The high-mobility-group domain of Sox proteins interacts with DNA-binding domains of many transcription factors

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ABSTRACT

Sox proteins are widely believed to team up with other transcription factors as partner proteins to perform their many essential functions during development. In this study, yeast two-hybrid screens identified transcription factors as a major group of interacting proteins for Sox8 and Sox10. Interacting transcription factors were very similar for these two group E Sox proteins and included proteins with different types of DNA-binding domains, such as homeodomain proteins, zinc finger proteins, basic helix–loop–helix and leucine zipper proteins. In all cases analyzed, the interaction involved the DNA-binding domain of the transcription factor which directly contacted the C-terminal part of the high-mobility-group (HMG) domain. In particular, the C-terminal tail region behind helix 3 of the HMG domain was shown by mutagenesis to be essential for interaction and transcription factor recruitment. The HMG domain thus not only possesses DNA-binding and DNA-bending but also protein-interacting ability which may be equally important for the architectural function of Sox proteins on their target gene promoters.

INTRODUCTION

Sox proteins are important transcriptional regulators of various developmental processes (1,2). Compared to other transcription factors, Sox proteins are unusual in that they introduce a strong bend into DNA upon binding to the minor groove. Sox proteins therefore alter DNA conformation and act as architectural proteins. A second characteristic feature of all Sox proteins is their dependence on other transcription factors as partner proteins for efficient target gene activation (3,4).

The 20 different Sox proteins that exist in mammals can be classified into several groups according to sequence similarities (5). Group E Sox proteins (also referred to as SoxE proteins) include Sox8, Sox9 and Sox10 in mammals. They exhibit partially overlapping expression patterns during embryogenesis and when co-expressed often exert similar functions arguing for at least partial functional redundancy among them (6–8). However, there are also developmental processes in which SoxE proteins have unique roles. Sox9, for example, is specifically required for chondrogenesis, whereas Sox10 alone drives gliogenesis in the peripheral nervous system (9,10).

It is astonishing that all three SoxE proteins control seemingly unrelated developmental processes in different tissues and can regulate consecutive steps during cell lineage progression (10–12). The pleiotropic actions of Sox proteins are usually attributed to their reliance on partner proteins. According to this model, Sox proteins acquire a function based on the transcription factors co-expressed in a particular tissue and change function with each shift in trancription factor patterns during cell lineage progression.

Only few of these partner proteins have so far been identified for SoxE proteins (13–19). Here we show that the high-mobility-group (HMG) domain of SoxE proteins establishes weak interactions with DNA-binding domains of numerous transcription factors in solution. These weak interactions may be the basis for the establishment of cooperativity between Sox proteins and their partners on target gene promoters and for the resulting synergistic gene activation.

MATERIALS AND METHODS

Plasmid constructs

To generate bait plasmids for yeast two-hybrid screening, fragments of the rat *Sox10* cDNA (GenBank accession no. AJ001029) were generated by PCR that code for amino

acids 1–230 or amino acids 133–203. These fragments were inserted into pGBKT7 (Clontech) using EcoRI and BamHI restriction sites. A fragment corresponding to amino acids 124–192 of mouse Sox8 (GenBank accession no. NM_011447) was analogously inserted into the pGBKT7 bait plasmid. Coding sequences from potential interaction partners identified in the yeast two-hybrid screen were retrieved from the pVP16 prey plasmid and cloned in frame behind a T7 epitope tag into the eukaryotic expression vectors pcDNA3 or pCMV5 using XhoI in combination with EcoRI or HindIII restriction sites. DNA-binding domains of additional transcription factors were obtained by PCR and similarly inserted behind a T7 epitope tag into eukaryotic expression vectors. The following primers were used: 5′-AGATCCCGGTGCAG-3′ and 5′-CCCACTGTTAACGTG-GTTC-3′ for c-Jun (accession no. X17163), 5′-CGGTGGA-TAAGAACAGCAAC-3′ and 5′-GCTCTCAGGCAGCTGG-3′ for C/EBPα (accession no. NM_012524), 5′-AGGA-AAGGCGGATGG-3′ and 5′-TTCTAGACTAAGGAT-GACTGC-3′ for REB (accession no. NM_013176), 5′-AGCTGCGCCTGAAGATCAAC-3′ and 5′-GGTGAG-CATGAGGATGTAGTTTC-3′ for Olig2 (accession no. AB038697), 5′-CGTACCCCTGCCCAG-3′ and 5′-GTGGA-TCTTGGTGTGGCG-3′ for Krox-20 (accession no. U78102), 5′-AGCATATTTGCCACATCCAAG-3′ and 5′-GTGGGTC-TTGATATGTTTTGA-3′ for Sp1 (accession no. J03133), 5′-CTCGCTACTGTGCAGT-3′ and 5′-GTCTTTTCGT-ATCCCACC-3′ for estrogen receptor (accession no. NM_000125), 5′-ACGAGCTCTGTGTAGTG-3′ and 5′-GT-CATCCAGCACCAAATC-3′ for thyroid hormone receptor β (accession no. NM_000461), 5′-CCGAGGAGTCCCAGG-3′ and 5′-TGTCGGCTTCCTCCACC-3′ for the POU-specific domain of Oct-3/4 (accession no. NM_013633) and 5′-TCGG-CCAGGGCCG-3′ and 5′-CCTCAGGATGCGACTGATGG-AAC-3′ for the paired domain of Pax3 (accession no. XM_343601). Coding sequences for Sox8 (amino acids 1–175) and Sox10 (amino acids 1–203), were placed in frame behind a myc epitope tag in pCMV5-based expression plasmids (20). pCMV-Sox10, pCMV-Sox8 and pCMV-Brn2 have been described previously (21–23). Luciferase reporter plasmids carried a multimerized AP1 response element (TRE-luc) or the Schwann cell specific enhancer of the mouse *Krox-20* gene (SCE-luc) (15) in front of the TATA-box containing minimal β-globin promoter (23).

