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Maximal Expression of the Evolutionarily Conserved *Slit2* Gene Promoter Requires Sp1

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Abstract

Slit2 is a neural axon guidance and chemorepellent protein that stimulates motility in a variety of cell types. The role of *Slit2* in neural development and neoplastic growth and migration has been well established, while the genetic mechanisms underlying regulation of the *Slit2* gene have not. We identified the core and proximal promoter of *Slit2* by mapping multiple transcriptional start sites, analyzing transcriptional activity, and confirming sequence homology for the *Slit2* proximal promoter among a number of species. Deletion series and transient transfection identified the *Slit2* proximal promoter as within 399 base pairs upstream of the start of transcription. A crucial region for full expression of the *Slit2* proximal promoter lies between 399 base pairs and 296 base pairs upstream of the start of transcription. Computer modeling identified three transcription factor binding consensus sites within this region, of which only site-directed mutagenesis of one of the two identified Sp1 consensus sites inhibited transcriptional activity of the *Slit2* proximal promoter (–399 to +253). Bioinformatics analysis of the *Slit2* proximal promoter –399 base pair to –296 base pair region shows high sequence conservation over twenty-two species, and that this region follows an expected pattern of sequence divergence through evolution.

Keywords

promoter; Slit2; transcription; phylogenetics; evolution

Introduction

The SLIT family of proteins are secreted by midline glial cells, while their receptor, Roundabout (Robo), is expressed on the axon growth cone. In mammals, Slit-Robo signaling is required for the proper development of the central nervous system (CNS), and organs including the lung, kidney, and mammary gland. Slit-Robo signaling also plays a role in leukocyte chemotaxis, and in the migration of endothelial cells (Ypsilanti et al. 2010). Recent studies suggest that slit-robo signaling affects tumorigenic properties such as angiogenesis, adhesion, apoptosis, and proliferation in complex ways that often contradict each other (Ballard and Hinck 2012).

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The membrane-bound Slit2 protein was originally thought of as a mid-line guidance molecule only; chemorepulsive to trunk neural crest cell migration (Kidd et al. 1999; De Bellard et al. 2003; Blockus and Chédotal 2014). Specifically, an *in vitro* extracellular Slit2 protein gradient results in a reversal in the direction of migration of neuronal precursor cells without affecting their migration speed (Xu et al. 2004). In fact, Slit2 gain-of-function mutations significantly impaired neural crest cell migration while Slit2 loss-of-function mutations favored migration (Giovannone et al. 2012). But, Slit2's role in nervous system development and function has extended far beyond migration and patterning to include the outgrowth of dendrites and the formation of the spine itself (Blockus and Chédotal 2014).

In original studies, *Slit2* gene expression was found in many human stationary tumors (Wang et al. 2003), but not in aggressive metastatic tumors (Dallol et al. 2002; Dallol et al. 2003). A recent compilation tumor types suggests that this categorization is much more complicated. In fact, *Slit2* expression is down-regulated in many tumor types including lung, brain, cervical, acute lymphocytic leukemia, and breast cancers (Blockus and Chédotal 2014). In fact, in ~50% of sampled human breast tumors, the *Slit2* gene was silenced. Additionally, breast cancer cells that actually over-expressed *Slit2* exhibited decreased proliferation and migration capabilities compared with control cells under *in vitro*, and *in vivo* conditions (Marlow et al. 2008). Increased levels of *Slit2* expression are also associated with colon and prostate cancers (Blockus and Chédotal 2014).

Appropriately controlling the expression of *Slit2* is absolutely critical for proper CNS formation and development, and for controlling its tumorigenic and metastatic potential. Here, we identify and characterize the *Slit2* gene promoter as an evolutionarily conserved, TATA-less, dispersive transcription start site promoter that relies on an Sp1 site for maximal transcription.

Materials and Methods

Rapid Amplification of cDNA ends

The Invitrogen 5' RACE system (Invitrogen) was used to perform 5' RACE as described. Gene Specific Primers GSP1 (Reverse), GSP3 (Forward) and GSP2 (nested, Reverse) (Integrated DNA Technologies) were designed using the Primer 3 software as per the Invitrogen 5' RACE system protocol.

GSP1: 5'-CCTTCCTTGGAATTGCTTGA -3',

GSP2 (nested): 5'-TCTCGATGGTGCTGATTCTG -3',

GSP3: 5'-CTCCTGCCCCATATCACTGT -3'.

