MOLECULAR AND CELLULAR BIOLOGY, June 1996, p. 2670–2677 Vol. 16, No. 6
0270-7306/96/$04.00+0

The WRPW Motif of the Hairy-Related Basic Helix-Loop-Helix Repressor Proteins Acts as a 4-Amino-Acid Transcription Repression and Protein-Protein Interaction Domain

ALFRED L. FISHER,[1] SHUNJI OHSAKO,[1,2] AND MICHAEL CAUDY[1]*

Department of Cell Biology and Anatomy, Cornell University Medical College, New York, New York 10021,[1] and Department of Cell Biology, Tokyo Metropolitan Institute for Neuroscience, Tokyo 183, Japan[2]

Received 11 January 1996/Returned for modification 21 February 1996/Accepted 8 March 1996

Hairy-related proteins include the *Drosophila* Hairy and Enhancer of Split proteins and mammalian Hes proteins. These proteins are basic helix-loop-helix (bHLH) transcriptional repressors that control cell fate decisions such as neurogenesis or myogenesis in both *Drosophila melanogaster* and mammals. Hairy-related proteins are site-specific DNA-binding proteins defined by the presence of both a repressor-specific bHLH DNA binding domain and a carboxyl-terminal WRPW (Trp-Arg-Pro-Trp) motif. These proteins act as repressors by binding to DNA sites in target gene promoters and not by interfering with activator proteins, indicating that these proteins are active repressors which should therefore have specific repression domains. Here we show the WRPW motif to be a functional transcriptional repression domain sufficient to confer active repression to Hairy-related proteins or a heterologous DNA-binding protein, Gal4. This motif was previously shown to be necessary for interactions with Groucho, a genetically defined corepressor for *Drosophila* Hairy-related proteins. Here we show that the WRPW motif is sufficient to recruit Groucho or the TLE mammalian homologs to target gene promoters. We also show that Groucho and TLE proteins actively repress transcription when directly bound to a target gene promoter and identify a novel, highly conserved transcriptional repression domain in these proteins. These results directly demonstrate that Groucho family proteins are active transcriptional corepressors for Hairy-related proteins and are recruited by the 4-amino acid protein-protein interaction domain, WRPW.

Basic helix-loop-helix (bHLH) transcription factors control cell fate decisions, such as myogenesis or neurogenesis, in many animal species (9–11, 21, 33, 34, 38, 60, 80, 81). These proteins can be classified into two groups, the activator bHLH proteins and the repressor bHLH proteins, on the basis of biological function (56). Remarkably, the DNA binding specificities of the activator and repressor bHLH proteins directly correlate with their biological functions (56). The activator proteins, such as MyoD or the proteins of the Achaete-Scute complex, promote differentiation by binding to class A binding sites and activating transcription (21, 50, 52, 53, 56, 77, 81). The repressor bHLH proteins are Hairy-related proteins such as Hairy, the proteins of the Enhancer of Split [E(spl)] complex, and the homologous mammalian Hes proteins (2, 19, 23, 32, 41, 43, 56, 61, 62, 70, 71). These proteins antagonize the activator proteins and prevent differentiation by binding to specific class B or C sites and repressing transcription (2, 32, 54, 56, 70, 73, 75). Thus, Hairy-related proteins are distinct from the emc and Id HLH repressors, which lack basic regions and repress by forming non-DNA-binding heterodimers with the activator bHLH proteins (7, 17, 22, 25, 76, 77).

Drosophila neurogenesis is regulated by both activator and repressor bHLH genes (9–11, 33, 34, 38). The activators are proneural genes (33) and include *daughterless* (13), the four genes of the *achaete-scute* complex (3, 78), and *atonal* (35). Heterodimers between Daughterless and Achaete-Scute proteins bind and activate the transcription of target genes such as *achaete* (50, 76, 77). The repressors are Hairy-related proteins, such as Hairy or the proteins of the E(spl) complex (19, 41, 43, 61). The repressors act to prevent neuronal development at two levels. Hairy acts as a prepattern gene (33) that ensures that proneural clusters arise in the correct locations by restricting the global pattern of expression of proneural genes such as *achaete* (8, 16, 66). Subsequently the Enhancer of Split proteins mediate lateral inhibition within a proneural cluster as part of the Notch signaling pathway to locally repress neurogenesis in all but one or two cells within the proneural equivalence group (20, 42, 54, 65).

The control of cell fate decisions in mammals is under similar control by opposing activator and repressor bHLH proteins. Muscle development is controlled by members of the activator bHLH MyoD family (21, 60, 80, 81). Forced expression of MyoD family members in many cell lines in culture has been shown to commit these cells to the myogenic lineage (21, 60, 80, 81). In addition, mice lacking specific combinations of the MyoD family members have been shown to have early defects in myogenesis (60, 80). In contrast, the Hairy-related protein Hes-1 can prevent differentiation by MyoD in cell culture, as expression of Hes-1 along with MyoD in fibroblasts prevents the myogenic conversion that would normally occur with MyoD expression alone (2). Mammalian neurogenesis has also been shown to be under control of activator and repressor bHLH proteins. The activator bHLH protein Mash-1 is expressed in the developing mammalian nervous system (26, 48) and is essential for the development of olfactory and autonomic neurons in the mouse (27). In contrast, forced expression of Hes-1 in the central nervous system by use of a retroviral expression vector disrupts neurogenesis in the infected cells (31).

In both *Drosophila melanogaster* and mammals, some Hairy-related proteins control cell fate decisions by acting as part of the Notch signaling pathway (42, 47). While it is well established that the *Enhancer of Split* genes act downstream of

* Corresponding author. Phone: (212) 746-6160. Fax: (212) 746-8175.

Notch and are essential for Notch function in *D. melanogaster* (42, 47, 58, 69), recent work has further defined the link between Enhancer of Split and Notch in both *D. melanogaster* and mammals. Several of the *Enhancer of Split* genes have been shown to be direct targets for the Suppressor of Hairless protein (6, 46), which is a DNA-binding protein that associates with the intracellular domain of Notch and becomes activated following Notch activation (24). Once activated, the Suppressor of Hairless protein stimulates the transcription of the *Enhancer of Split* genes (6, 46). Similarly, the promoter of the mammalian *Hes-1* gene has been shown to be activated in cells by expression of an activated Notch homolog from mammals (36). This promoter contains sites for the mammalian homolog of the Suppressor of Hairless protein, KBF2/RBP-J_κ (36). Taken together, these results for both *D. melanogaster* and mammals suggest that the *E(spl)* and *Hes* genes are the major, and possibly the only, direct nuclear targets which mediate Notch signaling.

Because Hairy-related proteins repress transcription by binding to specific hexameric sites and not by competing with activator proteins for binding sites (2, 56, 71, 75), they function as active repressors and therefore should have specific repression domains (15, 37, 56). We previously hypothesized that the WRPW (Trp-Arg-Pro-Trp) motif might function as a transcriptional repression domain because this motif is found at the carboxyl termini of all of the repressors and none of the activators (56). The importance of the WRPW motif was initially shown by Wainwright and Ish-Horowicz, who sequenced *hairy* alleles and found two separate mutations which specifically affect the WRPW motif (79). Subsequently the motif was shown to be required for interactions with the WD40 repeat protein Groucho both in vivo and in vitro (57). *groucho* was also shown to function genetically as a repressor in developmental pathways regulated by Hairy-related proteins (20, 57, 65). In addition to having a role in neurogenesis, *groucho* was shown to be required for the proper function of the Hairy-related proteins Hairy and Deadpan in segmentation and sex determination, respectively (57). This observation led to the proposal that Groucho is a corepressor recruited to DNA by DNA-bound Hairy-related proteins (57).