Bacterial expression plasmids for glutathione-*S*-transferase (GST) fusion proteins were created by placing PCR fragments corresponding to amino acids 17–147 or 133–203 of rat Sox10 (Sox10 A and Sox10 B), amino acids 96–124 or 122–175 of mouse Sox8 (Sox8 A and Sox8 B) as well as amino acids 58–84 or 84–133 of human SRY (SRY A and SRY B) in frame behind the GST coding sequence into pGEX-KG. Amino acid substitutions were introduced into the Sox10 sequences of pGEX-KG-Sox10 B using the QuickChange mutagenesis Kit (Stratagene) to generate mutants m1 (R176E) and m2 (R161E, K165E). GST fusions were also generated with the HMG domain of mouse LEF-1 (amino acids 297–365 according to accession no. NM_010703), the second HMG domain of mouse HMGB1 (amino acids 94–162, according to accession no. Z11997), and with the third and sixth HMG domains of mouse UBF1 (amino acids 298–362 and 568–634 according to accession no. X60831).

Yeast two-hybrid screen

A pVP16-based cDNA-library prepared from 10.5 days post coitum (dpc)-old mouse embryos where preys are expressed as fusions with the VP16-transactivation domain (24) was introduced by transformation into yeasts (strain AH109) that already carried pGBKT7-based plasmids and expressed the N-terminal region of Sox10 (amino acids 1–230) or the C-terminal part of the HMG domain of Sox8 (amino acids 124–192) or Sox10 (amino acids 133–203) as baits in frame with the Gal4-DNA-binding domain. In total, 8×10^5 independent colonies were screened with Sox10 (amino acids 1–230), 1×10^6 colonies with Sox10 (amino acids 133–203) and 3×10^6 colonies with Sox8 (amino acids 124–192). Transformants were plated on medium lacking tryptophane, leucine and histidine. Growth was evaluated after five days of incubation at 30°C. Yeast colonies were further analyzed by filter-lift assays for β-galactosidase reporter activity. Prey plasmids were retrieved from β-galactosidase-positive colonies and retransformed with the bait to verify the interaction. Nineteen percent of verified preys corresponded to transcription factors and 27% to other proteins with nuclear localization. Thirty-three percent were predominantly localized outside the nucleus and the remaining 21% were proteins of unknown function and subcellular localization.

Cell culture, transfections, extract preparations and luciferase assays

HEK 293 and S16 cells were kept in DMEM supplemented with 10% (v/v) fetal calf serum (FCS). For the production of Sox proteins and DNA-binding domains of various transcription factors, HEK 293 cells were transiently transfected by the calcium phosphate technique using 10 μg plasmid DNA per 100 mm plate. After 48 h post transfection, cells were harvested for extract preparation. Briefly, whole cell extracts from HEK 293 cells were prepared using 2 μg leupeptin and aprotinin each in ice cold 20 mM HEPES (pH 7.9), 10 mM KCl, 5 mM MgCl₂, 0.5 mM EDTA, 10% glycerol, 2 mM DTT, 0.1% Triton X-100 and 300 mM NaCl. After extraction for 15 min under constant rotation, cell debris was removed from the extract by centrifugation (25). For luciferase assays, S16 cells were transfected in 35 mm plates using Superfect reagent (Qiagen) with 0.5 μg of luciferase reporter plasmid and 0.5 μg of pCMV5 effector plasmid, if not stated otherwise. Cells were harvested and reporter gene expression was analyzed 48 h post transfection (25).

GST pulldowns, co-immunoprecipitations and western blotting

For pulldown experiments, GST or GST fusion proteins with parts of Sox8, Sox10, SRY, LEF-1, UBF1 and HMGB1 were produced in *Escherichia coli* strain BL21 DE3 pLysS and bound in the presence of DNase I to glutathione Sepharose 4B beads as described (26). An aliquot of the washed and equilibrated beads, now carrying GST or the GST fusion protein, was incubated with one-tenth of the HEK 293 extract obtained from a 100 mm plate in interaction buffer [20 mM HEPES (pH 7.9), 10 mM KCl, 5 mM MgCl₂, 0.5 mM EDTA, 5% glycerol, 1 mM DTT, 0.05% Triton X-100 and 100 mM NaCl]. After centrifugation and washing, bead-bound proteins

from the HEK 293 extract were separated by SDS–PAGE and transferred to a nitrocellulose membrane.

For co-immunoprecipitation, one-seventh of the extract obtained from one 100 mm plate was incubated for 2 h at 4°C in 300 µl interaction buffer with mouse monoclonal anti-myc-antibodies (clone 9E10, hybridoma supernatant) already coupled to 30 µl of protein A-Sepharose CL-4B beads (Amersham Biosciences). After centrifugation, the beads were washed, before precipitated proteins were separated by SDS–PAGE and transferred to a nitrocellulose membrane.

Western blotting was performed as described (27) using a mouse monoclonal antibody against the T7 epitope tag (1:10 000 dilution, Novagen) or a rabbit antiserum against Sox10 (1:3000 dilution) (21) as primary antibodies, and horse-radish peroxidase (HRP) coupled goat-anti-mouse antibodies or HRP coupled protein A as secondary detection reagents with enhanced chemiluminescence.

RESULTS

Many transcription factors interact via their DNA-binding domain with Sox10 and Sox8 in yeast two-hybrid screens

To identify interaction partners for the HMG domain of SoxE proteins, we performed yeast two-hybrid screens with the N-terminal region of Sox10 (Figure 1a). This bait includes the HMG domain as well as the DNA-dependent dimerization domain that is specific to SoxE proteins (25,28). Using a library prepared from mouse 10.5 dpc-old embryos, we identified several transcription factors including the homeodomain proteins Meox1 and Pax6 and the zinc-finger protein Hivep1 (Figure 1b). In all cases, the identified region of the interacting protein contained at least part of the homeodomain or several zinc fingers indicating that the DNA-binding domain mediates the interaction with SoxE proteins.

