

A novel 9-amino-acid transactivation domain in the C-terminal part of Sox18

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Abstract

Sox transcription factors are members of the Sry-related protein family that play multiple roles mainly during development. Sox18 has been implicated in the development of hair follicles as well as the blood and lymphatic vasculature, due to the identification of mutations that result in the *ragged* phenotype in mice, and in the hypotrichosis lymphedema telangiectasia syndrome in humans. Sox18 consists of an N-terminal high-mobility group DNA binding and a central transactivation domain, followed by a C-terminal region of unknown function. We show here that this C-terminal domain consists of three blocks that are highly conserved within a subgroup of the Sox family, and that the central so-called charged block comprises an additional strong transactivating domain. This activity can be pinpointed to a recently described 9aa transactivation motif that mediates the interaction with the transcriptional cofactor TAF9. These result can explain previously controversial data on the functional consequences of Sox18 mutations in mice and humans.
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The Sox family of high mobility group (HMG) box transcription factors that are homologous to the Y-chromosome encoded sex-determining factor SRY plays important roles in embryonic development [1–4]. Sox18, together with Sox7 and -17, constitutes the subgroup F within this family. Mutations in Sox18 are the underlying cause of the *ragged* phenotype in mice, which is characterized not only by the name-giving coat defects but also by cardiovascular abnormalities, e.g., edema, cyanosis, dilation, distention, and rupture of peripheral blood vessels, as well as lymphatic defects [5–9]. A corresponding phenotype, hypotrichosis–lymphedema–telangiectasia (HLT), is correlated with Sox18 mutations in humans [10]. Previous structure–function studies have revealed an N-terminal HMG DNA binding domain (DBD), a central transactivation domain (TAD), followed by an uncharacterized C-terminal region [11]. All cognate *ragged* mutations, as well as

some in the HLT affected families, are located in a short stretch at the very end of the central TAD and the beginning of the C-terminal region, and lead to premature stop codons [6]. However, although these mutants retain most of the central TAD, they display strongly impaired transactivating functions [6,9]. Furthermore, Sox18^{-/-} mice show only a very mild phenotype [8], suggesting that the naturally occurring *ragged* mutants act as dominant-negative proteins and that other transcription factors, possibly members of the Sox subgroup F family, have redundant function. However, the generation of Sox18/Sox17 double mutant mice has only partially supported this view [12].

This apparent discrepancy between the dn function of the *ragged* mutants and the mapping of the TAD to the central part of the protein prompted us to analyze the previously uncharacterized C-terminal region of Sox18 in more detail. Bioinformatic analysis of the C-termini of subgroup F Sox family members revealed three conserved blocks including highly conserved residues. Generation of deletion mutants and reporter gene analysis revealed a strong TAD within one of the conserved blocks that could

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be attributed to the presence of a recently discovered 9aa transactivation motif [13] present in a subset of transcription factors. These results demonstrate an important and previously unrecognized function of the C-terminal domain of Sox18.

Materials and methods

Bioinformatics. Multiple sequence alignment of Sox18, -17, and -7 from human, mouse, rat, *Xenopus laevis*, and chicken was done using ClustalW (<http://www.ebi.ac.uk/clustalw>). The degree of conservation of amino acids within the three blocks was visualized with the aid of the programs Boxshade (http://www.ch.embnet.org/software/BOX_form.html) for Fig. 1, and WebLogo (<http://weblogo.berkeley.edu/>) for Fig. 4. The prediction of the 9aa TAD was done online from the National EMBnet-Node Austria (<http://embl.bcc.univie.ac.at/toolbox/9aatad/>).

