2 environment in a PROOX *In Vitro* Chamber (BioSpherix), controlled by the PROOX, model 110 (BioSpherix), for 24 h. Firefly and *Renilla* luciferase activities were assayed with the Dual-luciferase reporter assay system (Promega) in the Monolight 2010 luminometer. *CA9* promoter activity was expressed as the average of ratios of firefly:*Renilla* luciferase activities from three independent experiments.

EMSA. NEs were prepared from 3×10^6 cells, seeded at 80,000/cm^2, and incubated for 16 h. Cells were washed with ice-cold PBS, resuspended in 1 ml of buffer A [10 mM HEPES (pH 7.9) and 1.5 mM $MgCl_2$], and kept on ice for 15 min. The nuclei were pelleted by centrifugation for 5 min at $4{,}000 \times g$ at 4°C, resuspended in 200 μl of buffer B [30 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 0.2 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and leupeptin, 500 mM KCl, 10% glycerol], and kept on ice for 15 min. After clearing by centrifugation for 5 min at $12{,}000 \times g$ at 4°C, the supernatant was recovered as NE and stored in aliquots at −80°C. Protein concentrations were determined with the bicinchoninic acid protein assay reagent. Double-stranded probe PR5 (TGCCCCTCACTCCACCCCCATCCTAGCT and its complement; Ref. 12) was end-labeled with T4-polynucleotide kinase and purified with QIAquick Nucleotide Removal kit (Qiagen). As competitors PR5, PR1 (CCAGGCTTGCTCCTCCCCCACCCAG and its complement), SP1 (GC-box-ATTCGATCGGGGCGGGGCGAGC and its complement), transforming growth factor-β RCE (CGCCCCCGGCCCCACCCCAGGAAG and its complement), and AP-1 (CGCTCTGTGAGTCAGCCTG and its complement) oligonucleotide probes were used. Binding reactions, supershifts with SP1 and SP3 Ab, electrophoretic separation, and detection of complexes were performed as described previously (13).

Results

PR5 within the CA9 Promoter Is a SP1/SP3-binding Site. Inspection of the PR5 sequence in the *CA9* promoter revealed significant identity with PR1 (86% in the 14 base overlap; Fig. 1*A*), identified previously as an SP1/SP3-binding site (13). Competition EMSA of PR5 revealed that PR1 and PR5 oligonucleotides equally competed PR5 binding (Fig. 1*B, Lanes 2* and *3*), suggesting binding of similar factors to these PRs. The GC-box and RCE probes (SP1-binding competitors) also mounted efficient competition, but, unlike PR1 and PR5, neither was able to completely eliminate the formation of complex 3 (Fig. 1*B, Lanes 4* and *5*). Competition with an unrelated AP-1 probe did not affect PR5 binding (Fig. 1*B, Lane 6*). On the basis of competition analysis, we conclude that in addition to the factors bound by GC-box and RCE, both PR1 and PR5 are recognized by an additional, as yet uncharacterized transcriptional factor. The assumption regarding the same factors binding to PR1 and PR5 was confirmed by supershift EMSA; SP1 and SP3 Abs recognized the PR5

