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Induction of the reelin promoter by retinoic acid is mediated by Sp1

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Abstract

We have previously described the cloning of the human reelin promoter and provided evidence that it is regulated, in part, through changes in methylation. Results from our current studies provide a more detailed analysis of this promoter and the interactions of the transcription factors Sp1 and paired box gene 6 (Pax6) with their recognition sites. The promoter was studied in NT2 cells which are a neuroprogenitor line that undergoes differentiation *in vitro*. We examined reelin mRNA and promoter induction following a 6-day treatment of these cells with retinoic acid (RA). Deletion and site-directed mutations showed functionally relevant sequences necessary for regulation. Gel-shift assays demonstrated that the main site of action of the promoter lies within a closely packed (~25 bp) region in which these transcription factors likely bind, possibly forming a DNA/protein complex. Based on our results, it appears likely that RA-induces reelin expression through a critical Sp1 site that resides adjacent to the Pax6 site within this multisite enhancer region. We show that induction of the reelin promoter with RA is accompanied by higher amounts of Sp1 and Pax6 binding to this region. Finally, we show that while mutations in the Sp1 site prevent the RA-mediated promoter induction, similar mutations in the Pax6 site do not. The data suggest that while the Pax6 site plays a role in modulating reelin expression, it is not absolutely required for induction by RA.

Keywords: chromatin, gene expression, promoter, reelin, schizophrenia, transcription factors.

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Reelin and glutamic acid decarboxylase 67 are consistently down-regulated in post-mortem cortical and hippocampal regions of schizophrenia patients at both the mRNA and protein levels (Fatemi *et al.* 2000; Guidotti *et al.* 2000; Eastwood and Harrison 2003). The mechanism responsible for this down-regulation may be related to a simultaneous increase in DNA methyltransferase I mRNA in GABAergic interneurons of these patients (Veldic *et al.* 2004). It has recently been shown that the reelin promoter is hypermethylated in patients with schizophrenia suggesting an epigenetic origin of this disease (Abdolmaleky *et al.* 2005; Grayson *et al.* 2005). Interestingly, the down-regulation of reelin mRNA associated with human metastatic pancreatic cancers is also epigenetic in origin and drugs that activate reelin expression in these cells tend to be useful in preventing the metastasis of pancreatic tumors (Sato *et al.* 2006).

Several studies have shown that histone deacetylase inhibitors, such as valproic acid, trichostatin A, and the benzamide *N*-(2-aminophenyl)-4-[*N*-(pyridine-3-ylmethoxy carbonyl)aminomethyl]benzamide, activate reelin gene expression in neural progenitors *in vitro* (Chen *et al.* 2002; Mitchell *et al.* 2005; Marija Kundakovic, unpublished results) and *in vivo* (Tremolizzo *et al.* 2002, 2005; Dong *et al.* 2005; Simonini *et al.* 2006). These inhibitors induce a simultaneous return of reelin promoter methylation to baseline levels (Mitchell *et al.* 2005; Dong *et al.* 2005). Similarly, it has been shown that methylation inhibitors added to hippocampal slice cultures induce the demethylation of the reelin and brain-derived neurotrophic factor promoters and also block the induction of long-term potentiation at Schaffer collateral synapses (Levenson *et al.*

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Abbreviations used: Ac-H3, acetylated histone H3; CASK, human homolog of CASK, Drosophila Camguk and *C. elegans* Lin-2 genes; ChIP, chromatin immunoprecipitation; GC, guanosine cytosine; hNT, differentiated NT2 neurons; mPax6, mutant Pax6; mSp1, mutant Sp1; Pax6, paired box gene 6; PBS, phosphate-buffered saline; RA, retinoic acid; RAR, retinoic acid receptors; RXR, retinoid X receptors; Sp1, transcription factor Sp1; Tbr-1, transcription factor T brain-1.

2006). More recently, bidirectional changes in the methylation patterns of the reelin and protein phosphatase I promoters in response to fear-conditioning provides a direct link between epigenetics and memory consolidation (Miller and Sweatt 2007). It is not known whether this occurs through the activation of a neuronal DNA demethylase, but it is becoming clear that methylation patterns are reversible in post-mitotic cells. It also seems clear from recent studies that the reduced reelin mRNA expression that accompanies increased promoter methylation is associated with an increased binding of methyl-cytosine-phospho-guanine dinucleotide-binding protein to the methylated promoter (Dong *et al.* 2005; Grayson *et al.* 2005; Kundakovic *et al.* 2007).

The cloning of the mouse (Royaux *et al.* 1997) and human (Chen *et al.* 2002) reelin promoter showed the corresponding regulatory sequences were part of a very guanosine cytosine (GC)-rich genomic region. Expression of the human reelin promoter as it relates to a pronounced cytosine-phospho-guanine dinucleotide island which spans about 1000 bp of upstream sequence and extends through the first exon was described several years ago (Chen *et al.* 2002). We provided data to show that neural progenitor cells (NT2) that do not express reelin mRNA exhibit a closed chromatin conformation and that agent that induce chromatin remodeling, such as valproic acid and trichostatin A, activate expression and reverse promoter methylation patterns (Chen *et al.* 2002; Mitchell *et al.* 2005). Interestingly, these same sites are more heavily methylated in patients that have been diagnosed with schizophrenia and the increased methylation correlates with reduced reelin mRNA levels (Abdolmaleky *et al.* 2005; Grayson *et al.* 2005).

Retinoic acid (RA) is a vitamin A derivative and powerful morphogen that plays distinct roles in CNS development through its action at members of the retinoic acid receptors (RAR) and retinoid X family of receptors (Mey and McCaffery 2004; Lane and Bailey 2005; Mark *et al.* 2006). RA is abundant in the developing nervous system and it is widely used to differentiate neural progenitor cells *in vitro* (Pleasure and Lee 1993; Guillemain *et al.* 2000; Chen *et al.* 2002; Misiuta *et al.* 2006). In the present manuscript, we have used RA treatment of NT2 cells as a paradigm to stimulate reelin mRNA expression. Moreover, this induction occurs prior to the onset of neuronal differentiation which generally takes several weeks of persistent RA treatment. We have defined regions of the promoter relevant for transient expression and RA-induced promoter activity. Of the three putative Sp1 sites previously identified, only the most proximal was functional with respect to basal and RA-induced expression. While the adjacent paired box gene 6 (Pax6) site was important for basal levels of expression, mutations in this site failed to affect the RA responsiveness of the promoter.

Experimental procedures

Cell culture and RNA isolation

NT2 cells were maintained and differentiated as previously described (Chen *et al.* 2002; Mitchell *et al.* 2005). Cells were plated at low density (25% confluence) and treated with 10 μmol/L RA for various times (24, 48, 72, 96, and 144 h) for analysis. At the desired times, cells were harvested for total RNA isolation, reporter measurements, or nuclear extract preparation. For each condition, a minimum of three 10 cm dishes of cultured cells were used. To extract total RNA, ultracentrifugation through CsCl was employed as described (Chen *et al.* 2002).

Immunohistochemistry

NT2 cells were plated onto coverslips in a 6-well culture plates. After several washes in 0.1 mol/L phosphate-buffered saline (PBS), pH 7.4, cells were fixed in cold 4% paraformaldehyde for 15 min. Coverslips were again rinsed in PBS and pre-incubated in 10% normal goat serum and 0.03% Triton X-100 in PBS for 1 h at RT. These were then incubated overnight at 4°C with the mouse anti-Ki67 (diluted 1 : 200; NCL-Ki67-MM1, Novocastra laboratories Ltd, Newcastle upon Tyne, UK). For the control, the Ki67 antibody was omitted. Following antibody incubation, coverslips were rinsed and incubated with the biotinylated anti-mouse made in goat (diluted 1 : 250, Vector Laboratories, Burlingame, CA, USA) in 1% normal goat serum for 1 h. After a brief rinse in PBS, the avidin–biotin-peroxidase complex was added (ABC; Vector Laboratories) for 1 h. After several more washes, sections were allowed to react with 3-3′-diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories) with 0.1% nickel ammonium sulfate for 1 min. This step was followed by a final rinse for 5 min in ddH_2O. Coverslips were allowed to dry, and mounted with Permount (Fisher-Scientific, Pittsburgh, PA, USA). Digital photomicrographs of DAB (Sigma, St Louis, MO, USA) stained images were captured.

Competitive RT-PCR with internal standards

Internal standard templates were generated by site-directed mutagenesis using PCR overlap extension (Grayson and Ikonomovic 1999; Auta *et al.* 2006). The primers used for RT-PCR amplify both the mRNA of interest and the cRNA generated from the internal standard. Primers used for each mRNA are listed in Table 1 (RT-PCR) as are the sequences amplified referenced to the indicated Genbank accession numbers. The primers used to generate the internal standard templates corresponding to each mRNA amplified are also listed in Table 1 (internal standard). Following RT-PCR, amplicons were digested with either *Ban*I (reelin) or *Bgl*II (Pax6) and products were separated by electrophoresis on agarose gels. For Sp1, Tbr1, and human homolog of CASK, Drosophila Camguk and *C. elegans* Lin-2 genes (CASK) mRNA quantitation, internal standards were generated by deleting a middle portion of the amplicon using overlap extension PCR. This allows for the separation of the amplification products on agarose gels without post-amplification restriction digestion (Noh *et al.* 2005). cRNA from these internal standards was subsequently synthesized using Ampliscribe T7 Transcription kit (Epicentre, Madison, WI, USA).

