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PRINCIPAL INVESTIGATOR: Dr. Jan Kitajewski
Dr. Hendrik Uyttendaele

CONTRACTING ORGANIZATION: Columbia University
New York, New York 10032

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Dr. Jan Kitajewski
Dr. Hendrik Uyttendaele

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New York, New York 10032

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Elongation and branching of epithelial ducts is a crucial event during the development of the mammary gland. Branching morphogenesis of the mouse mammary epithelial TAC-2 cell line was used as an assay to examine the role of Wnts, HGF, TGF-β and the Notch receptors in branching morphogenesis. Wnt-1 was found to induce the elongation and branching of epithelial tubules, like HGF and TGF-β2, and to strongly cooperate with either HGF or TGF-β2 in this activity. The Notch4(int-3) mammary oncoprotein, an activated form of the Notch4 receptor, inhibited the branching morphogenesis normally induced by HGF and TGF-β2. The minimal domain within the Notch4(int-3) protein required to inhibit morphogenesis consists of the CBF-1 interaction domain and the cdc10 repeat domain. Co-expression of Wnt-1 and Notch4(int-3) demonstrates that Wnt-1 can overcome the Notch-mediated inhibition of ductal morphogenesis. These data suggest that Wnt and Notch signaling may play opposite roles in mammary gland development, a finding consistent with the convergence of the wingless and Notch signaling pathways found in Drosophila.

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Role of the *int-3* Oncogene in Mammary Gland Development and Tumorigenesis

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Introduction

I. Nature of the problem

In the past several years, it has been shown that oncogenes contribute to the pathobiology of breast cancer. Mutational activation of the *int-3* oncogene, has been shown to contribute to experimental mammary gland tumorigenesis in mouse. Several human orthologues of the *int-3* gene have been implicated in human cancers. There is strong evidence that the int-3 protein regulates the cell fate decisions required for the morphogenesis and functional differentiation of the mouse mammary gland. Despite this evidence, work on the role of the *int-3* gene in breast cancer is still in its infancy. Extensive studies on other int-3 family members (lin-12/Notch) in organisms more tractable to genetic analysis such as Drosophila and C. elegans, demonstrates the evolutionary conservation of these proteins and their fundamental importance in cell fate decisions.

The proposed research will investigate the role of the int-3 protein in the normal physiology of breast development and study the biochemical properties that are important for int-3 transforming activity. This information will broaden our understanding about the events which control normal mammary gland development and how alterations in those events can lead to neoplastic growth of the mammary gland.

II. Background

Mouse mammary tumor virus induces breast cancer in mouse by insertional mutagenesis. In tumors, viral integration can result in activation of the *int-3* gene by promoter insertion and results in expression of a truncated *int-3* gene product (2.3 kb RNA) (1). The nucleotide sequence of this truncated cDNA revealed homology with the Notch/lin-12 gene family (2). However the full length cDNA of the *int-3* gene has not been cloned. Several lines of evidence confirm a role for int-3 in mammary tumorigenesis. Transfection of a recombinant *int-3* genomic DNA fragment, encoding the truncated oncoprotein, into the HC11 mouse mammary epithelial cell line induces anchorage-independent growth in soft agar (2). Expression of this same genomic fragment *in vivo* as a transgene in a transgenic mouse strain is associated with arrest of normal mammary gland development and impaired differentiation (3), intraductal hyperplasia of mammary epithelium, and a high incidence of focal mammary tumors

(adenocarcinomas) (4). It has also been reported that the normal *int-3* gene is endogenously expressed in the mouse mammary gland (5).

Int-3 is related to the Notch/lin-12 family of proteins. The Notch/lin-12 protein family currently consist of eleven members, Notch (Drosophila) (6), lin-12 and glp-1 (C. Elegans) (7, 8, 9), Xotch (Xenopus) (10), Notch 1, 2, 3 and int-3 (Mouse) (2, 11-15), Notch 1 and 2 (Rat) (16,17), NOTCH 1 and 2 (Human) (18, 19). These genes encode for transmembrane receptor proteins. The extracellular domain of Notch/lin-12 family members contains variable numbers of EGF (epidermal growth factor) like repeats and other cysteine rich repeats named lin-12/Notch repeats(26). The intracellular domain of all Notch/lin-12 family members contains several copies of a repeat sequence, named cdc10 or ankyrin repeat. The cdc10 repeats have recently been implicated as a protein-protein interaction domain. The intracellular domain of this family of proteins also contains a PEST sequence, a nuclear localization signal, and an opa repeat. PEST sequences are found in proteins which are rapidly degraded or may also represent potential phosphorylation sites. An opa repeat is a protein domain that is rich in glutamine and is commonly found in transactivating domains of transcription factors or transcription factor binding proteins (27). The Notch and lin-12 proteins are required for cell-cell interactions that play a pivotal role in cell-fate decisions. For instance, the mechanisms that control how a group of equivalent progenitor cells give rise to a group of cells each with their particular fate. The fundamental importance of these genes during development has been demonstrated by genetic analysis of lin-12, Notch and Xotch mutants (20-24). In the mouse, null mutants of Notch 1 and 2 are lethal during embryonic development, although the exact cause for this premature death is not known (25).

Genetic and molecular analysis have identified several proteins that participate in Notch signaling. Drosophila *Delta* (28) and *Serrate* (29) and C. elegans *Lag-2* (30)and *Apx-1* encode a family of structurally related ligands for the Drosophila Notch and C.elegans lin-12 and glp-1 receptors respectively. These ligands are transmembrane proteins, containing EGF-like domains and a cysteine rich DSL (Delta-Serrate-Lag-2) domain within the extracellular part of the protein. Recently, mouse homologues of these ligands have been cloned, *Jagged-1* (31) and *Dll-1* (32). These ligands have been demonstrated to regulate Notch receptor activity through cell-cell interactions. The products of three Drosophila genes, deltex, disheveled and suppressor of hairless (Su(H))have been shown to interact with the intracellular domain of Notch and may thus participate in the intracellular signaling pathway of Notch (33,34) Furthermore, genetic analysis has revealed similar phenotypes in certain Deltex and Notch mutants.

Deletion of the extracellular part of Notch, Xotch and lin-12 proteins results in a dominant gain of function mutation (20-22). The truncated gene product encoding for the intracellular part of the receptor exhibits constitutively activated protein function. The phenotype observed in this class of mutants suggests that the truncated gene products delay cell determination and thereby increase the proportion of uncommitted stem cells, leading to a prolonged lifetime of the cell or to a greater number of descendants (20-22). By analogy to the function of other Notch/lin-12 family members in lower organisms, one can speculate that delay in differentiation and accumulation of pluripotent proliferative stem cells would result in a growth advantage, thereby increasing the probability for secondary oncogenic mutations. This model would propose that Notch proteins contribute to oncogenesis by stimulating stem cell growth and blocking differentiation.

Studies on the Notch protein in Drosophila, demonstrated that the intracellular part of the Notch protein is translocated to the nucleus when a truncated Notch protein (corresponding to the intracellular part of the protein) is expressed as a transgene in Drosophila embryos (20). Based on the hypermorphic effect of the deletion mutants, and on the presence of a nuclear translocation signal in the intracellular domain of the protein, a hypothetical model would be that ligand binding to the receptor would result in cleavage of the intracellular domain of the receptor and subsequent translocation to the nucleus, where it could interact with its substrate.

Notch/lin-12 gene family members have been implicated in human tumorigenesis. Alteration of NOTCH-1 (also named TAN-1) has been associated with a T lymphoblastic neoplasm (18). The mutation of the NOTCH-1 gene in T lymphoblastic lymphomas is caused by a translocation that results in expression of a truncated gene product. TAN-1 mutations are analogous to the *int-3* activating mutations as a result of MMTV insertion, as well as to the dominant gain of function mutations of Notch, lin-12 and Xotch. Furthermore, human NOTCH-1 and NOTCH-2 (also named hN) were found to be overexpressed in human cervical carcinomas (19).

III. Purpose

The *overall goal* of the work proposed here is to understand in molecular detail the function of the int-3 protein in mammary epithelial cells and during mouse mammary gland development, with the *long term goal* of understanding the role of the *int-3* gene in mammary tumorigenesis.

Body

I. Technical Objectives

The *overall goal* of the work proposed here is to understand in molecular detail the function of the int-3 protein in mammary epithelial cells and during mouse mammary gland development.

The approach I propose will have two *major objectives*. First, the biochemical and biological properties of the int-3 protein will be investigated. I have cloned the part of the *int-3* gene that encodes for the putative intracellular domain of the int-3 protein, which is thought to be constitutively active. Initially, this truncated protein will be used as a probe to study the signaling pathway and the mechanisms of action of the int-3 protein. These studies will be further expanded once the full-length *int-3* is cloned. Second, I will clone, sequence and characterize the full length *int-3* cDNA and study int-3 function and expression pattern in mammary epithelial cells as well as during mammary gland development.

I propose five *specific aims* to pursue the mechanisms of action of the int-3 protein in a relatively simple and biological context:

1. Characterize the full length *int-3* cDNA and determine if it encodes for a secretory, transmembrane protein. A truncated int-3 protein will also be cloned to be used in the biological and biochemical studies outlined in aim 2 and 3.

2. Determine the biological activity of the int-3 protein in mammary epithelial cells. Using biological and molecular methods, I will study the transforming potential of the int-3 protein.

3. Define the biochemical properties of the int-3 protein in mammary epithelial cells. Biochemical strategies will be used to analyze the production, processing and subcellular localization of the int-3 protein .

4. Analyze the signaling pathway activated by int-3. Molecular and biochemical strategies will be developed and used to detect interacting proteins, and to isolate and characterize these interacting molecules.

5. Analyze the int-3 expression pattern in mouse tissues, in mammary epithelial cells and during mouse breast development. I will generate polyclonal antibodies against polypeptide components of the intra and extracellular domain of int-3. Molecular, biochemical and histological methods will be used to determine int-3 protein as well as *int-3* mRNA in various cells and tissues.

II. Experimental Results

This annual report describes the progress I have made during the first 36 months of this fellowship. As described in the Statement of Work of the original fellowship application, the experimental work proposed in aims 1, 2, 3 and part of aims 4 and 5 of the fellowship application were proposed to be executed during the initial 36 months.

1. Characterization of the full length *int-3* cDNA (months 1-12)

The *int-3* oncogene has been classified in the lin-12/Notch protein family solely on the basis of its homology to the intracellular part of the lin-12/Notch family members. There has been no direct evidence that demonstrates that the full length *int-3* encodes for a transmembrane protein. By cloning the full length *int-3* cDNA I have demonstrated that the *int-3* gene encodes for a transmembrane protein, homologous to the lin-12/Notch family of transmembrane proteins. We have proposed to name the full length gene *Notch4*, and reserve the *int-3* nomenclature when referring to the truncated and oncogenic form of the gene.

A. Cloning truncated *int-3.* I have cloned the truncated *int-3* gene that encodes for the intracellular part of the protein and corresponds to the int-3 mammary oncogene (for a more detailed description see Appendix A)

B. Cloning of *Notch4* I have cloned the full length *int-3* cDNA that encodes for a transmembrane protein with an intracellular domain containing six ankyrin repeats, a transmembrane domain, and an extracellular domain containing three Notch/lin-12 repeats and twenty nine EGF-like repeats (for a more detailed description see Appendix A).

2. Determination of the biological activity of the int-3 protein. (months 3-21)

The *int-3* gene was discovered in mouse mammary tumors, induced by MMTV infection. Insertional mutagenesis by MMTV results in expression of a truncated int-3 protein product that is able to transform mammary epithelium cells both in vivo and in vitro.

Analysis of truncated int-3:

A. Epitope tagging of int-3. For a detailed description of the generation of epitope tagged int-3 cDNA and epitope tagged int-3 deletion mutant cDNA's, see Appendix B. In addition to the proposed aim in the research proposal, I also generated a Wnt-1 cDNA that is also epitope tagged (see Appendix B).

B. Construction of int-3 expressing cell lines. As described in detail in Appendix B, TAC-2 mammary epithelial cell lines were generated that were programmed to express the above described epitope tagged cDNA's, either singularly or combinationarly. In addition to the proposed aim in the research proposal, I also generated TAC-2 cell lines that were programmed to express Wnt-1 (see Appendix B).

C. Biological activity of int-3. As described in detail in Appendix B, the biological activity of int-3 was studied in the TAC-2 mouse mammary epithelial cell line. A structure function analysis of the int-3 oncoprotein was done using the above described int-3 deletion mutants (Appendix B). In addition to the proposed aim in the research proposal, I also examined the biological activity of the Wnt-1 oncoprotein in TAC-2 mammary epithelial cell lines. These experiments demonstrate that the int-3 and Wnt-1 oncoproteins have opposing activities in the branching morphogenesis pathway in TAC-2 mammary epithelial cell line, and also demonstrate that the Wnt-1 oncoprotein can act as a morphogen (as opposed to mitogen) in the TAC-2 assay.

Analysis of full length int-3:

The full length Notch4 cDNA was epitope tagged at the carboxy terminus. Since the *Notch4* transcript is 6.5 kb, retroviral vectors can not be used to generate stable cell lines expressing Notch4. I'm in the process to use lipofection, electropolation and Ca/Phosphate mediated transfection, as methods to introduce a eukaryotic vector containing Notch4 directly into TAC-2 mammary epithelial cell lines. The biological activity of the Notch4 protein will be evaluated in TAC-2 cells. If an int-3 like activity of the full length protein is observed in the TAC-2 branching morphogenesis assay, then this observation may suggest that overexpression of the Notch4 protein results in activation of the Notch4 pathway in a ligand independent matter, or it might suggest that TAC-2 cells express a ligand for the Notch4 protein. These possibilities will be studied in media transfer and co-culturing experiments.

Although not described in the specific aims of this research proposal, I have generated stable cell lines expressing either int-3, Notch4, or Jagged-1 (a putative Notch ligand) using a rat brain endothelial cell line. I chose this cell line because of the endothelial specific expression of Notch4 (see Appendix A). An int-3 specific biological phenotype was observed in this cell line, and which is identical to the observed Jagged-1 phenotype in this same cell line. Endothelial cells programmed to express Notch4 did not have a biological phenotype. Based on this results, these experiments suggest that overexpression of Notch4 does not result in a gain of function phenotype. In addition, these experiments suggest that endothelial cells express an endogenous Notch (possibly Notch4) that can be activated by Jagged-1, which result in a phenotype identical to the phenotype of a constitutive activated Notch (i.e. int-3). The above described preliminary experiments will be repeated in other cell lines (such as TAC-2).