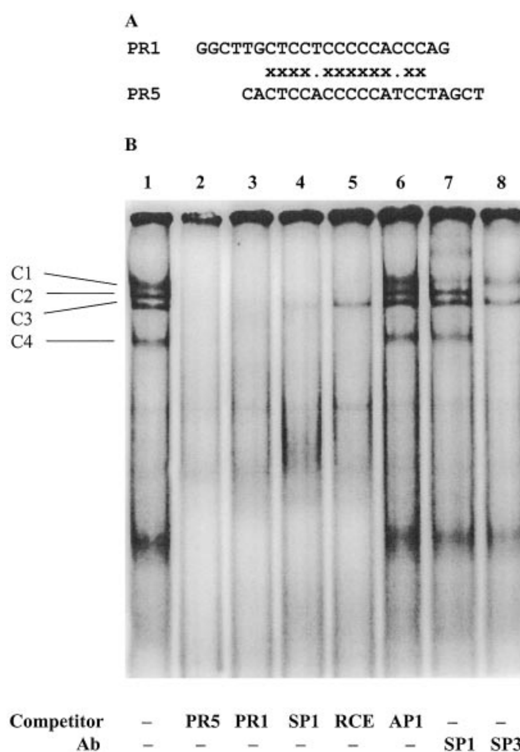

Fig. 1. *A,* alignment of PR1 and PR5 sequences. *B,* EMSA of PR5. Binding reactions contained end-labeled PR5 probe and 10 μg of HeLa NE. Competitors were used in 100-fold excess. For supershifts, 1 μg of Ab was used. Complexes were resolved in a nondenaturing 5% polyacrylamide gel in 0.5 × Tris-borate EDTA at 4°C.

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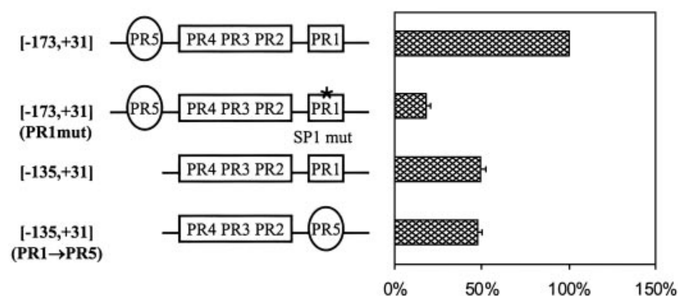

Fig. 2. The effect of PR5 on *CA9* promoter activity. HeLa cells were cotransfected with a *CA9* reporter construct (expressing firefly luciferase) and pRL-CMV (expressing *Renilla* luciferase). Transfectants were harvested 48-h post-transfection, the ratio of firefly:*Renilla* activity was calculated for each construct, and the activity of each construct is expressed as the percentage of the control full-length, wild-type construct. Each of the bars represents the mean value (X ± SD) from at least three individual experiments.

complexes in the same way as the PR1 complexes (13). Complex 1 contains SP1 (Fig. 1*B, Lane 7*), and complexes 2 and 4 contain SP3 (Fig. 1*B, Lane 8*), whereas complex 3 is recognized by neither Ab (Fig. 1*B*). In this way we proved that the binding capacities of PR5 and PR1 are equivalent. The *CA9* promoter thus contains two SP1/SP3-binding sites: (a) the proximal PR1 and (*b*) the distal PR5.

Functional Analysis of the Roles of PR5 and PR1 in the CA9 Promoter. Next, we analyzed the role of the PR5 SP1/SP3-binding site in *CA9* activation. Deletion of PR5 led to a 50% drop in promoter activity in transiently transfected HeLa cells (Fig. 2). The presence of proximal and distal SP1/SP3-binding sites raised questions about their relative contribution to *CA9* transcriptional activity. The elimination of SP1/SP3 binding in PR1 by point mutations led to a more dramatic decrease in promoter activity (<20% of the wild-type promoter activity) than the elimination of SP1/SP3 binding in PR5 by deletion (Fig. 2). Therefore, there appears to be an unequal contribution of the proximal (PR1) and distal (PR5) SP1/SP3-binding sites to *CA9* promoter activity.

Although the EMSA results indicated identical binding to PR1 and PR5, quantitative differences in their contribution to *CA9* promoter activity induced us to test to what extent PR5 and PR1 are functionally equivalent. A block-replacement promoter mutant with PR5 in place of PR1 generated as much reporter activity as the control construct in transiently transfected HeLa cells (Fig. 2). The results with the PR5 mutant constructs thus confirm that the PR1 and PR5 SP1/SP3-binding elements are functionally equivalent, and the differences in their contribution to *CA9* promoter activity are attributable to their relative distance from the transcription start. The *CA9* promoter function is more dependent on SP1/SP3 binding in the PR1 than in the PR5 position, and SP1/SP3 binding from the PR5 position cannot compensate for SP1/SP3 binding in the PR1 position.