Total RNA was extracted from NIH3T3 cells using the RNeasy mini prep kit (Qiagen) as per the protocol. 5' RACE parameters were as per protocol using total RNA and primers with $T_m < 60^\circ\text{C}$.

Plasmid construction and mutagenesis

The mouse *Slit2* putative promoter sequence was obtained using a mouse cDNA for *Slit2* (Accession number NM_178804.4) and the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Protocol (BLAST) algorithm (Altschul et al. 1990; Altschul et al. 1997; Zhang and Madden 1997). Putative transcription factor binding sites in the mouse *Slit2* promoter were identified using the following transcription factor analysis databases: TF SEARCH, MATCH public version 1.0, Alibaba 2.1, ConSite, PROMO, and Motif Viz. The *Slit2* promoter region was PCR amplified from mouse genomic DNA using primers at -1023 bp upstream and +239 bp downstream (just before the first ATG start of translation) of the Genbank recorded transcription start site. -1023 F: 5'-GAAACTTCCTGCACTTTGAGGCTGAGGAC-3', +200 R: 5'-CTTCCCTAATAGACTCCTTGCTTTGAAGG-3'. The 1223bp fragment was directionally cloned into the pGL3 Basic Vector (Promega). *Slit2* promoter deletion constructs were generated using existing or by creating restriction sites using the Quik-change site-directed mutagenesis kit (Stratagene), excising the internal fragment, and religating the vector. Complementary oligonucleotides for site-directed mutagenesis are as follows:

NheI-153: 5'-ACTGGAATCGGATTTGCTAGcGGGTTTCGTGGGATCA-3',

XhoI-674: 5'-GCTCTTCTGGGCTCGagTATCTGCAAATCTTGC-3',

MluI-490: 5'-GCGTCGCCAAGGACGCgtGGAGGGAGGCGGGATTG-3',

MluI-399: 5'-GGAAGTGGAGAAGACGcgTGAGGCTGCTGCTGT-3',

MluI-296: 5'-GTGCGCCCCACGCGtGCCATGACCAGTCTC-3',

PstI-100: 5'-GCAACCAGCCTCTGCAgCTTCGCGGACACTGC-3'.

The *Slit2* constructs with mutated Sp1 or USF sites were created using the Quik-change site directed mutagenesis kit (Stratagene, La Jolla, CA, USA) and the following complimentary oligonucleotides:

Sp1 (-359bp): 5'-GAGCCCTGAGaaCCGCATCACCGCCCGGCACCCCC-3',

Sp1 (-345bp): 5'-GCATCACCGCtCGGCACtCCAAGGCTGTCTGAATACC-3',

USF (-305bp): 5'-GAATACCCGGTGCgCCCCCAatCGCGCCATGACCAGTCTC-3'.

Cell Culture, Transfections, and Dual Luciferase assays

Cell culture media was supplemented with 3.7g NaHCO₃, 1X Sodium Pyruvate, 1X Non-essential Amino Acids, 1X Penicillin-Streptomycin, and 10% Fetal Bovine Serum. HEK 293T cells were grown in RPMI, and NIH 3T3 cells were grown in DMEM. Cell lines were co-transfected using 2-3.5 ul Effectene transfection reagent (Qiagen), 0.6 micrograms pGL3 constructs, 0.4 micrograms pRL SV40 for normalization, and assayed as described using the Dual Luciferase assay kit (Promega). Results are the averages of at least three independent

transfections using at least two preparations of Mini-prep DNA (Qiagen). Statistical analysis was performed using the Student's two-sided t-test in Microsoft Excel.

Multiple sequence alignment

The *Slit2* promoter sequence was used in conjunction with a Basic Local Alignment Search Protocol (BLAST) to obtain orthologous *Slit2* promoter sequences from other species in the NCBI nucleotide database. A total of 21 species with a highly conserved DNA sequence (>50%) in the -399 to -296bp *Slit2* promoter region were found in NCBI (not including the mouse *Slit2* promoter query sequence). The species and accession numbers the BLAST protocols are listed in Supplemental Table 1. A Multiple Sequence Alignment (MSA) was constructed using CLUSTAL W 3.2 (<http://workbench.sdsc.edu>), and BioEdit 7.0.9.0 software (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>).

