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The *Nrarp* Gene Encodes an Ankyrin-Repeat Protein That Is Transcriptionally Regulated by the Notch Signaling Pathway

Luke T. Krebs,*,[1] Michael L. Deftos,†,[1] Michael J. Bevan,† and Thomas Gridley*,[2]

**The Jackson Laboratory, Bar Harbor, Maine 04609; and †Department of Immunology and Howard Hughes Medical Institute, University of Washington, Seattle, Washington 98195*

We have identified a gene encoding a novel protein that is transcriptionally regulated by the Notch signaling pathway in mammals. This gene, named *Nrarp* (for Notch-regulated ankyrin-repeat protein), encodes a 114 amino acid protein that has a unique amino-terminus and a carboxy-terminal domain containing two ankyrin-repeat motifs. A *Xenopus* homolog of the *Nrarp* gene was previously identified in a large-scale *in situ* hybridization screen of randomly isolated cDNA clones. We demonstrate that in T-cell and myoblast cell lines expression of the *Nrarp* gene is induced by the intracellular domain of the Notch1 protein, and that this induction is mediated by a CBF1/Su(H)/Lag-1 (CSL)-dependent pathway. During mouse embryogenesis, the *Nrarp* gene is expressed in several tissues in which cellular differentiation is regulated by the Notch signaling pathway. Expression of the *Nrarp* gene is downregulated in *Notch1* null mutant mouse embryos, indicating that expression of the *Nrarp* gene is regulated by the Notch pathway *in vivo*. Thus, *Nrarp* transcript levels are regulated by the level of *Notch1* signaling in both cultured cell lines and mouse embryos. During somitogenesis, the *Nrarp* gene is expressed in a pattern that suggests that *Nrarp* expression may play a role in the formation of somites, and *Nrarp* expression in the paraxial mesoderm is altered in several Notch pathway mutants that exhibit defects in somite formation. These observations demonstrate that the *Nrarp* gene is an evolutionarily conserved transcriptional target of the Notch signaling pathway.

Key Words: **Notch signaling pathway; downstream target gene; ankyrin repeat.**

INTRODUCTION

The Notch signaling pathway is an evolutionarily conserved intercellular signaling mechanism, and mutations in its components disrupt cell fate specification and embryonic development in organisms as diverse as insects, sea urchins, nematodes, and mammals (Artavanis-Tsakonas *et al.*, 1999). Genes of the Notch family encode large transmembrane receptors that interact with membrane-bound ligands encoded by Delta/Serrate/Jagged family genes. The signal induced by ligand binding is transmitted intracellularly by a process involving proteolytic cleavage of the receptor and nuclear translocation of the intracellular domain of the Notch protein (for recent reviews, see Kadesch, 2000; Mumm and Kopan, 2000; Weinmaster, 2000). In the nucleus, the Notch intracellular domain (Notch-IC) interacts with a sequence-specific DNA-binding protein termed CSL (for CBF1/Su(H)/Lag-1; also known as CBF1 and RBPJκ in vertebrates, Suppressor of hairless [*Su(H)*] in *Drosophila*, and Lag-1 in *Caenorhabditis elegans*). In the absence of Notch signaling, CSL binds to specific DNA sequences in the regulatory elements of various target genes and represses transcription. Following activation of the Notch pathway, nuclear Notch1-IC displaces a corepressor complex from CSL and activates transcription via its transcriptional activation domain, thereby converting CSL from a repressor to an activator of gene transcription (Hsieh *et al.*, 1996; Kao *et al.*, 1998; for reviews, see Kadesch, 2000; Mumm and Kopan, 2000). Although the majority of Notch

[1] These authors contributed equally to this work.

[2] To whom correspondence should be addressed at The Jackson Laboratory, 600 Main Street, Bar Harbor, ME 04609-1500. Fax: (207) 288-6077. E-mail: gridley@jax.org.

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signaling appears to occur via a CSL-dependent pathway, some developmental decisions in both *Drosophila* embryos and mammalian cultured cells can occur via a CSL-independent Notch signaling mechanism (Shawber *et al.*, 1996; Matsuno *et al.*, 1997; Wang *et al.*, 1997; Ligoxygakis *et al.*, 1998; Rusconi and Corbin, 1998; Nofziger *et al.*, 1999; Zecchini *et al.*, 1999).

A number of target genes whose expression appears to be transcriptionally regulated by Notch signaling in vertebrates have been identified. These include genes encoding basic helix–loop–helix transcription factors of the *Hes* (Jarriault *et al.*, 1995; Nishimura *et al.*, 1998; Kuroda *et al.*, 1999) and *Hey/Hrt* (Kokubo *et al.*, 1999; Leimeister *et al.*, 1999; Nakagawa *et al.*, 1999, 2000; Maier and Gessler, 2000) families, NFκB (Oswald *et al.*, 1998), and the locus control region of the β-globin locus (Lam and Bresnick, 1998). As part of our analysis of the role of Notch signaling in T-cell development (reviewed in Deftos and Bevan, 2000), we have been using representational difference analysis to isolate cDNA clones that are transcriptionally upregulated in thymoma cell lines overexpressing the intracellular domain of the Notch1 protein (Notch1-IC) (Deftos *et al.*, 1998, 2000). Genes induced under these conditions include the Notch pathway components *Notch1*, *Deltex*, and *Hes1*, as well as *Meltrin* β (which encodes an ADAM family metalloprotease), *Pre-T*α (a component of the pre-T-cell receptor complex), and members of the *Ifi-200* gene family (which encode nuclear proteins implicated in transcriptional regulation and cell cycle control). We describe here the cloning and analysis of another gene induced under these conditions. This gene, which we termed *Nrarp* (for Notch-regulated ankyrin-repeat protein), encodes a small protein containing two ankyrin repeats. We demonstrate that the levels of *Nrarp* transcription are regulated by Notch signaling both in tissue culture cells and in mouse embryos. We also show that the *Nrarp* expression pattern during mouse embryogenesis and the alteration of this expression pattern in Notch pathway mutants suggest a possible role for this gene in development of the central nervous system and somites.