CAIX Expression Is Dependent on SP1 Activity. The demonstration that there are two SP1/SP3-binding elements (PR1 and PR5) within the *CA9* promoter, as well as the strong dependence of the PR1 position on SP1/SP3 binding, prompted us to investigate the importance of SP1 activity for CAIX expression. MMA, an anticancer drug, specifically inhibits transcription of multiple genes by preventing SP1 binding (19, 20). We tested the effects of MMA on CAIX expression induced by cell density and the hypoxia-mimic DFO in HeLa and HT 1080 cell lines. Western blotting revealed that cell density-induced CAIX expression was effectively abrogated by ≥100 nM MMA concentrations (Fig. 3*A*). Although more refractory than cell density-induced expression (maximal concentrations were required for complete suppression), hypoxia-mimic-induced CAIX expression was also sensitive to inhibition by MMA (Fig. 3*B*). The MMA concentrations used were not toxic to any cell line, and β-actin levels were unaffected by MMA treatment (Fig. 3, *A* and *B*). Because of the higher sensitivity of cell density-mediated CAIX expression to MMA inhibition, we conclude that in the absence of HIF-1α stabilization, SP1 activity plays a more prominent role in the corresponding activating mechanism.

The inhibitory effect of MMA on *CA9* transcription was also studied in transiently transfected HeLa and HT 1080 cells, using the [−173, +31] and [−46, +14] *CA9* reporter constructs. The former contains two SP1 sites (PR1 and PR5), whereas in the latter, only PR1 is present. Reflecting the pattern of endogenous CAIX expression, the hypoxia-mimic DFO induced activity of the [−173, +31] and [−46, +14] *CA9* constructs in HeLa and HT 1080 cells (Fig. 4*A*). The fold of induction was considerably higher for the [−173, +31] fragment (Fig. 4*A*). DFO-induced reporter activities were inhibited by 500 nM MMA, but the residual activity was higher compared with control activity (Fig. 4*A*). As shown in Fig. 4*B,* both constructs were also activated by high cell density (160,000 cells/cm^2). Although the absolute values were higher for the [−173, +31] construct, the fold of induction was comparable with that of the [−46, +14] construct (Fig. 4*B*). In the presence of 500 nM MMA, the activation by cell density was prevented, and the reporter levels produced were comparable with those produced in control sparse cells (Fig. 4*B*).

The inhibitory effect of MMA on CAIX expression and *CA9* promoter activity demonstrated the importance of SP1 activity in transcriptional regulation of *CA9*. Although some quantitative differences exist in the sensitivity of cell density- and hypoxia-mimic-induced CAIX expression and promoter activity, collectively, our data show that CAIX expression, regardless of the mode of induction, is sensitive to inhibition of SP1 activity by MMA and that this inhibition is mediated at the level of SP1 sites in the *CA9* promoter.

The Hypoxia-inducible [−36, +14] CA9 Fragment Contains an SP1/SP3-binding Site. The minimal *CA9* sequence, tested previously for hypoxic inducibility, was the [−36, +14] fragment (4). However, in addition to the core HRE sequence (Fig. 5*A,* TACGTGCA on the antisense strand, underlined bold), this fragment contains a considerable part (boxed) of the SP1-binding site within PR1 (13). We asked

