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Promoter-specific co-activation by *Drosophila* Mastermind

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Abstract

Mastermind (Mam) is a co-activator protein of binary complexes consisting of Suppressor of Hairless (Su(H)) and Notch Intracellular Domain (NICD) proteins assembled on *cis*-regulatory regions of target genes activated by *Notch* signaling. Current evidence indicates that Mastermind is necessary and sufficient for the formation of a functional Su(H)/NICD/Mam ternary complex on at least one specific architecture of Su(H) binding sites, called the SPS element (Su(H) Paired Sites). However, using transcription assays with a combination of native and synthetic *Notch* target gene promoters in *Drosophila* cultured cells, we show here that co-activation of Su(H)/NICD complexes on SPS elements by Mam is promoter-specific. Our novel results suggest this promoter specificity is mediated by additional unknown *cis*-regulatory elements present in the native promoters that are required for the recruitment of Mam and formation of functional Su(H)/NICD/Mam complexes on SPS elements. Together, the findings in this study suggest Mam is not always necessary and sufficient for co-activation of binary Su(H)/NICD complexes on SPS elements.

Keywords

Mastermind; Notch; transcription; proneural; neurogenesis; promoter-specificity

Introduction

The *Notch* pathway is an evolutionarily conserved signaling pathway that is essential for multiple developmental processes [1;2;3;4]. *Notch* signaling is initiated by ligand binding to the Notch receptor protein, which results in the proteolytic release of the Notch Intracellular Domain (NICD). NICD is a non-DNA binding co-activator protein that translocates to the nucleus and binds the DNA-bound protein Suppressor of Hairless (Su(H); also called CSL). Typically, NICD de-represses target genes by displacing co-repressors initially bound to Su (H) and forming an NICD/Su(H) binary complex. The binary complex further activates target gene expression by recruiting the co-activator, Mastermind, to form a ternary Notch transcription complex (NTC).

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Given the multi-functional role of *Notch* signaling in development, it is important to understand the molecular mechanisms by which specific subsets of Notch target genes are selectively activated in the proper cell types. During *Drosophila* neurogenesis, *Notch* signaling is necessary for the cell-specific expression of several *Enhancer of split Complex* (*E(spl)-C*) genes in "proneural clusters." These clusters of adjacent cells are defined by the expression of proneural bHLH A (basic Helix-Loop-Helix Activator) proteins, which activate target genes by heterodimerizing with the ubiquitously expressed Daughterless bHLH A protein (Da). Within proneural clusters, typically only one or a few cells become a neural precursor cell (NPC), and the remaining cells, which are non-precursor cells (non-NPCs), adopt a non-neural cell fate. In the non-NPCs, several *E(spl)-C* bHLH repressor (bHLH R) and Bearded-like (Brd-like) genes are specifically up-regulated during Notch-mediated lateral inhibition. The function of these *E(spl)-C* proteins is to repress proneural gene expression in the non-NPCs. However, the non-NPC-specific expression of several *E(spl)-C* genes is mediated by a strong "Notch-proneural" synergistic interaction between the bHLH A and *Notch* signaling pathway proteins on the *cis*-regulatory regions [5;6].

Recent studies have provided insight into the precise "DNA transcription codes" that are used by *Notch* signaling to generate these cell-specific gene expression patterns during neurogenesis in *Drosophila* [5;6;7]. DNA transcription codes are *cis*-regulatory modules comprised of particular combinations and orientations of transcription factor binding sites that provide a heritable mechanism for encoding gene expression patterns [8;9]. We have previously shown that an "SPS+A" transcription code is critical for the Notch-proneural transcriptional synergy that drives the up-regulation of the *E(spl)-C m8* bHLH R gene in non-NPCs [5]. This transcription code is comprised of bHLH A binding sites ("A" sites) and an SPS DNA element, which is a precisely spaced inverted repeat of Su(H) binding sites ("S" site). A key functional feature of the SPS+A code is that the inverted orientation architecture of the SPS element is critical for transcriptional synergy between NTCs and proneural bHLH A proteins bound to SPS and A sites, respectively [5]. The SPS+A code is also predicted to mediate the non-NPC expression of the *E(spl)-C m7*, *mγ* and *mδ* genes [5], although a different "logic" or code regulates *mα*[7].