MATERIALS AND METHODS

Cell Lines and Retrovirus Infection

The AKR1010 and AKR1 murine thymoma cell lines and the 2B4.11 T-cell hybridoma cell line were described previously (Deftos *et al.*, 1998, 2000). The C2C12 mouse myoblast cell line was obtained from American Type Culture Collection (Rockville, MD). Cell lines were cultured in DMEM containing 10% FCS, 2 mM glutamine, 25 mM HEPES, 50 mM β-mercaptoethanol, 100 U/ml penicillin, and 100 mg/ml streptomycin. Production of polyclonal cell lines expressing Notch1-IC, Notch1-IC/mM2–2, and Notch1-IC/ΔAnk using the pMI retroviral vector was previously described (Deftos *et al.*, 1998).

Identification of the Mouse Nrarp cDNA

cDNA prepared from AKR1010 and AKR1010 expressing Notch1-IC (AKR1010/Notch1-IC) was used for representational difference analysis (RDA) as previously described (Deftos *et al.*, 1998, 2000). Analysis of one of the RDA products revealed a 482-bp sequence that matched several mouse ESTs in the GenBank database but did not match any known genes. The 5′ end of the corresponding cDNA was obtained by RACE PCR using the Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA) and an oligonucleotide primer designed within the RDA fragment (5′-gaagtcactaggaagggtaccatgc-3′). A 1.6-kb amplification product that hybridized to the RDA difference product was cloned into pCR3.1 (Invitrogen, San Diego, CA) and the consensus sequence of five clones determined. The 3′ end of the cDNA was deduced from the consensus of multiple overlapping murine ESTs, several of which contained an apparent poly(A) tail. The sequence of the complete *Nrarp* cDNA (GenBank accession number AY046077), including the deduced 3′ untranslated region, was confirmed by sequence analysis of a BAC clone containing the *Nrarp* gene.

Northern and Western Blot Analyses

Preparation of RNA from cell lines and Northern blot analysis were performed as previously described (Deftos *et al.*, 1998). The *Nrarp* cDNA probe consisted of a PCR product amplified from the cloned cDNA using primers flanking the coding region (5′-gatcgcggccgcctgcggcaacatgagcca-3′ and 5′-gatcgtcgactccgggtggctatcaccggc-3′). Multiple-tissue Northern blots were obtained from Clontech. Western blot analysis was performed as previously described (Deftos *et al.*, 1998) using antisera directed against the carboxy-terminal portion of the Notch1 protein.

In Situ Hybridization

Embryos from timed matings were dissected and fixed overnight with 4% paraformaldehyde in phosphate-buffered saline (PBS). The *Nrarp in situ* probe consisted of a 1.1-kb *Eco*RI fragment, which contained 300 nucleotides encoding the carboxy-terminus of the protein and 800 nucleotides of the 3′ untranslated region. *In situ* probes for the *Mesp2* (Saga *et al.*, 1997) and *Uncx4.1* (Neidhardt *et al.*, 1997) genes were previously described.

Whole-mount *in situ* hybridization with digoxigenin-labeled antisense RNA ribobprobes was performed as previously described (Jiang *et al.*, 1998). For two-color whole-mount *in situ* hybridizations, embryos were processed according to the standard protocol and were then hybridized with both digoxigenin- and fluorescein-labeled antisense riboprobes at 70°C overnight (digoxigenin-UTP, fluorescein-12-UTP; Roche Molecular Biochemicals, Indianapolis, IN). After washing and blocking with 10% sheep serum, embryos were incubated with 1:2000 anti-digoxigenin-alkaline phosphatase (Roche) at 4°C overnight. Embryos were washed extensively with TBST (140 mM NaCl, 2.7 mM KCl, 0.1% Tween 20, 25 mM Tris–HCl, pH 8.0), then developed using NBT/BCIP (Roche) following the manufacturer's protocol. Development was stopped and embryos were incubated for 1 h in 100 mM glycine, pH 2.2. Embryos were then washed with TBST and blocked with 10% sheep serum before incubating with 1:2000 anti-fluorescein-alkaline phosphatase (Roche) at 4°C overnight. After washing with TBST, embryos were developed using INT/BCIP (Roche) according to the manufacturer's instructions. Development was terminated

and the embryos were placed through a graded series of glycerol/PBS/0.1% Tween 20 washes before taking photographs.

Mutant Mouse Strains

Official nomenclature and references for the mutant alleles used in these studies are *Lfng*^{lacZ} (Zhang and Gridley, 1998); *Lfng*^{tm1Grid}; *Notch1*^{in32} (Swiatek *et al.*, 1994); *Notch1*^{tm1Grid}; *Dll3*^{pu} (Kusumi *et al.*, 1998); and *Dll1*^{tm1Go} (Hrabé de Angelis *et al.*, 1997).

RESULTS

Identification of the Mouse Nrarp cDNA

We previously studied the effects of Notch signaling on thymocyte development (Deftos *et al.*, 1998, 2000). During these studies, we used representational difference analysis (Hubank and Schatz, 1994) to identify genes induced by *Notch1* signaling in the murine AKR1010 thymoma cell line (Deftos *et al.*, 1998, 2000). One of the cDNA fragments identified by this approach encoded a 482-bp sequence without homology to any known genes. We cloned the full-length 2.6-kb cDNA of this gene by RACE PCR and analysis of the murine EST database (Fig. 1A). Conceptual translation of this cDNA revealed an open reading frame of 114 amino acids preceded by a Kozak consensus translational start sequence. The predicted amino acid sequence consists of an amino-terminal domain that is not homologous to any defined protein domain and a carboxy-terminal domain that contains two ankyrin-repeat motifs (Fig. 1B). We named this gene *Nrarp*, for Notch-regulated ankyrin-repeat protein. A search of the GenBank database with the complete cDNA sequence of *Nrarp* revealed that its coding region is highly homologous to a cDNA previously identified in *Xenopus* (Fig. 1C). This gene (termed 5D9) was identified during a large-scale *in situ* hybridization screen of over 1700 randomly isolated cDNA clones (Gawantka *et al.*, 1998).