Model Testing

To determine the best-fit substitution model for the MSA, model testing was performed in the Model Test Program within MEGA 5.1. Model testing included the Bayesian information criterion (BIC) and the Akaike information criterion (AIC) to assess a substitution model's goodness of fit to the dataset (Guindon and Gascuel 2003; Posada and Buckley 2004; Erb and van Nimwegen 2011). The Model test data indicate that the Tamura 3-parameter with Gamma distributed rates (T92+G) model, having the lowest Akaike Information Criterion (AIC) score, will provide the best DNA model for the pairwise distances of the DNA sequences. (Supplemental Table 2). Each parameter of the T92+G model including the individual base frequencies, nucleotide substitution rates, transition/transversion bias (R), and the gamma distribution shape parameter (+G), which was determined to be essentially one (1.08). Invariable sites (+I) were not detected. Base substitution rates were also calculated.

Tree Construction

Bayesian Analysis was performed using Mr. Bayes 3.1.2 (Huelsenbeck and Ronquist 2001). The Bayesian analysis parameters include three million generations, generation of a tree every 100 generations, and a "burn-in" value of 7,000 generations. MEGA 5.1 was used to produce a Maximum Likelihood tree using parametric bootstrapping at 5000 replicates. Maximum Parsimony and Neighbor-Joining trees were both produced with bootstrapping for 5000 and 10,000 replicates using MEGA software.

Results

The *Slit2* gene has multiple, dispersed transcription start sites

Analyses of core promoters have led to the discovery of consensus sequence motifs such as the TATA box, the Initiator element (Inr), the motif ten element (MTE), the downstream core element (DCE), and the downstream promoter element (DPE) that drive basal levels of transcription from focused single transcription start sites (TSS) (Juven-Gershon and Kadonaga 2010). These elements are generally not found or not functional in promoters with multiple dispersed TSS (Juven-Gershon et al. 2008). Using the *Slit2* gene mRNA sequence, NCBI BLAST was performed and the genomic DNA region upstream of the 5'UTR was

identified. Computer analyses of the putative promoter region using multiple transcription factor scanning software failed to identify a consensus TATA box sequence, an Inr, an MTE, a DCE, or a DPE in the *Slit2* putative promoter. The lack of these consensus sequences suggests that transcription initiation of the *Slit2* promoter occurs at multiple dispersed sites (Juven-Gershon and Kadonaga 2010). 5' RACE confirmed multiple dispersed TSS in the putative *Slit2* promoter, confirming the computer analyses (Figure 1). The TSS suggested by mRNA sequences submitted to GenBank™ is located 253 bp upstream of the ATG translation start site. 5' RACE analysis identified six independent TSS in the *Slit2* promoter that were located 38 bp, 57 bp, 68 bp, 72 bp, 202 bp, and 314 bp upstream of the first ATG (Figure 2) and are consistent with a dispersed mode of transcription initiation. Interestingly, none of the experimentally identified TSS aligned with the suggested start site from mRNA sequences submitted to GenBank™ (253 bp).

Identification of the *Slit2* gene proximal promoter

To define the boundaries of the most active *Slit2* gene promoter sequences and to identify *cis* elements that may govern the transcriptional activity of *Slit2*, a series of deletion constructs was prepared (Figure 1) and tested for promoter activity using transient transfection of a mouse fibroblast cell line (NIH 3T3) and the dual luciferase assay system (Figure 2). The ten constructs tested, –1075 bp, –674 bp, –607 bp, –490 bp, –469 bp, –399 bp, –296 bp, –153 bp, and –100 bp all showed significant activity over promoterless control (Figure 2). The *Slit2* promoter sequence for each deletion construct is delineated in Figure 1.

The *Slit2* –296 bp, –153 bp, and –100 bp constructs all showed a statistically significantly lower activity than the *Slit2* –399 bp construct (Figure 2). The significant drop in activity between the *Slit2* –399 bp construct and the *slit2* –296 bp suggests the putative transcription factor binding sites within this region may be essential for maximal proximal promoter transcription of *Slit2*. Therefore, the *Slit2* –399 bp and –296 bp region was analyzed for putative transcription factor-binding sites using TFSEARCH, AliBaba2.1, MATCH, ConSite, PROMO, and Motif Viz databases.

