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MASTERMIND-LIKE 1-Dependent Notch Target Gene Activation Requires a Sequence-Paired Site and a TATA Box

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MASTERMIND-LIKE 1-DEPENDENT NOTCH TARGET GENE ACTIVATION
REQUIRES A SEQUENCE-PAIRED SITE AND A TATA BOX

A Thesis

Presented to

The Faculty of the Department of Biological Sciences

San José State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Cassandra Agbayani Ramos

August 2013

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The Designated Thesis Committee Approves the Thesis Titled

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by

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August 2013

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ABSTRACT

MASTERMIND-LIKE 1-DEPENDENT NOTCH TARGET GENE ACTIVATION REQUIRES A SEQUENCE-PAIRED SITE AND A TATA BOX

by Cassandra Agbayani Ramos

Notch signaling plays an important role in mammalian cellular proliferation, apoptosis, and differentiation. To activate target genes, the Notch intracellular domain (NICD) forms a complex with CBF1 and Mastermind-like protein (MAM). The Notch activation complex binds to a response element containing the consensus sequence RTGRGAR (R= purine) on target gene promoters. The promoter of one well characterized target gene, Hes1, contains four Notch response elements (NREs). Site-directed mutagenesis and reporter gene assays were used to examine the activation of Hes1 by mutating individual and combinations of NREs. Results indicated that NREs 2 and 4 are important for Hes1 activity, confirming previous results that this sequence-paired site (SPS) is an important feature of Notch target genes. Orientation and spacing between elements on the SPS were mutated in these studies. Other mutations included the Hes1 TATA box and the spacing between the SPS and TATA box. Additionally, a charged residue on NICD was mutated; this residue is believed to be responsible for Notch complex dimers. These results showed that SPS spacing and orientation as well as dimerization of Notch complexes are important for the promoter activity of Hes1. The TATA box was found to be necessary for promoter activation, and this activity is independent of spacing between the SPS and TATA box up to 173 base pairs. Taken together, these findings suggest that NICD- and MAM-mediated activation of target genes requires orientation- and spacing-dependent SPS and TATA box elements.

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DEDICATIONS

This thesis is dedicated to my mom, Lorraine Agbayani. She is my personal hero and lifelong teacher. Her immense courage, strength, and love are always my inspiration. She reminds me that biomedical research is about helping the people that we hold dearest in our hearts.

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ABBREVIATIONS

Ac/Da: Achaete and Daughterless

ADAM: A Disintegrin And Metalloprotease

ANK: ankyrin

bHLH: basic helix-loop-helix

CBF: C-promoter binding factor

CBP: c-AMP response element binding protein

CDK: cyclin dependent kinase

CIR: CBF1 interacting corepressors

CREB: c-AMP response element binding protein

CSL: CBF1/Su(H)/Lag-1

DMEM: Dulbecco's modification of Eagle's medium

dNICD: *Drosophila* Notch intracellular domain

DSL: Delta/Serrate/LAG-2

EGF: Epidermal Growth Factor

EMSA: electrophoretic mobility shift assay

E(spl): Enhancer of split

GTF: general transcription factor

H₀: null hypothesis

Hairy/Enhancer of Split: Hes

HD: Notch heterodimerization domain

HDAC: histone deacetylase

LB: lysogeny broth

LNR: Lin12/Notch repeats

MAM: Mastermind-like protein

MAM1: Mastermind-like protein 1

mHes: mouse Hairy/Enhancer of Split

NECD: Notch extracellular domain

NIICD: Notch1 intracellular domain

NICD: Notch intracellular domain

NRE: Notch response element

NRR: negative regulatory region

NT: NRE-to-TATA

NTM: Notch transmembrane subunit

NZY: NZ-amine and yeast

OFUT: O-fucosyltransferase

PCAF: p300/CBP-associated factor

PEST: proline, glutamate, serine, and threonine rich domain

RAM: Recombination Binding Protein-associated module

RNAPII: RNA polymerase II

SCF: S-phase kinase-association protein/Cullin/F-box

SEL10: Suppressor/Enhancer of lin-12 10

SHARP: SMRT/HDAC-1 Associated Repressor Protein

SKIP: Ski-interacting protein

SMRT: Silencing Mediator of Retinoid and Thyroid hormone receptor

SPS: sequence-paired site

TACE: tumor necrosis factor- α converting enzyme

T-ALL: T-cell acute lymphoblastic leukemia

TAF: TBP-associated factor

TBP: TATA-binding protein

TE: Tris-EDTA

TFII: Transcription factor II

TSS: transcription start site

INTRODUCTION

Notch is a transmembrane protein with an extracellular receptor used to communicate with adjacent cells (1). When the receptor binds to ligands from the Delta/Serrate/LAG-2 (DSL) family, there is a conformational change of Notch, allowing its cleavage by a gamma-secretase complex. This proteolytic cleavage releases the Notch intracellular domain (NICD), which translocates to the nucleus to bind to the promoters of target genes and initiate transcription (Fig. 1) (2, 3). In mammals, NICD binds to the transcription factor, C-promoter binding factor (CBF) 1, and the coactivator, Mastermind-like protein (MAM). This ternary complex binds to a Notch response element (NRE) containing the consensus sequence RTGRGAR (R=purine) on target gene promoters (4). This complex is essential for promoter activation. In mammals, there are four Notch proteins and three MAM proteins; however, it is still unclear how these homologs differentially modulate Notch signaling.

The Notch ternary complex recruits additional coactivators for transcription initiation. MAM recruits p300, a histone acetyltransferase, which is essential for transcription initiation (5). MAM also recruits cyclin dependent kinase (CDK) 8, as it is necessary for Notch turnover (6). CDK8 can associate with the Mediator coactivator complex, which recruits the basal transcription machinery. The working model of Notch and MAM mediated target gene activation postulates that Mediator is responsible for recruiting RNA polymerase II (RNAPII) in order to initiate transcription (Fig. 2) (6).

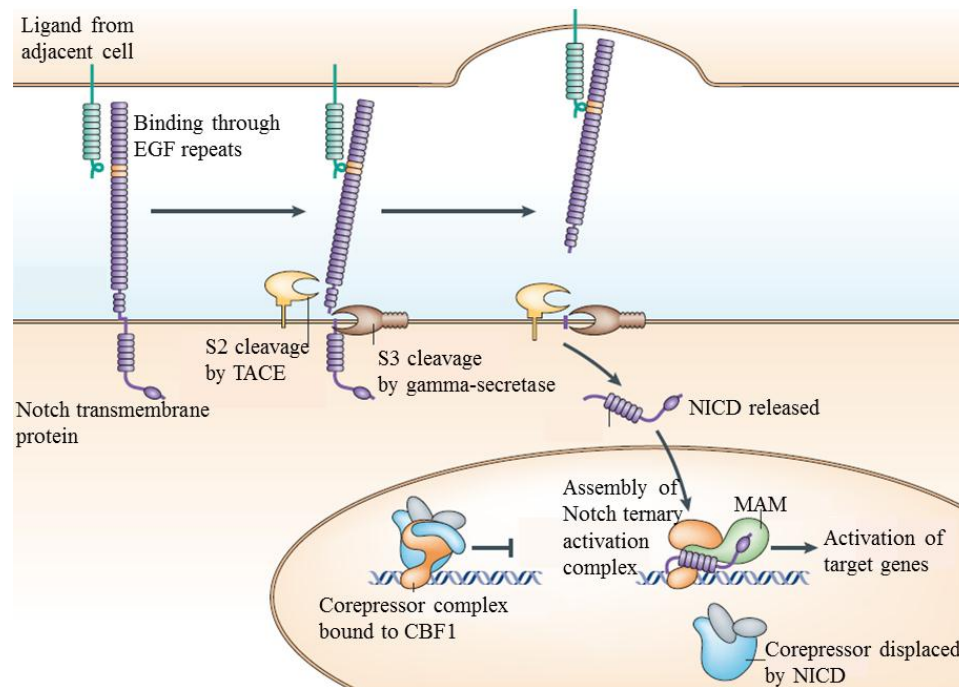


FIGURE 1: Overview of Notch signaling. The mature Notch transmembrane is cleaved after binding to ligands from the DSL family of proteins in adjacent cells. The NICD is released from the cell membrane and translocates to the nucleus. The assembly of NICD and MAM on CBF1 displaces a corepressor complex and activates target genes. Adapted by permission from Macmillan Publishers Ltd: *Nat. Rev. Mol. Cell Biol.* (3), copyright 2006.

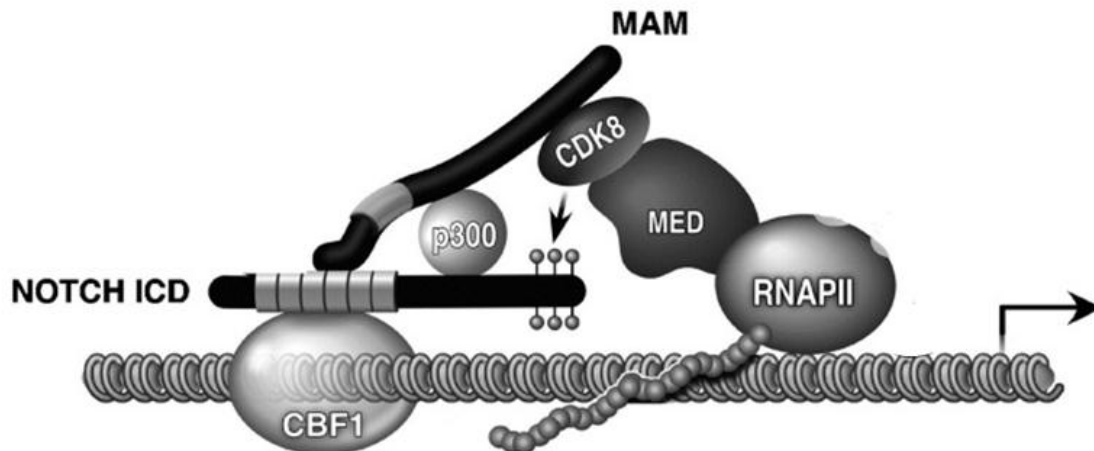


FIGURE 2: Model of transcription initiation by Notch complex. The Notch signaling complex binds to an NRE on target gene promoters. MAM recruits CDK8, which may recruit RNAPII through Mediator. Adapted by permission from Elsevier Ltd.: *Mol. Cell* (6), copyright 2004.

In the studies presented here, Notch target genes were investigated by studying the promoter of mouse Hairy/Enhancer of Split (mHes) 1 (Fig. 3). mHes1 has over 95% homology with the human Hes1 promoter. The mHes1 (hereinafter referred to as Hes1) promoter has four NREs, which are conserved in all vertebrates. Three of the Hes1 NREs have been studied by other research groups, whereas one NRE has not been investigated in previous literature until now (7-9). These NREs have a specific orientation depending on whether the consensus sequence is located on the forward or reverse strand of the promoter. NREs on the forward strand are designated as Head sites, whereas NREs on the reverse strand are Tail sites. The Hes1 promoter also has a putative TATA box, a region rich in thymines and adenines that binds to the Transcription Factor II (TFII) D complex. TFIID is a component of the pre-initiation complex that assembles the core transcription machinery. TFIID has been shown to recruit RNAPII to the promoter (10). The NRE-to-TATA (NT) region on the Hes1 promoter refers to the 24 base pairs between the NREs and the TATA box.