Previously we have shown that the SPS architecture is critical for co-activation of NTCs by Mam [5]. Recent structural studies have also indicated that Mam is necessary for cooperative assembly of ternary NTCs to each of the S sites in SPS elements [10]. Thus, current models of *Notch* signaling indicate that Mam is an essential co-activator of gene transcription regulated by all promoters containing the SPS+A transcription code. However, in this study, we show that co-activation of SPA+A modules by Mam is promoter specific and Mam does not co-activate all promoters containing functional SPS+A modules. These findings suggest, in contrast to current models, that Mam is not always necessary and sufficient for co-activation of binary NTCs bound to SPS elements.

Materials and Methods

Details of the S2 cell culture transfection protocol have been described elsewhere [5]. All protein expression plasmids for S2 cell culture were constructed using pAc 5.1/V5-HisA plasmids (Invitrogen). The Mam expression plasmid used in this study was generated by isolating the Mam cDNA from pNB40 (B. Yedvobnick, Emory Univ.) using NheI and BamHI, and then ligating this cDNA into pAc 5.1/V5-HisA which had been digested with XbaI and BamHI. The MamΔC expression plasmid was generated by digesting the Mam pAc 5.1/V5-HisA plasmid with SacI and then religating the plasmid. The MamΔC-VP16 fusion protein was generated by isolating the VP16 activation domain coding sequence from pVP16 (Clontech) and inserting this coding sequence, in frame, with the MamΔC pAc 5.1/V5-HisA

expression plasmid. Construction of all other expression plasmids used for S2 cell culture have been previously described [5].

All transcription reporters for S2 cell culture were constructed with pGL2-basic *luciferase* plasmids (Promega). Details about the construction of the native *m8* and SPS-4A promoters have been given elsewhere [5]. Insertion of the second S site to create the *ac*-SPS promoter was generated by PCR. The *mγ* promoter contained nucleotides –319 to +84 of the native gene. This *mγ* promoter was isolated from a pGL2-basic plasmid containing –1210 and +84 of the native *mγ* gene (C. Delidakis, Inst. Mol. Biol. and Biotech.). Proper construction of all plasmids in this study were confirmed by DNA sequencing.

Results and Discussion

We have previously shown that Mam can strongly enhance Notch-proneural transcriptional synergy on the native *m8* promoter in transcription assays with cultured S2 cells (Figure 1B *cf.* expts. 7 *vs*. 8) [5]. We have now confirmed our prediction [5] that Mam also strongly enhances Notch-proneural transcriptional synergy on the native *mγ* promoter (Figure 1B *cf.* expts. 15 *vs*. 16), which contains an SPS+A module and is specifically expressed in the non-NPCs of proneural clusters by *Notch* signaling.

We next used rationally-designed synthetic reporters, which are useful for addressing whether specific transcription factor binding sites hypothesized to function are actually sufficient for promoter activation. The SPS-4A synthetic promoter contains an SPS element placed adjacent to 4 A sites and a minimal *Hsp70* basal promoter (Figure 1A). As a result of this design, there should be no other functional binding sites for other transcription factors that could influence or contribute to Notch-proneural transcriptional synergy and co-activation by Mam.