Nrarp Expression Is Induced by Notch1 Signaling via a CSL-Dependent Pathway

To confirm that *Nrarp* expression is induced by *Notch1* signaling, we performed Northern blot analysis of RNA isolated from three mouse T-cell lines and the C2C12 myoblast cell line following expression of Notch1-IC. An approximately 2.6-kb transcript was induced in all four cell lines, confirming that *Nrarp* expression is induced by *Notch1* signaling in multiple mammalian cell lineages (Fig. 2A). As mentioned previously, Notch signaling can occur by both CSL-dependent and CSL-independent pathways. To determine whether the induction of *Nrarp* is mediated by the CSL-dependent pathway, we determined whether mutations in Notch1-IC that disrupt its interaction with CSL affect its ability to induce *Nrarp* expression (Fig. 2B). As expected, expression of Notch1-IC strongly induced *Nrarp* expression in the AKR1010 cell line. However, expression of Notch1-IC/mM2–2, which contains mutations in the RAM domain that disrupt interaction with CSL (Tamura *et al.*, 1995), did not induce detectable *Nrarp* expression. Similarly, expression of Notch1-IC/ΔANK, which deletes the first two ankyrin repeats of Notch1-IC, did not induce *Nrarp* expression. Expression of the various Notch1-IC polypeptides was confirmed by Western blot analysis, which revealed that the mutant Notch1-IC polypeptides were expressed at higher levels than those of wildtype Notch1-IC (Fig. 2C). Together, these results demonstrate that Notch signaling induces *Nrarp* expression via a CSL-dependent pathway.

We previously showed that infection of AKR1010 cells with a Notch1-IC-expressing retroviral vector conferred dexamethasone resistance to the infected cells (Deftos *et al.*, 1998). To determine whether overexpression of the *Nrarp* gene was sufficient to confer dexamethasone resistance to these cells, AKR1010 cells were infected with a retroviral vector expressing the *Nrarp* cDNA. No dexamethasone-resistant cells were isolated under these conditions, indicating that *Nrarp* expression alone was not sufficient to confer dexamethasone resistance to AKR1010 cells (data not shown).

Nrarp Is Highly Expressed in Tissues Regulated by Notch Signaling and Is Downregulated in Notch1 Mutant Embryos

To characterize the pattern of expression of the *Nrarp* gene in adults, we performed Northern blot analysis using multiple-tissue blots containing RNA isolated from a variety of adult mouse and human tissues. This revealed expression of the 2.6-kb *Nrarp* transcript at low levels in most tissues examined, with the highest expression observed in the brain, heart, colon, kidney, liver, lung, and small intestine (Fig. 3).

We determined the spatial and temporal localization of *Nrarp* transcripts in mouse embryos by whole-mount *in situ* hybridization between 8.5 and 10.5 days of gestation (Fig. 4). *Nrarp* expression was prominent in several tissues in which cellular differentiation is regulated by the Notch signaling pathway. High levels of *Nrarp* expression were observed in the central nervous system and in the paraxial mesoderm. Whereas *Nrarp* expression was observed throughout most of the central nervous system, at E9.5 *Nrarp* expression was excluded from the midbrain region (Figs. 4B and 4C). The mesodermal expression consisted of an anterior stripe and a more extensive posterior domain (Figs. 4A, 4B, 4E, 4F; also see below). At E10.5, *Nrarp* expression was observed in the mesonephros (Fig. 4D), where expression of both the *Notch1* (Franco del Amo *et al.*, 1992) and *Jag1* genes (Mitsiadis *et al.*, 1997) was also observed. Sectioning of the whole-mount *in situ* embryos revealed that in the neural tube, *Nrarp* expression was downregulated in the floor plate and in differentiating motor neurons in the ventral horn (Figs. 4G and 4H). *Nrarp* expression also was observed in blood vessels (Fig. 4I).

A

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1     GAACTATTTTGCAAAGGGACGCGGGTGGAGTTCAGCTGGAGCGAAGCCTAACCTCGAAGGGACCACGACTGCGAGTGTCGCGGCCTCCGGCTGCCCCAGG  100
101   TTCTGCGAACCAGCACCCAGCCCTGTCGCCCGCCCGCTTGGGCGCGGCTGGATGCAGCAGGGGTCCCGCGCCAGCGGCCCTCGGCCCCGAACGCCCGGAG  200
201   TGCGCAGGGGCGGCGTGCGGGGCCCGAGGGCGCAGCGCCCTCCATGCGCCGAGGCGTACCTCGAGACACGCCCCGAGACAGCCCGGGGCCCGCACTGCAG  300
301   CCGCCGCCCGCGCTAAGCCGGGGCGCGGCTCGCAGCCCTAGCTCTGCGGCAACATGAGCCAAGCCGAGCTGTCCACCTGCTCGGCGCCACAGACGCAGCG  400
                                                    M  S  Q  A  E  L  S  T  C  S  A  P  Q  T  Q  R

401   CATCTTCCAGGAAGCGGTGCGCAAGGGCAACACGCAGGAGCTGCAGTCGCTGCTGCAGAACATGACTAACTGCGAATTCAACGTGAACTCGTTCGGGCCG  500
        I  F  Q  E  A  V  R  K  G  N  T  Q  E  L  Q  S  L  L  Q  N  M  T  N  C  E  F  N  V  N  S  F  G  P

501   GAGGGCCAGACAGCACTACACCAGTCAGTCATCGACGGCAACCTGGAGCTGGTGAAGCTGTTGGTCAAGTTCGGAGCCGACATCCGCCTAGCTAACCGCG  600
        E  G  Q  T  A  L  H  Q  S  V  I  D  G  N  L  E  L  V  K  L  L  V  K  F  G  A  D  I  R  L  A  N  R

601   ACGGCTGGAGCGCGCTACACATCGCCGCTTTCGGGGGCCACCAGGACATCGTGCTCTATCTCATCACCAAGGCCAAGTACGCGGCCAGCGGCCGGTGATA  700
        D  G  W  S  A  L  H  I  A  A  F  G  G  H  Q  D  I  V  L  Y  L  I  T  K  A  K  Y  A  A  S  G  R  *