To extend our screen and simultaneously analyze whether interaction is primarily mediated by the HMG domain or rather by the protein region preceding the HMG domain, we repeated the yeast two-hybrid screen with a shorter bait from the HMG domain. The region chosen for this screen consisted of amino acids 133–203 and contained helices 2 and 3 and the C-terminal tail of the Sox10 HMG domain (Figure 1a). Helix 3 and the C-terminal tail are still solvent exposed when the HMG domain binds to DNA and should therefore be accessible to interacting proteins (29,30). Using this bait, we again identified interactions with several homeodomain proteins. Identified interactors include Dlx5, Hhex, Alx4, Hoxa3 and Brn-1 (Figure 1b). The interacting region again contained at least part of the homeodomain. In case of Brn-1, both the homeodomain and a small part of the POU-specific domain were present in the interacting region, which is furthermore almost completely conserved in the related Brn-2 and Oct-6 proteins. Our ability to detect homeodomain-containing interactors both with the HMG-specific bait and with the complete N-terminal region is compatible with the conclusion that on the side of the Sox protein, the HMG domain is involved in the interaction. As the identified homeodomain proteins belong to diverse groups of the homeodomain superfamily, the interaction between
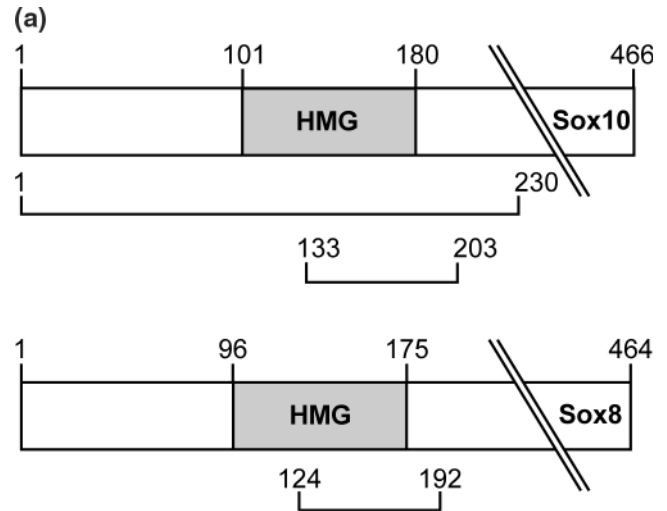
(b) bait: Sox10 (1-230)

name	acc.no.	residues	domain
Pax6	NM_013627	152-303	homeo
Meox1	NM_010791	154-253	homeo
Hivep1	NM_007772	2059-2140	zinc finger

(c) bait: Sox10 (133-203)

name	acc.no.	residues	domain
Dlx5	NM_010056	119-243	homeo
Hhex	NM_008245	85-229	homeo
Alx4	NM_007442	187-399	homeo
Hoxa3	NM_153632	23-113	homeo
Brn-1	NM_008900	357-437	POU
UTF1	NM_009480	240-345	bHLH

(d) bait: Sox8 (124-192)

name	acc.no.	residues	domain
Prrx1	NM_175686	91-185	homeo
Hoxc4	NM_013553	138-153	homeo
Prrx2	NM_009116	105-192	homeo

Figure 1. Interaction of SoxE proteins with other transcription factors. (**a**) Schematic representation of Sox10 and Sox8 with their HMG domain and delineation of the regions used as bait in yeast two-hybrid screens. Numbers above bars or lines represent amino acids at the beginning or end of proteins, protein domains and baits. (**b–d**) Summary of transcription factors identified in yeast two-hybrid screens with a Sox10 bait containing amino acids 1–230 (b), a Sox10 bait containing amino acids 133–203 (c) and a Sox8 bait containing amino acids 124–192 (d). In addition to transcription factor names, accession numbers and obtained amino acid residues are listed as well as the domain identified in the screen.

homeodomain and HMG domain appears to be a general feature. Additionally, we isolated a region from UTF1 that corresponded to its bHLH domain (Figure 1c), indicating that the range of interactors might extend to members of the bHLH family of transcription factors.

When the yeast two-hybrid screen was repeated with a bait that corresponds to the C-terminal region of the HMG domain of Sox8 including helix 2, helix 3 and the C-terminal tail (amino acids 124–192, Figure 1a), we also obtained several homeodomain proteins. Specifically, Prrx1, Prrx2 and Hoxc4 were identified with at least part of their homeodomain present in the preys (Figure 1d). The HMG domains of the two related SoxE proteins Sox8 and Sox10 thus interact with at least similar sets of transcription factors. Whether interacting transcription factors are only similar or even identical, cannot be concluded from our yeast two-hybrid screens because the number of screened yeast colonies was too low to achieve complete coverage of the cDNA library.

In addition to transcription factors, many other proteins were identified as potential interaction partners in our screen (see Materials and Methods), including subunits of chromatin remodelling complexes, chromatin modifiers, transcriptional cofactors, protein kinases and in accord with recent studies (31,32) components of the sumoylation system.

Interaction with SoxE proteins is mediated through the C-terminal part of the HMG domain

To confirm the interactions, we performed GST-pulldown experiments for a selected set of candidates representing the different transcription factor families from which members were identifed in the yeast two-hybrid screens. GST fusion proteins were used that either contained N-terminal Sox10 regions including the front part of the HMG domain up to helix 2 (Sox10 A in Figure 2a), or the hind part of the HMG domain with helices 2 and 3 and the C-terminal tail (Sox10 B in Figure 2a). In agreement with the results from yeast two-hybrid screens, all homeodomain proteins tested in GST pulldown experiments, including Dlx5, Hhex and Brn-1, interacted specifically with the GST fusion carrying the C-terminal part of the Sox10 HMG domain, not however with GST alone or the GST fusion carrying the N-terminal part of the HMG domain and preceding amino acid sequences (Figure 2b). The bHLH protein UTF1 also interacted specifically with the C-terminal part of the Sox10 HMG domain (Figure 2b). In general, GST pulldown experiments involving DNA binding proteins are prone to artefacts, as binding of both proteins to contaminating DNA is difficult to distinguish from bona fide protein–protein interactions. However, most of our GST pulldown experiments were carried out with GST–Sox10 fusion proteins that lacked an intact HMG domain and thus were unable to bind to DNA. Results were furthermore identical when DNase I was continuously present throughout the experiment (data not shown and Figure 4b), indicating that pulldowns indeed reflect true protein–protein interactions.