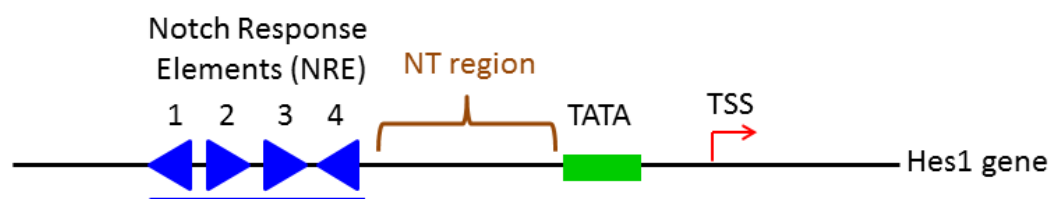


FIGURE 3: Hes1 gene fragment used for promoter analysis studies. Hes1 contains four Notch Response Elements (NREs) and a TATA box. The 24 base pairs between the NREs and the TATA box comprise the NT Region. Triangles pointing right signify a Head site and triangles pointing left signify a Tail site. TSS represents the transcription start site.

Previous research groups have shown that two of the four NREs recruit Notch activation complexes that form a dimer, which is an essential element of transcription initiation of *Hes1* (8, 9). NREs 2 and 4 comprise a sequence-paired site (SPS), a response element architecture for two transcription factors. The orientation of the NREs within the SPS is Head and Tail. The Notch dimer crystal structure was solved on a synthetic promoter, and this dimerization appears to depend on residue 1985 in the ankyrin (ANK) domain of NICD (Fig. 4) (9, 11). A mutant form of NICD with this residue altered from an arginine to an alanine showed a decrease in *Hes1* promoter activity compared to activity of wildtype NICD. In addition, altering the spacing and orientation of the SPS was shown to reduce dimerization activity in electrophoretic mobility shift assays (EMSAs) (9).

To further elucidate the essential promoter elements of *Hes1* and other Notch target genes in the studies reported here, the promoter activity was examined directly through reporter gene assays. Because MAM has been shown to recruit important proteins that are essential for proper transcriptional regulation, these experiments examined the promoter activity in the presence of both NICD and MAM. Mutation analysis was used to determine which promoter elements are necessary for transcriptional activation of *Hes1*. Individual and combinations of NREs were mutated along with the SPS spacing and orientation. The residue responsible for forming a NICD dimer was also mutated. The *Hes1* TATA box was mutated, as well as the TATA box in Hairy/Enhancer of Split 5 (*Hes5*), another Notch target gene that contains an SPS. The

number of base pairs between SPS and the TATA box were both increased and decreased in mutant Hes1 promoters.

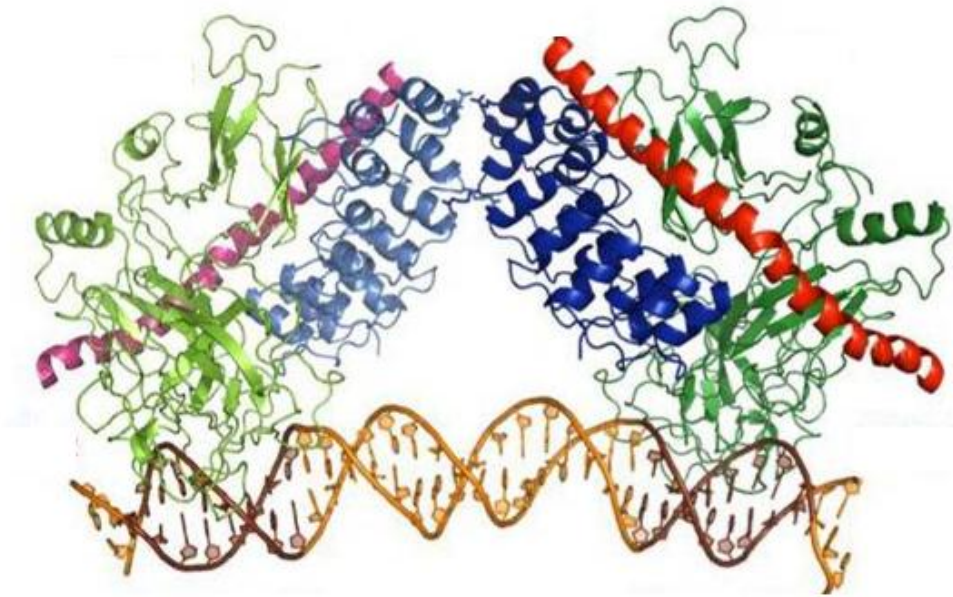


FIGURE 4: Crystal structure of Notch complex dimers assembled on the Hes1 promoter. Dark and light green ribbons represent CBF1 protein bound to NREs 2 and 4. Red and pink ribbons represent the N-terminal helix of MAM. Light and blue ribbons represent ANK domain on NICD. Adapted by permission from Macmillan Publishers Ltd.: *Nat. Struct. Mol. Biol.* (11), copyright 2010.

These findings demonstrated that the SPS on NREs 2 and 4 on Hes1 are both essential to promoter activity since mutations of individual or both SPS elements abolished activation. NREs 1 and 3 did not appear to contribute to activation. Perturbing the spacing between the SPS elements produced reduced promoter activity, so proper SPS spacing is essential for promoter activation. The NICD mutant data confirmed that the dimer between the activation complexes is essential for promoter activity. Changing the SPS orientation to Tail-Head and Head-Head from the wildtype Head-Tail orientation significantly reduced promoter activity, suggesting that SPS orientations are crucial for

transcription. The Tail-Tail orientation showed promoter activity at the same level as wildtype. Results also showed that the TATA box is crucial for Hes1 and Hes5 activation, meaning that the TFIID complex or TATA-binding protein (TBP) alone is necessary for transcription. The SPS was effective in activating the promoter when moved up to 150 base pairs upstream from the TATA box, suggesting that Notch activation complexes may stabilize the TFIID complex through other proteins bridging their interaction, or directly through a DNA looping mechanism. Taken together, these results suggest that NICD- and MAM-mediated transcription activation requires a TATA box and a spacing- and orientation-dependent SPS.

LITERATURE REVIEW

Overview of Notch Signaling

Notch was first discovered 100 years ago when a mutation that disrupted the gene produced a notched wing phenotype in *Drosophila melanogaster* (12). Since then, Notch has been genetically and molecularly characterized in various organisms. Notch signaling regulates important cellular processes, such as differentiation, growth, and death (1). This pathway is named after Notch, a transmembrane protein with an extracellular receptor used to communicate with adjacent cells. Notch signaling is found in all metazoans; the number of Notch receptors, however, varies with the complexity of the organism. For example, while the fruit fly *Drosophila melanogaster* has only one Notch receptor, mammals have four Notch receptors to accommodate more diversified pathways. The pathway serves important and diverse functions, and aberrancies in Notch signaling often result in disease and dysfunction in many types of tissues. In aberrant Notch signaling, Notch functions as an oncoprotein or tumor suppressor depending on the cellular context (13). Mutated Notch has been linked to cancers, such as T-cell acute lymphoblastic leukemia and mammary tumorigenesis, and neurological disorders, such as CADASIL and Alzheimer's disease (14-17).

Synthesis of the Notch protein is synthesized in the endoplasmic reticulum, where the protein O-fucosyltransferase (OFUT) 1 binds to Notch. The fucosyltransferase activity of OFUT1 is responsible for the proper folding of Notch (18). After Notch is transported to the Golgi body, it is cleaved by a furin-like convertase, resulting in a Notch extracellular subunit (NECD) and a Notch transmembrane subunit (NTM) that are

tethered together non-covalently at a site called S1 in the heterodimerization (HD) domain (19, 20). Notch is transported to the cell membrane, where it functions as a single-pass transmembrane protein that appears at the cell surface (20). The NECD also contains Epidermal Growth Factor (EGF)-like repeats and three conserved Lin12/Notch repeats (LNR). The LNR and HD domains together comprise the negative regulatory region (NRR), which is essential for the prevention of non-specific, ligand-independent proteolytic cleavage that prematurely dissociates the NECD and NTM (21).

Notch communicates with cells through direct contact by binding its NECD to ligands expressed as transmembrane proteins in adjacent cells. This selective cell-cell interaction is believed to mediate spatiotemporal Notch signaling in vertebrate development. Notch ligands are members of the Delta/Serrate/LAG-2 (DSL) family of proteins. Delta and Serrate were the first ligands to be identified as Notch ligands in *Drosophila* (22). In mammals, Notch ligands include Jagged 1 and 2, homologs of Serrate, and Delta-like 1, 3, and 5. The DSL ligands bind to the NECD on its EGF-like repeats. The Golgi body contains Fringe, which can site-specifically glycosylate Notch EGF-like repeats during protein processing; modifications by Fringe appear to adjust Notch responsiveness to different ligands (23). When a ligand binds to Notch, Notch becomes sensitive to cleavage by tumor necrosis factor- α converting enzyme (TACE), a protease from the ADAM (A Disintegrin And Metalloprotease) family, at a site called S2 in the Notch HD domain (24). When the NECD is dissociated from NTM after proteolytic cleavage, this results in an intermediate form of Notch that acts as a substrate for a presenilin-dependent gamma-secretase. This enzyme cleaves NTM inside the cell

membrane between G1743 and V1744 on a site called S3. Cleavage at S3 releases the Notch intracellular domain (NICD) (2, 25).

The NICD contains a Recombination Binding Protein-associated module (RAM) domain and several ANK repeats for interactions with other proteins. The NICD also has a proline, glutamate, serine, and threonine rich (PEST) domain that is necessary for degradation. When released, NICD proteins form multimers within the cell in an antiparallel orientation through its N- and C-terminal regions. Ski-interacting protein (SKIP) binds to the NICD multimers, forming a docking site for MAM (26). Next, the NICD is then released from SKIP as a monomer bound to MAM (26). This forms the Notch transcription activation ternary complex consisting of CBF1, NICD, and MAM. SKIP has been shown to associate with CBF1 upon activation by NICD, and it is postulated that the NICD RAM domain contains a region that can bind to CBF1 at its four amino acid $\Phi W \Phi P$ (Φ = hydrophobic residue) motif and also a separate region necessary for multimerization with other NICD proteins (6, 26, 27). CBF1 may displace SKIP from the RAM domain by steric crowding; therefore, the NICD RAM domain could be responsible for toggling between the pre-activation complex and the ternary activation complex. Because SKIP delivers NICD and MAM to CBF1, SKIP appears to be a limiting factor in Notch transcriptional activation. It has been shown that addition of SKIP significantly increases target gene promoter activation by NICD and MAM *in vivo* (6).