Plasmids. The cDNA comprising the coding region of human Sox18 was isolated by RT-PCR from human umbilical vein endothelial cells and cloned into the vector pCMV-myc (Clontech). Deletion mutants lacking the serine (ΔS), serine plus charged (ΔS/C), and the serine, charged, and

proline blocks (ΔS/C/P), the respective Gal4-based deletion mutants with and without the central TAD, as well as a construct representing the human HLT mutant (family 3; [10]) that contains a cystein to stop mutation at position 240 were all generated by PCR. Point mutations were introduced using the QuikChange site-directed mutagenesis kit (Stratagene). For generation of the 9aa construct, an EcoRI–PstI fragment where positions YL330/331 were mutated to QS was generated by PCR followed by replacing the respective fragment in the Gal4-based ΔS mutant without the central TAD (Gal4ΔS). The Sox18 dependent luciferase reporter construct was generated by insertion of two copies of an oligonucleotide with SpeI compatible ends containing three Sox18 binding sites (AACAAAG; [11]) into the vector pUBT-Luc containing a minimal (–37 bp) thymidine kinase promoter [14]. All primer sequences are given in the Supplementary Material. All constructs were verified by sequencing.

Cell culture and reporter gene analysis. HEK293 cells were obtained from the American Type Culture Collection and propagated in DMEM containing 10% FCS. They were transfected in triplicate with the different reporter constructs by the calcium-phosphate method. Luciferase levels were normalized for β-gal expression from a cotransfected CMV-βgal vector.

Western blotting. Sox18 mutants and Gal4-fusion proteins were detected after transfection into HEK293 cells by Western blotting after

