

# The DNA-binding specificity of SOX9 and other SOX proteins

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## ABSTRACT

**SOX (SRY-related HMG box) proteins are transcription factors that have critical roles in the regulation of numerous developmental processes. They share at least 50% homology in their HMG domains, which bind the DNA element AACAAT. How different SOX proteins achieve specific regulation of target genes is not known. We determined the DNA-binding specificity of SOX9 using a random oligonucleotide selection assay. The optimal SOX9 binding sequence, AGAACAATGG, contained a core DNA-binding element AACAAT, flanked by 5' AG and 3' GG nucleotides. The specific interaction between SOX9 and AGAACAATGG was confirmed by mobility shift assays, DNA competition and dissociation studies. The 5' AG and 3' GG flanking nucleotides enhance binding by SOX9 HMG domain, but not by the HMG domain of another SOX factor, SRY. For SRY, different 5' and 3' flanking nucleotides are preferred. Our studies support the notion that SOX proteins achieve DNA sequence specificity through subtle preferences for flanking nucleotides and that this is likely to be dictated by signature amino acids in their HMG domains. Furthermore, the related HMG domains of SOX9 and Sox17 have similar optimal binding sites that differ from those of SRY and Sox5, suggesting that SOX factors may co-evolve with their DNA targets to achieve specificity.**

## INTRODUCTION

SOX (SRY-related HMG box) proteins have critical functions in a number of developmental processes, including sex determination, skeleton formation, pre-B and T cell development and neural induction (1). Mutations in SOX9 result in Campomelic Dysplasia (CD), a severe dwarfism syndrome associated with gonadal and brain abnormalities. SOX9 plays a direct role during chondrogenesis by binding and activating the chondrocyte-specific enhancer of the *Col2a1* gene (2–5). Interestingly, SOX9 is also expressed in cells of the brain and testis, which do not express *Col2a1*, suggesting that SOX9 activates other target genes (6,7).

At least 20 SOX proteins have been identified, all of which contain an HMG domain, which facilitates sequence-specific DNA-binding and shares >50% identity with the HMG domain of SRY, the human testis-determining factor. The high affinity DNA-binding sites of SRY (8), Sox5 (9) and Sox17 (10) have been defined to be (A/T)(A/T)CAA(A/T), using *in vitro* random

oligonucleotide selection procedures. Given that SOX proteins bind this common DNA element, how is target gene specificity achieved? While specificity can be achieved through restricted tissue distribution (1) and protein interaction (11–13), we sought to understand better the intrinsic specificity between SOX9 and DNA. We have defined the preferred DNA-binding site of SOX9 to be AGAACAATGG, which contains the SOX core-binding element (SCBE), AACAAT. Flanking 5' AG and 3' GG nucleotides enhance binding by SOX9, but not SRY. For SRY, different flanking 5' and 3' nucleotides are preferred which contribute to DNA-binding affinity. These results show that SOX proteins achieve DNA sequence specificity through subtle preferences for nucleotides flanking the SCBE and that this specificity is likely to be dictated by signature amino acids in the HMG domain.

## MATERIALS AND METHODS

### Production of recombinant SOX9 and SRY proteins

pT7-SOX9box plasmid: DNA encoding SOX9 HMG box (amino acids 101–184) was generated by PCR using SOX9 cDNA (14) as a template and PCR primers (5'-CCACATCATATGAACAAGCC-GCACGTCAAG-3') and (5'-CGCTAAGCTTAGTTCTTCCACC-GACTTCCTC-3'), and subcloned into *NdeI* and *HindIII* digested pT7-7 (United States Biochemicals). pT7-hSRYbox plasmid: encoding SRY amino acids 55–143 has been described (11). Plasmids were transformed into *Escherichia coli* BL21 (DE3) and soluble protein extracts prepared (15). Extracts were analysed by SDS-polyacrylamide gel electrophoresis (PAGE) and gels stained with Coomassie Brilliant Blue. The concentration of active HMG domain protein in the soluble extract was determined from the *x*-axis intercept of Scatchard plots (see later, 16).

pBS-HA-SOX9 plasmid was constructed for the production of full-length human SOX9 by amplification from SOX9 cDNA (14) by PCR, using primers (5'-ATCATAGATCTGCCACCATGTA-CCCATAACGATGTTCCGGATTACGCTAGCCATATGAATCT-CCTGGACCCCTTC-3') and (5'-CGTCTTGCTGAGCTCGG-CGT-3'), and subcloned into pBluescript (Stratagene). SOX9 protein (509 amino acids) was produced using the TNT T3-coupled rabbit reticulocyte lysate system (Promega). Yields of SOX9 were estimated from the incorporation of <sup>35</sup>S-methionine in reactions which were performed in parallel with non-radiolabelled syntheses. Non-radiolabelled SOX9 was utilised for electrophoretic mobility shift assay (EMSA) gels (17) where phosphorimage analysis of free and bound DNA probe was required.