The four mammalian Notch receptors have the same major domains, but vary slightly in their structure. These differences appear to play a role in differential

expression, both throughout different types of tissues and within the same tissue. The extracellular portion of Notch contains between 29 and 36 EGF-like repeats, and these help to determine binding to specific ligands (28). Only Notch 1 and 2 contain a transactivation domain (TAD), and it has been postulated that the phosphorylation sites on TAD allows Notch 1 and 2 to selectively modulate signaling activity (29). It has also been postulated that the number of ANK repeats dictates target gene activation (30). With different combinations of ligands, four Notch receptors, and three MAM coactivators, this suggests that the Notch signaling pathway can elicit differential responses depending on cellular context. However, additional data suggests that different MAM proteins dictate activation strength of target genes, regardless of which Notch homolog is used.¹

CBF1 is the mammalian version of the CBF1/Su(H)/Lag-1 (CSL) protein, which is named for the orthologs in mammals, *Drosophila*, and *C. elegans*, respectively. CBF1 is made up of an N-terminal domain, which interacts with the major groove of DNA, a β -trefoil domain that interacts with DNA minor grooves, and a C-terminal domain that does not bind DNA. CBF1 binds to target genes at the consensus sequence RTGRGAR. This sequence varies in different target genes, but the internal guanines of the consensus sequence are essential for CBF1 binding (4). In the absence of Notch, CBF1 binds to a transcriptional corepressor complex comprising Silencing Mediator of Retinoid and Thyroid hormone receptors (SMRT). The SMRT complex recruits histone deacetylase (HDAC)-1 and SMRT/HDAC-1 Associated Repressor Protein (SHARP), and

¹ T. M. Burke and J. B. White, unpublished data

CBF1 Interacting Corepressor (CIR) with HDAC-2 (31-33). Interaction of CBF1 with amino acids 640-811 in SMRT is also necessary for correct nuclear translocation of CBF1 (34). It is unclear how the presence of NICD displaces the corepressors, but it has been hypothesized that NICD competes with the corepressor complex for binding to CBF1. CBF1 is responsible for the interaction of the activation complex with target genes and also for mediating the protein interactions within the complex. The CBF1 β -trefoil domain interacts with a 23-residue segment of the RAM domain in NICD, while the CBF1 C-terminal domain simultaneously interacts with the NICD ANK domain and MAM N-terminal helix. In addition, the CBF1 N-terminal interacts with MAM at its C-terminal helix (35). Notch is essential for the correct conformation of CBF1 in the activation complex. Without the Notch RAM domain, the CBF1 N-terminal loop has a closed configuration that cannot interact with MAM. Furthermore, when Notch RAM binds to the CBF1 β -trefoil domain, the CBF1 N-terminal changes into an open conformation that is suitable for binding to MAM (36).

MAM, a component of the Notch ternary activation complex, has been identified by its similar functionality to its orthologs: LAG-3 in *C. elegans* and Mastermind in *Drosophila*. MAM was discovered to only form a complex with CBF1 in the presence of Notch (37). MAM contains a recognition motif with a 52 residue helix that detects binding sites on both CBF1 and Notch simultaneously; NICD with CBF1 without MAM is not sufficient to activate transcription, nor is MAM alone or MAM with CBF1 sufficient (5, 38, 39).

MAM has been shown to bind to p300 and c-AMP response element binding protein (CBP), which is necessary for p300- and p300/CBP-associated factor (PCAF)-acetylation of nucleosomes at Notch target genes. This histone acetyltransferase activity is necessary for activation at chromatin templates (40). Only p300, not CBP, appears to strongly associate to MAM in the presence of NICD (6). CBP is recruited by c-AMP response element binding protein (CREB) transcription factor on Notch responsive genes, as it has been shown on Hes1 (41). Nuclear MAM also selectively recruits CDK8, which hyperphosphorylates NICD by targeting serines at the PEST domain. This Notch ternary complex interaction with CDK8 has been shown to have an inhibitory effect on Notch signaling (6, 42). Hyperphosphorylated NICD is a target for SEL10 (Suppressor/Enhancer of lin-12 10), which contains seven regions with about 40 repeats of tryptophan and aspartate that bind directly to phosphorylated NICD (43). SEL10 is a component of the S-phase kinase-association protein/Cullin/F-box (SCF) complex that functions as an E3 ubiquitin ligase. When ubiquitin is transferred to the phosphorylated proteins, they subsequently become targets for proteasome-mediated degradation. In protein immunoblots, proteasome inhibitors and dominant negative SEL10 without E3 ubiquitin ligase activity stabilized NICD (44). While SEL10 has been previously shown to reduce presenilin activity, SEL10 does not modulate Notch signaling through the presenilin-dependent gamma-secretase cleavage of the receptor (44).

MAM's role in Notch signaling, therefore, appears to activate expression of target genes by integrating with the Notch ternary activation complex and recruiting chromatin modifiers. Upon activation, MAM appears to immediately inactivate gene expression by

recruiting CDK8, whose phosphorylation of NICD PEST domain becomes the target of proteasomal degradation. This dual role of MAM reflects an important aspect of Notch signaling, highlighting the significance of proper timing of target gene activation. When this simultaneous on-and-off switch cannot occur, diseased states in humans often results, usually when Notch signaling is overexpressed. The first Notch mutation found in humans was identified in T-cell acute lymphoblastic leukemia (T-ALL) patients who had a t(7;9)(q34;q34.3) translocation (14). The disrupted locus on chromosome 9 contained a mutant Notch1 gene that transcribed truncated forms of Notch1 that resembled NICD. NICD is expressed in T-ALL cells regardless of ligand interaction with the Notch receptor, and constitutively active Notch signaling was shown to transform T-cells. Because degradation of NICD is arranged by MAM and CDK8, overexpressed Notch signaling may be due to disruption of the proteasomal pathway.

Overview of Transcription in Class II Promoters

A protein-encoding gene is transcribed by RNA polymerase, the enzyme that polymerizes nucleotides to form an RNA molecule. In mammals, there are three types of RNA polymerases that encode different RNA molecules: RNA polymerase I produces ribosomal RNA, RNAPII transcribes messenger RNA (mRNA), small nuclear RNA, and microRNAs, and RNA polymerase III synthesizes transfer RNA and other small RNAs (45). RNA polymerase I, II, and III transcribe Class I, II, and III genes, respectively. Target genes of Notch signaling are Class II genes, which are the most well characterized group of genes.

On Class II genes, RNAPII binds to a region called a core promoter. Some core promoters contain a TATA box, which typically has the consensus sequence TATAA (46). TBP interacts with the TATA box, causing the bound DNA to bend nearly 90 degrees (10). The TATA sequence becomes distorted and general transcription factors (GTFs) are allowed to bind to the core promoter. TBP along with TBP-associated factors (TAFs) make up one GTF, the TFIID complex. Some other GTFs include TFIIA, which stabilizes the interaction between TFIID and DNA, TFIIB, which recruits RNAPII, and TFIIH, which contains helicase activity that unwinds DNA to create the transcription bubble. Together, the GTFs and RNAPII form the pre-initiation complex (10). In core promoters that do not contain a TATA box, the pre-initiation complex can assemble on other promoter elements, such as the Initiator sequence, which contains the consensus sequence YYANWYY (Y= C or T; W= A or T), where A is the transcription start site. The pre-initiation complex can also bind to upstream and downstream promoter elements.

In addition to a pre-initiation complex, other activators can stimulate transcription initiation. This can occur when gene-specific transcription factors, or activators, bind to the promoter to help stabilize or recruit TFIID to the core promoter. One important coactivator is Mediator. Mediator is not necessary for the function of the basal transcription machinery of the pre-initiation complex, but it increases basal transcription levels. It has been shown in mammalian cells that abolishing Mediator results in a loss of transcription (47, 48). Mediator is 1.4 MDa and made up of over 30 subunits in mammals. The coactivator consists of a core complex with a head, middle, and tail module, and a kinase module that is loosely associated with the core complex (49). Due

to Mediator's large size, it can act as a linker between the pre-initiation complex and other gene-specific transcription factors and coactivators. Some researchers refer to Mediator as a GTF since Mediator can act as an adaptor between transcription activators and the core promoter factors. Mediator has been seen to have direct interactions with TBP, TFIIE, and TFIIH, suggesting that Mediator plays a role in recruiting RNA polymerase and helps stabilize the pre-initiation complex (50-52). Mediator has been seen both as a required activator in housekeeping genes that are constitutively expressed and as a recruited activator in target gene activation. Mediator can also interact synergistically with transcriptional coactivators that are involved in chromatin remodeling, such as p300, a histone acetyltransferase (53). Mediator has also been shown to direct the assembly of the pre-initiation complex by binding p300 to TFIID (54). Nucleosomes are acetylated by p300, causing the DNA to dissociate from the histone and subsequently allowing TFIID to bind.

In addition to Mediator's extensive role as a transcriptional coactivator, Mediator has also been shown to repress transcription when it is associated with the kinase module. The Mediator kinase module is composed of four subunits: Med12, Med 13, CDK8, and CycC. Mammalian CDK8 has been shown to be associated with inactive transcription complexes, and leaves when the transcription is activated (55). Structural studies have shown that CDK8 binds to the core region of Mediator, preventing RNA polymerase binding (56). However, CDK8 has also been shown to positively influence transcription initiation in both mouse *in vivo* and *in vitro* studies (57). Mediator has been shown to activate and repress transcription initiation activities; however, the roles of each subunit

and module in transcription initiation and activator recruitment are still not clearly defined. The Notch activation complex utilizes MAM to recruit CDK8, a component of the Mediator kinase module, but it is still unclear whether or not Notch- and MAM-mediated activation is Mediator-dependent.

Class II promoters can also be activated by enhancer elements that recruit proteins that activate transcription. Enhancers are defined as elements that are located upstream or downstream of the core promoter and can activate transcription regardless of their orientation (58). The distance from the core promoter can vary greatly among genes. One enhancer for the Sonic hedgehog gene in developing mouse limb buds has been located over 1 Mb away from the promoter (59). One mode of action used by enhancers is the looping of DNA so that the activator and the GTFs on the core promoter are brought close together. DNA looping was first discovered in the L-arabinose operon in *E. coli*, and can be determined today using chromosome conformation capture to examine if a distal element is brought closer to the promoter (60). One simple mechanism of enhancers utilizes free diffusion in the nucleus to join the activator and core promoter complex. Activators bound to enhancers can also dynamically scan DNA until interacting with the core promoter complex to initiate transcription (61).

