INTERACTIONS BETWEEN GRG AND HES PROTEINS

INTERACTIONS BETWEEN GRG (GROUCHO RELATED GENE) AND HES (HAIRY/ENHANCER OF SPLIT) PROTEINS IN THE NOTCH SIGNALLING PATHWAY

By

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ABSTRACT

The Notch signalling pathway is a lateral inhibition pathway that serves to limit the number of cells in a proneural cluster (a group of equipotent cells) that will adopt a neural cell fate during neurogenesis in *Drosophila.* The proper segregation of neural and epidermal progenitor cells during neurogenesis requires the expression of both the proneural genes and the neurogenic genes. Expression of proneural genes, such as *achaete*, gives cells the potential to commit to a neural cell fate. The neurogenic genes encode proteins that act in the Notch signalling cascade and are required for cell fate determination during Drosophila neurogenesis. *Notch* and *Delta* are neurogenic genes that encode large transmembrane proteins. Interaction between the extracellular domains of Notch and Delta is thought to transmit a signal to the nucleus by way of the DNA-binding Suppressor of Hairless protein. In response to Notch activation Suppressor of Hairless is translocated to the nucleus where it activates the transcription of the neurogenic genes of the *Enhancer of split complex (E(spl)-C).* The products of the *E(spl)-C* are bHLH transcription factors. They possess a C-terminal tryptophan-arginine-proline-tryptophan (WRPW) motif that interacts with the product of another neurogenic gene, *groucho.* The *groucho* gene product encodes a protein containing a WD40 repeat element. When bound to Groucho, E(spl) bHLH proteins are able to repress transcription of proneural genes, such as *achaete,* thereby directing the cell to adopt a non-neural cell fate.

A number of murine *groucho* homologues have been identified and named Grg's (Groucho related genes). Three full length Grg proteins have been

identified which contain all five domains found in the *Drosophila* Groucho protein. Two short Grg proteins have also been identified which only contain one of the domains found in the full-length Grg proteins. A number of murine homologues of the *Drosophila E(spl)-C* have also been identified and named Hes (Hairy/Enhancer of split) proteins. Like the gene products of the *Drosophila E(spl)-C,* the Hes proteins are bHLH proteins containing a C-terminal WRPW motif. One of the Hes proteins, Hes3, is lacking a basic domain and therefore lacks the DNA-binding activity possessed by the other Hes proteins.

Attempts were made to detect interactions between Grg and Hes proteins using co-immunoprecipitation techniques. The anti-WD40 antibody, which recognizes the long WD40-containing Grg proteins, was able to specifically immunoprecipitate ^{35}S-labelled Grg1 . This antibody was also able to recognize WD40-containing Grg proteins present in P19 cell extracts. However, attempts to co-immunoprecipitate radiolabelled Hes1 and AML1b proteins with Grg proteins present in P19 cell extract were unsuccessful due to the low affinity of the anti-WD40 antibody and the background caused by the binding of the test proteins to Sepharose. A second method of co-immunoprecipitation was attempted using an HA-tagged Grg1 fusion protein and a commercially available anti-HA antibody. The attempt to co-immunoprecipitate ^{35}S-labelled Hes1 with radiolabelled HA-tagged Grg1 was unsuccessful due to a high degree of background caused by Hes1 binding to protein G Agarose. Using the Yeast Two-Hybrid interaction assay, the WD40-containing Grg proteins, Grg1 and Grg4, were found to interact with Hes1. However, using the same assay WD40-containing Grg proteins were found not to interact with Hes3, which lacks DNA-binding activity. A Western blot was performed to determine if the Hes3 fusion proteins were being expressed

in transformed yeast but none were detected. This may have been due to the poor affinity of the anti-GAL4 activation domain antibody. A similar Western blot demonstrated that the Grg proteins, fused to the GAL4 DNA binding domain, were being expressed in transformed yeast extract. The WD40-containing Grg proteins, Grg1 and Grg4, were also found not to interact with AML1b, a protein which contains a C-terminal VWRPY domain which is reminiscent of the C-terminal WRPW interaction domain found in Hes proteins and *Drosophila* E(spl) proteins. However, WD40-containing Grg proteins were able to interact with an AML1b mutant in which the VWRPY motif was mutated to VWRPW in the Yeast Two Hybrid assay.

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LIST OF ABBREVIATIONS

A	alanine
AML	acute myeloid leukemia
Amp	amperes
AP	alkaline phosphatase
APS	ammonium persulphate
ASC	achaete-scute complex
bHLH	basic helix-loop-helix
BSA	bovine serum albumin
C	cysteine
CcN	casein kinase II-cdc2 kinase-nuclear localization sequence
cDNA	complementary deoxyribonucleic acid
CIP	calf intestinal phosphatase
CNS	central nervous system
D	aspartic acid
da	daughterless
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
dGTP	deoxyguanosine 5'-triphosphate
Dl	Delta
DNA	deoxyribonucleic acid
dpc	days post conception

DTT	1,4-dithiothreitol
dTTP	deoxythymidine 5'-triphosphate
E	glutamic acid
EGF	epidermal growth factor
emc	extramacrochaetae
E(spl)	Enhancer of split
E(spl)-C	Enhancer of split complex
G	glycine
GP	glycine-proline
Grg	groucho related gene
GST	glutathione S-transferase
H	histidine
Hes	Hairy-Enhancer of split
HLH	helix-loop-helix
I	isoleucine
K	lysine
kD	kilodaltons
Leu	leucine
mAmp	milliamperes
MASH	mammalian achaete-scute homologue
MATH	mammalian atonal homologue
mV	millivolts
MyoD	myogenic protein D
N	Notch
NLS	nuclear localization sequence

Notch-IC	untethered intracellular fragment of Notch
P	proline
PEG	polyethylene glycol
PCI	phenol-chloroform-iso amyl alcohol
PNS	peripheral nervous system
Q	glutamine
R	arginine
Resp	rat Enhancer of split
RNA	ribonucleic acid
S	serine
SP	serine-proline
SS-DNA	single-stranded deoxyribonucleic acid
Su(H)	Suppressor of Hairless
T	threonine
Trp	tryptophan
TEMED	N,N,N',N',-Tetramethylethylenediamine
TLE	transducin-like element
UV	ultraviolet
V	valine
WD40	tryptophan-aspartate repeat every 40 amino acids
WRPW	tryptophan-arginine-proline-tryptophan
VWRPY	valine-tryptophan-arginine-proline-tyrosine
X-gal	5-bromo-4-chloro-3-indolyl β-D-galactosidase
Y	tyrosine

I. INTRODUCTION

1.1 Neurogenesis in *Drosophila melanogaster*

Much of the work that has been done in studying the development of the central nervous system (CNS) has been carried out in *Drosophila melanogaster* due to its well characterised genetics. The beginning of neurogenesis in *Drosophila* is marked by the separation of the neural progenitors (neuroblasts) from the epidermal progenitors (epidermoblasts). Initially, all the cells of a proneural cluster have the potential to become either a neuroblast or an epidermoblast. The Notch signalling pathway plays a key role in helping these cells choose between two alternative developmental fates. The Notch pathway is a lateral inhibition pathway, thus a developing neuroblast will use cell-cell interactions to inhibit neighbours from also becoming neurons and these neighbouring cells are therefore directed towards the secondary epidermal fate (Heitzler and Simpson 1991). Many of the molecular mechanisms involved in cell fate choices in the development of the CNS are shared in the development of the peripheral nervous system (PNS) (Ghysen and Dambly-Chaudiere 1989).

1.1.1 The Proneural Genes

The central nervous system of *Drosophila* arises from the ventral neurogenic region of the ectoderm during embryogenesis. The expression of two groups of

genes, the proneural genes and the neurogenic genes, is required for the proper segregation of neural and epidermal progenitor cells during the formation of the nervous system. The proneural genes are so-called because their expression gives cells the potential to commit to a neural fate, whether it be neuroblasts in the CNS or sensory organ progenitor cells in the PNS (Ghysen and Dambly-Chaudiere 1989). Proneural genes, such as the genes of the *achaete-scute complex* (*AS-C*) and *daughterless*, play an important role in the development of the CNS and PNS. The *AS-C* is a complex consisting of four genes: *achaete*, *scute*, *lethal of scute*, and *asense*. Elimination of individual genes of *AS-C* reveals that the product of *lethal of scute* plays an important role in the development of the CNS (Jiminez and Campos-Ortega 1987) while *achaete, scute,* and *asense* are required for the development of the PNS (Dambly-Chaudiere and Ghysen 1987). The gene product of *daughterless* (*da*) is ubiquitously expressed and is required for the formation of all sensory organs (Jan et al. 1987). *Da* mutants exhibit a complete absence of the PNS and a reduction in the size of the CNS (Caudy et al. 1988). The products of the *AS-C* and *da* genes are basic-helix-loop-helix (bHLH) transcription factors. In general, bHLH proteins are able to homo- or hetero-dimerize through their HLH domain thus uniting two regions rich in basic amino acids that make sequence-specific contacts to DNA (Ferre-d'Amare et al. 1993). The four bHLH proteins encoded by the *achaete-scute complex* are tissue-specific transcription factors that can heterodimerize with the ubiquitously expressed

Daughterless protein. Da-AS-C heterodimers can then bind to E boxes (CANNTG) in the promoter region of target genes to initiate the development of neuroblasts (Cabrera and Alonsa 1991; Martinez et al. 1993).

1.1.2 The Neurogenic Genes

The second group of genes that is required for neurogenesis in *Drosophila melanogaster* are the neurogenic genes. The neurogenic genes, such as *Notch*, *Delta*, *suppressor of Hairless*, *hairy*, *groucho* and *enhancer of split*, are so-called because null mutations in any one of these genes results in hypertrophy of the nervous system at the expense of epidermal structures (Lehman et al. 1993). The Notch protein is a large transmembrane receptor which is expressed on the cell surface of both epidermal and neuronal precursor cells during *Drosophila* development (Johansen, Fehon, and Artavanis-Tsakonas 1989). The *Notch* transcript encodes a 2703 amino acid transmembrane protein with an extracellular domain containing 36 epidermal growth factor (EGF)-like repeats and three cysteine-rich Notch/Lin-12 repeats. The Notch protein also contains a 1000 amino acid intracellular domain containing a nuclear localisation signal, a PEST sequence and 6 tandem ankyrin repeats. The *Delta* gene encodes the ligand for Notch and is initially expressed in all cells with neurogenic potential (Kopczynski and Muskavitch 1989; Haenlin, Kramatschek, and Campos-Ortega 1990). As development proceeds, the *Delta* transcript becomes localised to cells that adopt the neural fate. Conversely, cells which express higher levels of Notch relative to

neighbouring cells adopt a non-neural cell fate whereas cells expressing lower levels develop a neural fate (Heitzler and Simpson 1991). Notch interacts with its membrane-bound ligand, Delta, through EGF repeats located in the extracellular domains of both proteins (Fehon et al. 1990; Lieber et al. 1992). *Notch* transcripts are ubiquitously expressed during the early embryonic stages (Hartley, Xu, and Artavanis-Tsakonas 1987). Later in development, Notch is expressed in uncommitted and proliferative cell populations which is consistent with its role in neurogenesis (Fehon et al. 1991; Johansen, Fehon, and Artavanis-Tsakonas 1989). Notch can be constitutively activated by deletions which result in an untethered intracellular fragment (Lieber et al. 1993). Intracellular fragments of Notch (Notch-IC) contain nuclear localisation signals and are found in the nucleus where they have been shown to inhibit myogenesis in *Drosophila* (Kopan et al. 1996). Expression of an untethered cytoplasmic fragment of Notch during the development of the *Drosophila* embryo blocks neurogenesis, whereas epidermal development proceeds normally (Struhl, Fitzgerald, and Greenwald 1993).

A second neurogenic gene present in *Drosophila* is *hairy*, a segmentation gene. Hairy participates in the formation of alternate embryonic segments early on in development and is also required later in development during neurogenesis (Ingham 1985; Jan and Jan 1990). Hairy is thought to act during neurogenesis by suppressing the proneural gene, *achaete*, thereby controlling the number of sensory organs that are formed (Botas, Moscoso del Prado, and Garcia-Bellido 1982; Skeath and Carroll 1991). The *hairy* gene encodes a bHLH transcription

factor which is thought to heterodimerize with Daughterless, bind to N boxes in the promoter region of proneural genes such as *achaete*, and repress transcription (Sasai et al. 1992). Since Daughterless is also required as a dimerization partner for the E-box-binding bHLH transcriptional activators such as Achaete, Hairy-like transcription factors can also repress transcription by sequestering dimerization partners. The ability of Hairy to repress transcription is dependent upon the presence of its corepressor, Groucho (Fisher, Ohsako, and Caudy 1996). Groucho, also encoded by a neurogenic gene, has been shown to physically interact with Hairy. A serine/proline-rich domain in Groucho and a C-terminal tryptophan-arginine-proline-tryptophan (WRPW) motif in Hairy have been shown to be crucial for this interaction (Paroush et al. 1994). The ability of Hairy to repress transcription has been mapped to its WRPW motif and a novel transcriptional repression domain has been identified in Groucho (Fisher, Ohsako, and Caudy 1996). Therefore, the function of Hairy seems to be to bind N boxes in the target promoter and recruit Groucho which can then repress transcription of that gene, perhaps by interacting with the basal transcription machinery.

Another group of neurogenic genes in *Drosophila* is encoded by the *Enhancer of split complex [E(spl)-C].* The *E(spl)* locus encodes 13 transcripts, seven of which encode related bHLH proteins (*mγ, mβ, mδ, m3, m5, m7, and m8*) (Knust et al. 1987c, 1992; Ziemer et al. 1988; Klambt et al. 1989). Genetic analysis suggests that there is some functional overlap in the activity of the E(spl) proteins since targeted deletions of any one of these genes results in only a very

mild or even undetectable neurogenic phenotype Deletion of more than one *E(spl)* gene is required to obtain an obvious neurogenic phenotype and even then, the extent of neural hypertrophy corresponds to the number of *E(spl)* genes deleted (Delidakis et al. 1991; Schrons, Knust, and Campos-Ortega 1992). The expression patterns of the different *E(spl)* bHLH genes are very similar during *Drosophila* embryonic neurogenesis, consistent with the proposed functional redundancy of these genes (Knust et al. 1987, 1992). The proteins encoded by the *E(spl)-C* are Hairy- like transcription factors because in addition to a bHLH domain, they also contain a C-terminal WRPW motif such as the one found in Hairy. The effects of the E(spl) bHLH proteins on transcription likely involve interaction with the product of the neighbouring gene, *groucho*. Groucho binds specifically to certain bHLH proteins including those encoded by *hairy* and certain members of the *E(spl)-C* (Paroush et al. 1994). The gene products encoded by *E(spl)-C* are expressed in the ventral neurogenic region of *Drosophila* and expression is restricted to those ectodermal cells that surround a neuroblast in the process of delaminating (Jennings et al. 1994). There is no expression of E(spl) proteins in the neuroblasts themselves. The E(spl) transcription factors accumulate in response to Notch signalling and may function to temporarily maintain cells in an undifferentiated state so that they can respond to later differentiation signals (Jennings et al. 1994). Studies examining the effects on neurogenesis of an E(spl) derivative containing a heterologous transcriptional activation domain demonstrated ectopic transcription of the proneural genes *achaete* and *scute*

(Jimenez and Ish-Horowicz 1997). Mutations in the basic domain of this chimeric transcriptional activator that disrupt DNA binding also blocks its transcriptional activity. These results suggest that E(spl) proteins normally function as promoter-bound transcriptional repressors of *achaete* and *scute*.

During the initial characterization of the *E(spl)* locus, a second functional group encoded by the *m9/m10* transcripts was identified (Hartley, Preiss, and Artavanis-Tsakonas 1988; Delidakis et al. 1991). However, due to its non-redundant function and unique characteristics the *m9/m10* transcript is no longer considered a member of the *E(spl)-C* and has been given the name *groucho*. Early in development, *groucho* is ubiquitously expressed in the neurogenic region, an expression pattern which is consistent with its role in neurogenesis (Hartley, Preiss, and Artavanis-Tsakonas 1988). The *groucho* gene encodes a 771 amino acid protein which has some homology in its C-terminus to the β subunit of transducin. A repeat element known as the WD40 repeat element found at the C-terminal end of the Groucho protein is the region containing the homology. The WD40 repeat is a forty amino acid repeat element that ends with a tryptophan followed by an aspartate and is found four times in tandem at the end of the Groucho protein. The WD40 repeat element is also found in the general yeast repressor Tup1 and the cell cycle protein CDC4 where it is involved in mediating protein-protein interactions which are important in the normal function of these proteins (Yochem and Byers 1987; Williams and Trumbly 1990; Komachi, Redd, and Johnson 1994). As described earlier, Groucho acts as a corepressor for the

Hairy-like bHLH transcription factors. Using the yeast two hybrid system, a direct interaction has been shown between Groucho and Hairy-like transcription factors including Hairy, certain E(spl) proteins, and Deadpan (a sex determination gene) (Paroush et al. 1994). In conjunction with their corepressor Groucho, these Hairy-like transcription factors have diverse functions during embryonic development. Hairy represses the pair-rule gene *fushi-tarazu* during embryonic segmentation, the *E(spl)* gene products repress *AS-C* genes (such as *achaete* and *scute*) during neurogenesis, and Deadpan represses *sex lethal* during sex determination (Howard and Ingham 1986; Ish-Horowicz and Pinchin 1987; Campos-Ortega 1993).

1.1.3 The Notch Pathway

During *Drosophila* neurogenesis, groups of equipotent cells (proneural clusters) acquire the potential to become neural. The Notch pathway acts to restrict this neural fate to a single cell from each proneural cluster through a process of lateral inhibition (Ruiz-Gomez and Ghysen 1993). Proneural gene products, such as Achaete, are first expressed in these proneural clusters. The cell which accumulates the highest levels of Achaete expression corresponds to the developing sensory organ precursor cell (Cubas et al. 1991; Skeath and Carroll 1991). Disruption of this lateral inhibitory signal through loss of Notch function leads to extreme hypertrophy of the embryonic nervous system because all the cells of a proneural cluster adopt a neural fate (Brand and Campos-Ortega 1988). Conversely, expression of an activated form of the Notch protein prevents all the

cells of a proneural cluster, including the presumptive sensory organ precursor cell, from adopting a neural cell fate (Lieber et al. 1993).

At the beginning of neurogenesis in *Drosophila melanogaster*, all the cells of a proneural cluster express Delta, the ligand for Notch, on their cell surface (Kopczynski and Muskavitch 1989; Haenlin, Kramatschek, and Campos-Ortega 1990). It is possible that small differences in the amount of Delta expressed by neighbouring cells accounts for some cells becoming neurons while their neighbours assume the secondary epidermal fate. Ligand binding is thought to expose the intracellular domain of Notch to proteolytic processing such that an intracellular fragment of Notch is released into the cytoplasm (Kopan et al. 1998; Schroeter, Kisslinger, and Kopan 1998). This intracellular fragment is then translocated to the nucleus where it is thought to bind and activate the DNA-binding protein, Suppressor of Hairless [Su(H)] (Tamura et al. 1995). Su(H) has been shown to be retained in cytoplasm through direct interaction with the ankyrin repeats located in the intracellular domain of Notch. (Fortini and Artavanis-Tsakonas 1994). Upon ligand binding and Notch activation, Su(H) is released and localised in the nucleus (Fortini and Artavanis-Tsakonas 1994). Thus, the proteolytically processed form of Notch may travel to the nucleus as a complex with Su(H). In response to ligand binding, Su(H) binds to the promoter regions of the *Enhancer of split complex [E(spl)-C]* and activates transcription (Bailey and Posakony 1995). The Hairy-like bHLH transcription factors encoded by the *E(spl)-C* then act with their corepressor Groucho to negatively regulate

transcription of the proneural genes. A feedback loop exists in which the proneural gene product, Achaete, activates transcription of *Delta* (Heitzler et al. 1996). Thus cells in which the Notch pathway has been activated turn off expression from the proneural genes and, as a result, express decreasing amounts of the ligand Delta on their cell surface. Activation of the Notch pathway thereby directs the cell to assume a non-neural cell fate. The lateral inhibition process mediated by the neurogenic genes of the Notch signalling pathway can be seen as the restriction of expression of genes of the *achaete-scute complex* which are initially expressed in every cell of a proneural cluster but, as a result of the action of the neurogenic genes, continue to be expressed only in the neuronal precursor cell (Cubas et al. 1991).

1.2 The Mammalian Notch Pathway

1.2.1 Conservation of the Notch Pathway From Invertebrates to Vertebrates

Many members of the mammalian Notch signalling pathway have been identified based on their conservation from *Drosophila*. Four homologues of Notch have been isolated in both mouse and humans (Lardelli and Lendahl 1993; Lardelli et al. 1994; Weinmaster et al. 1991, 1992; Stifani et al. 1992). Mammalian Notch homologues are expressed in many tissues early in development and in proliferating cell layers later on in development (Coffman, Harris, and Kintner 1990; Weinmaster et al. 1991, 1992). Thus the expression

pattern of vertebrate homologues of Notch is very similar to Notch expression in *Drosophila*. In *Drosophila*, Notch functions in the neural-epidermal cell fate decision of neural competent cells. However, in vertebrates Notch appears to be involved in a decision between becoming a fully differentiated post-mitotic neuron or remaining in a proliferative state (Coffman et al. 1993; Nye et al. 1994). Vertebrate homologues of Notch ligands have also been isolated in Xenopus (Chitnis et al. 1995), rat (Lindsell et al. 1995), and mouse (Bettenhausen et al. 1995). The vertebrate Notch ligands closely resemble the *Drosophila* ligands Delta and Serrate. Also, like their fly counte`parts, functional analysis of the vertebrate homologues of Delta indicate that they are involved in a lateral inhibition pathway during neurogenesis (Chitnis et al. 1995; Lindsell et al. 1995). *Suppressor of Hairless [Su(H)]* is represented in mammals by a single gene, *RBP-Jκ (Recombination signal sequence binding protein for Jk genes)*. *Hes1*, a mammalian homologue of the *Hairy and Enhancer split* genes, has been shown to contain binding sites for RBP-Jk in its regulatory sequences (Jarriault et al. 1995) and the binding of a RBP-Jk/Notch-IC (constitutively active intracellular domain of Notch) complex to these binding sites has been shown to activate transcription of *Hes1* (Jarriault et al. 1995). A truncated, active form of human Notch1 has also been shown to bind RBP-Jκ and to activate transcription through a RBP-Jκ-responsive promoter (Lu and Lux 1996). The bHLH transcriptional repressors encoded by *hairy* and the *Enhancer of Split Complex* are conserved in mice as the *Hes (Hairy/Enhancer of split)* gene family consisting of Hes1 to Hes 5.