We also included GST fusion proteins in our pulldown experiments that carried parts of the HMG domain of Sox8 and the more distantly related SRY. Interestingly, all candidates that interacted with the C-terminal part of the Sox10 HMG domain also interacted specifically with the corresponding region of Sox8 and SRY (Figure 2b), thus indicating that the interactors identified in our screen are probably common interactors for all Sox proteins. In contrast, no interaction was
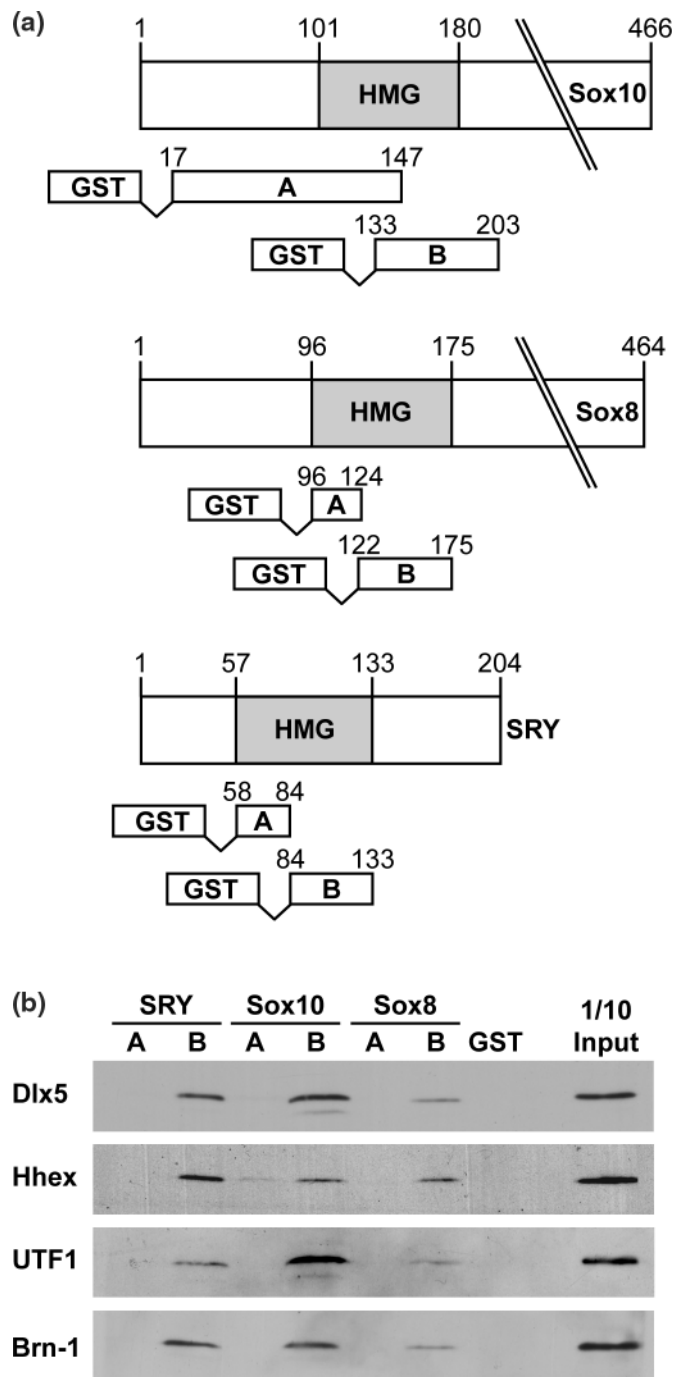

Figure 2. Confirmation of yeast two-hybrid interactions in GST pulldown experiments. (**a**) Schematic representation of Sox10, Sox8 and SRY with their HMG domain and GST fusion proteins GST-Sox10A, GST-Sox10B, GST-Sox8A, GST-Sox8B, GST-SRYA and GST-SRYB. Numbers above bars represent amino acids at the beginning or end of proteins, domains and protein fragments. (**b**) Regions from several transcription factors identified in the yeast two-hybrid screen (Dlx5, Hhex, UTF1 and Brn-1) were tagged with a T7 epitope, expressed in HEK 293 cells and analyzed for their ability to interact with GST or the GST-Sox10A, GST-Sox10B, GST-Sox8A, GST-Sox8B, GST-SRYA and GST-SRYB fusions. The amount of each transcription factor present in one-tenth of the extract before pulldown (1/10 input) is shown in the right lane of each panel. Transcription factor regions were detected on western blots via their T7 epitope tag.

observed with the N-terminal part of the HMG domain of Sox8 and SRY.

Next we investigated whether the Sox HMG domain also interacted with other transcription factors with which Sox proteins are known to be co-expressed in various cell types during different phases of development. These transcription factors covered a broad spectrum of different DNA-binding domains. With Olig2 and REB, two additional bHLH proteins were tested that are co-expressed with Sox10 and Sox8 in oligodendrocytes and with Sox10 in Schwann cells, respectively (33–35). Both interacted with the HMG domains of Sox10, Sox8 and SRY in a manner similar to UTF1 (Figure 3). We also included bZip proteins in our study. Although no bZip transcription factor was originally obtained in our yeast two-hybrid screen, both c-Jun and C/EBPα interacted with the HMG domain of Sox10. Again this interaction occurred specifically with the C-terminal region of the HMG domain. It was similarly observed with the HMG domains of Sox8 and SRY. Among zinc finger proteins, both Krox-20 and Sp1 interacted with the HMG domain of Sox10, Sox8 and SRY. These interactions may also be relevant under physiological conditions as Krox-20 is co-expressed with Sox10 in myelinating Schwann cells, whereas Sp1 has been proposed to support Sox10 in its activation of the myelin gene MBP in oligodendrocytes (36,37). Among the tested zinc finger proteins, there were also two nuclear receptor proteins. These were the estrogen receptor and the thyroid hormone receptor β (Figure 3). Both reproducibly failed to interact via their DNA-binding domain with the HMG domain of Sox10, Sox8 or SRY. These results do not exclude the possibility that these nuclear receptors might establish contacts with Sox proteins either through different regions outside the DNA-binding domain or through one of the many co-factors for nuclear receptors.

In our original yeast two-hybrid screen, a Pax protein and a POU protein were identified. As evident from the preys, the interaction with the HMG domain was likely mediated in both cases by the homeodomain. In addition to the homeodomain, these proteins possess a second DNA-binding domain which in case of the Pax protein is a paired domain and in case of the POU protein is a POU-specific domain. To analyze whether these DNA binding domains also possess the capability to interact with the HMG domain of Sox10, we performed GST pulldown assays with the paired domain of Pax3 and the POU-specific domain of Oct-3/4. In both cases interaction was detected with the C-terminal part of the Sox HMG domain (Figure 3).

