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Different transcriptional properties of mSim-1 and mSim-2

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Abstract **The *mSim-1* and *mSim-2* gene products are mammalian homologues of the *Drosophila Sim* gene. The *dSim* gene product transactivates through a DNA binding site known as the CNS midline enhancer (CME) element. We have investigated the transcriptional properties of mSIM-1 and mSIM-2 mediated through the CME element in concert with their dimerization partners, ARNT and ARNT-2. The mSIM-1/ARNT heterodimer transactivates reporter constructs via the ARNT carboxy-terminus. However, mSIM-2 quenches ARNT transactivation. We find that mSIM-2 competes with mSIM-1 for binding to ARNT, suggesting a possible antagonism between these transcription factors.**

Key words: Sim; Arnt; Transcription; DNA binding; Basic helix-loop-helix PAS

1. Introduction

The basic helix-loop-helix PAS [Per-Arnt-Sim] (bHLH-PAS) proteins contain a bHLH dimerization and DNA binding motif [1] as well as a second dimerization domain known as the PAS domain. The PAS domain is designated as a conserved region among the first three proteins (PER, ARNT and SIM) found to contain amino acid similarity over 250–300 amino acids including two degenerate hydrophobic repeats of approximately 50 amino acids termed PAS A and PAS B. These individual repeats exhibit homology to a much broader family of PAS containing proteins involved in a wide variety of physiological and functional roles [2].

Members of the bHLH-PAS protein family include both subunits of the dioxin receptor complex, AHR (aryl hydrocarbon receptor) and ARNT (aryl hydrocarbon receptor nuclear translocator) [3], the hypoxia-inducible factors, HIF-1α and EPAS1/HIF-2α [4,5], as well as the *Drosophila single-minded* (*dSim*) gene product which is critical to the development of the midline cells of the central nervous system [6]. Several groups have reported on the cloning of mammalian *Sim* homologs [7–11]. The *Sim-1* gene has been shown to be critical for the development of neuroendocrine lineages [12] and the *Sim-2* gene maps to the Down syndrome critical region [13]. Both mammalian *Sim* homologs are expressed in the adult kidney and the developing central nervous system in overlapping regions [8,9]. The *Sim-1* gene is also expressed in the somites where it is an early marker of presomitic mesoderm and lateral dermatome [9,14]. The *Sim-2* gene is strongly expressed in a number of cartilage primordia, particularly in a number of craniofacial structures deriving from the first pharyngeal arch as well as ribs and vertebrae [9,10,15]. The murine mSIM-1 and mSIM-2 proteins form heterodimers with the ARNT and ARNT-2 proteins [7,8,10,16–18].

dSim upregulates gene expression through binding of the CNS midline enhancer (CME) element ($^{5'}$G/ATACGTGA$^{3'}$) [19–21] and has transcriptional activation properties mapping to its carboxy-terminus [8,22]. In contrast, we and others have shown, using GAL4 fusion proteins, that both mSIM proteins have repressive functions in their carboxy-termini [8,17]. However, the transcriptional properties of these proteins have not been investigated in their wild type form, with a heterodimerization partner, in the context of a native DNA binding site.

Herein we report that mSIM-1 and mSIM-2, in concert with either ARNT or ARNT-2, are able to regulate reporter constructs containing CME sites. Our data suggest that the mSIM-1/ARNT heterodimer can strongly transactivate transcription via the transactivation domain of ARNT. In contrast, mSIM-2/ARNT heterodimers do not transactivate, due to the repression activity present within the carboxy-terminus of mSIM-2. We also find that mSIM-2 can interfere with the transactivational properties of mSIM-1/ARNT through competition for dimerization with ARNT as well as for DNA binding site occupation.

2. Materials and methods

2.1. Cell lines, transfections and CAT assays

C33A and 293 cells were maintained in α-modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL), penicillin, and streptomycin. Cells were transfected by the calcium phosphate precipitation method [23]. To normalize for transfection efficiency, cells were co-transfected with 0.5 μg of pcDNA3/β-gal. At 48 h after transfection, cells were harvested and assayed for β-galactosidase and luciferase activity [23].

2.2. Recombinant plasmids

Subcloning and mutagenesis were carried out according to standard protocols [23]. All constructs made via PCR were sequenced to ensure the absence of mutations. To make reporter constructs containing the *toll* 4 CME site [20] two tandem repeats of the sequence $^{5'}$GGAGCATGCAAGCTTAGAT(CTAGAAATTTGTACGTGCCACAGA)$_3$GGATCCGTG$^{3'}$, where the core CME consensus sequence is underlined, were cloned into the *Hin*dIII/*Bgl*II sites of pML [24]. Reporter constructs containing mutated versions of the CME element (pML/6C-X and pML/6CAM) were constructed using the same strategy with mutations in the core CME (Fig. 1).