In this study, we directly test the hypotheses that the WRPW is the repression domain for Hairy-related proteins and that Groucho is a transcriptional corepressor that is recruited by Hairy-related proteins. We find that the repression domains of Hairy-related proteins map to the WRPW motif found at the carboxyl termini of the all of the family members. We then show that this motif is both necessary and sufficient to form protein-protein interactions with Groucho and the TLE mammalian homologs of Groucho. Finally, we show that both Groucho and the TLE proteins are corepressors with a novel amino-terminal repression domain and are able to repress transcription when directly bound to DNA in the absence of binding by Hairy-related proteins.

MATERIALS AND METHODS

Plasmids. All expression vectors were based on plasmid pAct5CPPA (28). Plasmids pA*cda*, pA*csc*, and pA*cac* were generously provided by M. Van Doren and J. Posakony (77). pA*ch* was previously described (56). Gal4 fusion constructs for Schneider cells were based on pActGal4 NS or pActGal4 ΔKpn (29), generously provided by J. Colgan and J. Manley (Columbia University, New York, N.Y.). All expression constructs were made by using restrictions sites or in one case PCR and in another case site-directed mutagenesis. The details of how each plasmid was constructed are available upon request. pActGal4 H constructs were made from pA*ch* or BSD2E6 (56). pActGal4 Hes-1 constructs were made from a full-length Hes-1 cDNA provided by John Feder and Yuh Nung Jan (University of California at San Francisco) (23). pActGal4 Gro constructs were made from NB5 *groucho*, a full-length *groucho* cDNA, provided by Christos Delidakis and Spyros Artavanis-Tsakonas (Yale University).

HeLa expression constructs were made by using the pCDNAIII vector (Invitrogen), which uses the cytomegalovirus promoter. pCDNAIII Gal4 was made by subcloning the Gal4 DNA binding domain from pActGal4 as a *Hin*dIII-*Bgl*II fragment into *Hin*dIII-*Bam*HI-digested pCDNAIII. pCDNAIII H267-337, Gro 1-719, and Gro 1-264 were made by subcloning *Bam*HI-*Kpn*I fragments from the appropriate pActGal4 plasmid into *Bam*HI-*Kpn*I-digested pCDNAIII Gal4. pCDNAIII Gal4WRPW was made by subcloning a *Hin*dIII-*Bgl*II fragment from pActGal4 WRPW into *Hin*dIII-*Bam*HI-digested pCDNAIII. pCDNAIII Gal4 TLE1 1-770 and TLE1 1-400 were made from a TLE1 cDNA provided by S. Stefani (Montreal Neurological Institute) in Bluescript (68). The TLE1 cDNA was digested with *Ban*II, blunted with T4 DNA polymerase, and digested with *Not*I. The resulting fragment was subcloned into pGex4T-1 digested with *Sma*I and *Not*I. This plasmid was digested with *Bam*HI and *Not*I, and the resulting fragment was subcloned into pCDNAIII Gal4 to produce pCDNAIII Gal4 TLE1 1-400. The TLE 1-770 version was made by inserting a *Not*I fragment from the TLE1 cDNA into pCDNAIII Gal4 TLE 1-400.

Reporter plasmids were based on the pGL2 vectors (Promega) which encode the luciferase reporter gene. The reporter pT5-0.9 5X/luc, which contains five Gal4 binding sites inserted into the mutated Hairy binding site of pT5-0.9 mut/luc (56), was made by inserting a PCR-generated DNA fragment containing the five Gal4 binding sites into the *Sma* site of pT5-0.9 mut/luc. The reporter 5× Gal4 Promoter was provided by C. Abate (UMDNJ-Robert Wood Johnson Medical School, Piscataway, N.J.) (12).

Glutathione *S*-transferase (GST) fusion protein expression constructs were made from the pGex4T plasmids (Pharmacia). pGexH267-337, pGex m7 142-186, pGex Hes-1 193-281, pGexWRPW, and pGexH267-333 were all made by digesting the appropriate pActGal4 plasmid with *Bam*HI and *Bgl*II and subcloning the *Bam*HI-*Bgl*II fragments into pGex4T-2 digested with *Bam*HI.

DNA transfection and transient expression assay. Transient transfection of Schneider L2 cells and luciferase activity assays were performed as previously described (56) except that 1 μg of pActGal4 expression construct was used and the cells were lysed in 100 μl instead of 250 μl of cell lysis buffer.

Pairs of HeLa cell plates were transfected with 5 μg of expression construct, 5 μg of reporter, 1 μg of pCMV-beta-galactosidase internal control, and 9 μg of Bluescript carrier by means of calcium phosphate precipitation by a standard protocol (4). After 20 h, the precipitates were removed and the medium was changed. The next day, luciferase activity was assayed as described above.

Yeast two-hybrid analysis. Yeast two-hybrid analysis was performed with the Clontech Match-maker yeast two-hybrid system as instructed by the manufacturer. The appropriate fragments were subcloned from pActGal4 into pGBT9, which encodes the Gal4 DNA binding domain, and Groucho was subcloned from pActGal4 Gro into pGAD424, which encodes the Gal4 activation domain. *Saccharomyces cerevisiae* SFY526 was transformed with the plasmids and selected on appropriate media. At least two independent colonies were tested for β-galactosidase activity with 5-bromo-4-chloro-3-indolylphosphate-β-D-galactopyranoside (X-Gal). A score of +++ was defined as the activity from yeast cells expressing pGBT9 H 267-337 and pGAD424 Gro. Additional details are available upon request.

Protein preparation. GST proteins were expressed in *Escherichia coli* DE3 pLysS (Novagen). A 5-ml overnight culture was grown in LB with ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml). The overnight culture was added to 500 ml of medium with antibiotics and grown until the optical density was 0.6. The culture was then transferred to a room temperature shaker, and isopropylthiogalactopyranoside (IPTG) was added to 0.4 mM. The bacteria were induced for 2.5 h before being pelleted and resuspended in 8 ml of NETN (20 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) and freeze-thawed three times. A brief sonication was used to reduce the viscosity, and the sonic extract was clarified by centrifugation at 12,000 × *g* for 10 min. To the clarified sonic extract, 1 ml of a 1:1 slurry of glutathione-Sepharose beads (Pharmacia) in NETN with 0.5% powdered milk was added. The mixture was rotated in the cold for 30 min before the beads were pelleted and washed three times with 12 ml of NETN. Proteins were eluted in 1 ml of elute buffer (100 mM Tris [pH 8.0], 120 mM NaCl) with 8 mg of reduced glutathione per ml. The eluted protein was dialyzed against 50 mM Tris (pH 8.0)–100 mM NaCl–1 mM EDTA buffer. The expression and quantity of proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Coprecipitation and Western blotting (immunoblotting). Interaction assays were performed by immobilizing 20 μg of GST fusion protein on glutathione beads and adding 1 mg of cell lysate prepared with phosphate-buffered saline (PBS) with 0.5% Triton X-100 and 800 μl of binding buffer (20 mM Tris [pH 8.0], 50 mM KCl, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 5 mg of bovine serum albumin per ml). After 12 h at 4°C, the beads were washed three times with 1 ml of PBS before being boiled in sample buffer and subjected to SDS-PAGE followed by Western blotting. The cell lysate lanes contained 125 and 100 μg of cell lysate for the Groucho Western and TLE Western blots, respectively.