HMG domains are not only present in Sox proteins, but also in other sequence-specific and non-sequence-specific DNA-binding proteins. We therefore tested HMG domains from LEF-1, HMGB1 and UBF1 (Figure 4a) in GST pulldown experiments for their ability to interact with a representative set of DNA-binding domains. In contrast to the HMG domain of Sox10, none of these HMG domains exhibited significant binding to the bHLH region of Olig2, the bZip region of C/EBPα, the Krox-20 zinc fingers, the paired domain of Pax3 or the homeodomain of Dlx5 (Figure 4b). We therefore conclude that the interactions are specific for the HMG domain of Sox proteins.
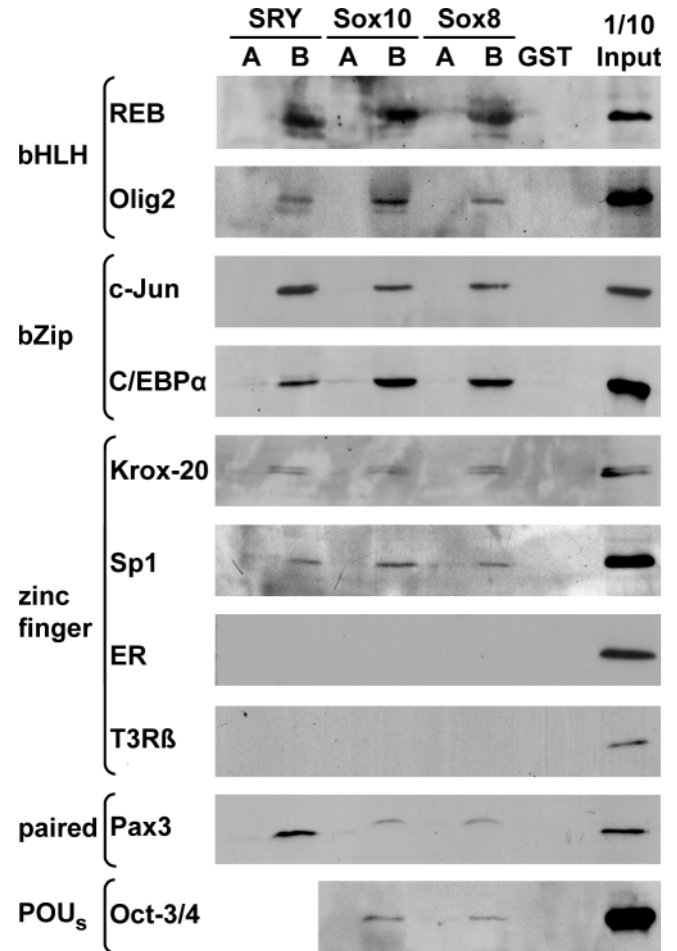

Figure 3. Interaction of the Sox HMG domain with DNA-binding domains of many other transcription factors in GST pulldown experiments. DNA-binding domains of additional transcription factors which are co-expressed with SoxE proteins *in vivo* were tagged with a T7 epitope, expressed in HEK 293 cells and analyzed for their ability to interact with GST or the GST-Sox10A, GST-Sox10B, GST-Sox8A, GST-Sox8B, GST-SRYA and GST-SRYB fusions (see Figure 2a). Analyzed DNA-binding domains include: bHLH domain (REB, Olig2), bZip domain (c-Jun, C/EBPα), C2H2 zinc finger (Krox-20, Sp1), C4 zinc finger (estrogen receptor ER, thyroid hormone receptor T3Rβ), paired domain (Pax3) and POU-specific domain (Oct-3/4). The amount of each DNA-binding domain present in one-tenth of the extract before pulldown (1/10 input) is shown in the right lane of each panel. DNA-binding domains were detected on western blots via their T7 epitope tag.

Interaction with the HMG domain of SoxE proteins is weak

As most of our GST pulldown studies were performed with GST fusions carrying HMG domain fragments that may not fold properly, we tried to reproduce the interaction by co-immunoprecipitation from extracts of transfected HEK293 cells that contained a SoxE protein with intact HMG domain. In these experiments SoxE proteins carried an N-terminal myc-tag which was used for immunoprecipitation. Detection of the co-immunoprecipitated proteins was through their T7-epitope. As evident from Figure 5a, previously identified interactors were co-immunoprecipitated with Sox10 from extracts in which both were co-expressed, including the zinc finger protein Krox-20, the homeodomain protein Dlx5
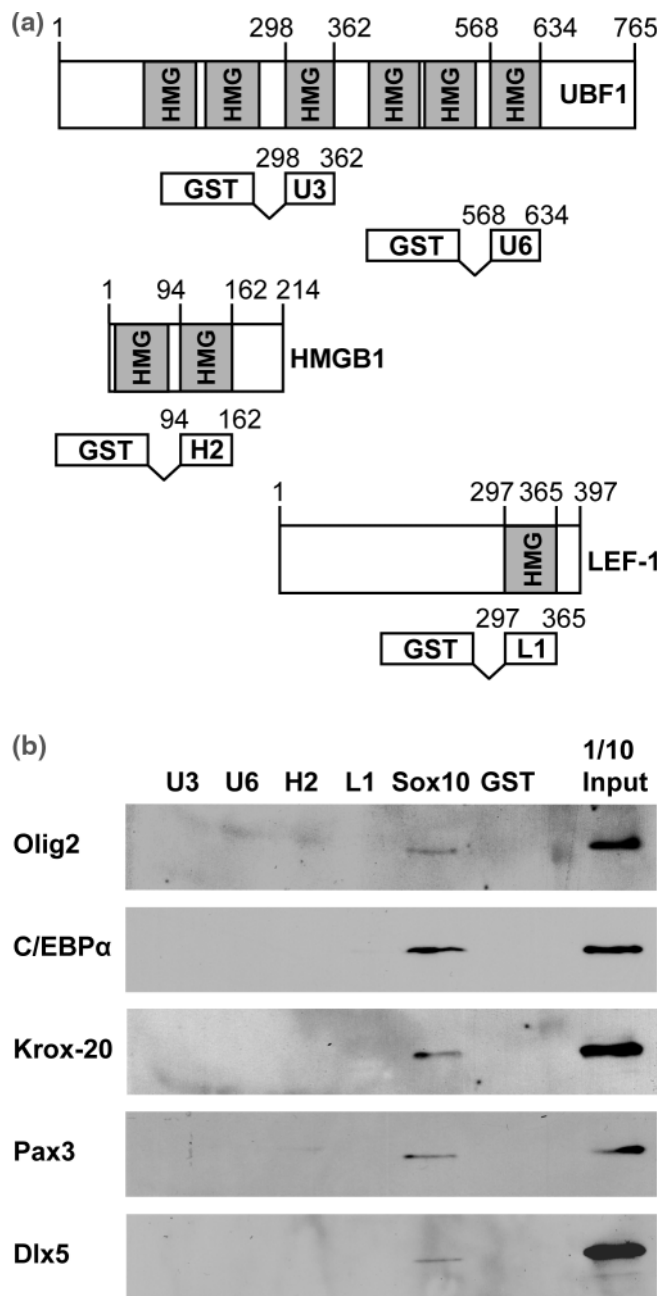
Figure 4. Interaction of other HMG domains with DNA-binding domains of transcription factors in GST pulldown experiments. (**a**) Schematic representation of UBF1, HMGB1 and LEF-1 with their HMG domains and GST fusion proteins GST-U3, GST-U6, GST-H2 and GST-L1. Numbers above bars represent amino acids at the beginning or end of proteins, domains and protein fragments. (**b**) T7-tagged DNA-binding regions from several transcription factors (Olig2, C/EBPα, Krox-20, Pax3 and Dlx5) were expressed in HEK 293 cells and analyzed for their ability to interact with GST or the GST fusions with HMG domains from UBF1 (U3, U6), HMGB1 (H2) and LEF-1 (L1). DNase I was present throughout the experiment. The amount of each transcription factor present in one-tenth of the extract before pulldown (1/10 input) is shown in the right lane of each panel. Transcription factor fragments were detected on western blots via their T7 epitope tag.