The SPS-4A synthetic promoter mediates strong Notch-proneural synergy when NICD and Ac/Da are co-expressed (Figure 1C *cf.* expts. 3, 5 and 7), as previously shown [5]. By contrast, co-expression of Mam did not co-activate a synthetic SPS-A promoter (Figure 1C *cf.* expts. 7 *vs*. 8). The ability of co-expressed NICD and bHLH A proteins to synergistically activate the SPS-4A promoter confirmed that the Su(H)/NICD binary complexes bound to the SPS element were functional. However, the inability of Mam to co-activate this Notch-proneural synergy on the SPS-4A promoter was unexpected since current models predict that Mam should co-activate all functional Su(H)/NICD binary NTCs. These new findings indicate that the SPS+A module is sufficient to mediate Notch-proneural transcriptional synergy, but not to mediate co-activation by Mam.

To further examine the promoter specificity of co-activation by Mam on SPS+A modules, we tested the ability of Mam to co-activate the *achaete* (*ac*) promoter. The *ac* gene encodes a proneural bHLH A protein that is not activated by *Notch* signaling in proneural clusters even though the *ac* proximal promoter contains an single S site (Figure 1A). In fact, *ac* is indirectly repressed *in vivo* by NICD *via* recruitment of *E(spl)-C* bHLH R proteins [11; 12]. In S2 cells, expression of NICD or Mam either separately or together did not synergistically activate the *ac* promoter when co-expressed with the Ac/Da bHLH A proteins (Fig. 1D *cf*. expts. 5–8). These results are consistent with our previous findings that single S sites do not mediate NTC-bHLH transcriptional synergy [5]. By contrast, if an additional S site is added to the *ac* promoter in order to create an SPS element (Figure 1A), then the resulting *ac*-SPS promoter is strongly and synergistically activated by co-expression of NICD and Ac/Da (Fig. 1D *cf*. expts. 11, 13 and 15). However, similar to the SPS-4A promoter, co-expression of Mam did not generate additional *ac*-SPS promoter activity despite the presence of functional NTCs that can mediate transcriptional synergy with bHLH A proteins (Figure 1D *cf*. expts 15 *vs*. 16).

Together, these studies show that Mam co-activated only the native *m8* and *mγ* promoters, which are activated by *Notch* signaling *in vivo*. By contrast, Mam did not co-activate either the synthetic SPS-4A promoter, the native *ac* promoter or the modified *ac*-SPS promoter, which are not activated by *Notch* signaling *in vivo*. These results indicate that co-activation by Mam is promoter-specific. This promoter-specificity of co-activation was unexpected since all of the promoters containing an SPS+A module were sufficient to mediate strong Notch-proneural transcriptional synergy, which indicated that functional NTC complexes had assembled on the SPS elements. Thus, these novel findings indicate that there are promoter contexts in which Mam does not co-activate of NTCs bound to SPS elements.

Previous studies have reported that Mam mediates cooperative assembly and binding of Su (H)/NICD/Mam ternary NTCs on SPS elements in the absence of combinatorial cofactors, such as the proneural proteins [13]. Consistent with these findings, co-expression of NICD and Mam activated the *m8* and *mγ* promoters in the absence of Ac/Da proteins (Figure 2 expts. 3 and 8). These findings indicate that the Su(H)/NICD/Mam ternary complex formed on these native promoters and activated reporter gene transcription. However, formation of the ternary complex in absence of bHLH A proteins mediates substantially lower levels of reporter gene expression than when bHLH A are co-expressed (*cf*. Figure1 expts. 4 vs. 8 and 12 vs. 16). In contrast to the native *m8* and *mγ* promoters, neither the SPS-4A or *ac*-SPS promoters were activated when NICD and Mam were co-expressed in the absence of bHLH A proteins (*cf*. Figure 2 expts 3 and 8 *vs*. 13 and 18). Although the functional Su(H)/NICD complexes can form on the SPS-4A and *ac*-SPS promoters and mediate NTC-bHLH transcriptional synergy (Figure 1C and 1D), these findings suggest that functional Su(H)/NICD/Mam ternary complexes do not form on these promoters.