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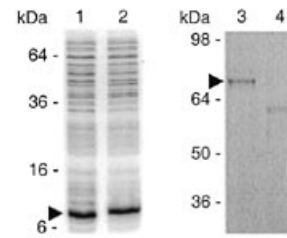
### Random oligonucleotide selection assay

The double-stranded oligonucleotide, R76, contains a 26 bp random sequence flanked by two PCR primer sites containing *Bam*HI and *Eco*RI restriction endonuclease sites, to facilitate the cloning of selected sequences (18). Typical binding reactions (10  $\mu$ l) containing 3 nM SOX9 or luciferase control, 9 nM of  $^{32}$ P-labelled DNA and binding buffer (19) were incubated for 20 min on ice and analysed by EMSA. The location of the SOX9–DNA complex in the early rounds was located using an oligonucleotide of the same length which contained the *in vivo* preferred DNA-binding site of the T cell-specific transcription factor, TCF-1 (19). This DNA probe was also incubated with SOX9 and electrophoresed in an adjacent lane for reference. Selection and amplification of selected DNA was repeated for seven cycles after which DNA inserts were sequenced. Sequences were aligned using PileUp (Wisconsin Package), a computer program which creates a multiple sequence alignment from a group of sequences using progressive pairwise alignments.

### DNA probes and EMSA

Oligonucleotides were synthesised on an Applied Biosystems 394 DNA/RNA Synthesiser. DNA probes were prepared by annealing complementary oligonucleotides, prior to a fill-in reaction using AMV Reverse Transcriptase and [ $\alpha$ - $^{32}$ P]dCTP. Ten 29mer double-stranded DNA probes were prepared. The upper strands were as follows: Probe S9WT (5'-GGGTAAAC-**AGAACAATGGAATCTGGT**AGA-3') contains the wild-type SOX9 preferred DNA-binding site (bold) with an SCBE. Probe S9M1 (5'-GGGTAAAC**GTAAACAATAA**ATCTGGTAGA-3') contains a mutated SOX9 preferred DNA-binding site with 5' and 3' flanking mutations (bold). Probe S9M2 (5'-GGGTAAAC-**GTAACAATGGAATCTGGT**AGA-3') contains a mutated SOX9 preferred DNA-binding site with 5' flanking mutations (bold). Probe COL2C1 (5'-GGGCCCTCTCC**ACAATG**CCCCCTGTC-3') and Probe COL2C2 (5'-GGGTCGAGAAA**AGCCCATTCATG**AGAGC-3') contain SOX9 binding sequences (bold) within the *Col2a1* enhancer (2). Probe CD3- $\epsilon$  (5'-GGGA-GACTGAGA**CAAA**AGCGCTCTCACAC-3') contains the TCF-1 DNA-binding site (bold) within the *CD3- $\epsilon$*  enhancer (19). Probe SRYWT (5'-GGGTAACTAA**ACAATAGA**ATCTGGTAGA-3') contains the wild-type SRY preferred DNA-binding site (bold) with an SCBE. Probe SRYM1 (5'-GGGTAAAC**GCAACA**ATCTGGTAGA-3') contains a mutated SRY preferred DNA-binding site with the 5' and 3' flanking nucleotides mutated (bold) to those which are least preferred by SRY for DNA-binding (8). Probe SRYM2 (5'-GGGTAAAC**GCAACA**ATAGAATCTGGTAGA-3') contains a mutated SRY preferred DNA-binding site with 5' flanking mutations (bold). Unlabelled Probe AllMut (5'-GGGTAAAC**GTCGCGG**TAATCTGGTAGA-3') where the SCBE is fully mutated (bold).