The anti-Groucho monoclonal antibody was generously provided by S. Stefani (Montreal Neurological Institute) (67). The antibody was used at a dilution of 1:20 and followed by an anti-mouse alkaline phosphatase conjugate (Promega). The pan-TLE monoclonal antibody c597.4A (68) was generously provided by S. Stefani. The antibody was used at a dilution of 1:20 and followed by an anti-rat alkaline phosphatase conjugate (Promega). The bands were visualized using with

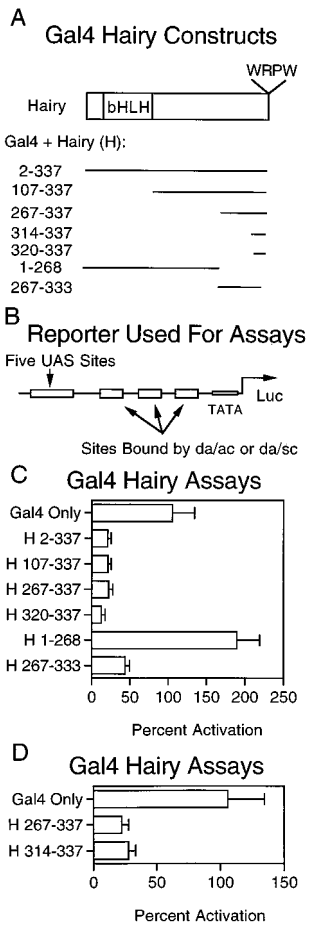

FIG. 1. Mapping of the transcriptional repression domain of the bHLH repressor protein Hairy. (A) Expression constructs encoding the Gal4 DNA binding domain fused to various full-length or truncated Hairy proteins were made in the vector pActGal4 for expression in Schneider cells. (B) The reporter consisted of a modified *achaete* (ac) promoter, in which five UAS sites have been substituted for the single Hairy binding (class C) site present in the native promoter. *achaete* was used as the reporter for all *Drosophila* Schneider cell transfection experiments because it is a well-characterized in vivo target for Hairy. da, Daughterless; sc, Scute. (C) *Drosophila* Schneider cells were transfected with the reporter and plasmids encoding Daughterless, Achaete, and the indicated Gal4 fusion protein. The activity of the reporter when activated by Daughterless and Achaete alone is defined as 100% activity. Each bar represents the average of at least three pairs of trials, and each error bar represents the standard deviation of the trials. (D) Results for activation of the *achaete* reporter by expression of Daughterless and Scute proteins were similar to those for activation by Daughterless and Achaete protein. One hundred percent activity is defined as the activity seen when the reporter is activated by Daughterless and Scute alone. The standard deviation seen for activation by either Daughterless and Achaete (C) or Daughterless and Scute (D) was about 20% (not shown).

nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate toluidinium as instructed by the manufacturer.

RESULTS

Function of the WRPW motif as the repression domain for Hairy-related proteins. To directly test whether the WRPW motif is a transcriptional repression domain, we made expression constructs encoding proteins with the Gal4 DNA binding domain fused to various full-length or truncated Hairy proteins (Fig. 1A). These fusion proteins were tested for transcriptional repressor activity by using a modified *achaete* promoter as the reporter (Fig. 1B), as the *achaete* promoter is a well-defined in vivo target for Hairy (8, 56, 75). Instead of the single binding site for Hairy-related proteins, the modified reporter has five Gal4 upstream activation sequences (UASs) substituted at the same position. In control experiments, this reporter was not repressed by wild-type Hairy-related proteins (data not shown). In all Schneider cell experiments, transcription of the reporter was activated by cotransfection of plasmids encoding the activator bHLH proteins Daughterless and Achaete or Daughterless and Scute, which activate the promoter as heterodimers (50, 77). Cotransfection of plasmids encoding the Gal4 DNA binding domain alone along with those encoding the activator proteins results in no repression of the reporter (Fig. 1C). In contrast, a fusion protein containing Hairy 2-267, a protein with the full coding sequence of Hairy (amino acids 2 to 337) (Fig. 1C), behaved as an efficient repressor and showed repression equivalent to that of wild-type Hairy acting on the native *achaete* promoter (56). Similar activity was seen for a series of amino-terminal deletion mutants containing Hairy 107-337, 267-337, or 320-337. These constructs all contain the WRPW motif. However, constructs lacking the WRPW motif such as the fusion protein containing Hairy 1-267 or 267-333 show no activity or reduced activity, respectively. To ensure that constructs with no or reduced activity were not artifacts due to poor expression or expression of incorrect proteins, Western blotting with an anti-Gal4 antibody was performed. These blots showed the production of fusion proteins of the correct molecular weight and at expression levels at least equivalent to that of Hairy 2-337 (data not shown). Additionally, these blots show that the constructs encoding Hairy 1-267 and 267-333 were expressed at levels that were severalfold higher than those of any of the other constructs, which may account for the activation and repression, respectively, seen with these two constructs. Equivalent repression results were seen for activation by either Daughterless-Achaete (Fig. 1C) or Daughterless-Scute heterodimers (Fig. 1D).

The region of Hairy needed to give efficient repression is small and contains the WRPW motif found in all family members. To generalize this result to other Hairy-related proteins, a similar deletion analysis was performed with mammalian Hes-1. Wild-type Hes-1 was shown in control experiments to act as a repressor on promoters containing class C binding sites in *Drosophila* Schneider cells (data not shown). A Gal4–Hes-1 fusion protein and two amino-terminal deletion fusion proteins (Fig. 2A) were constructed and tested in Schneider cells in a fashion similar to that used to test the Gal4-Hairy fusion proteins. All of the fusion proteins (Fig. 2B) acted as repressors and showed the same level of repression. Western blots of transfected cells showed that all three constructs were expressed at similar levels (data not shown). Additionally, a fusion protein containing the carboxyl terminus, with the WRPW motif, of the Hairy-related protein *Drosophila* Enhancer of Split m7 was made. This protein was also an efficient repressor (Fig. 2C).

Given the complete conservation of the WRPW motif in all of the Hairy-related proteins and in all of the minimal repression domains, a fusion protein containing the WRPW motif only was constructed. Remarkably, this fusion protein showed repressor activity nearly equivalent to that of full-length Hairy (Hairy 2-337) or the carboxyl-terminal 70 amino acids of Hairy (Hairy 267-337) (Fig. 3). The repression activity shown by the Gal4-WRPW construct is comparable to the repression shown by any of the minimal repression domain constructs, demonstrating that the 4-amino-acid WRPW motif found at the carboxyl terminus of all family members is sufficient for transcriptional repression.