Evolutionarily conserved transcription factor consensus sequences

Three putative transcription factor-binding site consensus sequences were identified within the *Slit2* –399 bp and –296 bp region, two Sp1 sites (–359 bp and –345 bp) and a USF site (–305 bp), by at least two of the above six search algorithms (See Figure 1). Both Sp1 and USF are well known transcription factors that commonly interact with sequences in TATA-less promoters like *Slit2* (Workman et al. 1990; Emami et al. 1998; Erb and van Nimwegen 2011). Other putative transcription factor-binding site consensus sequences identified within the entire *Slit2* promoter –1075 bp are shown in Figure 1 as well. Although using the CLUSTALW Algorithm and BioEdit7.0.9.0 to create a Multiple Sequence Alignment (MSA) of the human, rat and mouse *Slit2* –1023 promoter regions revealed high conservation (>70%, data not shown), adding additional species to the alignment resulted in a loss of conservation. These additional orthologous species were identified by NCBI BLAST analysis using the mouse promoter sequences as a reference. An MSA of the *Slit2* –399bp to –296bp region of the proximal promoter revealed 21 species with highly conserved DNA sequences in this region (data not shown) (Brown et al. 1998). The MSA of

the mouse *Slit2* proximal promoter showed a 51.5% conservation of sequence in the least related species, the Giant Panda, with the highest conservation of sequence seen in the Norway Rat sequence (90.4%). A high level of conservation among all of the Primate species, with the exception of the Galago, and a high level of sequence conservation among the Ungulates was also found (see phylogenetic tree in Figure 4).

Several transcription factor binding site search algorithms were used to identify conserved transcription factor consensus binding sequences in the areas of highest conservation among the 21 species. Figure 1 shows the identified transcription factor consensus binding sites for Sp1(–359), Sp1(–345) and USF1(–305). Among the 21 species, the Sp1(–359) site had only 2/8 identical base pairs (25%) and 3/8 base pairs that were conserved (37.5%), suggesting this consensus sequence was conserved, but not as highly conserved through evolution as the other two sites. Both the Sp1(–345) site and the USF1 site showed higher conservation of identical and conserved sequences among the 21 species analyzed. The Sp1(–345) site was 5/13 identical base pairs (38.5%) and 9/13 conserved base pairs (69%). The USF1 site was 6/12 identical base pairs (50%) and 7/12 conserved base pairs (58%). These data lend strong evidence for evolutionary conservation of these transcription factor consensus binding sites, and for the conclusion that this region is in fact the proximal promoter of the *Slit2* gene. Figure 4 illustrates this *Slit2* –399bp to –296bp promoter region MSA graphically in phylogenetic tree form showing the evolutionary relationships among the 21 species.

Phylogenetic analysis of the *Slit2* –399bp to –296bp proximal promoter

Model testing was performed on the MSA to determine the evolutionary best-fit model for phylogenetic tree construction (Supplemental Table 2). Bayesian inference was performed using the Tamura 1992 model with Gamma distributed rates (T92+G) substitution model for tree construction (Figure 4). All of the primate clades show high Bayesian support values save for the clade containing the Marmoset and the Bolivian Squirrel Monkey, due to a higher degree of divergence between the two species' sequences. As expected, the Mouse and Norway rat are grouped in a single clade with a high Bayesian support value, as is the clade containing the Orca, Sheep, and Cow. The branch that gives rise to the Horse, Walrus, White Rhinoceros, and African Elephant has a much lower Bayesian support value than most of the clades in the tree, as does the branch that separates the Galago, Mouse, and Norway rat from the Giant Panda, European Shrew, Star-nosed mole, Sheep, Cow, Orca, Horse, Walrus, White Rhinoceros, and African Elephant. This is likely due to a higher degree of divergence in the *Slit2* –399 to –296 bp proximal promoter sequence between these species. As the degree of divergence in the nucleotide sequence increases, the Bayesian support of species relatedness decreases. These data support the sequence conservation identified by MSA (data not shown and Figure 4).

A single Sp1 site is essential for maximal *Slit2* proximal promoter activity

The three most highly conserved transcription factor consensus binding sites were mutagenized to determine their importance in the *Slit2* –399 bp proximal promoter. Site-directed mutagenesis of the Sp1(–359 bp), Sp1(–345 bp) and USF1(–305 bp) sites, using defined sequences known to abolish binding of the respective transcription factors (REFS), were introduced into the *Slit2* –399 bp promoter construct and tested for activity in transient

transfection. Figure 5 shows that mutations in the Sp1(–359 bp) site resulted only in a slight inhibition of transcriptional activity that was not statistically significant ($p>.05$). Surprisingly, mutations in the USF1 site did not affect promoter activity either, even though it is highly conserved through evolution (Figure 3). When the Sp1 (–345 bp) with high sequence identity and conservation through evolution was mutated, transient transfection of the *Slit2*–399 bp promoter construct resulted in a statistically significant inhibition of transcriptional activity ($p<.001$), indicating that this site is crucial for maximal transcription of the *Slit2*–399 bp proximal promoter.