We previously developed the use of competitive RT-PCR to make quantitative measurements of selected mRNAs in total cellular RNA using specific primer pairs and corresponding internal standards

Table 1 Complete list of primers used in this work. Sequences are from the following accession numbers: Genbank numbers; Pax6 NM_001604, Sp1 NM_138473, Tbr1 U49250, and CASK BC117311

Assay	Name	Primer oligonucleotide
RT-PCR	Pax6	5′-TTCTGCAGACCCATGCAGATGCAA
		3′-CTTCAGCTGAAGTCGCATTTGAGC
	Sp1	5′-TGCAGCAGGATGGTTCTGGTCAAA
		3′-TGGAACAGCCTGAAGCTGGAGGTT
	Tbr1	5′-AAGAACTGCGCAAGGACATTAGC
		3′-TCCATCCATCCATCCATCCATCCA
	CASK	5′-TGTTCGAGGATGTGTACGAGCTGT
		3′-GCGTAGAGCTTCCAGTATCTGTCT
Internal standard	Pax6	5′-AACGATAACATACCAAGATCTCATCAATAAACA
		3′-CTCTGTTTATTGATGAGATCTTGGTATGTTAT
	Sp1	5′-TCTCAGGCAGGCACGATC/CAGACACCCCAGAGGGTC
		3′-GACCCTCTGGGGTGTCTG/GATCGTGCCTGCCTGAGA
	Tbr1	5′-ATGGCCGTCTGCAGCGTA/CCTCCTACTTCTCT
		3′-AGAGAAGTAGGAGGT/ACGCTGCAGACGGCCAT
	CASK	5′-CAAGTCCAGGGTTAAGTA/ATAGCTCAGATGGAATGC
		3′-GCATTCCATCTGAGCTAT/TACTTAACCCTGGACTTG
Mutation	mSp1-230	5′-GGCGGGGCGGCGCGCAGATCTACTACAGCGGCCGGGACACG
		3′-CGTGTCCCGGCCGCTGTAGTAGATCTGCGCGCCGCCCCGCC
	mSp1-180	5′-CGGGGGGGACGCGAAGATCTTACATTTAAGAAGGTGGAG
		3′-CTCCACCTTCTTAAATGTAAGATCTTCGCGTCCCCCCCG
	mSp1-150	5′-TAAGAAGGTGTGAGCAGATCTATCATGTTTCCCAGGCCTGGC
		3′-CGGAGGCCTGGGAAACATGATAGATCTGCTCACACCTTCTTA
	m-Pax6	5′-GAGCGGGGCGGGCGCAGATCTTAATTGCCAGAGGGGCGTCGCGCA
		3′-TGCGCGACGCCCCTCTGGCAATTAAGATCTGCGCCCGCCCCGCTC
	m-Tbr1	5′-CGGCCGGGCTTTAAGAATTAACATTACGGGGCGGGC
		3′-GCCCGCCCCGTAATGTTAATTCTTAAAGCCCGGCCG
	m-141/-136	5′-GGCGGGCGCTTTCCAAGGCATGGCCGAGGGGCGTCGCGC
		3′-GCGCGACGCCCCTCGGCCATGCCTTGGAAAGCGCCCGCC
	m-141	5′-GGCGGGCGCTTTCCAAGGCCTGGCCGAGGGGC
		3′-GCCCCTCGGCCAGGCCTTGGAAAGCGCCCGCC
	m-136	5′-GGGCGCTTTCCCAGGCATGGCCGAGGGGCGTG
		3′-GACGCCCCTCGGCCATGCCTGGGAAAGCGCCC
Gel shift	Reln-Pax6	5′-CGGGCGCTTTCCCAGGCCTGGCCGAGGG
		3′-CCCTCGGCCAGGCCTGGGAAAGCGCCCG
	Consensus Pax6	5′-CCTCGGGCTTGATGCGTGAAAATTGCGC
		3′-GCGCAATTTTCACGCATCAAGCCCGAGG
	Sp1/Pax6	5′-CGGGGCGGGCGCTTTCCCAGGCCTGGCCGA
		3′-TCGGCCAGGCCTGGGAAAGCGCCCGCCCCG
	Tbr1/Sp1	5′-GAAGGTGTGGAGCGGGGCGGGCGCTTT
		3′-AAAGCGCCCGCCCCGCTCCACACCTTC
	Sp1 only	5′-CGGGGCGGGCGCTTT
		3′-AAAGCGCCCGCCCCG
	Pax6 only	5′-CCCAGGCCTGGCCGA
		3′-TCGGCCAGGCCTGGG
	Tbr1 only	5′-GAAGGTGTGGAGCGG
		3′-CCGCTCCACACCTTC

Pax6, paired box gene 6; mSp1, mutant Sp1; CASK, human homolog of CASK, Drosophila Camguk and *C. elegans* Lin-2 genes.

(Grayson and Ikonomovic 1999; Auta *et al.* 2006). Reverse transcription of total RNA with random hexamers and M-MLV RT was performed as previously described (Noh *et al.* 2005). Increasing amounts of cRNA synthesized from individual internal standards were added to total RNA prior to RT and all subsequent steps were carried out in the same tube. PCR annealing temperatures for each of the templates are indicated in Table 1.

Amplification products were subsequently analyzed electrophoretically and visualized by ethidium bromide staining. Images of gels were digitally captured through a Kodak EDAS 290 (Kodak, New

Haven, CT, USA). Individual bands were quantified using Kodak 1D 3.6 image analysis software. All data were plotted as previously described and analyzed for midpoint determinations (Grayson and Ikonomovic 1999). Quantitative RNA analyses were performed three times per sample from a minimum of three different RNA isolations. The data were analyzed for significance using a one-way ANOVA.

Site-specific mutagenesis of the reelin promoter

To generate site-specific promoter mutants, we designed primers to PCR amplify the region of interest using overlap extension PCR (Grayson and Ikonomovic 1999). The primers used to generate each of the promoter mutations are listed in Table 1 (mutation). Following PCR, amplicons were subcloned into the pGL-3/reelin −514 promoter construct. Mutant promoter constructs were transfected and reporter assays were used to assess the effects of the mutations relative to the activity of −514 reelin promoter. All mutations were sequenced to verify the accuracy of both the inserted and surrounding sequences.

Co-transfection assays and reporter measurements

Cells were transfected at 80% confluence using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA). We used the dual-luciferase reporter assay system (Promega/Fisher, Pittsburgh, PA, USA) and routinely transfected from 3.5 to 4 μg reelin promoter/promoter mutant DNA for each well of a 6-well plate. Small amounts (30 ng) of the pRL-TK vector (*Renilla* luciferase/thymidine kinase) were used per well to control for variations in transfection efficiency (Chen *et al.* 2002). Cell lysates were prepared 48 h after transfection and aliquots (∼20 μL) were used for determination of luciferase activity in a TD20/20 luminometer (Turner Design, Sunnyvale, CA, USA). The SV40 promoter/luciferase vector (pGL3-control vector) was transfected in parallel for signal normalization. Renilla luciferase (transfection efficiency) corrected values were normalized to the pGL3 control to assess promoter integrity, except where noted. Data (minimum of 3–5 transfections/construct) are expressed as a ratio of the test signal (reelin promoter) to the signal obtained with the pGL3-control vector (Promega/Fisher).

To test for the effects of Pax6, Tbr1, and Sp1 on endogenous reelin mRNA expression, NT2 cells were co-transfected with each (1 μg) expression vector. Transfections performed with transcription factor T brain-1 (Tbr-1)/CASK were not different than with Tbr-1 alone. Since the amounts of CASK were initially abundant and did not change over the duration of the RA treatments, it seemed likely that the presence of CASK was not rate limiting in terms of Tbr-1 activity (data not shown). To test the synergistic action of various combinations of transcription factors: 1 μg reelin minimal promoter (reelin −514) was used to transfect NT2 cells with 1 μg each of Pax6, Tbr1, SP1 alone or in the indicated combinations. Total transfected DNA was adjusted to 4 μg/well by including additional empty expression vector. For the promoter deletion construct co-transfections, 1 μg of each construct was used to transfect NT2 cells with 1 μg of each Pax6, Tbr1 and SP1 expression vectors. Cells were lysed after 48 h and reporter activity was measured.

For the pGEX-RAR co-transfection experiment, each well contained 2 μg each promoter/reporter construct, 1 μg Sp1 expression vector plus 30 ng RAR expression vector along with pRL-TK (Renilla luciferase, 10 ng/dish) to normalize for transfection efficiency. Total DNA amounts were adjusted to 3.5 μg/well by adding empty expression vector (pBluescript, Stratagene, La Jolla, CA, USA). For dose–response assays, NT2 cells were incubated with the transfection mix for 12 h, washed with PBS and then treated or untreated with increasing concentrations of (1 nmol/L to 10 μmol/L) RA for another 48 h. NT2 cells lysates were used to measure luciferase activity.

To assess the integrity of the mutant Sp1 (4 μg of each of mSp1-230, mSp1-180, and mSp1-150) and Pax6 (4 μg: mPax6) promoter variants, these constructs were transfected in parallel with the reelin −514 promoter for comparative purposes. The mSp1-150 was used for the Sp1 co-expression studies because it showed the lowest promoter activity of the three. Two micrograms of the promoter construct (mSp1-150) and 2 μg of Sp1 expression vector were co-transfected into NT2 cells for 48 h to examine the effects of excess Sp1 on this mutant.

Nuclear extract preparation, electrophoretic mobility shift assays, and *in vitro* translation

Nuclear extracts were prepared using the NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL, USA) with minor modifications. 8×10^6 treated (RA, 6 days) or untreated NT2 cells were washed with cold PBS (2X) before they were scraped. Cells were spun down at 500 *g* for 2 min and the packed cell volume was about 50 μL. Protein concentrations were determined using the Bradford G-250 protein assay (Bio-Rad, Hercules, CA, USA).

Reelin promoter gel shifts

The double-stranded oligonucleotide probes used for the various reelin-binding sites are listed in Table 1 (gel shift). Hybridized oligos were labeled with γ-^{32}P ATP using T4 polynucleotide kinase to a specific activity of $\sim 1 \times 10^8$ dpm/μg. Labeled probe (2 ng) was incubated with 10 μg of nuclear extract and electrophoresed on non-denaturing gels. The consensus Pax6 site (Table 1), has been shown to bind human Pax6 (Epstein *et al.* 1994; Czerny and Busslinger 1995). For some competitions, a non-specific oligo was used: forward 5′-CTCGATCATACGTCCCTCACA-3′ and reverse 5′-TGTGAGGGACGTATGATCGAG-3′. Gel-shift assays were performed as originally described (Grayson *et al.* 1988) as modified according to the manufacturer's instructions (Promega).