The promoter-specific formation of functional Su(H)/NICD/Mam ternary NTCs was due either to Mam being in an inactive state when bound the Su(H)/NICD binary complex on the SPS-4A and *ac*-SPS promoters or the lack of recruitment of Mam on to the SPS-4A and *ac*-SPS promoters. To test these possibilities, we removed the previously identified Mam transcription activation (TA) domain [14;15] and replaced it with the strong constitutively active viral VP16 protein TA domain to create a MamΔC-VP16 fusion protein (Figure 2A). Co-expression of MamΔC, which lacked the entire Mam TA domain, showed no co-activation on either the *m8* and *mγ* promoters (Figure 2B, expts. 4 and 9). However, co-expression of MamΔC-VP16 and NICD co-activated the *m8* and *mγ* promoters at levels comparable to those by MamWT (*cf*. Figure 2 expts 3 *vs*. 5 and expts 8 *vs*. 10). By contrast, MamΔC-VP16 did not co-activate either the SPS-4A or *ac*-SPS promoters (Figure 2). Together, these results suggest that Mam is selectively recruited by Su(H)/NICD binary complex on the *m8* and *mγ* promoters, but not on the SPS-4A or *ac*-SPS promoters, even though binary NTC complexes are present and functional for mediating strong Notch-proneural transcription synergy on all four promoters.

Summary and conclusions

The findings in this study indicate that co-activation of binary Su(H)/NICD complexes on SPS elements by Mam is promoter specific. The MamΔC-VP16 data suggest that selective recruitment and formation of functional Su(H)/NICD/Mam ternary NTCs underlie this promoter specificity. Since all the promoters in this study were tested in an identical cellular context, the differential co-activation by wild-type Mam is not likely to be due to a post-translational mechanism. Rather, the promoter-specificity of co-activation by Mam is likely mediated by DNA sequences specific to the native *m8* and *mγ* promoters that contain cryptic *cis*-regulatory binding sites for additional combinatorial cofactor proteins. These cryptic sites are missing from the synthetic SPS-4A promoter as well as the *ac*-SPS promoter, which is not a native *Notch* target gene *in vivo*.

The apparent requirement for additional regulatory elements to mediate co-activation by Mam was unexpected since *in vitro* studies have indicated that the Su(H)/NICD/Mam ternary NTC can self-assemble on SPS elements [10]. However, our findings suggest that in a cellular context additional factors are required to either recruit or stabilize functional ternary NTC complexes containing Mam. Furthermore, the ability of the SPS-4A and *ac*-SPS promoters to mediate Notch-proneural transcriptional synergy in the absence of co-activation by Mam indicates that the functional Su(H)/NICD binary complexes can assemble and bind the SPS element without Mam. These findings differ from recent mammalian studies reporting that Mam is required for the assembly and binding of homologous Su(H)/NICD complexes on SPS elements in the absence of proneural proteins [10]. It is possible that physical interactions between Su(H) and the bHLH A Da protein programmed by assembly on an SPS module can stabilize the Su(H)/NICD complex bound to the SPS element in the absence of Mam [5].

Together, the findings in this study indicate that co-activation of SPS+A modules by Mam is promoter specific, and requires additional unknown cofactors that are bound to unknown sites in the native promoters. Our results also provide preliminary evidence that Mam is not always necessary to co-activate Su(H)/NICD binary complexes, and that other cofactors, such as Ac/Da, can co-activate these binary NTCs in the absence of Mam. Thus, these findings suggest that Mam is not always necessary and sufficient for co-activation of Su(H)/NICD complexes bound to SPS elements. These results suggest that the mechanisms regulating cell-specific regulation of *Notch* target gene expression are more complex than the current models, and involve additional unknown combinatorial cofactors.