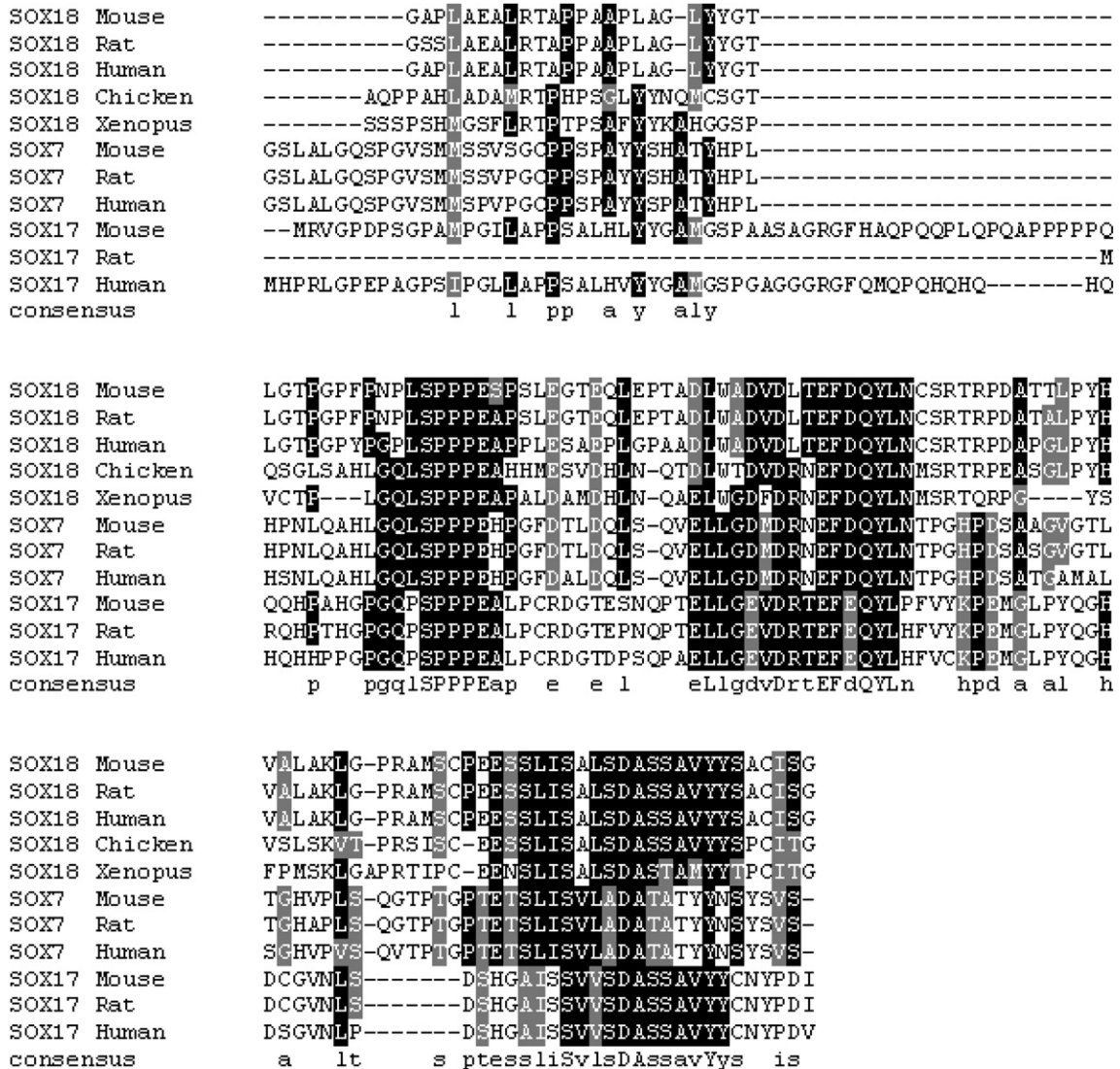


Fig. 1. Multiple sequence alignment of the C-terminal regions of Sox18, -7, and -17 from human, rat, mouse, chicken, and *Xenopus*. The program ClustalW was used, conserved residues are shaded, and the three conserved blocks (serine, charged, and proline block, respectively) are boxed.

separation on 12% PAGE and transfer to Hybond C Extra (Amersham) membranes using polyclonal rabbit anti-Sox18 or anti-Gal4 antibodies (Santa Cruz) at a dilution of 1:5000, followed by HRP-linked anti-rabbit IgG, and detection by Super Signal West Pico (Pierce).

Results

Identification of three highly conserved blocks in the C-terminus of Sox18

Bioinformatic analysis of the C-terminal region of the three subgroup F family members Sox18, -17, and -7 from different species including humans, mice, rat, chicken, and *Xenopus* revealed the presence of three highly conserved blocks. They were termed proline, charged, and serine block according to the predominance of the respective amino acids (Fig. 1). We generated a series of deletion mutants of Sox18 lacking either the C-terminal serine block (ΔS), the middle charged plus serine ($\Delta S/C$), or all three blocks ($\Delta S/C/P$; Fig. 2A). Following confirmation of expression (Fig. 2B), the activity of these mutants was analyzed by transient transfection into HEK293 cells using a Sox18 responsive reporter construct. As shown in Fig. 2C, deletion of the serine block caused an approximately 50% reduction of luciferase activity; additional deletion of the charged block resulted in a further reduction by again >50%, whereas deletion of also the proline block had no additional effect. Truncation of the Sox18 by a stop

codon at position 240 (the human HLT mutant) also resulted in almost complete loss of activity (Fig. 2C).

In order to uncouple possible effects of DNA binding from transactivation, and to analyze the contribution of the transactivating properties of the newly discovered blocks in relation to the published central transactivation domain, we replaced the Sox18 DBD with the heterologous Gal4 DBD in all three constructs. In addition, all deletion constructs were generated without the central TAD (Fig. 3A). Again, expression was confirmed by Western blotting (Fig. 3B), and the constructs analyzed by Gal4-dependent reporter gene analysis. Here, deletion of the serine block caused a small gain of activity, whereas deletion of the charged block resulted in strongly impaired transactivation. Deletion of the proline block did not further reduce the activity of this Sox18 mutant significantly. The latter two mutants retained only approximately 80% of the activity of the wt Sox18. A similar picture was seen in the corresponding mutants lacking the central TAD: whereas deletion of the serine block led to a small increase in activity, a strong reduction was caused by deletion of the charged block (Fig. 3C).