701   GCCACCCGGACTCCGGCCCCTGGCCCCCACCCGCGTCGTCTCTGCTGTACCTTCCCGCCAACTACCTCGGTGTGCGCCAGGTTTGCTGGTCCGCGTGGAA  800
801   ATCGGGCTAAGTCTCTACGTCCGCGGCCACGACCAAAGTAGCGCATCCGTGGACCCCAGGGTTCGGCTCTCCGCAAAAGAGCGCCTCCCCTTGGCAGTCC  900
901   TCTGAGCCCCGCGGGGCCCCAGAGCCTTCCCACGGCCCCGACCGCCTAGAGGGTGGGGGCGGGGCGCAGGCTCCAGTCCGTTTTGAAATTTGAGTCTCAC 1000
1001  ACCGGGAGACTTCGGAATCCCGAGATACCGGATCCTCCGCTTGAAATGTTTTCTCCGGAAGGTGAAAGGCGCGGGCGGACTCGCGCTCCAGCCGCCGCTC 1100
1101  GTGAGACCCGGCCGCTCACAGTGCGGCGCCCGCCGCTGGCAGCCGCCAAACAACCATTACCGGGCTCCACCATCTGCGCGCCCTCGCACTTAGGAAGGGA 1200
1201  AGGGGACGCTCCGGGTTCCTGATGTCCTCAACTATTTATCACGTGTGTGTATATTTGTGGGTGAAGTTTGTGCCGCCTGAGTTTTTTGTTTGGAAATATTG 1300
1301  TGCGTGGTTATGGGAGAAAGATGCATTTTTTTTCCTTTAAAACTAAAAACTTGAGTCTACCATTTTTTGGTTGCACTGAAAATACCGCCTAGCCCTATGG 1400
1401  TTTTCCCATTGCTAGCCCCTCCCCCACCGAGTCCCTAATCTTCCACACCTCATTCCTCTTTTCCTCTCCCTGGATTCTGAGTTTAGAGAGCCTAGGATCT 1500
1501  GTCTCCCTTTCCCCTCCCGTGAGAGGCCACCATTCCCCACACAGGCTGGTTTAGCAAAGTTCCAAAGGGCTCTTGAAGCCCGCGTGGCTGGGACGTGGGG 1600
1601  ATTCTGAGAGCATGGTACCCTTCCTAGTGACTTCTATTATAGTTAATAGTCGGTTGCACACTTTTTTAAAAAAGTAAATGAATTTGCCACGATTAAATGT 1700
1701  CATAACATTTATGACAGAATATAAAATATTAACATATTTTAAGCCAAGTTTTAGGTGTATTTTTTTGAATCTTGGTTATGAACCCAATTTTAAAGGGCGT 1800
1801  TGTATCCAGCGTTGTGAAGGCTGTTGTGTACCCATATTTATATTTTTATAAAATTCCTATAAAGACTGTGAATCTCTCATACTATTGCTGAATGAGTGGA 1900
1901  AGGGCTGCTGTGCTCTTTCTGCCTTCCCCAATCCCCATCCCCACCCTCAAACCCTGCTCCAAGGCCCTATTATGATCTGAGGATCCAGCCATGAGGGAGG 2000
2001  GGCTGTGCCAAGGACCAGGGCTGGAGGAGGTGCTGGAGCGTCACTGGCTGTCCCAGAAAGGAGACTTCCTGGTTTCTGTGGTTCCAATTTTCTATGCATC 2100
2101  TCCATTCCCCTTCTTGTTTTGATCCTGGGAAATAAAAGGGAGGCTGAATTATTCAAATTTAAATGAGGTTTCCCCTTCGTAGAAGTGCTGCTGACCCTGC 2200
2201  GTGCAAAAATGGGGAGCACTTGAGGACACAGGTGGGTGGAGCCCTTTGTGCCGCCTGGCCATATTCTTCGGTCTTCTGCGCTTTTATGAACCAGTGTGGG 2300
2301  AGGAATAGTGATAATGTCATGAGAATGGCAGACACTGAGACAAGCACAGATTACTTCAAAAGGAGTTCTGTATGGTTTTTCTACACGCAAATGCCTTTTT 2400
2401  AATTATGTTAATTAATGTTAAAACGTACTGGGCCTATGTTGAAGCTTCAGATGGGTCCTGGTTGGTCTCTTCTATCTGTATGAAGTCTGTGGCACACTCC 2500
2501  TAAGCGTACGATATAGACTTGTAGCCCATCGTGAAAAATTTATAAATAAATTTTTCATTGGTCTTTTTATATTAAAAAAAAAAAAAAAA
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B

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-G-TPLHLA---G---VV-LLL--GADVNA-D-     ANK Consensus
EGQTALHQSVIDGNLELVKLLVKFGADIRLANR     Nrarp ANK A (50-82)
DGWSALHIAAFGGHQDIV*IYLITKAKYAASGR     Nrarp ANK B (83-114)
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C

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1     MSQAELSTCSAPQTQRIFQEAVRKGNTQELQSLLQNMTNCEFNVNSFGPE   Mouse
      -----M----M-H---V------N---K--H--------------------   Xenopus

51    GQTALHQSVIDGNLELVKLLVKFGADIRLANRDGWSALHIAAFGGHQDIV
      ----------------------------------------Y-------

101   LYLITKAKYAASGR  114
      ---------SS-S-
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FIG. 1. The *Nrarp* gene encodes an ankyrin repeat-containing protein. (A) Complete nucleotide sequence and deduced amino acid sequence of the mouse *Nrarp* cDNA. The two ankyrin-repeat motifs are boxed and the polyadenylation signal is underlined. The GenBank accession number for the mouse *Nrarp* cDNA sequence is AY046077. (B) Comparison of the ankyrin-repeat motifs of mouse *Nrarp* with the consensus ankyrin-repeat sequence (Sedgwick and Smerdon, 1999). ANK A and ANK B refer to the first and second ankyrin repeats of the Nrarp protein. (C) Comparison of mouse and *Xenopus* Nrarp amino acid sequences. For the *Xenopus* sequence, amino acids identical to the mouse sequence are indicated by a dash (–).