3. Define the biochemical properties of the Notch4 protein. (months 6-24)

The deduced amino acid sequence of the full length Notch4 protein predicts that Notch4 is a putative transmembrane protein. I have investigated this hypothesis by studying the intracellular localization of the Notch4 protein. A full length Notch4 transcript was assembled and the Notch4 protein was detected in protein lysates of transiently transfected 293T cells. The molecular weight of epitope tagged Notch4 is approximately 215 kD. Immunofluorescence experiments on 293T cells transiently transfected with epitope tagged Notch4, cells demonstrated plasma membrane staining (as reported in annual report 9/96). The intracellular localization of truncated int-3 was investigated in transiently transfected cells (293T and HeLa cells). I have investigated the intracellular localization by indirect immunofluorescence (45) using the anti HA antibodies, and have found nuclear localization of truncated int-3 (as reported in annual report 9/95).

In the research proposal, I suggested to investigate the proteolytic processing, glycosylation and half life determination of Notch4. In the last several months, several manuscripts have been published that describe and provide evidence of complex proteolytic processing of Notch proteins. I have decided to focus my research on the study of the biological activity of Notch4, and the proposed experiments on the biochemical properties of Notch4 will be postponed.

4. Analysis of the signaling pathway mediating int-3 action. (months 24-48)

The experiments proposed in this aim, such as the identification of Notch4 binding proteins using biochemical and molecular techniques , will be abandoned for the following reasons. First,

my research has become more focused on the biological roles of Notch4 in mammary gland development. Second, as mentioned as a critique by the reviewers of my pre-doctoral fellowship proposal, the identification of Notch4 binding proteins is unrealistic to be accomplished within the time limits of this fellowship. Thirdly, other members of this laboratory have identified a novel Notch4 binding protein, and they will focus their studies on the intracellular signal transduction pathway of Notch4.

As described in Appendix B, I have analyzed the role of several different signal transduction pathways (Notch, Wnt, receptor tyrosine kinase and cytokine mediated pathways) in the branching morphogenesis of TAC-2 mammary epithelial cells. These experiments have demonstrated the functional interactions between these pathways. More specifically, these experiments have demonstrated for the first time in a mammalian system, that the Wnt pathway can override the Notch pathway as was previously described in Drosophila. For a more detailed discussion of the functional interactions between the different pathways studied in TAC-2 mammary epithelial cell lines, see Appendix B.

5. Analysis of the int-3 expression pattern. (months 12-48)

I will analyze the expression pattern of the Notch4 protein as well as the *Notch4* mRNA. Such analysis will give me further insights in the function of the Notch4 protein. I will study the expression pattern of *Notch4* in different mouse tissues to determine if the Notch4 protein is tissue specifically expressed, and I will investigate whether *Notch4* is expressed in the mammary gland and whether this expression is developmentally regulated.

A. Generating int-3 specific antibodies. To study the endogenous and recombinant expression pattern of the int-3/Notch4 protein, polyclonal antibodies against a GST fusion protein (fused to the intracellular part of the int-3 protein) have successfully been generated in two rabbits. The specificity of these antibodies as well as their ability to recognize int-3/Notch4 was analyzed in immunoblot analysis. Ectopically expressed int-3 and Notch4 proteins, were used to test specificity of antibodies (as described in annual report 9/96). The appropriate total immune serum titer for detection in immunoblot experiments has also been determined.

B. Expression analysis of *Notch4*. *Notch4* mRNA expression was studied by Northern blot analysis and in situ hybridization using probes derived from the 3' UTR (Appendix A). The *Notch4* gene encodes for a 6.5 kb transcript that is highly expressed in lung, heart and kidney in adult tissues. Several shorter *int-3* transcripts were observed in adult testis, and are the products of aberrant transcriptional events in post-meiotic spermatids (Appendix A). The *Notch4*

transcript is expressed at all stages of mouse development (day 6.5 to 15.5). In situ hybridization (using the same probe as in the Northern blot analysis) was performed to determine the cellular origin of *Notch4* expression during mouse development, and revealed endothelial specific expression. In situ hybridization on adult lung tissue was performed and revealed endothelial cell specific expression of *Notch4*. (for a more detailed description see Appendix A). I'm in the process of using the polyclonal antibodies against Notch4 in immunohistochemistry experiments. Preliminary data confirms the endothelial cell specific expression of Notch4 in adult mouse kidney glomeruli. These experiments will be repeated and optimized in order to perform a detailed analysis of Notch4 expression in adult mouse tissues such as the developing mammary gland.

Conclusions

The data presented in this annual report represent my progress in the experiments outlined in the specific aims of the research proposal. As outlined in the statement of work in the research proposal, I have largely completed the aims as scheduled for months 1-36. As explained in Experimental Results, some of the proposed experiments in aim 3 have been abandoned because these experiments have been done by others, so instead of repeating these experiments, I think it would be more interesting to continue my studies on the biological activities of Notch4. As explained in Experimental Results, some of the proposed experiments in aim 4 have been accomplished by other members of this laboratory, and hence will be their research focus. I have extended the aim of my research proposal by studying the role of a different mammary oncoprotein in the branching morphogenesis of mammary epithelial cell lines, and have studied the functional interactions of the Notch4 signal transduction pathway with other signal transduction pathways that are known to regulate mammary gland development. In this last year of my pre-doctoral fellowship, I will complete the experiments outlined in aims 2 and 5, and as described in Experimental Results, I will expand aim 2 in a more detailed analysis of the biological activity of Notch4.

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Appendices

Appendix A Notch4/int-3, a mammary proto-oncogene, is an endothelial cell specific mammalian Notch gene.
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Appendix B Notch and Wnt proteins function to regulate branching morphogenesis of mamary epithelial cells in opposing fashion.
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Notch4/int-3, a mammary proto-oncogene, is an endothelial cell-specific mammalian *Notch* gene

Hendrik Uyttendaele[1], Giovanna Marazzi[2], Guangyu Wu[1], Qingyou Yan[1], David Sassoon[2] and Jan Kitajewski[1,*]

[1]Department of Pathology in the Center of Reproductive Sciences, Columbia University, College of Physicians and Surgeons, New York, NY 10032, USA
[2]Brookdale Center for Molecular Biology, Mount Sinai Medical Center, New York, NY, 10029, USA

*Author of correspondence (e-mail: jkk9@columbia.edu)

SUMMARY

The *int-3* oncogene was identified as a frequent target in Mouse Mammary Tumor Virus (MMTV)-induced mammary carcinomas and encodes the intracellular domain of a novel mouse *Notch* gene. To investigate the role of the *int-3* proto-oncogene in mouse development and carcinogenesis, we isolated cDNA clones corresponding to the entire coding potential of the *int-3* proto-oncogene. We propose to name this gene *Notch4* and reserve the *int-3* nomenclature for references to the oncogenic form. The deduced amino acid sequence of Notch4 contains conserved motifs found in Notch proteins; however Notch4 has fewer epidermal growth factor (EGF)-like repeats and a shorter intracellular domain than other mouse Notch homologues. Comparison of the coding potential of the *int-3* gene to that of *Notch4* suggests that loss of the extracellular domain of Notch4 leads to constitutive activation of this murine Notch protein. In situ hybridization revealed that *Notch4* transcripts are primarily restricted to endothelial cells in embryonic and adult life. Truncated *Notch4* transcripts were detected in post-meiotic male germ cells. The distinct Notch4 protein features and its restricted expression pattern suggests a specific role for *Notch4* during development of vertebrate endothelium.

Key words: Notch, int-3, endothelial cells, mammary oncogene

INTRODUCTION

The *int-3* gene was originally identified on the basis of its oncogenic effects in the mouse mammary gland. *int-3* is a frequent target for insertional activation by MMTV proviral DNA in MMTV-induced mammary gland tumors (Gallahan and Callahan, 1987; Robbins et al., 1992; Sarkar et al., 1994). Tumor-specific transcripts derived from the *int-3* gene encode a protein homologous to the intracellular part of the Notch family of cell surface receptors. Exogenous expression of the int-3 oncoprotein has been shown to affect the growth and development of mammary epithelial cells. Overexpression of the int-3 oncoprotein in mouse mammary epithelial cells (HC11) promotes anchorage-independent growth (Robbins et al., 1992). Expression of *int-3* as an MMTV-LTR-driven transgene in the mouse mammary gland results in abnormal development of the mammary gland and rapid development of undifferentiated mammary carcinomas (Jhappan et al., 1992). In the normal mouse mammary gland, endogenous int-3 protein has been detected in mammary stroma and epithelium (Smith et al., 1995).

Members of the *Notch/lin-12* gene family were first identified in *Drosophila* and *Caenorhabditis elegans* through genetic analysis of mutations that alter cell fate decisions (for reviews see Artavanis-Tsakonas et al., 1995; Artavanis-Tsakonas and Simpson, 1991; Greenwald and Rubin, 1992). *Drosophila Notch* regulates multiple cell fate decisions that involve cell-cell interactions during fly development, for instance, control of cell fate decisions involving neural/epidermal specification in proneural clusters (Artavanis-Tsakonas and Simpson, 1991). The *C. elegans* lin-12 and glp-1 proteins are structurally related to Notch and are also involved in cell fate specifications during development in the nematode (Greenwald, 1985; Yochem and Greenwald, 1989). Genetic analysis of *Notch/lin-12* genes suggests that this family of genes controls binary cell fate decisions and inductive signaling that depend on cell-cell interactions (reviewed by Artavanis-Tsakonas et al., 1995; Greenwald, 1994; Greenwald and Rubin, 1992). Alternatively, *Notch/lin-12* genes have been proposed to block cell differentiation, thus maintaining the competence of cells for subsequent cell-fate determination (Coffman et al., 1993; Fortini et al., 1993).

Notch/lin-12 genes encode transmembrane receptor proteins characterized by highly repeated, conserved domains. The amino terminus of Notch proteins encodes the extracellular domain and contains as many as 36 repeats of an EGF-like motif involved in ligand binding (Rebay et al., 1993) and three tandem copies of a Notch/lin-12 sequence motif of unknown function. The intracellular portion of Notch proteins is characterized by six tandem copies of a cdc10/ankyrin motif, thought

to be a protein-protein interaction domain (Michaely and Bennett, 1992) and a PEST sequence motif which may represent a protein degradation signal (Rogers et al., 1986). In several systems, truncated forms of Notch/lin-12 proteins that contain an intact intracellular domain without most of the extracellular domain behave as constitutively activated receptors (reviewed by Artavanis-Tsakonas et al., 1995; Greenwald, 1994). The human Notch 1 orthologue, TAN-1, was first identified in independently isolated translocation breakpoints in acute T lymphoblastic leukemia, and is predicted to encode a truncated product that has an intact intracellular domain but lacks most of the extracellular domain (Ellisen et al., 1991). Similarly, the int-3 oncoprotein encodes the intracellular domain of a Notch-like protein and thus has been proposed to act as an activated Notch receptor (Robbins et al., 1992).

Based on sequence similarity to *Drosophila Notch*, additional *Notch*-related genes have been isolated from mammals, including mouse (Franco Del Amo et al., 1993; Lardelli et al., 1994; Lardelli and Lendahl, 1993; Reaume et al., 1992), rat (Weinmaster et al., 1992; Weinmaster et al., 1991) and human (Ellisen et al., 1991; Stifani et al., 1992; Sugaya et al., 1994). To date, three *Notch* homologues, *Notch1*, *Notch2* and *Notch3*, have been identified in the mouse, and their embryonic expression patterns display partially overlapping but distinct patterns of expression that are consistent with a potential role in the formation of the mesoderm, somites and nervous system (Williams et al., 1995). Abundant expression of *Notch1*, *Notch2* and *Notch3* is found in proliferating neuroepithelium during central nervous system development. Targeted disruption of the *Notch1* gene in mice results in embryonic death during the second half of gestation (Conlon et al., 1995; Swiatek et al., 1994) and homozygous mutant embryos display delayed somitogenesis as well as widespread cell death, preferentially in neuroepithelium and neurogenic neural crest (Conlon et al., 1995; Swiatek et al., 1994).

The gene products of *Drosophila Delta* (Vassin et al., 1987) and *Serrate* (Fleming et al., 1990) and *C. elegans Lag-2* (Henderson et al., 1994; Tax et al., 1994) and *Apx-1* (Mello et al., 1994) are thought to act as ligands for Notch proteins. In the mouse, the orthologue of *Delta*, referred to a *Dll1* (*Delta-like gene 1*), is expressed during embryonic development in the paraxial mesoderm and nervous system in a pattern similar to that of mouse *Notch1* (Bettenhausen et al., 1995). A murine *Serrate*-related gene named *Jagged* has been identified and is partially co-expressed with murine *Notch* genes in the developing spinal cord (Lindsell et al., 1995).

We report here the identification and expression analysis of a fourth murine *Notch* homologue, which we propose to name *Notch4*, reserving the *int-3* nomenclature for the truncated oncogene. Although the intracellular domain of the int-3 oncoprotein shares homology with the Notch/Lin-12 protein family, we now provide a comparison of the full-length Notch4 protein with that of the int-3 oncoprotein. The activated int-3 protein contains only the transmembrane and intracellular domain of the Notch4 protein. The predicted amino acid sequence of Notch4 includes the conserved features of all Notch proteins, but Notch4 has seven fewer EGF-like repeats compared to Notch1 and Notch2 and contains a significantly shorter intracellular domain. Notch4 is expressed primarily in embryonic endothelium and in adult endothelium and male germ cells.

