Characterization of the *Drosophila* SLOWPOKE binding protein (SLOB) promoter

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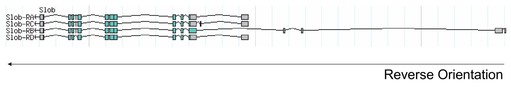
Introduction

Drosophila SLOWPOKE (SLO) is a voltage- and calcium-dependent, large conductance potassium channel important for action potential repolarization, neuronal excitability, neurotransmitter release, and hormone secretion. SLO binding protein (SLOB) binds to and modulates SLO activity. We have shown previously that modulation of SLO by SLOB has profound effects on SLO channel currents, synaptic transmission, and metabolism. Multiple isoforms of SLOB exist and are encoded by multiple transcripts; the isoforms are named based on their predicted protein molecular weights, in kilodaltons. In the *Drosophila* brain, SLOB57/51 proteins are expressed especially prominently in insulin producing neurons of the *pars intercerebralis*, while SLOB71/65 proteins are enriched in the lateral neurons that participate in the generation of circadian rhythms. Here we sought to determine the transcription initiation sites in the *slob* gene and investigated promoter elements responsible for expression of the different *slob* transcripts.

Genomic Organization of Slob Variants



Reverse Orientation

Slob-RA/RC → SLOB51 and SLOB57

Slob-RB → SLOB65 and SLOB71

= UTR

= Exons

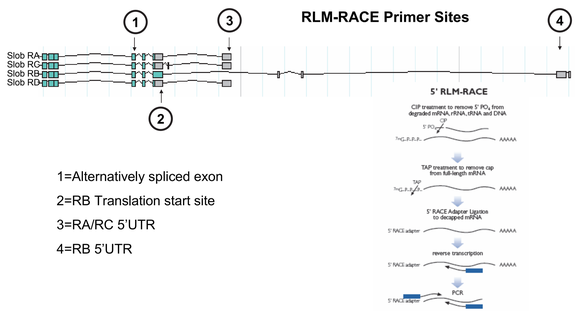
Methods

•Transcription start sites (TSS) were mapped using RNA ligase mediated 5' rapid amplification of cDNA ends (RLM-RACE) (Ambion). Total RNA was extracted from heads of *yw* fruit flies, and RLM-RACE was performed with 4 reverse primers targeted to different regions of Slob RA/RC and Slob RB. RLM-RACE products were run on agarose gels, and individual products were extracted. Products were then TOPO cloned and sequenced to identify TSSs.

•Luciferase reporter constructs were created by sequential cloning of regions upstream of the 2 identified TSSs and inserting sequences into the pGL4.10 luciferase reporter vector or the minimal promoter (minP) pGL4.23 vector (Promega).

•Sequences within the downstream promoter element (DPE) and motif ten element (MTE) were mutated in the RB-1500→+81 luciferase construct using Quikchange site-directed mutagenesis (Agilent Technologies). Hunchback (hb) and mirror (mir) target sequences were also mutated with Quikchange.

•Drosophila S2 cells were transfected with the luciferase reporter constructs along with a vector expressing beta-galactosidase in duplicate using lipofectamine (Invitrogen). S2 cells were lysed 48 hrs post-transfection, incubated with luciferase reagent (Promega), and luminescence was measured using a luminometer. Activity of the beta-galactosidase enzyme was measured in a spectrophotometer after addition of substrate, and luciferase activity was normalized to beta-galactosidase activity to control for transfection efficiency. Fold changes were calculated by dividing normalized luciferase activity measures for the Slob RA/RC and Slob RB promoter constructs by the luciferase activity measured in S2 cells transfected with the pGL4.10 or pGL4.23 empty vectors. Data are the mean ± SEM. * indicates p<0.05, ** indicates p<0.01, and *** indicates p<0.001, one way ANOVA with Bonferroni post-hoc test or one-sample t-test.