Mammalian homologues of the corepressor Groucho have also been identified in several species. A family of murine *groucho* homologous genes named *Grg (groucho related gene)*, a rat family of *groucho* homologous genes named *Resp (rat enhancer of split)*, and a family of human *groucho* homologous genes named *TLE (transducin like element)* have been identified. Two murine (Franco del Amo et al. 1993; Guillemot et al. 1994), two rat (Johanson et al. 1990), and a human (Ball et al. 1993) homologue of the *Drosophila AS-C* genes have been isolated and named *MASH (mammalian achaete-scute homologue)*. Like their *Drosophila* counterparts, the *MASH* gene products form heterodimers with E12 and E47, the vertebrate homologues of the ubiquitous proneural gene *daughterless,* and activate transcription by binding to E-boxes (Johanson et al. 1992a). However, unlike the *Drosophila AS-C* genes, the gene products of *MASH1* and *MASH2* are insufficient in promoting neurogenesis (Johanson et al. 1990, 1992a; Ferreiro et al. 1994). The expression of the vertebrate homologues of the proneural and neurogenic genes suggest a conserved role for these genes in neurogenesis but their precise function in these processes may not have been conserved from invertebrates to vertebrate.

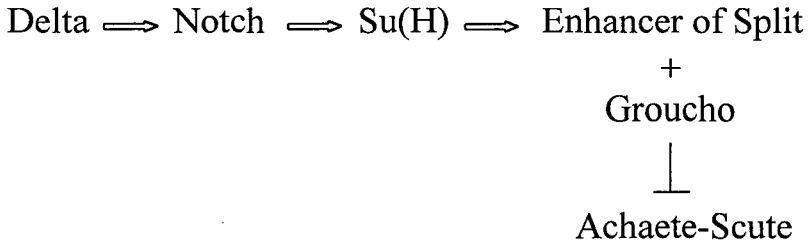
1.2.2 The Groucho-Related Gene (Grg) Family

Five mouse homologues of the *Drosophila groucho* gene have so far been identified (Figure 1.2). Three of these Groucho-related genes products (Grg), Grg1, 3, and 4 contain all five domains that are found in Groucho. At the amino

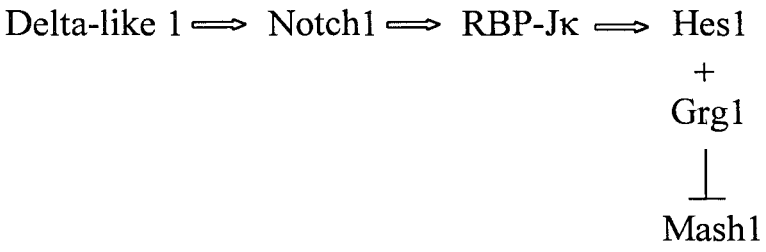
Figure 1.1 The Notch Signalling Pathway

The proteins acting in the Notch signalling pathways of *Drosophila melanogaster* and mammals are shown in figures A and B, respectively. The members of the Notch pathway in *Drosophila* are Delta, Notch, Suppressor of Hairless [Su(H)], Enhancer of Split, Groucho, and Achaete-Scute. The proteins acting in the mammalian Notch pathway include the Delta-like protein 1, Notch1, Recombination signal-binding protein Jκ (RBP-Jκ), Hairy/Enhancer of Split 1 (Hes1), Groucho-related gene 1 (Grg1), and mammalian achaete-scute homologue 1 (Mash1).

A


Delta
Notch
Su(H)
Enhancer of Split
+
Groucho
Achaete-Scute

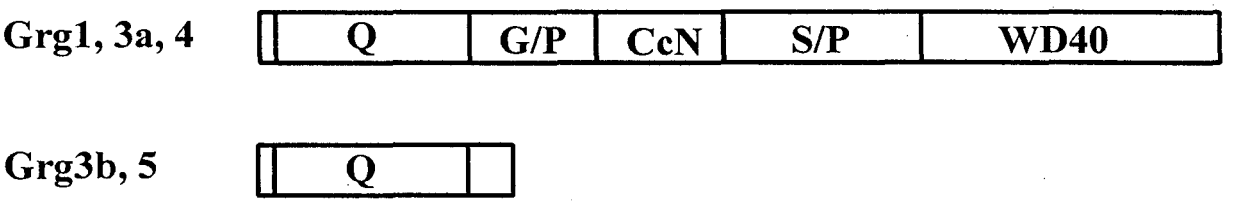
B


Delta-like 1
Notch1
RBP-Jκ
Hes1
+
Grg1
Mash1

terminal end of the mammalian Grg proteins is a glutamine-rich Q domain that has been shown to be necessary for dimerization between Grg proteins (Pinto and Lobe 1997) The Q domain is followed by a glycine/proline-rich (G/P) domain and the CcN domain which contains a putative nuclear localisation signal followed by consensus sequences for phosphorylation by casein kinase II and cdc2 kinase (Jans et al. 1991). Phosphorylated forms of Groucho and TLE1 (human paralogue of Grg1) exhibit a higher affinity for the nuclear compartment than faster migrating forms. These studies also showed that the slower migrating nuclear form of TLE1 is induced during neural determination of P19 embryonic carcinoma cells. Phosphorylation of Groucho and its mammalian homologues may be important for the nuclear function of these proteins (Husain et al. 1996). The fourth domain found in Grg proteins is a serine/proline-rich (S/P) region which, in Groucho, is the domain responsible for interaction with Hairy (Paroush et al. 1994). The S/P domains are not well conserved between mouse Grg family members, but are quite conserved between mouse and human paralogues suggesting that each Grg protein may have different specificities for binding to Hairy-like transcription factors. The fifth domain of Grg proteins consists of a WD40 domain which was first described in the β subunits of G proteins (Fong et al. 1986). The WD40 domain consists of four tandem repeats of a conserved sequence of 40 amino acids and is thought to be involved in protein-protein interactions.

Figure 1.2 The domains of Grg proteins.

The long Grg proteins, Grg1, Grg3a, and Grg4 contain a Q dimerization domain, a glycine/proline-rich (G/P) domain, a CcN domain containing a nuclear localisation signal, a serine/proline-rich domain, and a WD40 domain. The truncated Grg proteins, Grg3b and Grg5, contain only an amino terminal Q domain and part of the G/P domain.


Grg1, 3a, 4
Q
G/P
CcN
S/P
WD40
Grg3b, 5
Q

A gene encoding a truncated mouse homologue of *groucho*, known as *Grg5*, is also known to exist (Mallo, Franco del Amo, and Gridley 1993). Furthermore, one of the mouse *groucho-related genes, Grg3*, encodes both a long (Grg3a) and a short (Grg3b) protein (Leon and Lobe 1996). The short Grg5 and Grg3b proteins contain only the Q domain and part of the G/P domain. Despite the fact that Grg5 lacks the lysine-arginine-rich nuclear localisation signal found in the long Grg proteins, it has been shown by immunofluorescence analysis to be localised to the nucleus (Mallo et al. 1995). Since the short Grg proteins lack an S/P domain with which to interact with Hairy-related transcription factors but are still able to dimerize with the long Grg proteins (Pinto and Lobe 1997) through their intact Q domain, their function may be to regulate the activity of the long Grg proteins.

Resp 2, the rat homologue of Grg4, has a 25 amino acid gap in the region of the Q domain required for Grg dimerization (Schmidt and Sladek 1993). So far, all isolated Grg4 cDNAs extend only into the G/P domain (Koop, MacDonald, and Lobe 1996), so it is not known if a similar gap exists in the Q domain of Grg4. However, if Grg4 is lacking some of the sequence required for Grg dimerization, it may be free of any interaction with Grg 5 or Grg3b and therefore may not be subject to the same regulation as the other full-length Grg proteins.

1.2.3 Expression Patterns of Grg Proteins

Immunohistochemistry has revealed that TLE2, the human homologue of Grg2, is strongly expressed in areas of the developing brain and spinal cord

containing postmitotic neurons and, to a lesser degree, in regions containing neural progenitor cells (Koop, MacDonald and Lobe 1996; Grbavec et al. 1998). This may indicate a more predominant role for TLE2 in functions specific to the maturation and survival of postmitotic neurons rather than the differentiation of progenitor cells. In contrast, TLE1 and TLE3 are strongly expressed in neural progenitor cells suggesting a more important role for these proteins in the differentiation of progenitor cells (Dehni et al. 1995; Yao et al. 1998). TLE2 is co-expressed with Hes1 and Hes5 in cells of the developing mammalian nervous system (Grbavec et al. 1998).

In situ hybridisation analysis of *Grg4* expression revealed that *Grg4* is detected at 8.5 dpc (days post conception) in the rostral forebrain and the roof of the midbrain (Koop, MacDonald, and Lobe 1996). The expression of *Grg4* in the developing nervous system may reveal a role for *Grg4* in neuronal differentiation. *Grg4* expression overlaps with the expression of other mouse neurogenic genes such as *Grg3, Notch1*, and *Hes1* in proliferating epithelial tissues undergoing mesenchymal induction such as the salivary gland, lung and kidney epithelia, and the neural layer of the retina (Koop, MacDonald, and Lobe 1996). *Grg4* transcripts were detected in somites, but in cells adjacent to those cells expressing *Grg3, Notch1*, and *Hes1*. This may suggest a role for Grg4 in later stages of cell differentiation than for other mouse neurogenic genes (Koop, MacDonald, and Lobe 1996).

Grg5 RNA expression in mouse embryos and adults is first detected at 8.5

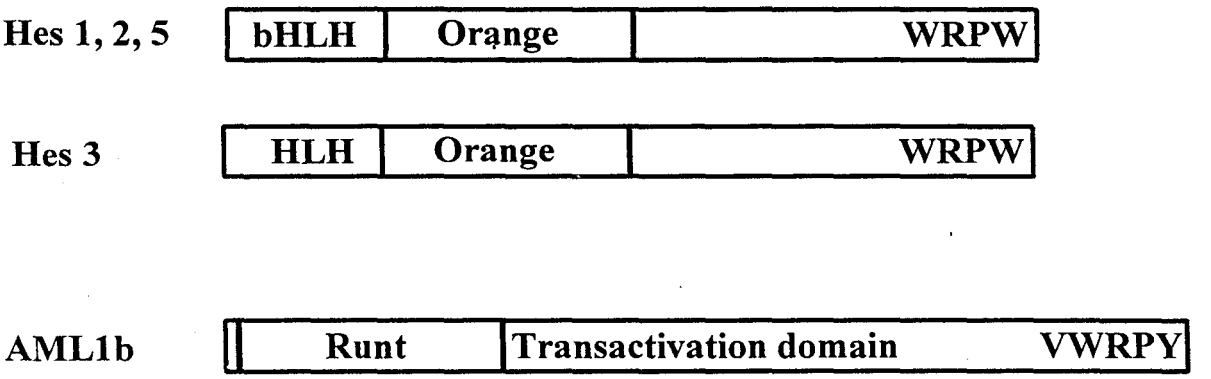
dpc in the yolk sac, the ventral floor of the fore- and hindgut, and the surface ectoderm region of the foregut (Mallo, Franco del Amo, and Gridley 1993). By 10.5 dpc *Grg5* RNA is ubiquitously expressed at different levels in all regions of the embryo with the exception of skeletal elements undergoing ossification. *Grg5* RNA is also widely expressed in the tissues of adult mice. *Grg5* appears to be expressed in a spatial and temporal progression that approximately coincides with organogenesis, suggesting that *Grg5* may play a role in the establishment or maintenance of the differentiated state (Mallo, Franco del Amo, and Gridley 1993).

1.2.4 The Hairy-Enhancer of Split (Hes) Gene Family

The *Hairy-Enhancer of Split (Hes)* family of genes consists of five mammalian homologues of the *Drosophila hairy* and *enhancer of split* genes (Figure 1.3). Like their counterparts in *Drosophila*, the *Hes* gene products are bHLH transcription factors which have a unique WRPW motif at their carboxy terminus (Figure 1.3). One member of the Hes family, Hes3, is lacking the basic domain at the amino-terminal end of the protein. Another domain present in Hes proteins is the loosely conserved Orange domain. This domain may confer specific interactions with E-box binding bHLH proteins (Heitzler et al. 1996). The Hes proteins are negative regulators of neurogenesis that can repress transcription from target promoters by binding to N boxes having the consensus sequence CACNAG (Sasai et al. 1992).The Hes1 proteins is capable of negatively

Figure 1.3 The Hairy/Enhancer of Split (Hes) and AML1b protein domains

The Hes proteins, Hes1, Hes2, and Hes5 have an amino terminal basic-helix-loop-helix (bHLH) domain, an Orange domain, and a C-terminal tryptophan-arginine-proline-tryptophan (WRPW) motif. The Hes3 protein lacks the basic domain but retains the HLH domain, the Orange domain and the WRPW motif. The Runt domain protein AML1b contains a DNA-binding Runt domain and a C-terminal valine-tryptophan-arginine-proline-tyrosine (VWRPY) motif.


Hes 1, 2, 5
bHLH
Orange
WRPW
Hes 3
HLH
Orange
WRPW
AML1b
Runt
Transactivation domain
VWRPY

autoregulating its own expression by directly binding to the multiple N-box elements present in its promoter (Takebayashi et al. 1994). *In vitro* assays have also shown that Hes proteins are capable of repressing transcription through E boxes (CANNTG), independently of N box binding. Although Hes proteins, with the exception of Hes2, have only a weak affinity for binding E boxes (Sasai et al. 1992), they are thought to repress E-box mediated transcription by forming non-functional heterodimers with E box-binding transcriptional activators, such E47, the mammalian homologue of Daughterless. Hes1 can antagonise the activity of the neural bHLH activators MASH1 and MATH1, mammalian homologues of the products of the *Drosophila* proneural genes *achaete-scute* and *atonal*, respectively, *in vitro* by preventing the latter from binding to E-boxes (Sasai et al. 1992; Akazawa et al. 1995). Hes proteins have also been shown to repress the myogenic factor MyoD using *in vitro* transcriptional assays (Sasai et al. 1992). Hes3 lacks the amino terminal basic domain present in other Hes proteins and therefore cannot bind N boxes to repress transcription. However, Hes3 is still capable of efficiently repressing transcriptional activation by E47 from an E box reporter *in vitro* (Sasai et al. 1992).

The ability of Hes proteins to repress transcription has been mapped to a conserved carboxy-terminal WRPW motif (Fisher, Ohsako, and Caudy 1996). This WRPW motif has been shown to be necessary and sufficient for interactions with the *Drosophila* Groucho protein (Paroush et al. 1994). Recently, interactions between the mammalian homologues Hes1 and TLE1 have been demonstrated

(Grbavec and Stifani 1996). Although Groucho and its mammalian homologues have no DNA-binding activity themselves, they are able to actively repress transcription when directly bound to a target promoter (Fisher, Ohsako, and Caudy 1996). Thus, the WRPW motif of the Hes proteins functions as a four amino acid protein interaction domain which serves to recruit the active transcriptional corepressors.

The Hes proteins have a conserved role in neurogenesis but appear to play a role in regulating neural differentiation rather than a role in determining a neural versus epidermal fate as in *Drosophila*. Introduction of a viral vector expressing Hes1 into cells of the central nervous system prevented neural and glial differentiation (Ishibashi et al. 1994). Targeted disruption of the *Hes1* gene in mice produces a lethal phenotype with severe abnormalities in the CNS, the most striking being the failure of the neural tube to close, apparently due to premature differentiation of neurons (Ishibashi et al. 1995). Hes1 therefore seems to be involved in delaying neuronal differentiation rather than selection of a non-neural cell fate. Hes1 also appears to play a role in myogenesis by inhibiting the function of MyoD (a myogenic bHLH transcriptional activator) and diminishes MyoD-induced myogenic conversion of C3H/0T1/2 cells (Sasai et al. 1992). Thus, in mammals, the proteins acting at the end of the Notch pathway may function to keep cells in an undifferentiated state so that they can respond to later differentiation signals.

1.2.5 Expression Patterns of Hes Proteins

The expression of patterns of the Hes genes are consistent with a conserved role in the Notch signalling pathway. Hes1, which shows the highest sequence homology to the *Drosophila hairy* gene product is the only family member that is widely expressed in both embryos and adults (Feder, Jan, and Jan 1993). In the developing CNS, *Hes1* is highly expressed in neural progenitor cells in the ependymal zone and is also found in epithelial tissues undergoing mesenchymal induction (Sasai et al. 1992). The epithelial cells of embryonal and adult respiratory and digestive organs express high levels of *Hes1*. *Hes1* is also highly expressed in the ventricular zone of the embryonal nervous system, where neural precursors proliferate. However, the expression of *Hes1* decreases to a low level in adult muscles and the differentiated nervous system (Sasai et al. 1992). The highest levels of *Hes1* expression are detected throughout the ventricular zone where the neural precursor cells are located. However, *Hes1* is not detected in the outer layers where differentiated neurons and glial cells are present. The observation that *Hes1* expression appears to negatively correlate with neural differentiation led to the proposal that *Hes1* may negatively regulate the development of the mammalian CNS. *Hes5* is also expressed in the ventricular zone of the CNS where it may play a similar role in negatively regulating neurogenesis (Takebayashi et al. 1995).

Hes3 has an expression pattern that is distinct from the other *Hes* genes.

Hes3 was initially thought to be expressed only in cerebellar Purkinje cells between 6 and 11 days after birth (Sasai et al. 1992). However, recent analysis of *Heq3* expression has shown that *Hes3* is also expressed during embryogenesis (Lobe 1997). By 8.5 dpc, *Hes3* is highly expressed in the region of the mesencephalic/metencephalic boundary and in two bands in the hindbrain corresponding to rhombomeres 1, 2, 4, 6, and 7. This pattern of expression suggests that *Hes3* may have a role in midbrain and hindbrain patterning and may reflect a conserved role in segmentation. *Grg3* has an overlapping expression domain in the midbrain and hindbrain and could potentially interact with *Hes3* in that region. *Hes3* transcripts were also detected later in embryogenesis in epithelial tissues and overlapped with the expression of other neurogenic genes which suggests a later role for *Hes3* in regulating cell differentiation.

1.3 The Acute Myeloid Leukemia (AML) Proteins

The AML transcription factor complex is composed of both an alpha and a beta subunit. The alpha subunit contains a Runt domain which confers the ability to heterodimerize with the beta subunit and to bind DNA. The beta subunit is thought to increase the DNA binding affinity of the alpha subunit. Three separate genes encoding the alpha subunit (*AML1, 2, 3*) have been identified in both humans and mice (Bae et al. 1993, 1994, 1995). The *AML1* gene product is highly homologous to the *Drosophila* transcription factor Runt, which regulates segmentation and cell determination during embryogenesis. Three distinct AML1

proteins have been identified (AML1a, AML1b, and AML1c). AML1b and AML1c differ significantly from AML1a in their C-terminal regions. Although all three proteins contain the Runt domain, AML1b and AML1c contain an additional large C-terminal region which is suggested to be a transcriptional activation domain (Miyoshi et al. 1995). Another interesting feature of AML1b and AML1c is that they contain a conserved Val-Trp-Arg-Pro-Tyr (VWRPY) motif at the C-terminal end of the protein. The *Drosophila* Runt protein acts in conjunction with Hairy to regulate segmentation during fly development and both Runt and Hairy contain very similar C-terminal sequences (VWRPY and WRPW, respectively). In mammals, AML1b has been reported to regulate the transcription of various genes important for hematopoiesis such as those for myeloperoxidase, neutrophil elastase, and the receptor for macrophage colony-stimulating factor (Nuchprayoon et al. 1994). Mice lacking AML1b die during midembryonic development due to the complete absence of liver-derived hematopoiesis (Niki et al. 1997).

1.4 The Yeast Two Hybrid System

The yeast two hybrid system is a genetic assay used to detect protein-protein interactions *in vivo* (Bartel et al. 1993). The yeast two hybrid system is useful because it is sensitive enough to detect weak or transient protein interactions. Also, because it is an *in vivo* assay, the proteins are more likely to be in their native conformations.

The two hybrid system makes use of the fact that GAL4 and other eukaryotic transcriptional activators consist of two physically discrete modular domains (Keegan, Gill, and Ptashne 1986). One of these domains acts as the DNA-binding domain, while the other acts as a transcriptional activation domain. The DNA binding domain is responsible for recognizing specific sequences in the region upstream of target genes, while the activation domain interacts with components of the basal transcription machinery in order to initiate transcription of the target gene. Normal transcription of target genes requires the presence of both domains, which are normally present in the same protein. However, using recombinant DNA technology, it has been shown that a functional transcriptional activator can be assembled *in vivo* from separated domains of the same or unrelated transcription factors (Ma and Ptashne 1988).

In order to detect an interaction between two proteins, the gene encoding protein (A) is subcloned into an expression vector containing the sequence for the GAL4 DNA-binding domain in order to create a fusion protein containing the GAL4 DNA-binding domain and the target protein. A second hybrid cloning vector is used to generate a fusion of the GAL4 activation domain and a potentially interacting protein (B). Both hybrid vectors are introduced into yeast and cotransformants are selected on the appropriate synthetic minimal medium. If proteins A and B interact with each other, then the GAL4 DNA-binding domain will be tethered to the GAL4 activation domain and a functional transcriptional activator will be reconstituted. Reconstitution of the GAL4 transcriptional

activator is indicated by transcription of a *lacZ* (or *HIS3*) reporter gene containing upstream GAL4 binding sites. Cotransformant colonies can therefore be assayed for β-galactosidase activity and if protein A and protein B interact, the colonies will stain blue.

1.5 Experimental Goal

The purpose of this study was to gain insight into the mechanism by which Grg (Groucho related gene) and Hes (Hairy/enhancer of split) proteins function at the end of the Notch signalling pathway to establish cell fate during embryonic development. Five Grg proteins (including an alternate splice) have been identified in mice. Three of the five Grg proteins (Grg1,3a,4) contain all five domains found in their *Drosophila* counterpart, Groucho. These domains include the Q dimerization domain, a G/P (glycine/proline-rich) domain, a CcN domain containing a nuclear localisation signal, an S/P (serine/proline-rich) domain, and a WD40 domain. Four murine Hes proteins have so far been identified. All have DNA binding capabilities with the exception of Hes3, which lacks the basic domain required to make sequence specific contacts with DNA. All four Hes proteins have a conserved WRPW motif at their C-terminus. In *Drosophila*, the DNA-binding transcription factor Hairy has been shown to bind N boxes in the promoter region of target genes and recruit its corepressor Groucho thereby forming a complex capable of repressing transcription. The interaction between Hairy and Groucho has been shown to require both the highly conserved WRPW

motif of Hairy and the S/P domain of Groucho (Paroush et al. 1994). Since the function of these proteins and the domains required for this interaction have been conserved from invertebrate to vertebrate, it is reasonable to postulate that there is an interaction between Hes and Grg proteins. However, since the S/P domain of the Grg proteins is not well conserved between family members but is well conserved between mouse and human paralogues, it may be that the Grg proteins have varying affinities for binding to different Hes family members. My thesis attempts to test this theory by asking some basic questions. Can Grg and Hes proteins interact? If so, do Grg proteins bind different Hes family members with varying affinities? Can Grg proteins interact with Hes-like proteins such as AML1b which contains a C-terminal VWRPY motif? Answers to these questions will provide an initial assessment for the possibility of the presence of additional levels of regulation of Grg proteins by limiting the number of Hes proteins with which they can interact. This study is intended to provide insight into the mechanisms of transcriptional repression used by Hes proteins via their interaction with Grg proteins.