Hes1 Promoter and Sequence-Paired Sites

One well characterized mammalian target gene of the Notch signaling pathway is Hes1, a paralog of *Drosophila* Hairless and Enhancer of Split. The HES1 protein in *Drosophila* inhibits neurogenesis. Additionally, HES1 has been shown to be a

transcriptional repressor in mouse models as well (62). HES1 is a basic helix-loop-helix (bHLH) protein that binds to N boxes (CACNAG) on its target gene promoters. This is a unique characteristic of HES1 since most bHLH proteins preferentially bind to promoter E boxes (CANNTG) (63). By directly binding to the DNA, HES1 can recruit additional repressors such as *Drosophila* Groucho or mammalian Grg (62). HES1 plays crucial roles in neural, muscle, endocrine, exocrine, and T-cell development, as well as other important functional tissues (64). HES1 is necessary for maintenance of undifferentiated cells. HES1-deficient mice show premature differentiation and developmental abnormalities in various tissues, such as brain, eye, and pancreas (65, 66). Therefore, HES1 is responsible for maintaining populations of precursor cells until signaled by differentiation cues. Because of its diverse functions, control of Hes1 is tightly regulated in a tissue-specific manner. Regulation of HES1 expression is heightened by its autoregulatory negative feedback loop in which HES1 protein binds to its own promoter at N boxes to repress transcription (67).

The Hes1 promoter has over 95% sequence homology with human and rat Hes1 (68). Structural characterization of the Hes1 showed that the gene is located on chromosome 16 and contains four exons. The first coding region begins 248 base pairs after the transcription start site (TSS). There is a TATA box at 31 base pairs upstream and a CAAT box for CAAT-protein binding at 151 base pairs upstream of the TSS. Hes1 contains four N boxes at -165, -132, -58, and +16, and HES1 protein binds to the Hes1 promoter at the three N boxes upstream of the TSS (69). In addition to the N boxes, Hes1 promoter contains four additional regulatory elements called Notch response

elements (NREs), where the Notch ternary activation complex binds through CBF1. These NREs are located upstream of the TSS at -107 (NRE 1 on the negative strand), -85 (NRE 2 on the positive strand), -71 (NRE 3 on the positive strand), and -62 (NRE 4 on the negative strand). NREs typically contain the consensus RTGRGAR; although NREs 3 and 4 contain a single base pair that do not match the consensus, all the NREs contain the guanines that are necessary for CBF1 binding (4). The putative CBF1 binding site at NRE 3 has not been addressed in previous literature, and it appears that this site being addressed for the first time in the experimental studies presented here.

The *Drosophila* promoter Enhancer of Split, or E(spl), is a homolog of Hes1 and a Notch signaling target gene. E(spl) contains two NREs oriented as a sequence-paired site (SPS), an evolutionarily conserved pattern of inverted repeats of NREs (70). The first NRE of the SPS is located on the positive strand and designated as a Head site, while the second NRE of the SPS is on the negative strand and designated as a Tail site. These NREs are separated by 17 base pairs. Cave *et al.* examined the importance of the SPS as well as bHLH binding sites in activation of E(spl) promoter by *Drosophila* NICD (dNICD) and the proneural bHLH proteins Achaete and Daughterless (Ac/Da) (71). They found that the native promoter is activated synergistically by dNICD and Ac/Da, but not by dNICD or Ac/Da alone. When either of the NRE sites are mutated, the E(spl) promoter is only weakly activated by dNICD and Ac/Da alone or together. This study framed a promoter code for Notch target gene activation in which the SPS is necessary for promoter activity, and the Notch complex can work synergistically with other proteins in order to activate transcription.

Like the synergistic activation of Notch target genes in *Drosophila*, Notch and MAM work together through Notch ternary complexes in order to stimulate transcription initiation (6, 72). On Hes1, the SPS motif appears on NRE 2, a head site, and NRE 4, a tail site, and these are separated by 16 base pairs. Studies have shown that a Notch complex binds to a single NRE site and many groups believe this assembly can activate transcription in mammals (11, 73). However, it has been shown that abolishing the SPS on Hes1, either by mutating a single NRE or by changing the orientation of the second NRE, produces a decreased level of promoter activity compared to wildtype when NICD is transfected into cell culture with Hes1 fused to a reporter gene (8). Nam *et al.* examined the importance of the SPS and reported that the Notch complexes cooperatively dimerize on the Hes1 SPS in order to induce transcription. This group solved a crystal structure containing dimerized Notch ternary complexes on a synthetic promoter derived from Hes1. The dimerization occurred on NREs 2 and 4 through key residues that form salt bridges between the Notch ANK domains. These residues are conserved across a wide range of animals, including *Drosophila* (9). When the native Hes1 promoter was transfected with a NICD construct containing one mutated ANK residue at amino acid 1985, promoter activity was abolished. Additionally, this study used EMSAs to show that proper dimerization cannot occur when NRE 2, NRE 4, or both are mutated, when NRE 4 is inverted, nor when the spacing between the SPS is increased by four base pairs or decreased by two.

These studies raised questions of whether or not the SPS is important for Notch- and MAM-mediated transcription activation of the native Hes1 promoter and other Notch

target genes. Hes1 has a TATA box which recruits the general transcription factor TFIID, but it is not been determined whether or not TATA boxes contribute to transcription activation on Notch target genes. It has been postulated that Notch- and MAM-mediated activation occurs when MAM recruits CDK8, which may recruit the Mediator complex to assemble the transcription pre-initiation complex (6). While the two NREs on Hes1 comprising an SPS were found to be necessary to activate the promoter, these tests had yet to be performed to assess synergistic activation by NICD and MAM together. Also, it has not yet been determined whether or not the two unpaired sites on Hes1 contributed to NICD- and MAM-mediated activation. Additionally, new research questions can assess whether Notch and MAM activation of Hes1 is dependent on the orientation of the SPS, the spacing between the SPS, and the spacing between the SPS and the TATA box. Ong *et al.* showed that increasing the distance between the SPS and the TSS by 1 kb decreased promoter activation by Notch; however, MAM was not included in these studies, so it was still undetermined whether or not the coactivators recruited by MAM account for activation by the SPS from a distance (8). Additionally, Notch has been shown to regulate position-dependent transcriptional enhancers in *Drosophila*, and therefore the role of Notch as a activator bound to enhancer sequences can be further studied in mammals (74).

EXPERIMENTAL PROCEDURES

Materials and Equipment

HeLa cells were purchased from the American Type Culture Collection. Cells were grown in Dulbecco's modification of Eagle's medium (DMEM). The DMEM was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (denoted as complete medium) and was stored at 37°C and 5% CO₂. DMEM with high glucose, L-glutamine, and sodium pyruvate was purchased from Mediatech (Manassas, VA).

Transfection reagent Lipofectamine 2000, Opti-MEM Reduced Serum Medium, Subcloning Efficiency DH5 α Competent Cells, and Gateway LR Clonase II Enzyme mix were purchased from Life Technologies (Carlsbad, CA). Custom DNA Oligos for use as mutagenic primers were also purchased from Life Technologies (Table 1). QuikChange II XL Site-Directed Mutagenesis Kit was purchased from Agilent Technologies (Santa Clara, CA). GenCatch Plasmid DNA Maxi Prep Kit was purchased from Epoch Life Science (Missouri City, TX). Buffers P1, P2, P3, QBT, QC, and QF were purchased from QIAGEN (Germantown, MD). Dual Luciferase Reporter Assay System, pRL-TK, and PureYield Plasmid Miniprep System was purchased from Promega (Madison, WI). pGL2-Hes1 was a gift from K.A. Jones (Salk Institute for Biological Studies), and this construct was originally cloned in Reference (7). pGL2-Hes5 was a gift from R. Kageyama (Kyoto University), as was originally cloned in Reference (75). Human Notch 1 intracellular domain and Mastermind-like 1 were cloned in frame to Myc tags in the pCS2-6MT vector, which was originally cloned in Reference (76).

Luminescence readings were obtained on two machines manufactured by Promega: the Veritas Microplate Luminometer and the Modulus II Microplate Multimode Reader. Data analysis was performed using Microsoft Excel and IBM SPSS Statistics 20 software.

Generation of Mutant Plasmids by Site-Directed Mutagenesis

Reagents from the QuikChange II XL Site-Directed Mutagenesis Kit were used for the site-directed mutagenesis reactions. One reaction contained the reagents and thermal cycler parameters listed in Tables 1 and 2.

TABLE 1
Reaction conditions for site-directed mutagenesis.

Volume (μl)	Reagent
5	10x Reaction Buffer
0.5	Template DNA [50 ng/μl]
1	Forward primer [150 ng/μl]
1	Reverse primer [150 ng/μl]
1	dNTP mix
3	QuikSolution
38.5	water
1	PfuUltra HF DNA polymerase

TABLE 2
Thermal cycler conditions for site-directed mutagenesis reactions.

Segment	Cycles	Temperature (°C)	Time
1	1	95	1 min
2	18	95	50 s
		60	50 s
		68	7 min
3	1	68	10 min

The cycled reaction was incubated with 1 μ l DpnI at 37°C for 2 h in order to digest template DNA. Next, the digested reaction was transformed into Subcloning Efficiency DH5 α competent cells. A volume of 3 μ l was pipetted into a microcentrifuge tube containing 25 μ l thawed cells, then the mixture was incubated on ice for 30 min. The mixture was incubated in a 42°C water bath for 20 s, then moved to ice for 2 min. Next, 475 μ l NZ-amine and yeast (NZY) broth was added to the cell mixture, and the tube was shaken at 37°C for 1 h at 225 rpm. On lysogeny broth (LB) and agar plates with 100 μ g/ μ l ampicillin, 100 μ l of the transformation reaction was plated and incubated at 37°C for 16 h. A single colony of bacteria was inoculated in 5 ml LB broth with 100 μ g/ μ l ampicillin and shaken at 37°C for 1 h at 225 rpm.

The plasmid was purified from 4.5 ml bacterial culture using PureYield Plasmid Miniprep System. The culture was centrifuged at 5,000 rpm. After discarding the supernatant, the bacterial pellet was resuspended in 600 μ l Tris-EDTA (TE) buffer. The cell lysate was prepared by adding 100 μ l Cell Lysis Buffer and 350 μ l of 4°C Neutralization Solution, mixing the solution after the addition of each buffer. The mixture was centrifuged at 13,000 rpm for 3 min, and then the supernatant was transferred to a minicolumn in a collection tube. The collection tube waste was discarded and replaced with the minicolumn. The minicolumn was washed with 200 μ l Endotoxin Removal Wash and 400 μ l Column Wash Solution, spinning at 13,000 rpm for 30 s after addition of each buffer. The minicolumn was removed from the collection tube and placed in a clean 1.5 ml microcentrifuge tube. Next, 50 μ l TE buffer was added directly to column matrix. After letting column stand for 1 min at 25°C, the microcentrifuge tube

was centrifuged at 13,000 rpm for 30 s. The eluted plasmid was submitted to Sequetech DNA sequencing service (Mountain View, CA) to verify that the plasmid had the correct mutation.