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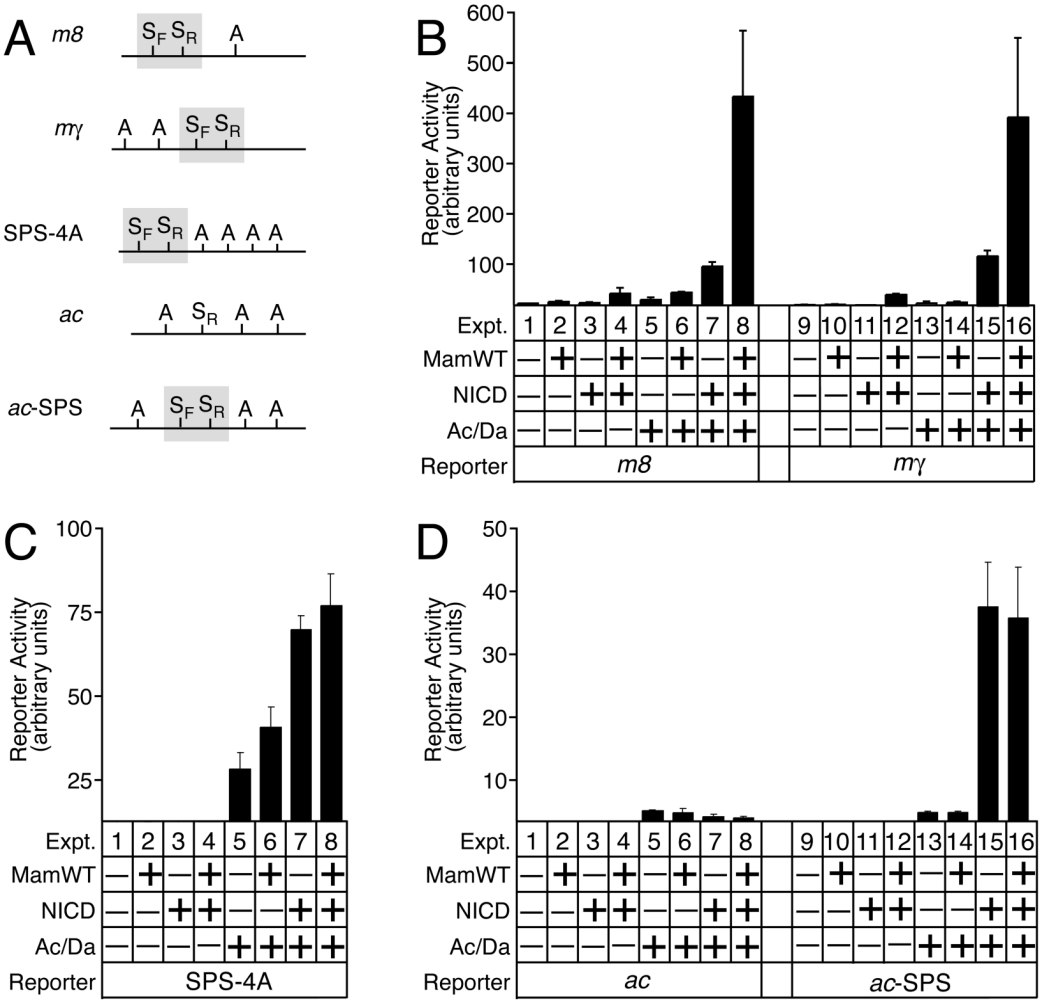

Figure 1.