Function of the WRPW motif as a protein-protein interaction domain for interactions between Hairy-related proteins and Groucho. Since several of these bHLH proteins have been

FIG. 2. Mapping the transcriptional repression domains of the Hairy-related proteins Hes-1 and Enhancer of Split m7. (A) Expression constructs encoding the Gal4 DNA binding domain fused to various full-length or truncated Hes-1 proteins were made for expression in Schneider cells. (B) Schneider cells were transfected with the reporter and plasmids encoding the indicated Gal4 fusion protein, Daughterless, and Achaete. The activity of the reporter when activated by Daughterless and Achaete alone is defined as 100% activity. (C) A Gal4 fusion protein containing the carboxyl terminus of Enhancer of Split m7 was made. Schneider cells were transfected with a plasmid encoding this construct, the reporter, and plasmids encoding Daughterless and Scute.

shown to biochemically interact with Groucho (57), we wanted to test whether the truncated proteins tested in the assays described above still retain this ability. To test for biochemical interactions, fusion proteins consisting of GST fused with Hairy 267-337, Hes-1 193-281, m7 142-186, or the WRPW motif alone were synthesized in bacteria and incubated with lysates from *Drosophila* Schneider cells. After extensive washing, coprecipitated proteins were subjected to SDS-PAGE and Western blotting and probed with an anti-Groucho monoclonal antibody (67) (Fig. 4). Groucho was abundant in these lysates (Cell Lysate lane; also see reference 67) and specifically interacted with GST fusion proteins containing Hairy 267-337, Hes-1 193-281, m7 142-186, and the WRPW motif alone. In contrast, neither GST alone nor GST–Hairy 267-333 coprecipitated Groucho.

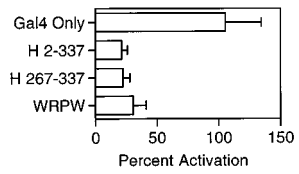

FIG. 3. Transcriptional repression by the 4-amino acid WRPW motif. A plasmid encoding the Gal4 DNA binding domain fused to the WRPW tetrapeptide was cotransfected into Schneider cells along with the reporter and plasmids encoding Daughterless and Scute. Parallel transfections with plasmids encoding Gal4–Hairy 2-337 and Gal4–Hairy 267-337 were performed to directly compare the WRPW activity with the previous results.

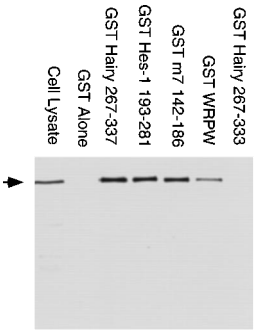

FIG. 4. Interaction of the WRPW motif with Groucho from *Drosophila* cell lysates. The indicated fusion proteins were expressed in bacteria, purified, and then incubated with Schneider cell lysates. The associated proteins were coprecipitated, subjected to SDS-PAGE and Western blotting, and then probed with an anti-Groucho monoclonal antibody (67). The arrowhead indicates the location of full-length Groucho in the control lane (Cell Lysate) and in lanes in which Groucho has been coprecipitated by one of the GST fusion proteins.

As an independent assay for protein interactions in vivo, we used yeast two-hybrid analysis. The Gal4 DNA binding domain alone (pGBT9 [Table 1]) did not interact with either the Gal4 activation domain (pGAD424) or a Gal4 activation domain-Groucho fusion protein (pGAD424 Gro). Likewise, Gal4 fusion proteins containing Hairy 267-337, Hairy 267-333, Hes-1 193-281, m7 142-186, and the WRPW motif alone did not interact with the Gal4 activation domain (Table 1). By contrast, Gal4 fusion proteins containing Hairy 267-337, Hes-1 193-281, m7 142-186, and the WRPW motif alone all interacted with a Gal4 activation domain-Groucho fusion protein, while Hairy 267-333, which lacks the WRPW motif, did not (Table 1). These results demonstrate that in both yeast and *Drosophila* cell lysates, the WRPW motif is not only necessary (57) but also sufficient to mediate protein-protein interactions with Groucho.

Active transcriptional repression by Groucho when bound to DNA by a heterologous DNA binding domain. Since the WRPW motif alone interacts with Groucho and acts as a repression domain, we tested if binding Groucho directly to DNA is sufficient for transcriptional repression. A Gal4 fusion protein containing full-length Groucho (Gro 1-719 [Fig. 5A]) repressed transcription to the same degree as any of the WRPW-containing constructs (Fig. 5B). Thus, Groucho is an active transcriptional repressor when bound directly to DNA. We then mapped the transcriptional repression domains within Groucho by using a series of Gal4 fusion proteins containing different full-length and truncated Groucho proteins (Fig. 5A). The results showed that fusion proteins containing the first 264 amino acids (Gro 1-264) behaved equivalently to the full-

TABLE 1. Interactions between Hairy-related proteins and Groucho in *S. cerevisiae*

Bait plasmid	Interaction with target plasmid pGAD424 Gro[a]
pGBT9	−
pGBT9 H 267-337	+++
pGBT9 H 267-333	−
pGBT9 Hes-1 193-281	++
pGBT9 m7 142-186	+++
pGBT9 WRPW	++

[a] None of the bait plasmids exhibited interaction with pGAD24 as a target plasmid.

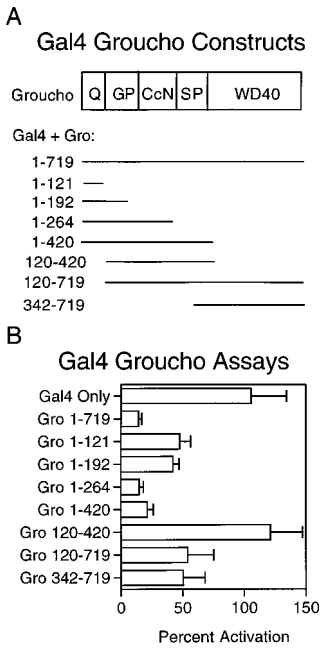

FIG. 5. Function of Groucho as a transcriptional repressor when directly bound to the *achaete* promoter. (A) The diagram of Groucho is based on a previously reported alignment of Groucho and the four mammalian TLE proteins (68). Q, GP, and SP refer to the predominance of glutamine, glycine and proline, and serine and proline found in these regions; WD40 and CcN refer to the WD40 repeats and the CcN motif found in Groucho and the mammalian homologs. The CcN motif is an approximately 60-amino-acid region that contains a nuclear localization signal, cdc2 phosphorylation site, and casein kinase II phosphorylation site located near each other and is found in other types of proteins (68). On the basis of the diagram, expression constructs encoding the Gal4 DNA binding domain fused to a series of full-length or truncated Groucho proteins were made. (B) Schneider cells were cotransfected with the appropriate plasmid and the reporter and plasmids encoding Daughterless and Achaete. One hundred percent activity represents the activity of the reporter when activated by Daughterless and Achaete alone.

length protein (Gro 1-719). Thus, most of the carboxyl-terminal region of the protein was not required for repressor activity (Fig. 5B). However, the WD40 repeats alone also showed partial repressor activity (Gro 342-719). Together these results indicate the presence of two repression domains, with amino acids 1 to 264 comprising the stronger repression domain and the WD40 repeats comprising a weaker domain, in this assay. Both of these domains are in regions that are highly conserved in homologs from other species (49, 51, 63, 68, 72). Western blots of transfected cells probed with anti-Gal4 antibodies showed expression of proteins of the correct molecular weights and expression of all proteins at levels at least equivalent to that of Gal4 Groucho 1-719 (data not shown).