MATERIALS AND METHODS

Isolation and sequencing of *Notch4* cDNA clones

A 1680 bp fragment was amplified by PCR from adult mouse testis cDNA (RT-PCR) using specific primers (5′ primer: CGTCCTGCTGCGCTTCCTTGCA and 3′ primer: CCGGTGCCTAGTTCAGATTTCTTA) designed from the *int-3* cDNA sequence (Robbins et al., 1992). This cDNA fragment corresponds to the previously cloned *int-3* oncogene. Two consecutive 5′ RACE reactions (5′-Amplifinder RACE kit, Clonetech) using testis and lung cDNA were done to obtain cDNA clones located 5′ of the *int-3* oncogene. The above described cDNAs were cloned into Bluescript KS (Stratagene) and the TA cloning vector (Invitrogen) and used to generate probes to screen a lung cDNA library (Clonetech). Briefly, nitrocellulose membranes (Schleicher&Schuell) were hybridized in a solution containing 50% formamide, 3× SSC, 100 mM Tris-HCl (pH 7.4), 5× Denhardt's solution, 0.2% SDS and 0.1 mg/ml salmon sperm DNA at 42°C for 14 hours. Filters were then washed in 1× SSC and 0.5% SDS at room temperature followed by washes at 65°C. Positive clones were purified and sequenced to confirm overlapping regions. Novel 5′ restriction fragments of these newly isolated clones were used in consecutive screens in order to obtain the full-length *Notch4* cDNA. All the above described clones were sequenced using the dideoxy termination method (Sanger) with an automatic DNA sequencer (Applied Biosystems). Sequence data from both strands were obtained for the entire *Notch4* cDNA and were analyzed and assembled using computer software (MacVector, Assemblylign).

Northern blot analysis

Total RNA was isolated from adult CD-1 mouse tissues and northern blot hybridization analysis was performed. 20 μg of total RNA was electrophoresed on a 1% agarose gel containing 6% formaldehyde. After electrophoresis RNAs were transferred to a nylon membrane (Duralon-UV membranes, Stratagene) by capillary blotting. ^{32}P-labeled riboprobes were transcribed (Maxiscript in vitro transcription kit, Ambion) from *Notch4* cDNA clones encoding the 5′ or 3′ UTR (untranslated region) or ORF (open reading frame). The 3′ UTR *Notch4* cDNA clone was isolated by RT-PCR and a 440 bp restriction fragment of this cDNA was used as riboprobe. Hybridization solution contained 60% formamide, 5× SSC, 5× Denhardt's solution, 1% SDS, 20 mM NaH_2PO_4 (pH 6.8), 0.1 mg/ml salmon sperm DNA, 100 μg/ml yeast tRNA, 10 μg/ml poly(A) mRNA and 7% dextran sulfate and was done for 14 hours at 65°C. Washing solution contained 2× SSC and 1% SDS and was done at room temperature and 50°C for 15 minutes each, followed by a 2 hour wash at 80°C with a solution containing 0.2× SSC and 1% SDS. Membranes were exposed to X-ray film (X-OMAT AR, Kodak). The integrity of the RNA, as well as comparable amounts of RNA, were tested by rehybridization with a GAPDH probe.

In situ hybridization

Staged embryos ranging from 9 days post-coitum (d.p.c.) to birth were obtained from timed breedings of CD-1 mice. The morning when the the vaginal plugs appeared was counted as 0.5 d.p.c. Lungs was obtained from adult CD-1 mice. Preparation of tissue and subsequent procedures for in situ hybridization were done as previously described (Marazzi and Buckley, 1993; Sassoon and Rosenthal, 1993). After hybridization, sections were dehydrated rapidly and processed for standard autoradiography using NTB-2 Kodak emulsion and exposed for 2 weeks at 4°C. Analyses were carried out using both light- and dark-field optics on a Leica DA microscope. To avoid potential cross-hybridization with homologous RNAs, we used an antisense ^{35}S-labeled RNA probe corresponding to the 3′ UTR of *Notch4*. Probes were used at a final concentration of 9×10^4 dpm/ml.

RESULTS

Isolation and analysis of *Notch4* cDNA clones

The *int-3* mammary oncogene encodes a truncated protein that is highly homologous to the intracellular part of the Notch receptor proteins. The full-length *int-3* gene, which we will refer to as *Notch4*, had been proposed to encode a novel member of the Notch protein family (Robbins et al., 1992). To prove this hypothesis, we have cloned cDNAs containing the complete coding potential of the *Notch4* gene. Using primers derived from the published sequence of the *int-3* oncogene, RT-PCR was used to isolate a 2.4 kb *int-3* cDNA encoding the putative intracellular portion of the receptor. To obtain cDNA clones encompassing the full coding potential of the normal *int-3* gene, cDNAs were isolated by 5′ RACE and by screening a mouse lung cDNA library. A total of 37 overlapping cDNA clones were analyzed and sequenced to obtain a 6677 bp cDNA sequence. This sequence encodes one long open reading frame of 1964 amino acids, starting with an initiator methionine at nucleotide 347 and terminating with a stop codon at nucleotide 6239. The 6677 bp cDNA corresponds in size to that of *Notch4* transcripts detected by northern blot analysis; thus, we believe the cloned cDNA represents the full-length *Notch4* gene.

Several differences (insertions, deletions and single nucleotide changes) were found between the nucleotide sequence of *Notch4* reported here and the previously published *int-3* nucleotide sequence (Robbins et al., 1992). These differences alter the reading frame in several locations within the intracellular domain and may be a result of differences in sequence analysis or, possibly, of mutations found in the tumor-derived *int-3* transcript (Robbins et al., 1992) that are not found in the *Notch4* gene. The nucleotide sequence of mouse *Notch4* has been deposited with GenBank under the Accession number U43691.

Analysis of the deduced *Notch4* amino acid sequence

Analysis of the deduced amino acid sequence of *Notch4* reveals the presence of conserved domains shared by all Notch proteins (see Fig. 1). Notch4 contains EGF-like repeats, Notch/lin-12 repeats, a transmembrane domain, cdc10/ankyrin repeats and a putative PEST domain. The overall homology between Notch4 and other Notch proteins was determined using GCG (Bestfit, gap weight 3.0, length weight 0.1). The Notch4 protein is approximately 60% similar and 43% identical to other vertebrate Notch proteins and 58% similar and 40% identical to *Drosophila* Notch. Lower homologies were found when compared with the *C. elegans* lin-12 and glp-1 proteins (49% similar and 29% identical).

Two hydrophobic regions in the Notch4 protein sequence were identified by hydropathy analysis (Kyte Doolittle algorithm, data not shown). The N-terminal region contains 19 hydrophobic residues that could function as a signal peptide sequence (Fig. 1) and a putative signal peptidase cleavage site

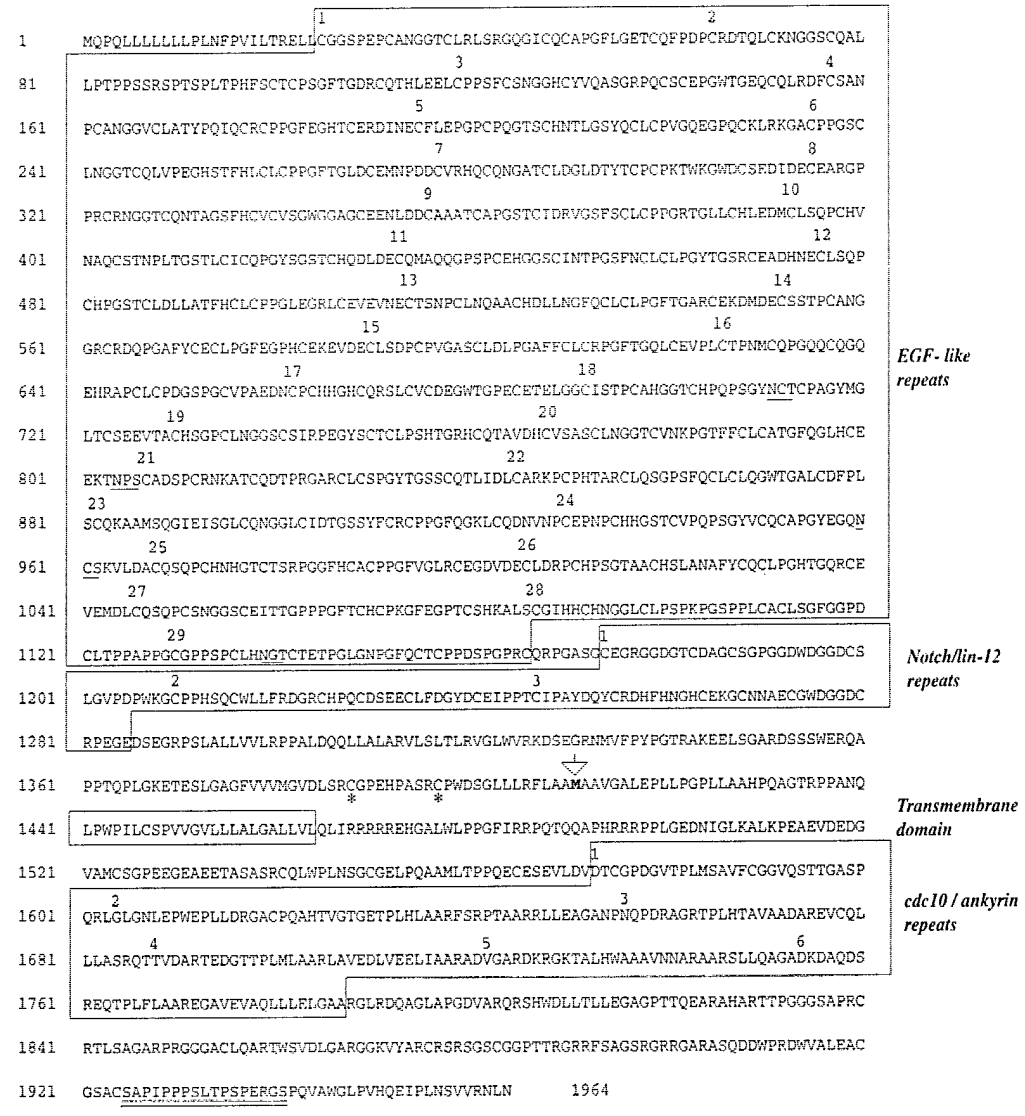

Fig. 1. Deduced amino acid sequence of Notch4 (GenBank accession number U43691). The boxed regions indicate the major structural elements of the Notch family of proteins, as follows: 29 epidermal growth factor(EGF)-like repeats; 3 Notch/lin12 repeats; a transmembrane domain; and 6 cdc10/ankyrin repeats. Putative glycosylation sites are underlined. A putative PEST domain is doubly underlined. The two cysteines thought to promote dimerization are marked with asterisks. The initiating methionine of the int-3 oncoprotein is in bold and marked by an arrow.

was identified at residue 20. A second hydrophobic region from amino acid residues 1441 to 1465 is of sufficient length (25 amino acids) to behave as a membrane-spanning domain and is immediately followed by five consecutive arginine residues that are consistent with a stop transfer signal (Fig. 1).

The extracellular domain of Notch4 contains 29 EGF-like repeats (Figs 1, 2), in contrast to the 36 EGF-like repeats found in murine Notch 1 (Franco Del Amo et al., 1993) and rat Notch 2 (Weinmaster et al., 1992) and to the 34 EGF-like repeats found in murine Notch 3 (Lardelli et al., 1994). EGF-like repeats are defined by a cysteine-rich consensus sequence and generally occur in analogous locations in two different Notch proteins. Since analogous repeats are more homologous to each other than to their neighboring EGF-like repeats, they have been referred to in Notch proteins as equivalent EGF-like repeats. We analyzed the relationship between particular EGF-like repeats of other Notch proteins and those of the Notch4 protein. Fig. 2 schematizes the relationship of EGF-equivalents between Notch4 and Notch1/Notch2. EGF-like repeats 1-13 of Notch4 are equivalent to EGF-like repeats 1-13 of Notch1/Notch2, EGF-like repeats 22-24 of Notch4 correspond to EGF-like repeats 28-30 of Notch1/Notch2 and EGF-like repeats 26-29 of Notch4 are equivalent to EGF-like repeats 33-36 of Notch1/Notch2. Comparison of Notch4 to other Notch proteins revealed no clear-cut identification of the seven particular equivalent EGF-like repeats that are absent in Notch4. The amino acid sequence of equivalent EGF-like repeats has diverged between different Notch homologues and orthologues (Maine et al., 1995), sometimes resulting in loss of a clear-cut equivalent repeat consensus. Six of the unassigned EGF-like repeats of Notch4 appear to be derived from EGF-like repeats 14-27 of Notch1 and Notch2 (Fig. 2). EGF-like repeat 25 of Notch4 may be a hybrid EGF-like repeat derived from parts of EGF-like repeats 31 and 32 of Notch1/Notch2 (Fig. 2). For a discussion of the relationship between Notch3 and Notch1/Notch2 (shown in Fig. 2), see Lardelli et al. (1994).

EGF-like repeats 11 and 12 of *Drosophila* Notch have been shown to be necessary and sufficient for Notch to bind Delta and Serrate proteins in vitro (Rebay et al., 1991). These two equivalent EGF-like repeats are present in Notch4 (Fig. 2). The putative calcium-binding residues (Handford et al., 1991) in EGF-like repeat 11 are also conserved in Notch4 (Fig. 3). The residues between the first and second cysteines of EGF-like repeat 11 have been shown in *Xenopus* Notch to be important in ligand binding and are divergent between Notch proteins (Fig. 3). In this region, Notch4 has additional residues and is unique when compared to other murine Notch proteins. In addition, EGF-like repeats 22-23 of Notch4 have been conserved among murine Notch proteins (EGF-like repeats 28 and 29 of Notch1) and equivalent EGF-like repeats in *Drosophila* Notch are implicated in the regulation of Notch protein function through genetic analysis of the *Abruptex* alleles of Notch (Kelley et al., 1987).

Notch4 also contains three Notch/lin-12 repeats, which are approximately 53% identical to the Notch/lin-12 repeats found in other murine Notch proteins. Between the Notch/lin-12 repeats and the transmembrane domain of Notch4 are two cysteines at positions 1388 and 1397 that are conserved among all Notch proteins and may promote receptor dimerization upon ligand binding (Greenwald and Seydoux, 1990).

The intracellular domain of Notch4 contains the six ankyrin/cdc10 repeats found in other Notch proteins. The ankyrin repeat domain of Notch4 is 48%, 52% and 55% identical to the ankyrin repeat domains of Notch1, Notch2 and Notch3, respectively. In all Notch proteins the number of amino acids between the transmembrane domain and the ankyrin/cdc10 repeats is 110 residues, as it is in Notch4 (Fig. 1). Like other Notch proteins, Notch4 contains a C-terminal PEST domain, albeit of shorter length. In addition, Notch4 lacks a recognizable opa repeat (Fig. 1), such as that found in *Drosophila* Notch. The carboxy-terminal end of Notch proteins, beyond the ankyrin/cdc10 repeats, is the least conserved region among Notch proteins. Within this C-terminal region, Notch4 displays little homology to other Notch proteins and no significant homology to other known proteins. This C terminus is also much shorter in Notch4 (177 residues), than in other Notch proteins (457 residues in Notch1, 437 in Notch2 and 329 in Notch3).