II. MATERIALS AND METHODS

2.1 Bacterial Culture Conditions

All plasmids were propagated in the rec- E. coli. strain DH5-α. Bacterial cultures were grown with agitation at 37 °C in YT, which was composed of 8 g/L bacto-tryptone, 5 g/l bacto-yeast extract, and 5 g/L NaCl. For drug resistance selection, ampicillin or kanamycin were added to a final concentration of 100 μg/ml or 30 μg/ml, respectively. In the case of large 200 ml cultures, a single colony was used for inoculation and the culture was grown, with agitation, for 12 to 16 hours. Single colonies were obtained by spreading bacteria over a YT/agar plate, composed of 8 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 5 g/L NaCl, and 15 g/L bacto-agar, which was incubated at 37 °C overnight or until colonies appeared. For drug resistance selection, the plates were made with ampicillin to a final concentration of 100 μg/ml or with kanamycin to a final concentration of 30 μg/ml.

2.2 Cloning Techniques

2.2.1 Restriction Enzyme Digests

The cloning techniques utilised are described in Sambrook et al. (1989). The restriction enzymes used were largely supplied by Boehringer Mannheim (BM)

New England BioLabs (NEB) and MBI Fermentas (MBI). DNA was digested by restriction enzyme in the buffer supplied by the manufacturer and following their recommended conditions.

2.2.2 Creating Blunt-Ended DNA Fragments

In cases where a 5' overhang needed to be converted into a blunt end, the Klenow fragment of DNA polymerase I (NEB) was used to fill in the protruding end. The reaction was carried out in the presence of 1 mM deoxynucleotides, in 1X Klenow buffer (10 mM Tris-HCl, 5 mM $MgCl_2$, 7.5 mM DTT, [pH 7.5 @ 25 °C]) and with 1 unit of Klenow per microgram of DNA (approximately 1 unit of Klenow per 5 x 10^{-13} moles of ends). After a 30 minute incubation at room temperature, the enzyme was removed by extraction with one volume of phenol:chloroform:iso-amyl alcohol (PCI, 25:24:1), and the DNA was precipitated by the addition of NaOAc and ethanol to a final concentration of 0.3 M and 70 % respectively.

In situations where a 3' overhang needed to be converted into a blunt end, T4 DNA polymerase (NEB) was used to remove the overhang, followed by a Klenow fill-in reaction to repair any excessive exonuclease activity. The initial T4 DNA polymerase reaction was carried out in 2 mM deoxynucleotides, 50 µg/ml BSA, 1X T4 DNA polymerase buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, [pH 7.9 @ 25 °C]), and with 1 unit of T4 DNA polymerase per µg of DNA (approximately 1 unit of T4 DNA polymerase per 5 x 10^{-13} moles of ends).

After a 20 minute incubation at 12 °C, the enzyme was removed by extraction with PCI and the DNA was recovered by ethanol precipitation. The DNA was then subjected to a Klenow fill-in reaction as described above.

2.2.3 Adapting DNA Ends with Linkers

Complimentary linkers (NEB) used for cloning were first annealed by heating them at 55 °C for 5 minutes in 1X ligation buffer (66 mM Tris-HCl [pH 7.6], 6.6 mM $MgCl_2$, 10 mM DTT) supplemented with 50 mM NaCl, and then allowing them to cool slowly to room temperature. Linkers and oligonucleotides not already phosphorylated were then treated with T4 polynucleotide kinase (Pharmacia). The kinase reaction was carried out in 1X ligation buffer supplemented with 50 mM NaCl, 1 mM dATP, and 1 unit of T4 polynucleotide kinase per mg of DNA (1 unit T4 polynucleotide kinase per 6.3×10^{-11} moles of ends for a 12 bp oligonucleotide). The reaction was allowed to continue for 30 minutes at 37 °C.

After phosphorylation, the linkers were ligated to blunt DNA fragments at a molar ratio of termini of approximately 100 (linker) to 1 (DNA fragments). The ligation reactions were performed in a 20 µl reaction volume in 1X ligation buffer supplemented with 1 mM dATP and 1 unit of T4 DNA ligase. Ligation reactions were left for 6 hours to overnight at 14 °C after which time an aliquot was removed and visualizedd on a 1 % agarose gel in order to determine if the ligation reaction was successful in producing a ladder of ligated DNA fragments. The rest

of the ligation reaction was expanded to 100 µl with water, and the ligated linker-DNA fragments were digested with the appropriate restriction enzyme (1 unit restriction enzyme per 0.5 µg of DNA) in order to remove oligomers and create the appropriate sticky end. The DNA was then purified twice by gel electrophoresis through an agarose gel. DNA fragments that were fractionated on a gel were purified using the QIAEX II gel extraction kit (QIAGEN) or by electroelution for one hour at 100 mV followed by PCI extraction and ethanol precipitation to recover the DNA.

Before ligating the DNA fragments into the appropriate vector, the linearized vector was treated with calf intestinal phosphatase (CIP) to prevent self-ligation. 1 to 3 µg of digested vector was mixed with 5 µl of 10X CIP buffer (500 mM Tris-HCl, 1 mM EDTA, [pH 8.5 @ 20 °C]) and 5 units of CIP (BM) in a total volume of 50 µl and incubated for one hour at 37 °C. The CIP was removed by PCI extraction followed by ethanol precipitation to recover the DNA. DNA fragments were ligated to CIP-treated vector at an approximate molar ratio of termini of 50 (DNA fragments) to 1 (vector). Generally, the amount of vector used in the ligation reactions was between 10 and 30 ng. After ligation at 14 °C overnight, an aliquot was visualizedd on an agarose gel to ensure that the ligation reaction worked, and the rest of the reaction volume was expanded to 100 µl and used to transform competent bacteria.

2.3 Preparation of Competent Cells

Frozen stocks of competent DH5-α cells were prepared by the calcium chloride method. An overnight bacterial culture was added to 500 ml of YT to an OD_{600} of 0.1. The cells were grown to an OD_{600} of 0.375, collected into 50-ml Falcon tubes, and incubated on ice for 10 minutes. The cells were then spun in a centrifuge at 1100 x g for 15 minutes at 4 °C, and the pellet was resuspended in 1/15 volume of an ice-cold solution of 60 mM $CaCl_2$, 10 mM Pipes [pH 7.0], and 15 % glycerol. The cells were aliquoted into microcentrifuge tubes, snap frozen in liquid nitrogen, and stored at -70°C.

2.4 Transformation of Competent Cells

Competent DH5-a cells were thawed on ice for approximately 10 minutes. 1 ng of intact plasmid DNA (for propagation) or 200 ng of DNA from a ligation reaction was mixed with 100 µl of cells and incubated on ice for 25 minutes. The cells were then heat shocked by incubation at 42 °C for 90 seconds and placed back on ice for 2 minutes. 500 µl of YT was added and the mixture was incubated for one hour at 37 °C. Meanwhile, sufficient YT/Amp plates were placed at 37 °C to warm and dry. After the incubation, the cells were pelleted by centrifugation at 13,000 x g for 30 seconds and 500 µl of the supernatant was removed. The pellet was resuspended by gentle pipetting and spread over a pre-warmed YT/Amp plate. The plates were incubated at 37 °C for 16 hours or until colonies appeared and could then be stored at 4 °C for several months.

2.5 Colony Hybridisation

Colony hybridisation experiments were performed to identify potential recombinant plasmids from a plate of transformed competent cells. A Hybond-N membrane (Amersham, 82 mm) was labelled asymmetrically on 3 sides with India ink and placed, labelled-side down, on a bacterial plate containing small colonies (< 1 mm) for one minute while the labels were traced onto the plate. The membrane was removed and placed colony-side up on a fresh, pre-warmed YT/Amp plate and incubated at 37 °C for 3 to 4 hours. If required, the master plate was also incubated at 37 °C to allow the colonies to regrow and was thereafter stored at 4 °C. After the incubation period, the membrane was placed colony-side up on Whatmann 3MM paper soaked with 10 % sodium docedyl sulfate (SDS) for 5 minutes, followed by 5 minutes on Whatmann 3 MM paper soaked with Denature (1.5 M NaCl, 0.5 M NaOH), followed by 10 minutes on Whatmann 3 MM paper soaked with Neutralize (1.5 M NaCl, 0.5 M Tris-HCl [pH 7.4], 1 mM EDTA). The membrane was then allowed to air-dry for 20 minutes before being baked for 90 minutes at 70 - 80 °C. The membrane was washed twice for 10 minutes at 50 °C in Prewash (20 mM Tris-HCl [pH 8.0], 1 M NaCl, 1.25 mM EDTA, 0.1 % SDS) and any bacterial debris that remained was wiped off the membrane. The membrane was then incubated for one hour at 50 °C in 50 ml of hybridisation solution (600 mM NaCl, 1.20 mM Tris-HCl [pH 7.5], 4 mM EDTA, 1 % SDS). The appropriate ^{32}P-labelled probe was added to the hybridisation solution at 100,000 cpm/ml (for a total of 5 μCi) and the

hybridisation was allowed to continue overnight at 50 °C. The next day, the membrane was washed three times for 20 minutes at 50 °C with a solution of 2X SSC (300 mM NaCl, 3 mM sodium citrate [pH 7.0], and 1% SDS). The membrane was then exposed to Kodak XAR-5 film for 3 hours at –70 °C. Positive clones were identified by aligning the developed film with the labelled membranes and subsequently with the corresponding labelled master plate.

2.6 Preparation of ^{32}P-labelled Probes

The Random Primers DNA Labelling System (BRL) was used to prepare the ^{32}P-labelled probes used in colony hybridisation experiments. 100 ng of the appropriate DNA fragment was dissolved in 6 µl of water and then denatured by heating at 95 °C for 5 minutes, spinning in the centrifuge for 15 seconds, and cooling immediately on ice. 2.2 µl of GAT mix (containing 0.5 mM each of dGTP, dATP, and dTTP) was added, followed by 4 µl (40 µCi) of (alpha-^{32}P)-dCTP (Dupont) and 7 µl of 3X Random Primers Buffer Mixture. The 3X Random Primers Buffer Mixture was made from 50 µl of 2X Random Primer Buffer (1.34 M Hepes, 0.34 M Tris, 34 mM $MgCl_2$, 66 mM 2-mercaptoethanol, 2.66 mg/ml BSA), 30 µl of water, and 20 µl of Random Primers (Promega). 4 units of Klenow was then added and the reaction was allowed to continue for two hours at room temperature.

After the two hour incubation, the probe was passed through a Sephadex G-50 spin column to remove unincorporated deoxynucleotides. The spin column

was made by plugging a 1 cc syringe with a small amount of siliconized glass wool and then filling the syringe with Sephadex G-50 equilibrated in TE (10 mM Tris-HCl [pH 7.6], 1 mM EDTA [pH 8.0]). The syringe was then placed in a 14 ml snap-cap tube and spun in the centrifuge for 1 minute at 200 x g to pack the column. The syringe was washed with 100 µl of STE (100 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]) and centrifuged for 1 minute at 200 x g. After inserting a microcentrifuge tube underneath the column to collect the radiolabeled DNA, the volume of the probe was brought up to 100 µl with water and the entire volume was added to the spin column. The column was then spun in the centrifuge for 1 minute at 200 x g. The probe that was collected in the microcentrifuge tube was transferred to a fresh microcentrifuge tube tube and a 1 µl sample was counted.

2.7 Purification of Plasmid DNA

2.7.1 Small Scale Preparations

Rapid plasmid preparations were used to identify recombinant plasmids. Single colonies from a bacterial plate transformed with a ligation reaction were used to inoculate 2 ml of YT/Amp and grown overnight. The overnight cultures were transferred to microcentrifuge tubes and centrifuged at 13,000 x g for one minute. The supernatant was poured off and the pellet was resuspended in 100 µl of solution 1 (50 mM glucose, 25 mM Tris-HCl [pH 8.0], 10 mM EDTA).

200 µl of solution 2 was added (1 % SDS, 200 mM NaOH) and the tubes were gently mixed by inversion. 150 µl of solution 3 (3 M KOAc, 11.5 % glacial acetic acid) was immediately added and mixed by vigorous shaking. The tubes were then centrifuged for 5 minutes at 13,000 x g and the supernatant was poured off into fresh microcentrifuge tube tubes. 500 µl of PCI was added, mixed by vortexing, and centrifuged for 5 minutes at 13,000 x g. The aqueous phase was transferred into fresh microcentrifuge tubes and the DNA was precipitated by adding 1 ml of ethanol, mixing by inversion and letting the tubes sit 2Xfor 5 minutes at room temperature. The tubes were then centrifuged for 10 minutes at 13,000 x g. The supernatant was removed and the DNA pellet was resuspended in 50 µl of water supplemented with 50 µg/ml of RNase A and incubated at 37 °C for 30 minutes. Recombinant plasmids were then identified by restriction enzyme analysis.

2.7.2 Large Scale Preparations

A single bacterial colony was grown in an overnight culture of 200 ml of YT/Amp. The next day, the culture was transferred to a Nalgene tube and centrifuged for 15 minutes at 1400 x g. The supernatant was removed and the bacterial pellet was resuspended in 5 ml of solution 1. 10 ml of solution 2 was added and the tube was gently inverted 6 times before adding 7.5 ml of solution 3 and mixing vigorously. After centrifugation at 1400 x g for 20 minutes, the supernatant was filtered through cheesecloth into a 50 ml tube. An equal volume

of isopropanol was added and the mixture was left at room temperature for 5 minutes, then centrifuged for 30 minutes at 1400 x g at 4 °C to pellet the DNA. The supernatant was poured off and the DNA pellet was allowed to air-dry for 15 minutes before being dissolved in 3 ml of sterile water. 3.4 g of CsCl was added and mixed, followed by 250 µl of ethidium bromide (10 mg/ml). The DNA/CsCl solution (density = 1.6 g/ml) was then transferred to a small Nalgene ultracentrifuge tube and spun in a centrifuge at 55,000 rpm at 20 °C for approximately 20 hours. The lower, supercoiled band of DNA was removed from the centrifuge tube with a syringe and transferred to a 14 ml snap-cap tube. The ethidium bromide was removed by extracting three times with water-saturated butanol and the DNA was ethanol precipitated twice. After the second ethanol precipitation, the DNA pellet was resuspended in 100 - 200 µl of sterile water. The final concentration of the DNA was determined by reading the absorbance at 260 nm of a 1:100 dilution.

2.8 Gel Electrophoresis

2.8.1 Agarose Gels

Agarose gels were used to examine restriction enzyme digests of plasmid DNA and to isolate DNA fragments for use in cloning or as probes. Gels were made using 1X TAE (40 mM Tris base, 1 mM EDTA, 0.11% glacial acetic acid), 0.1 µg/ml ethidium bromide and 0.6 - 1.2 % agarose, depending on the fragment

sizes to be separated. Before loading, samples were made 1X in gel loading buffer (5 % glycerol, 0.025 % bromophenol blue, 0.025 % xylene cyanole). Agarose gels were run at 100 mV for 1 to 3 hours, depending on the degree of separation required. After electrophoresis, the ethidium bromide-bound DNA in the gels was visualized under UV light.

2.8.2 SDS-Polyacrylamide Gels

Proteins generated in a cell free system and proteins from cell extract were analyzed by electrophoresis through SDS-polyacrylamide gels. The proteins in the gel could then be visualized by autoradiography in the case of ^{35}S-labelled proteins, or by Coomassie staining or Western blot analysis in the case of cell extract proteins. A 30 % acrylamide stock was prepared containing 29.2 % (w/v) acrylamide and 0.8 % (w/v) N',N'-bis-methylene-acrylamide and was stored at 4 °C. 12% acrylamide separating gels were prepared from 30 % acrylamide stock, 0.375 mM Tris-HCl [pH 8.8], 0.1 % SDS, 0.1 % ammonium persulfate, and 0.01 % TEMED. The separating gel was overlaid with water and allowed to polymerize for 30 minutes. The water was then poured off and replaced by a 4 % stacking gel made from stock acrylamide, 125 mM Tris-HCl [pH 6.8], 0.1 % SDS, 0.1 % ammonium persulfate, and 0.01 % TEMED. The comb was then inserted into the stacking gel and the gel was left to polymerize for one hour. Before loading, the protein samples were made 1X in SDS gel-loading buffer (50 mM Tris-HCl [pH 6.8], 100 mM 1,4-dithiothreitol, 2 % SDS, 0.1 % bromophenol

blue, 10 % glycerol) and boiled for 5 minutes at 95 °C. Electrophoresis of the gel was performed in 1X running buffer (50 mM Tris base, 384 mM glycine, and 0.1 % SDS) at 18 milliamps through the stacking gel and 30 milliamps through the separating gel.

After electrophoresis, SDS-polyacrylamide gels containing nonradiolabeled proteins were either subjected to a Western transfer or were stained with Coomassie blue. Gels were stained with Coomassie blue by immersing the gel in stain solution (46 % ethanol, 8.4 % glacial acetic acid, 0.23 % Coomassie blue) for half an hour per mm of gel thickness. The stain was removed and the gel was rinsed in water and soaked in destain solution (23 % ethanol, 8.4 % glacial acetic acid) until the protein bands were plainly visible. Gels were then dried at 60 °C for 90 minutes.

SDS polyacrylamide gels containing radiolabeled proteins were first fixed for 30 minutes in 10 % methanol, followed by three 15 minute washes in DMSO, and finally the gels were left for 45 minutes in a solution of 22 % PPO in DMSO. The gels were then washed for 30 minutes under running water, dried for 2 hours at 60 °C and exposed to Kodak XAR-5 film.

2.9 Mammalian Tissue Culture

P19 cells, a mouse embryonic carcinoma cell line, were used as a source of cell extract. The cells were maintained in Dulbecco's modified eagle medium (DMEM) (Gibco) supplemented with 10 % fetal calf serum, 2 mM L-glutamine,

100 U/ml penicillin G, and 100 μg/ml streptomycin sulphate. Cells were grown as monolayer culture in Falcon 100x20 mm tissue culture dishes at 37 °C, in a humidified environment in the presence of 5 % CO_2. Once the cells reached confluence (approximately 2 days) they were split by removing the media and adding 3 ml of 1X trypsin (Gibco) in PBS to disadhere the cells from the plate. After sitting for approximately 5 minutes in trypsin, the cells were pipetted several times to break up clumps and 0.3 ml of the trypsinized cells were transferred into 10 ml of media in a fresh gelatinized tissue culture dish.

2.10 Preparation of Cell Extract

P19 cell extract was used in immunoprecipitation and Western blot experiments. Cell extract was prepared by washing each confluent plate (approximately 1 X 10^7 cells) twice with cold PBS and then adding 1 ml of cold lysis buffer. One of two lysis buffers was used, EIA lysis buffer (50 mM Hepes [pH 7.0], 250 mM NaCl, 0.1 % NP40) or PBS/0.5% Triton X-100. Protease inhibitors (2 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A) and 100 mM DTT, were added to both lysis buffers immediately before use. The cells were left in lysis buffer for 10 minutes, then the plates were scraped and the cells were collected into a microcentrifuge tube. The microcentrifuge tubes were centrifuged at 4 °C for 10 minutes at 13,000 x g to pellet the cellular debris, and the supernatant was transferred to a fresh microcentrifuge tube. Cellular extract that was to be used immediately was kept on ice, while the remainder was flash

frozen and stored at –70 °C. The protein concentration of the P19 extract was determined in two ways. The protein concentration of cell extract that was prepared using the EIA lysis buffer was determined by reading the absorbance of a 1:100 dilution at 260 nm and 230 nm and using the following formula:

$$[\text{protein}] \text{ in mg/ml} = 183 * A230 - 75.8 * A260$$

The protein concentration of cell extract that was prepared using the PBS/0.5 % Triton X-100 lysis buffer was determined by the Bradford assay (Bio-Rad) since Triton X-100 absorbs at 260 nm thus the spectrophotometric protein determination method could not be used.

2.11 Freezing Cells

P19 cells that had grown to confluence in 100 mm plates were trypsinized and resuspended in 2 ml of freezing media (DMEM + 20 % FCS). 200 µl of dimethyl sulfoxide was added and the suspension was quickly aliquoted into two cryogenic tubes. The cells were frozen slowly by placing them within a Styrofoam container at –70 °C. After 24 hours at –70 °C, the cells were stored in liquid nitrogen.

To culture stocks of frozen cells, cells were thawed quickly and transferred to a gelatinized plate containing 10 ml of fresh media. The cells were incubated at 37 °C for several hours. Once the cells had adhered to the plate, the media was aspirated and replaced with fresh media.

2.12 Preparation of Radiolabeled Proteins in a Cell-Free System

In vitro translation in reticulosyte lysate was used to prepare ^{35}S-labelled proteins for immunoprecipitation experiments. Plasmids to be used for *in vitro* transcription were first linearized using a restriction enzyme that cut downstream of the coding sequence and produced a 5' protruding end. The digested plasmid was purified by extraction with PCI and ethanol precipitation. To produce capped mRNA, 1 μg of linearized plasmid was mixed with 1X transcription optimized buffer (40 mM Tris-HCl [pH 7.5], 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl) (Promega), 10 mM 1,4-dithiothreitol, 27 units RNase inhibitor (RNasin, Promega), 1 mM of each ribonucleotide, and 0.5 mM m7G(5')ppp(5')G. 20 units of either T3 or T7 RNA polymerase (Promega) was added and reaction mixtures were incubated for one hour at 37 °C. The transcripts could then be stored at –20 °C for future use.

The RNA transcripts were used to synthesize ^{35}S-labelled proteins using the Rabbit Reticulosyte Lysate System (Promega). The translation reactions were performed in 25 μl reticulosyte lysate, 1 μl amino acids (minus methionine), 27 units RNasin, 40 μCi ^{35}S-methionine, and 4 μl of the appropriate mRNA transcript. The reaction volume was then brought up to 50 μl with water and incubated for 2 hours at 30 °C. The translation products were analyzed by SDS-PAGE.

2.13 Immunoprecipitation

Immunoprecipitation reactions were performed in order to characterise the antibody as well as to detect the presence of target protein in cell extract. Radiolabeled proteins that were produced in a cell-free system were immunoprecipitated by mixing them with either 150 or 500 μg of protein from P19 extract depending on the experiment being performed. The mixture was incubated with gentle shaking at 4°C for 30 minutes. 100 μl of affinity purified antibody was added and the mixture was incubated for an hour with shaking at 4 °C. The affinity purified antibody was prepared by passing the serum through a peptide-coupled Sulfo-Link affinity purification column (Pierce) and dialyzing the eluant overnight in phosphate-buffered saline. The antibody/protein complexes were isolated by adding 100 μl of a 3 % slurry of Protein A Sepharose (Sigma) and incubating at 4 °C with shaking for 2 hours to overnight, depending on the experiment being performed. The beads were then washed three times with cold lysis buffer and twice with cold PBS. Several different lysis buffers were used for these washes and they included EIA lysis buffer (50 mM Hepes [pH 7.0], 250 mM NaCl, 0.1 % NP-40), PBS + 0.5 % Triton X-100, and NET-gel buffer (50 mM Tris.Cl [pH 7.5], 150 mM NaCl, 0.1% NP-40, 1 mM EDTA [pH 8.0], 0.02 % sodium azide, and 0.25 % gelatin). The bound proteins were eluted from the beads by resuspending them in 36 μl of 1X SDS gel loading buffer (50 mM Tris-HCl [pH 6.8], 100 mM 1,4-dithiothreitol, 2 % SDS, 0.1 % bromophenol blue, 10 % glycerol) and boiling each sample for 5 minutes. The samples were then analyzed

by electrophoresis through a 12% SDS-polyacrylamide gel and autoradiography. Co-immunoprecipitation experiments were performed exactly the same way as the immunoprecipitations except that the radiolabeled proteins that were used in co-immunoprecipitations were not recognized by the antibody and had to be co-precipitated along with target proteins present in the cell extract.