Discussion

Gene expression is generally governed by the presence of certain cis-acting motifs within a promoter, including the TATA box, the downstream core promoter element (DPE), and the Initiator (Inr), that interact with transcription initiation proteins (Juven-Gershon and Kadonaga 2010). When transcription initiation occurs from promoters that lack these motifs, multiple transcription start sites result (Anish et al. 2009). Dispersed transcription initiation from multiple TSS occurs in about 70–72% of promoters in vertebrates, while focused initiation is the predominant mechanism in simple organisms (Juven-Gershon et al & Anish et al). These data are consistent with what we have found for the *Slit2* promoter lacking the common transcription initiation motifs, TATA, DPE, and Inr and having multiple transcription start sites. Maximal activity of the mouse *Slit2* (mu*Slit2*) gene promoter lies in the –399 bp region, suggesting important transcription factors interact there. Although computer analyses found many transcription factor binding consensus sequences, only ten were found commonly among the four algorithms used to analyze this region. The three consensus sequences found in the region between the –399bp and –296 bp of the *Slit2* promoter were subject to scrutiny because this region harbored the highest levels of promoter activity. Of the identified two Sp1 sites and USF1 site, mutations at only one of the Sp1 sites, Sp1(–345 bp), showed a statistically significant reduction in transcriptional activity. The mutations created in both of the Sp1 sites and the USF site were previously shown to reduce binding of these transcription factors and to reduce transcriptional activity (Sims et al. 1993; Tamaki et al. 1995; Jiang et al. 1997; Huang et al. 2013).

The Sp1 transcription factor is a zinc-finger protein that can recruit the basal transcription factor TBP/TFIID to the initiation machinery, explaining previously characterized ability of Sp1 to induce transcription of TATA-less genes (Brandeis et al. 1994; Emami et al. 1998; Paonessa et al. 2013). The Sp1 core consensus sequence, CCGCCC or GGGCGG, has been further defined as (G/T)GGGCGG(G/A)(G/A)(C/T) (Song et al. 2001), although the Sp1 transcription factor is known to bind a variety of GC-rich sequences known as GC boxes (Solomon et al. 2008). In fact, just the core sequence, CGCC or GGcG was sufficient for DNA binding in vitro (Thiesen and Bach 1990). Sp1 sites tend to occur and contribute to the unmethylated state of CpG islands and therefore gene expression levels (Futscher et al. 2002) in TATA-less promoters (Brandeis et al. 1994; French et al. 2003; Cheng et al. 2004). CpG islands are generally at least 200 bp in length where the overall GC content is greater than 55%, and includes more CpG sequences than by random chance compared to the rest of the genome (Deaton and Bird 2011). While the majority of CG dinucleotides outside CpG islands in the human genome are methylated, those within the islands are not (Bird 1980;

Gardiner-Garden and Frommer 1987). CpG island promoters typically initiate from multiple start sites, unlike the single start sites characteristic of TATA box, DPE, and Inr promoter types (Suzuki et al. 2001), and are the most common type of promoter in the mammalian genome (Gagniuc and Ionescu-Tirgoviste 2012). The *Slit2* promoter classifies as a CpG island due to its high GC content (62 %) and 26 CpG sites within the –399 bp promoter alone. In fact, the *Slit2* gene was silenced by promoter hypermethylation in many aggressive metastatic tumors (Dallol et al. 2002; Dallol et al. 2003), including 29% of neuroblastomas, 38% of Wilms' tumors, and 25% of renal cell carcinomas studied (Astuti et al. 2004), while it was expressed in many stationary tumors (Wang et al. 2003). Taken together, these data are consistent with our mutagenesis results showing that Sp1(–345) is critical for *Slit2* gene expression from the –399 bp promoter. The USF transcription factor is a basic helix-loop-helix leucine zipper family protein that activates transcription at initiator (Inr) elements (C/T C/T A N A/T C/T C/T) and E-box motifs (5'-CACGTG-3') (Roy et al. 1991; Jones 2004). The *Slit2* promoter contains an evolutionarily conserved non-canonical E-box motif (5'-CACGCG-3') that when mutated (5'-CAatCG-3') did not affect transcription of the *Slit2* –399 proximal promoter, suggesting that it was not a functional E-box in our experiments (Carr and Sharp 1990; Gregor et al. 1990; Sims et al. 1993; Chen et al. 2012; Huang et al. 2013). Because USF is known to interact with other transcription factors, such as Sp1 (Ge et al. 2003), it is possible that we did not see any difference in activity without combinatorially mutating the Sp1 and USF sites. The region just 5' adjacent to the non-canonical E-box was highly evolutionarily conserved as well, but the transcription factor consensus sequence analyses did not consistently identify any transcription factor binding consensus sites there (See Figures 1 and 3). Further mutational analyses of this region would be necessary to uncover the significance of the evolutionary conservation.