The columns from the GenCatch Plasmid DNA Maxi Prep Kit with QIAGEN buffers were used to obtain transfection quality plasmids. The remaining 500 µl bacterial culture was inoculated in 200 ml LB medium and 100 ng/µl ampicillin. This culture was shaken for 16 h at 225 rpm. After incubation, the culture was centrifuged at 5,000 rpm for 10 min and the supernatant was discarded. The bacterial pellet was resuspended in 10 ml Buffer P1, Buffer P2, and Buffer P3, with mixing by inverting after addition of each buffer. The column was washed with 30 ml Buffer QBT. The bacteria and buffer mixture was centrifuged at 15,000 rpm for 15 min at 4°C, and the supernatant was poured into the maxiprep column. The column was washed with 30 ml of Buffer QC and then eluted with 15 ml of Buffer QF. The eluted plasmid was mixed with 10.5 ml isopropanol and centrifuged at 15,000 rpm for 30 min at 4°C. The supernatant was discarded and the remaining DNA pellet was mixed with 5 ml 70% ethanol and centrifuged at 15,000 rpm for 10 min at 4°C. The supernatant was discarded and the DNA was allowed to dry. The purified plasmid DNA was resuspended in TE buffer.

Transfection into HeLa Cells

HeLa cells were plated in complete medium 24 h prior to transfection in a 96-well plate at 2.5×10^4 cells per well. On day of transfection, the complete medium was replaced with 50 µl Opti-MEM. Expression constructs (Table 4) for one well were

diluted in 25 µl Opti-MEM in the following amounts: 100 ng promoter DNA fused to Firefly luciferase, 10 ng NICD, 100 ng MAM, and 10 ng pRL-TK to express Renilla luciferase. pCS2-6MT was added DNA mixture to bring the total DNA amount to 200 ng. Transfection reagent for one well was diluted by adding 0.5 µl Lipofectamine 2000 in 25 µl of Opti-MEM and incubating for 5 min. The Lipofectamine mixture was then mixed with the DNA dilution and incubated for 20 min. The Lipofectamine and DNA mixture was transferred to one well of HeLa cells. Transfections were performed as technical triplicates.

```

GCTCAGGCGCGCGCCATTGGCCGCCAG -168

ACCTTGTGCCTAGCGGCCAATGGGGGGGCGCAGT -134
      N box          CAA box          NRE 1
CCACGAGCGGTGCCGCGTGTCTCTTCCTCCCATT -100
      N box          NRE 2          NRE 3
GGCTGAAAGTTACTGTGGGAAAGAAAGTTTGGGA -66
      NRE 4
AGTTTTCACACGAGCCGTTTCGCGTGCAGTCCCAGA -32
      TATA box          N box
TATATATAGAGGCCGCCAGGGCCTGCGGATCACA 3
      CAGGATCTGGAGCTGGTGCTGATAACAGCGGAAT 37
      N box
CCCCTGTCTACCTCTCTCCTTGGTCCTGGAATAG 71
TGCTACCGATCACTAAGTAGCCCTAAGACATAAT 105
AAACCTTCAACTGCTCAGTAGTTTTTCTTATGAA 139
AGTCAAGTAAAAGGACGTAAG 160

```

FIGURE 5: Hes1 promoter fragment used in reporter gene studies. This construct was originally cloned into the pGL2-Basic vector with KpnI and BglII in Reference (7). The TSS is denoted as A.