Pax6 binding-site affinity test

Dissociation rate analysis was performed by gel-shift competition assays. The Reln-Pax6 labeled probe was competed with an excess of the unlabeled consensus Pax6 binding site. Similarly, the consensus Pax6 probe was competed using the Reln-Pax6 binding site oligo. The density of each of the shifted bands was quantified and dissociation rates were calculated as described previously (Chen *et al.* 2004; Grayson *et al.* 2005). The competitions show that the consensus Pax6 probe has a higher binding affinity for the nuclear protein than does the Reln-Pax6 probe.

In vitro *translation of Pax6*

The recombinant Pax6 protein was made from the pSP64-Pax6 plasmid *in vitro* using the TNT T7 translation system (Promega) according to the manufacturer's recommended protocol. One microgram of purified pSP64-Pax6 was used as DNA template. Two reactions were carried out. The first included ^{35}S-methionine so that the size of the protein could be checked by gel electrophoresis. A

second reaction was carried out using 1 μL, 1 mmol/L amino acid mixture minus leucine instead of the labeled methionine for the generation of larger amounts of the recombinant protein. The reactions were incubated at 30°C for 90 min. The ^{35}S-methionine-labeled translation products were analyzed on a 4–20% denaturing sodium dodecyl sulfate/Tris/glycine gel. The gel showed that the major product was a single band of ~46 kDa molecular size. Recombinant Pax6 protein was stored at −80°C for subsequent gel-shift assays.

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays were performed as previously described (Kundakovic *et al.* 2007), with the exception that anti-Pax6 polyclonal antibody (Abcam, Cambridge, MA, USA), anti-Sp1 and anti-acetyl-histone H3 polyclonal antibodies (Upstate, Lake Placid, NY, USA) were used to immunoprecipitate chromatin preparations. Samples incubated without antibodies (no Ab) served as negative controls. The immunoprecipitated DNA was analyzed by semi-quantitative PCR using the primers indicated in Table 1 (ChIP). For all experiments, non-immunoprecipitated input and immunoprecipitated DNA samples were below saturation levels.

Statistical analyses

All experimental results are expressed as mean ± SEM of three independent experiments (a minimum of three separate measurements were obtained per experiment). For most of the transfection experiments, data are expressed relative (%) to the data obtained from an SV40 containing construct transfected in parallel (pGL-3 control vector; Promega/Fisher) after normalizing for transfection efficiency. Student's *t*-test (for ChIP results) and one-way ANOVA followed by the Fisher least significant difference method (for all other results) were used to assess significance of the differences between groups. Analyses were conducted using SigmaStat software (SysStat, Richmond, CA, USA). In addition, dose–response curves for reelin promoter induction in response to RA were obtained using GraphPad Prism Version 4 (GraphPad Software, San Diego, CA, USA). EC_{50} values were calculated from RA dose–response curves analyzed by the Probits test using the SPSS statistical package software (SPSS, Chicago, IL, USA). Statistical comparisons among the different EC_{50}s were performed by *t*-test. Differences between constructs were considered significant at $p < 0.05$.

Results

Induction of reelin and transcription factor mRNAs by RA in NT2 cells

We had previously shown that a 6-week treatment of NT2 cells with 10 μmol/L RA which converts these neural progenitors to differentiated NT2 neurons (hNT) neurons is accompanied by a 100-fold increase in reelin mRNA (Chen *et al.* 2002). We have subsequently used a similar treatment dose but shortened the incubation period from between 24 and 144 h (6 days). This time frame allows for maximal reelin mRNA induction and at the same time does not cause significant morphological differentiation of the NT2 cells. Moreover, the treatment does not force these cells to exit the cell cycle. Staining of these cultures for the proliferation marker Ki-67 (Scholzen and Gerdes 2000; Kee *et al.* 2002) showed that RA-treated NT2 cells immunostain less intensely than untreated cultures. However, as compared with the terminally differentiated hNT neurons that have been treated with RA for 6 weeks and then replated, Ki-67 immunostaining is readily apparent suggesting that these cells are still dividing and have not reached the post-mitotic stage. As shown in Fig. 1, Ki-67 immunoreactivity is most intense for the non-treated NT2 cells (Fig.1a and b). NT2 cells treated with RA for 6 days still show substantial staining (Fig. 1c and d) while differentiated hNT neurons show little if any Ki-67 immunoreactivity (Fig. 1e and f). This suggests that while the cells are starting to differentiate after only a 6-day treatment with RA they are still proliferating.

Quantitative analysis of mRNAs using competitive RT-PCR with sequence-specific internal standards showed that reelin mRNA increases 130-fold after a 6-day RA treatment

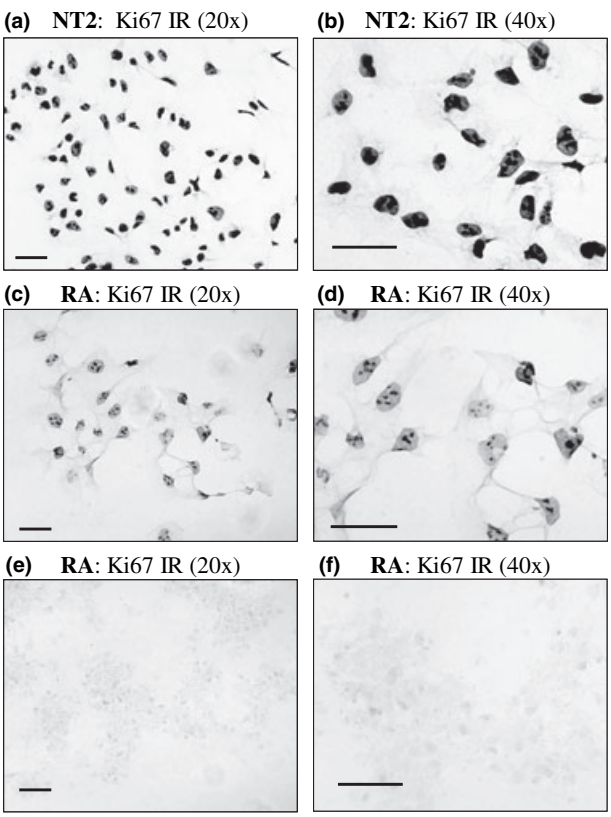

Fig. 1 Ki67 immunoreactivity (IR) in NT2 cells treated with retinoic acid (RA). (a and b) Ki67 immunostaining of NT2 cells with no RA treatment (0 days). Note the intense IR associated with Ki67, a cell proliferation marker. (c and d) NT2 cells treated with RA for 6 days. RA induces cell differentiation decreasing the expression of Ki67 protein. (e and f) NT2 cells treated with RA for 6 weeks. Cells are totally differentiated and Ki67 is not expressed. a, c, and e, 20x magnification; b, d, and f, 40x magnification (scale bars, 50 μm).

Table 2 Induction of reelin and various transcription factor mRNAs in NT2 cells treated with retinoic acid for 6 days

Time (hours)	Reelin mRNA[a]	Pax6 mRNA[a]	Sp1 mRNA[a]	CASK mRNA[a]	Tbr1 mRNA[a]
0	0.0064 ± 0.0009	0.0013 ± 0.0003	40.0 ± 1.6	40.0 ± 1.8	0.0051 ± 0.0026
24	0.0061 ± 0.0009	0.016 ± 0.003	36.5 ± 9.1	33.3 ± 1.9	0.0056 ± 0.0025
48	0.0070 ± 0.0003	1.22 ± 0.24	35.7 ± 7.4	34.4 ± 3.0	0.0532 ± 0.0270
72	0.027 ± 0.004	14.6 ± 1.19	42.0 ± 9.8	42.2 ± 3.9	0.999 ± 0.198
96	0.42 ± 0.08	58.7 ± 4.56	38.8 ± 8.0	44.9 ± 3.9	1.342 ± 0.338
144	0.84 ± 0.12	58.4 ± 6.15	38.8 ± 7.2	43.0 ± 4.4	1.416 ± 0.475

Values are means ± SEM.

[a]Quantitive measurements of mRNA were performed by competitive RT-PCR. Each measurement was made three times using three independent RNA isolates.

Results shown as pg RNA/μg total RNA.

Pax6, paired box gene 6; CASK, human homolog of CASK, Drosophila Camguk and *C. elegans* Lin-2 genes.

(Table 2 and Fig. 2). The time course profile shows that the increase is first evident at 96 h and increases further to maximum 2 days later. Neuron-specific enolase mRNA values stayed constant throughout the treatment (Fig. 2). We have previously provided preliminary evidence that the reelin promoter contains recognition sites for the transcription factors Sp1, Tbr1, and Pax6 (Chen *et al.* 2002). To gain insight into the role that these proteins play in the temporal induction of reelin mRNA, we also quantitated the time course of the corresponding mRNAs using internal standards for each of these factors (Fig. 2 and Table 2). Pax6 mRNA increased initially after 48 h and stayed elevated through 6 days. Tbr1 mRNA did not increase until 72 h post-treatment and then stayed elevated for the duration of the time course. Amounts of the Tbr-1 associated MAGUK protein, CASK, mRNA were abundant in non-treated NT2 cells and did not change during the RA treatments. mRNA corresponding to Sp1 was also high at the start of the treatment and remained fairly constant throughout (Fig. 2, Table 2).

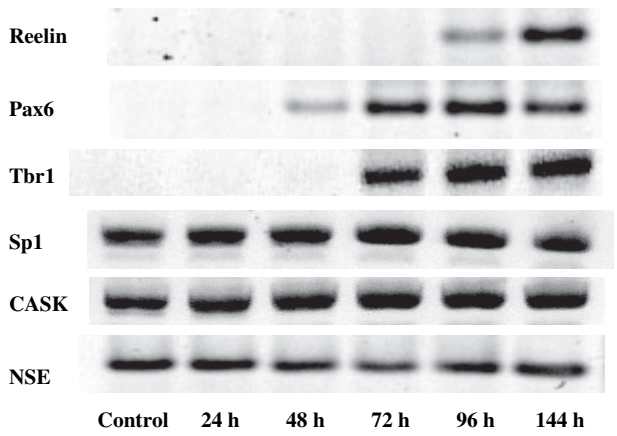

Fig. 2 Time course of retinoic acid (RA) treatment induced paired box gene 6 (Pax6), Tbr1, and reelin mRNA expression. Gels show representative results of the RT-PCR analysis of Reelin, Pax6, Tbr1, Sp1, CASK, and neuron specific enolase (NSE) mRNAs using primers listed in Table 1. NT2 cells were treated with 10 μmol/L RA for the indicated times and the gels show relative changes in individual mRNAs. Absolute amounts of each mRNA were quantified using internal standard cRNA for each transcript measured (see Table 2).