Identification of the 9aa motif in the charged block

Given the high conservation of certain amino acids within the three blocks, we mutated several of them in parallel to the experiments described above (Fig. 4A). However, no significant effect of any of these mutants on the transactivating properties could be detected, including the tyrosine in the charged block (not shown). Recently, one of us (M.P.) has bioinformatically defined a short 9-amino acid motif with transactivating properties, termed 9aa, that is contained in a subset of transcription factors [13]. This 9aa TAD was found within the charged block (Fig. 4A). In p53, it has been shown that changes of the hydrophobicity profile of the 9aa motif rather than individual amino acids are the important parameter for transactivating activity [15]. Mutation of two amino acids within this motif in p53 to Q and S resulted in greatly diminished transactivation [15]. We therefore mutated the corresponding amino acids Y and L (pos. 330/331) in Sox18 in the same way. In contrast to the single Y mutation described above, this double mutation changed the hydrophobicity profile of the 9aa motif in the charged block (see [Supplementary Material](#)) and reduced the transactivating activity of the Gal4-based Sox18 TAD construct (ΔS) by approximately 85% as compared to the non-mutated form (Fig. 4C). This demonstrates that the 9aa TAD within the charged block is the major element contributing to the transactivating properties of Sox18.

Discussion

The four cognate allelic *ragged* mutants of Sox18 in mice (*ragged*, *ragged-like*, *ragged-Jackson*, and *ragged opossum*) are all located within 30 amino acids at the end, or in

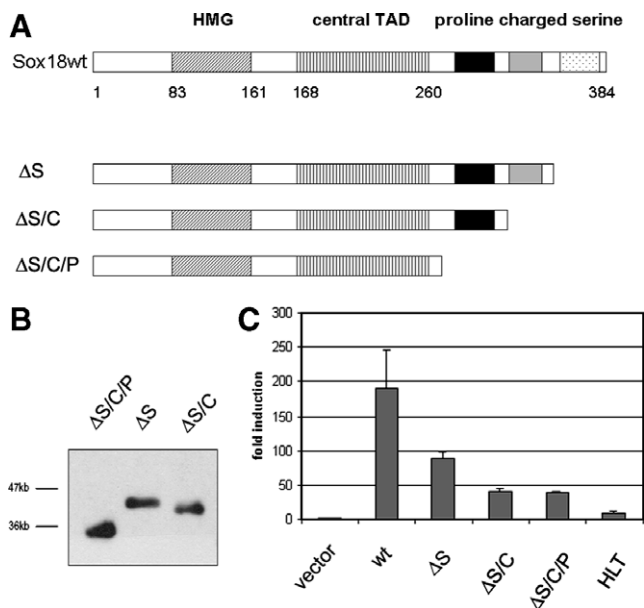


Fig. 2. Construction and analysis of Sox18 C-terminal mutants. (A) Schematic representation. (B) Expression of the constructs was confirmed by Western blotting after transient transfection into HEK293 cells. (C) Reporter gene analysis. The three constructs, the wild-type (wt) Sox18, and the empty parental vector were transiently transfected into HEK293 cells together with an Sox18-dependent minimal promoter-luciferase reporter and CMV- β gal as internal control. Luciferase levels were measured 2 days after transfection and normalized for β -galactosidase.