Proteins were immunoprecipitated from P19 extract in much the same way. 150 μg of protein from P19 extract was mixed with 100 μl of affinity purified antibody and incubated with shaking at 4 °C. 100 μl of Protein A Sepharose was added and the mixture was incubated for another hour with shaking at 4 °C. The beads were then washed, eluted, and loaded onto an SDS-PAGE gel as described above. After SDS-PAGE, the proteins were transferred to a nylon membrane and Western blotted.

2.14 Western Blotting and Detection

Immunoprecipitations followed by Western blot analysis were used to test antibodies for the ability to recognize proteins of a specific size in P19 cell extract. The immunoprecipitation reactions were performed as described above. After electrophoresis through a 12% SDS-polyacrylamide gel, the gel was soaked for 15 minutes in cold transfer buffer (25 mM Tris, 192 mM glycine, 20 % methanol) along with two pieces of Whatmann 3MM paper, two sponges, and one piece of nitrocellulose cut to the size of the gel. One of the sponges was then placed on the cathode side of the cassette (Bio-Rad), a piece of Whatmann 3MM

paper was placed over it, followed by the gel itself. The soaked nitrocellulose membrane was carefully positioned over the gel and all air bubbles were removed. A piece of Whatmann 3MM paper was placed on the nitrocellulose, followed by the second sponge and the cassette was closed. The cassette was placed in the transfer apparatus which had been filled with cold transfer buffer and the transfer was allowed to proceed overnight at 50 V using circulating cold water to keep the transfer buffer cool.

After the transfer was complete, the nitrocellulose membrane was incubated for 2 hours in a blocking solution consisting of 2.6 % skim milk powder and 0.02 % sodium azide in PBS. Meanwhile, the primary antibody was preincubated in the same blocking solution at a dilution of 1:200. After the blocking solution was removed, the primary antibody, in its blocking solution, was added to the membrane and incubated overnight with light shaking. The next day the membrane was washed three times for 15 minutes with a solution of 0.05 % Tween-20 in PBS and once for 15 minutes with a solution containing 150 mM NaCl and 20 mM Tris-HCl [pH 7.5]. Alkaline phosphatase conjugated anti-rabbit IgG (Fc) (Promega) was used as the secondary antibody. The secondary antibody was diluted 1:5000 in a solution containing 1 % nonfat dried milk, 150 mM NaCl, and 20 mM Tris-HCl [pH 7.5] and incubated with the membrane for 4 hours. After the incubation, the membrane was washed three times for 10 minutes in a solution containing 150 mM NaCl and 20 mM Tris-HCl [pH 7.5]. An alkaline phosphatase colour development solution containing 3.3 mg of nitroblue

tetrazolium and 1.65 mg of 5-bromo-4-chloro-3-indolyl phosphate per 10 ml of alkaline phosphatase buffer (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 5 mM $MgCl_2$) was prepared and added to the membrane after the final wash. The membrane was incubated with this solution in the dark until bands were clearly visible (approximately 20 minutes). The reaction was stopped by incubating the membranes in a solution of 2 mM EDTA [pH 8.0] in PBS. A permanent record was obtained by allowing the membrane to air-dry overnight.

2.15 PCR Amplification

PCR was used to amplify fragments of known sequence from cDNA plasmids for subcloning. PCR amplification was performed by mixing 30.4 µl of uater with 1 µl containing either 10 pg, 1 ng, or 100 ng of the cDNA plasmid containing the sequence to be amplified. The mixtures were overlaid with 2-3 drops of mineral oil and placed in a thermocycler. The tubes were heated to 95 °C for 10 minutes to denature the template and then paused at 85 °C while 18.2 µl of PCR mix was added. The PCR mix contained 1X PCR buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl), 1.48 mM $MgCl_2$, 0.19 mM deoxynucleotides, 0.91 µM 5' PCR oligo, 0.91 µM 3' PCR oligo, and 1.95 U of Taq DNA polymerase (Gibco). The reactions were then incubated for 30 cycles of 1 minute at 94 °C, 1 minute at 50 °C or 55 °C (depending on the primer used), and 2 minutes at 72 °C. After the 30 cycles, the reactions were extended for 8 minutes at 72 °C and then allowed to

sink to 4 °C. Following PCR amplification, the reaction mixtures were analyzed by agarose gel electrophoresis.

2.16 Yeast Two-Hybrid System

2.16.1 Cotransformation of Yeast with Two Hybrid Vectors

Yeast were transformed using the LiAc/SS-DNA/PEG transformation protocol developed by Schiestl et al. (1993). The yeast strain used in these experiments was the yeast strain Y190 (MATa ade2-101 gal4-D gal80-D his3 leu2-3, 112 trp1-D901 ura3-52, LYS2::GAL1-HIS3 URA3::GAL1-lacZ; gift of J. Capone). Yeast cells were maintained on YPD plates containing 20 g/L Difco peptone, 10 g/L yeast extract, 20 g/L Agar, and 2 % dextrose. Ppior to transformation, yeast cells were inoculated into 5 ml of liquid YPD medium (20 g/L Difco peptone, 10 g/L yeast extract, 2 % dextrose) and incubated overnight at 30 °C with shaking. The cell titre of the overnight culture was determined by measuring the optical density (OD_{600}) of a 1 in 5 dilution of the inoculated culture. An OD_{600} of 0.1 is equivalent to approximately 1 X 10^6 cells/ml for most yeast strains (Schiest et al. 1993). The overnight culture was used to seed 50 ml of YPD media to a final cell titre of 5 X 10^6 cells/ml. The 50 ml culture was incubated at 30 °C with shaking until the cell titre reached 2 X 10^7 cells/ml (approximately 3 to 5 hours). The cells were then centrifuged at 3000 x g for 5 minutes. The pellet was resuspended in 20 ml of water and centrifuged again at

3000 x g for 5 minutes. The water was poured off and the pellet was resuspended in 1 ml of water and transferred to a microcentrifuge tube. The cells were pelleted by centrifugation at 13,000 x g for 15 seconds and the water was removed. The yeast cells were resuspended in 100 mM LiAc to give a final volume of 500 µl and then incubated at 30 °C for 15 minutes.

While the cells were incubating, a sample of single stranded carrier DNA (1 mg/ml) was boiled for 10 minutes and then placed on ice. For each transformation to be performed, the following volumes of reagents were added to a microcentrifuge tube, vortexing after each addition: 240 µl of PEG (50% w/v), 36 µl of 1.0 M LiAc, 20 µl of water, 50 µl of carrier DNA (1 mg/ml), and 2.5 µl of each of the DNA samples (1 mg/ml) to be co-transformed for a total of 5 µl.

After the 15 minute incubation, 50 µl aliquots of the cell suspension were dispensed into microcentrifuge tube tubes. The microcentrifuge tubes containing the cells were then centrifuged for 15 seconds at 13,000 x g and the LiAc was removed. The yeast pellets were each resuspended in 350 µl of the transformation mix and then incubated at 30 °C for 30 minutes. The cells were then heat shocked at 42 °C for 20 minutes. The cell suspensions were centrifuged for 15 seconds at 13,000 x g and the supernatant was removed. The cells were resuspended in 1 ml of water and 100 µl of each transformation was plated onto SD plates lacking the appropriate nutritional requirements. The SD plates contained 6.7 g/L Difco yeast nitrogen base without amino acids, 20 g/L agar, 2 % dextrose, 200 µg/mL

Adenine hemisulfate salt, 200 μg/ml L-Histidine, and 200 μg/ml L-Uracil. The plates were incubated at 30 °C until colonies were 1-2 mm in diameter (usually 4-7 days).

2.16.2 β - Galactosidase Lift Assay

Interactions between GAL4 hybrid proteins were detected by assaying cotransformed yeast colonies for β-galactosidase activity. A piece of nylon membrane (Hybond-N) was placed over the surface of an agar plate containing transformant yeast colonies 1-2 mm in diameter. The membrane was lifted carefully off the plate and placed, colonies side up, in a pool of liquid nitrogen. The membrane was submerged for 10 seconds and then removed and allowed to thaw at room temperature. This freeze/thaw cycle was repeated twice more before the membrane was placed, colonies-side up, in a petri dish on top of a piece of Whatmann 3MM paper soaked in Z buffer/X-gal solution. The Z buffer/X-gal solution contained 16.1 g/L $Na_2HPO_4.7H_2O$, 5.5 g/L $NaH_2PO_4.H_2O$, 0.75 g/L KCl, 0.246 g/L $MgSO_4.7H2O$, 0.27 % β-mercaptoethanol, and 1.5 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) from a stock solution of 20 mg/ml X-gal in N,N-dimethylformamide. Colonies containing interacting proteins turned blue 6 to 48 hours later.

2.17 Preparation of Yeast Lysate

Transformed yeast colonies were tested for expression of the introduced

fusion proteins by Western blot analysis of the yeast lysate. A single transformed yeast colony was used to inoculate 50 ml of SD synthetic media (6.7 g/L Difco yeast nitrogen base without amino acids, 2 % dextrose) supplemented with 200 μg/ml Adenine hemisulfate salt, 200 μg/ml L-Histidine, and 200 μg/ml L-Uracil. The cells were grown to an OD_{600} of 0.8 and then pelleted by centrifugation at 800 x g for 5 minutes at 4 °C. The pellet was resuspended in 1.5 ml of water and transferred to a microcentrifuge tube. The cells were pelleted again by centrifugation at 800 x g for 5 minutes at 4 °C. The supernatant was removed and an equal volume of acid-washed glass beads was added to the pellet. Two volumes of 2 X SDS gel loading buffer was then added. The cell suspension was vortexed twice for 30 seconds and once for 15 seconds with incubations of 1 minute on ice in between vortexing. The suspension was then boiled for 3 minutes and incubated on ice for 5 minutes. The debris was pelleted by centrifugation at 13,000 x g for 30 seconds and the supernatant was transferred to a fresh microcentrifuge tube. The yeast lysate could then be loaded directly onto an SDS-PAGE gel or stored at -20 °C.

III. RESULTS

3.1 Experimental Approach

The goal of this study was to gain insight into whether or not members of the Grg and Hes families of proteins interact at the end of the Notch pathway to regulate transcription. Two different approaches were used to characterise the physical interactions between the Hes and Grg proteins. In one approach, co-immunoprecipitation techniques were used to detect direct interactions between Grg and Hes proteins. The second approach used to detect these interactions required fusion of Grg and Hes proteins to the DNA binding and activation domains of GAL4, respectively. Interactions between Grg and Hes proteins were then tested using the Yeast Two-Hybrid System, an *in vivo* genetic assay. The Yeast Two-Hybrid system was also used to detect interactions between Grg proteins and AML1b since AML1b contains a C-terminal VWRPY motif and mutations which remove the C-terminus of this protein are associated with acute myeloid leukemia.

3.2 Co-Immunoprecipitation Experiments

3.2.1 α-Grg 5 and α-WD40 Antibodies

Antibodies directed against Grg5 or all WD40-containing Grg proteins were generated by injecting rabbits with synthetic peptides specific to the carboxy terminals of Grg5 or long WD40-containing Grg proteins such as Grg1, Grg3, and Grg4, respectively (Fig. 3.1). Prior to injection, the synthetic peptides were conjugated to Keyhole limpet hemocyanin. The antibodies were generated and characterised by Madalena Pinto.

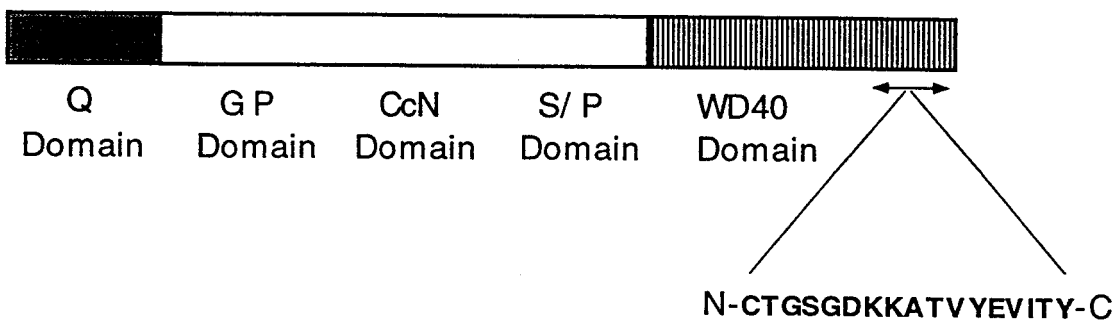
3.2.2 α-WD40 Immunoprecipitates ^{35}S-labelled Grg1

Prior to use, the antibodies were immunoaffinity purified on an antigen column (the WD40 peptide was used as the antigen in the column) and dialysed against PBS. The specificity of the antibody directed against WD40-containing Grg proteins (α-WD40) was tested by immunoprecipitating Grg1 protein prepared in a cell-free system. Prior to *in vitro* transcription, the Grg1 plasmid was linearized with Xho1. T7 RNA polymerase was used to generate transcript from the linearized plasmid. Grg1 and Luciferase (Promega) transcripts were translated into ^{35}S-labelled proteins by *in vitro* translation in reticulosyte lysate. The labelled proteins were then incubated with P19 cell extract, α-WD40, and Protein A Sepharose. Mock immunoprecipitations were also performed by leaving α-WD40 out of the reaction. After washing and eluting the beads, the supernatant was analyzed by SDS-PAGE and autoradiography (Fig. 3.2). It was found that the α

Figure 3.1 Synthetic Peptides

The synthetic peptide that was used to generate the α-WD40 antibody has a sequence unique to the carboxy terminus of long WD40-containing Grg proteins. A second synthetic peptide was used to generate the α-Grg5 antibody and the sequence of this peptide is unique to the carboxy terminus of Grg5. Both synthetic peptides were obtained from Chiron Mimotopes Peptide Systems (USA). The amino acid sequence of each peptide is shown. The N- on the left side of each sequence signifies the amino terminus and the C- on the right side of each sequence signifies the carboxy terminus. The antibodies were generated by Madalena Pinto.

mouse Grg 1,2,3,4



mouse Grg 5

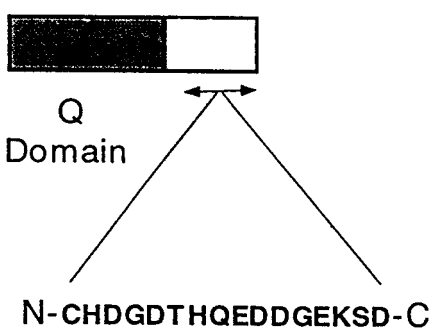
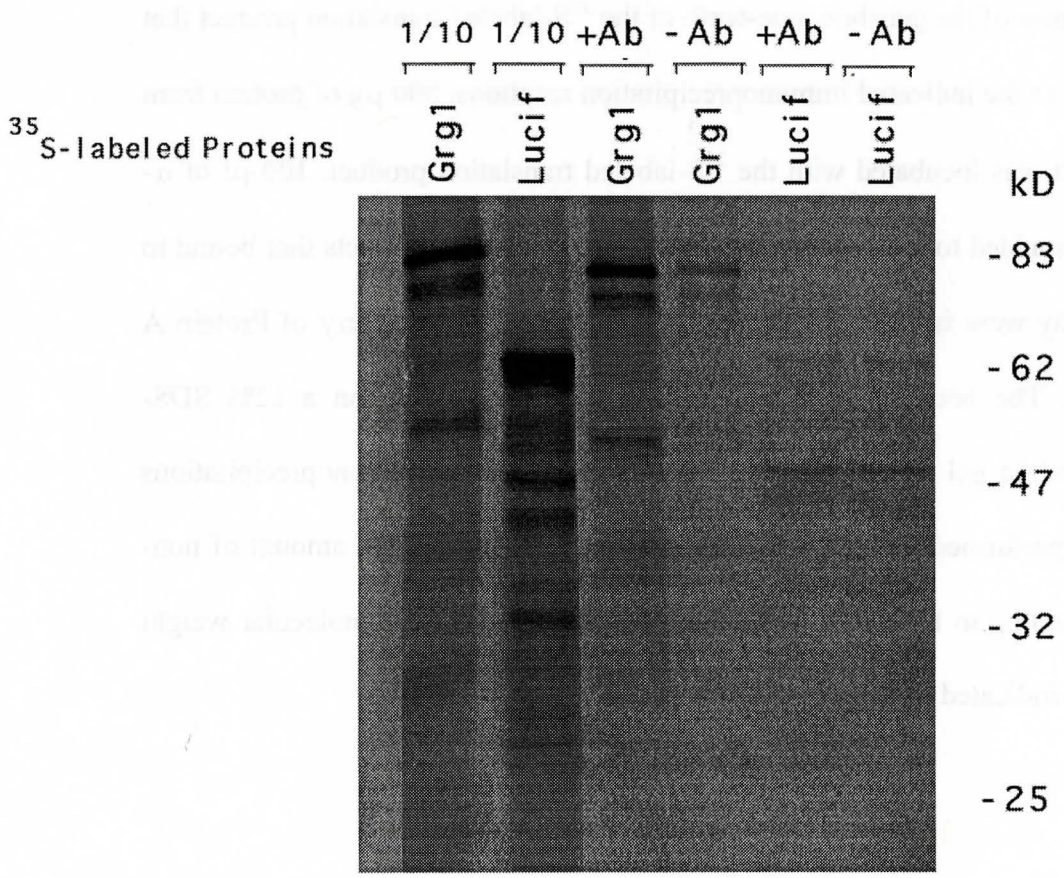


Figure 3.2 Immunoprecipitation of ^{35}S-labeled Grg1 with α-WD40

The immunoprecipitations were performed in P19 extract made from lysis buffer containing 50 mM Hepes [pH 7.0], 250 mM NaCl, and 0.1 % NP40. The first two lanes of the gel show one-tenth of the ^{35}S-labeled translation product that was added to the indicated immunoprecipitation reactions. 500 μg of protein from P19 extract was incubated with the ^{35}S-labeled translation product. 100 μl of α-WD40 was added to the appropriate tubes. The translation products that bound to the antibody were isolated by reaction with 100 μl of a 3 % slurry of Protein A Sepharose. The bound labeled protein was eluted, separated on a 12% SDS-polyacrylamide gel and exposed to film for 3 days. The immunoprecipitations were also performed without α-WD40 in order to determine the amount of non-specific binding to Protein A Sepharose. The migration of the molecular weight markers is indicated on the right.

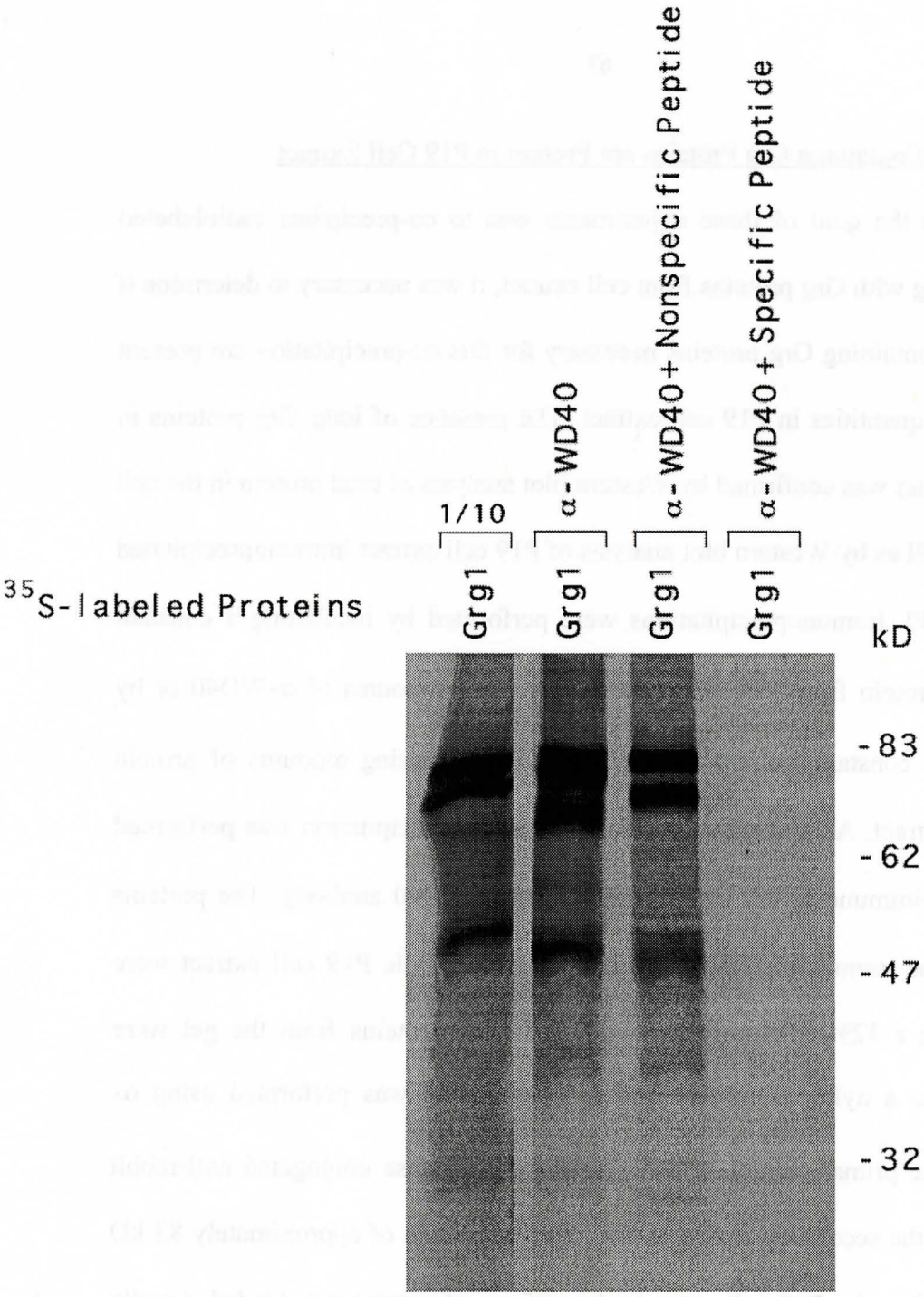

1/10
1/10
+Ab
-Ab
+Ab
-Ab
Grg1
Lucif
Grg1
Grg1
Lucif
Lucif
35S-labeled Proteins
kD
-83
-62
-47
-32
-25

WD40 antibody was able to immunoprecipitate the Grg1 translation product which ran at 80 kD as expected. However, α-WD40 was not able to recognize the unrelated Luciferase protein. The immunoprecipitations were performed in the absence of the α-WD40 antibody in order to determine if the radiolabeled translation products were being precipitated non-specifically by binding directly to the Protein A Sepharose beads. A small amount of Grg1 and Luciferase bound non-specifically to the beads. By comparing the amount of immunoprecipitated Grg1 (Fig. 3.2, lane 3) with the lane containing 1/10th of the Grg1 protein available for immunoprecipitation (Fig. 3.2, lane 1), it was determined that approximately 5 % of the total ^{35}S-labelled Grg1 present in the reaction mixture was immunoprecipitated specifically by α-WD40.