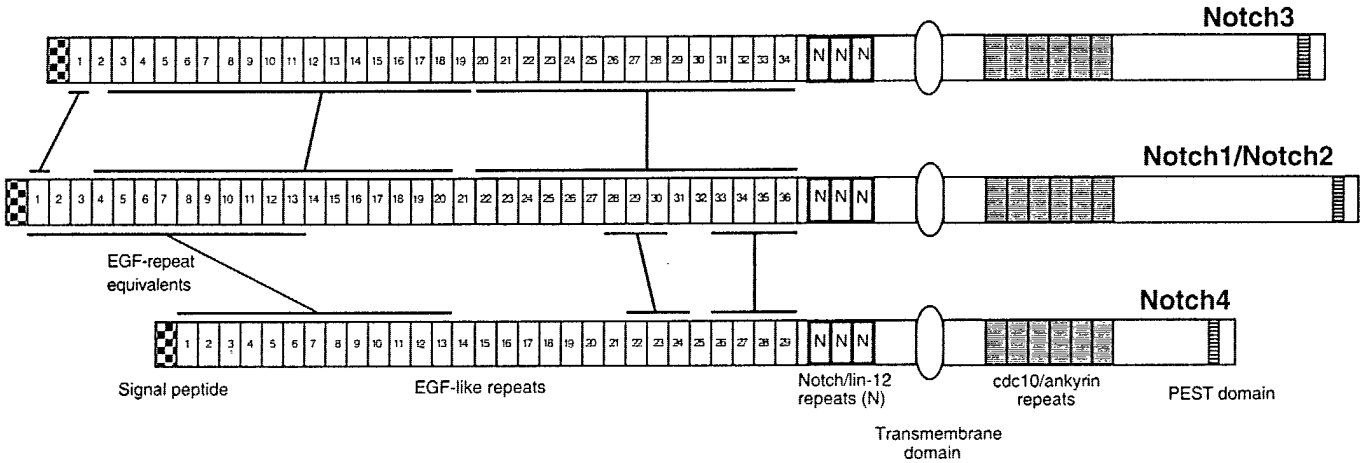

Fig. 2. Schematic structural comparison of the four murine Notch proteins. The EGF-like repeats are numbered according to their position in each different protein. Where equivalent EGF-like repeats can be identified, connecting lines are placed to compare the relationship between these repeats in different Notch proteins (see EGF-repeat equivalents). Notch4 contains seven EGF-like repeats, fewer than Notch1 and Notch2. One of the missing EGF-like repeats (#25) in Notch4 is derived from equivalent repeats #31 and #32 of Notch1/Notch2, creating a novel and hybrid EGF-like repeat. Eight of the EGF-like repeats of Notch4 (#14 to #21) have no identifiable equivalent repeats in Notch1/Notch2. The region of Notch4 from the end of the cdc10/ankyrin repeats to the carboxy terminus is shorter when compared to Notch1, 2 and 3.

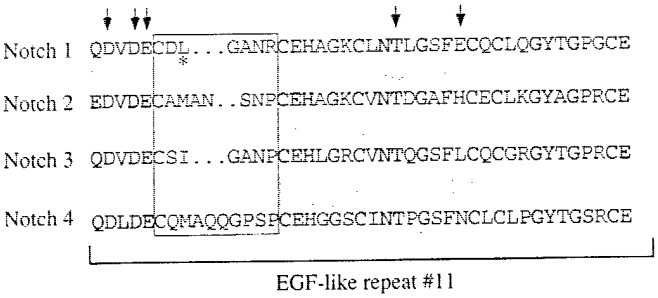

Fig. 3. Amino acid sequence comparison of EGF-like repeat #11 of mouse Notch1, 2, 3 and 4. Residues conserved between the mouse Notch proteins are shaded and the putative calcium-binding sites are marked with arrows. A region within EGF-like repeat #11 of the Notch proteins containing non-conserved and variable numbers of residues is boxed. The leucine to proline mutation in Xenopus Notch that obliterates binding to Delta is marked with an asterisk (*).

Analysis of *Notch4* transcripts in adult tissues

Several adult tissues were examined for the presence of *Notch4* transcripts by northern blot analysis. To minimize cross-hybridization with other mouse *Notch* transcripts, we used a riboprobe derived from the 3′ UTR of *Notch4*. In most tissues analyzed, a single hybridizing species of 6.7 kb was detected (Fig. 4), which roughly corresponds in size to the cloned *Notch4* cDNA. The 6.7 kb transcript is most highly expressed in lung, at lower levels in heart and kidney and at detectable levels in ovary and skeletal muscle. Very low levels of the 6.7 kb transcript were observed in several other adult tissues, including brain, intestine, liver, testis (Fig. 4) and spleen (data not shown). In adult testis, two abundant transcripts of 1.5 kb and 1.1 kb were observed. Thus, *Notch4* expression varies widely in adult tissues. Other than in testis, we did not detect transcript size variation in different tissues.

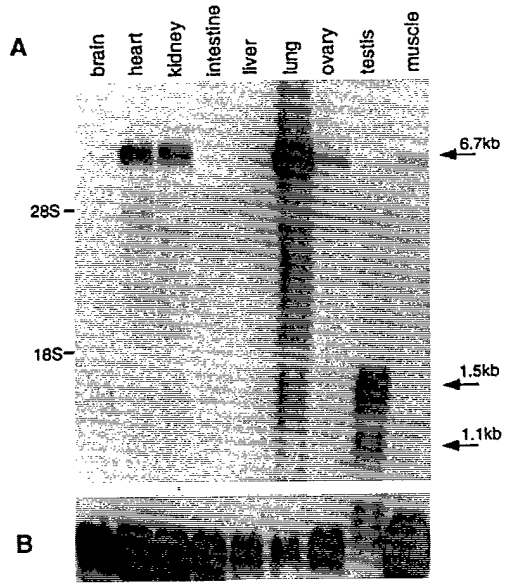

Fig. 4. Expression analysis of *Notch4* in adult mouse tissues. (A) Northern blot using a riboprobe transcribed from the 3′ UTR of *Notch4* (probe D in Fig. 5). (B) The same blot reprobed with a GAPDH probe. The transcript sizes of 6.7 kb, 1.5 kb and 1.1 kb are indicated and were estimated with reference to 28 S and 18 S rRNA migration.

Analysis of testis-specific truncated *Notch4* transcripts

To determine the cell lineage specificity of *Notch4* expression in the murine testis, RNA was analyzed in the germ cell-deficient mouse testis (Fig. 5). Mice that carry two mutations at the white-spotting locus (W/W^v) are devoid of germ cells, but have the normal complement of somatic cell types, including Leydig, Sertoli and peritubular myoid cells (Mintz and Russell, 1957). Heterozygous litter mates ($W/+$) have normal somatic and germ cell complements. Northern blot analysis of total RNA from germ cell-deficient testes (W/W^v) and testes with normal germ cells [$W/+$ and adult (+/+)] was done using a riboprobe derived from the 3′ UTR (probe D in Fig. 5C). Transcripts of 1.5 kb and 1.1 kb were detected in RNA from the testes of adult wild type and $W/+$ mice (Fig. 5A). However, neither transcript was detected in RNA from homozygous mutant testes, suggesting that these transcripts were likely to be specific to the germinal compartment.

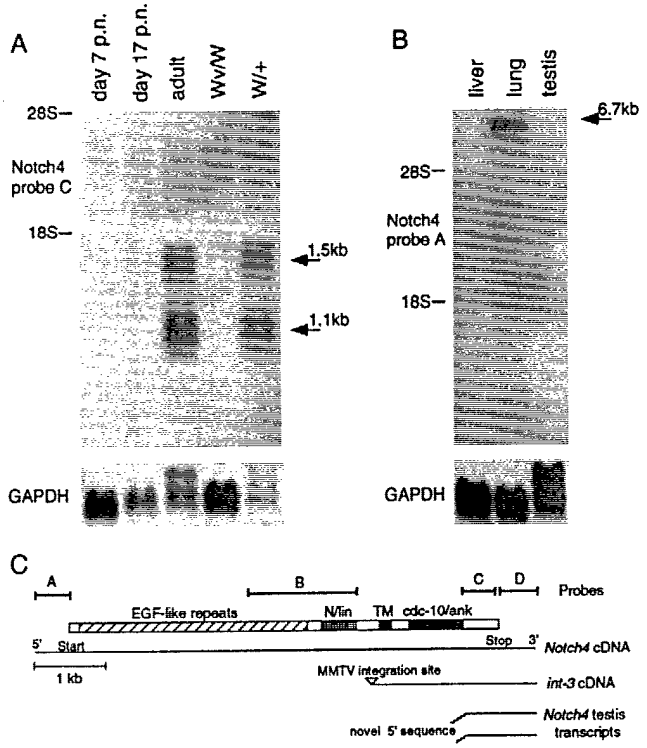

Fig. 5. Expression analysis of *Notch4* testis transcripts. (A) *Notch4* testis transcripts are expressed in post-meiotic germ cells. Northern blot analysis from staged and germ cell-deficient testes with probe C and a GAPDH probe. Note that GAPDH transcripts appear as two isoforms in the adult testis. RNA was isolated from testes of day 7 p.n., day 17 p.n., adult, W^v/W and $W/+$ mice, as indicated. (B) Northern blot analysis of several adult tissues with probe A, derived from the 5′ UTR of Notch4 and a GAPDH probe. (C) Schematic representation of truncated *Notch4* transcripts as compared to the full-length coding potential. Relative positions of probes used in the northern blot analysis are shown. Conserved elements of Notch family proteins are indicated. The MMTV integration site reported by Robbins et al. (1992) is indicated by an arrow. Novel 5′ sequences of testes cDNAs are indicated.

Since spermatogenic differentiation undergoes a characteristic temporal progression, one can use mice testes at specific days of postnatal development to enrich for or eliminate particular germ cell types. Testes from day 7 of postnatal development (day 7 p.n.) mice contain mitotic spermatogonia, while testes from day 17 p.n. mice have entered meiosis and have progressed to spermatocytes (Nebel et al., 1961). Both day 7 p.n. and day 17 p.n. testes lack post-meiotic spermatids. Total RNA from immature and adult testes was analyzed by northern blot hybridization to determine stage-specific expression of *Notch4* transcripts during male germ cell development. Both *Notch4* transcripts of 1.5 kb and 1.1 kb are absent in day 7 p.n. and day 17 p.n. testis, but are present in adult testis (Fig. 5A). These results indicate that the expression of the 1.5 kb and 1.1 kb *Notch4* transcripts is restricted to post-meiotic germ cells.

To determine the nature of the short *Notch4* transcripts in adult mouse testis, northern blot analysis was done using riboprobes derived from different regions of the *Notch4* coding sequence, as well as from 5′ and 3′ UTR (Fig. 5B). A riboprobe derived from the 5′ UTR (probe A in Fig. 5C) failed to hybridize to either the 1.5 kb or the 1.1 kb transcripts (Fig. 5B), whereas this probe did hybridize to the 6.7 kb transcript found in lung RNA (Fig. 5B). However, riboprobes derived from the 3′ UTR (probe D in Fig. 5C) or from cDNA encoding part of the intracellular domain of Notch4 (probe C in Fig. 5C) hybridize to the testis transcripts (Fig. 5A and data not shown). Probes derived from the coding sequence of the extracellular domain of Notch4 (probe B in Fig. 5C) did not hybridize to the testes transcripts (data not shown). To characterize the transcripts expressed in the adult mouse testis, a cDNA library prepared from adult mouse testes RNA was screened using probe C of Fig. 5C. All the clones analyzed encoded the most C-terminal coding sequence and the 3′ untranslated region of Notch4. Two independent clones of distinct size contained novel 5′ sequences unrelated to that found in the full-length *Notch4* cDNA (schematized in Fig. 5C, *Notch4* testis transcripts). Based upon the northern blot analysis described above and the sequence of the cloned testis cDNAs, we believe that *Notch4* transcripts are either derived from an alternate intronic promoter that is active in post-meiotic germ cells or that they may be driven by the same promoter as the 6.7 kb transcript and consist of spliced products derived from a 5′ untranslated region upstream of what we have currently identified. The predicted amino acid sequence of the testis *Notch4* transcripts with the novel 5′ sequence does not contain a methionine that could function as a translation initiator; therefore, these transcripts are unlikely to encode protein products. The testis transcripts may thus represent aberrant transcriptional events in post-meiotic germ cells, as has been described previously (Davies and Willison, 1993).