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TCCTCTGGAAGTCGCTGTCTGTTCAATTGTAAACTCTCATTTCTCT -408
GCGCCTGTGGCCTCTGCCAGCCAGCGGTGGGGAGCCTCTGGGGAGTGGGA -358
GGGAAGAAGGGAGAGAAGGGGGGGGAGAGCACTCCTTCCTGCCCTCCCC -308
ACCTCCCCGCGGCCTGGGAAAAGGCAGCATATTGAGGCGCGGGGCTCTCA -258
GCATCAGGCCCCGGGATGCTAATGAGGGCGAGCGCGTTCCACAGCCCGG -208
ACATTGTGCCGCGCGGCCACCTGCTCCTCGGGGAGCGACCATTGTGCCC -158
GCGCCAATTACAGGCAATTTAGCGTGCGCTAATGGGCCGGCGCCTTTGT -108
GCGGCCGGCGCCGCCATTGGCCGCCGAGTGTGGGAACGGCCGCGGCGCCC -58
GGACCCCAGGCGCCGGGCCGCTGCCCGCGCCTATATAGGGCTGGCGTGCT -8
GGGGTCCAGGTCGCGCCAGTCCGGGACGCCTGGCTCTGTCCGCTCCGCTC 43
CGCTCCGCTCGCTAATCGCCTCCAGAGCTCCAGGCATGGCCCCAAGTACC 93
GTGGCGGTGGAGATGCTCAGTCCCAAGGAGAAAAACCGACTGCGGAAGCC 143
GGTGGTGGAGAAGATGCGTCGGGACCGCATCAACAGCAGCATAGAGCAGC 193
TGAAGCTGCTGCTGGAGCAGGAGTTCGCGCGGCACCAGCCCAACTCCAAG 243
CTGGAGAAGGCCGACATCCTGGAGATGGCCGTCAGCTACCTGAAACACAG 293
CAAAGCCTTCGCCGCGGCCGCGGCCCAAGAGCCTGCACCAGGACTACA 343
GCGAGGGCTACTCCTGGTGCCTGCAGGAGGCGGTACAGTTCCTGACCCTG 393
CACGCCGCCAGCGACACGCAGATGAAGCTGCTT 427

```

FIGURE 6: **Hes5 promoter fragment used in reporter gene studies.** This construct was originally cloned into the pGL2-Basic vector in Reference (77). The TSS is denoted as A.

Mutant promoter sequences

^a Sequences shown here include relevant promoter elements. The full promoter sequences can be found in Figs. 5 and 6.

^c 10 bp insert sequence: AGAAAGTTTG

e 20 bp insert sequence: AGAAAGTTTGAGAAAGTTTG

^g 50 bp insert sequence: AGAAAGTTTGAAAGTTTGAAAGAGAAAGTTTGAGAAAGTTTGGAAC

h 150 bp insert sequence: AGAAAGTTTGAGAAAGTTTGAGAAAGTTTGAGAAAGGAGCCGTTCGCGTGCAGTCCAGA
AGTTTGAGTTTGAGAAAGTTTGAGAAAGGAGTTTGAGAAAGTTTGAGAAAGTCCCAGA

Reporter Gene Assays

The reporter gene assay was performed 48 h after the transfection reaction using reagents from the Promega Dual Luciferase Reporter Assay System. The Opti-MEM was aspirated from the well and replaced with 30 μ l 1X Passive Lysis Buffer, which is incubated with the cells at room temperature with light shaking for 15 min. After the cells were lysed, 20 μ l cell extract was transferred to an opaque plate and read on the luminometer. The luminescence readings were measured under the following conditions: 50 μ l Luciferase Assay Reagent II, 50 μ l Stop & Glo Reagent, 5 s delay between injection and measurement, 10 s integration time. The readings were obtained in Microsoft Excel software. The measurement readings were given as relative light units, and analyzed as a ratio of the Firefly luciferase measurements to the control Renilla luciferase measurements.

Statistical Analysis

For comparison between activation levels of the wildtype promoter and one other mutant promoter, the data were analyzed using two-tailed independent samples t-tests, and a p value less than 0.05 indicated a statistically significant result. When comparing activation levels of the wildtype promoter and two or more mutant promoters, pairwise independent samples t-tests were performed with the wildtype data. A statistically significant result was indicated by a p value less than a Bonferroni-adjusted α , where $\alpha = 0.05/k$ and k = total number of promoters compared in the dataset.

In the studies reported here, the null hypothesis (H_0) stated that the activation of the mutant promoter and Notch mutants are not different from the wildtype expression constructs. The minimum acceptable risk for a Type I false positive error, where H_0 is true but is incorrectly rejected, is 5% for experimental studies, therefore α was set to 0.05. When analyzing the mean values of two samples, a p value less than α indicates that the H_0 was rejected and the values from one sample set were significantly different from the values of the set to which it was being compared. A p less than α is indicated by an asterisk (*) on the reported figures. Many of the datasets included more than two samples, such as the NRE site mutants which have several samples from the different mutant NRE sites. When multiple samples are compared to wildtype, the risk of a Type I error was increased. In order to combat the problem of multiple comparisons, a Bonferroni correction was applied to α values to restrict the conditions in which H_0 was rejected. The smaller α value, which is indirectly proportional to the number of samples, accounted for the H_0 that none of the samples is significantly different from wildtype (78). It has been debated that using Bonferroni corrections is a very conservative method for multiple comparisons (79). However, this methodology places much importance on the promoter elements that are found to be statistically significant and assists in powerfully supporting the conclusions presented here.

RESULTS

Activation of Hes1 with Notch 1 and Mastermind-like 1

A luciferase reporter construct containing the wildtype Hes1 promoter was transfected into HeLa cells with either coactivator expression plasmid Notch1 intracellular domain (N1ICD), Mastermind-like 1 (MAM1), or both. The promoter activity of Hes1 alone was normalized to 1; the activity of Hes1 with N1ICD and MAM1 are shown as fold promoter activities compared to Hes1 alone (Fig. 7). These results are consistent with other research groups that have shown that NICD and MAM work synergistically to activate Hes1 transcription, but show very weak activation individually (6, 72).

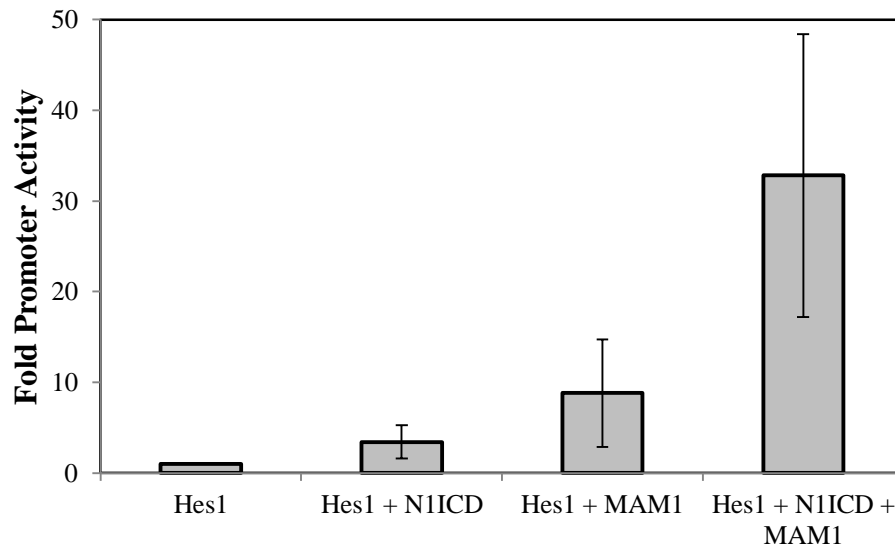


FIGURE 7: Fold promoter activities of Hes1 with N1ICD and MAM1. Fold promoter activities of are normalized to Hes1 promoter alone. Data are shown from at least three independent experiments. Standard deviation bars are shown.

Notch 1- and Mastermind-like-1-Mediated Hes1 Activation with Mutated Notch Response Elements

The NREs on Hes1 were mutated by changing the internal guanines to cytosines so that CBF1 could no longer bind. N1ICD and MAM1 were transfected with Hes1 promoter and Hes1 promoters with mutant NRE sites (Fig. 8). N1ICD- and MAM1-mediated activations of Hes1M1, Hes1M3, and Hes1M1M3 were not significantly different from Hes1, demonstrating that NREs 1 and 3 do not contribute to activation on the Hes1 promoter. Activations of Hes1M2, Hes1M4, and Hes1M2M4 were significantly decreased compared to wildtype, showing that NRE 2 and NRE 4 are necessary for Hes1.

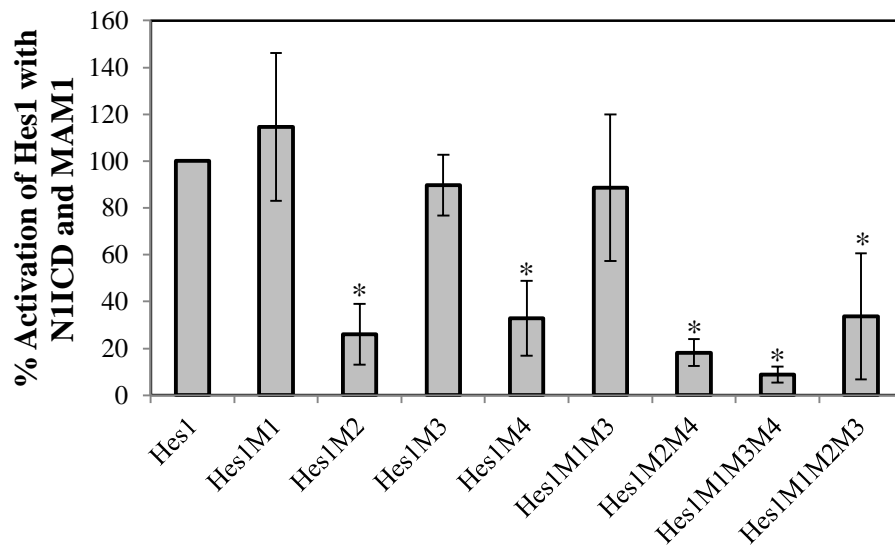


FIGURE 8: Activation of Hes1 NRE site mutants. The Hes1 promoter activation by MAM1 and N1ICD is normalized to 100% and the activity of the mutant promoters with MAM1 and N1ICD is shown as a percentage compared to wildtype. Data are shown from at least three independent experiments. Standard deviation bars are shown. * denotes $p < 0.0055$.

NRE 2 and 4 together comprise the SPS motif that was proposed by Cave *et al.* to be important for Notch target gene activation (71). Activation by N1ICD and MAM1

produced significantly decreased promoter activity of Hes1M1M3M4 and Hes1M1M2M3. NRE 2 and NRE 4 separately are not sufficient to activate Hes1.

Hes1 Promoter Activity with a Notch Dimerization Mutant

Site-directed mutagenesis was used to create a mutation in N1ICD by changing the arginine on residue to 1985 to an alanine. Nam *et al.* proposed that this residue was necessary for the Notch complexes to dimerize on the SPS (9).

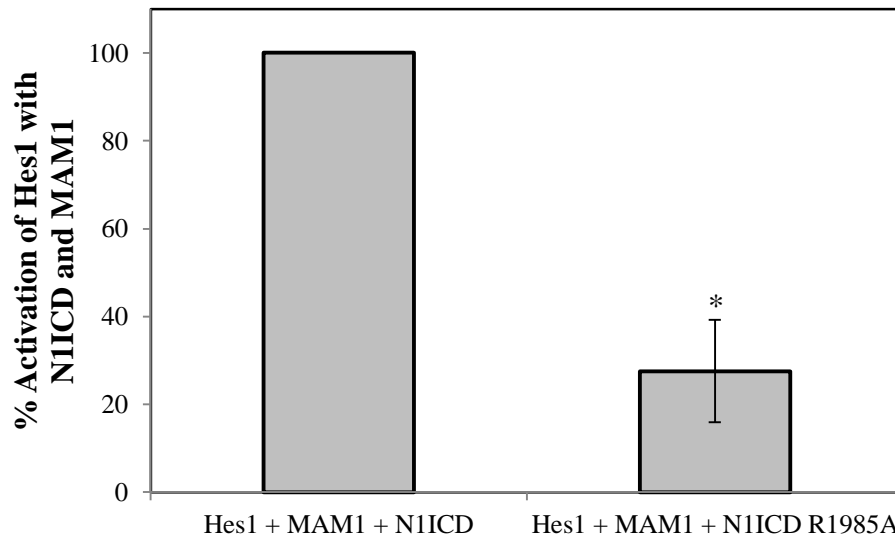


FIGURE 9: Activation of Hes1 with N1ICD R1985A. The Hes1 promoter activation by MAM1 and N1ICD is normalized to 100%, and the activity of Hes1 with MAM1 and the mutant N1ICD is shown as a percentage compared to wildtype. Data are shown from at least three independent experiments. Standard deviation bars are shown. * denotes $p < 0.05$

Hes1 activation by MAM1 and N1ICD R1985A was significantly less than activation with the wildtype expression constructs (Fig. 9). This result is in agreement

with Nam *et al.* and confirms that the dimerization of the Notch complexes assembled on the SPS is important for Hes1 activation.

Notch- and Mastermind-like-Mediated Activation of Hes1 with a Sequence-Paired Site Separated by Additional Base Pairs