The expression construct pcDNA3/mSIM-1 was obtained as a gift from Dr. C.-M. Fan (Carnegie Institute of Washington, Baltimore, MD). A series of deletion mutants of the mSIM proteins was constructed as shown schematically in Fig. 2A as well as series of chimeric constructs between mSIM-1 and mSIM-2 as described in Section 3.4 (Fig. 3A) were generated using standard PCR mutagenesis and subcloning procedures [17,23]. Detailed information on plasmid construction is available from the authors upon written request. Ex-

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pression vectors for ARNT have been previously described [25]. To make pcDNA3/hHIF-1αΔC (aa 1–403), pcDNA3/hHIF-1α [5] was digested with *Pfl*MI and *Xba*I, followed by repair with T4 polymerase, and ligation-recircularization with a linker containing an in frame stop codon ($^{5'}$pCTAGTCTAGACTAG$^{3'}$ [New England Biolabs]). To construct pcDNA3/dSIM the *Nhe*I/*Sal*I fragment of pNB40/dSIM (a gift from Dr. S. Crews, University of North Carolina, Chapel Hill, NC) was subcloned into the *Nhe*I/*Xho*I sites of pcDNA3. The construct pcDNA3/ARNT-2 was made by transferring the *Eco*RI fragment of pBSK/mARNT-2 [16] into the *Eco*RI site of pcDNA3.

2.3. Antibodies and Western blots

Whole cell extracts were fractionated by SDS-PAGE and transferred to Immobilon-P membrane (Millipore). Membranes were preblocked with 6% bovine serum albumin and incubated with indicated antibodies in TBST. Hemagglutinin (HA) epitope tagged proteins were visualized with HA.11 (BabCo, Richmond, CA) and mSIM-2 proteins were visualized using Ab 1850, a polyclonal rabbit antiserum raised against amino acids 359–566 of mSIM-2. Primary antibodies were detected with a peroxidase-conjugated sheep anti-mouse or donkey anti-rabbit immunoglobulin (Amersham) and detected by chemiluminescence (NEN).

3. Results

3.1. Mammalian SIM proteins can activate through a CME site

To investigate the transcriptional properties of the mammalian SIM proteins we constructed reporter plasmids containing multimerized CME sites, as well as mutant derivatives thereof (Fig. 1A). When a CME containing reporter construct (pML/6C-WT) was cotransfected into C33A cells with ARNT and mSIM-1 or mSIM-2, strong transactivation was observed with mSIM-1 (Fig. 1B, lane 3). However, the activity seen with mSIM-2 (Fig. 1C, lane 3) was not significantly greater than that observed with the negative control reporter, pML/6C-X (Fig. 1C, lane 6). No activation by mSIM-2 was seen in other cell lines (CHO, 293 and COS-7) with this, or other, reporter construct (Figs. 3–5 and P. Moffett, data not shown). As expected, the positive control, dSIM, was also able to activate this reporter (Fig. 1D, lane 3). Since the consensus DNA binding site for HIF-1α ($^{5'}$T/GACGTGCGG$^{3'}$) [4] is similar to the CME, we also tested a constitutively active version of HIF-1α (HIF-1αΔC) [26] on this reporter construct. This construct is also able to transactivate pML/6C-WT (Fig. 1E, lane 3) demonstrating that the SIM and HIF-1α proteins can activate through similar DNA binding sites. None of the SIM-like proteins could effect a significant response from the pML parent vector lacking CME sites or from reporters containing mutant derivatives (pML/6C-AM or pML/6C-X) (Fig. 1B–D, lanes 5–7; Fig. 1E, lanes 4–6). The mutation in pML/6C-AM disrupts the ARNT binding half site of the CME and in pML/6C-X, the SIM binding half site has been mutated to resemble a xenobiotic response element (XRE) [3].

Activation was also dependent on inclusion of ARNT (Fig. 1B–E, lane 2). Presumably, endogenous ARNT is limiting in cotransfection experiments, where exogenous proteins are expressed to high levels and reporters are at high copy number. This allowed us to investigate the contribution to transactivation by the ARNT carboxy-terminus [27]. When ARNTΔC was used in place of ARNT, dSIM still transactivated, albeit to a lesser extent (Fig. 1D, lanes 3 and 4). In contrast, mSIM-1 and mSIM-2 were unable to activate transcription in concert with ARNTΔC (Fig. 1C,D, lane 4), suggesting that activation by mSIM-1/ARNT is dependent on the ARNT transactivation domain. Similar results were also obtained in C33A, CHO and COS-7 cells with both luciferase and CAT based reporters (P. Moffett, data not shown), indicating that this is not a cell type or reporter specific phenomenon.

3.2. Deletion analysis of mammalian SIM proteins.

We undertook structure–function analysis of mSIM-1 and mSIM-2 in an attempt to determine the contribution of the carboxy-termini and basic DNA binding domains of the mSIM proteins (Fig. 2). As a further control to demonstrate that the transcriptional effects of mSIM-1 and mSIM-2 were due to DNA binding, we cotransfected mSIM constructs lacking the basic DNA binding domains (aa 1–13) with ARNT and the pML/6C-WT reporter into 293 cells (Fig. 2A). As expected, neither mSIM-1 nor mSIM-2 deletion mutant is able to effect transactivation in combination with ARNT (Fig. 2B, compare mSIM-1 versus mSIM-1Δb and mSIM-2 versus mSIM-2Δb). Western blotting showed similar levels of expression of the full length and Δb mutant proteins (Fig. 2C, lanes 2, 3, 8, and 9).