Given that expression of the *Nrarp* gene was induced by overexpression of Notch1-IC in both T-cell and myoblast cell lines, we hypothesized that *Nrarp* expression might be downregulated in *Notch1* loss-of-function mutants. We therefore analyzed *Nrarp* expression in mouse embryos homozygous for a null mutation in the *Notch1* gene (Swiatek *et al.*, 1994) and found that *Nrarp* expression was downregulated in the *Notch1* mutant embryos (Fig. 5). *Nrarp* expression was undetectable in the paraxial mesoderm of *Notch1* mutant embryos. Some *Nrarp* expression

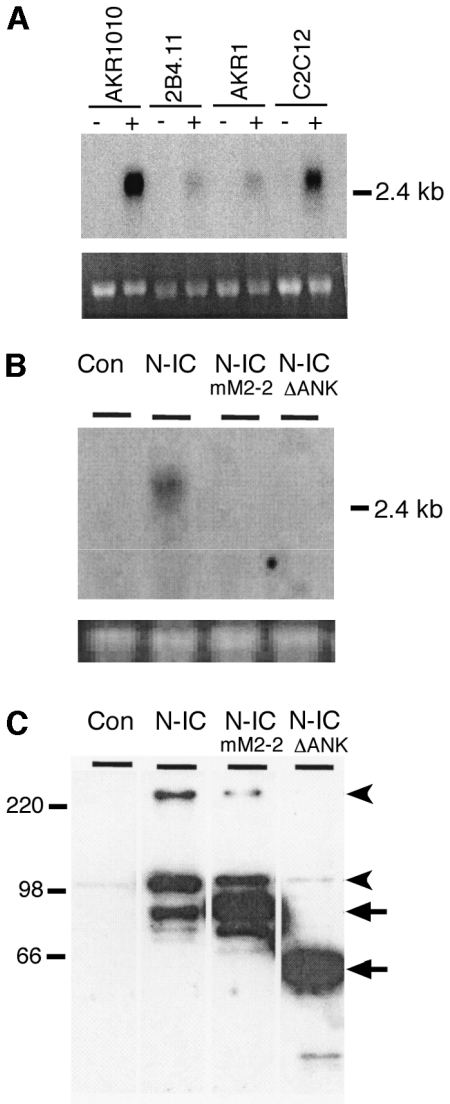


FIG. 2. *Nrarp* expression is induced by Notch1-IC in T-cell and myoblast cell lines via a CSL-dependent pathway. (A) Northern blot analysis of *Nrarp* expression in three T-cell lines and the C2C12 myoblast cell line following expression of Notch1-IC. RNA prepared from the parental cell lines and their Notch1-IC-expressing derivatives was analyzed by Northern blot using a *Nrarp* probe. Equivalent loading was confirmed by visualization of 28S ribosomal RNA. (B) Northern blot analysis of *Nrarp* expression in AKR1010 following expression of mutant forms of Notch1-IC. The exposure time for this blot was shorter than that for A. (C) Western blot analysis of wild type and mutant Notch1-IC polypeptides. Lysates from AKR1010 cells expressing the various Notch1-IC constructs were examined by Western blot using a polyclonal antibody directed against the carboxy-terminal portion of Notch1. The arrows indicate the various Notch1-IC constructs and the arrowheads indicated full-length and proteoytically processed forms of endogenous Notch1 induced by Notch1-IC.

was observed in the neural tube of the *Notch1* mutant embryos, although expression was less intense than that in either heterozygous (Fig. 5A) or wild type (not shown) littermates. These data demonstrate that *Nrarp* transcript levels are responsive to the levels of *Notch1* signaling during early mouse embryogenesis.

Localization of Nrarp Transcripts during Somitogenesis and Expression in Notch Pathway Mutants Exhibiting Defects in Somitogenesis

In the paraxial mesoderm, the *Nrarp* gene was expressed in two domains. The anterior domain consisted of a narrow stripe of *Nrarp* expression. Caudal to this stripe was a region devoid of *Nrarp* expression. Caudal to this nonexpressing region, *Nrarp* expression resumed and was maintained through the posterior end of the paraxial mesoderm. However, examination of the *Nrarp* expression pattern in a large number of similarly staged embryos did not reveal an obviously oscillating pattern of expression, similar to that observed for the chicken Hairy1 gene (Palmeirim *et al.*, 1997) or the chicken and mouse Lunatic fringe (*Lfng*) genes (Forsberg *et al.*, 1998; McGrew *et al.*, 1998; Aulehla and Johnson, 1999).

We were interested in determining the positions of the *Nrarp* mesodermal expression domains relative to the positions of previously characterized genes expressed in the paraxial mesoderm. We therefore performed two-color *in situ* hybridization with antisense riboprobes for *Nrarp* and either *Mesp2* or *Uncx4.1* (Fig. 6). The *Uncx4.1* gene encodes a paired-related homeobox protein that is expressed in the caudal compartment of the formed somite but is not expressed in presomitic mesoderm (Mansouri *et al.*, 1997; Neidhardt *et al.*, 1997). The *Mesp2* gene encodes a basic helix–loop–helix protein that is expressed in a band in the rostral presomitic mesoderm (Saga *et al.*, 1997; Takahashi *et al.*, 2000). This band of *Mesp2* expression demarcates the anterior half of the second presumptive somite in the presomitic mesoderm. The two-color analysis revealed that the anterior stripe of *Nrarp* expression coincided with the most caudal *Uncx4.1* stripe, indicating that this stripe of *Nrarp* expression marks the most recently formed somite (Figs. 6B and 6C). The anterior boundary of the caudal *Nrarp* expression domain coincided with the domain of *Mesp2* expression in the rostral presomitic mesoderm (Fig. 6D).