Transcription factor co-transfection experiments

To evaluate the role of the previously identified transcription factors in regulating reelin expression, we co-transfected vectors expressing each factor (Pax6, Tbr1, or Sp1) along with the reelin −514 construct/reporter (Chen *et al.* 2002). As shown in Fig. 3a, each transcription factor by itself had about a twofold effect in terms of inducing reelin promoter-directed luciferase activity. The double transcription factor combinations were approximately 8- to 10-fold greater than the normalized −514 promoter activity. In contrast, the triple combination enhanced the effect synergistically, increasing luciferase activity by some 30-fold (Fig. 3a). These data suggest that when the triple combination of transcription factors are co-expressed simultaneously, they act cooperatively to activate expression of the reelin −514 promoter.

As indicated above, we previously identified several putative recognition sites for these factors based on both reported sequence preferences and preliminary deletion studies (Grayson *et al.* 2006). Within the reelin enhancer region (approximately −300 bp to −130 bp), there are three potential sites for Sp1 and potential single sites for Tbr1 and Pax6 that are closely spaced within this functionally defined region. We co-transfected the triple combination of transcription factors with a series of reelin promoter constructs which were designed to more closely localize the recognition sites within the reelin promoter/enhancer (Fig. 3b). The deletions are conventional progressive 5′–3′ loss of sequence mutants. Fold increase refers to the signal obtained from each promoter construct alone relative to the signal obtained using the triple (Sp1/Pax6/Tbr1) transcription factor combination. The combination of Sp1/Pax6/Tbr1 factors did not show

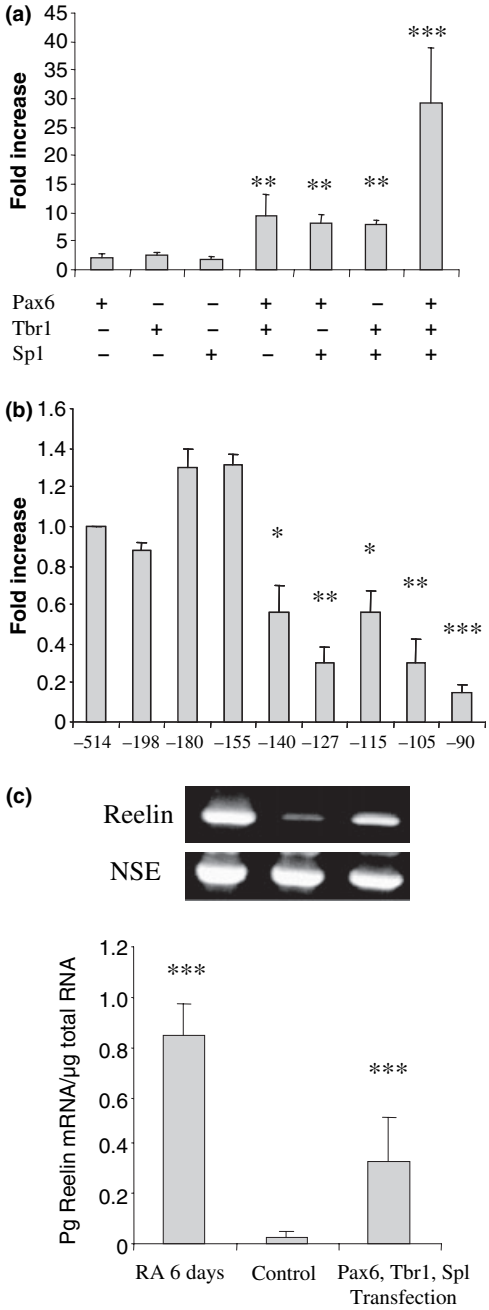

Fig. 3 Co-transfection of Sp1, Pax6 and Tbr-1 activates the reelin promoter. (a) NT2 cells were transfected with the reelin −514 promoter and expression vectors corresponding to Pax6, Tbr1, and Sp1 either alone or together. For purposes of this experiment, the value measured for the −514 construct without Pax6, Tbr1, and SP1 expression vector was designated as onefold. All other values represent a ratio of the respective signal (in the presence of the indicated constructs) to that obtained from the −514 promoter alone. Data are mean ± SEM versus single factor transfection. (b) Deletion constructs co-transfected with all three transcription factor expression vectors (Pax6, Tbr1, and SP1). Data are expressed as the fold increase over the −514 reelin promoter as described in Experimental procedures and are compared statistically with that value. (c) RT-PCR assay of endogenous reelin mRNA measured following the transfection of NT2 cells with the Pax6, Tbr1, and SP1 triple combination of expression vectors. Data are mean ± SEM. ***$p < 0.001$; versus control or non-treated group (one-way ANOVA followed by Fisher least significant difference method).

much difference with respect to boosting activity with constructs containing more than −155 bp of the promoter (compare −514, −198, −180, and −155 bp; Fig. 3b). However, the activity dropped to about ∼50% when sequences 3′ to the −155 bp promoter were deleted. That is, the 15 bp loss between −155 and −140 was responsible for more than half the activity as determined by this assay. The remaining promoter constructs appeared to have about the same level of reporter activity (i.e. compare −140, −127, −115, −105, and −90 bp; Fig. 3b). This suggests that the main segment of the promoter responsible for the enhancer activity lies between −155 and −140 bp upstream of the RNA start site. Finally, we compared the action of these three transcription factors on the activity of the endogenous promoter. As shown in Fig. 3c, the transfection of all three transcription factors into non-treated NT2 cells boosted expression of the endogenous reelin mRNA nearly 10-fold over mock-transfected NT2 cells. The levels of reelin mRNA in NT2 cells treated with 10 μmol/L RA for 6 days are shown for comparison.

Mutations defining the Sp1 site

We have previously identified three putative Sp1 recognition sites within the reelin promoter based on sequence similarities to known GC box containing Sp1-binding sites (Grayson *et al.* 2006). For clarity, these sites are referred to as the Sp1-230, Sp1-180, and Sp1-150 sites based on their proximity to the transcriptional start site (as indicated by the site numbering relative to the start site: that is, −230, −180, and −150 bp). We generated a series of site-directed mutations to examine the effect of sequence loss and loss of function on the otherwise intact promoter. Specific mutations for each site (mSp1-230, mSp1-180, and mSp1-150) were designed such that the GC box-binding site was changed but not the distance between other sites or the remainder of the promoter sequences. These were then built back into the reelin −514 promoter as described in Experimental procedures. The mutations used for each site are listed below the top panels in Fig. 4. Transfection of these constructs into NT2 cells suggested that only the 3′ most proximal Sp1 site is functional. That is, only when the Sp1 site at −150 (Sp1-150) was altered (mSp1-150) was there a significant loss of function (to about 35%) relative to the reelin −514 promoter reporter. The level of activity arising from the mSp1-230 (96%) and mSp1-180 (90%) is nearly the same as that arising from the −514 bp promoter construct (100%).

Sequential deletion mutants were also constructed and these were co-transfected into NT2 cells along with an Sp1

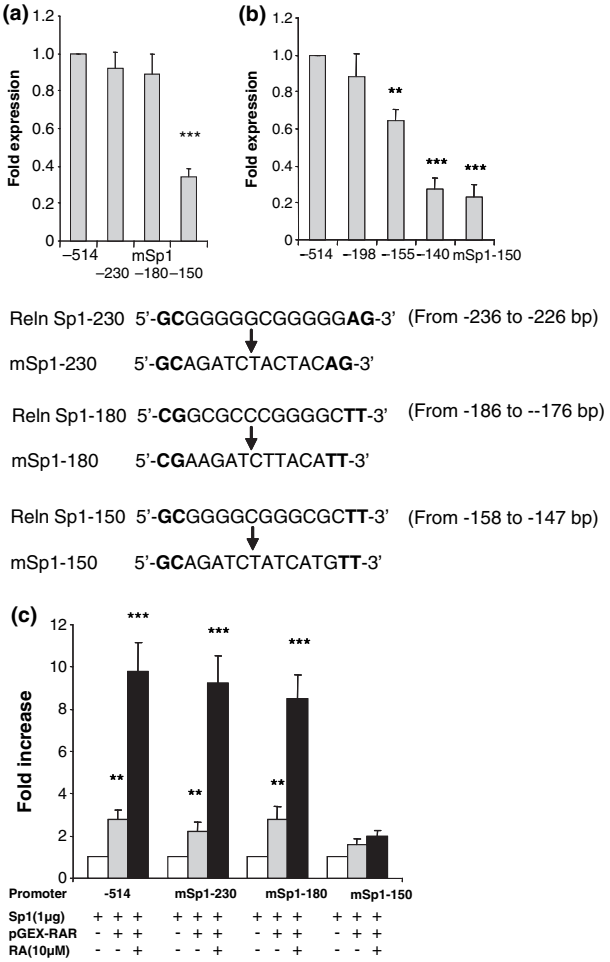

Fig. 4 Identification and location of the Sp1-binding site and its responsiveness to retinoic acid (RA). (a) Three Sp1 mutant constructs (mSp1-230, mSp1-180, and mSp1-150) and the reelin −514 promoter were transfected into NT2 cells. The positions of the Sp1 sites relative to the start site are shown below Fig. 4a and b. For each mutant construct, those specific residues that were mutated are indicated with the remainder of the parental reelin −514 promoter left intact. (b) Results obtained from co-transfection of an Sp1 expression vector with selected reelin promoter constructs and the mSp1 site at −150 (mSp1-150) are shown. Data obtained from the reelin promoter deletion series were performed in parallel for comparison and to confirm location identity. These deletion constructs support the results obtained with the putative upstream Sp1 sites (mSp1-230 and mSp1-180). That is, whether the sites are deleted or mutated, there is no loss of function. The data are expressed relative to the intact reelin −514 promoter reporter (onefold). (c) Reelin −514 promoter, Sp1 and pGEX-RAR co-transfection: Reelin −514 promoter, mSp1-230, mSp1-180, and mSp1-150 were co-transfected with an Sp1 expression vector and the pGEX-RAR vector and then treated with RA. Following plasmid transfection, cells were washed and treated with RA (or vehicle) for an additional 48 h. For each indicated (−514, mSp1-230, mSp1-180, and mSp1-150) construct, white bars indicate results obtained following co-transfection with the Sp1 expression vector. Gray bars indicate co-transfection of the indicated construct with both the Sp1 expression vector and the pGEX-RAR. Black bars show the indicated construct co-transfected with the Sp1 expression cassette, the pGEX-RAR and RA (10 μmol/L). Data are mean ± SEM. $^{***}p < 0.001$; $^{**}p < 0.01$, versus the reelin −514 promoter (one-way ANOVA followed by Fisher least significant difference method).