We investigated whether the carboxy-terminus of ARNT is sufficient for transactivation, and if the carboxy-termini of the mSIM proteins contribute to transcriptional control. To this end, 293 cells were cotransfected with pML/6C-WT, ARNT, and various mSIM expression vectors. In this context, mSIM-1 effected potent activation of the reporter construct (Fig. 2B, ∼46-fold). A similar degree of activation (Fig. 2B, ∼23-fold) with mSIM-1ΔC (aa 1–384) was seen, confirming that ARNT is likely responsible for most of the observed transactivational properties. The mSIM-2/ARNT complex does not transactivate in this context (Fig. 2B). The difference between mSIM-1 and mSIM-2 is not due to differences in expression levels (Fig. 2C, lanes 5 and 6). However, when the carboxy-terminus of mSIM-2 is deleted, mSIM-2ΔC (aa 1–360) can activate to a level comparable to mSIM-1ΔC (Fig. 2B, compare mSIM-2ΔC and mSIM-1ΔC), suggesting that mSIM-2 has repressive activity in its carboxy-terminus, which quenches the transactivation properties of ARNT. Using fusion proteins, we have previously identified two independent repression domains in the mSIM-2 carboxy-terminus [17]. To determine the regions of the mSIM-2 carboxy-terminus necessary for quenching ARNT we employed several deletion constructs (Fig. 2A). Deleting amino acids 504–657 (Fig. 2A, mSIM-2ST-PS) results in a polypeptide containing a proline/serine (P/S) rich region (aa 384–503) which has been shown to have a repressive function [17]. However, in this context the P/S region alone is unable to quench the transactivation of ARNT since mSIM-2ST-PS is able to effect transactivation to a similar degree as mSIM-2ΔC (Fig. 2B). The adjacent proline and alanine (P/A) rich region has also been demonstrated to repress transcription when fused to a heterologous DNA binding domain [17]. Deletion mutants mSIM-2ΔS and mSIM-2Δ10 (lacking amino acids 609–657 and 648–657 respectively; Fig. 2A) have properties intermediate to mSIM-2ΔC and mSIM-2 (Fig. 2B). Western blotting with an anti-SIM-2 antibody did not show significantly different levels of mutant proteins (Fig. 2C, lanes 8–12). These results demonstrate that the full P/A, if not the entire carboxy-terminal region, is necessary for the quenching activity of mSIM-2. As well, we find that the mSIM-1 and mSIM-2 proteins and their deletion derivatives can form complexes with ARNT-2 with transcriptional activities similar to that obtained with ARNT (Fig. 2B,

compare ARNT with ARNT-2). Similar results were also obtained in C33A, CHO and COS-7 cells (P. Moffett, data not shown).

3.3. Activities of mSIM-1 and mSIM-2 chimeras

Having shown differences in the transcriptional output of mSIM-1/ARNT and mSIM-2/ARNT, we wished to define the structural elements required for this phenomenon. To this