Functional conservation of the interaction between WRPW and Groucho family corepressors in mammals. Mammalian homologs of both Hairy-related proteins and Groucho have been identified (2, 23, 32, 49, 51, 62, 63, 68, 70–72). We therefore wished to test whether our finding of interactions between the WRPW motif of *Drosophila* Hairy-related proteins and the Groucho corepressor is also true for the mammalian proteins. To do so, we transfected human HeLa cells with both WRPW-containing Gal4 fusion proteins and fusion proteins containing Groucho or the human homolog TLE1 (Fig. 6). HeLa cells were used because they have been shown to express several of the TLE proteins (68). The reporter gene used consisted of the simian virus 40 promoter with five UAS sites inserted upstream fused to the luciferase gene (12). The simian virus 40 promoter

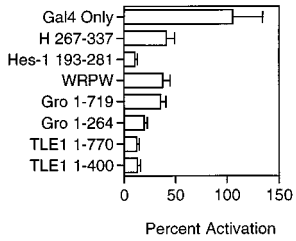

FIG. 6. Transcriptional repression by Hairy-related proteins and Groucho family proteins in mammalian cells. Human HeLa cells were cotransfected with plasmids encoding the indicated Hairy, Hes-1, Groucho, or TLE1 (human Groucho homolog) proteins or fragments or the WRPW tetrapeptide fused to the Gal4 DNA binding domain. The reporter contained the simian virus 40 promoter with five UAS sites inserted upstream, fused to the luciferase reporter gene. The simian virus 40 promoter contained in this reporter produces basal activity (12). One hundred percent activity is defined as the level of activity observed when the reporter alone was transfected into the HeLa cells. Each bar represents the average of at least three pairs of trials, and each error bar represents the standard deviation of the trials.

contained in this construct produces basal activity (12). However, when the reporter plasmid was cotransfected with plasmids expressing Gal4 fusion proteins containing Hairy 267-337, WRPW alone, or Hes-1 193-281, transcription was repressed (Fig. 6). Furthermore, Gal4 fusion proteins containing Groucho 1-719, Groucho 1-264, full-length TLE1 protein, or the amino terminus of TLE1 (TLE1 1-400) also repressed transcription when expressed in Hela cells. These results demonstrate that the WRPW motif functions as a transcriptional repression domain in mammalian cells as in *Drosophila* cells. These results also show that Groucho family members can repress both activated and basal transcription and possess a functionally conserved amino-terminal repression domain, as the amino-terminal regions of both Groucho (Gro 1-264) and TLE1 (TLE1 1-400) show repressor activity equivalent to or better than that of the full-length proteins.

We then tested for conservation of the biochemical interaction between the WRPW motif and the mammalian Groucho homologs by incubating bacterially expressed and purified GST fusion proteins with HeLa cell lysates (Fig. 7). After extensive washing, coprecipitated proteins were subjected to Western analysis with a pan-TLE monoclonal antibody (68). As with Groucho, interactions were observed between TLE proteins and GST–Hairy 267-337, GST–Hes-1 193-281, GST–m7 142-186, and GST-WRPW alone but not GST alone or GST–Hairy 267-333. Together with the transcription repression data, these results demonstrate that the mammalian homologs of Hairy-related proteins and Groucho are indeed functional corepressors. Additionally, these results show the WRPW motif to be both necessary and sufficient to mediate protein-protein interactions between Hairy-related proteins and members of the Groucho protein family in both insect and mammalian cells.

DISCUSSION

Groucho family proteins as active transcriptional corepressors. Our results demonstrate that Groucho family proteins function as active transcriptional corepressors which are recruited to target gene promoters by interactions with the WRPW tetrapeptide domain of Hairy-related proteins. These results confirm and extend the previous findings by Paroush and coworkers (57) which showed that Groucho functions genetically as a corepressor for Hairy-related proteins, that Groucho protein interacts with *Drosophila* Hairy-related pro-

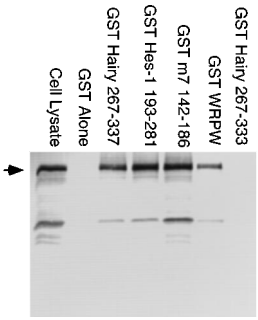

FIG. 7. Interaction of the WRPW motif with TLE proteins from human HeLa cell lysates. The indicated fusion proteins were expressed in bacteria, purified, and then incubated with HeLa cell lysates. The associated proteins were coprecipitated, subjected to SDS-PAGE and Western blotting, and then probed with a pan-TLE monoclonal antibody against human Groucho homologs (68). The arrowhead indicates the location of full-length TLE proteins in the control lane (Cell Lysate) and in lanes in which TLE protein has been coprecipitated by one of the GST fusion proteins. The lower bands are apparent degradation products of the TLE proteins.

teins, and that this interaction is disrupted if the WRPW motif is mutated. Because of its ability to interact with Groucho family proteins the WRPW motif of Hairy-related proteins functions as a transcriptional repression domain. Our assay shows no requirement for domains other than the WRPW motif for transcriptional repression. However, this finding does not exclude a function for other domains, and recent genetic evidence indicates that another conserved domain is necessary for function in other contexts and may mediate interactions with other proteins (18). Consistent with its role as a corepressor, Groucho is able to repress transcription when directly bound to target promoters and possesses a specific repression domain. This domain is highly conserved in other Groucho family members (49, 51, 63, 68, 72) and shows conservation of function, as the repression domain from Groucho also acts as a repressor in HeLa cells. These results suggest that the mechanism by which these corepressors affect transcription also is highly conserved. Given the ability of the Groucho family members to repress basal transcription as well as activated transcription, it seems likely that these proteins affect the basal transcription complex. Whether this mechanism involves direct contacts between this domain and components of the basal transcription complex or interactions with an intermediary protein is not known, but future experiments will address this question.

Recent work has shown that several other transcriptional repressors consist of a DNA-binding protein and a non-DNA-binding corepressor. Examples of such pairs of proteins are E2F and Rb (1, 82); thyroid hormone and retinoic acid receptors and the TRACs (14, 30); Mad or Mxi and mSin3 (5, 64); and TUP1/SSN6 and several types of DNA-binding proteins (40, 44, 59). Such corepressor systems have important functional properties not found in single-protein transcriptional repressors.

Recruitment of a corepressor in some cases allows an additional level of regulation because the interaction between the DNA-binding protein and the corepressor can be regulated. Both E2F and the nuclear receptors have regulated interactions with their corepressors, and this interaction allows them to rapidly switch from activators to repressors while remaining bound at the same DNA site. E2F is a DNA-binding protein and transcriptional activator which can be converted into a repressor when it is associated with the Rb corepressor protein (1, 82, 83). Phosphorylation of the Rb protein by cyclin-dependent kinase proteins then causes the dissociation of E2F and Rb and allows E2F to stimulate the transcription of target genes (1, 82, 83). The thyroid hormone and retinoic acid receptors are DNA-binding proteins which are constitutively bound to DNA. In the absence of the ligands, these proteins are associated with the TRAC proteins, which act as corepressors (14, 30). Once the ligand binds to the receptor, the TRAC protein dissociates from the receptor which then becomes a transcriptional activator (14, 30). It is not known if Hairy-related proteins have the ability to act as both repressors and activators or if the interactions between Hairy-related proteins and either Groucho or TLE proteins are regulated.