expression vector to test the functionality of the respective recognition sites. Deletion of the first two (most upstream) sites (−198 and −155) had little effect on promoter activity as compared with the reelin −514 promoter construct (Fig. 4b). That is, deletion to −198 bp and to −155 bp did not affect the level of activity as compared with the −514 bp promoter. However, deletion of the third site to −140 bp resulted in a significant loss of function in the presence of Sp1. Interestingly, the −140 bp deletion and the mSp1-150 had the same level of activity as determined by transient transfections. Collectively, these data suggest that the only functional Sp1 site of those previously identified is that located at −150 bp relative to the RNA start site.

We also evaluated the responsiveness of the three Sp1 sites to RA using a co-transfection assay (Fig. 4c). Reelin promoter constructs (−514 bp, mSp1-230, mSp1-180, and mSp1-150) were co-transfected with vectors driving expression of the RAR alpha (pGEX-RAR) and Sp1. Within each promoter set, the presence of the RAR increased expression. Moreover, the presence of RA boosted this expression even higher. However, the maximal induced activity occurred when the reelin promoter construct contained an intact Sp1 site (at −150 bp) in the presence of the RAR and RA. Of the promoters tested, the reelin −514, mSp1-230, and mSp1-180, all had intact Sp1 sites at −150 bp. The Sp1 mutant at −150 bp (mSp1-150) showed no responsiveness in this assay. These data suggest that RA normally activates expression through an interaction of the RAR and Sp1 protein acting at this Sp1 site and not the other GC-box Sp1 sites (at −230 and −180 bp). This result is consistent with what has been observed with other RA-responsive promoters that are Sp1 dependent (Suzuki *et al.* 1999; Husmann *et al.* 2000; Shi *et al.* 2001).

Mutations and binding to the Pax6 site

The putative site for the action of Pax6 in regulating reelin expression was positioned between −147 and −130 bp based on preliminary deletion analysis (Grayson *et al.* 2006). To examine this in more detail, we studied the effects of an expression vector on the ability of Pax6 to activate various reelin promoter constructs. We first tested some of the deletion constructs using a co-transfection assay. As shown in Fig. 5a, constructs extending from −514 to −140 bp all have approximately the same amount of reporter activity (relative to the −514 promoter). However, a marked loss of function occurred when sequences between −140 and −127 bp were deleted. Additional deletions upstream of this point failed to restore function (data not shown). To further

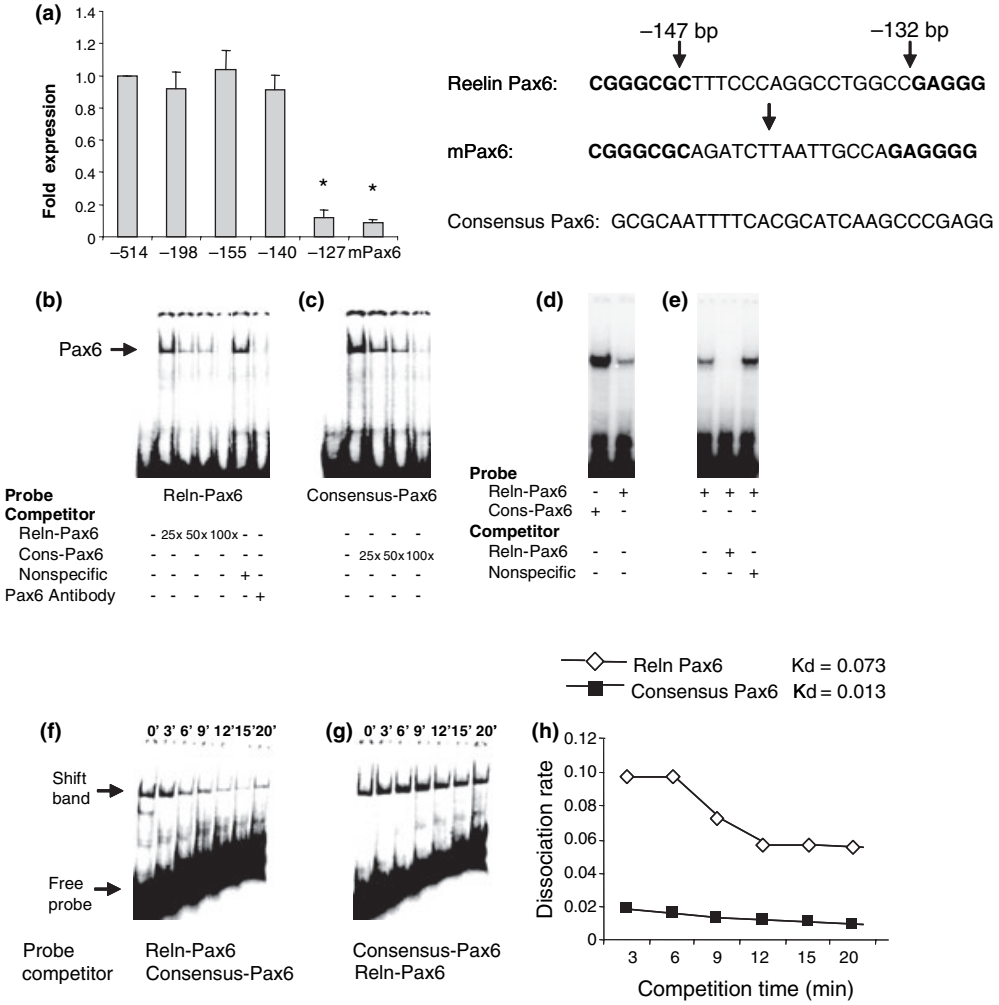

Fig. 5 Paired box gene 6 (Pax6) binding site mapping, gel shifts, and binding affinity measurements of recombinant Pax 6 for the reelin-Pax6 binding site. (a) Reelin promoter deletion constructs and the mPax6 promoter construct were co-transfected in parallel with the Pax6 expression vector. Values are expressed relative to the reelin −514 promoter (1.0). Data are mean ± SEM. $^*p < 0.001$ versus the reelin −514 promoter, (one-way ANOVA followed by Fisher least significant difference method). Sequences that were mutated in the mPax6 construct are shown to the right of Fig. 5a along with the consensus Pax6 site that was used in the shift assays. (b) The reelin-Pax6 probe was used to examine binding of nuclear proteins present in NT2 cells to this site using a gel-shift assay. In panels b–e, the probes for each shift and the various competitors used are indicated; 25X means that a 25-fold molar excess of competitor was used. The final lane shows diminution of the shifted band in the presence of the Pax6 antibody (Fig. 5b). (c) In this gel shift, a consensus-Pax6 probe was constructed and used as a measure of consensus Pax6 binding present in NT2 nuclear extracts. (d and e) Recombinant Pax6 protein binds to both the reelin-Pax6 (d) and consensus-Pax6 probes (e). (f and g) Shows a representative binding affinity test: (dissociation rate analysis) using a gel-shift competition assay. (h) The affinity of the recombinant Pax6 for the consensus Pax6 (Kd = 0.013) was higher for than it was for the reelin-Pax6 binding site (Kd = 0.073).

narrow the localization of the Pax6 binding site, we mutated a 15 bp stretch within the putative binding site leaving the remainder of the −514 bp promoter construct intact (Fig. 5a, mPax6). The precise location of the mutated residues and a comparison to a consensus Pax6 binding site are shown to the right. The activity of the mPax6 construct was reduced to that of the −127 bp promoter (Fig. 5a) showing that simply substituting 15 bp within this region was sufficient to reduce activity to minimal levels.

We next prepared double stranded oligonucleotides which corresponded to the region of the Pax6 recognition site (−140 to −127 bp deletion) and used this to shift proteins present in RA-treated NT2 cell nuclear extracts. Shown in Fig. 4b is the gel shift obtained from RA-treated NT2 cell extracts using the reelin-Pax6 recognition site. The first lane shows probe alone. The second lane shows the labeled reelin-Pax 6 site plus nuclear extract. The next three lanes (see below photo) exhibit the same gel shift in the presence of increasing

amounts of cold competitor DNA. The presence of a 100-fold excess of specific competitor DNA completely prevents the reelin-Pax6 shift. Lane 7 shows that a non-specific competitor fails to reduce the intensity of the gel-shifted band, while lane 8 shows that co-incubation of the nuclear extract with a human Pax6 antibody disrupts the factor-probe interaction. Rather than super shifting the complex, the Pax6 antibody has been shown to disrupt band formation (Scardigli *et al.* 2003) as we show here. The gel shifts shown in Fig. 5c were obtained using a consensus Pax6 binding site (Scardigli *et al.* 2003). The use of this site with the RA-treated NT2 nuclear extracts results in a similar pattern of shifted bands as the reelin-Pax6 probe. Moreover, this main Pax6 band self-competes. Fig. 5d shows a side by side shift using first the consensus Pax6 probe and then the reelin-Pax6 binding site probe. The same amount of labeled probe (at the same specific activity) is used in each lane indicating that the affinity appears to be higher for the consensus Pax6 binding site. In Fig. 5e, the reelin-Pax6 probe is shown first with no competitor, then with an excess (50X) of cold reelin-Pax6 competitor and then with a non-specific competitor DNA.