1=Alternatively spliced exon

2=RB Translation start site

3=RA/RC 5'UTR

4=RB 5'UTR

Fig 1: RLM-RACE Products

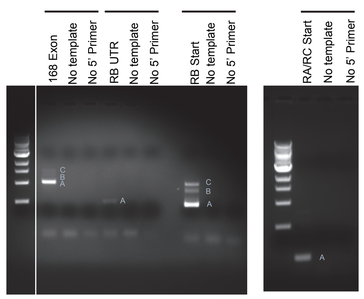


Fig 1: RLM-RACE products were separated on 1% agarose gels. Product bands were extracted, TOPO cloned, and sequenced.

Fig 2: Two unique TSSs were identified for Slob RA/RC and Slob RB

RLM-RACE Defined Transcription Start Sites: Summary

TSS1

RB-UTR
Clone 1: AATCCAGTTCGAGTCAGCCAG
Clone 2: AATCCAGTTCGAGTCAGCCAG
Clone 6: AATCCAGTTCGAGTCAGCCAG
RB-Start
B-Clone 1: AATCCAGTTCGAGTCAGCCAG
B-Clone 2: AATCCAGTTCGAGTCAGCCAG
B-Clone 3: AATCCAGTTCGAGTCAGCCAG
C-Clone 8: AATCCAGTTCGAGTCAGCCAG
C-Clone 9: AATCCAGTTCGAGTCAGCCAG
C-Clone 10: AATCCAGTTCGAGTCAGCCAG
168 Exon
B-Clone 7: AATCCAGTTCGAGTCAGCCAG
B-Clone 1: AATCCAGTTCGAGTCAGCCAG
C-Clone 4: AATCCAGTTCGAGTCAGCCAG
C-Clone 2: AATCCAGTTCGAGTCAGCCAG

Black sequences = Maintained intronic sequence between UTR regions 1 and 2
Blue sequences = Spliced out intronic sequence between UTR regions 1 and 2

TSS2 (***Predominant Product)

RB Start-A
A-Clone 6: ATTAACGGACGACCCGAGCA
A-Clone 7: ATTAACGGACGACCCGAGCA
A-Clone 5: ATTAACGGACGACCCGAGCA
168 Exon-A
A-Clone 1: ATTAACGGACGACCCGAGCA
A-Clone 2: ATTAACGGACGACCCGAGCA
A-Clone 10: ATTAACGGACGACCCGAGCA
RARC UTR
A-Clone 6: ATTAACGGACGACCCGAGCA
A-Clone 2: ATTAACGGACGACCCGAGCA
A-Clone 8: AACGGACGACCCGAGCA

Fig 2: The TSS for Slob RA/RC is located within what was previously identified as the RA/RC 5' UTR. The TSS for Slob RB is located 21 base pairs upstream of the previously identified 5' UTR.

Fig 3: The MTE and DPE core promoter elements are present in Slob RB

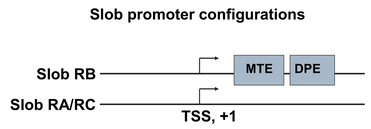


Fig 4: A construct containing 1 kb upstream from the Slob RA/RC TSS is sufficient for promoter activity

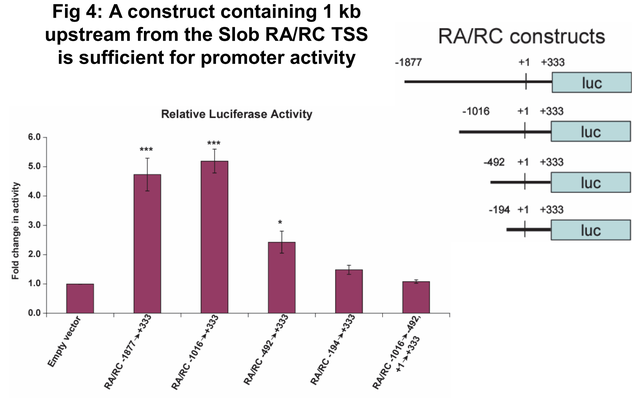


Fig 6: Slob RB constructs exhibit more robust promoter activity

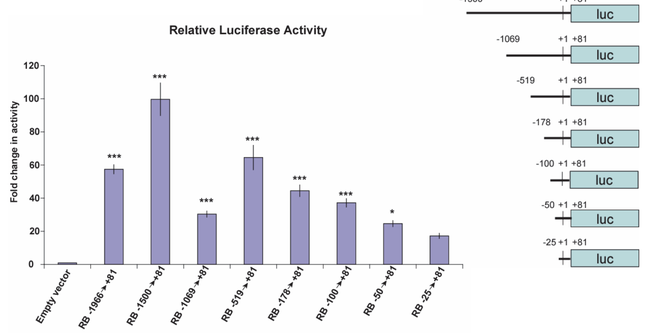


Fig 6: Transcription of Slob RB is regulated by the MTE and DPE core promoter motifs