In order to confirm that the radiolabeled Grg1 protein was being immunoprecipitated specifically by the α-WD40 antibody, the antibody was blocked with the peptide against which it was generated. As a negative control, α-WD40 was also preincubated with the Grg5 peptide, which should not block the antibody. Radiolabeled Grg1 was immunoprecipitated in P19 extract using α-WD40, or α-WD40 that had been preincubated with either the Grg5 peptide or the WD40 peptide (Fig. 3.3). The ^{35}S-labelled Grg1 could be immunoprecipitated by α-WD40 and by α-WD40 that had been preincubated with the non-specific Grg5 peptide, but not by α-WD40 that had been preincubated with the specific WD40 peptide. These results confirm that the α-WD40 antibody can specifically recognize and precipitate Grg1.

Figure 3.3 Immunoprecipitation of Grg1 with α-WD40 preincubated with specific and nonspecific peptides.

Immunoprecipitations were performed in P19 extract made from lysis buffer containing 50 mM Hepes [pH 7.0], 250 mM NaCl, and 0.1% NP40. The first lane of the gel shows one-tenth of the ^{35}S-labeled Grg1 that was added to each immunoprecipitation reaction. 500 μg of protein from P19 extract was incubated with radiolabeled Grg1. 100 μl of α-WD40, or 100 μl of α-WD40 that was preincubated with 7 μg (3.92 nmoles) of non-specific (Grg5) peptide, or 100 μl of α-WD40 that was preincubated with 7 μg (3.81 nmoles) of specific (WD40) peptide was added to the appropriate tubes. The ^{35}S-labeled Grg1 that bound to the antibody was isolated by reaction with 100 μl of a 3% slurry of Protein A Sepharose. The bound protein was eluted, separated on a 12% SDS-polyacrylamide gel and exposed to film for 6 days. The migration of the molecular weight markers is indicated on the right.

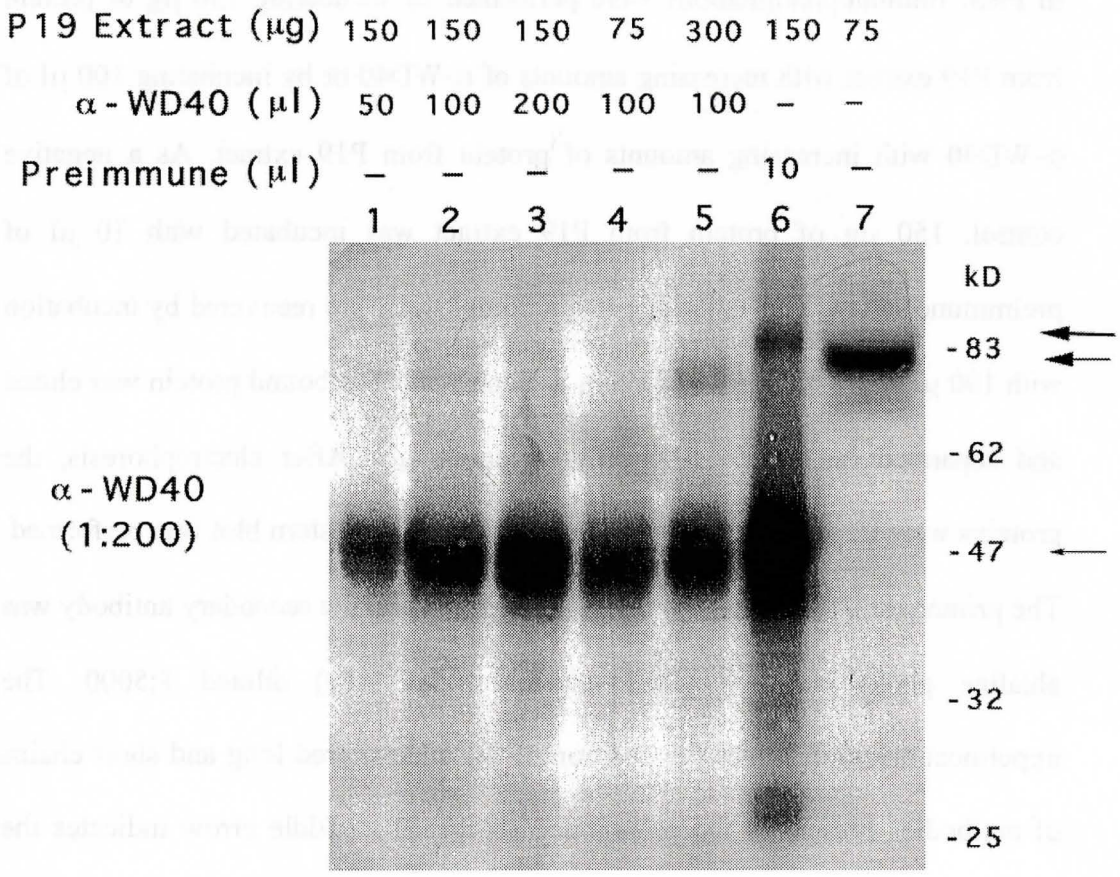

35S-labeled Proteins
1/10
α-WD40
α-WD40+ Nonspecific Peptide
α-WD40+ Specific Peptide
Grg1
Grg1
Grg1
Grg1
kD
-83
-62
-47
-32

3.2.3 WD40-Containing Grg Proteins are Present in P19 Cell Extract

Because the goal of these experiments was to co-precipitate radiolabeled proteins along with Grg proteins from cell extract, it was necessary to determine if the WD40-containing Grg proteins necessary for this co-precipitation are present in sufficient quantities in P19 cell extract. The presence of long Grg proteins in P19 cell extract was confirmed by Western blot analysis of total protein in the cell extract as well as by Western blot analysis of P19 cell extract immunoprecipitated with α-WD40. Immunoprecipitations were performed by incubating a constant amount of protein from P19 extract with increasing amounts of α-WD40 or by incubating a constant amount of α-WD40 with increasing amounts of protein from P19 extract. As a negative control, an immunoprecipitation was performed in which preimmune serum was substituted for α-WD40 antibody. The proteins present in the immunoprecipitation reactions or in whole P19 cell extract were separated on a 12% SDS-polyacrylamide gel. The proteins from the gel were transferred to a nylon membrane and a Western blot was performed using α-WD40 as the primary antibody and alkaline phosphatase conjugated anti-rabbit IgG (Fc) as the secondary antibody (Fig. 3.4). A protein of approximately 83 kD was detected in the P19 cell extract when 75 μg of extract was loaded directly onto the gel (Fig. 3.4, lane 7). An 80 kD protein was also immunoprecipitated from P19 cell extract by α-WD40 and detected on the gel (Fig. 3.4, lane 5). The WD40-containing protein detected in total P19 cell extract migrated slightly slower on the SDS-polyacrylamide gel than did the immunoprecipitated WD40-

Figure 3.4 Immunoprecipitation of WD40-containing Grg proteins from P19 extract with α-WD40.

P19 extract was collected using a lysis buffer containing 0.5 % Triton-X 100 in PBS. Immunoprecipitations were performed by incubating 150 μg of protein from P19 extract with increasing amounts of α-WD40 or by incubating 100 μl of α-WD40 with increasing amounts of protein from P19 extract. As a negative control, 150 μg of protein from P19 extract was incubated with 10 μl of preimmune serum. The antibody/protein complexes were recovered by incubation with 100 μl of a 3 % slurry of protein A-Sepharose. The bound protein was eluted and separated on a 12% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to a nylon membrane and a Western blot was performed. The primary antibody was α-WD40 diluted 1:200 and the secondary antibody was alkaline phosphatase-conjugated anti-rabbit IgG (Fc) diluted 1:5000. The uppermost arrow indicates the position of the undenatured long and short chains of antibodies present in the preimmune serum. The middle arrow indicates the position of the WD40-containing Grg proteins. The small arrow indicates the position of the long antibody chain. The migration of the molecular weight markers is indicated on the right.


P19 Extract (μg) 150 150 150 75 300 150 75
α-WD40 (μl) 50 100 200 100 100 – –
Preimmune (μl) – – – – – 10 –
1 2 3 4 5 6 7
α-WD40 (1:200)
kD
-83
-62
-47
-32
-25

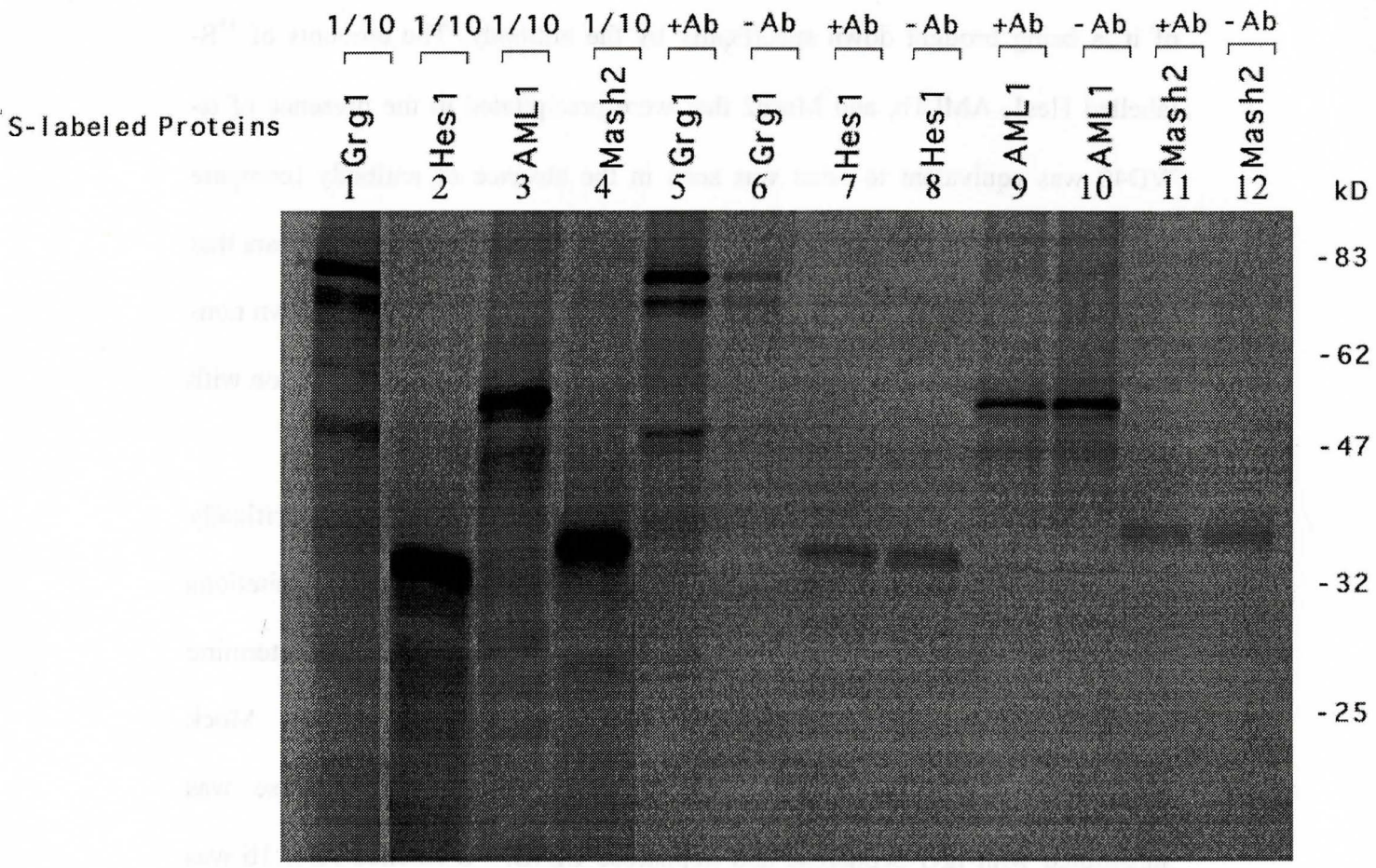
containing protein (Fig. 3.4, compare lanes 5 and 7). One possible explanation for this is that the antibody may have a greater affinity for one specific Grg protein present in the extract that is slightly smaller in size than other Grg proteins also present in the extract. The antibody may be only able to immunoprecipitate one Grg isolog while still being able to detect other denatured WD40-containing Grg proteins present in the total extract. Thus WD40-containing Grg proteins are present in P19 cell extract and are recognized by the α-WD40 antibody.

3.2.4 bHLH and AML1b Transcription Factors Bind to Sepharose

In previous co-immunoprecipitation experiments in which α-WD40 was used to co-immunoprecipitate WD40-containing proteins from P19 cell extract and radiolabeled proteins being tested for interaction, significant quantities of ^{35}S-labelled Mash2 appeared to be co-precipitating with Grg proteins from cell extract (data not shown). Mash 2 (mammalian achaete scute homologue 2) is a bHLH transcriptional activator and unlikely to physically interact with long Grg proteins since Mash2 does not have a WRPW motif. In order to determine whether the co-precipitation of Mash2 was due to non-specific binding to Protein A Sepharose beads or to an interaction occurring between Mash2 and long Grg proteins, co-immunoprecipitations were performed with, and without, the α-WD40 antibody. Equivalent amounts of ^{35}S-labelled translation product were mixed with P19 extract, Protein A Sepharose, and with or without α-WD40 antibody. The beads were washed, eluted, and the supernatant was subjected to SDS-PAGE and

Figure 3.5 Co-immunoprecipitation of WD40-containing Grg proteins and ^{35}S-labeled Hes1, AML1, and Mash2.

The co-immunoprecipitations were performed in P19 extract made from lysis buffer containing 50 mM Hepes [pH 7.0], 250 mM NaCl, and 0.1 % NP40. The first four lanes show one-tenth of the ^{35}S-labeled translation product that was added to the appropriate co-immunoprecipitation reaction. 500 μg of protein from P19 extract was incubated with radiolabeled translation product. 100 μl of α-WD40 was added to the appropriate tubes. The translation products that bound to the antibody were isolated by reaction with 100 μl of a 3% slurry of Protein A Sepharose. The beads were eluted and the supernatant was separated on a 12% SDS-polyacrylamide gel and exposed to film for 3 days. The migration of the molecular weight markers is indicated on the right.


1/10
1/10
1/10
1/10
+Ab
-Ab
+Ab
-Ab
+Ab
-Ab
+Ab
-Ab
Grg1
Hes1
AML1
Mash2
Grg1
Grg1
Hes1
Hes1
AML1
AML1
Mash2
Mash2
1
2
3
4
5
6
7
8
9
10
11
12
kD
-83
-62
-47
-32
-25
S-labeled Proteins

autoradiography (Fig. 3.5). The amount of Grg1 precipitated in the presence of α-WD40 was much greater than in the absence of the antibody as indicated by the intensity of the band in lane 5 versus lane 6. Therefore, although a small amount of Grg1 is binding non-specifically to the Protein A Sepharose beads, much more of it is being brought down specifically by the antibody. The amounts of ^{35}S-labelled Hes1, AML1b, and Mash2 that were precipitated in the presence of α-WD40 was equivalent to what was seen in the absence of antibody (compare lanes 7, 9, and 10 with lanes 8, 10, and 12 respectively). Therefore, it appears that the radiolabeled Hes1, AML1b, and Mash2 proteins are being brought down non-specifically by binding to Protein A Sepharose and not through an interaction with the WD40-containing Grg proteins present in the P19 cell extract.

Because the radiolabeled translation products were binding non-specifically to the Protein A Sepharose beads, mock immunoprecipitations (immunoprecipitations performed without antibody) were performed to determine which component of the beads was responsible for this activity. Mock immunoprecipitations were performed in which Protein A Sepharose was substituted with IgG Sepharose and Sepharose CL4B. ^{35}S-labelled AML1b was used in these experiments since it showed the highest levels of non-specific binding to Protein A Sepharose (Fig. 3.5). Equivalent amounts of radiolabeled AML1b were incubated with P19 extract and with equivalent amounts of either Protein A Sepharose, IgG Sepharose, or Sepharose CL4B. The beads were washed, eluted, and the supernatant was subjected to SDS-PAGE followed by

autoradiography (Fig. 3.6). It was shown that AML1b binds equally well to Protein A Sepharose, IgG Sepharose, and Sepharose CL4B. These results suggest that AML1b, as well as the other translation products, are binding directly to Sepharose.

3.2.5 Immunoprecipitation of HA-tagged Grg1

The α-WD40 antibody specific for the WD40-containing Grg proteins proved to have a low affinity for binding Grg1 produced by *in vitro* traslation in rabbit reticulosyte lysate since it bound only approximately 5 % of available Grg1 using the conditions described above (Fig. 3.2). Successful co-immunoprecipitations require a high affinity antibody and for this reason a commercially prepared polyclonal rabbit antisera α-HA (BAbco), which is specific for the influenza virus hemagglutinin (HA) epitope tag, was acquired. A 1625 bp fragment of Grg1 which lacked the amino-terminal Q and G/P domains but retained the CcN, S/P, and WD40 domains was subcloned into the pSPUTKHA vector (gift of Dr. Andrews, McMaster University). The pSPUTKHA vector contains SP6 and T7 promoters, a UTK 5' untranslated leader sequence containing a Kozak initiation site (Nco1) for efficient translation, and an HA epitope tag followed by the multiple cloning site. The Grg1 (754-2389) DNA fragment was generated by PCR amplification of the Grg1 cDNA using primers (Life Technologies) incorporating Apa1 and EcoR1 sites. The forward primer used to amplify Grg1 was

Figure 3.6 Mock immunoprecipitation of ^{35}S-labeled AML1b

The mock immunoprecipitations were performed in P19 cell extract made from lysis buffer containing 50 mM Hepes [pH 7.0], 250 mM NaCl, and 0.1 % NP40. The first lane of the gel shows one-tenth of the ^{35}S-labeled AML1b that was added to each immunoprecipitation reaction. 500 μg of protein from P19 extract was incubated with radiolabeled AML1b. 100 μl of a 3 % slurry of Protein A Sepharose, or 100 μl of a 3 % slurry of IgG Sepharose, or 100 μl of a 3 % slurry of Sepharose CL4B were added to the appropriate tubes. The beads were eluted and the supernatant was separated on a 12% SDS-polyacrylamide gel and exposed to film for 3 days. The migration of the molecular weight markers is indicated on the right.

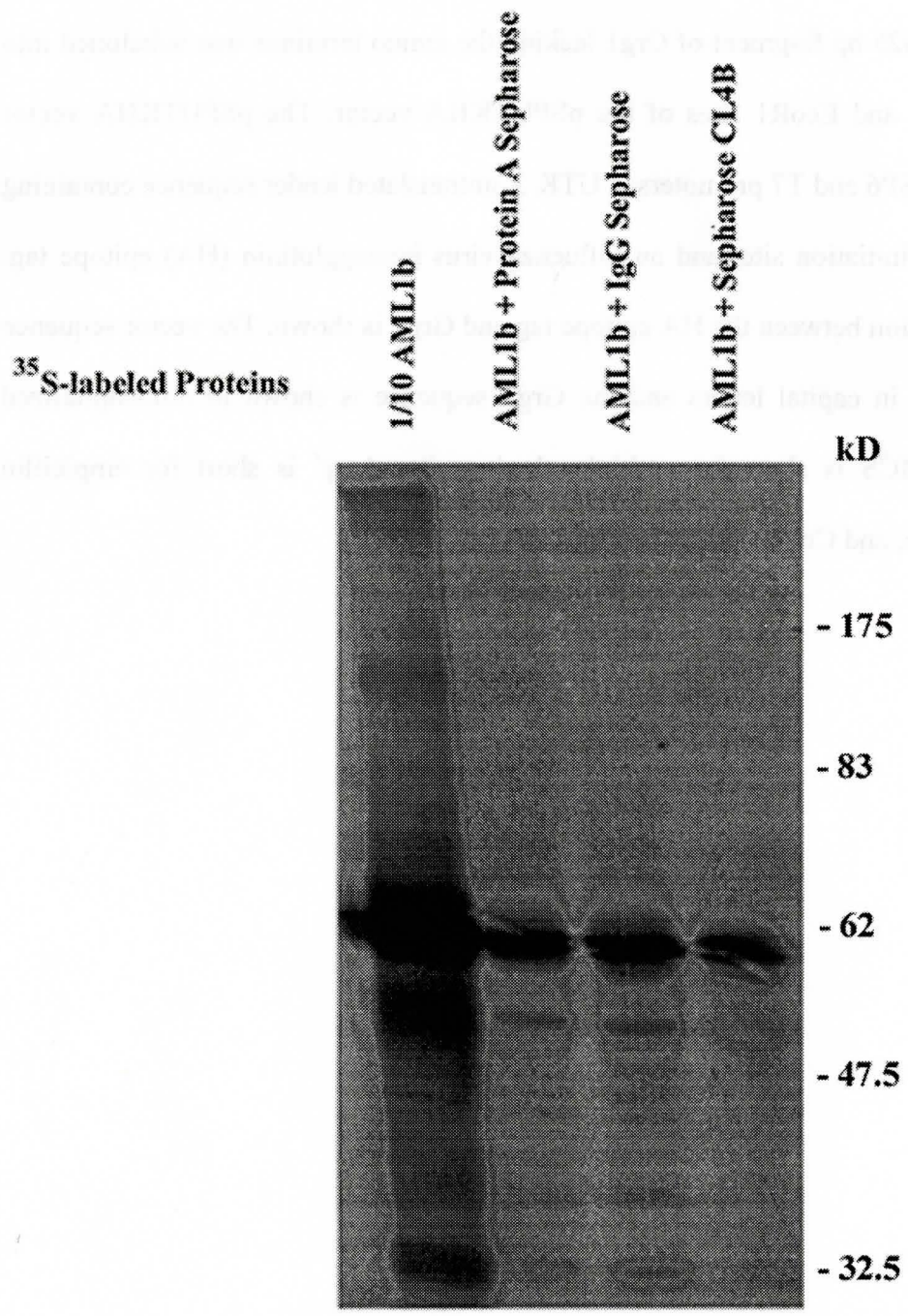
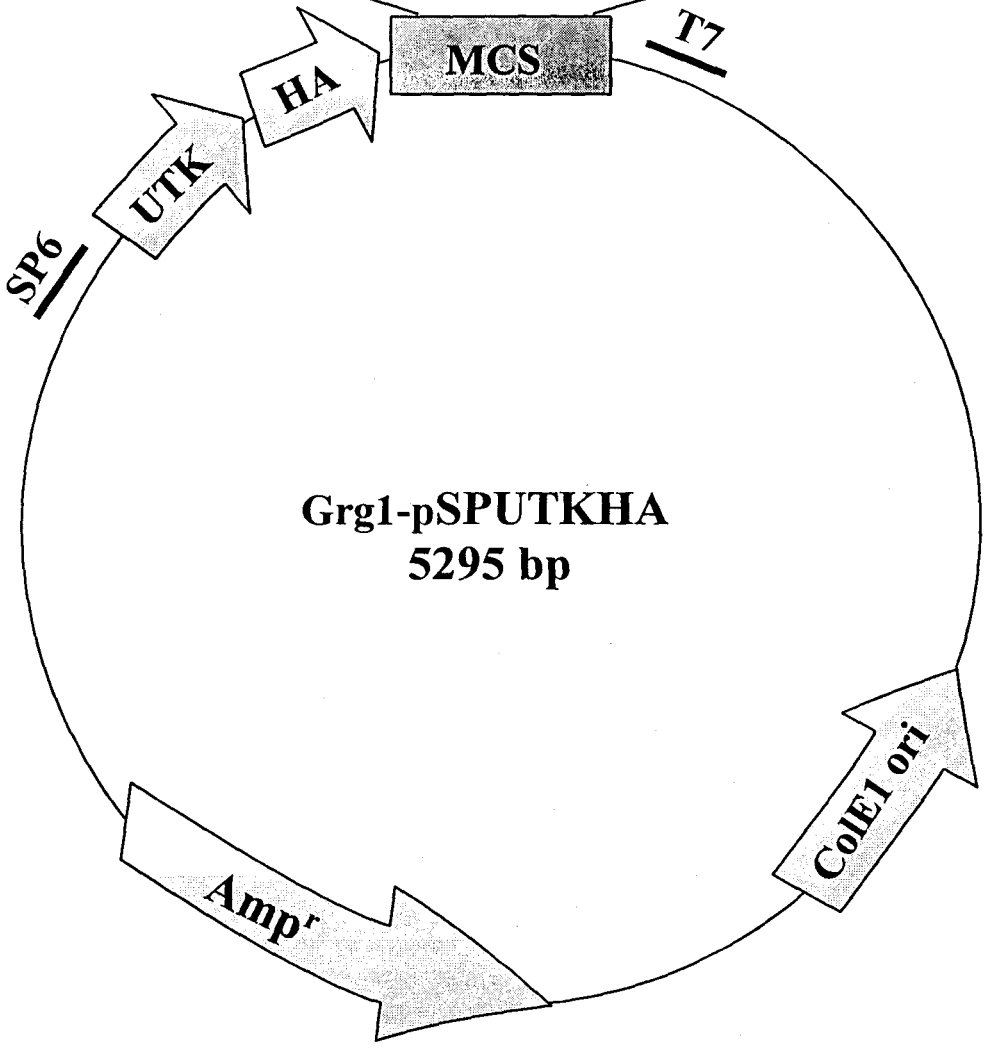

35S-labeled Proteins
1/10 AML1b
AML1b + Protein A Sepharose
AML1b + IgG Sepharose
AML1b + Sepharose CL4B
kD
- 175
- 83
- 62
- 47.5
- 32.5

Figure 3.7 The Grg1-pSPUTKHA construct.