The Pax6 site recognition site within the reelin promoter did not show strong sequence identity with other Pax6 binding sites (Scardigli *et al.* 2003). For this reason, we compared the binding affinities of recombinantly expressed Pax6 protein to the reelin-Pax6 and consensus Pax binding sites using a dissociation rate analysis (Chen *et al.* 2004; Grayson *et al.* 2005). With this type of assay, labeled probe is incubated with recombinant protein for a specified period of time. Cold competitor is added at various times (indicated at the top of each gel) and the dissociation of the complex as a function of time in the presence of the cross-competitor is measured by scanning the dried gels. Figure 5f shows the dissociation data obtained using labeled reelin-Pax6 with cold consensus Pax6 competitor. The sister analysis (labeled consensus Pax6 with cold reelin-Pax6 competitor) is shown in Fig. 5g. From these data it seems clear that the unlabeled consensus Pax6 binding site is better at competing the labeled reelin-Pax6 site (Fig. 5f) than the converse (Fig. 5g). This is more readily evident when the data are plotted (Fig. 5h) and the dissociation rate is determined by fitting the points to an exponential regression ($y = \text{span} \cdot e^{-kx}$). The data show that the reelin-Pax6 binding site has a lower affinity for recombinant Pax6 than does the consensus Pax6 binding site.

RA dose response and Sp1

Because the functionally defined Sp1 site (at −150 bp) mapped adjacent to the Pax6 site, we sought to determine if RA was acting through the Sp1 site alone or whether there might also be some involvement with Pax6. For these experiments, transient transfections were carried out using the −514 bp promoter, the mSp1-150 and the mPax6 promoters. Cells were then treated with RA for 48 h and reporter activity was measured. The data are plotted using a semi-logarithmic scale (dose) as a function of maximal increased response (Fig. 6a and b). As shown in Fig. 6a, the reelin −514 bp promoter showed a typical dose–response relationship following RA treatment. An analysis of these data showed the EC_{50} to be $\sim 1.2 \times 10^{-7}$ mol/L RA. The co-transfected mPax6 promoter construct was also RA responsive with an EC_{50} of 1.9×10^{-7} mol/L (Fig. 6b). A statistical analysis (Probit followed by *t*-test) of the data showed these two EC_{50} values were not significantly different. These results are consistent with RA acting only at the level of the Sp1 site located at −150 bp and not through other transcription factor binding sites. In contrast, when the mSp1-150 construct was used, there was no increase in activity in response to the RA treatment (Fig. 6c). In Fig. 6c, data from all three dose–response curves are shown side by side using conventional representations. As indicated, the mSp1-150 construct displayed ∼50% of the activity in the absence of RA (as compared with the −514 bp promoter) and this did not increase following addition of the morphogen.

Mutations defining the Tbr-1 site

Previous work reported that the reelin promoter contained two sites for Tbr1 and the associated CASK protein (Hsueh *et al.* 2000; Wang *et al.* 2004). These studies had positioned the Tbr-1/CASK binding sites at −4.3 kb and −2.1 bp relative to the start site. Our sequence data confirm the location of these half-T elements (Wang *et al.* 2004). We have tried to replicate these data using our reelin promoter/reporter constructs with only limited success. For example, the use of promoter constructs with >4.5 kb of upstream or 5′ flanking sequence (both sites) and multiple upstream deletion mutants did not show clear loss of function using co-transfection assays. For example, co-transfection of the Tbr-1 and CASK expression vectors along with the reelin −2.5 kb promoter construct did not boost expression over that obtained without the Tbr-1/CASK vectors (data not shown). We also observed little difference with the Tbr-1/CASK expression vectors and our deletion constructs which extended to within −155 bp of the RNA start site (Fig. 7a). This includes constructs with these putative sites (−4.3 and −2.1 kb) clearly missing (i.e. −514, −198, and −155 bp). Based on an evaluation of these data in the context of the previous report (Hsueh *et al.* 2000) along with some initial confusion regarding the location of the sites (Hsueh *et al.* 2002), we tentatively mapped the Tbr-1 element within the reelin promoter based on homology to consensus T-box binding sites (Tada and Smith 2001; Grayson *et al.* 2006), with the closest sequence identity match placing the Tbr-1 site just upstream of the −150 bp Sp1 site. We tested site-directed mutants of this site which failed to affect the −514 promoter/reporter activity in the presence of co-transfected Tbr-1/CASK (Fig. 7b, mutant Tbrain-1). Interestingly, we found that sequences between −155 and −140 bp showed reduced activity in this assay. Reporter activity is normalized

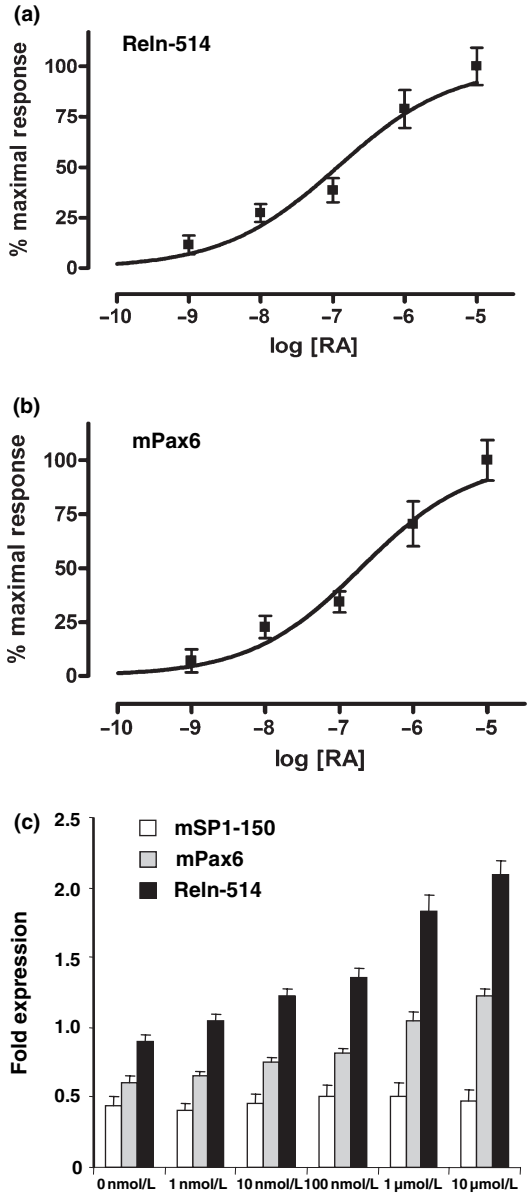

Fig. 6 Dose–response curves for retinoic acid (RA) induction of the reelin −514, mPax6, and mSp1-150 promoter constructs following RA treatment of transfected cells. NT2 cells were transfected (6 h) with either the reelin −514 promoter (a) or the mPax6 construct (b) and RA dose–response curves were measured 48 h later. Data are normalized to the lowest (minimal) and highest (maximal) responses. (c) Similar dose–response bar curves for RA induction following transfection of the mSp1-150 (white bars), mPax6 (gray bars), and reelin −514 promoter (black bars) at the indicated doses of RA. The data are presented as percent of the SV40 promoter used as a reference in parallel. Data were expressed as bars because the mSp1-150 construct did not respond to RA induction of reporter activity. These data are consistent with the action of RA requiring an intact Sp1 site at −150 bp relative to the transcriptional start site. A statistical analysis (Probit followed by *t*-test) comparing the responsiveness (EC_{50}) of the reelin −514 promoter and the mPax6 promoter to RA of these two constructs to RA was not significantly different.

to that measured from the −514 bp construct. There was additional further loss when sequences between −140 and −127 bp were removed (Fig. 7a). This finding is consistent with an indirect site of action of Tbr-1 acting at the levels of the Pax6 site. This can be seen in Fig. 7b, where each of the constructs are co-transfected with expression vectors encoding Tbr-1 and CASK. The construct that contained the mPax6 site showed the lowest activity suggesting that Tbr-1 might be acting in concert with Pax6.

The pGL3-control vector contains the SV40 promoter driving expression of luciferase. We modified the pGL-3 promoter construct such that it contained two intact Tbr-1 sites upstream of the SV40 promoter (pGL3-promoter Tb1+). We then used this construct as a control to show that our expression vectors were able to activate reporter activity (Fig. 7c). The effect of Tbr1 on the pGL3-promoter Tbr1+ construct is clearly greater than on the basal SV40 promoter and there seems to be a clear positive effect on the reelin −514 promoter. The CASK expression vector by itself did not boost reporter activity from any of the promoters. The final two parts of Fig. 7c showed the results of varying the ratio of the Tbr-1 and CASK expression vectors on a constant amount of SV40 promoter, Tbr-1 (2X) pGL-3 or reelin −514 promoter.