and the bZip proteins C/EBPα and c-Jun. When Sox10 was exchanged by Sox8, co-immunoprecipitation was still observed thus confirming that Sox8 is as much an interaction partner as Sox10 (Figure 5b). In the reciprocal experiment,

SoxE proteins were also co-immunoprecipitated with these transcription factors as evidenced from the presence of Sox10 in Olig2 immunoprecipitates (Figure 5c). However, only a small percentage of the interaction partner was detected in the immunoprecipitates, suggesting a fairly weak interaction between both proteins. The assumption of a weak interaction was also supported by two other observations. Already moderate increases in the salt concentration from 100 to 150 mM NaCl led to strong reductions in the amount of co-precipitated or pulled-down partner protein (data not shown). Additionally, interactions were not stable under electrophoretic mobility shift conditions. No changes in complex formation between Sox10 and its binding sites were observed in the presence of increasing amounts of its interaction partners. In particular, no ternary complex formed that contained the interaction partner in addition to Sox10 and its binding site (data not shown).

Despite their weakness, interactions strongly influenced promoter activities in reporter gene assays. A promoter that contained multiple AP1-binding sites, but no Sox binding site was efficiently activated by c-Jun only as long as Sox10 was absent. Co-transfection of even sub-stoichiometric amounts of Sox10 or Sox8 expression plasmid efficiently abolished c-Jun-dependent reporter gene activation (Figure 6a and data not shown). In contrast, regulatory regions with binding sites for both Sox protein and its interaction partners were cooperatively activated as shown for the Schwann-cell specific enhancer of the *Krox-20* gene (Figure 6b), which is jointly activated by Sox10 and Brn-2 or Oct-6 in Schwann cells (15). Interestingly, Sox8 cooperated as efficiently with Brn-2 in activating this enhancer as Sox10 (Figure 6b). Sox8 also activated the *Connexin-32* promoter in cooperation with Krox-20 (data not shown) as previously shown for Sox10 (14).

Interaction requires specific contacts with the C-terminal tail of the SoxE HMG domain

Previous studies on the POU protein Oct-3/4 and Sox2 had identified interactions between the POU-specific DNA-binding domain and the HMG domain under conditions where both proteins were bound to DNA on adjacent binding sites (29). Depending on the exact spacing and orientation of binding sites and therefore depending on the relative orientation of POU and HMG domains, interactions were disrupted by amino acid substitutions in helix 3 (R100E and M104E) or in the C-terminal tail of the HMG domain (R115E).