DNA-binding reactions (10  $\mu$ l) containing binding buffer (19), SOX9 or SRY protein and radiolabelled DNA were analysed by EMSA. In competition experiments, binding buffer, protein and unlabelled competitor DNAs were incubated on ice for 10 min then radiolabelled DNA probe S9WT added and incubated for a further 20 min before analysis by EMSA. For the determination of dissociation constants ( $K_d$ ), binding reactions contained a fixed amount of protein incubated with increasing amounts of DNA probe ranging from 10 to 162 nM (16). Quantitation of



**Figure 1.** Production of SOX9 and SRY proteins. Coomassie Brilliant Blue stained 18% SDS-PAGE gel of soluble lysate from *E. coli* harbouring *pT7-SOX9box* or *pT7-hSRYbox* plasmids. Following IPTG induction, cell lysates show a major band at 9 kDa, corresponding to SOX9 HMG domain (lane 1, arrow) and at 10 kDa corresponding to SRY HMG domain (lane 2). Fluorograph of a 7.5% SDS-PAGE gel of radiolabelled coupled transcription/translation reactions programmed with the full-length SOX9 gene or the luciferase gene. SOX9 migrates at ~69 kDa (lane 3, arrow), and luciferase migrates at 56 kDa (lane 4). Molecular weight standards are shown to the left of each panel.

radiolabelled DNA was by phosphorimage analysis of free and bound DNA probe (Analytical Imaging Station; FUJIX BAS 2000). Dissociation constants were calculated according to the method of Scatchard (16). The mean concentration of active protein was estimated from the  $x$ -axis intercept, derived from three experiments performed in duplicate.

## RESULTS

### Production of full-length SOX9 and HMG domains of SOX9 and SRY

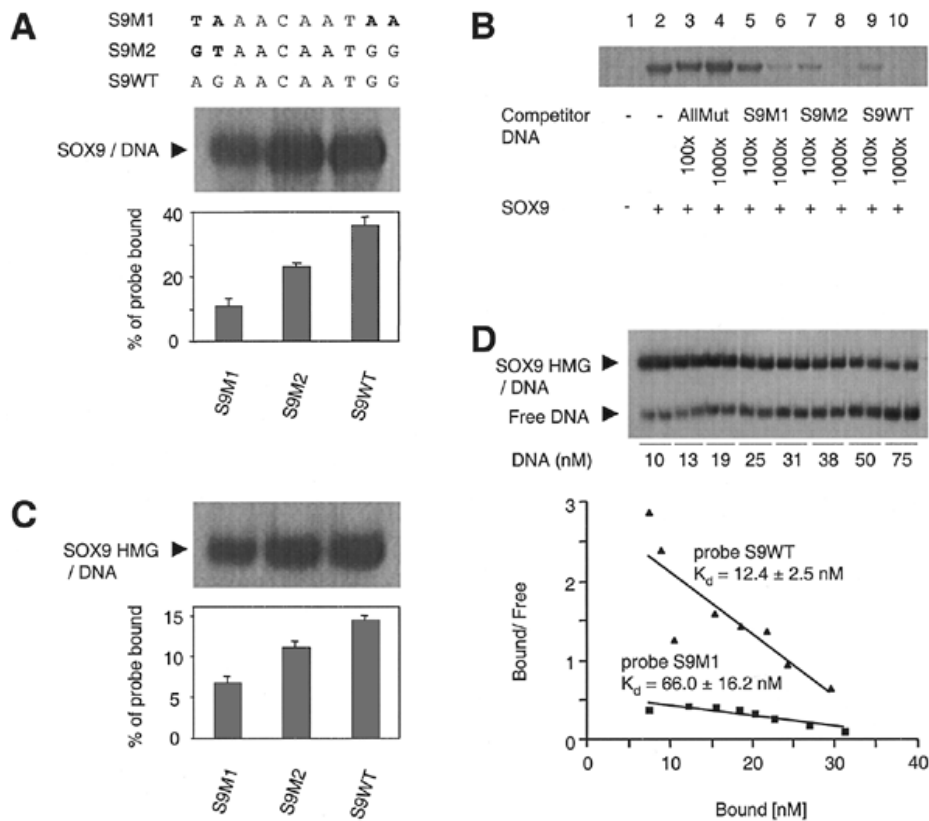
SDS-PAGE analysis of cell lysates following IPTG induction show a major band at 9 kDa, consistent with the predicted molecular weight of SOX9 HMG domain (Fig. 1, lane 1). Yields of HMG domains were estimated to be 6–8 mg/l of bacterial cell culture. Radiolabelled full-length SOX9, analysed by SDS-PAGE and fluorography, migrates at 69 kDa, consistent with the predicted molecular weight of 61 kDa (Fig. 1, lane 3). Yields of SOX9 protein were estimated to be 0.4 ng/ $\mu$ l lysate.