Expression analysis of *Notch4* during development and in adult lung

A 6.7 kb *Notch4* transcript was detected by northern hybridization in RNA isolated from day 12.5 p.c. mouse embryos (data not shown and Sarkar et al., 1994) and adult lung (Fig. 4). To determine the spatial and temporal pattern of *Notch4* transcript accumulation during development, we examined mouse embryo tissue sections from 9.0 d.p.c. to birth using in situ hybridization. During embryonic development, as well as in postnatal tissues, *Notch4* is highly expressed in endothelial cells. Intense labeling for *Notch4* is observed in embryonic blood vessels at 9.0 d.p.c. (Fig. 6A,B). As shown in Fig. 6C,D, strong labeling is observed over the dorsal aorta, the aortic tract and the pulmonary artery in a 13.5 d.p.c. embryo, while no labeling is detected in the epithelial cells lining the gut (red arrow). At higher magnification, we note that labeling is restricted to the endothelial cells lining the embryonic vessels (Fig. 6D,E) and no labeling is detected in the red blood cells

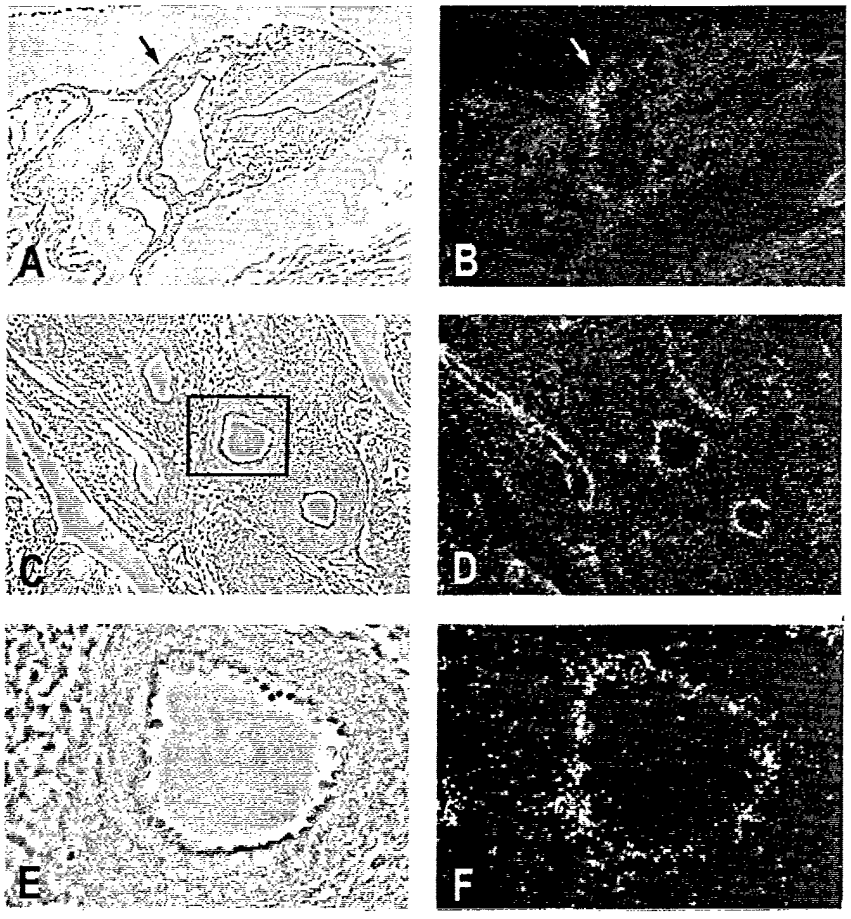

Fig. 6. *Notch4* is expressed in embryonic endothelial cells. (A,B) Phase contrast and dark-field photomicrograph of a horizontal section of a 9 d.p.c. embryo hybridized with a cRNA probe corresponding to *Notch4*. Strong labeling is detectable over the anterior cardinal vein (white/black arrows). Diffuse labeling is also present throughout the developing nervous system and at higher levels over the tip of the neural folds (red arrows). (C-F) Phase and darkfields images of a horizontal section of a 13.5 d.p.c. embryo hybridized for Notch4, showing the venous and arterial system anterior to the lung, including dorsal aorta arch, aortic and pulmonary tract. E and F are higher magnifications of the area framed in C. Embryonic vessels are labeled and, as shown in E and F, labeling is restricted to the endothelial cells lining the vessels. Arrows denote the gut, which does not have a detectable signal in the epithelium.

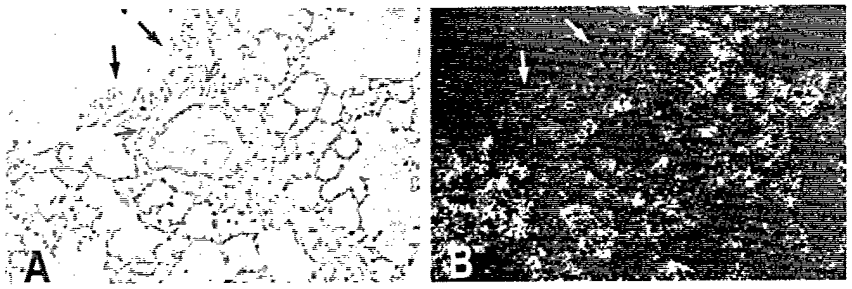

Fig. 7. *Notch4* is expressed in adult lung endothelial cells. (A,B) Phase contrast and dark-field photomicrographs of an adult mouse lung hybridized with a cRNA probe corresponding to *Notch4*. Punctate staining is observed over the alveolar walls, which are predominantly composed of capillaries. No labeling is observed over the pseudostratified squamous epithelium (black and white arrows) nor over the smooth muscle cells (red arrows).

in the vessel. A weak and transient signal is also detectable in the developing nervous system from 9.0 d.p.c. embryos. As shown in Fig. 6A,B, a light diffuse labeling is detected in the developing nervous system and a more distinct signal is observed at the tip of the neural folds. *Notch4* transcripts in the nervous system are still detectable at 11.5 d.p.c., but by 13.5 d.p.c. no labeling for *Notch4* is detectable in the nervous system (data not shown).

Since adult lung exhibited the highest levels of *Notch4* transcripts, in situ hybridization was performed on lung sections to determine whether *Notch4* expression remains endothelial cell-specific in adult life. Intense punctate staining was observed over the alveolar wall, indicative of capillary-specific expression (Fig. 7). The central component of the alveolar wall is the capillary flanked by pneumocyte type I epithelial cells, which line the alveolar lumen (Ross and Reith, 1985). Capillaries are highly localized in the alveolar wall and would give the punctate localized signal observed, as opposed to a more uniform pattern for epithelial cells lining the alveolar cavity. There is clearly no hybridization signal over other cellular components of the lung, that is, pseudostratified squamous epithelium, smooth muscle and connective tissue cells. The endothelial-specific expression probably underlies the abundance of *Notch4* transcripts found by northern blot analysis of highly vascularized adult tissues (lung, heart and kidney in Fig. 4).

DISCUSSION

We report here the identification of a novel mouse gene whose protein product exhibits structural homology with the vertebrate Notch protein family. We have named this gene *Notch4*, as it is the fourth murine *Notch* gene identified. Notch4 contains all the conserved domains characteristic of Notch proteins (Figs 1 and 2). However, Notch4 contains only 29 EGF-like repeats within its extracellular domain as compared to the 36 repeats found in Notch1 and Notch2. In addition, the C-terminal tail of Notch4, beyond the ankyrin/cdc10 repeats, is shorter and unique when compared to all other Notch proteins, but little is known of the function of this region in Notch proteins. Notch4 also contains a distinct EGF-like repeat 11, which has been proposed to be crucial for ligand binding. Structural variation in this repeat and differences in the number of EGF-like repeats between murine Notch proteins, may be important for ligand specificity among the different possible Notch ligands. It must be noted that Notch/lin-12 proteins of varying structure have been demonstrated to be functionally interchangeable; *C. elegans* glp-1 can fully substitute for lin-12 (Fitzgerald et al., 1993) for instance. Therefore, Notch4 may be functionally interchangeable with other murine Notch proteins, despite structural differences between them.

Notch4 is distinct from other Notch family proteins, based on its expression pattern during embryonic development and in the adult mouse. In situ hybridization demonstrates endothelial-specific embryonic expression of *Notch4*. This endothelial-specific expression of *Notch4* remains in the adult mouse. A weak and transient labeling is seen in the neural tube between day 9 p.c. and 11.5 p.c., with a more intense labeling at the tips of neural folds. This region of the neural tube is a highly plastic area where cells will probably participate in the fusion process of the neural tube and/or migrate as neural crest. The *Notch4* expression pattern is in sharp contrast to the expression patterns of *Notch1*, *2* and *3*. These *Notch* genes are expressed in a variety of different embryonic tissues such as the developing brain and spinal cord, presomitic and somitic mesoderm and a variety of epithelial cells and mesenchymal derived tissues (Weinmaster et al., 1991; Williams et al., 1995). *Notch1* is the only other *Notch* gene reported to be expressed in endothelial cells (Reaume et al., 1992; Bettenhausen et al., 1995; Lindsell et al., 1995). Expression of *Notch1* and *4* in endothelial cells might reflect either redundancy of function or distinct biological functions in endothelial development. Endothelial cell-specific expression has recently been reported for a putative Notch ligand, the chick *Serrate* homologue (Myat et al., 1996).

Since Notch proteins have been implicated in binary cell fate specification, regulating how equivalent cells can give rise to cells with different fates, a putative biological function of Notch4 might be to govern the cell fate decisions during endothelial growth and development. In amniotes, endothelial and hematopoietic cells appear synchronously in the blood islands. In zebrafish, lineage data have shown that individual cells of the early blastula can give rise to both endothelial and blood cells, suggesting a common embryonic precursor which has been referred to as the 'hemangioblast'. The occurrence of binary cell fate decision events in the hemangioblast is supported by analysis of the endothelial and/or hematopoietic cell lineages. *Cloche*, *bloodless* and *spadetail* are mutants isolated in zebrafish that display phenotypes defective in either hematopoietic development or both hematopoietic and endothelial development (Stainier et al., 1995). In the mouse, the *Flk-1* and the *Flt-1* genes encode receptor tyrosine kinases that are expressed in embryonic endothelium (Shalaby et al., 1995; Fong et al., 1995). Null mutants for the *Flk-1* gene are defective in endothelial and blood cell development (Shalaby et al., 1995), whereas null mutants for the *Flt-1* gene display only hematopoietic cell development defects (Fong et al.,

1995). Mutational analysis of the *Notch4* gene in whole animals would help to define the role of Notch4 in endothelial cell growth and development.

Alterations in stem cell fate decisions as a result of activated Notch proteins have been proposed to contribute to mitogenic growth of tumor cells. Blocked cell differentiation of fated daughter cells by activated Notch proteins may lead to an increase in the number of cells undergoing cell division, or a prolonged life of the cell. In these cells, the probability of secondary oncogenic mutations that contribute to neoplastic transformation would be enhanced. In the normal mouse mammary gland, endogenous int-3 protein has been detected at low levels in mammary stroma and epithelium (Smith et al., 1995). Although little is known about the nature of stem cells in the mammary epithelium, Notch4 might regulate the fate decisions of mammary epithelial cells. This hypothetical model may explain the phenotype that is observed in *int-3* transgenic mice, which display blocked development of the mammary gland and develop mammary carcinomas at high frequency.

The signal transduction pathways by which Notch proteins function are becoming understood through genetic studies in *Drosophila*. Deltex and Suppresser of Hairless [Su(H)] have been demonstrated to bind to the cdc10 repeats of the intracellular domain of *Drosophila* Notch (Diederich et al., 1994; Fortini and Artavanis-Tsakonas, 1994; Matsuno et al., 1995). More recently the mammalian Su(H) orthologue RBP-Jk, a transcription factor, has been shown to bind to the intracellular domain of Notch 1 (Jarriault et al., 1995). Since Notch4 contains the canonical ankyrin/cdc10 repeats, RBP-Jk or RBP-Jk homologues and mammalian Deltex homologues may interact with the cdc10/ankyrin repeats of Notch4. It has been proposed that upon activation of the Notch receptors, Su(H) or RBP-Jk are activated and translocate to the nucleus. where they may regulate transcription of target genes (Goodbourn, 1995). In fact, activated Notch proteins containing only the intracellular domain have been reported to localize to the nucleus (Kopan et al., 1994; Struhl et al., 1993), suggesting a nuclear function for this domain. We have found that the int-3 oncoprotein, modified to encode a flu epitope-tag at the C terminus, is also localized to the nucleus when expressed in cultured 293T cells, as determined by immunofluorescence (unpublished data). The activated int-3 protein lacks a signal peptide but contains a membrane-spanning domain and thus is not likely to enter the secretory pathway. This finding may indicate that int-3 can bind to cytoplasmic proteins that are then translocated to the nucleus.

We show that the *int-3* gene encodes a truncated Notch4 protein with the extracellular domain deleted (EGF-like repeats and Notch/lin-12 repeats), providing the first comparison of a naturally activated murine Notch protein and its normal counterpart. In MMTV-induced mouse mammary tumors with an activated Notch4, as described by Robbins et al. (1992), the oncogenic affects are probably the result of both overexpression or ectopic expression of *Notch4* mRNA as well as functional activation of the Notch4 protein. A structural comparison of the mutant int-3 protein to the normal Notch4 protein is reminiscent of the structural alterations reported to activate the effector function of *Drosophila* Notch and *C. elegans* lin-12 proteins (Greenwald, 1994) or oncogenic activation of TAN-1. Thus, loss of the extracellular domain is likely to lead to loss of the regulatory controls provided by the ligand-binding domain believed to reside in the EGF-like repeats of Notch4.

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Notch and Wnt proteins function to regulate branching morphogenesis of mammary epithelial cells in opposing fashion.

Hendrik Uyttendaele[1], Jesus V. Soriano[2], Roberto Montesano[2] and Jan Kitajewski[1*]

[1]Department of Pathology in the Center of Reproductive Sciences, Columbia University, College of Physicians and Surgeons, New York, NY 10032, USA
[2]Institute of Histology and Embryology, Department of Morphology, University Medical Center, 1 rue Michel-Servet, CH-1211 Geneva 4, Switzerland

*Author of correspondence- Phone/fax (212) 305-3624

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Correspondent Footnote
Mailing address: Department of Pathology
Columbia University
630 West 168th Street
New York, NY 10032
Phone/Fax: (212) 305-3624
e-mail address: jkk9@columbia.edu

ABSTRACT

Elongation and branching of epithelial ducts is a crucial event during the development of the mammary gland. Branching morphogenesis of the mouse mammary epithelial TAC-2 cell line was used as an assay to examine the role of Wnts, HGF, TGF-β and the Notch receptors in branching morphogenesis. Wnt-1 was found to induce the elongation and branching of epithelial tubules, like HGF and TGF-β2, and to strongly cooperate with either HGF or TGF-β2 in this activity. The Notch4(int-3) mammary oncoprotein, an activated form of the Notch4 receptor, inhibited the branching morphogenesis normally induced by HGF and TGF-β2. The minimal domain within the Notch4(int-3) protein required to inhibit morphogenesis consists of the CBF-1 interaction domain and the cdc10 repeat domain. Co-expression of Wnt-1 and Notch4(int-3) demonstrates that Wnt-1 can overcome the Notch-mediated inhibition of ductal morphogenesis. These data suggest that Wnt and Notch signaling may play opposite roles in mammary gland development, a finding consistent with the convergence of the wingless and Notch signaling pathways found in Drosophila.