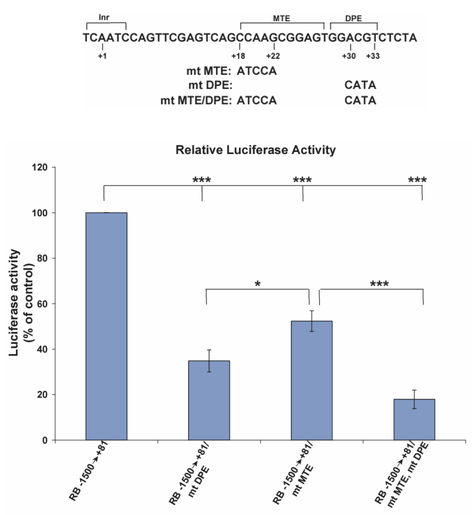


Fig 7: Promoter domains of Slob RB differentially repress or activate transcription from a minimal promoter

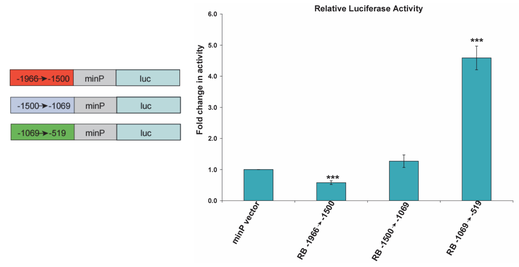
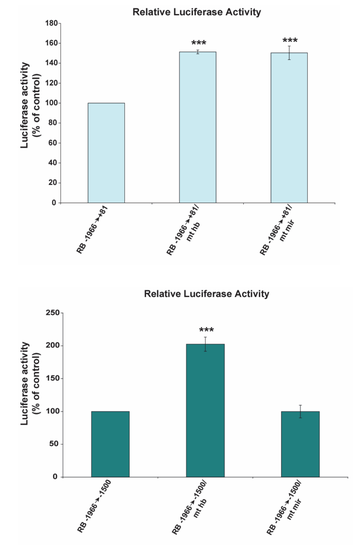


Fig 8: Mutation of hb or mir recognition sequences in Slob RB increases promoter activity



Conclusions

•TSSs for *slob57/51* and *slob71/65* transcripts were determined in *yw* flies. The TSSs are different from those previously identified for *y; cn bw sp* flies listed in FlyBase. This may be due to background differences between the 2 genotypes.

•By sequential analysis of promoter regions upstream of the 2 TSSs, we found that *slob71/65* promoters exhibit robust basal activity, whereas *slob57/51* promoters have relatively lower basal levels of promoter activity.

•*Slob71/65* promoters are regulated by core promoter elements (DPE and MTE), downstream of the site of initiation, whereas *slob57/51* promoters lack these core promoter elements.

•Insertion of different regions of the *slob71/65* promoters upstream of a minP differentially activates or represses promoter activity.

•Mutation of hb or mir target sequences increases *slob71/65* promoter activity, suggesting that hb and mir transcription factors act to repress transcription of *slob71/65*. Interestingly, in contrast to the effect on the *slob71/65* full length promoter, mutation of the *slob71/65* mir site has no effect on minP activity, suggesting that mir may repress activity at the core promoter of *slob71/65*.

•The high levels of promoter activity exhibited by *slob71/65* promoters are most likely due to regulation by the DPE and MTE core promoter elements, which *slob57/51* promoters lack. In future experiments, the interaction of enhancer elements with the core promoter will be further examined. In addition, we will create flies expressing *slob57/51* and *slob71/65* promoters to map the expression of different slob transcripts throughout development.