The Hes1SPS+7 promoter construct contained a seven base pair insertion on the 5' end of NRE 4, separating NREs comprising the SPS. According to Nam *et al.*, separating the SPS by an additional seven base pairs eliminates the ability of the Notch complexes to dimerize (9).

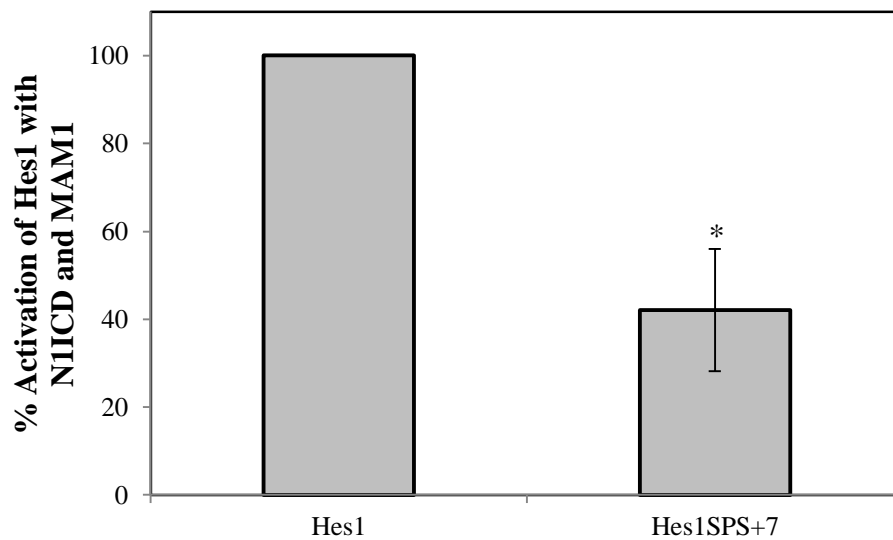


FIGURE 10: Activation of Hes1 with separated SPS elements. The wildtype Hes1 promoter activity is normalized to 100%, and the Hes1SPS+7 promoter activity is shown as a percentage compared to wildtype. Data are shown from at least three independent experiments. Standard deviation bars are shown. * denotes $p < 0.05$

When the Hes1 SPS is separated by 23 base pairs instead of 16 base pairs as it is in the native promoter, promoter activation was significantly decreased (Fig. 10). This

result strengthens the hypothesis that the dimer assembled on the SPS of Hes1 is necessary for maximum promoter activation by NICD and MAM.

Notch- and Mastermind-like-Mediated Activation of Hes1 with Different Orientations of the Sequence-Paired Site

To examine whether or not the orientation of the NREs within the SPS are important for Hes1 activation, site-directed mutagenesis was used to change the orientation of NRE 2, NRE 4, or both. The NREs in the wildtype Hes1 SPS are located 16 base pairs apart and are arranged as Head-Tail. When the SPS was arranged as Tail-Head in Hes1 T2-H4 or Head-Head in Hes1 H2-H4, the promoter activity was significantly decreased compared to Hes1. However, when the NREs were positioned as Tail-Tail in Hes1 T2-T4, promoter activity was not significantly different from wildtype (Fig. 11A).

NRE 1 and NRE 2 on Hes1 are located 15 base pairs apart. Although this differs from the SPS on the native Hes1 promoter that is separated by 16 base pairs, results from Nam *et al.* have shown that Notch dimers still form when there is a one base pair reduction between the SPS (9). NREs 1 and 2 are arranged as Tail-Head. In the Hes1 H1-T2 mutant promoter, NREs 1 and 2 were mutated so their orientation is Head Tail, and NRE 4 was mutated so that CBF1 cannot bind. Hes1 H1-T2 promoter activity did not differ significantly from wildtype Hes1 (Fig. 11B).

Taken together, these results suggest that the Head-Tail wildtype orientation or the Tail-Tail orientation is necessary for Hes1 activation.

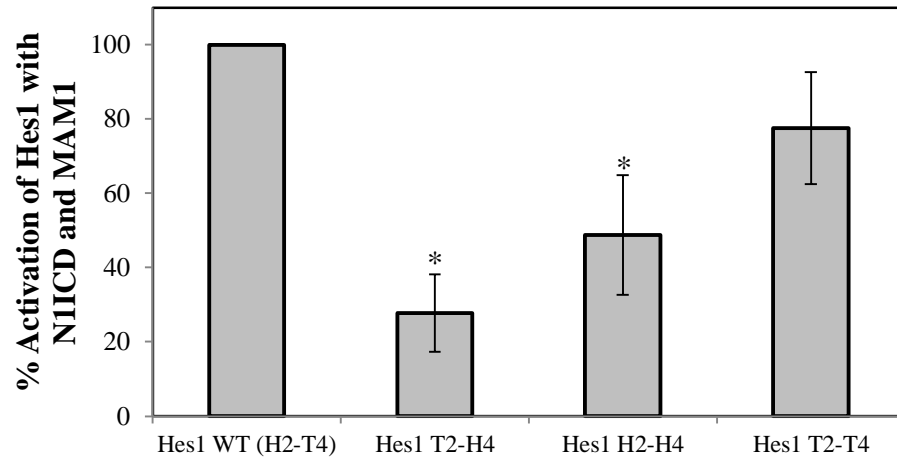
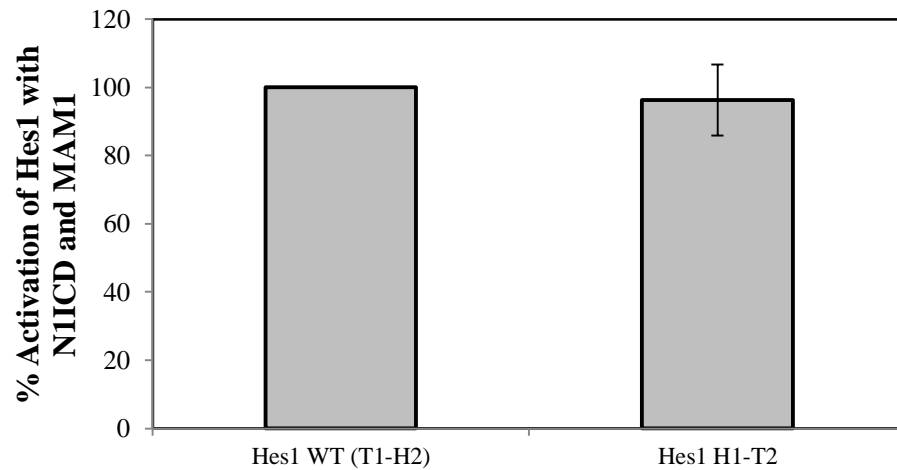
A**B**

FIGURE 11: Activation of Hes1 SPS orientation mutants. The wildtype Hes1 promoter activity is normalized to 100%, and the mutant promoter activities are shown as a percentage compared to wildtype. *A*, Activation is shown for mutant promoters where the NREs of the SPS were mutated to show different orientation combinations. *B*, Activation is shown for a mutant promoter in which NRE 1 and NRE 2 were mutated to a Head-Tail orientation to match the wildtype SPS orientation. Data are shown from at least three independent experiments. Standard deviation bars are shown. * denotes $p < 0.01$.

Notch- and Mastermind-like-Mediated Activation of Target Genes with a Mutated TATA Box

Hes1 contains a TATA box, which recruits RNA polymerase through the TFIID complex. In order to study the contribution of the TATA box to Hes1 activation, site-directed mutagenesis was used to mutate the TATA box sequence of TATATAT to GAGAGAG. Changing the TATA box thymines to guanines has been previously shown to abolish the functionality of the promoter element (81).

The Hes1 TATA-less promoter, Hes1TATA, had a significantly lower activation by N1ICD and MAM1 than Hes1 (Fig. 12A). This suggests that the pre-initiation complex assembles at the TATA box during transcription initiation. This also indicates that the activation complexes assembled at the NREs alone are not sufficient to activate transcription.

To assess whether or not a TATA box is necessary for other Notch target genes, the TATA box on Hes5 was examined. Hes5 is a Notch target gene that has a TATA box as well as an SPS (11). Hes5TATA is a mutant promoter in which the thymines of the TATA box are mutated to guanines. Activation of Hes5TATA was significantly lower than Hes5 (Fig. 12B).

Taken together, these results suggest that a TATA box is a necessary promoter element on Notch target genes that contain an SPS.

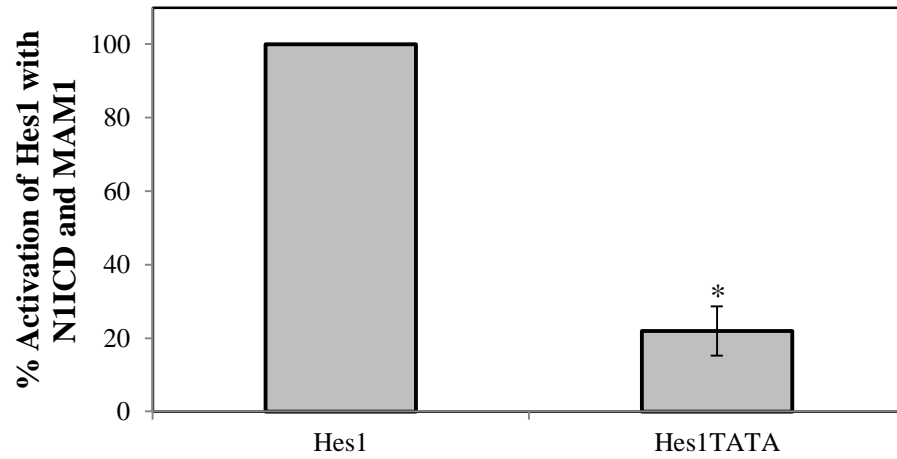
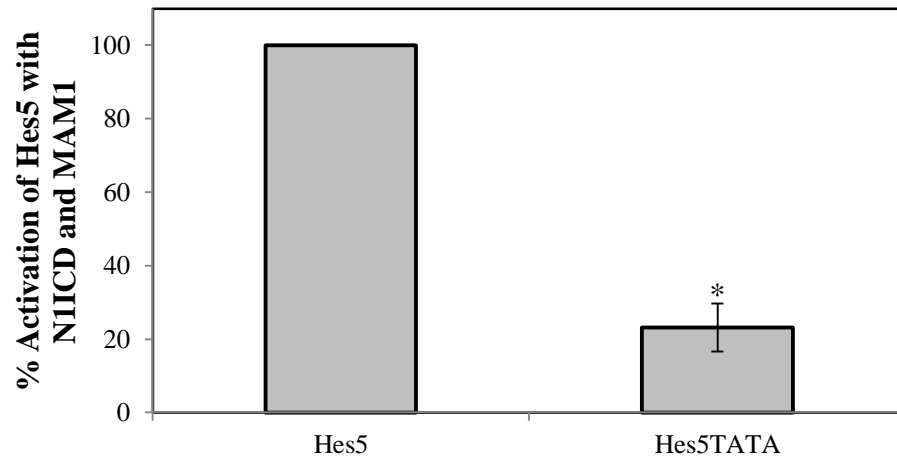
A**B**

FIGURE 52: Activation of Notch target genes with a mutant TATA box. The wildtype Hes1 promoter activity is normalized to 100%, and the TATA-less promoter activities are shown as a percentage compared to wildtype. *A*, TATA-less Hes1 promoter activity activation was compared to wildtype activation. *B*, Hes5 TATA box was mutated to assess promoter activity. Data are shown from at least three independent experiments. Standard deviation bars are shown. * denotes $p < 0.05$.

Notch- and Mastermind-like-Mediated Activation of Hes1 with Altered Base Pair Spacing between the Sequence-Paired Site and TATA Box

Since the Hes1 SPS and TATA box have been shown to contribute to promoter activation, altering the number of base pairs between these promoter elements can offer insight into the nature of the interactions between the Notch activation complexes and the pre-initiation complex. The 24 base pairs between the NREs and TATA box on Hes1 are designated as the NT region.

Hes1NT-10 and Hes1NT-20 mutants contained the Hes1 promoter with 10 and 20 base pairs, respectively, removed from the NT region. When activated by N1ICD and MAM1, both Hes1NT-10 and Hes1NT-20 had significantly reduced promoter activity compared to Hes1 (Fig. 13A). This suggests bringing the Notch activation complex closer to the protein complex bound to the TATA box on the Hes1 is detrimental for promoter activity.

Hes1NT+10, Hes1NT+15, Hes1NT+20, Hes1NT+25, Hes1NT+50, and Hes1NT+150 contained the Hes1 promoter with 10, 15, 20, 25, 50, and 150 base pairs, respectively, added to the NT region. All of these Hes1 promoters with an increased NT region did not have significantly different promoter activations from wildtype Hes1 (Fig. 13B). This suggests that the Notch activators on SPS can interact with the TFIID complex on the TATA box when the SPS is moved further upstream on the promoter.

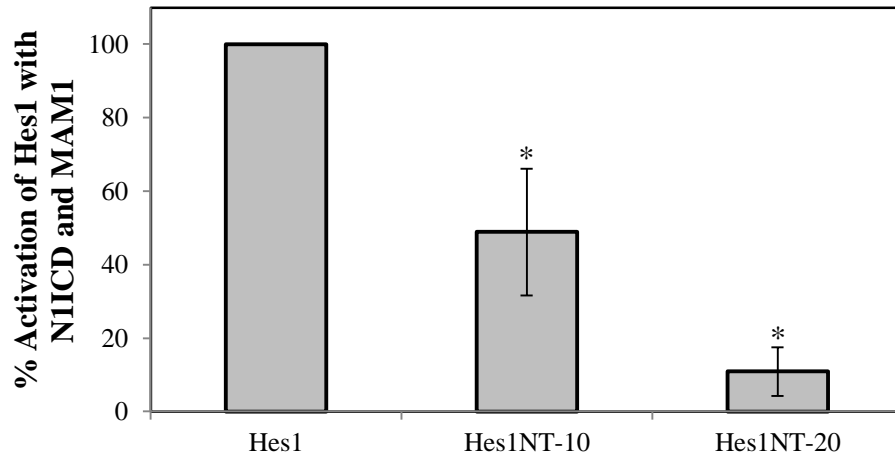
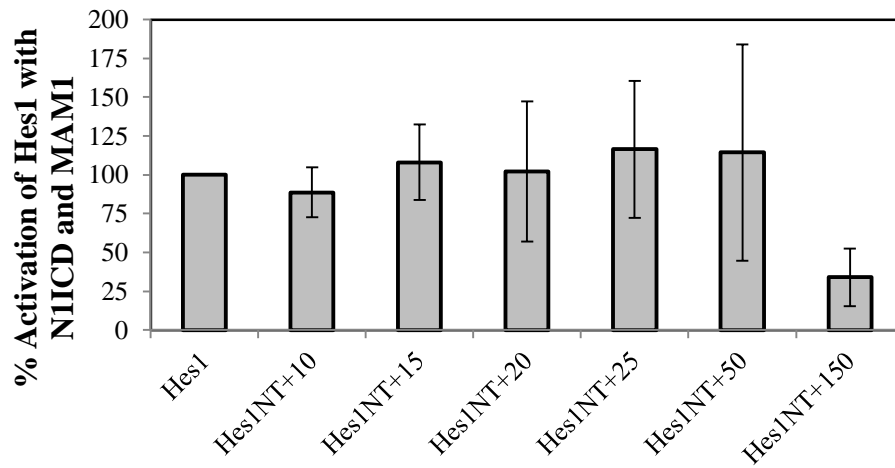
A**B**

FIGURE 13: Activation of Hes1 with modified spacing between SPS and TATA box. The wildtype Hes1 promoter activity is normalized to 100%, and the mutant promoter activities are shown as a percentage compared to wildtype. *A*, Activation is shown from mutant promoters where the Hes1 NT region had 10 and 20 base pairs removed. *B*, Activation is shown when up to 150 base pairs were added to the Hes1 NT region. Data are shown from at least three independent experiments. Standard deviation bars are shown. * denotes $p < 0.0055$.

DISCUSSION

Uncovering the mechanism of MAM-dependent Notch target gene activation is crucial for understanding Notch signaling gene regulation. This is important from a scientific discovery perspective because little is known about Notch signaling, including how target genes are activated, which functional domains contribute to activation, and how combinations of ligands and Notch homologs selectively modulate gene expression. Studying Notch has beneficial consequences for clinical research as well since Notch signaling influences cell immunity, tumor angiogenesis, and stem cell maintenance (81).

Transcriptional coactivators of Notch have been identified, but the mechanisms of transcription machinery assembly and initiation still need further clarification. Studying promoter elements of Notch target genes and their effect on activation provides insight into transcription initiation mechanisms. The results presented here show that two of the NREs on the Hes1 promoter are necessary for Notch- and MAM-mediated activation. NRE 2 and NRE 4 comprise the SPS and appear to contribute to wildtype levels of Hes1 promoter activity; however, neither NRE 2 nor NRE 4 alone is sufficient for activation. NRE 1 and NRE 3 do not appear to play any role in transcription. The lack of activation influence by NREs 1 and 3 is surprising given that these sequences contain the necessary components for CBF1 binding (4). Future studies can discern if these NREs do not contribute to activation because of their incorrect arrangement on the promoter or because there are other activators bound to the promoter that block CBF1 binding.