INTRODUCTION

The development of the murine mammary gland involves an intricate sequence of proliferative, morphogenic and differentiative events, which gradually results in the formation of an arborized tree-like structure of epithelial ducts. Postnatal development of the mammary gland is influenced by gonadal hormones, with distinct developmental stages occurring during puberty, estrous, pregnancy and lactation. At birth, the mammary epithelial ducts have few side-branches. During puberty, the epithelial ducts rapidly elongate and branch, and give rise to a highly organized epithelial structure with terminal end buds and lateral buds. The terminal end buds are the major sites of proliferation, whereas the lateral buds differentiate into alveoli during each estrous cycle. During pregnancy, the alveoli rapidly increase in size and number resulting in the development of fully differentiated lobules, which will produce milk at lactation. The mammary gland remodels after lactation ceases, and this process is characterized by the involution of the secretory lobules and regression to the ductal tree observed at puberty (review in (9, 38, 41)).

Mesenchymal-epithelial and epithelial-epithelial interactions are essential in the regulation of growth and development of the murine mammary gland. Peptide growth factors, such as Epidermal growth factor (EGF) (21), Fibroblast growth factors (FGF) (7), Hepatocyte growth factor (HGF), Insulin-like growth factor II (IGF-II) (3), Neuregulin (NRG) (51), and Transforming growth factor-β (TGF-β) (8, 37), have been implicated as regulators of mammary gland development based on their expression patterns and, in some cases, on their abilities to affect the development of the mammary gland. HGF (or scatter factor) is expressed in the mammary mesenchyme during ductal branching, whereas its tyrosine kinase receptor c-met is expressed in the mammary epithelial ducts at all stages (32). HGF can promote branching morphogenesis of the mammary ductal tree (32, 36, 47, 51) in several experimental settings. TGF-β1 is expressed in the epithelial compartment of the mammary gland at all stages, except during lactation (8,

44). In vivo, TGF-β1 has been shown to inhibit ductal out-growth from the mammary end buds (27, 37). In vitro however, TGF-β1 has been shown to induce opposite effects depending on its concentration. TGF-β1 at high concentrations (0.5-5 ng/ml) inhibit ductal elongation and branching of TAC-2 mammary epithelial cells, whereas at low concentrations (5-100 pg/ml) it is able to stimulate these biological processes (46).

The Wnt family of secreted growth factors are also implicated as regulators of the developing mouse mammary gland (34). *Wnt* genes are expressed during ductal development of the gland (*Wnt*-2, *Wnt*-5a, *Wnt*-7 and *Wnt*-10b) and during lobular development at pregnancy (*Wnt*-4, *Wnt*-5b and *Wnt*-6), and the expression of most *Wnt* transcripts is down regulated during lactation (16, 50). This pattern of expression during periods of morphogenesis has led to a proposed role for *Wnt* genes in morphogenic events during mammary gland development. *Wnt* gene expression has been documented in both the stromal and epithelial compartments of the mammary gland, raising the possibility of involvement in both stromal-epithelial and epithelial-epithelial interactions (5, 50). The *Wnt*-1 gene is not normally expressed within the mouse mammary gland, however its expression can contribute to tumorigenesis when activated by insertion of mouse mammary tumor virus (MMTV) proviral DNA in MMTV-induced mammary tumors (33). Mammary gland tumors develop in transgenic mice where ectopic *Wnt*-1 gene expression is controlled by the MMTV promoter; these mice display hyperplasia of the mammary epithelium and an increased incidence of tumors (48).

The *Notch4* gene was also identified as a frequent target for insertional activation by MMTV proviral DNA in MMTV-induced mammary gland tumors (40, 42). The *Notch4* gene encodes for a large transmembrane receptor protein (14, 49). The int-3 oncoprotein is activated by MMTV insertion and corresponds to a truncated form of Notch4 which has most of its extracellular domain deleted (49); this mutated version of Notch4 will be referred to as Notch4(int-3). In contrast to *Wnt*-1, expression of the

Notch4(int-3) oncogene as a transgene in the mouse mammary gland results in impaired development of the mammary gland which no longer generates a tree-like structure of epithelial ducts. Instead, a hyperproliferative mass of undifferentiated epithelial cells is observed near the nipple, from which undifferentiated mammary carcinomas rapidly develop (25).

The aim of this study was to define the roles of both Wnt and Notch signaling in mammary gland ductal morphogenesis. Using a previously described model in which TAC-2 mammary epithelial cells grown in collagen gels form branching cords or tubules in response to HGF or TGF-β1 (46, 47), we demonstrate that activation of the Wnt and Notch signaling pathways has opposite effects on branching morphogenesis. Wnt-1 acts to induce branching morphogenesis whereas Notch4(int-3) inhibits branching morphogenesis by either HGF or TGF-β. Wnt-1 has the capacity to overcome the Notch4(int-3) mediated inhibition of branching morphogenesis.

MATERIALS AND METHODS

Reagents. Recombinant human HGF (rhHGF) was provided by Genentech, Inc. (San Francisco, CA). Recombinant TGF-β2 was provided by Dr. G. Gunderson (Columbia University, New York, NY). Rat tail collagen solution was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-HA monoclonal antibody (12CA5) was from Berkeley Antibody Co. (Richmond, CA) and HRP-conjugated sheep anti-mouse immunoglobulin G from Amersham (Arlington Heights, IL).

cDNA clones. The murine *Notch4(int-3)* cDNA corresponds to a truncated *Notch4* cDNA; residues 4551 to 6244 of *Notch4* (Uyttendaele et al. 1996). An oligonucleotide encoding the haemagglutinin (HA) antigenic determinant was appended to the 3′ end of the *Notch4(int-3)* and *Wnt*1 cDNA's. Eighteen codons were added that specify the amino acid sequence SMAYPYDVPDYASLGPGP, including the nine residue HA epitope (underlined). HA-tagged *Notch4(int-3)* and *Wnt*1 cDNAs were created by subcloning each cDNA into Bluescript KS (Stratagene) with the coding region of the HA epitope situated downstream of the newly inserted cDNA. These two sequences were made co-linear by "loop-out" mutagenesis using oligonucleotides designed to eliminate the stop codon and non-coding 3′ sequence of the *Notch4(int-3)* and *Wnt*1 cDNAs. Oligonucleotides used in this procedure are as follows;

Notch4(int-3): CGG TTG TAA GAA ATC TGA ACT CCA TGG CCT ACC CAT ATG

Wnt-1: CGC GCG TTC TGC ACG AGT GTC TAT CCA TGG CCT ACC C .

The 5′ end of each oligo is complementary to the C-terminus of *Notch4(int-3)* or *Wnt*1 cDNA and their 3′ ends anneal to HA epitope-encoding sequence (underlined). Mutagenesis was done with the Muta-Gene*in vitro* mutagenesis kit (Bio Rad, Richmond, CA). The presence of each fusion was confirmed by DNA sequencing. *Notch4(int-3)* cDNA deletion mutants were generated from the epitope-tagged

Notch4(int-3) construct by restriction enzyme cloning, and were named ΔNT, ΔCDC, ΔCT and ΔNTΔCT. The ΔNT deletion mutant corresponds to nucleotides 4921 to 6244 of the *Notch4* sequence. The ΔCDC deletion mutant corresponds to nucleotides 4551 to 4864 and to nucleotides 5706 to 6244 of the *Notch4* sequence. The ΔCT deletion mutant corresponds to nucleotides 4551 to 5718 of the *Notch4* sequence. The ΔNTΔCT deletion mutant corresponds to nucleotides 4921 to 5718 of the *Notch4* sequence.

Cell culture. The TAC-2 cell line was derived from NMuMG cells as described previously (47). TAC-2 cell were grown on collagen-coated dishes in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% fetal calf serum (FCS, GIBCO). The Bosc 23 retrovirus packaging cell line (Pear et al. 1993) was obtained from Dr. W. Pear (MIT, MA) and grown in DMEM containing 10% FCS. Both culture media were supplemented with penicillin (500 i.u./ml) and streptomycin (100ug/ml). Both cell lines were grown at 37°C in 8% CO_2.

Cell line generation. HA-tagged cDNAs were inserted into the retroviral vector pLNCX (28) wherein neomycin phosphotransferase (neo) expression is controlled by the murine leukemia virus LTR, and cDNA transcription is controlled by the cytomegalovirus (CMV) enhancer/promoter. The retroviral vector pLHTCX was derived from pLNCX, however the neo gene is replaced by a hygromycin-resistence gene. Populations of TAC-2 cells, expressing either HA-tagged *Notch4(int-3)* or *Wnt1* cDNA, were prepared by retroviral infection. Recombinant retroviruses were generated by transiently transfecting constructs into the BOSC 23 cell line by calcium phosphate co-precipitation, as previously described (35). Retroviral infection of TAC-2 cells was done by culturing cells with viral supernatants collected from transfected BOSC 23 cells two day post-transfection. Infections were done in the presence of 4 μg/ml polybrene for 12 hours after which medium was replaced to DMEM + 10% FCS. One day post-

infection the culture medium was replaced to DMEM + 10% FCS containing 500 μg/ml Geneticin (GIBCO BRL Life Technologies, Grand Island, NY) or 200 μg/ml hygromycin B (Sigma Chemical Co.). Colonies appeared 5 days later and were pooled into medium containing 250 μg/ml Geneticin or 200 μg/ml hygromycin B. These resultant populations, each comprised of at least 50 clones, were used in assays described below.

Collagen cell culture assays. TAC-2 cell lines were harvested using trypsin-EDTA, centrifuged, and embedded in three-dimensional collagen gels as previously described (47). Briefly, 8 volumes of rat tail collagen solution (approximately 1.5 mg/ml) were mixed with 1 volume of 10x minimal essential medium (GIBCO) and 1 volume of sodium bicarbonate (11.76 mg/ml) in a sterile tube kept on ice to prevent premature collagen gellation. TAC-2 cells were resuspended in the cold mixture at cell densities of 2 or 4 x 10^4 cells/ml and 0.5 ml aliquots were dispensed into 16-mm wells of 24 multiwell plates (Becton Dickinson Labware). After the collagen mixture had gelled, 1ml of complete medium (DMEM + 10% FCS) with or without HGF or TGF-β2 was added to each well. TAC-2 collagen gel cultures were initially done in the presence and absence of 2mM sodium butyrate, but since no difference in phenotypes was observed, the sodium butyrate was omitted in all experiments. Media were changed every 2 days, and after 3 to 5 days, cell cultures were photographed with a Nikon ELWD 0.3 phase contrast microscope on Kodak T-Max film (100 X magnification).

Quantification of cord lenth and branching. TAC-2 cells were suspended at 5x10^3 or 1x10^4 cells/ml incollagen gels (500 μl) cast into 16-mm wells of 4-well plates (Nunc, Kampstrup, Rosklide, Denmark) and incubated in 500 μl complete medium in the presence or the absence of 10ng/ml HGF. After 7 days, the cultures were fixed with 2.5% glutaraldehyde in 0.1M cacodylate buffer, and at least 3 randomly selected fields (measuring 2.2 mm x 3.4 mm) per experimental condition in each of 3 separate

experiments were photographed with a Nikon Diaphot TMD inverted photomicroscope. The total length of the cords present in each individual colony was measured with a Qmet 500 image analyzer (Leyca Cambridge Ltd., Cambridge, UK). Cord length was considered as "0" in: a) colonies with a spheroidal shape, and b) slightly elongated structures in which the length to diameter ratio was less than 2. Quantification of branching was performed by counting all branch points in each colony. Values of cord length and branching obtained from the largest colonies are an underestimate, since in these colonies a considerable proportion of cords were out of focus and therefore could not be measured. Values were expressed either as mean cord length and number of branch points per photographic field (46) or as mean cord length and number of branch points per individual colony (47). The mean values for each experimental condition were compared to controls using the Student's unpaired T-test.

Immunoblot analysis. HA-tagged Notch4(int-3), Notch4(int-3) deletion mutants and Wnt-1 proteins from lysates of TAC-2 cell populations were analyzed by immunoblotting. To maximize protein expression, TAC-2 cells were treated with 2mM sodium butyrate for 16 hours prior to lysis. Cells were washed twice with cold PBS and then removed from dishes in 1.5 ml PBS using a rubber policeman. Cells were pelleted by centrifugation at 2,000x g at 4°C for 5 min. and lysed in 90 µl TENT buffer (20 mM Tris, pH 8.0, 2 mM EDTA, 150 mM NaCl, 1% Triton-X100) containing 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 2 mg/ml leupeptin, 1 mg/ml pepstatin, at 4°C for 30 min. Lysates were clarified by centrifugation at 10,000xg at 4°C for 10 min., and protein contents were determined using the BioRad Protein determination kit. Lysates containing 40 µg protein were electrophoresed in 10% SDS-polyacrylamide gels. Proteins were transferred from gels onto nitrocellulose by electroblotting, and then blocked overnight at 4°C in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.2% Tween-20) containing 1% bovine serum albumin (fraction V). Blots

were then incubated in anti-HA monoclonal antibody (12CA5) diluted 1:100 in TBST at room temperature. After four hours, the blot was washed three times for 5 min. each in TBST. Blots were exposed to a 1:16,000 dilution of HRP-conjugated sheep anti-mouse IgG. Blots were washed as above and then incubated 1-2 min. in enhanced chemiluminescence reagents (Amersham Inc, IL) and exposed to X-ray film (Fujifilm, Fuji Photo Film Co., LTD., Tokyo).

RESULTS

When suspended in collagen gels, TAC-2 mammary epithelial cells form small slowly growing colonies with a morphology ranging from irregular shaped cell aggregates to poorly branched structures. Under these same conditions, TAC-2 cells grown in the presence of either HGF or TGF-β1 develop an extensive network of branching cords that consist of elongated epithelial cords or tubules with multiple branch points (46, 47). This TAC-2 cell phenotype is thus reminiscent of the branching morphogenesis of epithelial ducts in the mammary gland and provides an experimental model to study the roles of growth factors in the development of the mammary gland. To investigate the role of Wnt and Notch signaling in mammary epithelial cell growth and morphogenesis we ectopically expressed either the Wnt-1 or activated Notch4(int-3) oncoproteins and analyzed their effects on branching morphogenesis of TAC-2 cells.

