Original Paper

Ophthalmic Research

Ophthalmic Res 2005;37:301–309
DOI: 10.1159/000087774

Received: November 19, 2004
Accepted after revision: April 18, 2005
Published online: August 23, 2005

Agonistic and Antagonistic Action of AP2, Msx2, Pax6, Prox1 and Six3 in the Regulation of *Sox2* Expression

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Key Words

AP2 · Msx2 · Pax6 · Prox1 · Six3 · *Sox2* · Reporter gene assay · Eye development · Transcription factor

Abstract

Sox2 transcription factor is expressed in neural tissues and sensory epithelia from the early stages of development. Particularly, it is known to activate crystallin gene expression and to be involved in differentiation of lens and neural tissues. However, its place in the signaling cascade is not well understood. Here, we report about the response of its promoter to the presence of other transcription factors, AP2α, Msx2, Pax6, Prox1 and Six3, in a transient reporter gene assay using HEK293 cells as recipient cells. Taking our data together, AP2, Pax6 and PROX1 can activate the *Sox2* promoter. Msx2 has an inhibitory effect, whereas Six3 does not affect the Sox2 promoter. These data indicate a common activating cascade at least for AP2, Pax6, Prox1 and Sox2.

Introduction

Sox2 is one of the very important transcription factors during eye development. First indication of its importance is the pathological situation demonstrated by its deletion or mutation: mutation analysis of 5 children suffering from bilateral anophthalmia discovered *de novo* point mutations in the SOX2 gene leading to truncation at various positions of the protein or even its entire deletion to be causative for the disorder [1]. Surprisingly, mice heterozygous with respect to a targeted *Sox2* null mutation showed no eye phenotype, whereas homozygotes died early in gestation [2]. Moreover, previous studies [3] demonstrated that SOX2 initiates (together with PAX6) lens development. Kamachi et al. [4] showed that in chicken Sox2 is involved in the regulation of δ-crystallins. In mouse, the expression of *Sox2* can be observed in the lens placode, but not in the mature lens. At E10.5, *Sox2* is transcribed in the optic stalk, in the presumptive neural retina, in the optic cup and in the lens pit; however, it is not observed in the presumptive pigmented retina [5].

From the studies mentioned above, some downstream targets of Sox2 are known, however, it is not clear, how the *Sox2* expression itself is regulated. There are a few reports dealing with far-distance enhancers [6, 7]. In agreement with previous studies [8] and based upon further primer extension assays, a single major transcription start site was determined approximately 406 bp upstream of the Sox2 protein coding region [9]. A basic characterization of this proximal promoter identified the *CAAT* box and two other unknown activating elements [9]. When looking for factors involved in Sox2 expression,

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one might get some ideas from overlapping expression patterns. Indeed, the *Sox2* expression pattern overlaps at least in part with the pattern of the transcription factors *AP2*, *Msx2*, *Pax6*, *Six3* and *Prox1*. *Pax6* as master control gene of eye development [10] is detected in the neural ectoderm as well as in the surface ectoderm, even before the formation of the lens placode [11]. *Six3* is present during early eye development (E9.5) in the optic stalk, in the presumptive retina and in the lens vesicle and later also in the lens [12]. At E9.5, *Prox1* is present in the lens placode and at E10.0 in the lens vesicle [13]. At later stages, it is translated in the lens and in horizontal and amacrine cells of the retina [14]. Similarly, *AP2α* is expressed in the developing lens placode and later on in the lens pit. At E12.5, it is expressed in the anterior lens epithelium, and at E15.5 AP2α is the only member of the AP2 transcription factor family, which is present in the developing lens. AP2α was also found in the developing neural retina and later in a subset of the ganglion cell layer and the inner nuclear layer [15]. In contrast, *Msx2* expression is observed in the surface ectoderm, in the lens placode and in the distal part of the optic vesicle (E 9.5). Later on, *Msx2* is transcribed in the lens [16].

The partial temporal and spatial overlapping expression of *AP2*, *Msx2*, *Pax6*, *Prox1* and *Six3* with *Sox2* suggested that these five transcription factors might be involved in the regulation of the *Sox2* gene promoter. To get an overview about the regulation of the *Sox2* gene expression, we dissected the *Sox2* core promoter into 3 fragments and tested their promoter activity in a luciferase reporter gene system depending on the cotransfection of expression vectors coding for the transcription factors AP2, Msx2, Pax6, Prox1, and Six3.

Material and Methods

Expression Systems and Western Blotting

Pax6 cDNA, which was kindly provided by R. Balling in the pBluescriptKS(+) vector, was digested with *Hin*dIII and *Bam*HI and subcloned into eukaryotic expression vector pcDNA3.1 (Invitrogen, Karlsruhe, Germany). For the cotransfection of *Pax6+exon5a*, cDNA was amplified from C3H mice using the primer pairs Pax6-L1 (5′-AAAGGCAGAAGACTTTAACCA-AGGGC-3′) and Pax6-R2 (5′-ACTGCTGTGTCCACATAGT-CATTGGC-3′); the PCR product was cloned into the pCR-TOPO vector (Invitrogen). Depending on its original orientation, the corresponding fragments were excised by *Bam*H1/*Not* or *Bst*X1/*Xba*I and subcloned into the pcDNA3.1 vector (Invitrogen); the correct cloning was confirmed by sequencing.

