Members of the HRT family of basic helix–loop–helix proteins act as transcriptional repressors downstream of Notch signaling

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The Hairy-related transcription-factor (HRT) genes encode three related basic helix–loop–helix transcription factors that show sequence similarity to the Hairy and Enhancer of split family of transcriptional repressors. HRT proteins are expressed in specific regions of the developing heart, vasculature, pharyngeal arches and somites, and the periodicity of their expression in somitic precursors mirrors that of Notch signaling-related molecules. In the present study, we show that the intracellular domain of the Notch1 receptor (Notch1 IC), which is constitutively active, up-regulates HRT2 expression in 10T1/2 fibroblasts. Luciferase reporter assays using the regulatory regions of the mouse HRT genes revealed that transcription of all three genes is stimulated by Notch1 IC. The promoters of the HRT genes share homology in a binding site for Suppressor of Hairless [Su(H)], a transcriptional mediator of Notch signaling. A dominant-negative Su(H) mutant abolished Notch-activated HRT2 expression, and mutation of the conserved Su(H) consensus site in the HRT2 promoter attenuated transcriptional activation by Notch. Ectopic expression of HRT proteins also blocked activation of HRT2 expression by Notch1 IC through a mechanism requiring the basic region, but not the conserved carboxyl-terminal YQPW-TEVGAF motif of HRT2. These findings identify HRT genes as downstream targets for Notch signaling and reveal a negative autoregulatory loop whereby HRT proteins repress their own expression through interference with Notch signaling.

The transmembrane receptor Notch participates in an evolutionarily conserved cell-interaction system that plays fundamental roles in embryonic patterning and development, including neurogenesis and somitogenesis (1, 2). Notch signaling is especially important in cell-fate specification and boundary formation in which clusters of undifferentiated cells are segregated into different cell lineages. Upon activation by ligands such as Delta or Jagged on neighboring cells, the intracellular domain of the Notch receptor (Notch IC) is cleaved and translocated to the nucleus together with Suppressor of Hairless [Su(H)] or related molecules. Su(H) provides DNA-binding specificity through recognition of the consensus sequence, whereas Notch IC functions as an activation domain (1–3). In response to Notch signaling, Su(H) activates transcription of the Hairy/Enhancer of split genes [H/E(spl)], which encode a family of basic helix–loop–helix (bHLH) transcriptional repressors (1, 2, 4). H/E(spl) proteins then inhibit transcription of their target genes, thereby preventing undifferentiated precursors from achieving differentiated phenotypes. The actions of Notch can also be mediated in Su(H)-independent pathways by the interaction of the Notch receptor with various molecules (1, 2).

We and others recently identified a subclass of Hairy-related transcription factors (HRTs), also called Hsr, Hey, CHF, and Gridlock (5–9). The HRT family consists of three proteins, HRT1, -2, and -3, which share structural similarity in their bHLH regions and contain a unique carboxyl-terminal domain similar to, but distinct from, the region of H/E(spl) proteins responsible for transcriptional repression (5). During embryogenesis, HRT genes show characteristic expression patterns that demarcate regions of the developing heart, vasculature, pharyngeal arches, and somites (5). Within the segmental-plate mesoderm, HRT gene expression exhibits a periodicity reminiscent of H/E(spl) and other components of Notch-signaling pathways.

Based on their embryonic expression patterns and on the importance of bHLH proteins for Notch signaling (4, 10), we investigated whether HRT genes might be downstream targets for Notch signaling. Here, we show that HRT gene expression in cultured cells is activated by Notch signaling and that HRT proteins interfere with Notch-dependent activation of HRT2 expression, thereby fulfilling a negative autoregulatory loop that tightly regulates HRT expression.

Materials and Methods

Expression Constructs. Plasmid expression constructs for Notch1 IC, mouse HRT (mHRT), and human HRT (hHRT) were prepared by the insertion of PCR fragments into pcDNA3.1 (Invitrogen, Carlsbad, CA) with an amino-terminal Myc-tag. The pCS2+mN1 IC(V1744)wt provided by R. Kopan (Washington University, St. Louis) was used as a template for Notch1 IC. All wild-type HRT constructs were designed to contain the entire coding region without the first methionine residue. The mHRT2 C(−) mutant construct was designed to delete the carboxyl terminus of mHRT2, KPYOPWT-TEVGAF, by introducing a premature stop codon. The mHRT2 B(−) construct was prepared by the ligation of two PCR fragments so that the basic domain of mHRT2 (RKKRGRGIEKKRR) was replaced with two amino acids, LE. The plasmids pCMX-RBP-J for wild-type RBPJ expression and pEF-BOSneocarboxyl-terminal YQPW-TEVGAF motif of HRT2. These findings identify HRT genes as downstream targets for Notch signaling and reveal a negative autoregulatory loop whereby HRT proteins repress their own expression through interference with Notch signaling.