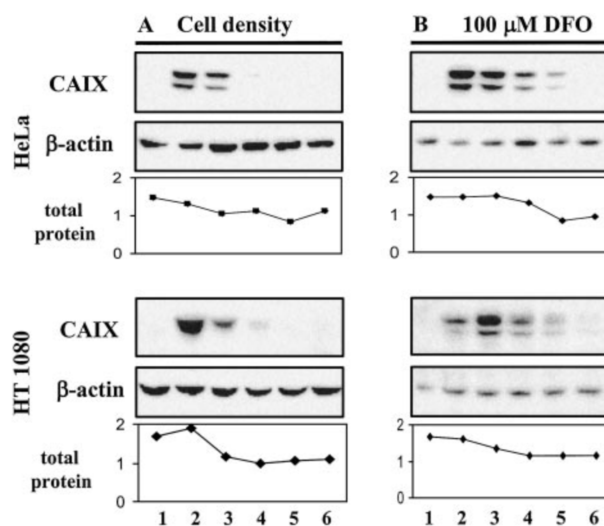

Fig. 3. Inhibitory effect of MMA on CAIX expression. Cells were kept in sparse culture (10,000/cm^2) for 3 days, seeded at the indicated densities, and incubated in the presence of MMA for 24 h. Western blot analysis was performed for CAIX and β-actin. Yield of total protein (mg/ml) is also indicated. *A,* cell density-induced CAIX expression. *1,* 40,000/cm^2; *2,* 160,000/cm^2; *3,* 160,000/cm^2 + 50 nM MMA; *4,* 160,000/cm^2 + 100 nM MMA; *5,* 160,000/cm^2 + 200 nM MMA; *6,* 160,000/cm^2 + 500 nM MMA. *B,* hypoxia-mimic-induced CAIX expression. Cells were seeded at 40,000/cm^2 and exposed to MMA for 30 min, followed by the addition of 100 μM DFO. *1,* control, no treatment; *2,* DFO only; *3,* DFO + 50 nM MMA; *4,* DFO + 100 nM MMA; *5,* DFO + 200 nM MMA; *6,* DFO + 500 nM MMA.

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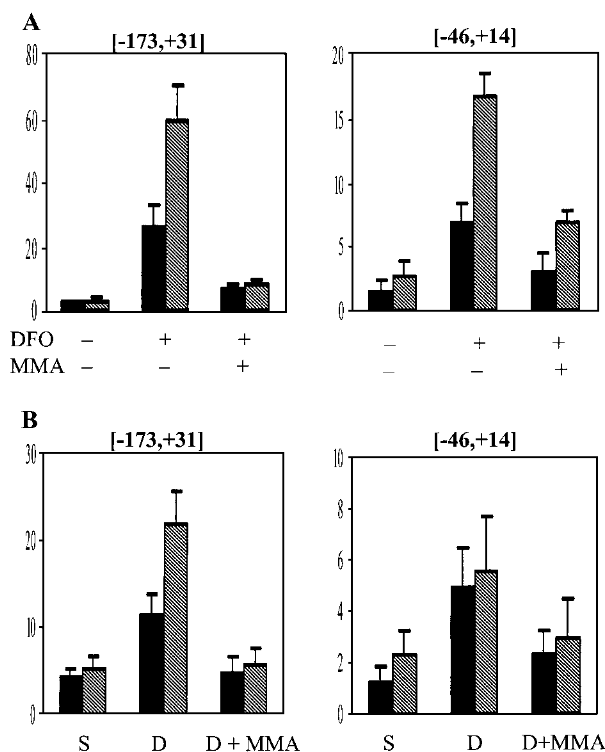

Fig. 4. Inhibitory effect of MMA on the activity of *CA9* promoter constructs. HeLa and HT1080 cells were cotransfected with the [−173, +31] (containing PR1 and PR5) or [−46, +14] (containing PR1) *CA9* construct fragments and pRL-CMV. After 7 h, the transfectants were trypsinized and seeded at the indicated density. The reporter assays were performed 42-h post-transfection, and the ratio of firefly:*Renilla* activity was calculated for each construct. The activity of the control in HeLa or HT1080 cells was set as 1, and the remaining activities were expressed as the fold induction against the control. *Closed bars,* HeLa cells; *hatched bars,* HT1080 cells. Each of the bars represents the mean value (X ± SD) from at least three individual experiments. *A,* hypoxia-mimic-induced *CA9* promoter activity. Cells were seeded at 40,000/cm^2 and, as indicated, pretreated with 500 nM MMA for 30 min, followed by the addition of 100 μM DFO. *B,* cell density-induced *CA9* promoter activity. Cells were seeded at 40,000/cm^2 (*S*) or 160,000/cm^2 in the absence (*D*) or presence of 500 nM MMA (*D + M*).