The generated *Pax6* and *Msx2* expression plasmids were taken for in vitro expression using the reticulocyte lysate of the TNT® Quick Coupled Transcription/Translation System (Promega, Mannheim, Germany) containing [^{35}S] methionine. An aliqout of 1 µl lysate was electrophoresed through a 7% SDS-polyacrylamide gel; the gel was dried under vacuum and radioactivity was detected overnight on a Fuji imaging plate using a PhosphorImager SI (Amersham Pharmacia Biotech, Freiburg, Germany).

For amplification of *AP2*, the primers Ap2a_f (5′-CGCAG-AGGGGCAAATCC-3′) and Ap2a_r (5′-TGAGGCAAGGCGCT-GAGTA-3′) were used. The PCR product was cloned into the pCR-TOPO vector and subsequently into the pcDNA3.1 vector.

In some cases, Western blot analysis was performed to detect the expressed gene products. Therefore, 10 µg of each cell preparation was electrophoresed through an SDS-12% polyacrylamide gel and electroblotted onto a PVDF membrane. The membrane was blocked overnight using 5% powdered milk in phosphate-buffered saline and incubated with a 1:200 dilution of an antibody raised against the C-terminus of the mouse Pax6 protein (H2N-QVPGSEPDMSQYWPRLQ-COOH) (HISS Diagnostics GmbH, Freiburg, Germany). Immunoreactivity was visualized with anti-rabbit Ig and enhanced chemiluminescence (Amersham). In the case of AP2α, the proteins are transferred from a PAA gel on a nitrocellulose membrane. The membrane was incubated with the primary (anti AP-2a, clone 8G8/5; Biomol, Hamburg, Germany) and the secondary antibody (goat anti-mouse IgG, HRP conjugate, Biomol) and washed 3 times with PBS buffer. After transferring the membrane into a fresh dish, 3 ml Visualizer™ Working Solution (Biomol) was added and incubated for 5 min at room temperature until staining was achieved.

For expression of *PROX1* and *Six3* the corresponding pcDNA3 or pcDNA3.1 expression constructs have already been described [17, 18].

Promoter Sequences

Sequences of interest were first analyzed using the MatInspector program [19] for putative binding sites of transcription factors; the Msx2 binding site was checked according to Ma et al. [20], and the Prox1- and Six3-binding sites according to Lengler et al. [17].

Genomic DNA was prepared from mouse spleen or tail tips of 3-week-old mice according to standard procedures. Forward primer covering the *Sox2* gene (GenBank Acc. No. AF118260) from position –426 to –403 contains a *Bam*HI site for cloning (5′-CCA-GC<u>GGATCC</u>ACAGTCGCCCTGAACCACCCATGG-3′), whereas the reverse PCR primer reaching from +9 to –12 bp has a *Hin*dIII restriction site (5′-CCAGC<u>AAGCTT</u>TTGAACAAGTTAATAGA-CAACC-3′). The PCR conditions have been described previously [18]. During the PCR of 40 cycles, the annealing temperature of 53°C was reduced to 45°C, 1°C per five cycles, in a 'touchdown' PCR [21].

The PCR products were cloned into the cloning site of the pPLLucII reporter vector [22] using *Bam*HI and *Hin*dIII restriction enzymes and then transformed into DH5α bacteria. DNA was prepared using a plasmid NucleoSpin column (Macherey Nagel, Düren, Germany). The inserted fragments have been confirmed by sequence analysis.

Transfection and Reporter Gene Assay

For luciferase *(Luc)* reporter assays, HEK293 fibroblast cells were cultivated in 96-well plates for 24 h and transfected by PolyFect transfection reagent (Qiagen, Hilden, Germany) using 0.7 µg plasmid DNA. The DNA mix in transfection reaction contained 200 ng reporter vector, a DNA fragment representing part of the

Sox2 promoter fused to the *Luc* reporter gene, 10–40 ng effector (either *AP2*-, *Pax6*-, *Six3*-, *Msx2*-pcDNA3.1 or *Prox1*-pcDNA3, or the parental plasmid pcDNA3.1 as negative control) and 20 ng pRL-SV40 for internal transfection control (Promega, Mannheim, Germany). It contains a cDNA encoding *Renilla* luciferase, which was originally cloned from the marine organism *Renilla reniformis* (R*luc*). The SV40 early enhancer/promoter region provides strong, constitutive expression of R*luc* in a variety of mammalian cell types.

Cells were harvested 48 h after transfection, and cellular extracts were assayed with the Dual-Luciferase Reporter Assay System (Promega) twice in triplicate, and the standard error of the mean was calculated (n = 6).