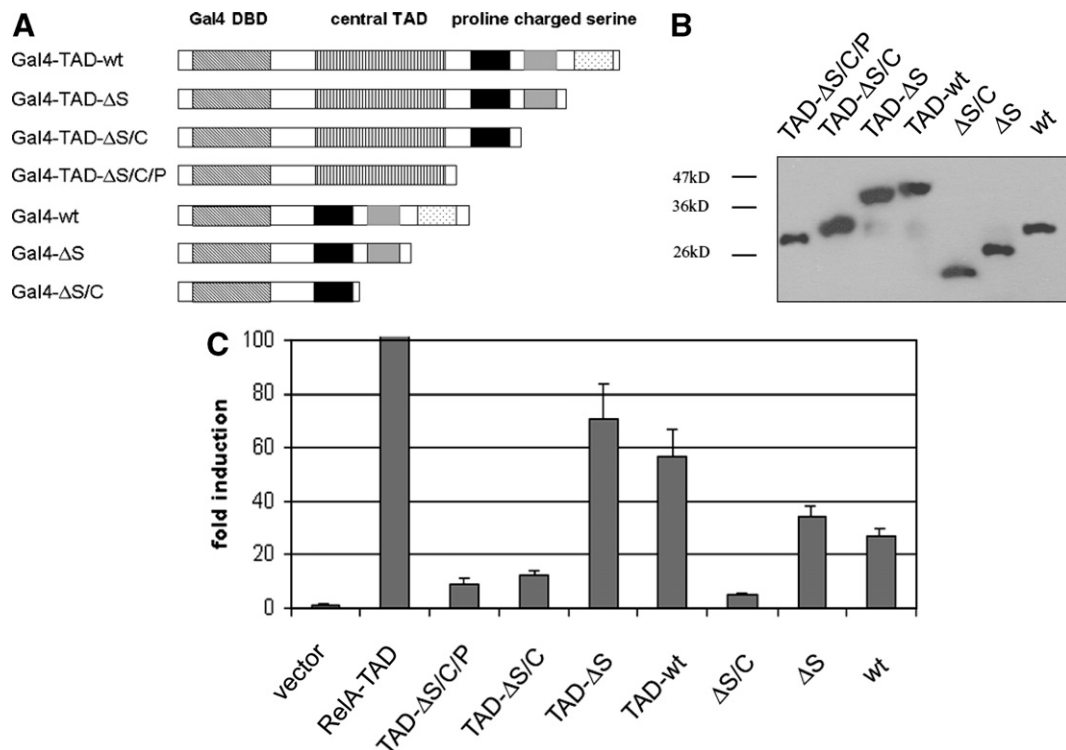


Fig. 3. Analysis of Gal4 fusion constructs. (A) Schematic representation of the three mutants with the Sox18 DBD replaced by the Gal4 DBD. Two sets were generated, one retaining and one without the central TAD. (B) Expression of the constructs was confirmed by Western blotting after transient transfection into HEK293 cells, using an anti-Gal4 antibody. (C) Reporter gene analysis was performed as in Fig. 2C, except that a Gal4-dependent luciferase reporter plasmid was used. RelA-TAD: NF- κ B-TAD construct used as positive control.

one case beyond the end, of the published central TAD. They cause frameshift mutations that result in a short missense sequence and a premature stop codon, and suggest that this region may be a hot spot for mutations in Sox18. One somehow paradox, yet interesting, aspect is that the severity of the *ragged* phenotype appears to increase the more downstream the mutation occurs, i.e., the more of the original Sox18 sequence around the end of the central TAD is retained [6], suggesting additional regulatory events to be operative in this region.

Therefore, all *ragged* mutants essentially contain the intact central TAD, which is not consistent with the observation that they result in a dominant-negative phenotype: cotransfected Sox18 *ragged* could inhibit the activity of the wild-type proteins in transient transfections, whereas knockout of Sox18 in mice showed a phenotype that is by far less severe as compared to the *ragged* mutants. In humans, three mutations in families affected by HLT are known [10], where two occur in the region of the HMG box, predicting impairment of DNA binding. One occurs in the same region as the *ragged* mutations in mice and has not been functionally characterized previously. In our experiments, this HLT mutant showed severely impaired activity (Fig. 2C). Taken together, this suggests a role for the hitherto uncharacterized C-terminus in the regulation of Sox18 activity.