Another potential consequence of having a separate DNA-binding protein and corepressor is the ability of the corepressor to interact with more than one type of DNA-binding protein. The yeast TUP1 corepressor is multifunctional because of its ability to interact with several different kinds of DNA-binding proteins (59). TUP1 is found in yeast cells complexed with the SSN6 protein (74), and together they interact with a variety of specific DNA-binding proteins (40). Since these DNA-binding proteins bind to the promoters of distinct target genes, TUP1 is able to function as a general transcriptional corepressor in different pathways as a result of the specificity conferred by the DNA-binding protein partners. It is possible that Groucho-like proteins have the ability to interact with several different kinds of DNA-binding proteins as well. These interactions may be mediated by WRPW motifs in other proteins or by motifs that resemble WRPW. For example, the *Drosophila* Runt protein and its mammalian homolog PEBP2a both contain the sequence WRPY at the carboxyl terminus (39, 55). Additionally, other types of DNA-binding proteins may utilize Groucho or the Groucho homologs by forming protein-protein interactions with other regions of the protein. One possible candidate for a second protein-protein interaction domain would be the WD40 repeats. In TUP1, these repeats have been shown to be a second interaction domain which can interact directly with the homeodomain protein α-2 (44).

WRPW as a 4-amino-acid protein-protein interaction domain. The use of four amino acids as a protein-protein interaction domain is remarkable but not unprecedented. Recently Kornau et al. described a small carboxyl-terminal tSXV motif which is found in *N*-methyl-D-aspartate receptor subunits as well as other proteins (45). This motif is involved in making protein-protein contacts between the *N*-methyl-D-aspartate receptor subunits and the synaptic density protein PSD-95. It is possible that more small protein-protein interaction domains will be found either at the protein termini or in loop regions. Such positioning of these domains might allow the remainder of the protein to be folded or otherwise inaccessible, while only the small sequence needs to be exposed. Additionally, the positioning these domains at the protein termini or in loops may allow the appropriate recognition domain to wrap around this accessible domain and form strong interactions. The small sizes of these motifs and their cognate recognition domains make them amenable to physical techniques like nuclear magnetic resonance spectroscopy or X-ray crystallography, which will allow direct determination of how such small sequences mediate specific, high-affinity protein-protein interactions.

ACKNOWLEDGMENTS

We are grateful to Stefano Stefani for the plasmids and antibodies that he generously provided. We also appreciate comments provided on versions of this paper by Claude Desplan, Robert Benezra, John Wagner, and Ulrich Hartl.

A.L.F. was supported by the Tri-Institutional M.D.-Ph.D. Program. S.O. was supported in part by a grant-in-aid for scientific research from the Ministry of Education, Science, and Culture of Japan. M.C. was supported by NIH grant R01 NS28652, the Alfred P. Sloan Foundation, the Pew Scholars in Biomedical Sciences Program, the Mather Foundation, the Cornell Scholar's Program, and an institutional grant from the Markey Foundation.