Interestingly, the region just upstream of the *Slit2* –399 region shows a much lower level of activity, suggesting the presence of silencer elements or negative regulation within the –469 to –400 *Slit2* promoter region (Malone et al. 2000; Malone et al. 2001; Malone et al. 2006). Another evolutionarily conserved E box is present within this region, potentially interacting with USF transcription factors (See Figure 1). Although most DNA interactions with USF promote gene activation, it can interact with negative cofactors (Anantharaman et al. 2011). In this case, however, USF and its negative cofactor repressed TATA-driven promoters, and the *Slit2* promoter does not contain a TATA box (Meisterernst et al. 1991). Again, a thorough mutational analysis of this region would be needed to implicate the perpetrators of the negative regulation in this region.

Model testing of the entire conserved *Slit2* –399 bp to –296 bp promoter MSA indicated that the T92+G model provided the best DNA model for pairwise distances. This method provided the lowest AIC score, likely because the T92 model accounts for the differences between rates of transition mutations (a change from one purine to another) and transversion mutations (change from a purine to a pyrimidine), allowing for variable substitution rates, unlike the Jukes-Cantor model, which has a higher AIC score. The T92 model also accounts for G/C content bias, which is a difference in the number of G and C nucleotides in a DNA sequence from that of A and T nucleotides, unlike the Kimura 2-parameter model which assumes that the frequencies of all four nucleotides A, T, C and G remains constant (.025) over evolutionary time (Tamura 1992).

Three methods of phylogenetic tree generation were performed that resulted in trees with similar topology. Maximum likelihood, Neighbor Joining, and Bayesian inference trees were generated, but only the Bayesian inference tree is shown in Figure 4 because it was produced using the Markov Chain Monte Carlo simulation. This process results in a posterior probability distribution and gives an actual statistical probability that the tree was accurately constructed (Huelsenbeck and Ronquist 2001). Each of the three methods grouped the primates in a single clade or, as is the case with the Bayesian inference tree (Figure 4) with the primates originating from the same branch, and then grouped the others into different clades according to the degree of species relatedness. Each of the trees grouped the ungulates in a clade, with the other orders grouped therein (Felsenstein 1981; Efron 1982; Felsenstein 1985). The Bayesian inference tree is presented here because overall, the bootstrapping values are highest for the Bayesian inference analysis compared to Maximum Likelihood and Neighbor Joining analyses (data not shown). The higher the percent value for Bootstrapping, the more confident the analysis was in the relatedness of species and how the tree is branched into clades. All phylogenetic trees generated from the MSA show that the *Slit2* promoter sequences of Primates, Ungulates, Carnivora, Rodentia, Soricomorpha, Lagomorpha and Proboscidea have differences that define them as separate orders, but the critical region of the *Slit2* promoter that drives maximal transcription is highly conserved through evolution. These data support the hypothesis that that while coding and non-coding DNA sequences are equally susceptible to mutation, selective pressures exist on protein coding and regulatory DNA sequences that reduce the mutation frequencies in populations, as compared to other non-coding sequences which have a smaller effect on fitness and therefore lack these selective pressures (Strachan and Read 2004; Kryukov et al. 2005). While sequence variability for transcription factor binding sites in promoter regions can vary greatly among different species, there tends to be some conservation of promoter sequences over broad phylogenetic groups (Florquin et al. 2005; Blanco et al. 2006; Doniger and Fay 2007; Eisermann et al. 2008). For example, high degrees of promoter conservation between species tend to be associated with genes for cell signaling, transcription factors, and genes that regulate development, like *Slit2*, and that lower degrees of conservation occur among the promoter regions of genes involved in metabolic processes (Chiba et al. 2008). It is clear from our genetic and phylogenetic data that the evolutionarily conserved *Slit2* –399 to –296 bp promoter region is necessary for maximal transcription and that the Sp1 (–345 bp) site is critical for activity in all the species analyzed, as is shown here for the mouse *Slit2* promoter.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. The *Slit2* gene promoter sequence identifying 5' RACE transcription start sites and construct lengths