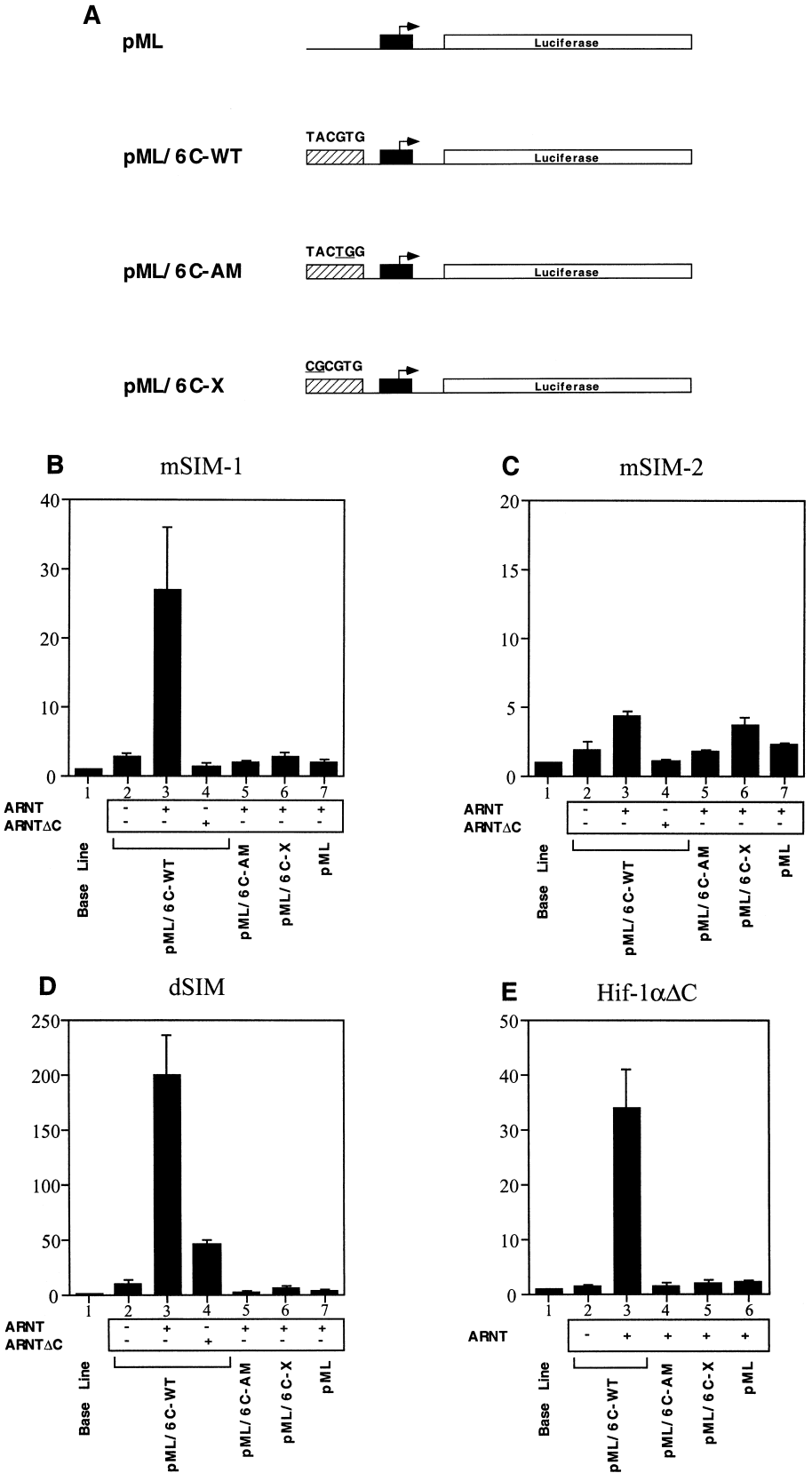

Fig. 1. Activation of transcription by SIM-like proteins. A: Schematic representation of reporter constructs. An open box represents the luciferase cDNA. The minimal adenovirus major late promoter (−34 to +33) is represented by a black box with the start site indicated by a right-angled arrow. A hatched box with the core CME or mutated derivative sequence indicated above represents a tandem array of six CMEs (or derivatives). Mutated bases are underlined. B–E: C33A cells were transfected with 2 μg of reporter construct (indicated below each graph) along with 4 μg of the indicated construct expressing SIM (indicated above each graph), pcDNA3 (lane 1), or HIF-1ΔC, as well as either 4 μg of ARNT, ARNTΔC, or pcDNA3 (lane 2). Graph bars indicate fold activation over baseline, which was arbitrarily set at one and represents the luciferase activity of the appropriate reporter vector alone with 8 μg of pcDNA3. Each transfection was repeated at least three times and error bars represent standard error.
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end, we constructed several fusion proteins exchanging various domains of the two SIM proteins (Fig. 3A). These constructs consisted of three classes: swapping the carboxy-termini (aa 348 to carboxy-terminus; mSIM-1/1/2 and mSIM-2/1/1), the PAS domains (aa 89–347; mSIM-1/2/1 and mSIM-2/1/2), or the bHLH domains (aa 1–88; mSIM-1/2/2 and mSIM-2/1/1). As well, mSIM-1 and mSIM-2 differ in their basic regions by a single amino acid (^{10}R in mSIM-1 and ^{10}K in mSIM-2), and a construct was made to express mSIM-2 with its DNA binding domain altered to be identical to that of mSIM-1 (mSIM-2 K→R). These fusion constructs were cotransfected into 293 cells in conjunction with pML/6C-WT and an ARNT expression vector. In this set of experiments mSIM-1 transactivated, on average, 16-fold over background whereas mSIM-2 had little effect. When the carboxy-termini were swapped, ARNT/mSIM-1/1/2 activity was reduced to 67% of wild type mSIM-1 but not reduced to the level of wild type mSIM-2 (Fig. 3B). Transferring the carboxy-terminus of mSIM-1 to mSIM-2 resulted in a fusion protein (mSIM-2/2/1) with essentially the same activity as mSIM-1 (Fig. 3B).