### The preferred DNA-binding site of SOX9

The preferred *in vitro* DNA target sequence of human SOX9 was determined using a random oligonucleotide selection assay. To monitor the progress of the selection procedure, SOX9 selected  $^{32}$ P-labelled DNA probes following 0, 1, 3, 5 and 7 cycles of selection were analysed by EMSA (Fig. 2A). The random oligonucleotide R76 (0 selection cycles) failed to form detectable protein–DNA complexes. Enrichment for SOX9-bound oligonucleotides was clearly evident following three cycles, after which the proportion of SOX9-specific DNA-binding sites in the pool continued to increase until the seventh cycle of selection. The DNA amplified after seven cycles of selection, failed to form protein–DNA complexes with the luciferase control lysate, indicating that only SOX9 protein present in the reticulocyte lysate, was responsible for the observed enrichment.

DNA from 79 of the selected clones was sequenced and aligned. Sixty-eight of the clones share a CAAT sequence—a feature of SOX binding sites (Fig. 2B). Of these, 14 contain two or more CAAT sites. The varied separation and orientation between these sites on different clones and the minority of these particular clones in the total population of selected sequences,





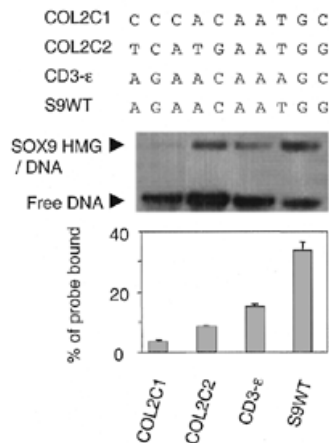
**Figure 3.** Contribution to binding of flanking nucleotides of the SOX9 preferred DNA-binding site. **(A)** EMSA of full-length SOX9 with three radiolabelled DNA probes (upper panel) representing versions of the SOX9 preferred DNA-binding site. Mutations within the site are shown in bold. Binding reactions contained full-length SOX9 (3 nM) and radiolabelled DNA probe S9WT, S9M1 or S9M2 (2 nM). Only the protein–DNA complex is shown. The bar graph shows the percentage of each DNA probe bound by full-length SOX9 ( $\pm$ SE). Values shown are the mean of three experiments. **(B)** Competition analysis of the specificity of full-length SOX9 for DNA probe S9WT. Full-length SOX9 (2 nM) was incubated with radiolabelled DNA probe S9WT (10 nM) with no competitor (lane 2) or in the presence of 100-fold (1  $\mu$ M) or 1000-fold (10  $\mu$ M) molar excess of unlabelled competitors: AllMut (lanes 3 and 4), S9M1 (lanes 5 and 6), S9M2 (lanes 7 and 8) and S9WT (lanes 9 and 10). Lane 1 contains radiolabelled DNA probe S9WT alone. Only the protein–DNA complex is shown. **(C)** EMSA of SOX9 HMG domain with three radiolabelled DNA probes [(A), upper panel]. Binding reactions contained SOX9 HMG domain (3 nM) and radiolabelled DNA probe S9WT, S9M1 or S9M2 (2 nM). Only the protein–DNA complex is shown. The bar graph shows the percentage of each DNA probe bound by SOX9 HMG domain ( $\pm$ SE). Values shown are the mean of three experiments. **(D)** Scatchard analysis of the equilibrium binding of SOX9 HMG domain to DNA probes S9WT and S9M1, in which the latter has mutated 5' and 3' nucleotides flanking the SCBE. Binding reactions, containing a fixed amount of SOX9 HMG domain ( $\sim$ 33 nM) and increasing concentrations of DNA probe (10–162 nM), were resolved by non-denaturing gel electrophoresis and bound and free DNA probe quantitated from three experiments performed in duplicate. An EMSA, representative of one experiment is shown in the upper panel. A Scatchard plot is shown in the lower panel. The points plotted are the mean of duplicate data points from one experiment and the  $K_d$ s given represent the mean from three experiments ( $\pm$ SE). The active protein concentration of SOX9 HMG domain was estimated from the average  $x$ -axis intercept value of six Scatchard plots to be  $32.8 \pm 7.7$  nM.