Pax6 and Sp1 gel shifts: effect of RA

We synthesized oligos spanning the 5′-Tbr1-Sp1-Pax6-3′ sites to evaluate the effects of RA treatment on factor occupancy at these recognition sites. We synthesized two sets of probes and these are shown with the location of the putative binding sites in Fig. 8 (bottom). The first (Fig. 8a) contained the Sp1 site 5′ to the adjacent Pax6 binding site (−160 to −129 bp), while the second (Fig. 8b) spanned the putative 5′ Tbr-1 site adjacent to the Sp1 site (−170 to −144 bp). We then used the double stranded probes in a series of gel-shift experiments with extracts prepared from either non-treated (left panels) or RA-treated Ntera-2 cells (right panels). Panels shown in Fig. 8a represent the shifts obtained when the more downstream Sp1/Pax6 probe was used with nuclear extracts from non-treated (left) and RA-treated cells (right), respectively. The non-RA-treated extracts showed only weak binding to the probe with competition to the Sp1 site of the oligo (Fig. 8a, left). Arrows point to the positions of the putative Sp1 and Pax6 shifted bands. The RA-treated cells show much clearer binding to the indicated recognition sites. The third lane of each shift (left and right) shows the results of self-competition in which cold competitor competes both shifted bands. It appears that the band located below the position of the Pax6 shift may be non-specific as it does not self-compete. This band is more prominent in the presence of non-specific competitor (lane 8). In the RA-treated series, the data show two abundant shifted bands. Based on competition studies (see Fig. 8), the uppermost band self-

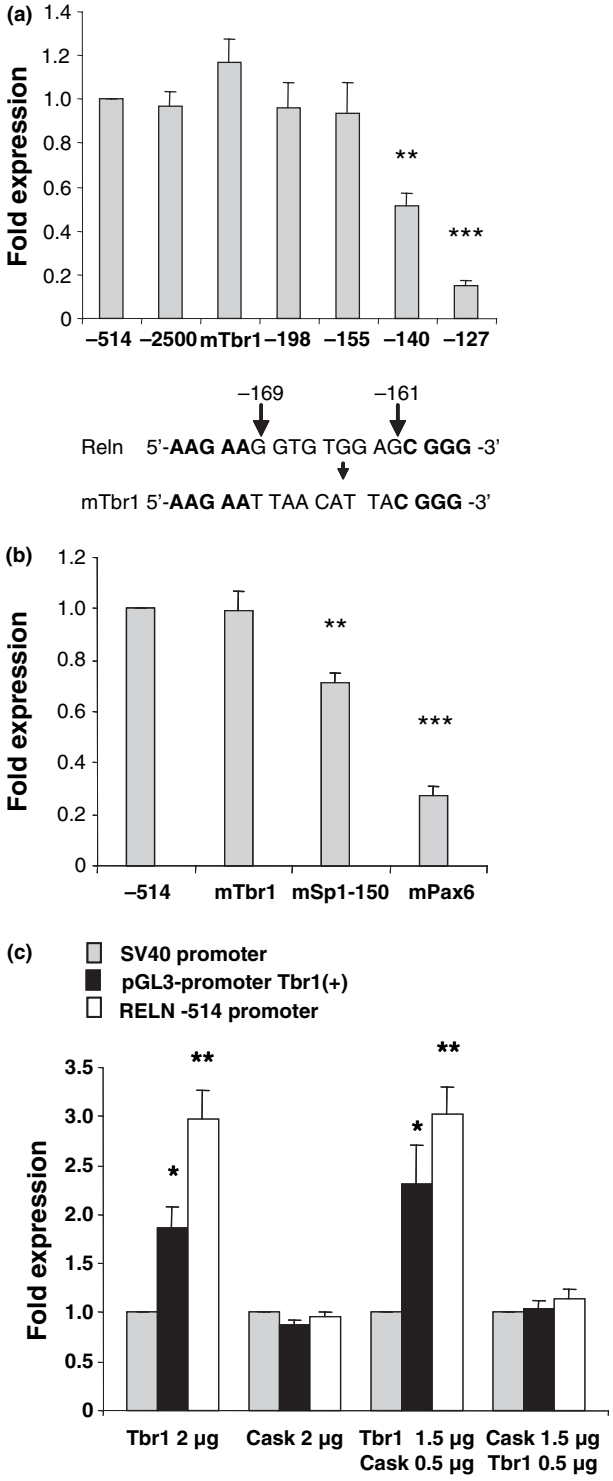

Fig. 7 Deletion and mutation assays examining the Tbr1-binding site. (a) A series of constructs were transfected into NT2 cells along with a Tbr-1 expression cassette to look for loss of function associated with these sequences. The tentative location and the bases changed in the mutant are shown below Fig. 7a. No loss of function was observed when sequences between −2500 and −514 bp were removed. Further deletion (to −155 bp) or replacement [mutant Tbrain-1 (mTbr1)] showed no loss of activity until the Sp1 site at −150 bp was deleted. Additional loss of function was observed in removing the Pax site (to −140 bp) in terms of the response to Tbr1. Values are compared with the reelin −514 promoter activity (1.0). (b) The mTbr1 construct is compared alongside the mSp1-150 and mPax6 mutants when co-transfected with a Tbr1 expression vector. Values are compared with the reelin −514 promoter activity represented as 1.0. A significant loss of function was observed upon separate mutations of the Sp1 and Pax6 sites. This suggests a possible interaction of Tbr1 with these factors rather than a direct interaction of Tbr1 with the promoter. (c) Co-transfection of Tbr1 with the pGL3-promoter (control), pGL3-Tbr1(+)-promoter and reelin −514 promoter show that Tbr-1 co-activates the reelin −514 promoter and the pGL3 Tbr-1 (2x) promoter with CASK having little effect. Values are normalized for transfection efficiency and compared with the SV40 promoter control (1.0). Data are mean ± SEM. ***$p < 0.001$; **$p < 0.01$, and *$p < 0.05$ expressed as fold expression of the reelin −514 promoter for statistical purposes (one-way ANOVA followed by Fisher least significant difference method).

competes with the entire oligo and also competes with the Pax6-only oligo. The lower band (labeled Sp1) both self-competes and competes with the 5′ half of the oligo (Sp1-only). There is little competition with the Sp1 mutant oligo (mSp1). The presence of Sp1 antibody in the shift mix (Fig. 8a, right, lane 7) weakens both shifted bands suggesting the possibility that disrupting the Sp1 shift could interfere with the Pax6 shift.

In Fig. 8b, results obtained with the 5′ most double stranded oligo which contained the putative Tbr-1 site adjacent to the Sp1 site are shown. The probes were designed in this way as the three sites were thought to exist side to side within the enhancer region of the reelin promoter (Grayson *et al.* 2006). The left portion of Fig. 8b shows results obtained from nuclear extracts prepared from non-treated cells, while the right hand shows a comparable set of shifts obtained using RA-treated nuclear extracts. With this probe, the pattern is somewhat simpler in the sense that there is only a single set of shifted bands. In non-treated NT2 cell nuclear extracts, there is no single shift that shows both self-competition and competition with either the Tbr-1 site or Sp1 site alone. There are a few additional bands that appear to be non-specific. In contrast, the RA-treated extracts contain a single prominent band that self-competes (compare lanes 2 and 3) and competes with the Sp1 only probe (compare lanes 2 and 4). The non-specific bands that were present with the non-treated extracts are also faintly evident. The major band is competed by the Sp1 site, but not the non-specific competitor site (compare lanes 2 with lanes 5 and 8). The Tbr-1 site alone does compete with this shift confirming our deletion studies and suggesting that this site is not the recognition site for Tbr-1.

Chromatin immunoprecipitation experiments

We also confirmed the results obtained from the gel-shift experiments using ChIP-based assays. The experiment was

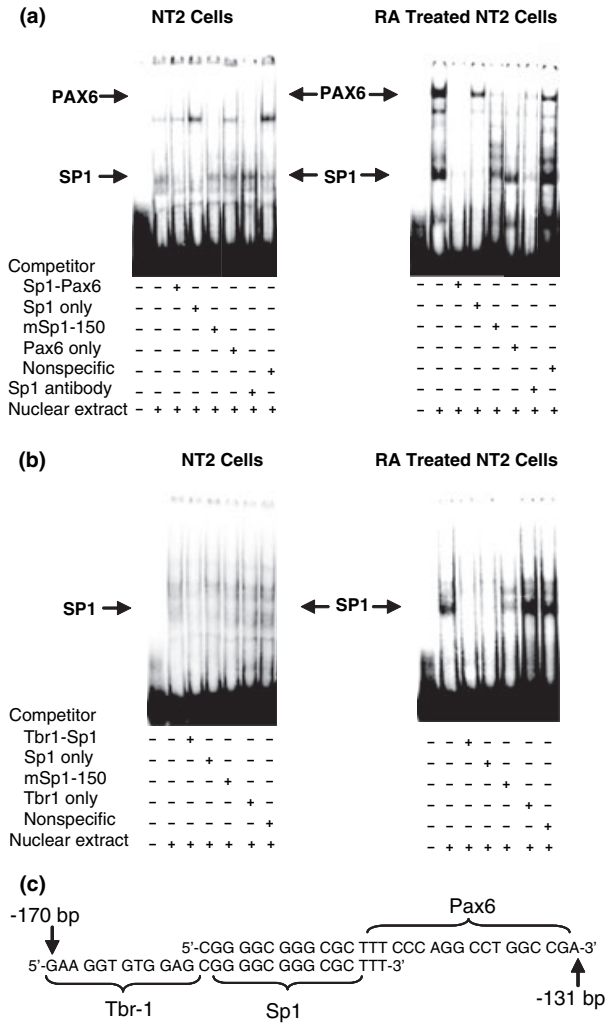

Fig. 8 Multiple binding site gel shift with extracts prepared from non-treated and retinoic acid (RA)-treated NT2 cells. (a) The Sp1-Pax6 probe bind proteins present in nuclear extracts prepared from NT2 cells treated with RA for 6 days (right). There were no specific Pax6- or Sp1-shifted bands in the non-treated NT2 cells nuclear extracts (left panels) as compared with extracts prepared after RA treatment for 6 days (right panels). The presence or absence of competitors (100X) are indicated in the table below each panel. Based on the competition experiments, the presence of specific bands are indicated by arrows. The probe shown in (a) spans both the Sp1 and Pax6 binding sites. (b) The upstream Tbr1-Sp1 probe binds to a protein present in RA treated (6 days) NT2 cell nuclear extracts. Competitor oligos: Pax6, Tbr1, Sp1, and mutant-Sp1 (5′-TAAGAAGGTGTGAGCAGATCTAT-CATGTTTCCCAGGCCTGGC-3′), were used to identify the binding sites as indicated.

based on the presumption that if RA-induced expression increased binding of Sp1 and Pax6 to sequences within the reelin promoter, then antibodies directed against the respective transcription factors should show increased binding following treatment. To this end, NT2 cells were treated with RA for 6 days and proteins were formalin cross-linked to genomic DNA. Nuclear extracts were prepared and the DNA was sonicated to fragments of some 500 bp or shorter. Acetylated histone H3 (Ac-H3), Sp1, and Pax6 specific antibodies were used to pull down protein DNA complexes and association with the reelin promoter was detected using a PCR-based assay. We have previously shown that when the reelin transcription unit is inactive (negative regulation), there is robust binding of methyl cytosine-phospho-guanine dinucleotide binding protein and DNA methyltransferase I to the promoter (Kundakovic *et al.* 2007). We have now looked at positive regulation in the same context. Figure 9 shows data obtained using antibodies against Ac-H3, Sp1, and Pax6 before and after RA treatment. Under control (non-treated) conditions, the data showed no specific interactions of Ac-H3, Sp1, or Pax to either reelin or the β-globin control locus (Fig. 9a and b). In contrast, following treatment with RA the ChIP data showed positive binding of each of the antibodies to a section corresponding to the reelin promoter that contains these defined recognition sites. Results from multiple experiments were obtained and gels were scanned. Quantitated results are shown (Fig. 9b) expressed relative to the amount of input DNA amplified in parallel. The β-globin locus control region was used as a negative control for any changes associated with RA treatment (Mitchell *et al.* 2005). The β-globin gene is inactive in these cells and should not show increased binding under these conditions.