REFERENCES

1. **Adnane, J., Z. Shao, and P. D. Robbins.** 1995. The retinoblastoma susceptibility gene product represses transcription when directly bound to the promoter. J. Biol. Chem. **270:**8837–8843.
2. **Akazawa, C., Y. Sasai, S. Nakanishi, and R. Kageyama.** 1992. Molecular characterization of a rat negative regulator with a basic helix-loop-helix structure predominantly expressed in the developing nervous system. J. Biol. Chem. **267:**21879–21885.
3. **Alonso, M. C., and C. V. Cabrera.** 1988. The achaete-scute gene complex of Drosophila melanogaster comprises four homologous genes. EMBO J. **7:**2585–2591.
4. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1992. Short protocols in molecular biology, 2nd ed. John Wiley & Sons, New York.
5. **Ayer, D. E., Q. A. Lawrence, and R. N. Eisenman.** 1995. Mad-Max transcriptional repression is mediated by ternary complex formation with mammalian homologs of yeast repressor Sin3. Cell **80:**767–776.
6. **Bailey, A. M., and J. W. Posakony.** 1995. Suppressor of Hairless directly activates transcription of *Enhancer of split* complex genes in response to Notch receptor activity. Genes Dev. **9:**2609–2622.
7. **Benezra, R., R. L. Davis, D. Lockshon, D. L. Turner, and H. Weintraub.** 1990. The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. Cell **61:**49–59.
8. **Botas, J., J. Mosoco del Prado, and A. García-Bellido.** 1982. Gene-dose titration analysis in the search of trans-regulatory genes in *Drosophila*. EMBO J. **1:**307–310.
9. **Campos-Ortega, J. A.** 1993. Mechanisms of early neurogenesis in Drosophila melanogaster. J. Neurobiol. **24:**1305–27.
10. **Campos-Ortega, J. A., and Y. N. Jan.** 1991. Genetic and molecular bases of neurogenesis in *Drosophila melanogaster*. Annu. Rev. Neurosci. **14:**399–420.
11. **Campuzano, S., and J. Modolell.** 1992. Patterning of the *Drosophila* nervous system: the *achaete-scute* gene complex. Trends Genet. **8:**202–208.
12. **Catron, K. M., H. Zhang, S. C. Marshall, J. A. Inostroza, J. M. Wilson, and C. Abate.** 1995. Transcriptional repression by Msx-1 does not require homeodomain DNA-binding sites. Mol. Cell. Biol. **15:**861–871.
13. **Caudy, M., H. Vaessin, M. Brand, R. Tuma, L. Y. Jan, and Y. N. Jan.** 1988. *daughterless*, a gene essential for both neurogenesis and sex determination, has sequence similarities to *myc* and the *achaete-scute* complex. Cell **55:** 1061–1067.
14. **Chen, J. D., and R. M. Evans.** 1995. A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature (London) **377:**454–457.
15. **Cowell, I. G.** 1994. Repression versus activation in the control of gene transcription. Trends Biochem. Sci. **19:**38–42.
16. **Cubas, P., J.-F. de Celis, S. Campuzano, and J. Modolell.** 1991. Proneural clusters of *achaete-scute* expression and the generation of sensory organs in the *Drosophila* imaginal wing disc. Genes Dev. **5:**996–1008.
17. **Cubas, P., and J. Modolell.** 1992. The *extramacrochaete* gene provides information for sensory organ patterning. EMBO J. **11:**3385–3393.
18. **Dawson, S. R., D. L. Turner, H. Weintraub, and S. M. Parkhurst.** 1995. Specificity for the Hairy/Enhancer of split basic helix-loop-helix (bHLH) proteins maps outside the bHLH domain and suggests two separable modes of transcriptional repression. Mol. Cell. Biol. **15:**6923–6931.
19. **Delidakis, C., and S. Artavanis-Tsakonas.** 1992. The *Enhancer of split* [E(spl)] locus of *Drosophila* encodes seven independent helix-loop-helix proteins. Proc. Natl. Acad. Sci. USA **89:**8731–8735.
20. **Delidakis, C., A. Preiss, D. A. Hartley, and S. Artavanis-Tsakonas.** 1991. Two genetically and molecularly distinct functions involved in early neurogenesis reside within the *Enhancer of split* locus of *Drosophila melanogaster*. Genetics **129:**803–823.
21. **Edmondson, D. G., and E. N. Olson.** 1993. Helix-loop-helix proteins as regulators of muscle specific transcription. J. Biol. Chem. **268:**755–758.
22. **Ellis, H. M., D. R. Spann, and J. W. Posakony.** 1990. *extramacrochaetae*, a negative regulator of sensory organ development in Drosophila, defines a new class of helix-loop-helix proteins. Cell **61:**27–38.
23. **Feder, J. N., L. Y. Jan, and Y. N. Jan.** 1993. A rat gene with sequence homology to the *Drosophila* gene *hairy* is rapidly induced by growth factors known to influence neuronal differentiation. Mol. Cell. Biol. **13:**105–113.
24. **Fortini, M. E., and S. Artavanis-Tsakonas.** 1994. The Suppressor of Hairless protein participates in Notch receptor signalling. Cell **79:**273–282.
25. **Garrell, J., and J. Modolell.** 1990. The Drosophila *extramacrochaete* locus, an antagonist of proneural genes that, like these genes, encodes a helix-loop-helix protein. Cell **61:**39–48.
26. **Guillemot, F., and A. L. Joyner.** 1993. Dynamic expression of the murine *achaete-scute* homolog in the developing (MASH-1) nervous system. Mech. Dev. **42:**171–185.
27. **Guillemot, F., L.-C. Lo, J. E. Johnson, A. Auerbach, D. J. Anderson, and A. L. Joyner.** 1993. Mammalian *achaete-scute* homolog 1 is required for the early development of olfactory and autonomic neurons. Cell **75:**463–476.
28. **Han, K., M. S. Levine, and J. L. Manley.** 1989. Synergistic activation and repression of transcription by Drosophila homeobox proteins. Cell **56:**573–583.
29. **Han, K., and J. L. Manley.** 1993. Transcriptional repression by the *Drosophila* Even-skipped protein: definition of a minimal repression domain. Genes Dev. **7:**491–503.
30. **Hörlein, A. J., A. M. Näär, T. Heinzel, J. Torchia, B. Gloss, R. Kurokawa, A. Ryan, Y. Kamei, M. Söderström, C. K. Glass, and M. G. Rosenfeld.** 1995. Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. Nature (London) **377:**397–404.
31. **Ishibashi, M., K. Moriyoshi, Y. Sasai, K. Shiota, S. Nakanishi, and R. Kageyama.** 1994. Persistent expression of helix-loop-helix factor HES-1 prevents mammalian neural differentiation in the central nervous system. EMBO J. **13:**1799–1805.
32. **Ishibashi, M., Y. Sasai, S. Nakanishi, and R. Kageyama.** 1993. Molecular characterization of HES-2, a mammalian helix-loop-helix factor structurally related to *Drosophila hairy* and *Enhancer of split*. Eur. J. Biochem. **215:**645–652.
33. **Jan, Y. N., and L. Y. Jan.** 1990. Genes required for specifying cell fates in *Drosophila* embryonic sensory nervous system. Trends Neurosci. **13:**493–498.
34. **Jan, Y. N., and L. Y. Jan.** 1993. HLH proteins, fly neurogenesis, and vertebrate myogenesis. Cell **75:**827–830.
35. **Jarman, A. P., E. H. Grell, L. Ackerman, L. Y. Jan, and Y. N. Jan.** 1993. *atonal* is a proneural gene that directs chordotonal organ formation in the *Drosophila* peripheral nervous system. Cell **73:**1307–1321.
36. **Jarriault, S., C. Brou, F. Logeat, E. H. Schroeter, R. Kopan, and A. Israel.** 1995. Signalling downstream of activated mammalian Notch. Nature (London) **377:**355–358.
37. **Jaynes, J. B., and P. H. O'Farrell.** 1991. Active repression of transcription by the Engrailed homeodomain protein. EMBO J. **10:**1427–1433.
38. **Jiménez, F., and J. Modolell.** 1993. Neural fate specification in *Drosophila*. Curr. Opin. Gen. Dev. **3:**626–632.
39. **Kania, M. A., A. S. Bonner, J. B. Duffy, and J. P. Gergen.** 1990. The *Drosophila* segmentation gene *runt* encodes a novel nuclear regulatory protein that is also expressed in the developing nervous system. Genes Dev. **4:**1701–1713.
40. **Keleher, C. A., M. J. Redd, J. Schultz, M. Carlson, and A. D. Johnson.** 1992. Ssn6-Tup1 is a general repressor of transcription in yeast. Cell **68:**709–719.
41. **Klämbt, C., E. Knüst, K. Tietze, and J. A. Campos-Ortega.** 1989. Closely related transcripts encoded by the neurogenic gene complex *Enhancer of Split* of *Drosophila melanogaster*. EMBO J. **8:**203–210.
42. **Knust, E., K. A. Bremer, H. Vässin, A. Ziemer, U. Tepas, and J. A. Campos-Ortega.** 1987. The *Enhancer of split* locus and neurogenesis in *Drosophila melanogaster*. Dev. Biol. **122:**262–273.
43. **Knust, E., H. Schrons, F. Grawe, and J. A. Campos-Ortega.** 1992. Seven genes of the *Enhancer of split* complex of *Drosophila melanogaster* encode helix-loop-helix proteins. Genetics **132:**505–518.
44. **Komachi, K., M. J. Redd, and A. D. Johnson.** 1994. The WD repeats of Tup1 interact with the homeo domain protein a2. Genes Dev. **8:**2857–2867.
45. **Kornau, H. C., L. T. Schenker, M. B. Kennedy, and P. H. Seeburg.** 1995. Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. Science **269:**1737–1740.
46. **Lecourtois, M., and F. Schweisguth.** 1995. The neurogenic Suppressor of Hairless DNA-binding protein mediates the transcriptional activation of the *Enhancer of split* complex genes triggered by Notch signaling. Genes Dev. **9:**2598–2608.
47. **Lieber, T., S. Kidd, E. Alcamo, V. Corbin, and M. Young.** 1993. Antineurogenic phenotypes induced by truncated Notch proteins indicate a role in signal transduction and may point to a novel function for Notch in nuclei. Genes Dev. **7:**1949–1965.
48. **Lo, L.-C., J. E. Johnson, C. W. Wuenschell, T. Saito, and D. J. Anderson.** 1991. Mammalian *achaete-scute* homolog 1 is transiently expressed by spatially-restricted subsets of early neuroepithelial and neural crest cells. Genes Dev. **5:**1524–1537.
49. **Mallo, M., F. Franco del Amo, and T. Gridley.** 1993. Cloning and developmental expression of *Grg*, a mouse gene related to the *groucho* transcript of the *Drosophila Enhancer of split* complex. Mech. Dev. **42:**67–76.
50. **Martinez, C., J. Modolell, and J. Garrell.** 1993. Regulation of the proneural gene achaete by helix-loop-helix proteins. Mol. Cell. Biol. **13:**3514–21.
51. **Miyasaka, H., B. K. Choudhury, E. W. Hou, and S. S.-L. Li.** 1993. Molecular cloning and expression of mouse and human cDNA encoding AES and ESG proteins with strong similarity to *Drosophila* enhancer of split groucho protein. Eur. J. Biochem. **216:**343–352.
52. **Murre, C., P. S. McCaw, and D. Baltimore.** 1989. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. Cell **56:**777–783.
53. **Murre, C., P. S. McCaw, H. Vaessin, M. Caudy, L. Y. Jan, Y. N. Jan, C. V.**

Cabrera, J. N. Buskin, S. D. Hauschka, A. B. Lassar, H. Weintraub, and D. Baltimore. 1989. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell **58:**537–544.