Results

Expression Analysis of the Recombinant Transcription Factors AP2, Msx2 and Pax6

HEK293 cells were transfected with pcDNA3.1 containing the coding region of *AP2α*. The corresponding protein lysates were analyzed by Western blot; the antibody recognized the AP2α band at 52 kDa, which was not present in the controls (fig. 1a).

The *Msx2*-pcDNA3.1 expression plasmid was checked using the reticulocyte lysate, and it yielded the proteins in their full size (29 kDa; fig. 1b). Similarly, the presence of Pax6 proteins was tested, however, we observed three different bands corresponding to various size products (fig. 1c). The middle band represents the correct Pax6 protein; its molecular weight was 48.2 kDa. The short fragment of 31.6 kDa is most likely due to the use of a second ATG as start codon during the in vitro translation. The larger fragment (63 kDa) is suggested to arise by ignoring the first stop codon; however, the in vitro translation machinery accepts the next stop codon 363 bp downstream, which is in the 3′ UTR of *Pax6* followed by two further stop codons within 40 bp. Therefore, Western blot analysis was performed in transfected and nontransfected HEK293 cells to confirm the correct size of the product derived from the transfected Pax6-pcDNA3.1 vector. Since only one signal of Pax6 was observed by Western blot analysis in the transfected cells, we conclude that the fidelity of the translation in cultured cells is better compared with the in vitro conditions, and that Pax6 is not expressed endogenously in HEK293 cells (fig. 1c). The correct expression of the *Prox1* and *Six3* expression constructs has been described previously [17, 18].

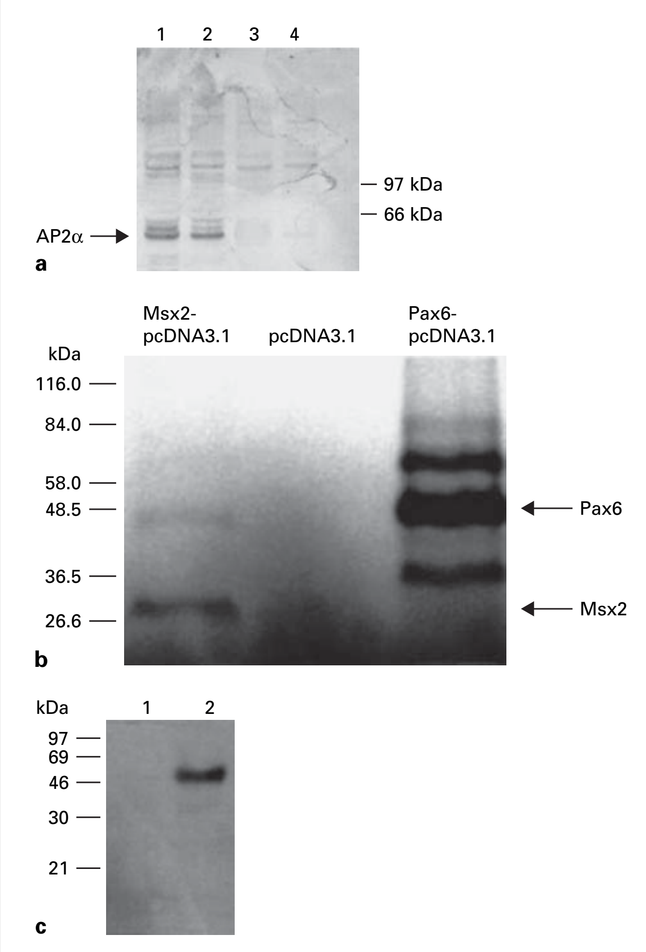

Fig. 1. Expression analysis of recombinant *AP2*, *Msx2* and *Pax6*. **a** Protein from lysates of HEK293 cells transfected with pcDNA3.1 for AP2 [1, 2] or control [3, 4] pcDNA3.1 were analyzed by Western blot; the antibody recognized the AP2 band at 52 kDa, which is not present in the two control lanes. The amount of protein loaded on the gel were 60 μg (lanes 1 and 3) or 30 μg (lanes 2 and 4). **b** Expression plasmids pcDNA3.1 containing *Msx2* or *Pax6* were transcribed and translated with reticulosyte lysate containing [^{35}S] methionine. A 29-kDa band for Msx2 is observed. Three signals were detected using the Pax6-pcDNA3.1, whereas the 48-kDa signal is the expected one and the bands above and below are considered to be additional products of the in vitro translation (see Discussion). For control, in vitro expression was performed with plasmid pcDNA3.1 without any cDNA sequence. **c** Lysates of 293 cells transfected with pcDNA3.1 for control [1] and Pax6-pcDNA3.1 [2] were immunostained with antibody raised against the C-terminal part of Pax6.