In order to activate Hes1, the Notch complexes on the SPS must be dimerized through the NICD proteins. This is shown by the N1ICD R1985A mutation on the ANK

domain presented here. This is an agreement with reporter gene results from Nam *et al.* (9). Unlike Nam *et al.*, the assay results of Hes1 with the N1ICD mutant shown here include MAM. The formation of the NICD dimer regardless of the presence of MAM signifies that the dimerization of the Notch complexes does not depend on MAM. Nam *et al.* also showed that a Notch complex dimer did not form when the SPS elements were spaced more than four base pairs apart. The results shown here provide the promoter activity of the Hes1 promoter where seven base pairs are inserted between the SPS. Along with previous results, these findings show that the Notch complex dimers bound to the SPS is necessary to activate the Hes1 promoter at wildtype levels.

In addition to the dimer, the orientation of the SPS plays a role in Hes1 promoter activity. The wildtype SPS orientation is Head-Tail. When site-directed mutagenesis was used to manipulate the promoter to form an SPS orientation of Tail-Head and Head-Head, Hes1 activation by N1ICD and MAM1 was significantly reduced. Interestingly, the Tail-Tail SPS orientation promoter activity is not statistically significant from the wildtype orientation. This is a more complete analysis of SPS orientation to supplement studies by Nam *et al.*, which used EMSAs to show that the Head-Head orientation did not form a Notch dimer on Hes1, as well as studies by Ong *et al.* that utilized reporter gene assays to show that the SPS in a Head-Head orientation gives a reduced activation of Hes1 (8, 9). Further studies are required to determine if a dimer is formed during activation of the Tail-Tail SPS.

The importance of SPS for transcription initiation was shown when a simulated SPS was created on NREs 1 and 2. On the wildtype promoter, the orientations of NREs 1

and 2 are Tail and Head, respectively. At 15 base pairs apart, NREs 1 and 2 meet the spacing requirements for the SPS (9). When they were changed to Head-Tail using site-directed mutagenesis techniques, the promoter activation was the same level as the wildtype promoter. The Hes1 SPS, a strict layout for orientation, spacing of response elements, and activator interaction, is a required element for Notch- and MAM-mediated transcription initiation. This work opens the possibility that the SPS promoter motif occurs in Notch responsive genes. Future research can utilize the SPS promoter architecture to discover novel direct target genes of the Notch signaling pathway.

In addition to the SPS on which the Notch activation complexes assemble, Hes1 promoter activity also depends on a TATA box. Eliminating the ability of TFIID to bind to Hes1 produces about 20% promoter activation by N1ICD and MAM1 compared to wildtype Hes1. The importance of the TATA box was also assessed in Hes5, a Notch target gene that contains both an SPS and TATA box. The Hes5TATA mutant also produced about 20% promoter activation compared to wildtype. This indicates that TFIID, the GTF bound directly to the TATA box, assembles the pre-initiation complex and recruits RNAPII.

Hes1 promoter activity is also regulated by the distance between the SPS and the TATA box. When spacing was reduced, fold promoter activity was proportionally decreased. This suggests that the Notch complexes and TFIID complex encountered steric crowding and therefore competed for binding sites on the promoter. When the spacing between the SPS and TATA was increased up to 150 base pairs, there was not a significant difference in activation compared to the wildtype promoter. This indicates

that the interaction between the Notch complexes and the pre-initiation complex may not depend on a fixed promoter distance. Since mechanisms such as DNA looping and promoter tracking are utilized by enhancer regions that are located upstream of the core promoter, these results suggest that the SPS is an enhancer element on the Hes1 promoter (61). However, previous studies have shown that the TATA box must remain at a fixed distance from other promoter elements in order to cooperatively initiate transcription. For example, activation of the Pal promoter of *Oryza sativa* depends on both a TATA box and Initiator element, and when six base pairs were inserted or deleted between the TATA box and Initiator sequence, TATA-dependent activation was abolished (82). Proper spacing of the TATA box has also been shown to be important for selective recognition by other promoter elements. Studies of the adenovirus major late promoter show that transcription does not begin on the preferred transcription start site when the TATA box is not located 21 to 25 base pairs from the Initiator sequence (83). Further investigation of the spacing between SPS and TATA box and the role of SPS as an enhancer can provide insight into additional regulatory mechanisms of Notch signaling.

The results proposed here provide evidence for a new model of Notch activation that may include the CDK8 module interacting with the core Mediator complex, which stabilizes TFIID at the TATA box (Fig. 14A). However, CDK8 only loosely associates with the core Mediator complex and may not recruit Mediator during Notch signaling. Instead of CDK8 bridging the connection between the Notch complexes and the pre-initiation complex, the interaction might occur through another protein, such as p300.

The histone acetyltransferase, p300, is recruited by MAM and *in vivo* and *in silico* research has shown that p300 interacts with many transcriptional coactivators, particularly with proteins associated with open chromatin (84-86). Another model of Notch- and MAM-mediated activation can include a transcription machinery assembly that does not depend on transcription factor stabilization by direct protein interactions, but instead depends on the chromatin remodeling activity of the Notch activation complexes (Fig. 14B).

It has not gone unnoticed that abolishing the SPS or TATA box on Hes1 decreases activation by NICD and MAM only by 80% and not a complete 100%. Further, although SPS manipulations, such as rearranging the orientation, produce statistically significantly less promoter activity than wildtype, the activation is decreased by only 40-50%. This launches new research questions about whether or not a single, unpaired Notch activation complex can substantially contribute to target gene activation. This may occur by the Notch complex synergizing with other transcription factors bound to the promoter. Alternatively, nuclear MAM separate from NICD may synergize with additional transcription factor complexes. Previous results suggest that MAM is targeted for a ubiquitination-dependent degradation pathway that is separate from NICD degradation.² This implies that MAM is involved in functional mechanisms outside its role of target gene activation with NICD and CBF1. Further studies are required to determine MAM's role with other transcription activation complexes.

² M. Farshbaf and J.B. White, unpublished data

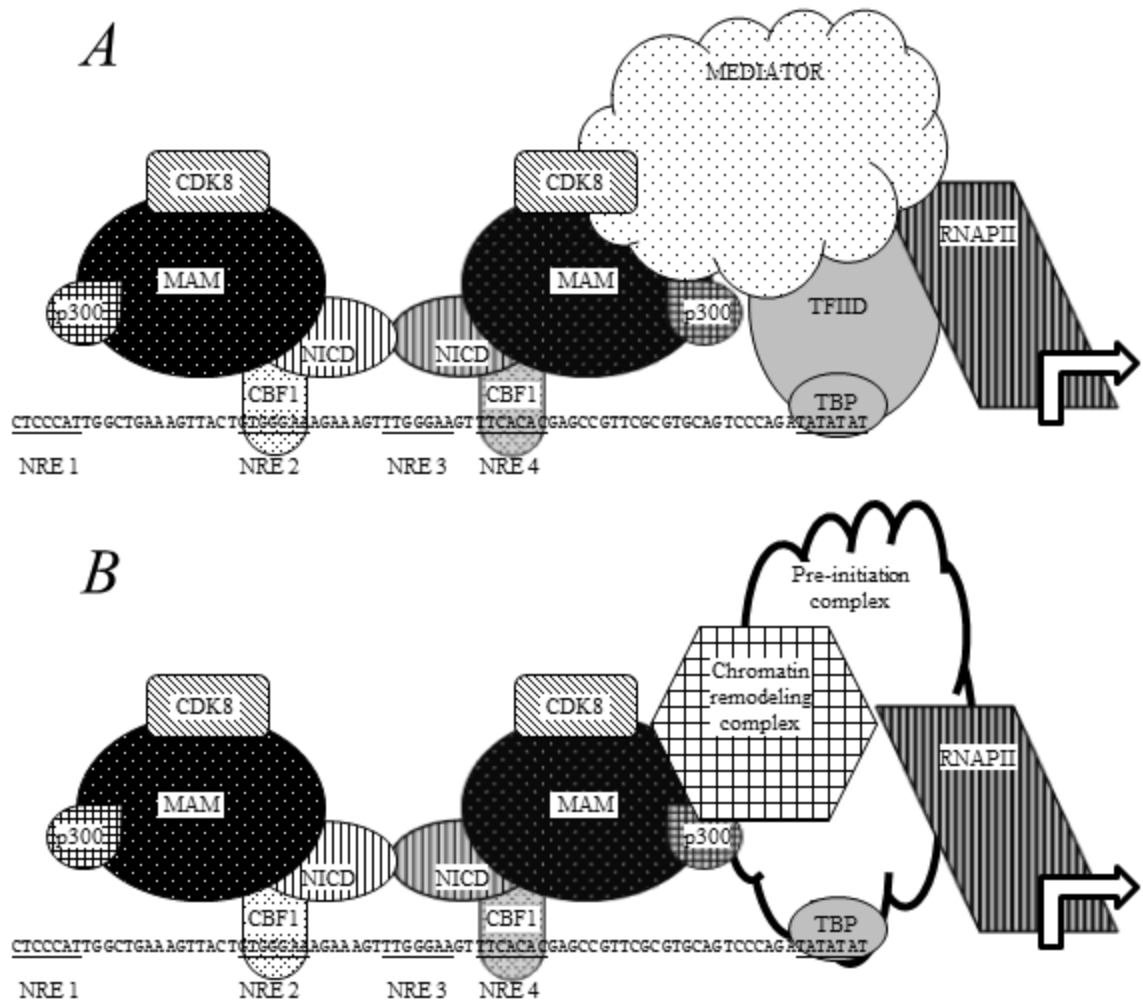


FIGURE 14: Proposed models of MAM-dependent Notch target gene activation. The Notch complexes dimerize through the ANK domain of NICD and assemble on the SPS of the gene promoter. The TFIID complex is bound to the TATA box through TBP. *A*, MAM recruits CDK8, which may recruit the Mediator core complex. Mediator can interact with TFIID and recruit RNAPII. *B*, MAM recruits p300, which may recruit a complex with the ability to remodel chromatin to an open conformation. This can allow the pre-initiation complex to assemble on TBP in order to recruit RNAPII.

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