Cell Culture and Plasmid Transfection. C3H10T½ (10T½) fibroblasts, COS7 cells, and C2C12 myoblasts were maintained as described (12). Plasmid transfection was performed by using Fugene 6 (Roche Diagnostics) or Lipofectamine Plus reagent (Life Technologies, Grand Island, NY). The total amount of plasmids was adjusted by using vector plasmids in each assay.

Abbreviations: bHLH, basic helix–loop–helix; H/E(spl), Hairy and Enhancer of split family; HRT, Hairy-related transcription factor; HRT1, HRT2, HRT3, Hairy-related transcription factor; HRT1, HRT2, HRT3, human HRT; mHRT, mouse HRT; Notch, Notch1 IC, intracellular domain of Notch1 receptor; Su(H), Suppressor of Hairless.

Data deposition. The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF311883, AF311884, and AF311885, for human cDNAs HRT1, HRT2, and HRT3, respectively).

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Northern Blot Analysis. Two days after transfection, RNA was purified from 10T½ cells and Northern blot analysis was performed by using mHRT1, -2, and -3 cDNA fragments. Positive genomic DNA fragments were analyzed by restriction-enzyme digestion, Southern blot analysis, and sequencing.

Isolation of Mouse HRT Genes. A mouse genomic DNA bacterial artificial chromosome library (Children’s Hospital Oakland Research Institute, Oakland, CA) was screened by using mHRT1, -2, and -3 CDNA fragments. Positive genomic DNA fragments were analyzed by restriction-enzyme digestion, Southern blot analysis, and sequencing.

Luciferase Reporter Analysis. A BamHI-MfI partial-digestion fragment of mHRT1, an EcoRI-NruI fragment of mHRT2, and a BamHI–BamHI fragment of mHRT3 were cloned into the pGL3 basic luciferase vector which lacks a promoter (Promega; Fig. 2A), and a series of 5’-deletion constructs was made for mHRT2. The mHES-1-luciferase construct provided by R. Kopan (13) was used as a positive control.

Results

Activation of mHRT2 mRNA Expression by Notch Signaling. We initially investigated whether Notch activation was sufficient to stimulate HRT gene expression in 10T½ fibroblasts by using expression of Notch1 IC, which mimics activation of Notch signaling triggered by ligand binding (1, 2). As shown in Fig. 1, Notch1 IC significantly induced endogenous HRT2 mRNA expression in 10T½ cells, indicating that mHRT2 is a target gene of Notch signaling.

Structure of Mouse HRT Genes. To determine whether the HRT genes are direct transcriptional targets for Notch signaling, we isolated genomic DNA fragments encompassing mHRT1, -2, and -3 and their 5’-flanking regions. Each gene comprised at least five exons and intervening introns (Fig. 2A) and all the introns had boundary sequences matching the GT-AG rule. Comparing the protein functional domains with the genomic structure, an intron was present in the middle of the basic domain and in the HLH domain, which is uncommon for other bHLH proteins.

Luciferase Reporter Assays. Luciferase reporter assays were performed as described (15) using in vitro-translated proteins and oligonucleotides containing a CACGTG motif (5’-TCGAGGTGACGTGCCATTG-3’, 5’-TCGACGTGCCACC-3’; ref. 16).

Structure-Acting Transcription of HRT Genes. We then analyzed the effects of Notch signaling on expression of luciferase reporters

Cultured cells were transfected with a luciferase reporter construct and various expression constructs. The CMV-LacZ expression construct (100 ng/35-mm well; ref. 12) was cotransfected for normalization. Two days after transfection, luciferase and LacZ activities in the cell extracts were assayed as described (12). All experiments were repeated at least twice, and the results from a representative experiment (n ≥ 3) are shown with standard deviations.

Site-Directed Mutagenesis. Mutation of the Su(H)-consensus site was introduced in the Nhel–NruI fragment of mHRT2 by PCR (CGTGGCAGAA to CGTGGGCAA; ref. 14) and the mutated fragment was ligated into several mHRT2-luciferase constructs.

Electrophoretic Mobility Shift Assay. DNA-binding assays were performed as described (15) using in vitro-translated proteins and oligonucleotides containing a CACGTG motif (5’-TCGAGGTGACGTGCCATTG-3’, 5’-TCGACGTGCCACC-3’; ref. 16).