S9WT is 9- and 4-fold higher than for the two type II collagen gene regulatory elements (DNA probes COL2C1 and COL2C2, respectively). DNA-binding to S9WT is 2-fold higher than for the TCF-1 site in the CD3- $\epsilon$  enhancer site (DNA probe CD3- $\epsilon$ ).

### SRY prefers particular nucleotides for DNA-binding

To establish whether a different SOX protein, SRY, also binds preferentially to the SOX9 preferred DNA-binding site, the binding affinities of SRY HMG domain for DNA probes S9WT, S9M1 and S9M2 were compared (Fig. 5A). In contrast to SOX9, SRY HMG domain exhibits comparable affinity for all three DNA probes suggesting that there is no observable preference for the particular flanking nucleotides that are selected by SOX9. Inspection of sequences selected by SRY in an *in vitro*

oligonucleotide selection assay (8) shows that flanking 5' TA and 3' AG nucleotides are more commonly selected by SRY. To test the contribution of these flanking nucleotides to binding by SRY HMG domain, three DNA probes were prepared and analysed by EMSA (Fig. 5B). Affinity of SRY HMG domain for its preferred DNA-binding site (SRYWT) is higher than for probe with mutated 5' and 3' flanking nucleotides (SRYM1). SRY HMG domain binds DNA probe containing the 3' AG but not the 5' TA nucleotides (SRYM2) with similar affinity to SRYWT, suggesting that only the 3' AG nucleotides contribute to binding by SRY HMG domain. Thus SRY prefers particular flanking nucleotides that differ from those preferred by SOX9. We investigated whether other SOX proteins show preferences for particular nucleotides flanking the SCBE.



**Figure 4.** Comparison of SOX9 binding to previously defined *in vivo* DNA-binding sites of SOX9 and CD3- $\epsilon$ . EMSA of SOX9 HMG domain with four radiolabelled DNA probes (upper panel). Binding reactions contained SOX9 HMG domain (17 nM) and radiolabelled DNA probe COL2C1, COL2C2, CD3- $\epsilon$  or S9WT (10 nM). Protein–DNA complex and free DNA are shown. The bar graph shows the percentage of each DNA probe bound by SOX9 HMG domain ( $\pm$ SE). Values shown are the mean of two experiments.

**Table 1.** Nucleotide preferences for DNA-binding of SOX proteins

Protein	<i>In vitro</i> preferred site	Reference
SOX9	<b>A G A A C A A T G G</b>	This study
Sox17	<b>A G G A C A A T G</b>	10
SRY	<b>T/A T/A A A C A A T A G</b>	8
Sox5	<b>T T/A A A C A A T A</b>	9

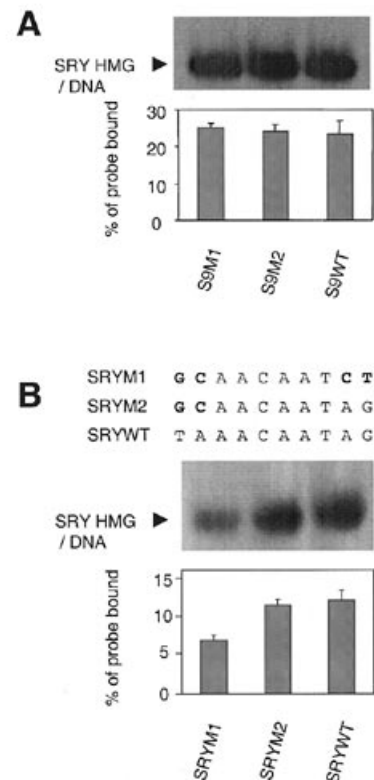
*In vitro* preferred nucleotide sequences of DNA-binding sites of SOX proteins, determined by *in vitro* oligonucleotide selection assays. The DNA-binding sequence of Sox17 is composed of two AACAAATs in opposite orientations with different flanking nucleotides. The preferred sequence of one of these is shown. Flanking nucleotides are shown in bold.

### DNA-binding preferences reflect sequence similarities between HMG domains of SOX proteins

Analysis of *in vitro* selected DNA sequences by other SOX proteins also suggest the preference for particular nucleotides flanking the SCBE (Table 1). SOX9 and Sox17 prefer 5' AG nucleotides, while SRY and Sox5 prefer 5' (T/A)(T/A) and 5' T(T/A), respectively. Further, SOX9 prefers 3' GG nucleotides and Sox17 a single 3' G, while SRY prefers 3' AG and Sox5 a single 3' A.