whether there is a contribution of this incomplete PR1 to transcriptional activity, and, if so, what is the activity of the *CA9* HRE in the absence of other upstream sequences (fragment [−25, +14])? To this end, we tested the activity of the [−46, +14], [−36, +14], [−25, +14], [−46, +14] (PR1mut), and SP1HRE constructs in transiently transfected HeLa cells. We found that the inducibility of the [−46, +14], [−36, +14], and SP1HRE constructs by cell density or the hypoxia-mimic DFO was similar, whereas the [−25, +14] and [−46, +14] (PR1mut) constructs were inducible only by DFO (Fig. 5*A*). The fold of induction by DFO for the [−46, +14], [−36, +14], and SP1HRE constructs was considerably higher (7–8-fold) than the [−25, +14] and [−46, +14] (PR1mut; 3-fold; Fig. 5*A*). Apparently, the [−46, +14], [−36, +14], and SP1HRE constructs respond in the same way to inducing signals, suggesting that even the incomplete SP1-binding site in the [−36, +14] fragment is functional, and the factors bound to it (SP1/SP3) are engaged in cooperation with factors bound to HRE.

SP1/SP3 Activity Modulates Induction of HRE by True Hypoxia. Finally, we compared the responsiveness of the *CA9* promoter fragments to true hypoxia (0.5% O_2) in transiently transfected HeLa cells. Under the conditions of lowered O_2 concentration in sparse culture (40,000cells/cm^2), only the [−46, +14] and SP1HRE minimal promoters were significantly induced (10-fold; Fig. 5*B*). The remaining [−25, +14], [−46, +14] (PR1mut), and [−46, +14] (HREmut) truncated promoter constructs responded to conditions of true hypoxia much less effectively (Fig. 5*B*). Thus, we observed that the juxtaposed SP1/SP3 activity considerably stimulates induction of the *CA9* HRE, even under conditions of true hypoxia. The *CA9* promoter thus contains a novel type of hypoxia-responsive enhancer comprising both the SP1/SP3-binding site and HRE.

Discussion

In the present study, we further expand our knowledge about transcriptional regulation of the gene coding for the tumor and hypoxia marker CAIX. Previous studies focused on the identification of *cis* elements and their cognate transcription factors within the *CA9* promoter. Our present study demonstrates the critical importance of SP1 for specific activation of the *CA9* promoter and identifies a novel type of hypoxic enhancer consisting of PR1 and HRE regulatory elements.

In addition to the SP1/SP3-binding site identified previously in the PR1 position (13), competition and supershift EMSA confirmed PR5 as another SP1/SP3-binding site in the *CA9* promoter. Deletion of the distal SP1/SP3-binding PR5 decreased *CA9* promoter activity, and PR5 could functionally replace PR1. It is of note, however, that PR5 from its original position cannot compensate for SP1/SP3 deficiency in the PR1 position in the mutant with disabled PR1. These results go beyond our previous conclusions about the essential role of SP1/SP3 activity in the PR1 position for *CA9* promoter activity (13), suggesting requirements for optimal spacing between SP1/SP3 binding and factor(s) binding to the juxtaposed HRE.

Except for SP1 and SP3 factors, both PR1 and PR5 are capable of binding another, as yet unidentified factor, designated previously as the complex 3-forming factor (13). The removal of this factor from the PR1 position by replacing PR1 with GC-box (that binds SP1/SP3 but does not bind complex 3-forming factor-13), within the [−173, +31] (13) or [−46, +14] (SP1HRE; this study) constructs, had no effect on promoter activity. Therefore, although the role of this factor in physiological transcriptional regulation of *CA9* is unknown at present, it seems to be dispensable for inducible *CA9* promoter activity under hypoxic conditions.