To investigate whether the PAS domains of mSIM-1 and

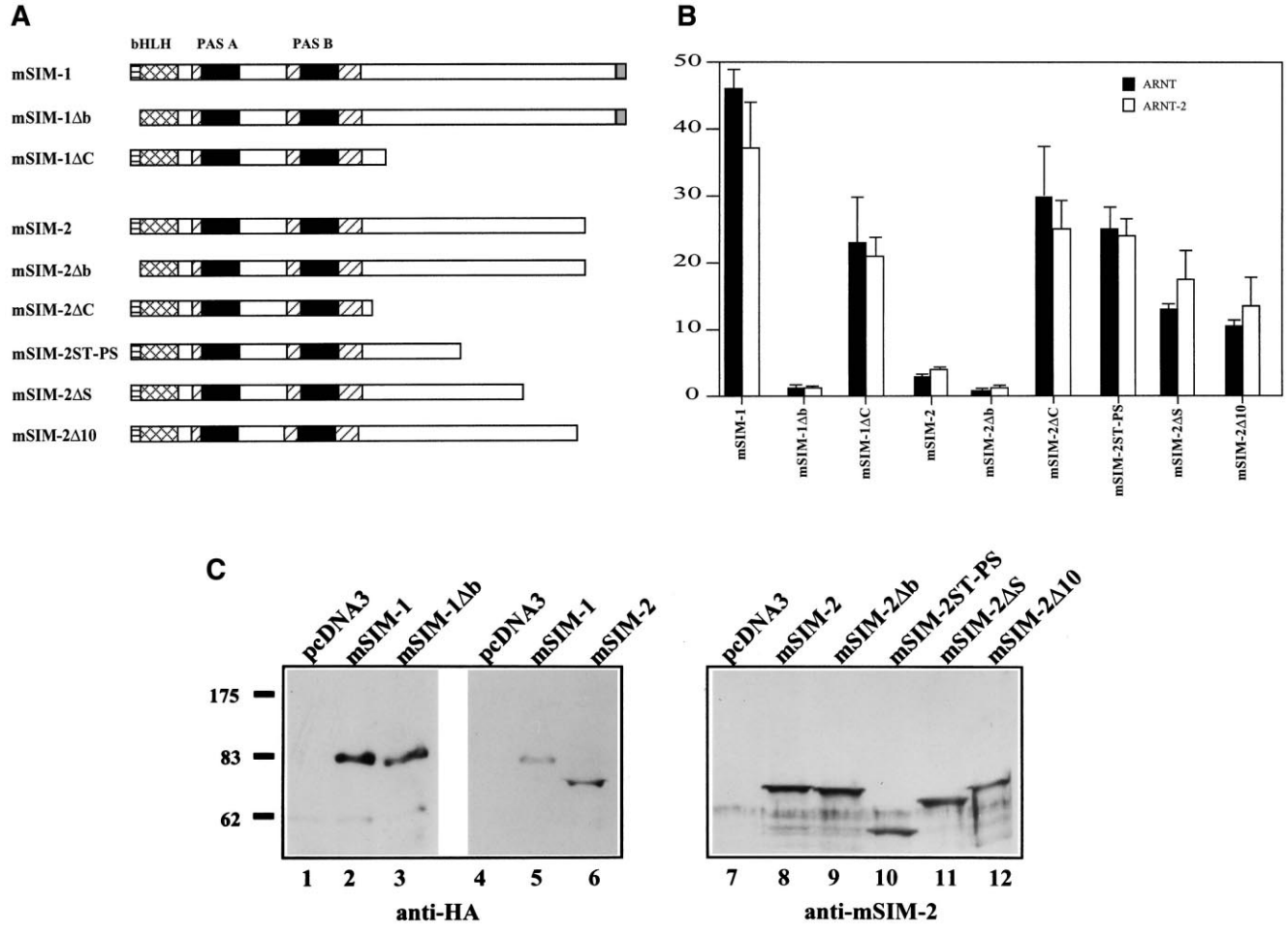

Fig. 2. Delineation of domains necessary for activation. A: Schematic representation of mSIM-1 and mSIM-2 deletion constructs. Solid boxes denote the PAS repeats and lightly hatched boxes represent extended sequence similarity to the PER, AHR and ARNT proteins. Crosshatched boxes represent the HLH region and horizontally hatched boxes represent the DNA binding basic region. Gray boxes represent the HA epitope tag. B: 293 cells were transfected with 2 μg pML/6C-WT, 4 μg ARNT or ARNT-2 expression vectors plus 4 μg pcDNA3 (basal level) or the indicated SIM construct. The vertical axis represents fold activation above basal level, which was arbitrarily set at one. Each transfection was repeated at least three times and error bars represent standard error. Solid bars represent transfections performed with ARNT and open bars represent transfections performed with ARNT-2. C: Western blot analysis of mSIM proteins. 293 cells were transfected with 4 μg of the appropriate expression vector (indicated at the top of each lane). Whole cell extracts were separated on a 9% SDS-PAGE and proteins detected as described in Section 2 using either an anti-HA epitope or anti-mSIM-2 antibody as indicated below each blot. Molecular weight markers (NEB) are indicated (in kDa) to the left of the blots. In lane 6, a HA epitope tagged mSIM-2 expression vector (see Fig. 4A) was transfected.