Promoter-specific co-activation by Mastermind. **A**, a diagram of the organization of bHLH A and Su(H) sites ("A" and "S", respectively) in the promoters used in this study. S sites and their respective "forward" and "reverse" orientations are indicated by "S_F" and "S_R" respectively. The S_F and S_R orientations are defined as 5'-GTGNGAA'3' and 5'-TTCNCAC-3' on the DNA strand containing the "ATG" start codon. Promoter size is not shown to scale. **B**, Mam selectively activates the native *E(spl)-C m8* and *mγ* promoter, which are activated *in vivo* by *Notch* signaling. **C**, by contrast, the synthetic SPS-4A promoter is not co-activated by Mam, even though this promoter can mediate strong Notch-proneural transcriptional synergy. **D**, the native *ac* promoter does not mediate either Notch-proneural transcriptional synergy or co-activation by Mam, but this promoter is also not an *in vivo* target gene for *Notch* signaling even though it has an S_R site in its proximal promoter. Creation of an SPS element in the native *ac* promoter (generating the *ac*-SPS promoter) allows for Notch-bHLH A transcription synergy, but not co-activation by Mam. The observed Notch-proneural transcriptional synergy on all the promoters containing SPS elements indicates that functional Su(H)/NICD complexes have formed on the S sites of the SPS elements. However, the promoter-specificity of co-activation by Mam suggests that additional cis-regulatory elements, present only in the *m8* and *mγ* promoters, are necessary to mediate co-activation by Mam.

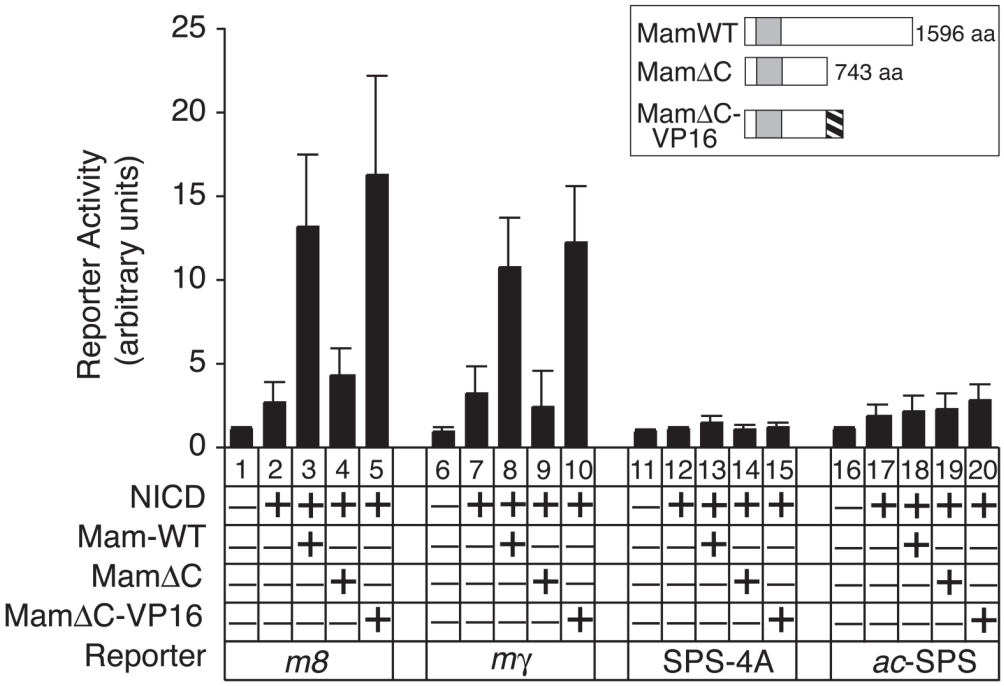

Figure 2.

Evidence for promoter-specific recruitment of Mam. In the absence of bHLH A proteins, co-expression of NICD with either wild-type Mam (MamWT) or a MamΔC-VP16 fusion protein activated the *m8* and *mγ* promoters. By contrast, co-expression of these proteins did not activate the SPS-4A or *ac*-SPS promoters. The ability of all these promoters to mediate Notch-proneural transcriptional synergy indicated that functional Su(H)/NICD complexes can form on the respective SPS elements of these promoters (Figure 1). However, the promoter-specific co-activation by Mam in the absence of bHLH A proteins suggests that recruitment of Mam and the formation of functional Su(H)/NICD/Mam ternary complexes occurs on the native *m8* and *mγ* promoters. Diagrams of the Mam proteins expressed are shown in the insert. The region within Mam required for binding both Su(H) and NICD is indicated by the shaded box.