To analyze whether the same residues were also involved in the interaction of the SoxE HMG domain with other DNA-binding domains in solution, the corresponding mutations were introduced into the C-terminal tail of the Sox10 HMG domain (R176E, m1 mutant in Figure 7) or into helix 3 (R161E and K165E, m2 mutant in Figure 7). When pulldown assays were performed with GST fusion proteins carrying the C-terminal part of the Sox10 HMG domain in the m1 mutant version, no interactions were observed with any of the bZip, bHLH, zinc finger or homeodomains (Figure 7). In contrast, mutant m2 interacted as efficiently with these DNA-binding domains as the wild-type. The same pattern was also observed in pulldown experiments with the POU-specific domain of Oct-3/4 arguing that the
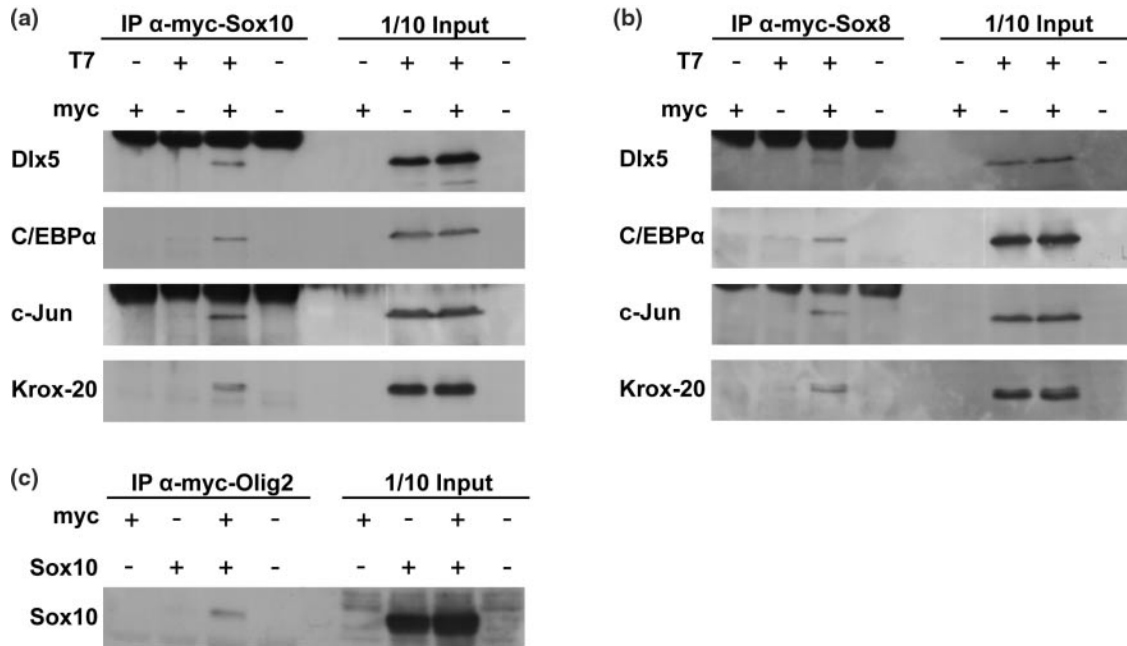
Figure 5. Confirmation of transcription factor interactions in co-immunoprecipitation experiments. (**a** and **b**) DNA-binding domains of several transcription factors (Dlx5, C/EBPα, c-Jun and Krox-20) were expressed in HEK293 cells in the presence or absence of myc-tagged Sox10 (amino acids 1–203) (a) or Sox8 (amino acids 1–175) (b) and precipitated from extracts by antibodies against the myc tag. Following immunoprecipitation (IP), transcription factors were detected in the precipitates on western blots with an antiserum directed against their T7 epitope. The presence of myc-tagged Sox10, myc-tagged Sox8 (myc) and T7-tagged transcription factors (T7) in the extract is indicated above each lane. The amount of each transcription factor present in one-tenth of the extract before precipitation (1/10 input) is shown in the right half. (**c**), Full length Sox10 was expressed in HEK293 cells in the presence or absence of myc-tagged Olig2 and precipitated from extracts by antibodies against the myc tag. Following IP, Sox10 was detected in the precipitates on western blots with a Sox10-specific antiserum. The presence of myc-tagged Olig2 (myc) and Sox10 in the extract is indicated above each lane. The amount of Sox10 present in one-tenth of the extract before precipitation (1/10 input) is shown in the right half.
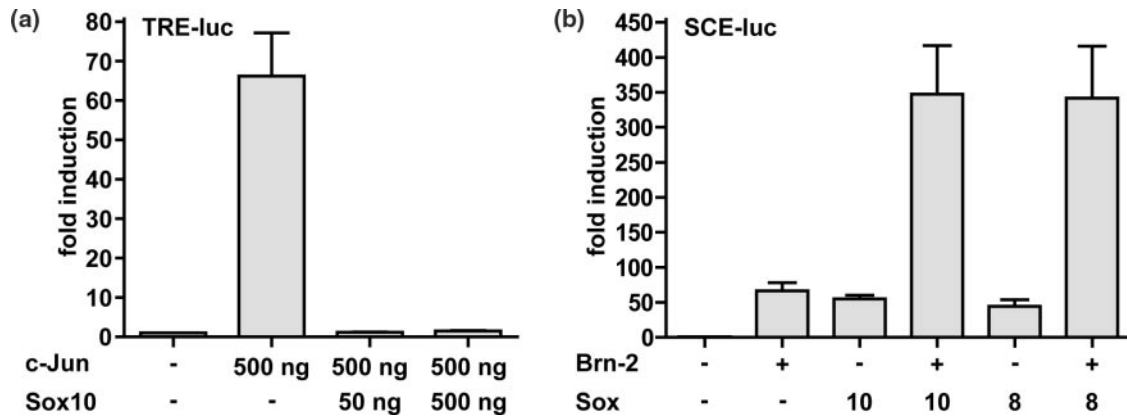

Figure 6. Gene expression in the presence of Sox proteins and their interaction partners. Reporter gene assays were performed as transient transfections in S16 cells. (**a**) A luciferase reporter with multimerized AP1-binding sites and minimal promoter (TRE-luc) was transfected alone or with c-Jun in the absence or presence of increasing amounts of Sox10 (one-tenth the amount of c-Jun and equal amounts) as indicated. (**b**) A luciferase reporter consisting of the Schwann-cell specific enhancer of the mouse *Krox-20* gene (15) and minimal promoter (SCE-luc) was transfected alone or with expression plasmids for Sox10, Sox8 and Brn-2 in various combinations as indicated. Activation rates for each promoter are presented as fold inductions ± SEM. Luciferase activities were determined in two experiments each performed in duplicates.

C-terminal tail of the HMG domain is generally essential for protein–protein interactions in solution. In contrast, the previously observed interaction with helix 3 of the HMG domain appears restricted in its importance to the interaction between Sox2 and Oct-3/4 or to protein interactions on DNA.