Characterization of the Sox2 *Promoter*

Using 2 kb of the 5′-flanking region of the mouse *Sox2* gene (GenBank Acc. No. AF118260), the computer program PromoterInspector (http://genomatix.gsf.de) predicts a promoter, which lies close to the start codon (bp –287 to –84) of the murine *Sox2* gene. Furthermore, we analyzed an extended region (till –426) of the *Sox2* pro-

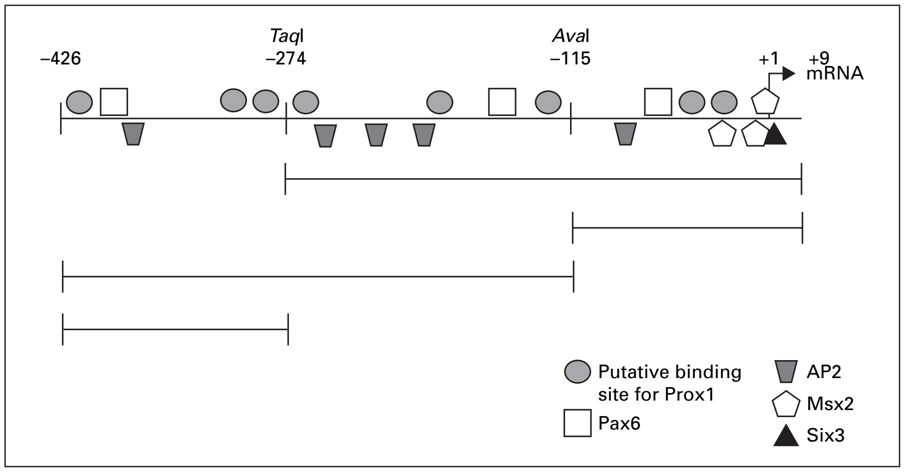

Fig. 2. Schematic overview of a 434-bp fragment of the murine *Sox2* core promoter including 426 bp upstream of the transcription start site (arrow) and 9 bp downstream. Additionally, 4 deletion constructs are indicated.

Table 1. Selected binding sites for transcription factors in the *Sox2* promoter – prediction by MatInspector

Transcription factor	Begins	Ends	Core	Matrix similarity	Consensus sequence[1]	*Sox2* promoter sequence[2]
AP2	–408	–396	0.904	0.861	MKCCCSCNGGCGN	CACCCATGGGCCT (+)
	–238	–226	0.976	0.870		CCCCCCCCGCCCC (–)
	–202	–190	1.000	0.921		GGCCCGCAGCCGG (–)
	–186	–174	0.976	0.897		CTCCCCCGCGCGG (+)
	–107	–95	0.857	0.918		AACCCTCTGGCGA (+)
Six3	–5	+3	1.000	0.962	NNSATTANN	TCTATTAAC (+)
Pax6	–392	–374	1.000	0.877	NNAGKKCCAGGNNMG	AAGCTGGGGCCAGGGTGGG (–)
	–160	–142	1.000	0.817		GAGCCGAGCCCAGCAGAGC (–)
	–98	–80	1.000	0.811		CGCCCGAGCCCAGCCTCGC (–)
Msx2	–62	–57	ND	0.833	CAATTA	CAATCA (–)
	–43	–38	ND	0.833		CATTTA (+)
	–4	+1	ND	0.833		CTATTA (+)
Prox1	–412	–389	ND	0.958	ANNNNANNNNNNNGNNNNNNCNNA	GAACCACCCATGGGCCTTGCCCCA (+)
	–313	–290	ND	0.958		ACAGCACCAAGACGACAGCTCCTT (–)
	–299	–276	ND	0.958		AAGGAAGTGGGTAAACAGCACCAA (–)
	–265	–242	ND	0.958		TTTTCAGCAACAGGTCACGGCGCA (–)
	–171	–148	ND	0.958		AGCCCAGCAGAGCGCTGTGCCCCG (–)
	–135	–112	ND	0.958		ATGAAAGGGGGCGGGGCCTGCCGC (–)
	–83	–60	ND	0.958		GGCGCAGGAGCCGGCGCTCGCTGA (+)
	–59	–36	ND	0.958		AATAAATGGGTTTCCGGCGGCCAA (–)

ND = Not determined; (+) = coding strand; (–) = antisense strand.
[1] Core sequence underlined.
[2] Genbank acc. No: AF118260 (1482–1916).

moter for transcription factor-binding sites using *MatInspector Professional* (http://genomatix.gsf.de) with respect to factors, which are known to be involved in eye development: the program predicted three Pax6-binding sites, 5 AP-2 binding sites and 8 Prox1-binding sites (fig. 2 and table 1). However, only one of these predicted Prox1-binding sites (between position –412 and –389) will be recognized also by prospero, the *Drosophila* ho-