54. **Oellers, N., M. Dehio, and E. Knust.** 1994. bHLH proteins encoded by the *Enhancer of split* complex of *Drosophila* negatively interfere with transcriptional activation mediated by proneural genes. Mol. Gen. Genet. **244:**465–473.
55. **Ogawa, E., M. Maruyama, H. Kagoshima, M. Inuzuka, J. Lu, M. Satake, K. Shigesada, and Y. Ito.** 1993. PEBP2/PEA2 represents a family of transcription factors homologous to the products of the *Drosophila* runt gene and the human *AML1* gene. Proc. Natl. Acad. Sci. USA **90:**6859–6863.
56. **Ohsako, S., J. Hyer, G. Panganiban, I. Oliver, and M. Caudy.** 1994. hairy function as a DNA-binding helix-loop-helix repressor of Drosophila sensory organ formation. Genes Dev. **8:**2743–2755.
57. **Paroush, Z., R. Finley, T. Kidd, S. M. Wainwright, P. Ingham, R. Brent, and D. Ish-Horowicz.** 1994. Groucho is required for Drosophila neurogenesis, segmentation, and sex determination and interacts directly with hairy-related bHLH proteins. Cell **79:**805–815.
58. **Rebay, I., R. G. Fehon, and S. Artavanis-Tsakonas.** Specific truncations of Drosophila Notch define dominant activated and dominant negative forms of the receptor. Cell **74:**319–329.
59. **Roth, S. Y.** 1995. Chromatin-mediated transcriptional repression in yeast. Curr. Opin. Genet. Dev. **5:**168–173.
60. **Rudnicki, M. A., and R. Jaenisch.** 1995. The MyoD family of transcription factors and skeletal myogenesis. Bioessays **17:**203–209.
61. **Rushlow, C. A., A. Hogan, S. M. Pinchin, K. M. Howe, M. Lardelli, and D. Ish-Horowicz.** 1989. The *Drosophila hairy* protein acts in both segmentation and bristle patterning and shows homology to N-*myc*. EMBO J. **8:**3095–3103.
62. **Sasai, Y., R. Kageyama, Y. Tagawa, R. Shigemoto, and S. Nakanishi.** 1992. Two mammalian helix-loop-helix factors structurally related to *Drosophila hairy* and *Enhancer of split*. Genes Dev. **6:**2620–2634.
63. **Schmidt, C. J., and T. E. Sladek.** 1993. A rat homolog of the *Drosophila* Enhancer of split (*groucho*) locus lacking WD-40 repeats. J. Biol. Chem. **268:**25681–25686.
64. **Schreiber-Agus, N., L. Chin, K. Chen, R. Torres, G. Rao, P. Guida, A. I. Skoultchi, and R. A. DePinho.** 1995. An amino-terminal domain of Mxi1 mediates anti-myc oncogenic activity and interacts with a homolog of the yeast transcriptional repressor SIN3. Cell **80:**777–786.
65. **Schrons, H., E. Knust, and J. A. Campos-Ortega.** 1992. The *Enhancer of split* complex and adjacent genes in the 96F region of *Drosophila melanogaster* are required for segregation of neural and epidermal progenitor cells. Genetics **132:**481–503.
66. **Skeath, J. B., and S. B. Carroll.** 1991. Regulation of *achaete-scute* gene expression and sensory organ pattern formation in the *Drosophila* wing. Genes Dev. **5:**984–995.
67. **Stifani, S.** Unpublished data.
68. **Stifani, S., C. M. Blaumueller, N. J. Redhead, R. E. Hill, and S. Artavanis-Tsakonas.** 1992. Human homologs of a Drosophila Enhancer of split gene product define a novel family of nuclear proteins. Nat. Genet. **2:**119–127.
69. **Struhl, G., K. Fitzgerald, and I. Greenwald.** 1993. Intrinsic activity of the Lin-12 and Notch intracellular domains in vivo. Cell **74:**331–345.
70. **Takebayashi, K., C. Akazawa, S. Nakanishi, and R. Kageyama.** 1995. Structure and promoter analysis of the gene encoding the mouse helix-loop-helix factor HES-5. J. Biol. Chem. **270:**1342–1349.
71. **Takebayashi, K., Y. Sasai, Y. Sakai, T. Watanabe, S. Nakanishi, and R. Kageyama.** 1994. Structure, chromosomal locus, and promoter analysis of the gene encoding the mouse helix-loop-helix factor HES-1. J. Biol. Chem. **269:**5150–5156.
72. **Tata, F., and D. A. Hartley.** 1993. The role of the *Enhancer of split* complex during cell fate determination in *Drosophila*. Dev. Suppl., p. 139–148.
73. **Tietze, K., N. Oellers, and E. Knust.** 1992. *Enhancer of split*^{D}, a dominant mutation of *Drosophila*, and its use in the study of functional domains of a helix-loop-helix protein. Proc. Natl. Acad. Sci. USA **89:**6152–6156.
74. **Tzamarias, D., and K. Struhl.** 1994. Functional dissection of the yeast Cyc8-Tup1 transcriptional co-repressor complex. Nature (London) **369:**758–761.
75. **Van Doren, M., A. M. Bailey, J. Esnayra, K. Ede, and J. W. Posakony.** 1994. Negative regulation of proneural gene activity: hairy is a direct transcriptional repressor of *achaete*. Genes Dev. **8:**2729–2742.
76. **Van Doren, M., H. M. Ellis, and J. W. Posakony.** 1991. The Drosophila extramacrochaete protein antagonizes sequence-specific DNA binding by daughterless/achaete-scute protein complexes. Development **113:**245–255.
77. **Van Doren, M., P. A. Powell, D. Pasternak, A. Singson, and J. W. Posakony.** 1992. Spatial regulation of proneural gene activity: auto- and cross-activation of *achaete* is antagonized by *extramacrochaete*. Genes Dev. **6:**2592–2605.
78. **Villares, R., and C. V. Cabrera.** 1987. The *achaete-scute* gene complex of D. melanogaster: conserved domains in a subset of genes required for neurogenesis and their homology to *myc*. Cell **50:**415–424.
79. **Wainwright, S. M., and D. Ish-Horowicz.** 1992. Point mutations in the *Drosophila hairy* gene demonstrate in vivo requirements for basic, helix-loop-helix, and WRPW domains. Mol. Cell. Biol. **12:**2475–2483.
80. **Weintraub, H.** 1993. The MyoD family and myogenesis: redundancy, networks, and thresholds. Cell **75:**1241–1244.
81. **Weintraub, H., R. Davis, S. Tapscott, M. Thayer, M. Krause, R. Benezra, T. K. Blackwell, D. Turner, R. Rupp, S. Hollenberg, Y. Zhuang, and A. Lassar.** 1991. The MyoD gene family: nodal point during specification of the muscle cell lineage. Science **251:**761–766.
82. **Weintraub, S. J., K. N. B. Chow, R. X. Luo, S. H. Zhang, S. He, and D. C. Dean.** 1995. Mechanism of active transcriptional repression by the retinoblastoma protein. Nature (London) **375:**812–815.
83. **Weintraub, S. J., C. A. Prater, and D. C. Dean.** 1992. Retinoblastoma protein switches the E2F site from positive to negative element. Nature (London) **358:**259–261.