DISCUSSION

So far the HMG domain of Sox proteins has been primarily perceived as a DNA-binding and DNA-bending domain (4,38). In agreement with previous findings (16,39,40), we show in this manuscript that the HMG domain also figures
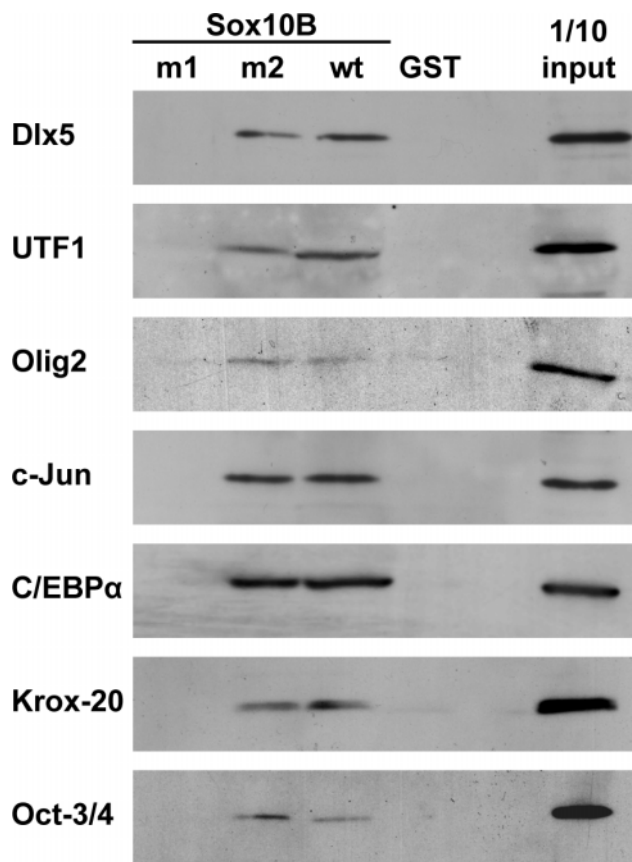
Figure 7. Mapping interactions to the C-terminal tail region of the SoxE HMG domain in GST pulldown experiments. DNA-binding domains of transcription factors Dlx5, UTF1, Olig2, c-Jun, C/EBPα, Krox-20 and Oct-3/4 were expressed in HEK293 cells and analyzed for their ability to interact with GST or the GST-Sox10B fusion carrying amino acids 133–203 of Sox10. GST-Sox10B was generated in a wild-type version (wt) and in mutant versions that either carried the R176E substitution (m1) or the R161E, K165E double mutation (m2). The amount of each transcription factor present in one-tenth of the extract before pulldown (1/10 input) is shown in the right lane of each panel. Transcription factors were detected on western blots via their T7 tag.

prominently as a protein–protein interaction domain that establishes contacts with a wide variety of DNA-binding domains found in many other transcription factors. This additional feature of the HMG domain may prove equally important for the architectural function of Sox proteins on gene regulatory sequences.

Protein–protein interactions were mediated by the C-terminal part of the HMG domain which includes helix 3 and the C-terminal tail region (29,30). Conspicuously, both regions are not involved in establishing DNA contacts indicating that they are still available for interactions with other proteins after Sox proteins bind to DNA. In particular, the C-terminal tail region which is unstructured in solution is a good candidate for these interactions. This was experimentally supported by the disruption of all interactions by the m1 mutation in which an arginine residue in the C-terminal tail region was substituted by a glutamate. The C-terminal tail region is furthermore specific to the HMG domain of Sox proteins and is not found in other HMG domains. Accordingly, no interactions were observed with the HMG domains of LEF-1, HMGB1 or UBF-1.

The fact that the HMG domain of Sox proteins interacts quite promiscuously with many different DNA-binding domains may explain why different Sox proteins exhibit at least partial redundancy in tissues where they are co-expressed, such as Sox8 and Sox9 in the developing testis and spinal cord, or Sox8 and Sox10 in oligodendrocytes or the developing enteric nervous system (6–8,41). It also argues that each Sox protein should be able to accept different transcription factors as interaction partners in different tissues or at different stages of development in a particular cell type. Accordingly, Sox10 is known to team up with Pax3 in neural crest-derived precursor cells, with Krox-20 in Schwann cells of the peripheral nervous system and with Mitf in melanocytes (13,14,16–18). This flexibility in the choice of interaction partners could be one of the reasons for the pleiotropic functions of Sox proteins.

Unfortunately, our results do not yield clues for the origin of specificity in the interaction between Sox proteins and its partners *in vivo*. Thus it needs to be clarified how promiscuous protein–protein interaction in solution is turned into specific functional cooperativity on target genes. It is important to stress that the contacts between HMG domain and DNA-binding domains of other transcription factors are fairly weak. Weak interactions are easily reversible and will allow the HMG domain to constantly change interaction partners in solution. It is likely that this weak protein–protein interaction has to be additionally strengthened to translate into functional consequences. This may be partially achieved through regions outside the DNA-binding domain of both the Sox protein and the partner transcription factor. Additionally, reinforcement may occur when the Sox protein and its partner transcription factor both bind to the same regulatory sequence. In the simplest case, physical contact between the DNA-binding domains of a Sox protein and its transcriptional partner may elicit cooperative binding of both proteins. This, however, requires that the binding sites for Sox protein and transcriptional partner are closely spaced in a composite recognition element (4). Such composite elements have indeed been identified in several gene regulatory sequences (16,19,39,40,42,43). For Sox10, only one composite element has been described in the *c-Ret* enhancer which is cooperatively bound and synergistically activated by Sox10 and Pax3 (16). Quite interestingly and in agreement with our findings, synergistic activation of the *c-Ret* enhancer required direct interaction of the paired domain of Pax3 with the HMG domain of Sox10 (16). However, other promoters which are synergistically activated by Sox10 and its partners do not exhibit this arrangement of binding sites in a composite element. This includes the *Mitf* promoter which is known to be synergistically activated by Sox10 and Pax3 (13,18), the *Dct* promoter which is synergistically activated by Sox10 and Mitf (17), the Schwann-cell specific enhancer of the *Krox-20* gene which is activated by Sox10 and Brn-2 or Oct-6 (15), and the *Connexin-32* promoter which is jointly activated by Sox10 and Krox-20 (14). Whether physical contacts between HMG domain and DNA-binding domain of partner transcription factor are only important on regulatory sequences with composite elements or also on those regulatory sequences that contain separate binding sites for SoxE proteins and their transcriptional partner proteins in greater distance, remains to be seen. Our attempts at resolving this issue by analyzing

the transcriptional activity of the Sox10 m1 mutant on various Sox10-dependent regulatory sequences failed, because the m1 mutation (and alternative substitutions of R176 by alanine or lysine, data not shown) not only abolished interactions with other DNA-binding domains in solution but also disrupted one of the two nuclear localization signals (44) and therefore interfered with efficient translocation into the nucleus (data not shown).

Whatever the exact mechanism, our finding of numerous interactions with DNA-binding domains of other transcription factors throws new light on to the HMG domain of Sox proteins. It may help to understand the pleiotropic nature of Sox protein function and functional equivalency among Sox proteins.

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