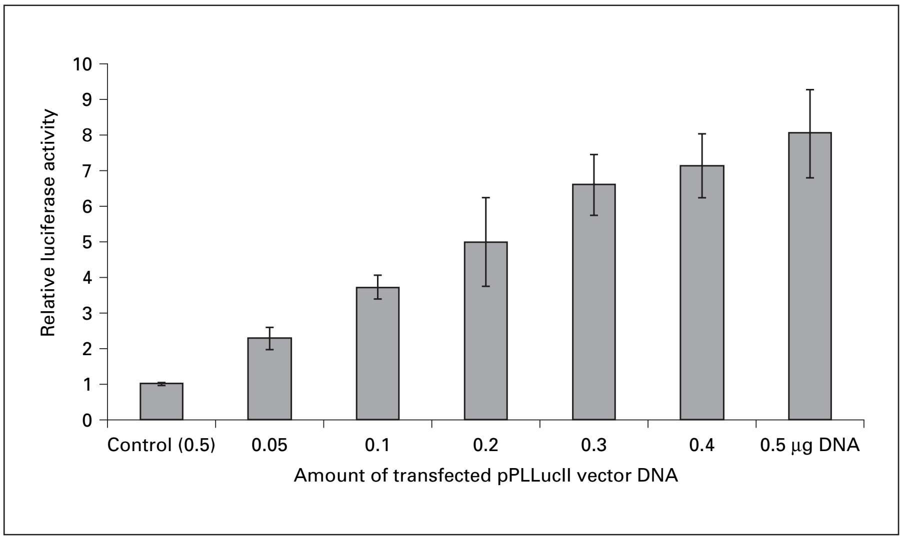

Fig. 3. Basal activity of the *Sox2* core promoter. Promoter activity is monitored after transfection of the entire *Sox2* promoter into HEK293 fibroblast cells. Reporter vector containing the –426/+9 *Sox2* promoter sequence exhibits in a dose-dependent manner up to 9-fold activity compared with reporter without any promoter sequence. Bars indicate SEM.

mologue of Prox1 [23]. Therefore, we suggest this particular site as the most likely Prox1-binding site in the *Sox2* promoter. Moreover, three putative Msx2-binding sites [20] were found in the *Sox2* core promoter between positions –62 and +1. Additionally, a Six3 responsive element [17] is suggested to be localized between –5 and +3 in the *Sox2* promoter (0.83% identity).

With regard to this in silico analysis, we cloned a 435-bp fragment directly upstream of the *Sox2*-coding region in front of the luciferase reporter gene. For cotransfection experiments, the kidney HEK293 epithelium cell line was used as transfection system [24], which is supposed to lack endogenous eye-specific factors. Transfection experiments including the *Sox2* core promoter revealed an activity, which is 8- to 9-fold higher compared with the reporter construct without promoter sequences used as control (fig. 3).

Activation of the Sox2 Promoter by AP2, Pax6 and PROX1

As outlined in figure 4a–d, the entire *Sox2* promoter can be stimulated by Pax6, AP2α and Prox1. The stimulating effect of Pax6 can be seen with both isoforms of Pax6, the canonical form (a) and the alternatively spliced Pax6-5a form (b). Increasing amounts of a cotransfected *Pax6* expression vector lead to constantly rising dose-response stimulation; we could not observe a saturation effect in our experiments. Pax6 showed its highest activity if the fragment –426/–274 was used (fig. 5d), which is in agreement with the prediction of a Pax6-binding site at –394/–376. Since the other fragments showed clear but weaker stimulations by Pax6 (fig. 5), the other Pax6-binding sites might have lower binding affinities.

AP2α is able to stimulate the entire *Sox2* core promoter in a dose-dependent manner up to 2.5-fold (fig. 4c). Moreover, compared with the core promoter, AP2α has a 4-fold higher stimulatory action at the deletion construct –426/–274 (fig. 5d), indicating that in this region an AP2-binding site is very likely. This is in agreement with the predicted AP2 site between positions –408 and –396. However, since also the other fragments exhibit stimulatory effects after cotransfection with AP2α, further binding sites with lower binding affinities might be considered.

Furthermore, PROX1 exhibits a similar activating effect on the *Sox2* promoter as described above for Pax6. Highest activation (4-fold) was observed using 40 ng of the *PROX1*-pcDNA3 expression vector (fig. 4d). In contrast to Pax6, the Prox1 activity is the lowest in the small 5′ fragment (–476/–274), but highest in the 3′ fragment (–115/+9). This is in agreement with the predicted presence of the two Prox1-responsive elements in this region (–83/–60 and –59/–36). Moreover, also both larger fragments contain remarkable Prox1-dependent stimulatory action. Since the small 5′-fragment does not respond to Prox1, this stimulatory action needs to be restricted to the middle fragment, which also includes the prospero-related binding site.

Six3 and Msx2 Inhibit Sox2 *Promoter Activity*

Since we identified a putative Six3 binding site in the *Sox2* promoter (fig. 2), we cotransfected the *Sox2* pro-