The presence of two SP1/SP3-binding sites (PR1 and PR5) indicated that SP1/SP3 factors may play an important role in specific regulation of *CA9* promoter activation. Indeed, by using the SP1 inhibitor MMA, we demonstrated that CAIX induction in HeLa and HT 1080 cell lines is dependent on SP1/SP3 activity. Quantitative differences in the sensitivity of hypoxia-mimic and mild hypoxia-induced CAIX expression to MMA inhibition may be reflective of distinct mechanisms driving CAIX expression under these conditions. We propose that the lower sensitivity of hypoxia-mimic-induced CAIX expression to MMA inhibition reflects the dominant role of HIF-1α stabilization in the hypoxic pathway (4) with concomitant less dependence on SP1/SP3 activity. On the other hand, mild hypoxia is not associated with HIF-1α stabilization (8), and, in the absence of HIF-1 signal, CAIX expression appears to rely more on SP1/SP3 activity. However, it should be noted that hypoxia alone (HIF-1α stabilization) induces a significantly lower level of expression than high density (inducing PI3-K activation) and hypoxia (HIF-1α stabilization) combined (8).

In the process of dissection of the minimal mild-hypoxia inducible *CA9* promoter fragment [−46, +14], comprising PR1 and HRE (8), we found that the hypoxia-inducible [−36, +14] *CA9* fragment described previously (4) contains a truncated but apparently functional SP1/SP3-binding site. Fold inductions of this construct by hypoxia-mimic and mild hypoxia, as well as absolute values, were comparable with the [−46, +14] and SP1HRE constructs that both harbor full-length SP1/SP3-binding sites. Conversely, the [−25, +14] and [−46, +14] (PR1mut) constructs with the SP1/SP3-binding site missing and

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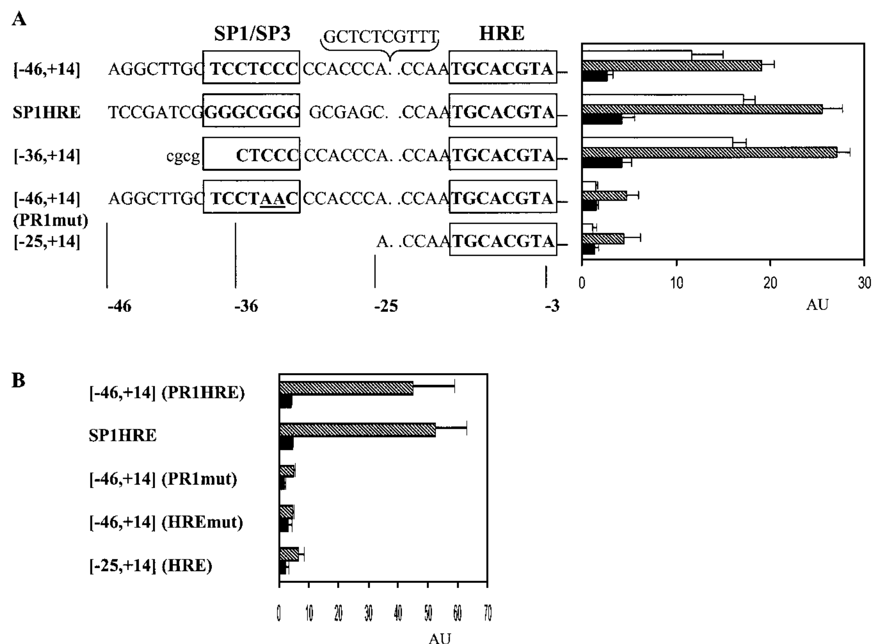

Fig. 5. Cooperation of SP1/SP3 factors in the PR1 position with *CA9* HRE. HeLa cells were transiently transfected with the indicated reporter constructs as described in Fig. 4. The activity of each construct is expressed in arbitrary units as the mean value (X ± SD) of the ratio of firefly:*Renilla* activity from three individual experiments. In *A,* SP1/SP3 factors are required for the induction of *CA9* promoter constructs by mild hypoxia. *Closed bars,* control (40,000/cm^2); *hatched bars,* hypoxia-mimic-induced activity (40,000/cm^2 + 100 μM DFO); *open bars,* cell density-induced activity (160,000/cm^2). *B,* juxtaposed SP1/SP3 site and HRE function as a true hypoxia-responsive enhancer. Transfectants were seeded at 40,000/cm^2 and exposed to either 21% (*closed bars*) or 0.5% O_2 (*hatched bars*) for 24 h.