Experiments were performed on three independent transfections with each plasmid are shown. Ethidium bromide staining of 28S rRNA is shown at the bottom and positions of 28S and 18S rRNA are shown on the left.
linked to the 5′-flanking regions of the HRT genes, using a 6.5-kb mHRT1 fragment, a 10-kb mHRT2 fragment, and a 6.5-kb mHRT3 fragment (Fig. 2A). As shown in Fig. 2B, overexpression of Notch1 IC significantly increased luciferase activity of all three HRT genes in different cell lines. Luciferase activity of an HES-1 construct was also stimulated by Notch1 IC as described (13). The fold-increase in activation for each gene varied among cell lines and was most prominent with mHRT2 in 10T½ cells. We, therefore, further characterized the regulatory region of mHRT2 by using these cells.

**Notch Activation of mHRT2 Is Mediated by the Su(H) Pathway.** Because the effects of Notch signaling can be fulfilled through Su(H)-dependent or -independent pathways (1–3), we next examined the involvement of RBPJ, a mammalian Su(H) orthologue, in regulation of HRT gene expression. As shown in Fig. 3A, coexpression of the RBPJ dominant-negative mutant, RBPJR218H (11), clearly interfered with mHRT2 mRNA expression in response to Notch1 IC. Furthermore, expression of RBPJR218H abolished Notch-dependent activation of mHRT2-luciferase expression (Fig. 3B).

To identify the cis elements responsible for the Notch responsiveness, we prepared a series of 5′ deletions of the mHRT2-luciferase constructs. As depicted in Fig. 3C, all the deletion constructs showed significant activation of transcription by Notch1 IC. The shortest, a 0.5-kb NheI-NruI fragment, was sufficient for the response to Notch activation, although the longer constructs yielded higher fold-increases.

Sequence analysis of the NheI-NruI fragment revealed two potential Su(H)-binding sites. The proximal motif, 140 bp upstream of the translation-initiation site, had a complete Su(H)-consensus sequence (CGTGGGAAA), whereas the distal one, located 203 bp upstream of the proximal site, was a complementary ATTCCCGG sequence with a single nucleotide difference from the classical Su(H)-consensus motif (T/C-GTG-G/A-GAA-C; ref. 3). Binding of RBPJ to the oligonucleotide fragment of the proximal Su(H) site was confirmed by gel mobility shift assay (data not shown). We, therefore, introduced a single nucleotide mutation in the proximal Su(H) site (CGT-GGGAAA). Binding of RBPJ to the mutated proximal Su(H)-site fragment was not detectable in the gel mobility shift assay (data not shown). As shown in Fig. 3D, mutation of the proximal Su(H) site abolished transcriptional activation of the NheI-NruI construct (1.5-fold increase versus 19-fold increase of wild type), and drastically decreased transcription of the longer constructs. These results indicated that the transcription of mHRT2 was up-regulated by Notch activation through a Su(H)-dependent pathway.

We also characterized the 5′ regulatory regions of mHRT1 and mHRT3, and found that a proximal Su(H)-binding site was present in a conserved position of the mHRT1 and -3 promoters (data not shown). In the mHRT3 promoter, one nucleotide mismatch to the Su(H)-consensus sequence was present (CCT-GGGAAA), which may possibly account for weaker activation of mHRT3 transcription (Fig. 2B).

The 3.6-kb SalI-NruI construct showed significantly higher transcriptional activation by Notch1 IC, compared with shorter constructs (Fig. 3C). In addition, mutation of the proximal Su(H) site did not abolish transcriptional activation with the 10-kb, 5.5-kb, or 3.6-kb construct (Fig. 3D). We, therefore, further sequenced the region between 3.6 kb and 0.5 kb upstream of mHRT2 and found two incomplete complementary Su(H)

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**Fig. 3.** Notch activation of mHRT2 is mediated by the Su(H) pathway. (A) 10T½ cells were transfected with a Notch1 IC expression plasmid (0.9 μg/10-cm dish) and/or 5.4 μg of dominant-negative RBPJ construct (RBPJR218H), and HRT2 mRNA was detected by Northern blot analysis. Note that less Notch1 IC expression plasmid was used compared with Fig. 1 because of the limitation of total plasmid amount in a transfection, and that the blot was exposed to x-ray film for 24 h. (B) The 10-kb mHRT2-luciferase construct (150 ng/35-mm well) was transfected with or without Notch1 IC and RBPJR218H constructs. (C) A series of 5′-deleted mHRT2-luciferase constructs (450 ng) was transfected with or without 450 ng of Notch1 IC plasmid. (D) Notch responsiveness of the luciferase constructs with a mutation in the proximal Su(H) site was examined as in C. Fold-increase in luciferase activity with Notch1 IC expression is shown in C and D.
sites, GTTCACACG and CTTCCCACT. These motifs may provide the Notch responsiveness of mHRT2 in addition to the proximal Su(H) site. mHRT1 also had an additional complete Su(H) site upstream of the conserved proximal Su(H) site within the approximately 1-kb region we sequenced.