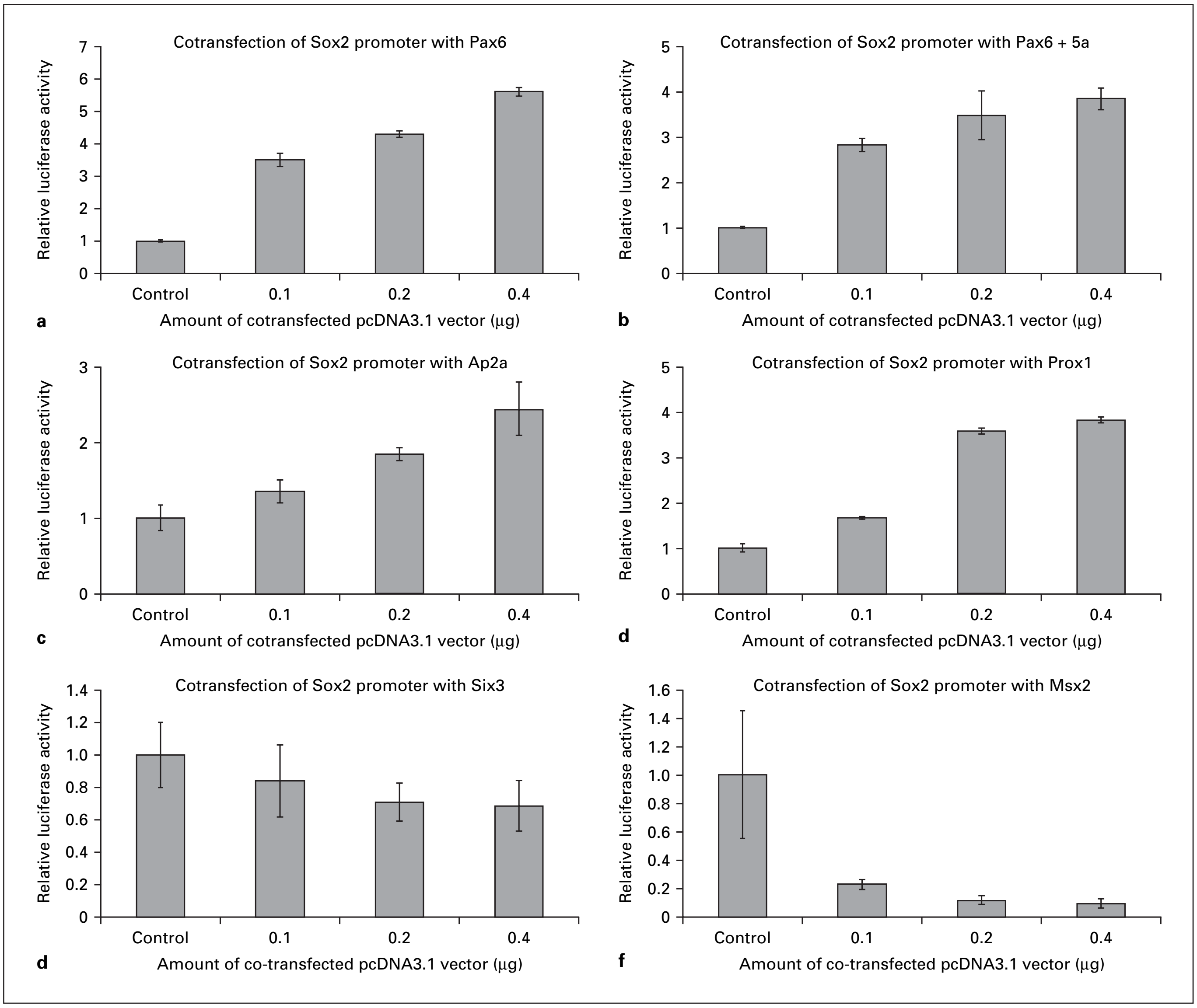

Fig. 4. Action of AP2, Pax6, Msx2, Six3 and Prox1 on the entire mouse *Sox2* promoter. The luciferase activity at the entire *Sox2* promoter can be stimulated additionally, if increasing amounts of Pax6 (**a**, **b**), AP2 (**c**), or Prox1 (**d**) are cotransfected together with 0.5 µg *Sox2*$^{-426/+9}$-luciferase plasmid. In contrast, cotransfection with Six3 (**e**) and Msx2 (**f**) inhibit the luciferase activity indicating a dose-dependent repressor effect. Bars indicate SEM.

moter with the Six3 expression vector. *Six3* weakly represses *Sox2* promoter activity (fig. 4e: ~60% rest activity after transfection of 40 ng DNA). Since the results show a remarkable variation as indicated by the high SD bars, this repression might not be statistically significant. In contrast to this overall effect at the entire promoter, Six3 slightly stimulates the luciferase activity, if the short 3′ fragment is used only (fig. 5b), supporting the prediction of a Six3-responsive element at position –5/+3. In all other fragments, Six3 has no obvious effect (fig. 5a, c, d).