mutated, respectively, were not induced by mild hypoxia. Although they retained inducibility by hypoxia-mimic, this was considerably lower compared with constructs containing an SP1/SP3-binding site. These results lend themselves to the following conclusions: (*a*) the [−36, +14] fragment contains a functional SP1/SP3 site; and (*b*) the SP1/SP3 binding is absolutely required for induction by mild hypoxia. Even under conditions of true hypoxia, induction of the promoter fragments with SP1/SP3-binding sites (the [−46, +14] and SP1HRE constructs) was much more efficient as opposed to the ones lacking the SP1/SP3-binding site (the [−25, +14] and [−46, +14] [PR1mut] constructs). Induction of the constructs without SP1/SP3-binding site was only slightly higher than the [−46, +14] (HREmut) construct with disrupted HIF-1 binding. Therefore, SP1/SP3 potently enhances the true hypoxia-stimulated response from HRE.

The *CA9* promoter lacks a TATA box, and it is GC rich (21). Clusters of SP1-binding sites in the proximity of the transcription start site appear to be typical of the TATA-less promoters (22). Earlier studies indicated that SP1 was responsible for recruiting the TATA-binding protein (23) and fixing the transcriptional start site at TATA-less promoters (22). These findings, together with the fact that SP1 sites are found in the promoters of many housekeeping genes, led to the widely held notion that SP1 acts as a basal transcription factor and that SP1 sites represent constitutive promoter elements that support basal transcription at these elements (24). More recently, however, it was realized that transcription from SP1 sites may be more complex than first envisioned, because these elements have been found to be involved in regulation of many processes, *e.g.,* tissue-specific gene expression, response to oncogenes, growth stimulation, and differentiation (Ref. 24 and references therein).

The strong requirement of CAIX expression and *CA9* promoter activity on SP1/SP3 raised questions about the possible regulation of SP1/SP3 activity in the process of true and mild hypoxic induction of CAIX. Previously, we failed to detect any changes with respect to SP1/SP3 activity in dense and sparse HeLa cultures; the EMSA indicated identical binding to PR1 and PR5 (12), and Western blotting with SP1 and SP3 Abs provided the same profile.[5] Apparently, in the process of cell density-induced CAIX expression, the activity of the PR1 and PR5-binding factors remains constitutive. Similarly, we were unable to reveal any differences in PR1 and PR5 binding in NE prepared from control and hypoxia-mimic-treated HeLa cells.[5] The conclusion that SP1/SP3 activity is not subject to regulation by hypoxia-mimic is supported by another study (25). Therefore, we conclude that the induction of *CA9* transcription by true or mild hypoxia is not associated with appreciable changes in either SP1/SP3 binding to PR1 and PR5 or SP1/SP3 activity. Rather, we propose that SP1/SP3 act as essential and necessary, but not sufficient, constitutive factors providing a platform for an inducible Factor X that will subsequently recruit the transcriptional machinery.

There is the formal possibility that SP1 may be required in the truncated promoter to fix the transcriptional start site (24). However, this is unlikely because we see the same requirement for SP1 in the context of a promoter construct containing a TATA box.[5]

Regulation of another hypoxia-inducible gene, the *VEGF* shares apparent similarities with *CA9*. Both *VEGF* and *CA9* genes possess SP1/SP3-binding sites close to the transcription start site (Ref. 26 and this study) and functional HREs (4, 27). However, in addition to the differences between the relative position of HRE (immediately upstream of the transcription start in *CA9* and ~1 kb upstream of the transcription start site in *VEGF*), there is a clear-cut difference in the role of HRE in each gene. Although transcription from the *CA9* promoter is critically dependent on the presence of both HRE and the proximal SP1/SP3-binding PR1 (Ref. 8; this study), *VEGF* HRE is dispensable for transcriptional activity, and it is required only for enhancement of transcriptional activation under conditions of true hypoxia (26). The outlined differences manifest in a relatively high constitutive transcription from the *VEGF* promoter, resulting in a moderate (compared with *CA9*) hypoxic induction. Consequently, despite apparent analogies, there is a distinction between these two genes, and the highly specific induction of *CA9* may be the result of a novel type of cooperation between SP1/SP3 and HIF1 factors attributable to the juxtaposed corresponding binding sites in the *CA9* promoter.