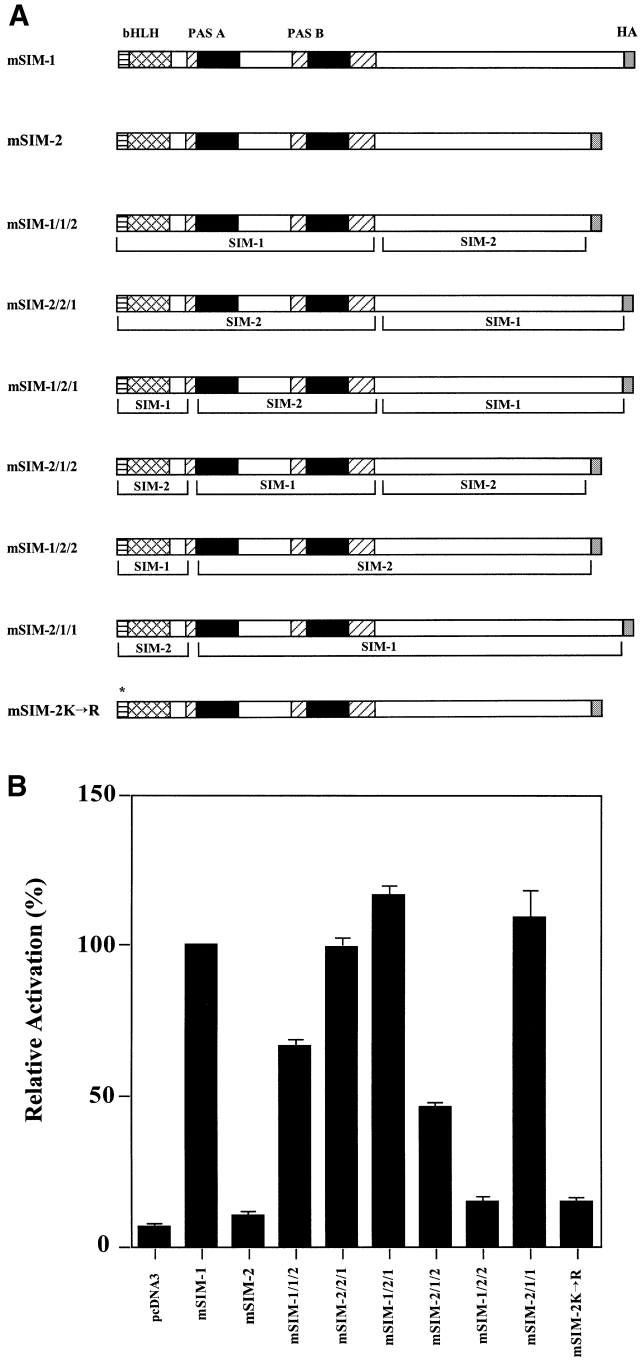

Fig. 3. Transcriptional activity of mSIM-1/mSIM-2 fusion proteins. A: Schematic representation of mSIM-1/mSIM-2 fusion constructs. Solid boxes denote the PAS repeats and lightly hatched boxes represent extended sequence similarity to the PER, AHR and ARNT proteins. Crosshatched boxes represent the HLH region and horizontally hatched boxes represent the DNA binding basic region. Gray boxes represent the HA epitope tag. The * represents a point mutation in the mSIM-2 DNA binding domain ($^{10}K \rightarrow {}^{10}R$). In the case of fusion proteins of mSIM-1 and mSIM-2, the portion of each protein originating from either mSIM-1 or mSIM-2 is indicated beneath the schematic diagram of the protein. B: 293 cells were transfected with 2 μg pML/6C-WT, 4 μg ARNT, and 4 μg pcDNA3 or the SIM expression construct indicated below the bar graph. The vertical axis represents fold activation relative to mSIM-1, which was arbitrarily set at 100%. Each transfection was repeated at least three times and error bars represent standard error.

mSIM-2 can affect ARNT transactivation properties, we exchanged the PAS domains of the two proteins. Placing the mSIM-2 PAS domain in the context of mSIM-1 (mSIM-1/2/1) did not significantly influence the activity of mSIM-1 (Fig. 3B). However, placing the mSIM-1 PAS domain in the context of mSIM-2 somewhat relieved the repressive activity of mSIM-2 such that the resulting fusion protein (mSIM-2/1/2) activated to a level 50% of wild type mSIM-1 (Fig. 3B).

When the bHLH domain of mSIM-1 was present in the context of mSIM-2, the resulting fusion protein (mSIM-1/2/2) acted analogously to wild type mSIM-2. Likewise, the bHLH domain of mSIM-2 did not affect mSIM-1 activity (mSIM-2/1/1). As well, mutating the basic domain of mSIM-2 to resemble that of mSIM-1 did not significantly affect the activity of mSIM-2 (mSIM-2K→R) either (Fig. 4B). Western blot analysis using an anti-HA epitope antibody showed that differences in fusion protein expression are not responsible for the observed differences in activity (P. Moffett, data not shown).

3.4. Competition between mSIM proteins

We sought to investigate whether the mSIM proteins could compete for limiting amounts of ARNT and/or binding site occupancy. To this end, 293 cells were cotransfected with pML/6C-WT, ARNT, and varying amounts of SIM proteins. Transfecting mSIM-1 alone with ARNT resulted in a reproducible 15-fold activation (Fig. 4A, compare lanes 1 and 2). Cotransfecting mSIM-1 and increasing amounts of mSIM-2Δb, which can bind ARNT but not DNA [17], resulted in a dose dependent decrease in mSIM-1 mediated activation, with up to a three-fold reduction of mSIM-1 mediated activation (Fig. 4A, lanes 3–7). However, transactivation by mSIM-1/ARNT was reduced to a greater extent even at relatively low amounts of co-transfected mSIM-2 (Fig. 4B, lane 3), and entirely abolished when twice as much mSIM-2 as mSIM-1 was transfected (Fig. 4B, lane 6).