Msx2 has a very strong inhibitory effect on the *Sox2* promoter, which becomes apparent using just 10 ng of the *Msx2*-pcDNA3.1 expression vector. Using 20 ng of *Msx2* effector, the *Sox2* promoter activity is completely inhibited. The rest activity is due to the reporter plasmid lacking any promoter sequence (fig. 4f). This repression is lost only if the most 5′ fragment is used, indicating that this

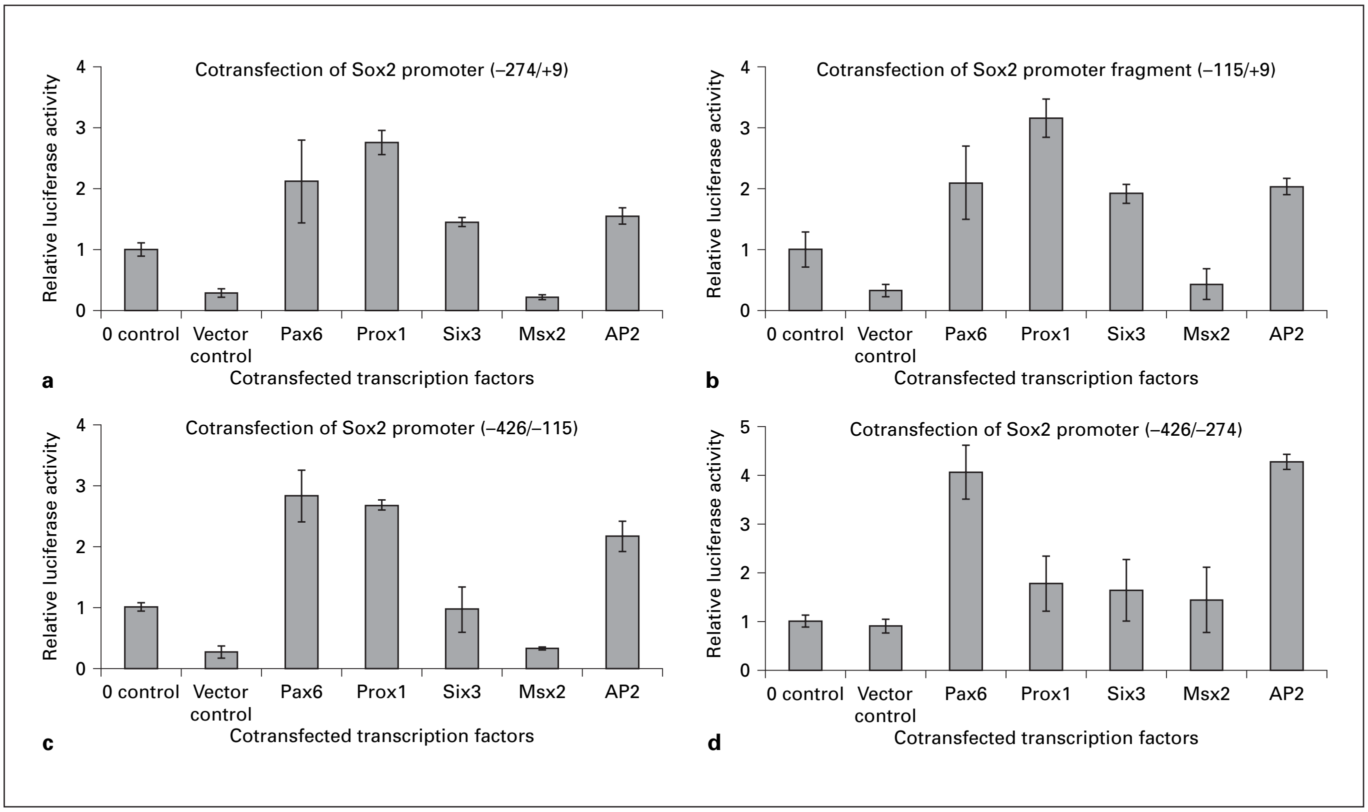

Fig. 5. Summary of the activities at the *Sox2* promoter. All five transcription factors (Pax6, Prox1, Six3, Msx2 and AP2α) are tested (in each case, 40 ng DNA was transfected) at the 4 fragments of the *Sox2* promoter: (**a**) –274/+9; (**b**) –115/+9; (**c**) –426/–115; (**d**) –426/–274. Data are given as stimulating factors of the basal activity at the corresponding fragment with an empty pcDNA3.1 vector (expression = 1); the additional 'vector control' indicates the empty luciferase vector. Values are given as mean ± SEM from 6 transfections (twice in triplicates).

particular fragment does not have a Msx2-responsive site. Moreover, the other two fragments showed an inhibition by Msx2, indicating that an additional Msx2 binding might be discussed upstream of position –274 (fig. 5a–d).

Discussion

In this communication we could characterize the *Sox2* promoter with respect to the activity of several transcription factors on a fragment covering more than 400 bp upstream of its transcription start site. We identified Pax6, AP2α and Prox1 as activators of the *Sox2* promoter and Msx2 as its strong inhibitor; the action of Six3 was not as clear as for the other four with a slight inhibitory trend at the entire promoter, but with a low stimulating activity at the small 3′ fragment.