Because expression of the *Nrarp* gene suggested that it might be involved in regulating formation of somites, we also examined *Nrarp* expression in several Notch pathway mutants that exhibit defects in somite formation. We analyzed expression of *Nrarp* at E9.5 in embryos homozygous for mutations in the *Dll1* (Hrabé de Angelis *et al.*, 1997), *Dll3* (Kusumi *et al.*, 1998), and *Lfng* (Zhang and Gridley, 1998) genes. This analysis revealed that *Nrarp* expression was altered in different ways in each one of these mutants. Unlike what we observed in *Notch1* mutant embryos, none of these mutants exhibited downregulation

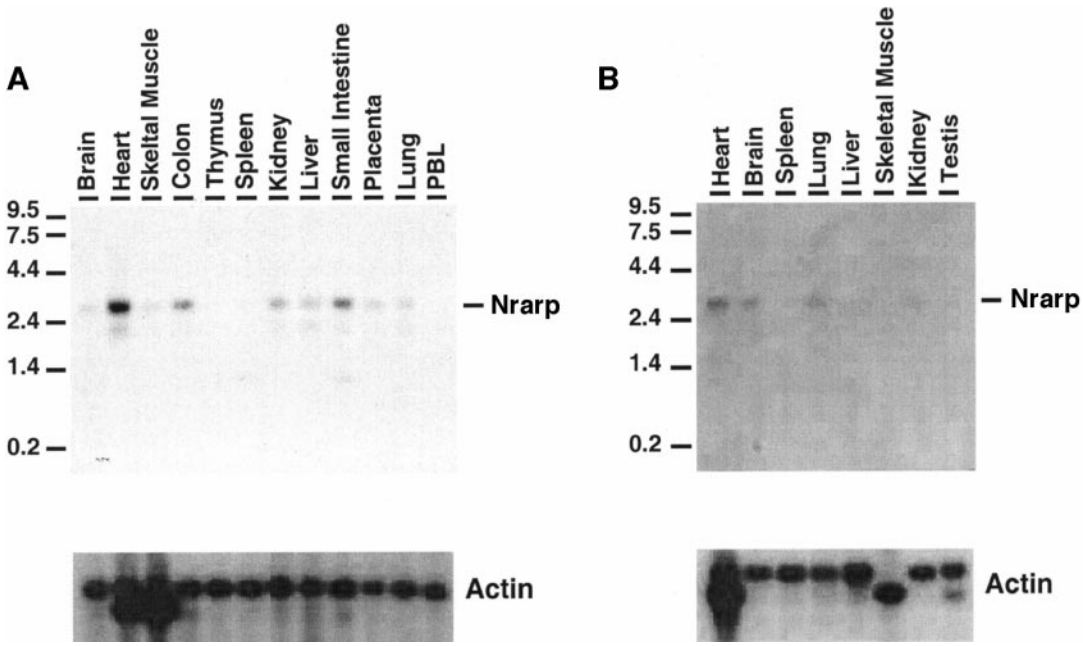


FIG. 3. The *Nrarp* gene is widely expressed in mouse and human tissues. Multiple-tissue Northern blots of human (A) and mouse (B) tissues were hybridized with a probe corresponding to the coding region of mouse *Nrarp*. To control for RNA integrity and loading, the blots were stripped and reprobed with a human β-actin probe.

of *Nrarp* expression in the central nervous system. However, each of the mutants exhibited alterations in *Nrarp* expression in the paraxial mesoderm. In *Dll1* mutant embryos, all *Nrarp* expression in the paraxial mesoderm was downregulated, similar to what was observed in the paraxial mesoderm of the *Notch1* mutant embryos (Fig. 7B). In *Lfng* mutant embryos, the anterior stripe of *Nrarp* expression was downregulated, although the posterior expression domain was unaffected (Fig. 7C). In $Dll3^{pu}$ mutant embryos, both the anterior and posterior expression domains were present, although *Nrarp* expression in the anterior stripe was diffuse and the stripe was expanded (Fig. 7D).

DISCUSSION

We report here the cloning and analysis of a gene encoding a novel protein, whose expression is regulated by the Notch signaling pathway in both tissue culture cell lines and in mouse embryos. The *Xenopus* homolog of this gene was originally identified by Niehrs and colleagues during a large-scale *in situ* hybridization screen of over 1700 randomly isolated cDNA clones (Gawantka *et al.*, 1998). They referred to this clone as 5D9; in this study, we refer to this gene as *Xenopus Nrarp*.

Nrarp Expression Is Regulated by Notch Signaling

We used representational difference analysis to clone a number of cDNAs, whose expression is induced by Notch1-IC in the thymoma cell line AKR1010 (Deftos *et al.*, 1998, 2000). In addition to *Nrarp*, other genes induced under these conditions include *Deltex*, *Hes1*, *Meltrin* β, *Pre-T*α, and members of the *Ifi-200* gene family. Our expression analysis demonstrates that, during early mouse embryogenesis, the *Nrarp* gene is expressed in a number of tissues in which differentiation is regulated by the Notch pathway, including the central nervous system, the somites, and the presomitic mesoderm. *Nrarp* was also expressed in other tissues in which a role for Notch signaling has not been demonstrated but which express other components of the Notch pathway. For example, *Nrarp* is expressed in the mesonephric tubules, which also express the *Notch1* and *Jag1* genes. Interestingly, in their original screen Gawantka *et al.* (1998) noted that the 5D9 cDNA (*Xenopus Nrarp*) was expressed in a pattern similar to that of other genes involved in the Notch signaling pathway, including *Xenopus Delta1* and three *Hes*-family genes. Our analysis of *Notch1* mutant embryos revealed that *Nrarp* transcription was downregulated. Thus, *Nrarp* transcript levels appear to be regulated by the level of *Notch1* expression in both T-cell and myoblast tissue culture cell lines and mouse embryos.