Negative Autoregulation of mHRT2 Gene Expression Induced by Notch Signaling. The structural similarity between HRT and H/E(spl) proteins suggested that HRT proteins might function as negative transcriptional regulators. Because mouse HES-1, a mammalian H/E(spl)-related bHLH protein, negatively autoregulates its gene expression (17), we investigated whether HRT proteins might behave similarly. As shown in Fig. 4A, cotransfection of a mHRT2 expression plasmid significantly down-regulated mHRT2 mRNA expression activated by Notch1 IC. Consistently, expression of mHRT2 markedly down-regulated Notch-stimulated activation of the mHRT2 promoter in luciferase assays (Fig. 4B). We also examined the effects of other HRT family members on Notch-activated HRT2 promoter activity. Interestingly, similar negative regulation was observed with hHRT1 and hHRT2, whereas hHRT3, a structurally divergent member of the family (5), showed a significantly weaker potency of negative regulation (Fig. 4C).

We next tested the effects of mHRT2 mutants to identify the domain(s) responsible for transcriptional repression. In the carboxyl-terminal regions, HRT proteins contain a YXPW motif that is structurally similar to the WRPW motif in H/E(spl) proteins, which is essential for their association with the Groucho family of corepressors (18). In addition, mouse and human HRT proteins possess a highly conserved TE(I/V)GAF motif at their carboxyl termini (5). Carboxyl-terminal elongation of Gridlock, a zebrafish orthologue of HRT2, results in impaired vessel formation (9), suggesting that the conserved carboxyl termini of HRT proteins are functionally important in vivo. As shown in Fig. 4D, a mHRT2 mutant lacking the carboxyl-terminal YQPW-TEVGAF motif [mHRT2 C(−)] repressed Notch-dependent activation of the HRT2 promoter. In contrast, a mutant lacking the basic domain [mHRT2 B(−)] showed no inhibition, indicating that the basic domain is essential for negative regulation (Fig. 4D). Protein expression with each construct was confirmed by Western blot analysis (data not shown).

Transcriptional Inhibition Independent of the HRT-Binding Site. HES-1 inhibits transcription of the HES-1 gene mainly by binding to N boxes (CACNAG) in the 5′ regulatory region of the gene (17). Because the consensus cis elements to which HRT proteins bind had not been determined, we examined the binding of in vitro-translated HRT proteins to several candidate motifs by using gel mobility shift assays. As shown in Fig. 5A, mHRT2, hHRT1, and hHRT2 preferentially bound to an E box motif, CACGTG, which was recently shown to be an additional cis element for E(spl) binding (16). hHRT3 showed no detectable binding to this site. The mHRT2 C(−) mutant also bound to the CACGTG fragment, whereas the mHRT2 B(−) mutant did not.

Fig. 4. Negative autoregulation of mHRT2 gene expression induced by Notch signaling. (A) 10T1/s cells were transfected with a Notch1 IC construct (0.9 μg/10-cm dish) and/or 5.4 μg of mHRT2 expression plasmid and HRT2 mRNA was detected by Northern blot analysis. The blot was exposed to x-ray film for 24 h. (B) The 10-kb mHRT2-luciferase construct (150 ng/35-mm well) was transfected with or without Notch1 IC and mHRT2 expression constructs. (C and D) 10T1/s cells were transfected with a 10-kb mHRT2-luciferase construct and a Notch1 IC plasmid (150 ng each), and the effects of cotransfection of various HRT constructs were observed. mHRT2 C(−), mHRT2 mutant without carboxyl-terminal YQPW-TEVGAF motif; mHRT2 B(−), mHRT2 mutant lacking basic domain. Luciferase activity without Notch1 IC or HRT cotransfection was given a value of 1 in B–D.
show detectable binding. Binding of mHRT2 to the N box (CACAAG) and other E box motifs (CACTTG and CATCTG) was not detectable by gel mobility-shift assays (data not shown).