A 1625 bp fragment of Grg1 lacking the amino terminus was subcloned into the Apa1 and EcoR1 sites of the pSPUTKHA vector. The pSPUTKHA vector contains SP6 and T7 promoters, a UTK 5' untranslated leader sequence containing a Kozak initiation site, and an influenza virus hemagglutinin (HA) epitope tag. The junction between the HA epitope tag and Grg1 is shown. The vector sequence is shown in capital letters and the Grg1 sequence is shown in non-capitalized letters. MCS is short for multiple cloning site, Amp^r is short for ampicillin resistance, and Col E1 ori is the origin of replication.

AGATCTACCATGGGGGCCcagagtgatgacaacttagttgtggatgtgtctaacga



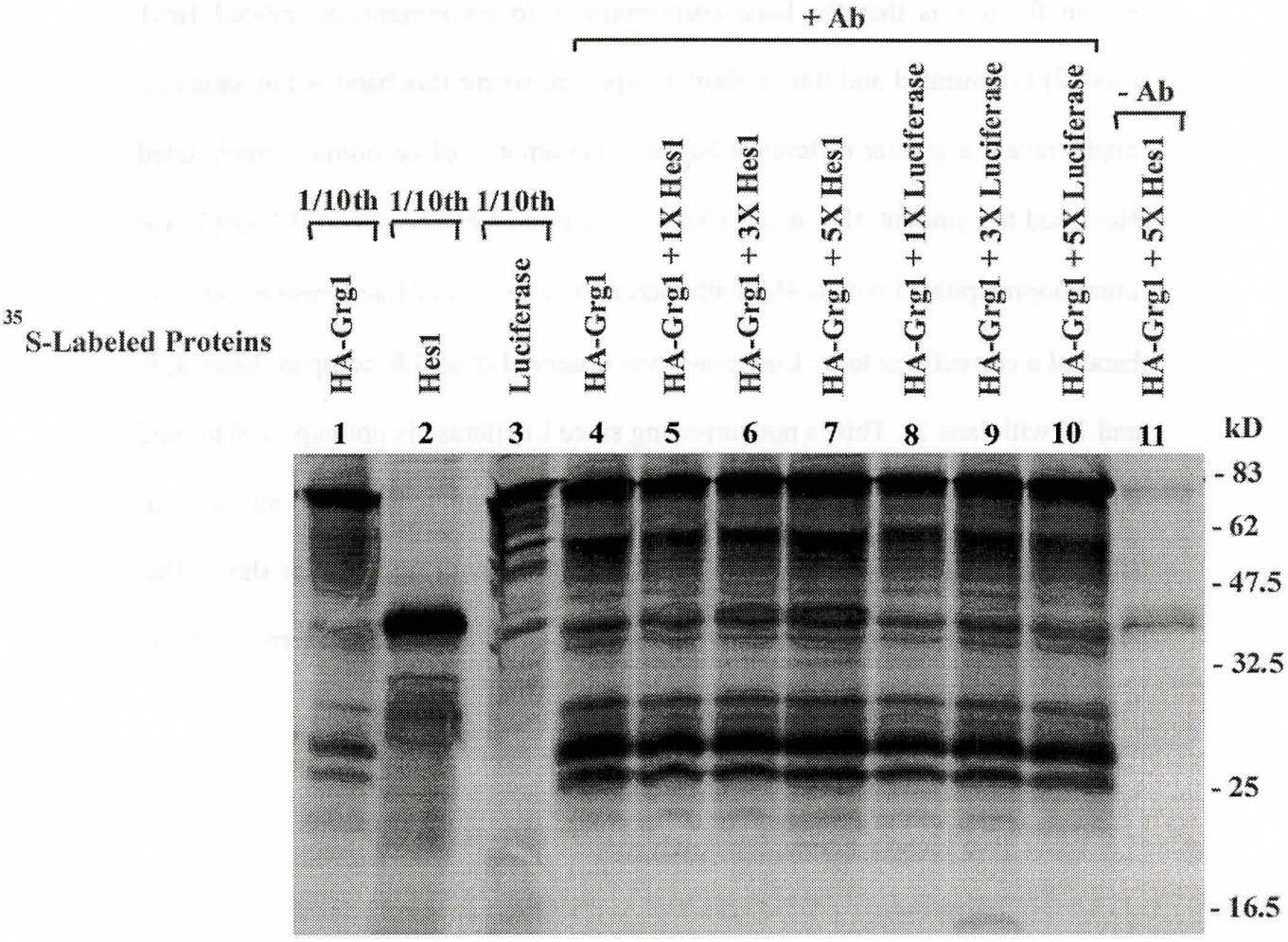
5' ATGGGGCCCAGAGTGATGACAACTTAGTTGTG 3' and the reverse primer was 5' AATGAATTCAGTAGATGACTTCATAGACTGTA 3'. The resulting PCR fragment was then subcloned into the Apa 1 and EcoR1 sites of pSPUTKHA to create Grg1-pSPUTKHA and the junction was verified by DNA sequencing (Mobix) (Fig. 3.7).

In order to produce a radiolabeled HA-tagged Grg1 protein to use in co-immunoprecipitation experiments the Grg1-pSPUTKHA plasmid was linearized with EcoR1 and *in vitro* transcribed using SP6 RNA polymerase. The resulting transcript was then *in vitro* translated in rabbit reticulosyte lysate (Promega) to produce the ^{35}S-labelled HA-Grg1 protein. Co-immunoprecipitation experiments were performed by mixing radiolabeled HA-Grg1 protein with increasing amounts of either radiolabeled Hes1 or Luciferase. Hes1 was used to determine the appropriate conditions for co-precipitation since Hes1 and TLE1, the human homologue of Grg1, have previously been shown to interact (Stifani, 1997). Luciferase was included as a negative control since it is not a member of the *Notch* signalling pathway and is unlikely to interact with Grg1. The volume of each sample was adjusted to 300 µl with NET-gel buffer, α-HA antibody was added and the tubes were rocked at 4°C for one hour. In certain samples the α-HA antibody was omitted from the previous step in order to determine the amount of non-specific precipitation of radiolabeled proteins. Protein G Agarose was added to collect the antibody-protein complexes and the tubes were incubated at 4°C overnight with rocking. The beads were collected, washed four times with NET-

gel buffer and then resuspended in 1X SDS gel loading dye. The radiolabeled proteins were eluted from the beads by boiling for 5 minutes and the supernatant was loaded onto a 12% SDS-PAGE gel. The labelled proteins were separated by electrophoresis and then subjected to autoradiography (Fig. 3.8). The first three lanes of Fig. 3.8 show 10% input of HA-Grg1, Hes1, and Luciferase. Approximately 10 % of available HA-Grg1 is precipitated by the α-HA antibody (Fig. 3.8, compare lanes 1 and 4). When HA-Grg1 was immunoprecipitated with α-HA with increasing amounts of Hes1 present, a band of the correct size to be Hes1 was found to increase in intensity in a parallel fashion (Fig. 3.8, compare lanes 5, 6, and 7 with lane 2). However, when HA-Grg1 was incubated with Hes1 and mock immunoprecipitated in the absence of α-HA, a small amount of Grg1 and Hes1 were found to precipitate non-specifically. Hes1 and Grg1 are thus binding to the Protein G Agarose beads. In previous experiments (Fig. 3.5), bHLH proteins such as Hes1 were found to bind Protein A Sepharose, resulting in a large amount of background that made it difficult to detect any specific interactions occurring between Grg1 and bHLH proteins. In this experiment (Fig. 3.8), Protein G Agarose was used to precipitate the antibody-protein complexes in the hope that bHLH proteins would have less affinity for binding Protein G Agarose than they did for binding Protein A Sepharose so that background levels could be reduced to an acceptable level. However, the amount of Hes1 being specifically co-immunoprecipitated along with HA-Grg1 is above background levels (Fig. 3.8, compare lanes 7 and 11). The difference between the amount of co-precipitated

Figure 3.8 Co-immunoprecipitation of ^{35}S-labeled HA-tagged Grg1 and Hes1 with the α-HA antibody.

The co-immunoprecipitations were performed in NET-gel buffer and with or without α-HA antibody. The first three lanes show one-tenth of the translation product that were added to the co-immunoprecipitaton reactions. Equivalent amounts of radiolabeled HA-Grg1 was added to each co-immunoprecipitation reaction. Increasing amounts of ^{35}S-labeled Hes1 or Luciferase were added and the volume was adjusted to 300 µl with NET-gel buffer. 2 µl of α-HA antibody was added to the appropriate reactions. 40 µl of a 3 % slurry of Protein G Agarosa was added to collect antibody/protein complexes. The beads were washed and eluted and the supernatant was separated on a 12% SDS-polyacrylamide gel and then exposed to film for 3 days. The migration of the molecular weight markers is shown on the right.


+ Ab
- Ab
1/10th
1/10th
1/10th
HA-Grg1
Hes1
Luciferase
HA-Grg1
HA-Grg1 + 1X Hes1
HA-Grg1 + 3X Hes1
HA-Grg1 + 5X Hes1
HA-Grg1 + 1X Luciferase
HA-Grg1 + 3X Luciferase
HA-Grg1 + 5X Luciferase
HA-Grg1 + 5X Hes1
35 S-Labeled Proteins
1
2
3
4
5
6
7
8
9
10
11
kD
- 83
- 62
- 47.5
- 32.5
- 25
- 16.5

Hes1 (lane 7) and the amount of non-specific precipitation of Hes1 (lane 11) might be greater if a shorter exposure of this experiment was available. The reason for this is that the band corresponding to co-immunoprecipitated Hes1 (lane 7) is saturated and thus a shorter exposure where this band is not saturated might reveal a greater difference between the amount of co-immunoprecipitated Hes1 and the amount of non-specifically precipitated Hes1. When HA-Grg1 was immunoprecipitated with α-HA with increasing amounts of Luciferase present, no band of a correct size to be Luciferase was observed (Fig. 3.8, compare lanes 8, 9, and 10 with lane 3). This is not surprising since Luciferase is not expected to bind Grg1 and because Luciferase has consistently shown a low amount of non-specific binding to Sepharose (Fig.3.2). This experiment (Fig. 3.8) has shown that Hes1 is co-immunoprecipitated by HA-tagged Grg1 at levels moderately above background.

3.3 Yeast Two Hybrid Interaction Analysis

3.3.1 Construction of the Yeast Two Hybrid Fusion Vectors

The yeast two hybrid system is a genetic assay which allows for detection of protein-protein interactions *in vivo*. (Fields and Sternglanz, 1994). The yeast two-hybrid system was used to detect possible interactions between Hes proteins (Hes1 and Hes3) and Grg proteins (Grg1 and Grg4). Interactions between Grg proteins (Grg1 and Grg4) and the AML1b transcription factor were also studied

using the yeast two hybrid system. GAL4 hybrid cloning vectors, pGBT9 and pGAD424, were obtained in the Matchmaker TM Two-Hybrid System (Clontech Laboratories Inc.). The pGBT9 expression vector contains the sequence for the GAL4 DNA binding domain and the pGAD424 vector contains the sequence for the GAL4 activation domain. These vectors also carry the genes for TRP1 and LEU2, respectively. Since the yeast strain Y190 that is used for the transformations is auxotrophic for Trp and Leu, it is possible to identify colonies that contain both types of vectors by plating transformed yeast on Trp and Leu deficient plates. The yeast strain Y190 also contains a lacZ reporter gene downstream of several GAL4 binding sites. Reconstitution of the GAL4 transcription complex through an interaction between hybrid proteins therefore results in lacZ expression. Transformed colonies can thus be assayed for β-galactosidase expression and colonies containing interacting proteins can be identified by their blue colour following the staining reaction.

This system requires that in-frame fusions of the proteins of interest to the DNA-binding or activation domains of GAL4 be made in the appropriate vectors. A fusion between Hes3 and the GAL4 activation domain was created by subcloning the entire coding sequence of Hes3 into the pGAD424 cloning vector carboxy terminal to the GAL4 activation domain. The pGAD424 vector contains the GAL4 (768-881) activation domain, LEU2, and amp^r. A DNA fragment containing the entire coding sequence of Hes3 flanked by EcoR1 restriction sites was generated by PCR amplification of the Hes3 cDNA using primers (Life

Technologies) with an incorporated EcoR1 site. The forward primer used in the amplification of the Hes3 cDNA had the sequence 5' TCCGAATTCCTGATGGAGAAGAAGCGTCG 3'. The reverse Hes3 primer had the sequence 5' TCTGAATTCTTGCCTAGGCCTCACCAG 3'. Following amplification, the Hes3 PCR fragment was digested with EcoR1 and subcloned into the EcoR1 site located in the multiple cloning site of the pGAD424 vector. Sequence analysis (Mobix) of the junction confirmed that the fusion between the GAL4 activation domain and Hes3 was in-frame (Fig. 3.9).

A fusion between Grg1 and the GAL4 binding domain was created by subcloning a large fragment of the Grg1 coding sequence lacking the amino terminus into the pGBT9 cloning vector carboxy terminal to the GAL4 binding domain. The pGBT9 vector contains the GAL4 (1-147) DNA-binding domain, TRP1, and amp^r. A Grg1 (754-2389) DNA fragment lacking the amino-terminal Q and G/P domains but containing most of the CcN domain and all of the S/P and WD40 domains was generated by PCR amplification of the Grg1 cDNA using primers (Life Technologies) incorporating EcoR1 restriction sites. The sequence of the forward primer 5' GTGGAATTCAAGGATAACTATGACAGTGATGGG 3'. The reverse Grg1 primer had the sequence 5' AATGAATTCAGTAGATGACTTCATAGACTGTA 3'. The Grg1 PCR fragment was digested with EcoR1 and subcloned into the EcoR1 site located in the multiple cloning site of the pGBT9 vector. Analysis of the sequence at the junction between the GAL4 activation domain and Grg1 confirmed that the fusion

Figure 3.9 The Hes3-pGAD424 construct.

A fusion between Hes3 and the GAL4 activation domain (GAL4 AD) was created by subcloning the entire coding sequence of Hes3 into pGAD424. The pGAD424 vector contains the GAL4 (768-881) activation domain, LEU2 (the gene for leucine), and amp^r (for ampicillin resistance). The coding sequence of Hes3 was subcloned into the EcoR1 site of the multiple cloning site (MCS) of the pGAD424 vector. The fusion between the GAL4 activation domain and Hes3 is shown. The vector sequence is shown in capital letters and the Hes3 sequence is shown in non-capitalised letters. P_{ADH1} and T_{ADH1} indicate the promoter and termination sequences of the ADH1 gene, respectively. Col E1 ori is the origin of replication.

GAGATCGaattcctgatggagaagaagcgtcgtgcccgcatcaacctgtcactggag

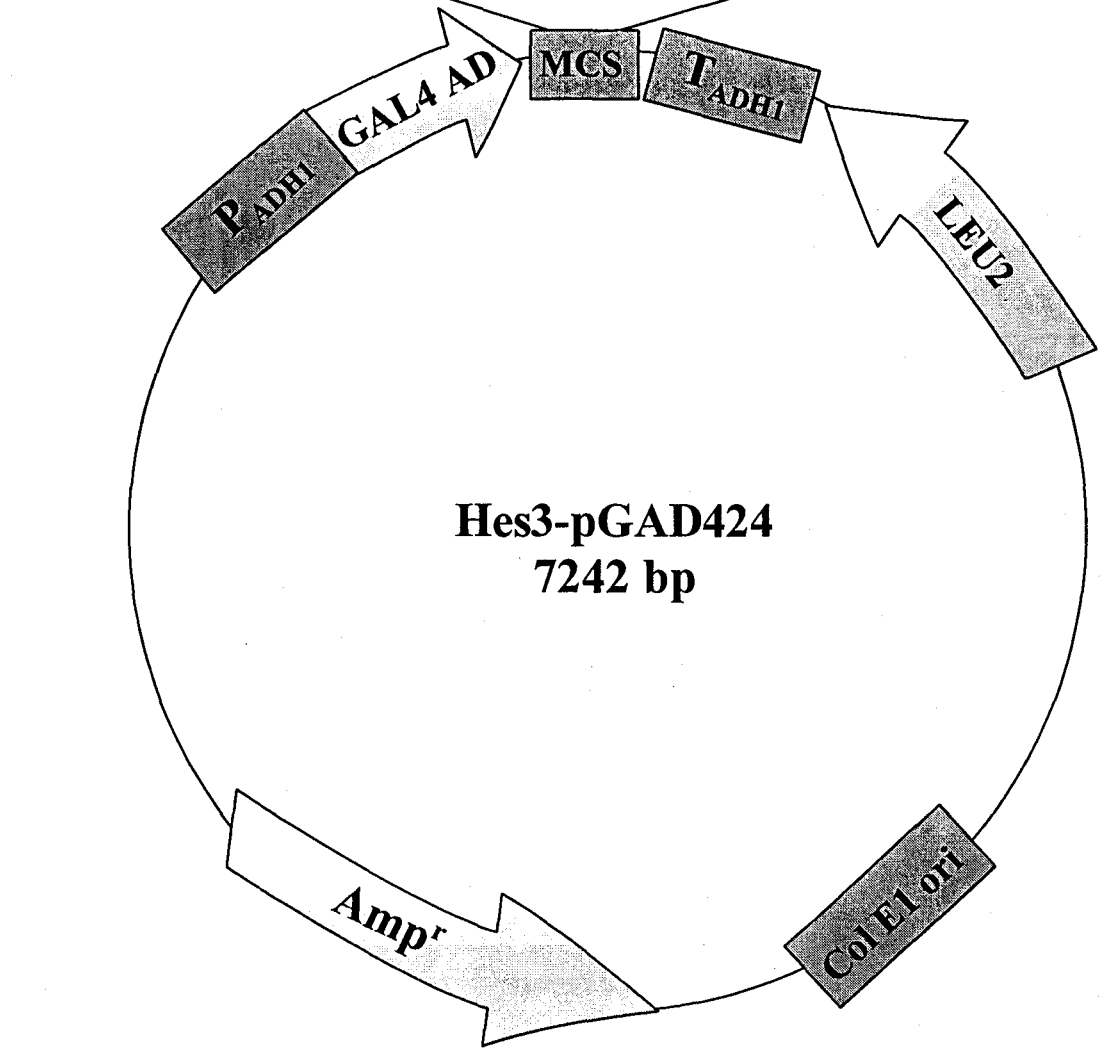
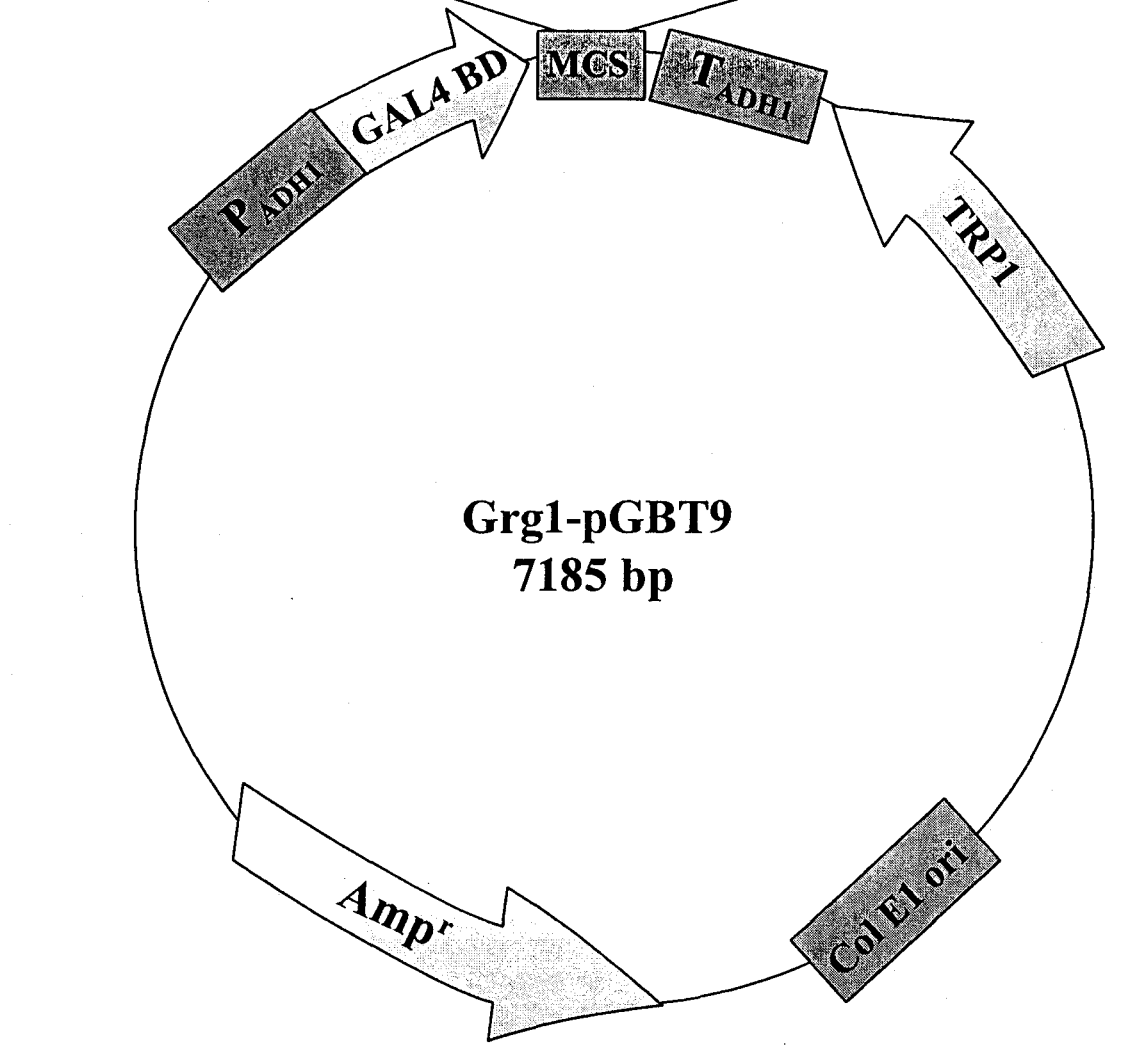


Figure 3.10 The Grg1-pGBT9 construct.