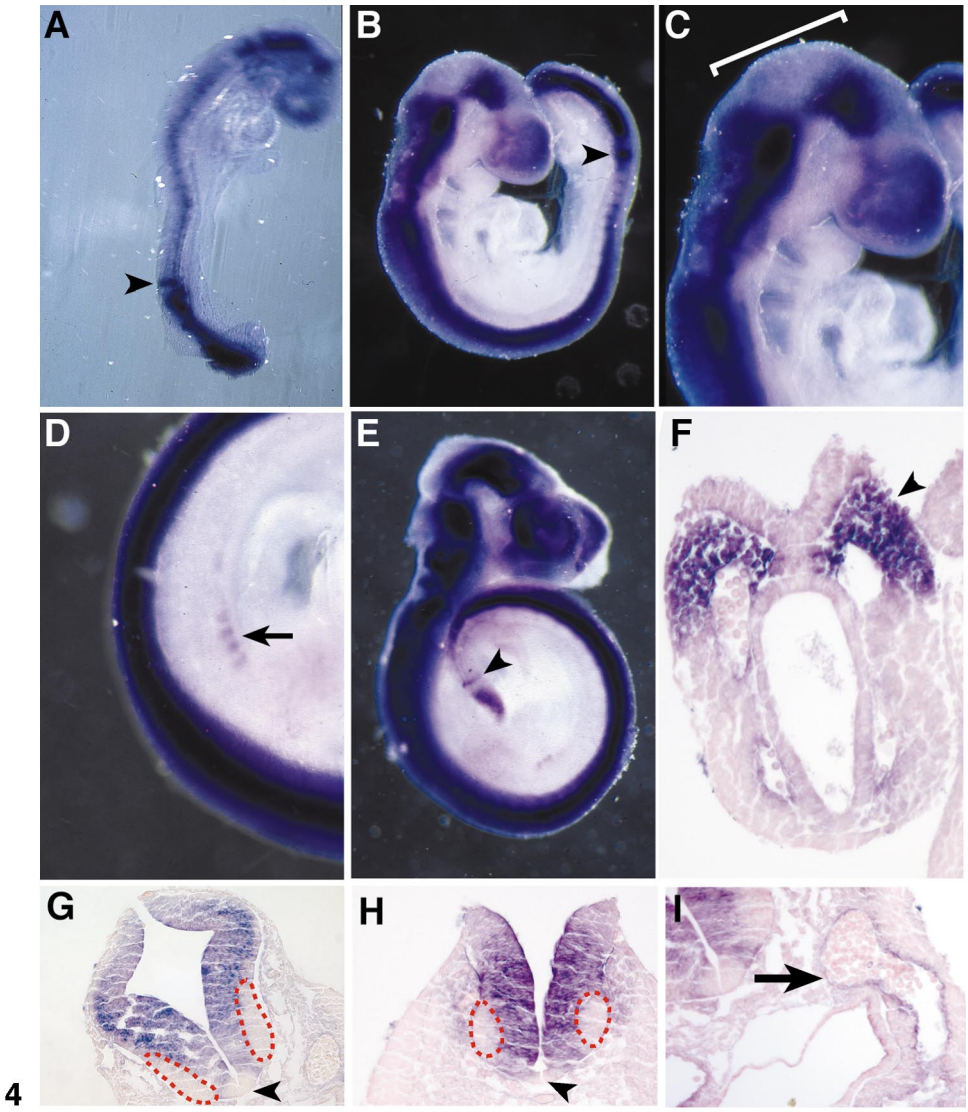
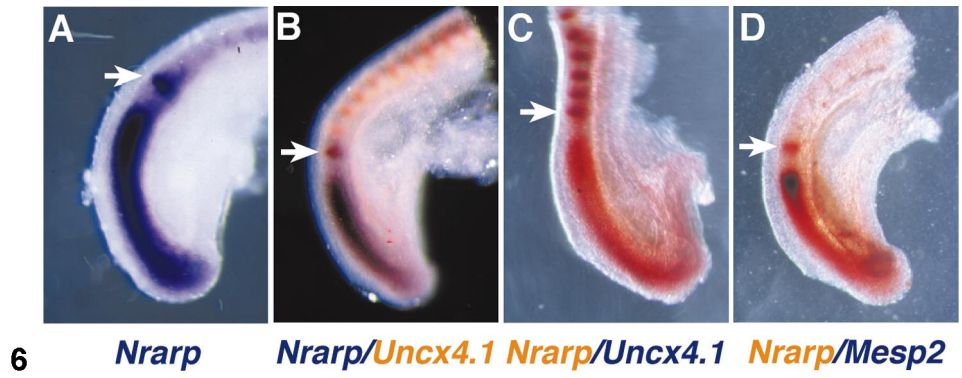


FIG. 4. Spatial localization of *Nrarp* RNA expression. (A–E) Whole-mount *in situ* hybridization with a *Nrarp* riboprobe. (F–I) Sections of the whole-mount *in situ* embryos: (A) E8.75; (B, C) E9.5; (D–I) E10.5. Two major domains of expression were observed in the central nervous system and in the paraxial mesoderm. The mesodermal expression consisted of an anterior stripe (arrowheads in A, B, and E) and a posterior domain. (C) In the central nervous system at E9.5, Nrarp expression was not observed in the midbrain region (bracket). (D) Expression was observed in mesonephric tubules (arrow). (F) Expression was observed in the paraxial mesoderm (arrowhead). (G, H) In the neural tube at E10.5, the *Nrarp* gene was not expressed in the floor plate (arrowhead) or in differentiating motor neurons in the ventral horn (circled in red). (I) The *Nrarp* gene was expressed in blood vessels, such as the anterior cardinal vein (arrow).

FIG. 5. *Nrarp* expression is downregulated in *Notch1* homozygous mutant embryos. Whole-mount *in situ* hybridization with a *Nrarp* riboprobe of embryos isolated at E9.0. (A) *Notch1* heterozygous embryo. (B, C) Two *Notch1* homozygous mutant embryos. *Nrarp* expression is downregulated throughout the mutant embryos. Some *Nrarp* expression is retained in the neural tube, particularly in the hindbrain region (asterisk). *Nrarp* expression is completely downregulated in the paraxial mesoderm (arrowhead) of the homozygous mutant embryos.