We also investigated whether mSIM-1 could interfere with the action of mSIM-2 by cotransfecting constructs expressing ARNT and mSIM-2ΔC along with excess amounts of mSIM-1Δb, which can still bind to ARNT, but not DNA [18]. Even when three-fold excess mSIM-1Δb expression vector is cotransfected with mSIM-2ΔC there is no decrease in activation by ARNT/mSIM-2ΔC (Fig. 4C, compare lanes 3 and 4).

4. Discussion

Binding to a CME site by mSIM-1/ARNT, but not mSIM-2/ARNT, has been demonstrated in vitro [18] but transcriptional regulation via this site has not been investigated for either protein. We find that, like dSIM, both mSIM-1 and mSIM-2 can interact with CME sites. This interaction is dependent on the presence of ARNT or ARNT-2 (Figs. 1 and 2), the DNA binding domains of the mSIM proteins (Fig. 2), and is abolished by introducing point mutations in the CME site (Fig. 1).

Whereas dSIM has transactivating properties [19,20], we find that the mSIM-1/ARNT complex also transactivates (Fig. 1B). However, this activity seems to be largely a result of the presence of the ARNT activation domain (Figs. 1B and 2B). Therefore, it appears that the mSIM-1 carboxy-terminus has neither activation nor repression activity in this context. In contrast, mSIM-2/ARNT does not activate transcription

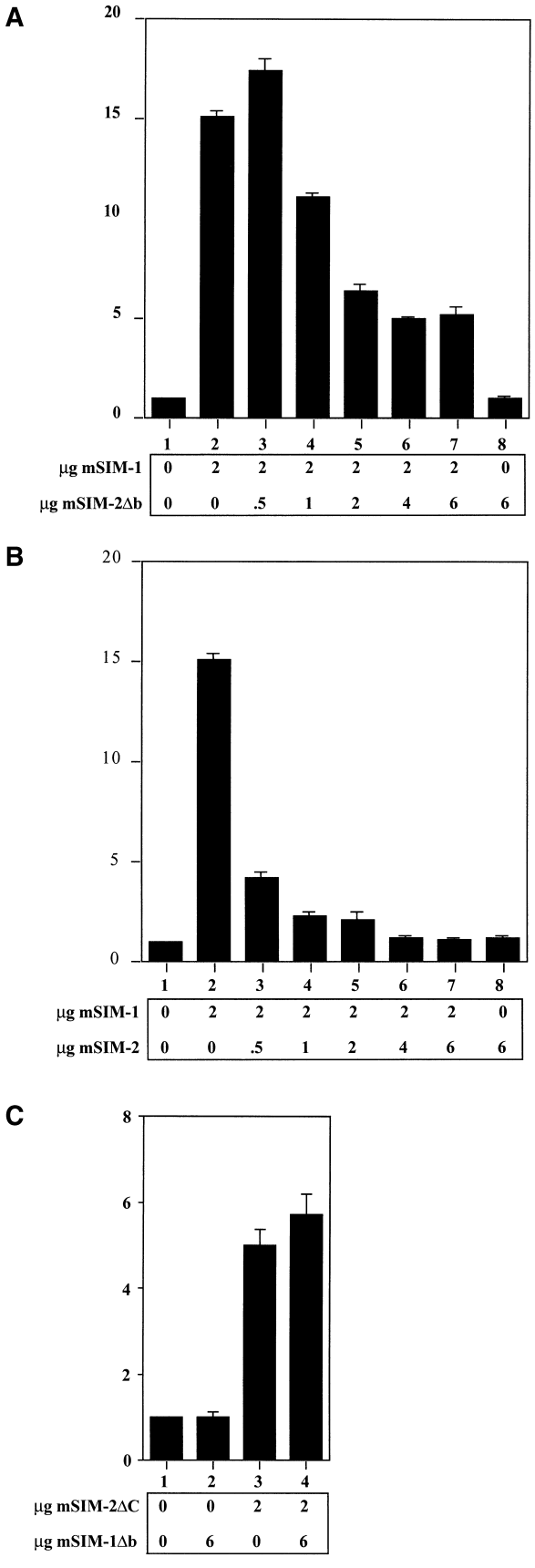

Fig. 4. Competition between mSIM proteins. A–C: 293 cells were transfected with 1 μg pML/6C-WT, 2 μg ARNT expression construct, and the indicated amount of pcDNA3/mSIM-1, pcDNA3/mSIM-2, pcDNA3/mSIM-2Δb or pcDNA3/mSIM-2ΔC expression constructs. The total amount of transfected DNA was made equal by compensating with appropriate amounts of pcDNA3. Numbers on the vertical axis represent fold activation above basal activity obtained with no SIM proteins or derivatives (lane 1) which is set at one. All transfections were performed at least three times and error bars indicate standard error.
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(Figs. 1–3) unless the carboxy-terminus is deleted (Fig. 2B). We interpret this to mean that the carboxy-terminus of mSIM-2 has a repressive function, which quenches the activity of the ARNT transactivation. Deletion analysis showed that the entire carboxy-terminus of mSIM-2 was necessary for this repressive effect (Fig. 2B).