### DISCUSSION

In the present study we define, *in vitro*, the high affinity DNA-binding site of SOX9 to be AGAACAATGG which includes the SCBE (bold) and flanking 5' AG and 3' GG. These flanking nucleotides enhance DNA-binding of SOX9, since affinity is reduced when these nucleotides are mutated. This is the first evidence that nucleotides flanking the SCBE play a role in DNA-binding. Both full-length SOX9 and its HMG domain alone show a preference for binding the 10 bp extended site. We find that the binding of SOX9 to its preferred DNA-binding site



**Figure 5.** SRY also prefers an extended DNA-binding site. (A) EMSA of SRY HMG domain with three radiolabelled DNA probes: S9WT, S9M1 and S9M2. Binding reactions contained SRY HMG domain (3 nM) and radiolabelled DNA probe S9WT, S9M1 or S9M2 (2 nM). Only the protein–DNA complex is shown. The bar graph shows the percentage of each DNA probe bound by SRY HMG domain ( $\pm$ SE). Values shown are the mean of three experiments. (B) EMSA of SRY HMG domain with three radiolabelled DNA probes (upper panel) representing versions of the SRY preferred DNA-binding site. Mutations within the site are shown in bold. Binding reactions contained SRY HMG domain (2 nM) and radiolabelled DNA probe SRYM1, SRYM2 or SRYWT (3 nM). Only the protein–DNA complex is shown. The bar graph shows the percentage of each DNA probe bound by SRY HMG domain ( $\pm$ SE). Values shown are the mean of four experiments.

( $K_d = 12.4 \pm 2.5$  nM) is comparable with that reported of SRY to its preferred DNA-binding site ( $K_d = 10$  nM) (22). In contrast, the HMG domain of SRY shows no preference for the 5' AG and 3' GG flanking nucleotides selected by SOX9. SRY prefers a DNA-binding site that includes flanking 3' AG nucleotides. The binding site of SRY on DNA, as revealed in our experiments, is longer than the 7 bp footprint defined by methylation interference (23). We conclude that the differences in the HMG domain amino acids of SRY and SOX9 produce different *in vitro* DNA sequence specificities which are likely to reflect different *in vivo* DNA targets.

Inspection of the NMR structure of the HMG domain of LEF-1 in complex with DNA suggests particular amino acid residues that might determine specificity for nucleotides flanking the SCBE. The sequence of the LEF-1 binding site in the TCR- $\alpha$  enhancer (TTCAAAGG) bears some similarity to the extended site bound by SOX9, in that it includes flanking 3' GG nucleotides (20). In the NMR structure of LEF-1 (24), amino acids Lys27, Glu28, Ser29 and Ala30 are involved in contacting one or both of the 3' GG nucleotides present in the LEF-1 DNA-binding site. An alignment

of the HMG domains of LEF-1, SOX9, Sox17, Sox5 and SRY reveal the corresponding residues in the SOX9 and Sox17 HMG domains to be Leu, His, Gln and Ala, respectively, and those in the HMG domains of SRY and Sox5 to be Met, Arg/His, Gln and Ser. Thus the specific residues in these four positions appear to correlate with the 3' flanking nucleotides preferred by each SOX protein. Furthermore, it may be worth noting that Ala30 is conserved in LEF-1, SOX9 and Sox17 which all prefer a 3' G following the SCBE, while this Ala is substituted with Ser in SRY and Sox5, which both prefer a 3' A following the SCBE. The similar DNA-binding preferences and residues at the region involved in contacting the 3' flanking nucleotides suggest that SOX proteins have co-evolved with their DNA targets to achieve DNA specificity.

The profiles of SOX9 expression in many adult and foetal tissues suggest that SOX9 acts upon several target genes *in vivo* (6,7). Compared to SOX9's *in vitro* preferred DNA-binding site, SOX9 binds sub-optimally to two SOX9 binding sites from the *Col2a1* enhancer. We postulate that the presence of enhancers with varying affinities for SOX9 might contribute to differential regulation of target genes.

SP1 and YY1 are two examples where their *in vitro* selected DNA-binding sites represent *in vivo* recognition sites (25–27). A database search for DNA regulatory elements bearing the high affinity SOX9 DNA-binding site revealed the best match to be AGAACAATGC (mismatch in bold) within the *Col4a2* enhancer. By EMSA, this element forms a complex, which is specific for cells that make collagen IV, and is therefore a promising candidate gene for regulation by SOX9 (28).

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