Discussion

We have focused on *cis*-acting elements and associated transcription factors that positively regulate reelin expression. Based on both transient transfections and gene knockout studies, the transcription factors Tbr-1 (Hsueh *et al.* 2000; Hevner *et al.* 2001) and Pax6 (Stoykova *et al.* 2003) have been implicated in regulating reelin expression. We sought to determine if these factors were important in the context of mediating the RA induction of the reelin promoter in the NT2 cell system. In addition, we evaluated the levels of Sp1 mRNA and protein as previous studies had identified several putative Sp1-binding sites within the reelin promoter/enhancer region and because of numerous reports showing the involvement of Sp1 proteins in RA-induced gene expression (Husmann *et al.* 2000; Safe and Kim 2004). Our data showed that RA treatment of NT2 cells up-regulated the levels of the Tbr-1 and Pax6 transcription factor mRNAs with a time frame that was comparable with that of the reelin mRNA. At the same time, these experiments showed no temporal change with respect to either CASK or Sp1 mRNAs. These studies (Table 2) show changes in some mRNAs encoding transcription factors (Tbr1, Pax6) relevant to reelin mRNA expression while others are already present (Sp1) and may undergo post-translational modifications prior to interacting with specific promoters.

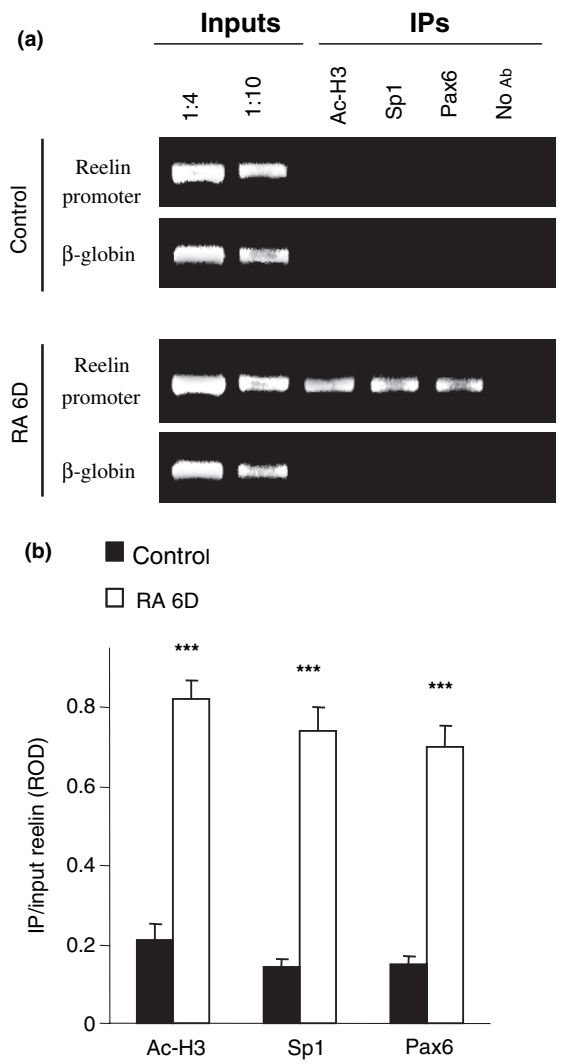

Fig. 9 Transcription factor Sp1 and paired box gene 6 (Pax6) bind to the reelin promoter following retinoic acid (RA) treatment as determined by chromatin immunoprecipitation (ChIP) assays. (a) Representative gels of the amplified reelin promoter region (482-bp band) and β-globin gene fragment (289-bp band) from non-immunoprecipitated input (1 : 10 and 1 : 4 dilutions), samples immunoprecipitated with anti-acetyl histone H3 antibody (Ac-H3 IP), anti-Sp1 (Sp1 IP) or anti-Pax 6 antibody (Pax6 IP), and negative control (no Ab). (b) Results of semi-quantitative analysis of the association of Ac-H3, Sp1, and Pax6 to the reelin promoter in vehicle- and RA-treated cells normalized to input DNA (1 : 10 dilution). Data are presented as ratio of relative optical densities (RODs) of the bands within the immunoprecipitated (IP) sample and input lanes from ethidium bromide-stained gels. The values represent mean ± SEM, ***$p < 0.001$, control versus RA (Student's *t*-test).

Using promoter/transcription factor co-transfection experiments, we demonstrated a role for Sp1 and Pax6 in regulating reelin promoter expression. Both Sp1 and Pax6 act within a short stretch of the reelin promoter that was previously shown to demonstrate enhancer-like properties (Chen *et al.* 2002). While the Sp1 site at −150 bp matches conventional Sp1 sites, this was not the case with the Pax6 site. For this reason, we performed competition experiments with a consensus Pax 6 site and showed that recombinant Pax6 binds to both its consensus site and the reelin-Pax6 site variant. This region of the promoter also contains a DNase I hypersensitive site that appears following RA treatment. This latter finding is consistent with a change in chromatin conformation in the promoter near the site of RA action. The observation that these two sites are adjacent suggests that a multimolecular protein complex may form in this region which is located some 130 bp from the RNA start site. While this bi-directional enhancer element is located within 130 bp of the RNA start site it is not known if the intervening sequences are dispensable for activity or not. However, the location implies that a short loop of DNA forms that brings the DNA/transcription factor complex proximal to the binding sites for protein components of the RNA polymerase II complex.

Data obtained with respect to the Sp1 and Pax6 recognition sites support the argument that chromatin in the vicinity of the reelin promoter undergoes a transition following RA treatment. Collectively, the deletion studies, mutation transfections, and gel-shift data indicate a role for these sites in modulating reelin promoter/reporter expression. Moreover, the ChIP data show that conditions which increase reelin mRNA expression also increase binding of these two factors to the promoter. Based on the data presented, it appears that the transcription factor primarily responsible for the RA induction of reelin promoter activity is Sp1. This was most clearly demonstrated through the dose–response relationship in terms of the −514 bp promoter and the mSp1-150 construct (Fig. 4). The mSp1-150 promoter construct was not responsive to the effects of RA. Other regions of the promoter such as the reelin −514, the mSp1-230, the mSp1-180, and the mPax6 promoter constructs all responded to the effects of RA. These data are consistent with findings of others that show that promoters lacking consensus RA-responsive elements are often indirectly activated through the action of Sp1 (Suzuki *et al.* 1999; Husmann *et al.* 2000; Shimada *et al.* 2001; Lee *et al.* 2005; Kwon *et al.* 2006). Presumably, the action of RA with the RA-receptor induces the phosphorylation of Sp1 which becomes active in promoting expression of responsive genes (Horie *et al.* 2001; Li *et al.* 2002). This is also consistent with our finding that RA-induces Pax6 mRNA levels but does not increase the expression of Sp1 mRNA.

Our evidence for a role for Tbr-1 indirectly acting at the reelin promoter seems less clear. Previous work has shown that Tbr-1 and CASK act to enhance reelin expression through two TCACAC sites located at −4309 and −2108 bp relative to the reelin mRNA start site (Hsueh *et al.* 2000; Wang *et al.* 2004). It remains possible that additional Tbr-1 sites located further upstream could play a

role in modulating reelin promoter activity. However, our data with the −2.5 kb construct and our co-transfection data with the −514 bp promoter suggests that the actual site of action is somewhat closer to the start site than previously identified. Results from co-transfection experiments suggest that the Tbr-1 site was just upstream of the Sp1 site. However, this was not confirmed with the deletion studies and no change was observed with the Tbr-1 site-directed mutant. The −155 deletion promoter removed the Tbr-1 site while maintaining the Sp1 site intact. This produced no loss of function. Deletion of sequences from −155 to −140 bp removed the Sp1 site which produced a loss of function so the precise mapping of the Tbr-1 site is not clear. The gel-shift data are not compelling either way as we have not been able to provide convincing evidence that the Tbr-1 site located just upstream of the Sp1 site shifts a band in our conditions. This would suggest that the Tbr-1 induction following RA treatment is coincidental and that it instead acts by driving the action of either Pax6 or Sp1 in the RA-mediated induction of reelin mRNA. Data supporting this include the loss of activity seen with the mPax6 site in the Tbr-1 co-transfection assays. In fact, this is the only mutant that showed loss of activity using this assay.

Much of our previous work describing the regulation of the reelin promoter focused on control of promoter activity through methylation (Grayson *et al.* 2006). The current set of results compliment those studies and provide new evidence establishing the interplay between negative and positive regulation of the reelin promoter. Our current hypothesis suggests that genes that are regulated by changes in methylation also rely on positive factors that engage and accompany alterations in chromatin structure. Using the RA-induction model, we provide evidence that support a model in which the promoter undergoes a combination of demethylation and detachment of repressor proteins. This is then followed by a more open chromatin conformation that allows access of positive transcription factors to this region promoting transcription.

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