A fusion between Grg1 and the GAL4 binding domain (GAL4 BD) was created by subcloning a large fragment of the Grg1 coding sequence lacking the amino terminus into pGBT9. The pGBT9 vector contains the GAL4 (1-147) DNA-binding domain, TRP1 (the gene for tryptophan), and amp^r (for ampicillin resistance) 3'. The Grg1 fragment was subcloned into the EcoR1 site of the multiple cloning site (MCS) of the pGBT9 vector. Analysis of the sequence at the junction between the GAL4 activation domain and Grg1 confirmed that the fusion was in-frame (Fig. 3.10). The fusion between the GAL4 binding domain and Grg1 is shown. The vector sequence is shown in capital letters and the Hes3 sequence is shown in non-capitalised letters. P_{ADH1} and T_{ADH1} indicate the promoter and termination sequences of the ADH1 gene, respectively. Col E1 ori is the origin of replication.

TCGCCGGaattcaaggataactatgacagtgatggggacaagagtgatgacaactta



was in-frame (Fig. 3.10). Four hybrid vectors were also obtained from Karen Koop, a former technician in our lab. These hybrid vectors included Grg4-pGBT9 (in which the amino terminal sequences for the Q and G/P domains are missing); Hes1-pGAD424 (containing the full coding sequence of Hes1); AML1b-pGAD424; and AML1b[Y480W]-pGAD424 (in which the WRPY motif of AML1b has been mutated to WRPW). Two other hybrid vectors constructed by Madalena Pinto, a former graduate student in our lab, were also obtained. These hybrid vectors were Grg3b-pGAD424 and Grg5-pGBT9. Since Grg3b and Grg5 have been shown to interact both in the yeast two hybrid system and in a second independent assay in which radiolabeled Grg3b was retained on Grg5-GST fusion proteins (Pinto, 1996), the Grg3b-pGAD424 and Grg5-pGBT9 hybrid vectors were used as a positive control in the yeast two hybrid experiments.

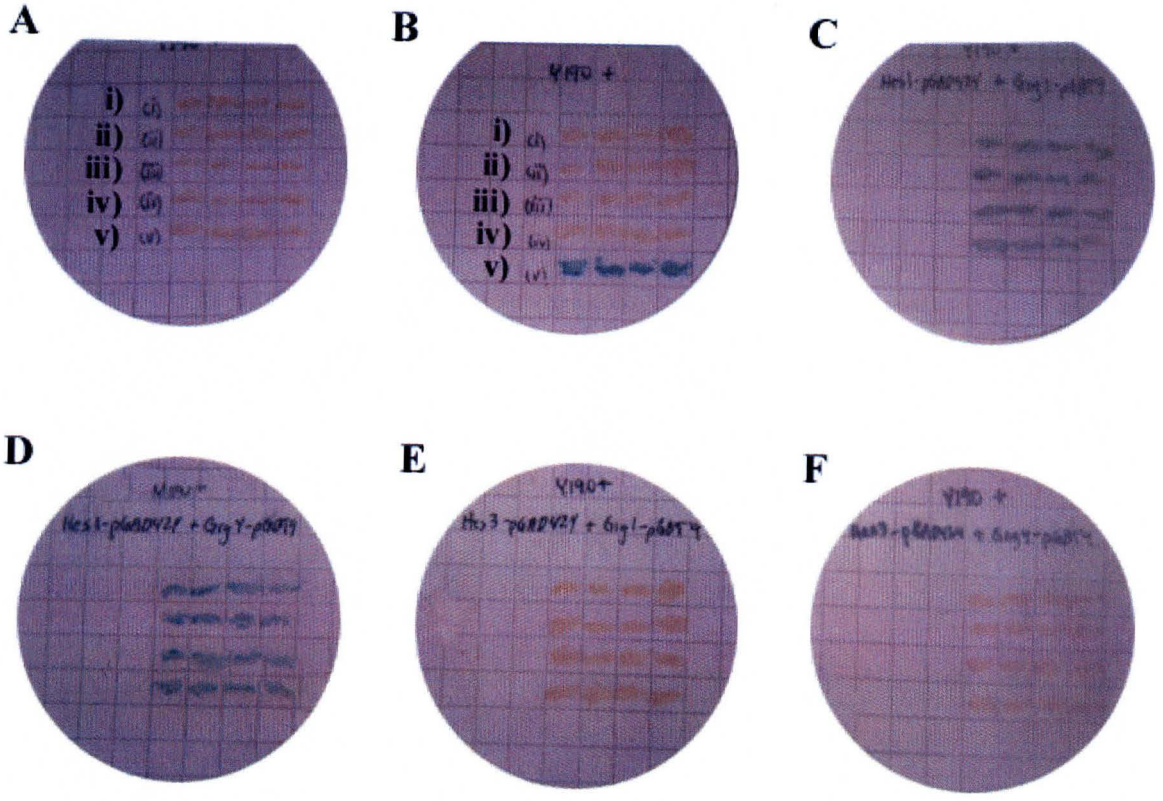
3.3.2 Hes1 Interacts with Grg1 and Grg4 in the Yeast Two Hybrid System

Yeast were transformed with individual or combinations of the hybrid vectors and parental vectors according to the protocol developed by Schiest et al (1993) and were then assayed for β-galactosidase expression (Fig. 3.11). Yeast co-transformed with the parental vectors pGAD424 and pGBT9 did not express β-galactosidase as indicated by the white colonies after the staining reaction [Fig. 3.11, A (i)]. Yeast transformed with Hes1-pGAD424 or Hes3-pGAD424 did not express β-galactosidase [Fig. 3.11, A (ii) & (iii)]. Transformation of yeast with Grg1-pGBT9 or Grg4-pGBT9 also resulted in white, non-β-galactosidase-

Figure 3.11 Yeast two-hybrid analysis of Hes and Grg interactions

Yeast were transformed with individual vectors or with a combination of vectors. Transformed yeast were streaked onto Hybond N and stained for β-galactosidase expression. The results of the yeast two-hybrid analysis are indicated in the figure and in the table below. White colonies are those that do not express β-galactosidase and blue colonies are those that do express β-galactosidase.

Plate#	Transforming Vector(s)	Colour of Colonies
A (i)	pGAD424 + pGBT9	white
A (ii)	Hes1-pGAD424	white
A (iii)	Hes3-pGAD424	white
A (iv)	Grg1-pGBT9	white
A (v)	Grg4-pGBT9	white
B (i)	Hes1-pGAD424 + pGBT9	white
B (ii)	Hes3-pGAD424 + pGBT9	white
B (iii)	pGAD424 + Grg1-pGBT9	white
B (iv)	pGAD 424 + Grg4-pGBT9	white
B (v)	Grg3b-pGAD424 + Grg5-pGBT9	BLUE
C	Hes1-pGAD424 + Grg1-pGBT9	BLUE
D	Hes1-pGAD424 + Grg4-pGBT9	BLUE
E	Hes3-pGAD424 + Grg1-pGBT9	white
F	Hes3-pGAD424 + Grg4-pGBT9	white


A
i)
ii)
iii)
iv)
v)
B
i)
ii)
iii)
iv)
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C
D
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F

expressing colonies [Fig. 3.11, A (iv) & (v)]. Thus, transformation of yeast with a combination of the parental vectors or with the individual hybrid vectors is not sufficient to initiate lacZ expression.

Yeast co-transformed with combinations of either Hes1-pGAD424 and pGBT9 or Hes3-pGAD424 and pGBT9 did not express β-galactosidase as indicated by the white colonies [Fig. 3.11, B (i) & (ii)]. Co-transformation of yeast with combinations of pGAD424 and Grg1-pGBT9 or pGAD424 and Grg4-pGBT9 also resulted in white colonies [Fig. 3.11, B (iii) & (iv)]. However, yeast co-transformed with the positive control vectors Grg3b-pGAD424 and Grg5-pGBT9 did express β-galactosidase as indicated by the blue colour of the colonies [Fig 3.11, B (v)]. These results indicate that the parental vectors are not capable of interacting with any of the hybrid vectors.

Co-transformation of yeast with Hes1-pGAD424 and Grg1-pGBT9 resulted in blue, β-galactosidase-expressing colonies indicating that an interaction between Hes1 and Grg1 is occurring [Fig. 3.11, C]. Similarly, co-transformation of yeast with Hes1-pGAD424 and Grg4-pGBT9 also resulted in blue colonies indicating an interaction between Hes1 and Grg4 [Fig. 3.11, D]. However, yeast co-transformed with Hes3-pGAD424 and Grg1-pGBT9 did not express β-galactosidase as indicated by the white colonies [Fig. 3.11, E]. Yeast co-transformed with Hes3-pGAD424 and Grg4-pGBT9 also did not express β-galactosidase [Fig. 3.11, F]. These results indicate that Hes3 does not interact with either Grg1 or Grg4 in the yeast two hybrid system.

Yeast co-transformed with the positive control vectors Grg3b-pGAD424 and Grg5-pGBT9 began to stain blue after approximately 6 hours and attained a maximum intensity after 12 hours. However, yeast co-transformed with combinations of Hes1-pGAD424 and either Grg1-pGBT9 or Grg4-pGBT9 did not begin to stain blue until after approximately 16 hours and achieved maximum intensity after approximately 24 hours. The lack of intensity in the blue staining of the cella and the length of time it took for colonies to turn blue may indicate a weak or transient interaction between Hes1 and Grg1. However, the light staining could also be a result of poor expression of one or both of the hybrid proteins in the yeast cells.

3.3.3 Expression of Hybrid Proteins in Yeast Extract

In 1994 Paroush et al. demonstrated that a WRPW motif is required for interaction with *Groucho* (Paroush, 1994). It is therefore surprising that Hes3, which contains a C-terminal WRPW motif, showed no interaction with either Grg1 or Grg4 in the yeast two hybrid system (Fig. 3.11, E and F). In order to determine whether this interaction was not detected because Hes3 is incapable of such an interaction or whether one or more of the hybrid proteins was simply not being expressed in yeast cells, transformed yeast extract was analyzed by Western blot. A monoclonal antibody specific to the GAL4 activation domain (8C1-21) and a monoclonal antibody specific to the GAL4 DNA binding domain (5C8) were obtained as a gift from Dr. Brenda Andrews' lab at the University of

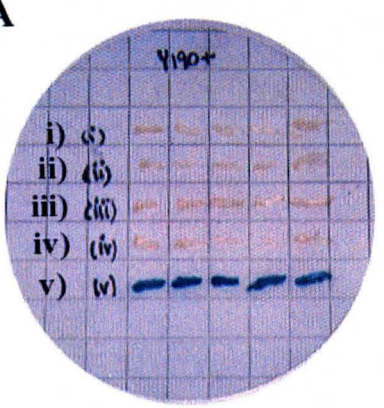
Toronto. Extract was prepared from untransformed yeast cells, or yeast cells co-transformed with combinations of either Hes1-pGAD424 or Hes3-pGAD424 and Grg1-pGBT9 or Grg4-pGBT9. 40 μg of yeast extract was loaded per lane on an SDS-PAGE gel and the proteins were separated by electrophoresis. The proteins were transferred to a nylon membrane and a Western blot was performed using the GAL4 activation domain antibody as the primary antibody and anti-mouse horseradish peroxidase-conjugated IgG antibody as the secondary antibody. The GAL4 activation domain antibody did not detect either the Hes1 or Hes3 hybrid proteins in transformed yeast extract (data not shown) even when used at a dilution of 1:100. However, the Hes1 hybrid protein showed an interaction with both Grg1 and Grg4 in the yeast two hybrid system (Fig. 3.11, C and D) and therefore it must be expressed in the transformed yeast extract even though it is not being detected. Therefore, due to the low affinity of the GAL4 activation domain antibody for the Hes1 and Hes3 hybrid proteins, it is not possible using this antibody to determine if Hes3 is being expressed in yeast.

A second Western blot was performed on untransformed yeast extract or extract from yeast transformed with either Hes1-pGAD424 or Hes3-pGAD424 and Grg1-pGBT9 or Grg4-pGBT9. 40 μg of yeast extract was loaded per lane on a 12% SDS-PAGE gel, the proteins were separated by electrophoresis and then transferred to a nylon membrane. The Western blot was performed using the GAL4 DNA binding domain antibody 5C8 as the primary antibody and anti-mouse horseradish peroxidase-conjugated IgG antibody as the secondary

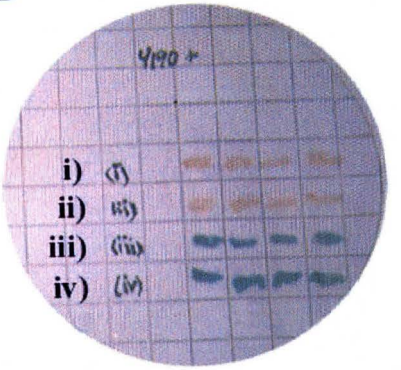
Figure 3.12 Western blot analysis of transformed yeast.

The Western blot was performed on extract prepared from untransformed yeast or from yeast transformed with combinations of Hes1-pGAD424, Hes3-pGAD424, Grg1-pGBT9, or Grg4-pGBT9. 40 μg of yeast extract was loaded per lane on a 12% SDS-polyacrylamide gel and the proteins were separated by electrophoresis. The proteins were transferred to a nylon membrane and a Western blot was performed. The primary antibody was the GAL4 DNA binding domain antibody 5C8 used at a dilution of 1:100 and the secondary antibody was horseradish peroxidase-conjugated anti-mouse IgG used at a dilution of 1:5000. The large arrow indicates the position of the GAL4 binding domain-Grg1 fusion proteins and the small arrow indicates the position of the GAL4 binding domain-Grg4 fusion proteins. The migration of the molecular weight markers is indicated on the right.

A



B



antibody. No proteins were detected by the GAL4 DNA binding domain antibody in the extract of untransformed yeast cells as expected (Fig. 3.12, lane 1). However, a protein of approximately 75 kD was detected in the extract of yeast transformed with either Hes1-pGAD424 and Grg4-pGBT9 or Hes3-pGAD424 and Grg4-pGBT9 (Fig 3.12, lanes 2 and 3). The expected size of the Grg4-GAL4 DNA binding domain hybrid protein is approximately 70 kD (64.7 kD for the Grg4 fragment and 5.4 kD for the GAL4 DNA binding domain). A 70 kD protein was also detected in the extract of yeast cells transformed with either Hes1-pGAD424 and Grg1-pGBT9 or Hes3-pGAD424 and Grg1-pGBT9 (Fig. 3.12, lanes 4 and 5). This protein is approximately the correct size to be the Grg1-GAL4 DNA binding domain hybrid protein which is estimated to be approximately 66 kD in size (60.4 kD for the Grg1 fragment and 5.4 kD for the GAL4 DNA binding domain). The detected proteins both ran at a slightly higher molecular weight than their predicted sizes (75 kD instead of 70 kD for Grg4 and 70 kD instead of 66 kD for Grg1). The proteins could be migrating slower on the gel due to phosphorylation. Thus, the GAL4 DNA binding domain hybrid proteins Grg1-pGBT9 and Grg4-pGBT9 are being expressed in yeast cells. Therefore, the lack of interaction between Hes3-pGAD424 and Grg1-pGBT9 or Grg4-pGBT9 in the yeast two hybrid system is not due to a lack of expression of the GAL4 DNA binding domain hybrid proteins in transformed yeast.

3.3.4 Analysis of Interactions Occurring Between Grg Proteins and AML1B.

The AML1b transcription factor contains a conserved VWRPY at its C terminal end which is very similar to the WRPW interaction motif found at the C-terminus of Hairy and its mammalian homologues, the Hes proteins. AML1b was tested for its ability to interact with Grg proteins in the yeast two hybrid system in order to determine if the VWRPY motif of AML1 could have a similar function to that of the WRPW motif found in Hes proteins. An AML1b protein in which the VWRPY motif was mutated to a VWRPW motif (AML1b[Y480W]) was also tested for its ability to interact with Grg1 and Grg4 hybrid proteins.

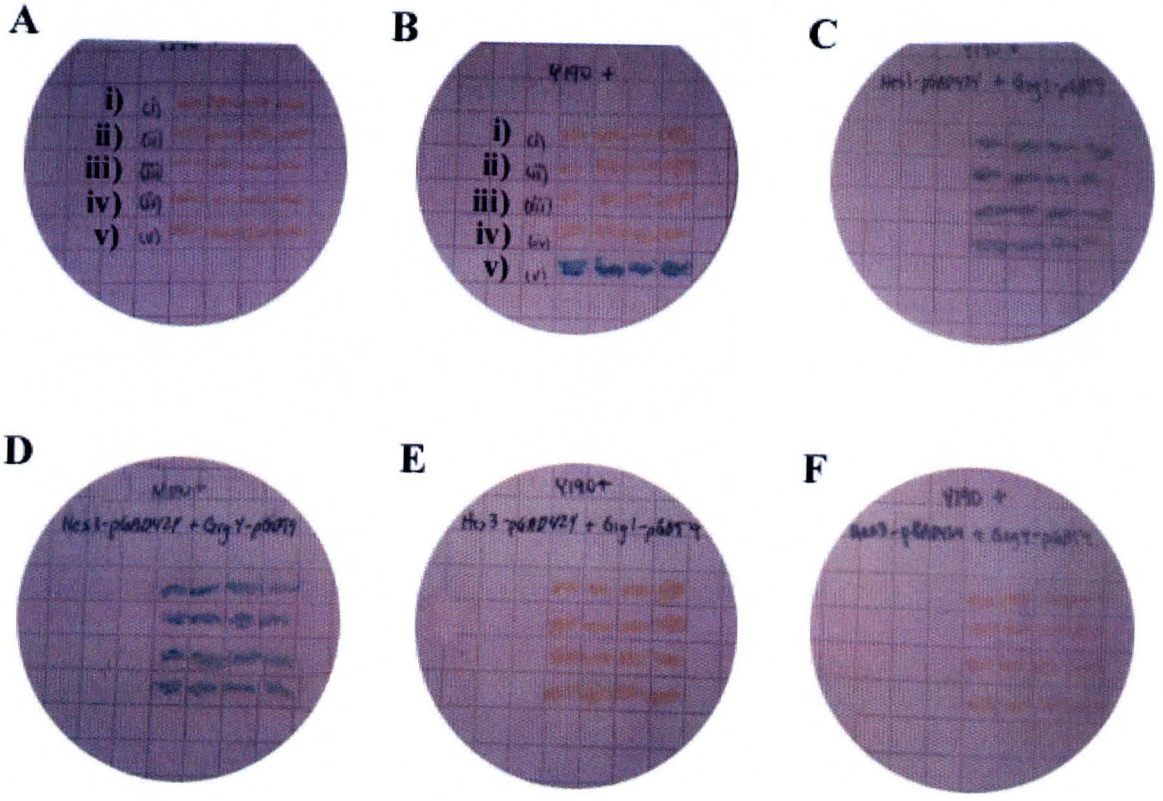
Yeast cells were transformed with combinations of the parental and hybrid vectors and were then assayed for β-galactosidase expression (Fig. 3.13). Yeast co-transformed with combinations of AML1b-pGAD424 and pGBT9 or AML1b[Y480W]-pGAD424 and pGBT9 did not express β-galactosidase as indicated by the white colonies [Fig. 3.13, A (i) & (ii)]. Similarly, co-transformation of yeast with combinations of pGAD424 and Grg1-pGBT9 or pGAD424 and Grg4-pGBT9 resulted in white colonies that did not express β-galactosidase [Fig. 3.13, A (iii) & (iv), respectively]. Yeast co-transformed with the positive control vectors Grg3b-pGAD424 and Grg5-pGBT9 did express β-galactosidase as indicated by the blue colonies [Fig. 3.13, A (v)]. These results indicate that the parental vectors were not able to interact with any of the hybrid vectors.

Co-transformation of yeast with AML1b-pGAD424 and Grg1-pGBT9 resulted in white colonies indicating that AML1b and Grg1 do not interact [Fig.

Figure 3.13 Yeast two-hybrid analysis of AML1b and Grg interactions

Yeast were transformed with individual vectors or with a combination of vectors. Transformed yeast were streaked onto Hybond N and stained for β-galactosidase expression. The results of the yeast two-hybrid analysis are indicated in the figure and in the table below. White colonies are those that do not express β-galactosidase and blue colonies are those that do express β-galactosidase.