In Fig. 6, we propose a model describing activation of transcription from the *CA9* promoter under various conditions. A key component of the transcription complex is the putative cofactor X. Among the transcription factors playing a role in this process, activity of the

[5] S. Kaluz, unpublished observations.

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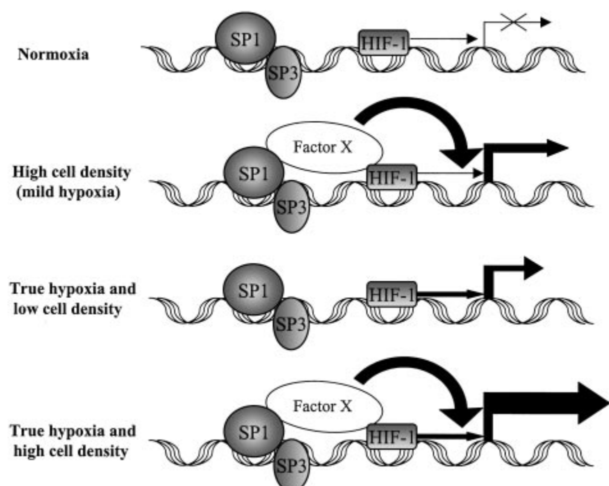

Fig. 6. A proposed model of assembling transcription factors on the minimal *CA9* promoter. Four different states of the *CA9* promoter are shown. Transcription from the *CA9* promoter is regulated by enhanced activity of HIF-1 under conditions of true hypoxia and/or the presence of the putative Factor X in the PR1-HRE region under conditions of high cell density. See text for additional details.

SP1/SP3 factors is constitutive, whereas activities of HIF-1 and Factor X are regulated by a combination of cell density-induced PI3-K activation and lowered oxygen tension, resulting in stabilization of HIF-1α. Under normoxic conditions, the *CA9* promoter is in the basal state; in the absence of Factor X and the presence of a minimal amount of HIF-1, SP1/SP3 factors are unable to initiate transcription on their own. Under conditions of high cell density (mild hypoxia), the presence of inducible Factor X, complexing with SP1/SP3 and HIF-1, initiates transcription even without a concomitant increase in the level of HIF-1α subunit. Under conditions of true hypoxia and low cell density, in the absence of Factor X, transcription is driven primarily by enhanced activity of HIF-1 attributable to HIF-1α stabilization. The maximal transcriptional activity is achieved under conditions of true hypoxia at high cell density; HIF-1α stabilization leads to increased HIF-1 activity that cooperates with SP1/SP3 and Factor X in recruiting the transcriptional machinery. It should be noted that there is a requirement for interaction between SP1, HRE, and Smad 3, as a coactivator, for transcriptional activation of the endoglin promoter (28). In our own studies, we have eliminated Smad 3/4 as a candidate for Factor X.[5] Experiments designed to reveal the identity of the putative inducible Factor X are under way.

Our results suggest that the *CA9* promoter contains a novel type of hypoxia-responsive enhancer consisting of the SP1/SP3-binding sites PR1 and HRE. As suggested by the results presented here, as well as the pattern of CAIX expression in tumors, the SP1/SP3-HRE enhancer from the *CA9* promoter could be unique with respect to being responsive to conditions of true hypoxia (associated with stabilization of HIF-1α and increased HIF-1 activity), as well as of mild hypoxia (intermediate O_2 tension that is not associated with HIF-1α stabilization). This type of enhancer could be useful in targeting expression to tumor regions with varying degrees of hypoxia.

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Expression of the Hypoxia Marker Carbonic Anhydrase IX Is Critically Dependent on SP1 Activity. Identification of a Novel Type of Hypoxia-responsive Enhancer

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