The fact that mSIM-2 quenches ARNT activation is consistent with our previous results demonstrating the presence of repression domains in its carboxy-terminus [17]. However, the involvement of mSIM-1 in transactivation is in contrast to previous results [8], as well as our own unpublished data, in a mammalian two hybrid system showing that full length mSIM-1 represses activation by a GAL4/ARNT fusion protein. The reason for the different transcriptional responses is unclear at this point. We feel that our results, using full length proteins on a native DNA binding site, more closely reflect the in vivo situation. However, mSIM-1 may have different properties depending on the manner by which it is recruited to a promoter. The resolution of this issue awaits the identification of downstream targets. At present, the only candidate for a target gene of a mSIM protein is the *brn-2* gene. Michaud et al. [12] found that *mSim-1* is required to maintain expression of the *brn-2* gene, which has a CME consensus site ($-368^{5'}$TTACGTGG$^{3'}-361$) present in its 5′ regulatory region [28]. However, in transient cotransfection assays with reporter constructs containing the 5′ regulatory region [28] of the human homolog of *brn-2*, we have not detected transactivation or repression by either mSIM-1 or mSIM-2 in concert with ARNT or ARNT-2 (P. Moffett, data not shown). The effect of *mSim-1* on *brn-2* may be indirect or may require additional factors not present in our experimental system. It is interesting, however, that our results showing that mSIM-1/ARNT can transactivate would be consistent with mSIM-1 being required for *brn-2* expression, whereas it was previously assumed to be a transcriptional repressor [8].

We employed a panel of domain swapping fusion proteins to investigate the structural basis of the difference in function of mSIM-1 and mSIM-2 (Fig. 3). Exchanging the basic region or the entire bHLH domain did not alter the properties of the mSIM proteins ruling out the possibility of differences in DNA binding properties (Fig. 3B). The carboxy-termini appear to account for the difference in transcriptional properties between the two proteins as their deletion results in proteins with similar activity (Fig. 2B). Transferring the mSIM-1 carboxy-terminus had little effect on mSIM-2 (Fig. 3B). However, the full repressive effect of mSIM-2 was not transferable to mSIM-1 (Fig. 3B). It is unclear why the mSIM-2 carboxy-terminus is not fully repressive when contiguous with the mSIM-1 PAS domain, but is possible that the PAS domain modifies the behavior of the carboxy-terminus of the mSIM proteins. In support of an extended role for the PAS domains in transcriptional regulation, Zelzar et al. [29] have shown that

the PAS domains of TRH and SIM determine target gene activation.

Several studies have indicated that competition for a common dimerization partner may contribute to gene regulation by bHLH-PAS proteins. For example, the AHR and HIF-1 signalling pathways have been shown to interfere with each other by competing for limiting amounts of ARNT [30–32]. On the other hand, Mimura et al. [33] showed that, as well as competing for ARNT binding, AHRR inhibits AHR more efficiently by competing for DNA binding, and repression of mutual target genes. Also, Gradin et al. [34] have shown that a factor that binds the XRE in concert with ARNT inhibits AHR, but not HIF-1α, signalling demonstrating that ARNT availability is not rate limiting. These studies imply that transcriptional antagonism between bHLH-PAS transcription factors may be as important as competition for a common dimerization partner.

Our interpretation of results presented in Section 3.4 is that excess mSIM-2Δb or mSIM-2 is able to sequester ARNT from mSIM-1 and thus inhibit mSIM-1/ARNT mediated transactivation. The fact that the DNA binding competent form of mSIM-2 is more effective at inhibiting mSIM-1 mediated transactivation suggests that the two proteins compete for DNA binding site occupation as well. When mSIM-1 and mSIM-2 are present at near equal levels (Fig. 2C, lanes 5 and 6) there is very little activation of a reporter construct (Fig. 4B, lane 5), implying that mSIM-2 repression is dominant over mSIM-1 mediated activation in this system. As well, even high levels of mSIM-1Δb do not affect mSIM-2ΔC activity (Fig. 4C) suggesting that the more important mechanism may be at the level of antagonistic transcriptional activities on the same DNA binding site rather than sequestration of ARNT.

Because ARNT is widely expressed in mammals [35–37] it is believed that it acts as a general dimerization partner for a number of bHLH-PAS proteins. Although *Arnt2* mRNA has a more restricted pattern of expression [16,37], the *Arnt2* gene product is very similar in structure and function to ARNT [16]. We have shown that ARNT and ARNT-2 are equally capable of transactivating in concert with the mSIM proteins (Fig. 2B) and are therefore both candidates for the role of in vivo partner in domains of mutual expression, such as the diencephalon where both *mSim* genes [7–12] and *Arnt2* are expressed [16,37].

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