To determine whether inhibition of mHRT2 transcription was mediated through the CACGTG element, we first examined the effects of mHRT2 on a series of 5'–9'-deletion HRT2 constructs. As shown in Fig. 5B, Notch-activated transcription of all the deletion constructs was clearly down-regulated by coexpression of mHRT2. The shortest NheI–NruI fragment did not contain a CACGTG motif but had two E box motifs (CAGGTG). To eliminate the possibility that repressive effects of HRT proteins occurred via these sites, we prepared a further 5'–9'-deletion construct with a 190-bp ApaI–NruI fragment that contained the proximal Su(H) site but no N box or E box motifs. As shown in Fig. 5C, transcription from the ApaI-NruI construct was significantly activated by Notch1 IC, and cotransfection of various HRT plasmids suppressed it to the same extent as we observed with the 10-kb luciferase construct (Fig. 4B–D). These results suggested that negative regulation of mHRT2 gene expression was independent of binding of HRT proteins to these consensus elements.

Stra13, a distantly related member of the H/E(spl) family, also negatively autoregulates its gene expression (19). Although the mechanism of negative regulation is unknown, the repressive effects of Stra13 are inhibited by treatment with trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor (19). In contrast, repression of the mHRT2 promoter activity by mHRT2 was maintained in cells treated with TSA (Fig. 5D), suggesting that the effects of mHRT2 did not occur by recruitment of HDACs. These results suggested that negative autoregulation of mHRT2 gene expression may be mediated by a mechanism distinct from those in the HES-1 and Stra13 genes.

**Discussion**

The present study demonstrates that the HRT genes are downstream targets for transcriptional activation by Notch signaling and that their responsiveness to Notch is mediated by binding of Su(H) to their 5' regulatory regions. Our results also reveal the existence of a negative-feedback loop in which HRT proteins interfere with Notch-dependent activation of HRT2 expression. This type of negative autoregulation may function to terminate or dampen Notch signaling, thereby resulting in a transient or periodic signal as is characteristic of Notch signaling in the developing paraxial mesoderm.

Within the presomitic mesoderm, the HRT genes show unique expression patterns similar to those of Notch-related molecules (5), suggesting their participation in Notch signaling pathways. Consistent with this notion, mouse embryos lacking the Notch-receptor ligand, Dll1, showed decreased expression of HRT1/Hesr1 in the somites (6). Conversely, transgenic overexpression of activated Notch1 in the cortex layer of hair follicles in mice results in misexpression of HRT3/HeyL (20). H/E(spl) proteins
Moreover, human mutations in vasculature (21, 22), and Notch4 receptor and Dll4 ligand are specifically expressed in the orthologue of \( HRT2 \) Notch ligand, cause defects in the outflow tract and aortic arch.

The finding that the basic region was required for the transcriptional inhibition by \( HRT2 \) suggests that DNA binding is important for this effect. The basic domain could also mediate the protein-protein interaction independent of DNA binding, analogous to that between MyoD and its transcriptional cofactor MEF2 (27). \( HRT2/CHF1 \) binds to the aryl hydrocarbon receptor nuclear translocator (ARNT) and inhibits ARNT-dependent transcription by dissociating the ARNT complex from DNA (8). \( HRT \) proteins may dimerize with other proteins and bind to unrecognized sites in the \( mHRT2 \) promoter.

The bHLH protein Stra13 directly associates with the promoter complex and inhibits the promoter activity of \( c-myb \) by a histone deacetylase (HDAC)-independent mechanism, like the autoregulation of \( mHRT2 \) in this study, whereas the effects of Stra13 on its own gene expression were suppressed by HDAC inhibition (19). It is also conceivable that \( HRT \) proteins inhibit \( mHRT2 \) expression through association with components of the basal transcriptional machinery.

Notch signaling pathways are characterized by multiple mechanisms of feedback regulation (1, 2). Activation of Notch signaling causes up-regulation of Notch receptor expression, reinforcing the responsiveness to Notch ligands. In contrast, Notch signaling down-regulates the expression of Notch ligands, which specifies the signaling and responding cells. Downstream of Notch signaling, negative autoregulation of \( HRT \) or \( HES \) gene expression can serve to spatially and temporally restrict the activation of Notch-dependent signaling. In light of the well known roles of Notch signaling in diverse developmental processes, it will be especially interesting to determine which of the activities of Notch rely on \( HRT \) proteins as essential downstream mediators and to identify target genes for \( HRT \) proteins in different cell types.

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