Primer sequences used for generating the *Slit2* -1075 to +253 construct are shown as black arrowhead underlines. The GenBank-assigned start of transcription is shown in red text labeled (+1) with a large red arrow above the sequence. The start of translation is shown in bold black text “ATG”. 5' RACE was used to experimentally identify the transcription start site(s) and are shown as labeled red sequences and small red arrows. PstI -607 and EcoRV -490 are endogenous restriction sites and site-directed mutagenesis was used to create the -674 (XhoI), -469 (MluI), -399 (MluI), -296 (MluI), -153 (NheI), and -100 (PstI) restriction sites for deletion of intervening sequence and re-ligation of deletion constructs. CpG dinucleotides are denoted in blue text. Putative transcription factor binding sites identified by database analyses are outlined in orange or green and labeled by putative transcription factor name.

Figure 2. The *Slit2* –399 to +253 promoter construct shows maximal activity and defines the *Slit2* proximal promoter

Transient transfection of the *Slit2* promoter deletion constructs –1075bp, –674bp, –607bp, –490bp, –469bp, –399bp, –296bp, –153bp, –100bp, and pGL3 basic control vector were performed in the HEK293T cell line. The *Slit2* promoter deletion construct lengths are represented by continually shorter labeled lines that are not drawn to scale. *Slit2* promoter sequences for each construct are shown in Figure 1. The activity of each construct is expressed as the fold activation over promoterless pGL3 basic vector. Luciferase activity is pRL SV40-luciferase promoter and enhancer vector normalized and are the average $\pm$ SD of at least four independent transfections using at least three preparations of DNA. All construct activities were significantly higher than pGL3 basic by the Student two-sided *t* test ($p < 0.05$). *Slit2* –399bp activity was significantly higher than all other constructs ($p < 0.05$) except for the *Slit2* –1075bp construct.

<i>Slit2</i> promoter		Sp1 (-359bp)	Sp1 (-345bp)		USF1 (-305bp)
Mouse	100.0%	CGGAGCCCGCATCA	CGCCCGGACCCCAA	-----	GGCTGTCTGAATACCGGTGCGCC
Norway rat	90.4%	CGGAGCCCGCATCA	CGCTCGGCGCCCAA	-----	GGCTGTCTGAATACCGGTGCGCC
Galago	64.5%	CCGCGAATCCGACAGCG	CGCCCGGCGCCCAA	-----	GGCTGTCTGAATACCGGTGCGCC
Horse	60.3%	CGGAGCCCGCATCA	CGCCCGGACCCCAA	-----	AGCTGTCTGAATACCGGTGCGCC
Orca	60.1%	CAGAGCCCGCATCA	CGCCCGGCGCCCAA	-----	GGCTGTCTGAATACCGGTGCGCC
Walrus	59.6%	CAGAGCCCGCATCA	CGCCCGGCGCCCAA	-----	GGCTGTCTGAATACCGGTGCGCC
Sheep	59.4%	CAGAGCCCGCATCA	CGCTCGGCGCCCAA	-----	GGCTGTCTGAATACCGGTGCGCC
Rhesus monkey	59.3%	CC-AGA-----	GGCTGTGACCCCAA	-----	AACTGTCTGAATACCGGTGCGCC
White Rhino	58.2%	CAGAGCCCGCATCA	CGCCCGGCGCCCAA	-----	GGCTGTCTGAATACCGGTGCGCC
Marmoset	57.9%	CC-AGA-----	GGCTGTGCGCCCAA	-----	AACTGTCTGAATACCGGTGCGCC
Squirrel monkey	57.9%	CC-AGA-----	GGCTGTGCGCCCAA	-----	AACTGTCTGAATACCGGTGCGCC
Gibbon	57.9%	CC-AGA-----	GGCTGTGCGCCCAA	-----	AACTGTCTGAATACCGGTGCGCC
Elephant	57.7%	CGGAGCTCCGACAGCG	CGCCCGGCGCCCAA	-----	GGCTGTCTGAATACCGGTGCGCC
Star-nosed mole	57.3%	GGGATCCCGCATCA	CGCCCGGCGCCCAA	-----	CTGGGCTGTGCGCCAGCTT
Human	56.4%	CC-AGA-----	GGCTGTGCGCCCAA	-----	AACTGTCTGAATACCGGTGCGCC
Cow	55.9%	CAGAGCCCGCATCA	CGCTCGGCGCCCAA	-----	CCGTTCTGTGCGCCAGCTT
Rabbit	55.7%	CC-AGA-----	GGCTGTGCGCCCAA	-----	GGCTGTCTGAATACCGGTGCGCC
Chimpanzee	55.7%	CC-AGA-----	GGCTGTGCGCCCAA	-----	AACTGTCTGAATACCGGTGCGCC
Pygmy chimp	55.7%	CC-AGA-----	GGCTGTGCGCCCAA	-----	AACTGTCTGAATACCGGTGCGCC
Giant panda	51.5%	CC-AGCCAG-----	GGCTGTGCGCCCAA	-----	GGCTGTCTGAATACCGGTGCGCC
Shrew	50.9%	GGGATCCCGCATCA	CGCCCGGCGCCCAA	-----	AACTGTCTGAATACCGGTGCGCC
CONSERVATION		..Y...TC.G.....GY..GCR.CCY.....R.CTY.Y.....RCY..GTGRCRCC..RCG..G.EA			
IDENTITY		C.G.....G...GC..CC.....CTG...CC..GTGC.CCC..CGC..G.EA			
Mutagenesis		CGGAGCCCGCATCA	CGCTCGGCGCCCAA	-----	GGCTGTCTGAATACCGGTGCGCC