Through comparison of the C-terminal sequences of all Sox F family members, including different species, we identified three conserved blocks. Although mutation of individual highly conserved amino acids within these blocks did not affect transactivation, we could by deletion analysis attribute the main transactivating function to the middle block, the so-called charged block. A difference in the contribution of the C-terminal serine block was observed depending whether the experiments were performed either with the intact Sox18 DBD or with constructs containing the heterologous Gal4 DBD: whereas deletion of this block in constructs containing the Sox18 DBD resulted in a 50% reduction of activity, this was not observed in the Gal4-based constructs. It could be hypothesized that this difference is due to intra-molecular interactions within Sox18 that cannot take place when the DBD is replaced.

The 9aa motif that occurs in a subset of mammalian, yeast, and viral transcription factors has been proposed to mediate the interaction with general co-factors. It is an essential part of the transactivating function of, e.g., p53, VP16, HSF1, NF- κ B, NF-IL6, and NF-AT [13], all of which utilize TAF9 [16]. The presence of this motif within the charged block of Sox18 suggests that it also belongs to this subgroup of transcription factors. Taken together, the identification of a strong TAD in the

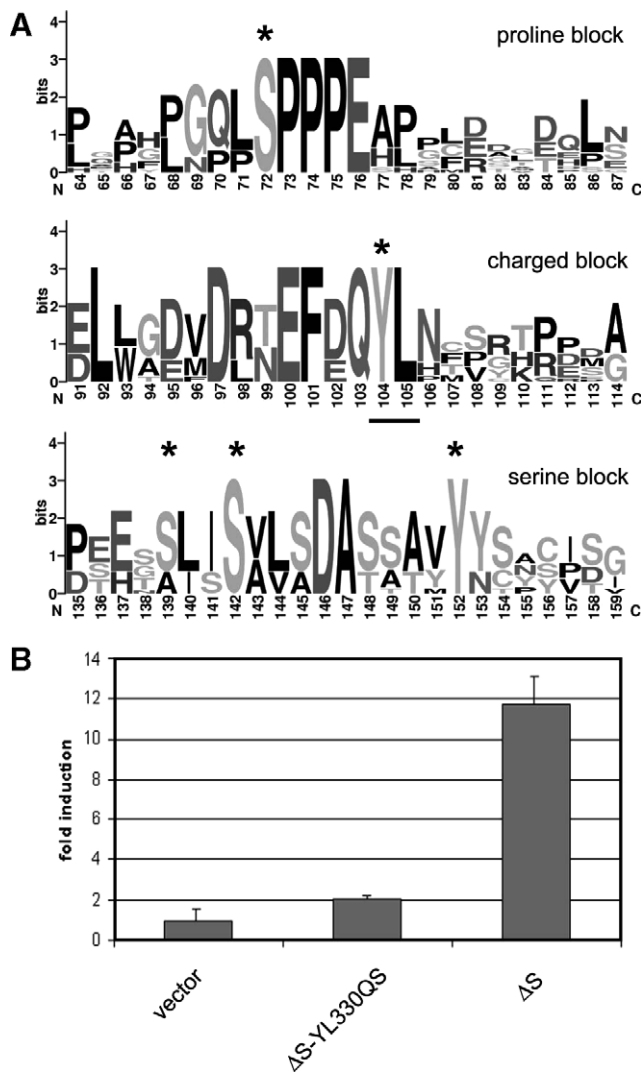


Fig. 4. Mutation of individual amino acid and identification of the 9aa domain. (A) Depiction of conservation of individual amino acids within the three blocks using the WebLogo program. The size of the letters indicates the degree of conservation (bits). The mutated residues are indicated by asterisks. The two residues mutated in the 9aa construct (Y and L in the charged block) are underlined. (B) Reporter gene analysis of the 9aa mutant YL330QS, as compared to the parental (Δ S) construct. Reporter gene analysis was performed as in Fig. 3C.

C-terminal part of Sox18 that contributes significantly to Sox18 transactivation can explain the dominant-negative nature of the *ragged* and corresponding human HLT mutants.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.06.095.

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