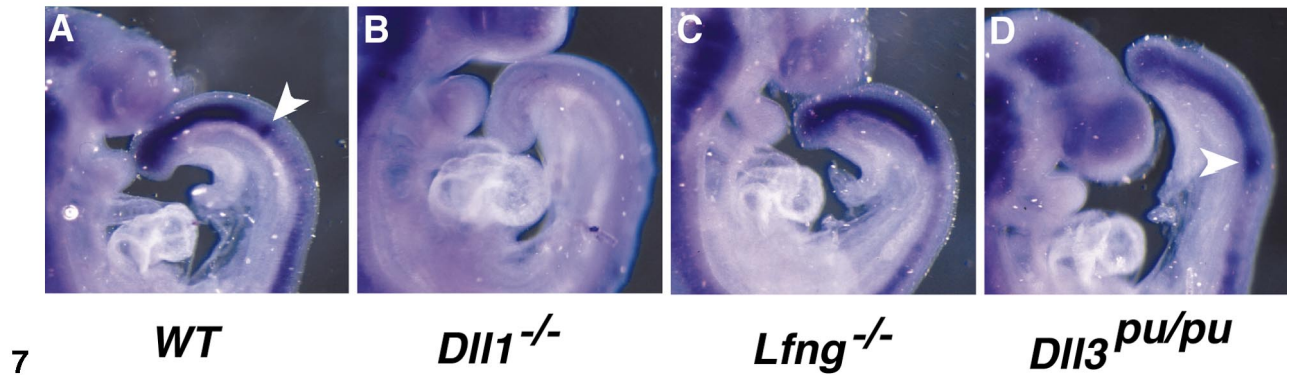


FIG. 6. Localization of *Nrarp* expression boundaries in the paraxial mesoderm of wild type embryos at E9.5. Double-label whole-mount *in situ* hybridization with riboprobes for *Nrarp* (A–D) and either *Uncx4.1* (B, C) or *Mesp2* (D). Beneath each panel, the digoxigenin-labeled probe is indicated in blue and the fluorescein-labeled probe is indicated in orange. In all panels, the white arrow indicates the anterior stripe of *Nrarp* expression. (B, C) *Nrarp/Uncx4.1* comparison. The anterior stripe of *Nrarp* expression coincided with the most caudal *Uncx4.1* stripe, indicating that this stripe of *Nrarp* expression marks the most recently formed somite. (D) *Nrarp/Mesp2* comparison. The anterior boundary of the caudal *Nrarp* expression domain coincided with the domain of *Mesp2* expression in the rostral presomitic mesoderm, which demarcates the anterior half of the second presumptive somite.

FIG. 7. *Nrarp* expression is altered in the paraxial mesoderm of Notch pathway mutants exhibiting defects in somitogenesis. (A) Wild type control embryo, exhibiting the two expression domains in the paraxial mesoderm: the anterior stripe (arrowhead) and the posterior domain. (B) *Dll1* homozygous mutant embryo. All *Nrarp* expression in the paraxial mesoderm was downregulated. (C) *Lfng* homozygous mutant embryo. The anterior stripe of *Nrarp* expression was downregulated, although the posterior expression domain was unaffected. (D) *Dll3*^{pu} homozygous mutant embryo. Both the anterior and posterior expression domains were present, but *Nrarp* expression in the anterior stripe was diffuse and the stripe domain was expanded (arrowhead). Embryos were isolated at E9.5.

Nrarp Expression during Somite Formation

Numerous studies have demonstrated that the Notch signaling pathway is involved in regulating somite formation and in partitioning somites into anterior and posterior compartments (reviewed in Maroto and Pourquié, 2001). In the paraxial mesoderm, the *Nrarp* gene is expressed in two domains: an anterior stripe, and a large posterior domain that extends to the caudal end of the embryo. Two-color *in situ* hybridization comparing *Nrarp* expression with the expression of the *Uncx4.1* gene demonstrated that the anterior stripe of *Nrarp* expression coincides with the most caudal *Uncx4.1* stripe, indicating that this *Nrarp* stripe is expressed in the posterior compartment of the most recently formed somite. Comparison with the pattern of *Mesp2* expression demonstrated that the anterior boundary of the caudal *Nrarp* expression domain coincided with the domain of *Mesp2* expression in the unsegmented paraxial mesoderm. Therefore, the *Nrarp* gene is expressed in sites consistent with the hypothesis that the *Nrarp* protein may play an important role in somite formation. Consistent with this idea, we found that expression of the *Nrarp* gene was altered in several Notch pathway mutants that exhibit defects in somite formation. These include mutants for the *Notch1, Dll1, Dll3,* and *Lfng* genes. Interestingly, all of these mutants exhibited distinct patterns of *Nrarp* expression.

Possible Functions of the Nrarp Protein

The *Nrarp* gene encodes a small protein containing two carboxy-terminal ankyrin repeats. The ankyrin repeat is one of the most common protein-sequence motifs and has been demonstrated to be a domain involved in mediating protein–protein interactions (Sedgwick and Smerdon, 1999). Orr-Weaver and colleagues cloned and analyzed a *Drosophila* gene encoding a similar two ankyrin repeat-containing protein. This protein is the product of the plutonium (*plu*) gene, which is required not only for the inhibition of DNA replication following the completion of meiosis but also for regulation of the oscillation of the S and M phases during the cell cycle (Axton *et al.*, 1994; Elfring *et al.*, 1997). The Nrarp and Plu proteins do not appear to be homologous, in that they show similarity only in evolutionarily conserved residues in the ankyrin-repeat domains. However, consideration of the biochemical properties of the Plu protein may give insight into possible roles of the Nrarp protein. Genetic analysis has shown that the pan gu (*png*) gene is also required for control of the same cell cycle processes as are regulated by the *plu* gene (Shamanski and Orr-Weaver, 1991). The *png* gene encodes a novel Serine/Threonine protein kinase (Fenger *et al.*, 2000). Biochemical analyses demonstrate that the Plu protein is found in a complex with Png protein. However, yeast two-hybrid studies indicate that the Plu and Png proteins do not bind directly to each other but, rather, may interact through a third molecule (Fenger *et al.*, 2000). The identity of this adapter molecule is currently unknown.

Given the small size of the Nrarp protein and the absence of any other conserved protein-sequence motifs, we hypothesize that the Nrarp protein functions via protein–protein interactions. It is possible that the Nrarp protein serves as an adapter molecule, holding different proteins together. Alternatively, the Nrarp protein might be a positive or negative regulator of proteins to which it binds. Further work will be required to identify proteins that bind to the Nrarp protein and to distinguish between these possibilities.

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