Initially, we used the MatInspector program to predict putative transcription factor-binding sites. Based upon this prediction we split the entire 435-bp fragment into three partially overlapping fragments to narrow the approximate position of the particular binding sites. Indeed, most of the predicted binding sites might be active, since the corresponding fragments showed a clear stimulatory or inhibitory action. However, there are two exceptions: first, there is obviously no Prox1 binding site in the small 5′ fragment –426/–274, and second, we observed an Msx2 response in the middle fragment of the Sox2 promoter between –274 and –115. It is noteworthy that the corresponding –426/–115 fragment shows a remarkable promoter activity even in the absence of the proximal promoter (which should include the 'core' promoter). Since the Sox2 promoter contains only a CCAAT box (on the reverse strand), but no TATA box, and since even the destruction of the CCAAT box reduces the activity of the

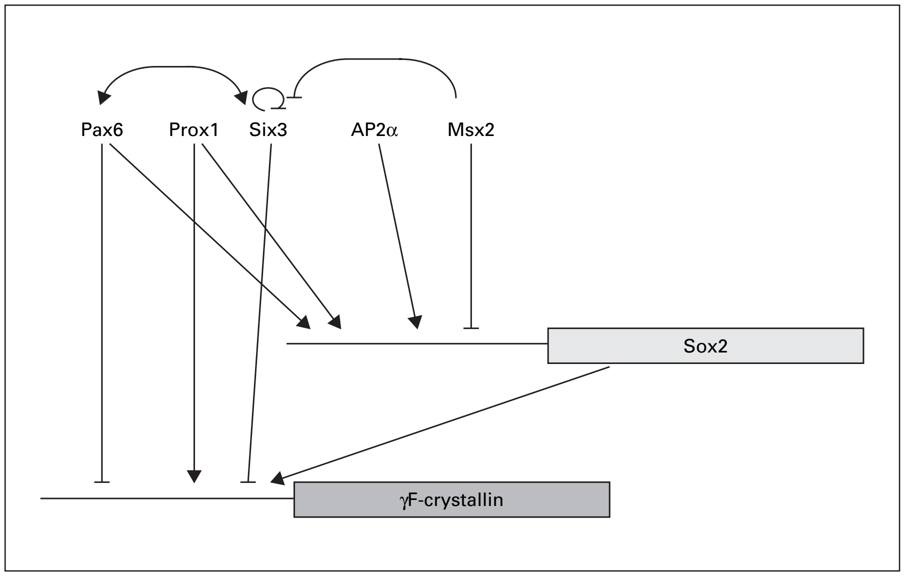

Fig. 6. *Sox2* as part of the regulatory network during eye development. The interaction of six transcription factors (Pax6, Prox1, Six3, Sox2, Msx2 and AP2α) and some of their target genes are demonstrated. Apart from the data reported here, some other reports are included [16, 17, 27–29]. Arrows indicate activating effects; lines with a stroke indicate inhibitory effects.

promoter only by ~60% [9], it might be speculated that in this particular fragment transcription might start from cryptic sites. However, for the detailed analysis and the exact identification of the particular binding sites a more sophisticated analysis would be necessary, e.g. by a random mutation approach as demonstrated previously for the action of Prox1 and Six3 at the γF-crystallin promoter [17] or gel shift assays [25].

Nevertheless, in our cell culture experiments we could demonstrate that *Sox2* is activated by AP2, Pax6 and Prox1, but inhibited by Msx2. Therefore, it should be expected that *Sox2* is expressed in those tissues where *AP2*, *Pax6* and *Prox1* are active [26], but not in *Msx2*-expressing tissues. However, the gene expression data strongly suggest that *Sox2* is expressed, even if *Msx2* is present, as it is apparent in the lens placode [16]. This might be due to further, up to now unknown factors, which bind to the putative Msx2-binding sites with higher affinity than Msx2 itself. Since both transcription factors are predicted to bind close to the CCAAT box, they might interfere with the trimeric transcription factor NF-Y [9].

Besides the *SIX3* promoter [18], the *Sox2* promoter is the second one being repressed by Msx2, but activated by Pax6 and Prox1. Obviously, AP2α acts in the same cascade because it has a similar effect as Pax6 and Prox1 at the *Sox2* promoter. Therefore, AP2α, Pax6, Sox2 and Prox1 are considered to be involved in the same pathway for lens development and differentiation. On the other hand, the strong repressor function of Msx2 and the different role of Six3 indicate that these two transcription factors may act in a different manner.

As outlined above, *Sox2* obviously has a critical role in eye development, because the loss of its function leads to anophthalmia in humans [1]. Obviously, *Sox2* is an essential part of the network regulating eye and lens development. Figure 6 summarizes the new aspects demonstrated here and integrates them into data available from the literature. However, further players in the field need to be identified and characterized with respect to their role until eye development can be fully understood.

Acknowledgements

We thank Erika Bürkle for expert technical assistance, and we gratefully acknowledge the receipt of clones from R. Balling (GBF, Braunschweig, Germany), D. Davidson (Edinburgh, Scotland/UK), P. Gruss (Göttingen, Germany), Y. Kamachi (Nagoya, Japan) and S.I. Tomarev (Bethesda, Md., USA). Oligonucleotides were synthesized by Utz Linzner (GSF Institute of Experimental Genetics). Part of this work was supported by the German Research Foundation (DFG) by a travel fellowship to A.A.G.

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