Wnt-1 stimulates TAC-2 cell branching morphogenesis. The biological activity of Wnt proteins was evaluated by generating TAC-2 cells ectopically expressing a *Wnt*-1 cDNA. TAC-2 cell lines programmed to express *Wnt*-1 (TAC-2 Wnt-1) were generated using the retroviral vector pLNCX to drive *Wnt*-1 expression from the CMV promoter . As a control, TAC-2 cells were generated that were programmed to express LacZ (TAC-2 LacZ). To evaluate the expression levels of Wnt-1 proteins in the cell lines generated, the *Wnt*-1 cDNA was fused at the carboxy terminus to the haemagglutinin-epitope (HA) tag, allowing us to detect Wnt-1 proteins in immunoblot analysis using the anti-HA monoclonal antibody (Fig. 1). Cell extracts from TAC-2 cell lines contained Wnt-1 proteins (Fig. 1) that migrated as a series of proteins with molecular weights between 41 and 45 kD, due to differential glycosylation. The ectopic expression of Wnt-1 proteins in TAC-2 Wnt-1 cells can be significantly increased by treating cells with sodium butyrate (2mM), which enhances transcription of the CMV promoter (Fig. 1). We found that addition of sodium butyrate to the TAC-2 branching morphogenesis assay did not

alter or enhance the TAC-2 cell phenotypes described below. The ensuing analysis of branching morphogenesis was conducted without sodium butyrate treatment.

TAC-2 cells programmed to express LacZ give rise to small colonies with poorly branched cords when grown in collagen gels for four days (Fig. 2A). Addition of either HGF (20 ng/ml) or TGF-β2 (50 pg/ml) to the culture induces pronounced changes in colony morphology, resulting in the formation of long branching cords or tubules (Fig. 2B and 2C). We utilized TGF-β2 in our assays, which we found has an identical activity as TGF-β1 in the induction of branching morphogenesis of TAC-2 cells (46). When TAC-2 cells are programmed to express Wnt-1 proteins, cell colonies form cords with moderate branching even in the absence of exogenous growth factors (compare Fig. 2A and 2D). When TAC-2 Wnt-1 cells are grown in the presence of either HGF or TGF-β2 (Fig. 2E and 2F), a highly extensive branching network is observed. This network of epithelial tubules is significantly more extensive when compared to control TAC-2 LacZ cells grown under identical conditions. An identical phenotype was observed in TAC-2 cell lines programmed to express either a non-epitope tagged *Wnt*-1 cDNA or a HA epitope tagged *Wnt*-1 cDNA transcribed from a SV40 based retroviral vector (data not shown). Thus, Wnt-1 activity was confirmed in at least three independently produced TAC-2 cell lines.

The morphological analysis of TAC-2 cell cultures suggested that Wnt-1 cooperates with either HGF or TGF-β2 in the induction of branching morphogenesis. To characterize combined effects of Wnt-1 and HGF, quantitative evaluation of cord length and the number of branch points was conducted (Fig. 3A and 3B). Both analyses showed that the branched network formed by TAC-2 Wnt-1 cells grown under control conditions was comparable to that found for HGF-treated TAC-2 LacZ cultures. When TAC-2 Wnt-1 cells are grown in the presence of HGF, both cord length and number of branch points is significantly greater than the combined values for TAC-2 Wnt-1 cells without HGF and TAC-2 LacZ cells grown with HGF (Fig. 3A and 3B). Thus, Wnt-1

and HGF act in a cooperative fashion to induce branching morphogenesis of TAC-2 cells.

To determine whether the effects of Wnt-1 on TAC-2 cell branching morphogenesis are due to effects on the growth characteristics of TAC-2 cells we compared the growth of the TAC-2 cell lines generated. TAC-2 cells were plated at different densities on collagen coated dishes, either in the presence or absence of HGF, and viable cell numbers were determined either two or six days after plating. No significant differences in cell number were found between control TAC-2, TAC-2 LacZ, or TAC-2 Wnt-1 cell lines grown in either the presence or absence of HGF (data not shown). When grown under these conditions, TAC-2 Wnt-1 cells and control TAC-2 cells both displayed contact inhibition at confluence and had similar morphological characteristics. Hence, the effects of Wnt-1 on TAC-2 branching morphogenesis are not correlated with mitogenic activity of Wnt-1 and are dependent on growth in three dimensional collagen gels. Identical results were obtained with TAC-2 cell lines that were programmed to express Wnt-1 using a SV40 based retroviral vector.

To further characterize morphogenic activities of Wnt-1 proteins, we analyzed TAC-2 cells induced to form cyst structures in collagen gel cultures. When TAC-2 LacZ cells are grown in collagen gels in the presence of hydrocortisone and cholera toxin, they form spheroidal cysts enclosing a widely patent lumen, as previously observed with non-transfected TAC-2 cells (47)(Fig. 4A). In contrast, under the same experimental conditions, TAC-2 Wnt-1 cells form branching structures (Fig. 4B). This clearly indicates that Wnt-1 expression modifies the spatial arrangement of TAC-2 cells and therefore has a morphogenic effect.

Notch4(int-3) inhibits TAC-2 cell branching morphogenesis. Notch activity in branching morphogenesis was evaluated by expressing an activated form of the Notch4 receptor, Notch4(int-3), in TAC-2 cells. TAC-2 cell lines programmed to express the Notch4(int-3) proteins were generated using the retroviral vector pLNCX and will be referred to as TAC-2 int-3. Notch4(int-3) was HA-epitope tagged at the carboxy terminus to allow detection of ectopically expressed proteins. Immunoblot analysis using anti-HA antibodies (Fig. 1) demonstrates that Notch4(int-3) proteins migrate with an approximately molecular weight of 60 kD, corresponding well to its predicted molecular weight. Notch4(int-3) expression is detected in extracts from TAC-2 int-3 cells and protein levels can be significantly increased by treatment with sodium butyrate (Fig. 1).

When TAC-2 int-3 cells are grown in collagen gels and incubated in the presence of either HGF or TGF-β2 (Fig 2H and 2I), cell colonies no longer form elongated cords like control cultures (Fig 2B and 2C). Instead, HGF- or TGF-β2-treated TAC-2 int-3 cell colonies form small aggregates or structures with rudimentary branches which are similar in appearance to those formed by either TAC-2 LacZ or TAC-2 int-3 colonies grown in the absence of HGF or TGF-β2 (Fig 2A and 2G). An identical phenotype was observed in at least three independently produced cell lines, including TAC-2 cells programmed to express a non-epitope tagged *Notch4(int-3)* cDNA or a HA epitope tagged *Notch4(int-3)* cDNA transcribed from a SV40 based retroviral vector (data not shown). Interestingly, we found that a smaller percentage of TAC-2 int-3 cells give rise to colonies in collagen gels with respect to TAC-2 LacZ cells (230±32 colonies/cm2 in TAC-2 int-3 cells versus 795±114 colonies/cm2 in TAC-2 LacZ cells), which suggests that Notch4(int-3) expression reduces plating (colony formation) efficiency in collagen gels. Accordingly, to avoid overestimating the inhibition of HGF-induced cord elongation in TAC-2 int-3 cells, the quantitative analysis of cord length and branching was carried out on a per colony basis, rather than on a per field basis (see Materials and

Methods). This analysis demonstrated that, despite the fact that colonies formed by TAC-2 int-3 cells are slightly more elongated and branched than those formed by TAC-2 LacZ cells, their morphogenic response to HGF is markedly decreased (Fig. 3C and 3D).

We analyzed the growth characteristics of the TAC-2 int-3 cell line, as described for TAC-2 Wnt-1 cells. TAC-2 int-3 cells plated on collagen coated dishes, either in the presence or absence of HGF, displayed no significant differences in cell number, morphology, or growth post-confluence when compared with TAC-2 controls (data not shown). Identical results were obtained with TAC-2 cell lines programmed to express Notch4(int-3) using a SV40 based retroviral vector (data not shown). Hence, the effects of Notch4(int-3) on TAC-2 branching morphogenesis are not correlated to changes in the growth properties in the cells.

The carboxy terminus of the Notch4(int-3) is not required for activity. The Notch4(int-3) oncoprotein, has most of the extracellular domain of Notch4 deleted and consists of the transmembrane and intracellular domains. To investigate which region(s) of Notch4(int-3) proteins are required and sufficient for activity, a series of Notch4(int-3) deletion mutants were generated (schematized in Fig. 5A). Four Notch4(int-3) deletion mutants were made and designated ΔNT (deletion of the amino terminal domain), ΔCDC (deletion of cdc10 repeat domain), ΔCT (deletion of the carboxy terminal domain) and ΔNTΔCT (N-terminal and C-terminal deletion) (Fig. 5A). All four mutant int-2 cDNAs were HA-epitope tagged at their carboxy termini and TAC-2 cell lines programmed to express each deletion mutant were generated using the retroviral vector pLNCX. Immunoblot analysis using anti-HA monoclonal antibodies demonstrated expression of Notch4(int-3) deletion proteins of appropriate molecular weight in each respective cell line; this expression can be increased by sodium butyrate treatment (Fig. 5B). The ΔNTΔCT Notch4(int-3) deletion protein with predicted molecular weight of 25 kD co-migrates with a non specific anti-HA antibody

background band, but is clearly visible over background when these TAC-2 cells are treated with sodium butyrate (Fig. 5B). TAC-2 cell lines expressing the four different Notch4(int-3) deletion mutants were grown in collagen gels as described above, and the ability of each Notch4(int-3) deletion mutant to inhibit HGF induced branching morphogenesis of TAC-2 cells was analyzed. As shown in Fig. 6, TAC-2 cells expressing either ΔNT (Fig. 6A and 6B), ΔCDC (Fig. 6C and 6D) or ΔNTΔCT (Fig. 6G and 6H) are responsive to HGF induced branching morphogenesis. In contrast, when grown in the presence of HGF, ΔCT expressing TAC-2 cells (Fig. 6E and 6F) display an identical phenotype as the TAC-2 int-3 cells. The carboxy terminus of the Notch4(int-3) is not required for Notch-mediated inhibition of TAC-2 branching morphogenesis. Thus, in this assay the activity of the Notch4(int-3) oncoprotein can be conferred by the amino terminus and cdc10 repeats.

Branching morphogenesis in cells co-expressing Wnt-1 and Notch4(int-3) oncoproteins. The activation of the Wnt-1 and Notch signaling pathways resulted in opposite effects on HGF- or TGF-β2-induced branching morphogenesis of TAC-2 cells. To explore the epistatic interactions between these two signaling pathways we investigated the effect of simultaneous expression of both Wnt-1 and Notch4(int-3) proteins on TAC-2 branching morphogenesis. The above described TAC-2 LacZ and TAC-2 int-3 cell lines, which were generated with the pLNCX expression vector, were now also programmed to express Wnt-1 using the retroviral vector pLHTCX. This vector drives gene expression from the CMV promoter and contains the hygromycin resistance gene. In this fashion, four additional TAC-2 cell lines were generated that were named TAC-2 LacZ/ctr, TAC-2 LacZ/Wnt-1, TAC-2 int-3/ctr and TAC-2 int-3/Wnt-1 (where ctr denotes control empty pLHTCX vector). To determine appropriate protein expression in each of these four cell lines, immunoblot analysis showed Notch4(int-3) and Wnt-1 proteins were expressed as expected and at levels similar to

those previously found to confer activity (data not shown). Each of the four cell lines were grown in collagen gels to determine their ability to undergo HGF- or TGF-β2-induced branching morphogenesis (Fig. 7), this assay was repeated three times with similar results. Doubly infected control cells TAC-2 LacZ/ctr (Fig. 7A, B, C) remained responsive to both HGF and TGF-β demonstrating that hygromycin and neomycin selection did not affect the phenotype of the TAC-2 cell lines. As observed previously for TAC-2 Wnt-1 cells, TAC-2 LacZ cells that are now programmed to express Wnt-1 (Fig. 7D, E, F) form small colonies that undergo modest branching even in the absence of HGF or TGF-β2; these cells form extensive elongated branches when grown in the presence of HGF or TGF-β2. The activity found for Notch4(int-3), that is the inhibition of HGF- and TGF-β-induced branching morphogenesis, was also found in the TAC-2 int-3/ctr cell line (Fig. 7G, H, I). Wnt-1 and Notch4(int-3) co-expressing cells, TAC-2 int-3/Wnt-1, are able to form colonies displaying branching and elongation and have an appearance similar to that of TAC-2/LacZ/Wnt-1 cells (Fig. 7J). An examination of several fields reveal that TAC-2 int-3/Wnt-1 cells displayed increased responses when treated with either HGF or TGF-β, thus these cells now regain responsiveness to these factors (Fig. 7K, L). Our results indicate that Notch activation attenuates responsiveness of TAC-2 cells to both HGF and TGF-β and that Wnt-1 can override the Notch activity in TAC-2 cells.

DISCUSSION

In this study, we have detailed a regulatory hierarchy involved in the branching morphogenesis of mammary epithelial cells. This regulation includes four distinct signaling pathways; the Wnt, Notch, HGF, and TGF-β signaling cascades. Using an in vitro model that reflects the branching morphogenesis exhibited during mammary gland development we have assessed the potential interactions between several different signaling pathways. This approach has allowed us to establish the epistatic relationships between these pathways. One remarkable feature of the regulation of branching morphogenesis we describe is its similarity to the regulatory pathways leading to morphogenic events during Drosophila development.

Wnt proteins as branching morphogens in the mammary gland. Formation of branching cords is induced in collagen gel cultures of TAC-2 cells by the addition of either HGF or low concentrations of TGF-β. Wnt-1 proteins induce moderate branching and elongation of TAC-2 cell tubules in the absence of added HGF or TGF-β2. The extent of Wnt-1 induced branching morphogenesis of TAC-2 cells is comparable to the induction by either HGF or TGF-β2. Our evidence suggests that Wnt-1 acts as a morphogen in this capacity. First, Wnt-1 induces a change in a morphogenic event, the formation of branched epithelial tubules or cords. Second, Wnt-1 does not appear on its own to alter the growth properties of the TAC-2 cells. Finally, Wnt-1 can induce branching in an environment where cysts typically form; that is, in hydrocortisone and cholera toxin treated cultures. Such cultures form spheroidal cysts enclosing a widely patent lumen. In this environment, peptide growth factors that display mitogenic and not morphogenic properties would increase the size of the cyst but the spheroidal structure would be maintained. In contrast, Wnt-1 alters the morphogenic behavior in such a way that new branch points are formed and the structures take on a tubular morphology.