Figure 3. Multiple Sequence Alignment of the -362bp to -303bp *Slit2* promoter reveals high evolutionary conservation of the Sp1 (-345) and USF1 (-305) sites

MSA using the *Slit2* proximal promoter -399bp to -296bp from 21 species was performed. The -362bp to -303bp region of the *Slit2* proximal promoter where the two Sp1 sites (-359 and -345, labeled, boxed, and shown in green text) and the USF1 site (-305, labeled, boxed, and shown in green text) is shown here. Conserved and identical sequences among species, omitting species with gapped sequences in the analyses, are labeled “Conservation” and “Identity” and shown in green text. Mutations incorporated into the *Slit2* proximal promoter -399bp to +253bp by site-directed mutagenesis are shown in bold, green, underlined text and labeled “Mutagenesis”.

Figure 4. Species relatedness by Bayesian inference analysis of the *Slit2* proximal promoter –399bp to –296bp from 21 species

The tree was produced using Mr. Bayes 3.1.2 Bayesian inference analysis demonstrating posterior probabilities, noted next to each clade between 0 and 100, with 100 equaling 100% agreement. Branch lengths indicate the amount of character change in proportion to estimated evolutionary distances. The scale bar represents units of genetic distance (0.05 mutations per site). Three million generations were performed with a tree produced every 100 generations and the first 7000 trees were discarded as “burn-in”.

Figure 5. The *Slit2* -399 to +253 mutant Sp1-345bp promoter construct shows loss of activity and confirms the evolutionary importance of the Sp1 -345bp site

Transient transfection of the *Slit2* promoter deletion construct -399bp, -399bp mutant Sp1-359bp, -399bp mutant Sp1-345bp, -399bp mutant USF1-305bp, -296bp, and pGL3 basic control vector were performed in the HEK293T cell line. *Slit2* promoter sequences for each deletion construct are shown in Figure 1 and mutations generated within the transcription factor consensus sequences are shown in Figure 3. The activity of each construct is expressed as the fold activation over promoterless pGL3 basic vector. Luciferase activity is pRL SV40-luciferase promoter and enhancer vector normalized and are the average $\pm$ SD of at least four independent transfections using at least three preparations of DNA. All construct activities were significantly higher than pGL3 basic by the Student two-sided *t* test ($p < 0.05$), except *Slit2* promoter deletion construct -399bp mutant Sp1-345. The only construct with significantly lower activity than the *Slit2* -399bp deletion construct was the *Slit2* promoter deletion construct -399bp mutant Sp1-345 ($p < 0.05$).