Plate # Transforming Vector(s)	Colour of Colonies
A (i) AML1b-pGAD424 + pGBT9	white
A (ii) AML1b[Y480W]-pGAD424 + pGBT9	white
A (iii) pGAD424 + Grg1-pGBT9	white
A (iv) pGAD424 + Grg4-pGBT9	white
A (v) Grg3b-pGAD424 + Grg5-pGBT9	BLUE
B (i) AML1b-pGAD424 + Grg1-pGBT9	white
B (ii) AML1b-pGAD424 + Grg4-pGBT9	white
B (Aii) AML1b[Y480W]-pGAD424 + Grg1-pGBT9	BLUE
B (iv) AML1b[Y480W]-pGAD424 + Grg4-pGBT9	BLUE


A
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3.13, B (i)] in the yeast two hybrid system. Similarly, yeast co-transformed with both AML1b-pGAD424 and Grg4-pGBT9 did not express β-galactosidase [Fig. 3.13, B (ii)]. However, when yeast were co-transformed with AML1b[Y480W]-pGAD424 and Grg1-pGBT9, the result was blue, β-galactosidase-expressing colonies [Fig. 3.13, B (iii)]. Also, when yeast were co-transformed with AML1b[Y480W]-pGAD424 and Grg4-pGBT9 blue, β-galactosidase-expressing colonies were observed [Fig. 3.13, B (iv)]. Although the lack of a suitable GAL4 activation domain antibody prevented testing transformed yeast extract for expression of the AML1b hybrid protein, these results suggest that AML1b is not capable of interaction with Grg1 and Grg4, but a single amino acid substitution (VWRPY to VWRPW) is sufficient to allow this interaction to occur.

IV. DISCUSSION

4.1 Grg Interactions with Hes and AML1b Transcription Factors

In this study, the interactions occurring between Grg proteins and Hes proteins were examined in order to better understand how these proteins may function during neurogenesis. To date, three full-length Grg proteins (Grg1, 3a, 4) containing all the domains found in their *Drosophila* homologue, Groucho, have been identified in mice (Figure 1.1). Two short Grg proteins containing only the amino terminal dimerization domain have also been identified in mice and have been proposed to negatively regulate the function of the long Grg proteins (Pinto and Lobe 1996). Neither Groucho or its mammalian homologues have DNA-binding activity. In *Drosophila*, Groucho has been shown to act as a corepressor which negatively regulates transcription of certain proneural genes by interacting with DNA-binding Hairy-like bHLH transcription factors. The Hairy-like bHLH transcription factors are conserved in mammals and are known as the Hes family of genes. To date, four Hes proteins have been identified (Hes1,2,3,5) in mice and humans. Since the domains responsible for interaction between Groucho and Hairy have been conserved in their mammalian homologues (Grg and Hes), this leads to the hypothesis that the interaction may have been conserved as well. Furthermore, since the Grg domain responsible for this interaction varies Hes proteins with varying affinities. It was also hypothesised that Grg proteins may be

able to interact with the Runt-domain protein AML1b, a transcription factor that significantly between Grg family members but is fairly conserved between mouse and human paralogues, this suggests that certain Grg proteins may bind different contains a C-terminal VWRPY domain which is very similar to the C-terminal WRPW interaction domain found in Hes transcription factors.

Interactions between Grg and Hes proteins were first studied using co-immunoprecipitation techniques. False positives are less likely to be obtained in co-immunoprecipitation experiments than in conventional pull-down assays since neither of the interacting proteins are present in overabundance. Co-immunoprecipitation experiments were performed in order to determine if Grg proteins present in P19 cell extract were able to interact with radiolabeled Hes1 and AML1b proteins produced in a cell-free system. The antibodies used in these experiments were α-WD40 and α-HA. The α-WD40 antibody, generated by Madalena Pinto, was directed against the C-terminal end of the long Grg proteins. In this study, the α-WD40 antibody was found to be able to specifically precipitate ^{35}S-labelled Grg1 produced in a cell-free system. However, the α-WD40 antibody only immunoprecipitated approximately 5 % of the total protein available for precipitation. One possible explanation for the poor performance of the antibody is that it is capable only of recognizing the denatured form of the protein. If this is the case then the antibody would only be able to precipitate the small fraction of denatured Grg1 which may be produced during an *in vitro* translation reaction. It was also determined in this study that a major set-back to

performing co-immunoprecipitation with bHLH transcription factors is that these proteins have an affinity for binding to Protein A Sepharose. Specifically, Hes1, Mash 2, and AML1b appear to bind directly to the Sepharose component of Protein A Sepharose. Attempts were made to diminish this activity by increasing the stringency of the buffers used to wash the Protein A Sepharose beads and by adding bovine serum albumin (BSA) to the immunoprecipitation reactions in order to block the binding sites. However, it was not possible to diminish the Sepharose-binding activity of these proteins using these methods.

Coimmunoprecipitation experiments were also attempted using ^{35}S-labelled HA-Grg1 and an α-HA antibody. It was hoped that the commercially available α-HA antibody would have a higher affinity for binding to HA-Grg1 than α-WD40 did for Grg1 in order to increase the likelihood of seeing an interaction between HA-Grg1 and radiolabeled Hes proteins. Protein G Agarose was substituted for Protein A Sepharose in the immunoprecipitation reactions since the Hes proteins had shown an ability to bind directly to Sepharose. However, the α-HA antibody was only able to immunoprecipitate approximately 10 % of available HA-Grg1 and background levels were high due to binding of Hes1 to Protein G Agarose. The amount of Hes1 co-precipitated with HA-Grg1 was above background levels but not dramatically. Therefore, due to the relatively low affinity antibodies as well as the high level of background as a result of the binding of the test proteins to Sepharose and Protein G Agarose, it was determined that co-immunoprecipitation techniques are not suitable for detecting interactions

between the Hes, AML, and Grg proteins.

The transcription-repressing activity of the Grg family of proteins likely requires interaction with Hairy-like bHLH proteins such as members of the Hes family. The yeast two hybrid experiments performed in this study indicate that Grg1 and Grg4 are capable of interaction with Hes1 but not with Hes3. An interaction between Grg1 and Hes1 was expected since TLE1 (the human paralogue of Grg1) has been shown to interact with Hes1 in the yeast two hybrid system as well as in an pull-down assay using GST fusion proteins (Grbavec and Stifani 1996). The interaction between Grg1 and Hes1 observed in this study appeared to be weak or transient as suggested by the light blue staining of the yeast colonies. However, the light staining could also be due to poor expression of one or both of the fusion proteins. The yeast colonies expressing both Grg4 and Hes1 stained a darker blue than yeast containing Grg1 and Hes1 indicating that Grg4 may have a higher binding affinity for Hes1 than Grg1. It would seem reasonable for different Grg proteins to have different binding affinities for Hairy-like transcription factors since the Grg domain responsible for this interaction, the S/P domain, varies significantly between Grg family members. However, differences in the intensity of the staining could also be a result of differences in the level of expression of these hybrid proteins in yeast.

The expression patterns of Grg1 and Grg4 also support a possible interaction with Hes1 due to their overlapping expression domains. Many neurogenic genes, including Notch 1, Grg1, Grg4, Hes1, and Hes3 are expressed during mid- to late

embryogenesis in several epithelial cell types undergoing mesenchymal induction (Dehni et al. 1995; Koop, MacDonald, and Lobe 1996; Weinmaster et al. 1991). Hes1 is expressed in many tissues in the developing embryo as well as in adults. Grg4 expression in the rostral mesencephalon overlaps Hes1 expression (Lobe 1996). Grg4 and Hes1 expression also overlap in the cells of the cranial and dorsal root ganglia in the peripheral nervous system (Franco del Amo et al. 1992; Koop, MacDonald, and Lobe 1996; Sasai et al. 1992; Weinmaster et al. 1991, 1992). Thus, due to overlapping expression domains, there are many tissues in which Hes1 could interact with either Grg1 or Grg4 to regulate cell differentiation.

Hes3 is a Hairy-like transcription factor that is lacking the amino terminal basic domain that is present in the other Hes factors. Hes3 is therefore incapable of binding DNA and thus cannot bind N boxes to directly repress transcription from target promoters. However, Hes3 can still repress transcription from E-box promoters by sequestering E box-binding transcriptional activators such as E47 (Sasai et al. 1992). Extramacrochaetae (emc) encodes a similar HLH protein that is lacking a basic domain. Emc can prevent Da-AS-C complexes from binding DNA *in vitro* (Garrell and Modolell 1990; Martinez, Modolell, and Garrell 1993). In this study neither Grg1 nor Grg4 were seen to interact with Hes3 in the yeast two hybrid system. Since Hes3 cannot bind DNA, and since Grg proteins can only repress transcription when bound to a target promoter, an interaction between these proteins might in fact be detrimental to their activity. An interaction

between Hes3 and Grg1 or between Hes3 and Grg4 would result in the formation of a non-functional heterodimer. Similarly, an interaction between Hes3 and a Grg protein might interfere with the ability of Hes3 to sequester E box-binding transcriptional activators. It is interesting to consider how Hes3 might escape interaction with Grg proteins since it contains a C-terminal WRPW motif that has been shown to be sufficient for interaction to occur. Fisher et al (1996) demonstrated that a WRPW-GST fusion protein is sufficient to retain radiolabeled *Groucho* and TLE1. The WRPW motif is located at the end of the C-terminus of the protein where it might be expected to be exposed on the surface of the protein, which is certainly the case in other Hes proteins that do interact with Grg proteins. In the case of Hes3, the tertiary structure of the protein may be such that the WRPW motif is either masked or buried within the protein so that it is not available for interaction. However, an alternate explanation of the failure of Grg1 and Grg4 to interact with Hes3 in the yeast two-hybrid system is that Hes3 is not being expressed in yeast cells or is not capable of translocating to the nucleus. It was not possible to test the transformed yeast cells for expression of Hes3 due to the lack of a suitable antibody.

AML1b is a transcription factor containing a Runt domain and a C-terminal VWRPY domain. Since mutations which remove the C-terminus of AML1 are associated with some forms of leukemia, it would be interesting to know if the VWRPY motif may allow AML1b to interact with, and thus be regulated by, Grg proteins. In this study, AML1b was not able to interact with either Grg1 or Grg4

in the yeast two-hybrid system. However, mutation of the C-terminal VWRPY motif of AML1b to VWRPW was sufficient to allow this interaction to occur. The observation that both Grg1 and Grg4 could interact with AML1b when its VWRPY motif was mutated to VWRPW supports the observation that an intact WRPW motif is essential for Grg interaction. Recently, *Groucho* has been shown to interact with Runt, the *Drosophila* homologue of AML1 (Aronson et al. 1997) through its C-terminal VWRPY motif. The interaction observed between Groucho and Runt is not contrary to the results observed in the yeast two-hybrid analysis performed in this study since another group (Aronson et al. 1997) observed that full-length Runt and Groucho, the *Drosophila* homologues of AML1b and Grg1/TLE1, do not interact in the yeast two-hybrid system. However, a Runt mutant with an N-terminal deletion that removes the Runt domain showed strong interaction with Groucho. Furthermore, a single amino acid change in the C-terminus (Y509W) which changes the pentapeptide VWRPY to VWRPW was also found to be sufficient to allow the interaction between Runt and Groucho to occur (Aronson et al. 1997). Aronson et al. also demonstrated that the VWRPY motif alone was able to interact with Groucho in the yeast-two hybrid system. These observations, as well as those described in this study, suggest that full-length Runt and AML1b may be in a conformation that inhibits interaction with Groucho and Grg1, respectively, and that small changes in the Runt domain and C-terminus are sufficient to relieve this conformational restrain. Since minor changes in the Runt and VWRPY domains allow interactions between

Runt/AML1b and Groucho/Grg1 to occur, it is possible that the Runt domain is acting to modulate these interactions, possibly through an interaction with the VWRPY sequence (Aronson et al. 1997). There is a need for such regulation since, unlike the bHLH Hairy-like proteins, Runt and AML1 proteins are capable of acting as transcriptional activators as well as transcriptional repressors. Thus, in order for Runt-domain proteins to act as transcriptional activators, they must be free of interaction with Groucho. Further work into the mechanism by which the Grg-binding ability of AML1b is modulated is warranted. It is possible that binding of AML1b to a protein partner may cause a conformational change in the Runt domain that allows binding with Grg proteins to occur.

Shortly after the work described above was completed, a physical and functional interaction between AML1b and TLE1, the human homologue of Grg1, was demonstrated (Imai et al. 1998). Using a GST fusion protein assay, Imai et al. demonstrated an interaction between the S/P domain of TLE1 and both the Runt domain and WRPY-containing C-terminus of AML1b. The C-terminus of AML1b bound the C-terminal end of the S/P domain of TLE1, while the Runt domain of AML1b bound the middle region of the S/P domain. These observations suggest two distinct modes of interaction between AML1b and TLE1. Moreover, TLE1 was able to inhibit AML1-induced transactivation of a target promoter and this repression was mediated by the C-terminal end of AML1b containing the VWRPY motif. AML1b has been shown to interact with Ets-1 (Zhang et al. 1996), Myb (Sun, Graves, and Speck 1995), and C/EBP

(Hernandez-Munain and Krangel 1994) and some of these interactions have been proposed to enhance AML1-induced transcriptional activation. TLE1 is capable of binding AML1b deletion mutants that lack the C-terminal VWRPY motif but is incapable of repressing its transcriptional activity. One possible explanation for this observation is that TLE1 may inhibit the interaction between AML1b and other proteins that may act as activators and that removal of the C-terminus of AML1b can disrupt this inhibition.

The yeast two-hybrid system is a very useful assay for detecting protein-protein interactions. However, this system has some potential drawbacks. For instance, complications may arise if the hybrid proteins are not stably expressed or if they are not localised to the yeast nucleus. Problems may also arise if the fused GAL4 domains interfere with the ability of the hybrid proteins to interact. Although this is an *in vivo* assay, the conditions might not allow certain protein-protein interactions to occur. For instance, the yeast may not provide the proper post-translational modifications required for correct protein folding of some mammalian proteins. The lack of apparent interaction between Hes3 and Grg1 or Grg4 may be a result of one or more of the above complications. False positives are also known to occur in the yeast two hybrid system, particularly if the test proteins have intrinsic DNA-binding or transcriptional activation properties. In this study, the DNA-binding Hes1 protein was fused to the GAL4 activation domain, which could potentially result in false positives. However, white, non β-galactosidase-expressing colonies were obtained with yeast transformed with the

Hes1 hybrid vector alone as well as with yeast cotransformed with various combinations of the Hes1 hybrid vector and parental vectors. This would suggest that the blue colonies that were obtained when yeast were cotransformed with Hes1-pGAD424 and Grg1-pGBT9 are not false positives. However, results obtained from a heterologous system such as the yeast two-hybrid system cannot be used to extrapolate the situation in the parent organism with certainty. The interactions must be considered as potential interactions only and must be confirmed by an independent biochemical assay such as a GST-fusion protein pull-down assay or affinity chromatography. A number of other parameters will determine whether these interactions occur *in vivo*, such as co-localisation and concentrations of the two partners, their relative interaction affinities, post-translational modifications, and the presence of competitor proteins (Alifragis et al. 1997). Many protein-protein interactions have successfully been detected by the yeast two hybrid assay using previously cloned proteins including interactions between Ras and Raf (Van Aelst et al. 1993) and Grb2 and Sos1 (Chardin et al., 1993).

4.2 Function of Grg and Hes Proteins in Neurogenesis

In *Drosophila*, the neurogenic genes act during the development of the nervous system in a lateral inhibition pathway that serves to limit the number of cells from a proneural cluster that adopt the neural cell fate. More specifically, the *Drosophila* neurogenic genes are involved in binary cell fate decisions such as the

choice between epidermal and neural cell fate. In mammals, the neurogenic homologues appear to act at the level of cell differentiation rather than cell determination as in *Drosophila*. Specifically, the mammalian neurogenic genes appear to act in a pathway that serves to delay cell differentiation so that the cell can respond to later differentiation signals.

Although the stage of neurogenesis at which these proteins are required differs from invertebrates to vertebrates, the mechanism by which they act may be quite similar. This idea is supported by the observation that the domain structures of *Drosophila* neurogenic proteins such as Hairy and Groucho have been well conserved in their mammalian homologues. The exceptions to this are the short Grg proteins, which contain only the dimerization domain, and Hes3, which lacks the basic domain required for DNA binding. Short Grg proteins have been shown to heterodimerize with long Grg proteins (Pinto and Lobe 1996). It has been postulated that the short Grg proteins, which lack the domains required for repression and interaction with Hairy-like proteins, may negatively regulate the long Grg proteins by forming non-functional heterodimers. Support for this hypothesis was provided by Mallo et al. when they observed that depletion of Grg5 protein from HeLa nuclear extracts inhibited the *in vitro* transcription activity of the extracts. The domains which were found to be required for the interaction between Groucho and Hairy are also found in their mammalian homologues, the Grg and Hes proteins. These interactions are also conserved since Hes1 has been shown to interact with both TLE1 and TLE2 in pull-down

assays (Grbavec and Stifani 1996; Grbavec et al. 1998) as well as with Grg1 and Grg4 in the yeast two-hybrid system. The work done in this study indicates that the interaction which is crucial for the function of Groucho is conserved in mammals. This suggests that in mammals, as in *Drosophila*, the long Grg proteins act as corepressors when sequestered to target DNA promoters by bHLH proteins such as Hes1. This is similar to an example of yeast transcriptional repression where a Tup 1/Ssn6 transcription repression complex is targeted to DNA by the sequence-specific DNA-binding protein alpha-2 (Komachi, Redd, and Johnson 1994). Groucho and Grg proteins share a similar structure with the yeast transcription factor Tup1 including an amino terminal glutamine-rich region that is involved in protein dimerization (Pinto and Lobe 1996; Tzamarias and Struhl 1995), an internal serine/threonine/proline-rich region (Stifani et al. 1992; Williams and Trumbly 1990), and carboxy-terminal tandem WD40 repeats (Stifani et al. 1992; Williams and Trumbly 1990). Like the Groucho/Grg proteins, Tup1 lacks a DNA-binding domain and is recruited to target DNA by interaction with DNA binding proteins such as the homeodomain factor α-2 (Komachi, Redd, and Johnson 1994). Upon recruitment to specific DNA sites, Tup1 can repress expression of a variety of genes. Genetic and molecular studies have revealed that the interaction of multimeric Tup1 complexes with nucleosomes is important for the general transcriptional functions of Tup1 (Roth 1995; Cooper, Roth, and Simpson 1994). The interaction between Tup1 and nucleosomes is mediated by the binding of Tup1 to the amino termini of histones H3 and H4 (Edmondson and

Roth 1996). These associations are thought to result in local chromatin remodelling effects that prevent access to transcriptional activators by establishing repressive structures (Edmondson and Roth 1996). Groucho/Grg proteins have also demonstrated an ability to interact with the amino termini of histone H3 *in vitro* (Palaparti, Baratz, and Stifani 1997). Therefore, Groucho and Grg proteins may use similar mechanisms as the yeast transcription factor Tup1 to negatively regulate transcription of target genes. Recent studies have also shown that oligomerization of Groucho may be important for its transcriptional repression activities (Chen, Nguyen, and Courey 1998). Chen et al. have shown that Groucho is a tetramer in solution and that two amphipathic α-helices in the amino terminus are required for tetramerization. Point mutations in these leucine zipper-like motifs which block oligomerization also block the transcriptional repression activity of Groucho (Chen, Nguyen, and Courey 1998). It has been postulated that Groucho oligomerization may serve to increase the concentration of DNA-bound transcription repression domains and thus increase the efficiency of repression (Chen, Nguyen, and Courey 1998). The leucine zipper-like motifs responsible for tetramerization are conserved in the mammalian homologues of Groucho and it is therefore probable that oligomerization is important for the transcriptional repression activity of these proteins as well.

Further evidence that the *Drosophila* lateral inhibition pathway is well conserved in mammals comes from the observation that Hes1 is able to transcriptionally regulate the human homologue of Achaete-Scute. Hes1 is present

in abundant levels in most non-neuroendocrine human lung cancer cells which do not express human achaete-scute homologue 1 (hASH1) but is virtually absent in lung cancer cell lines expressing hASH1 (Chen et al. 1997). Induction of Hes1 in a small cell lung cancer cell line has been shown to down-regulate endogenous hASH1 expression and this repression activity of Hes1 is mediated by binding of Hes1 to a class C site in the *hASH1* promoter (Chen et al. 1997). Another bHLH transcriptional activator, MATH1, which is structurally related to the product of the *Drosophila* proneural gene *atonal*, is also regulated by Hes1. MATH1 is an E box-dependent transcriptional activator that regulates transcription in conjunction with E47, the mammalian homologue of the ubiquitously expressed *Drosophila* bHLH protein Daughterless. The transcriptional activity of MATH1 and E47 was completely blocked by the presence of Hes1 in *in vivo* transcription assays (Akazawa et al. 1995). These observations suggest that Hes1 has a similar role to that of its *Drosophila* homologue - to transcriptionally repress Achaete-Scute and MATH1 expression.

Hes3 is unique among the Hes family of proteins due not only to its lack of a DNA-binding basic domain but also due to its unique expression pattern. Hes3 is expressed during embryogenesis in the region of the mesencephalic/metencephalic boundary and in two bands in the hindbrain corresponding to rhombomeres 1, 2, 4, 6, and 7 (Lobe 1996). This expression pattern suggests that Hes3 may have a role in midbrain and hindbrain patterning. The *Drosophila* homologue, Hairy, is a known segmentation gene and thus the

expression pattern of Hes3 may reflect a conserved role in segmentation. Hes3 is also present in epithelial tissues where its expression overlaps with that of other neurogenic genes. Hes3 is capable of acting as a transcriptional repressor without making sequence-specific DNA contacts by forming non-functional heterodimers with transcriptional activators such as E47 (Sasai et al. 1992). Other Hes proteins also appear to possess this activity in addition to their ability to repress transcription from a target promoter which requires interaction with corepressors, the Groucho homologues. Where it is expressed in the midbrain and hindbrain, Hes3 could repress transcription of segmentation genes by sequestering transcriptional activators. However, later in development when Hes3 is coexpressed with other Hes genes, such as Hes1, it is possible that Hes3 could negatively regulate the activity of the other Hes genes by forming non-functional heterodimers. In order to make sequence-specific contacts to DNA, bHLH proteins homo- or heterodimerize through their HLH domains, thus uniting two regions rich in basic amino acids that can bind DNA (Ferre-d'Amare et al. 1993). Heterodimers between Hes3 and other Hes proteins would not be able to make contact with DNA. Thus Hes3 could potentially act as a negative regulator of Hes, and indirectly, Grg activity. This hypothesis could be tested by several different experiments. First of all, Hes3 could be tested for its ability to dimerize with other Hes proteins using the yeast two hybrid system, GST fusion protein pull-down assays, or affinity chromatography. A good negative control for the GST fusion protein pull-down assays would be to include a deletion mutant of Hes1 or Hes3

in which the WRPW motif is lacking. If such an interaction was found to occur, the effect of Hes3 on the ability of Hes1 to bind DNA would need to be tested using a gel mobility shift assay. Finally, Hes1 and Hes3 could be transfected separately and in combination into an appropriate cell line expressing long Grg proteins and containing a suitable reporter gene to test the effect that Hes3 has on the ability of Hes1 to repress transcription from the reporter gene. The ability of the short Grg proteins, Grg3b and Grg5, to negatively regulate the transcriptional repression ability of long Grg proteins, Grg1 and Grg4, could be tested similarly in a transcriptional assay.

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