Several Wnt proteins are expressed in the mammary gland during periods of morphological changes of the ductal epithelium (16, 50). Ectopic expression of Wnt-1 *in vivo* suggests a role for Wnt proteins in cell proliferation during mammary gland development; however, morphogenic changes also occur in response to Wnt-1. It is has been proposed that the Wnt-1 expression mimics the activity of an endogenous mammary gland Wnt proteins. A transgenic line driving expression of *Wnt*-1 to the mammary gland displays a hyperplastic phenotype, indicative of increased proliferation (48). In addition, both virgin females and males display a marked increase in the number of terminal branches, and in fact resemble the hormonally stimulated glands normally observed in pregnant animals. Tissue reconstitution experiments in which *Wnt*-1 is ectopically expressed in mammary epithelium also result in a hyperplastic gland where duct epithelium show abundant fine side-branches, suggesting that Wnt-1 may instruct the epithelium to form branches (13). This phenotype is most likely not simply a consequence of proliferation as it is not seen with a variety of other oncogenic proteins which when ectopically expressed in the mammary gland induce hyperplasia without increasing branching (12).

The Wnt signal transduction pathway is mediated in part through β-catenin, a protein associated with cadherins, and which is necessary for the adhesive functions of adherens junctions (29). Wnt-1 signaling results in stabilization of the cytoplasmic pool of β-catenin, which then can associate with downstream targets in the cytoplasm to transduce signals that lead to regulation of target gene expression (30, 31). TAC-2 cells programmed to express Wnt-1 in fact display increased levels of cytosolic β-catenin, when compared to TAC-2 LacZ cells (our unpublished data). This stabilization may be regulated by the phosphorylation of β-catenin on serine/threonine residues, possibly by glycogen synthase kinase 3 (GSK-3) (52). Recent evidence has demonstrated the importance of β-catenin/cadherin interactions in regulating cell adhesion, cell migration and epithelial phenotype in embryonic development (19). The activation of β-catenin

by Wnt-1 induced signaling may result in changes of the adhesive and migratory characteristics of mammary epithelial cells and consequently affect ductal morphogenesis of TAC-2 cells.

Cooperative interactions between Wnt-1 and HGF or TGF-β. In response to the combined effects of Wnt-1 and HGF, TAC-2 cells form a network of elongated and branching tubules that is far more extensive than the branching cords observed when TAC-2 cells are grown in the presence of Wnt-1 or HGF singularly. We propose that the combined effect of HGF addition and Wnt-1 expression is not the result of the sum of their independent activities on branching morphogenesis, but that Wnt signaling synergizes with the HGF/c-met tyrosine kinase pathway. The possibility that Wnt proteins cooperate in vivo with the HGF/c-met pathway in the regulation of mammary morphogenesis is supported by the overlapping temporal patterns of *Wnt* genes and HGF/c-met expression (16, 36, 50).

One potential area where these two signaling pathways could converge might be through their effects on the catenin and cadherin proteins. The cooperation between Wnt-1 and HGF may be explained by their combined activation of β-catenin. β-catenin has been detected in a complex with the EGF receptor and can be phosphorylated in response to EGF and HGF (22, 43). In addition, the Ras pathway is essential for the biological activity induced by HGF/c-met (20) and β-catenin has been demonstrated to be a substrate for tyrosine kinases and to become tyrosine phosphorylated in cells expressing activated Src and Ras (4). Another catenin-like protein, p120, which was identified as a substrate of Src and several receptor tyrosine kinases, interacts with the cadherin-β-catenin complex and may participate in regulating the adhesive function of cadherins (10). EGF is also able to stimulate branching morphogenesis of TAC-2 cells, although not to the same extent as HGF, whereas NGF, bFGF, IGF-II and KGF can not (47). These activities correlate with the reported phosphorylation of β-catenin by the

EGF and HGF signal transduction pathways (43). It is yet unclear how tyrosine phosphorylation of β-catenin might regulate its activity. Tyrosine phosphorylated β-catenin is found in a detergent soluble pool (22, 26), which may reflect specific phosphorylation of a free pool of β-catenin. Since both Wnt-1 and HGF signaling can converge on β-catenin, it is therefore possible that the observed cooperation between HGF and Wnt-1 is due to their combined action on β-catenin activity.

Wnt-1, HGF, and TGF-β could induce branching morphogenesis by regulating the adhesive and migratory properties of TAC-2 cells through modulation of extracellular matrix components and their interaction with their receptors. Since HGF has been demonstrated to decrease adhesion of TAC-2 cells to collagen and to enhance the deposition of type IV collagen it is also possible that the observed cooperation between HGF and Wnt-1 is due to their combined effect on cell-substrate adhesion. TGF-β signaling involves receptors with serine/threonine kinase activity which are known to regulate the synthesis and degradation of extracellular matrix molecules and to induce matrix organization. Induction of branching morphogenesis by TGF-β could be mediated by a remodeling of extracellular matrix components and cell-substrate interactions. The Wnt signal transduction pathway may also regulate cell-substrate interactions, and the combined activity of both Wnt-1 and TGF-β may explain their cooperative activities on the branching morphogenesis of TAC-2 cells.

In *Drosophila*, wingless (*wg*) and the TGF-β homologue *Decapentaplegic* (*Dpp*) have been shown in some cases to act in combination to regulate gene transcription during inductive events. In particular, Wg and Dpp have been shown to act in combination during limb development (6, 11) and to induce *Ultrabithorax* expression during endoderm induction (39). Wnt-1 and TGF-β signaling may similarly converge to affect gene transcription during branching morphogenesis in the mouse mammary gland.

Notch inhibits branching morphogenesis of mammary epithelial cells. We demonstrate that Notch activation inhibits both the HGF and TGF-β induced branching morphogenesis of TAC-2 mammary epithelial cells. The precise mechanism of this inhibition is unclear. Activation of Notch signaling has been demonstrated to inhibit or alter the cell fate commitment or differentiation of a variety of different cell types (1, 18). For instance, *C. elegans* Lin-12 controls cell fate decisions during gonadogenesis, *Drosophila Notch* acts to control cell fate during neuroblast and photoreceptor cell differentiation, an activated *Xenopus Notch* can affect epidermal and neural crest cell development, and an activated mouse *Notch*1 can control cell fate during myogenesis and neurogenesis of cultured mouse cells. Transgenic mice that use the MMTV viral promoter to express the Notch4(int-3) oncoprotein, the activated form of Notch4, display severely impaired mammary ductal growth (25). When *Notch4(int-3)* is expressed from the whey acidic protein promoter, whose expression is restricted to the secretory mammary epithelial cells, the differentiation of the secretory lobules of the transgenic animals is profoundly inhibited (15). These experiments demonstrate that *Notch4(int-3)*, like many other activated *Notch* genes, can act as a regulator of cell fate decisions in the mammary gland of mice. Little is known about the spatial and temporal pattern of *Notch* gene expression in the mammary gland, however, *Notch4* is expressed *in vivo* in the murine mammary gland (42, 45). *Notch* genes may thus regulate the cell fate decisions occurring during mammary gland development that lead to the branched epithelial strutcture of the gland.

We have found that Notch activation can affect the response of TAC-2 mammary epithelial cells to either HGF or TGF-β. Since HGF acts through a tyrosine kinase receptor and TGF-β acts through serine/threonine kinase receptors, the affects of Notch activation may involve more than specific inhibition of a particular signaling cascade. Notch may regulate the competency of TAC-2 cells to respond to several different factors, possibly by shifting TAC-2 cells to a fate that is not predisposed to undergoe

branching morphogenesis. This model would be consistent with the proposed activites of Notch proteins in several different organisms. Alternately, these signaling pathways may be controlled by Notch at a point at which they may converge to induce expression of genes important for branching morphogensis. Recently, the intracellular domain of LIN-12, a *C. elegans* Notch, has been demonstrated to associate with EMB-5, which encodes for a cytoplasmic protein containing a SH2 domain (24). This finding raises the possibility that the Notch signaling proteins may interact directly with those elicited by tyrosine kinase receptors, such as the HGF receptor (c-met).

We have demonstrated that the domain, carboxy terminal to the cdc10 repeats, of the Notch4(int-3) oncoprotein is not required for biological activity. However, the amino terminal domain and the cdc10 repeats are required for Notch4(int-3) activity. These findings are consistent with previous observed data for other *Notch* genes. The RAM23 domain which is localized between the transmembrane and cdc10 repeats has been demonstrated to be the binding site of CBF-1, a downstream and essential element in Notch signaling (23). Deletion of the amino terminal domain of Notch4(int-3), which contains the RAM23 domain, may eliminate binding to CBF-1, and hence destroy Notch4(int-3) activity. The region of the LIN-12 protein that includes the RAM-23 domain and cdc10 repeats appears to interact with another downstream and positive regulator, EMB5 (24). Point mutations and deletions within the cdc10 repeats result in loss of function of Notch proteins (17). Our data thus indicates that Notch4 might interact and be regulated through similar mechanisms.

Competing influences of Wnt and Notch signaling in branching morphogenesis. When TAC-2 cells are programmed to express both Wnt-1 and Notch4(int-3), the cells are able to undergo branching morphogenesis. In Wnt-1 and Notch4(int-3) coexpressing TAC-2 cells, branching morphogenesis can be increased by either HGF or TGF-β; that is, the cells regain responsiveness to these factors. The phenotype observed

in Wnt-1 and Notch4(int-3) coexpressing cells was similar to that of TAC-2 cells expressing only Wnt-1. The opposite biological activities of Wnt-1 and Notch4(int-3) observed in the TAC-2 cell assay correlate well with the mammary gland phenotype observed in Wnt-1 and Notch4(int-3) transgenic mice that ectopically express these proteins in the mammary gland (25). Although both oncogenes increase mammary tumor development, Wnt-1 stimulates a hyperplastic phenotype with increased ductal development whereas Notch4(int-3) inhibits ductal development.

Wnt-1 can override the Notch4(int-3)-mediated inhibition of branching morphogenesis providing the first evidence of interaction between these two signaling pathways in vertebrates. The dominance of Wnt-1 over activated Notch we have seen in murine cells parallels the functional relationship proposed for *Drosophila Wnt* (*wingless*) and *Notch* during *Drosophila* development (2). In this study, genetic analysis suggests a pathway convergence between wingless and Notch signaling resulting in opposing effects during patterning of the developing *Drosophila* wing. Activation of the wingless signal leads to regulation of Notch activity, possibly by *Drosophila* dishevelled, a cytoplasmic protein that is also a positive mediator in the Wnt-1 signal transduction pathway. Analysis using a yeast interaction trap system suggested that Dishevelled may physically associate with the intracellular domain of Notch. The antagonism between Wnt-1 and Notch4(int-3) seen in branching morphogenesis may also be mediated by common regulators of the two signaling pathways such as Dishevelled.

During mammary gland development, the growth and differentiation of the gland is regulated by mesenchymal-epithelial and epithelial-epithelial interactions. Cells often receive different signals simultaneously and must integrate them in order to take on the correct proliferative or differentiative response. Notch inhibition of ductal morphogenesis may be an early event in ductal morphogenesis. An attractive mechanism for overcoming Notch and allowing ductal morphogenesis to initiate or progress would be to activate the expression of a *Wnt* gene(s). Wnt could then serve

the dual function of suppressing Notch activity and initiating branching morphogenesis. Wnt signaling may then cooperate with other signaling pathways, such as those mediated by HGF and TGF-β, in order to complete branching morphogeneis. Our study thus has revealed complex interactions between the signal transduction pathways of Wnt, Notch, HGF and TGF-β, in regulating the branching morphogenesis of mammary epithelial cells.

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FIGURE LEGENDS

Figure 1

Immunoblot analysis on TAC-2 cell lysates using anti-HA antibodies. TAC-2 cells programmed to express either LacZ, Wnt-1 or int-3 were grown in the presence or absence of sodium butyrate. Wnt-1 and int-3 proteins of appropriate molecular weight can be detected in the respective TAC-2 cell lines, and the level of protein expression can be increased by sodium butyrate treatment.

Figure 2

TAC-2 cell ductal morphogenesis assay. TAC-2 cells programmed to express LacZ (A,B,C), Wnt-1HA (D,E,F), or int-3HA (G,H,I) were grown in collagen gels either in the absence of exogenous growth factor (A,D,G), in the presence of HGF (B,E,H), or TGF-β2 (C,F,I). HGF and TGF-β2 induce branching morphogenesis of TAC-2 LacZ cells (compare A to B or C). Wnt-1 expression in TAC-2 cells induces modest branching morphogenesis of TAC-2 cells in the absence of either HGF or TGF-β2 (compare D to A), and robust branching is observed when TAC-2 Wnt-1 cells are grown in the presence of either HGF or TGF-β2 (compare E to B, and to C). TAC-2 cells programmed to express int-3 fail to undergo branching morphogenesis when grown in the presence of either HGF or TGF-β2 (compare G to H or I, H to B, and I to C).

Figure 3

Wnt-1 and HGF have cooperative effects on branching morphogenesis of TAC-2 cells, while expression of int-3 inhibits HGF-induced branching morphogenesis. TAC-2 LacZ, TAC-2 Wnt-1 and TAC-2 int-3 cells were suspended in collagen gels at $5x10^3$ cells/ml (A and B) or $1x10^4$ cells/ml (C and D) and incubated with either control medium or 10ng/ml HGF for 7 days. In each of 3 separate experiments, at least three randomly

selected fields per condition were photographed. The total additive length of all cords in each field (A), the number of cord branch points per field (B), the total additive length of all cords in each individual colony (C), and the number of cord branch points per colony (D) was determined as described in Materials and Methods. Values are mean ± s.e.m.; n=3. Values for HGF are significantly ($P<0.001$) different when compared to controls (except for TAC-2 int-3 cells) and values are significantly different ($P<0.001$) when TAC-2 LacZ and TAC-2 Wnt-1 cell lines are compared. Similar results were obtained by evaluating cord length and branching per individual TAC-2 LacZ andTAC-2 Wnt-1 colony (data not shown).

Figure 4

Differential behavior of TAC-2 LacZ cells and TAC-2 Wnt-1 cells in hydrocortisone-supplemented cultures. Cells were suspended in collagen gels at $5x10^3$ cells/ml and incubated for 10 days with 1 μg/ml hydrocortisone and 50 ng/ml cholera toxin. Under these conditions, TAC-2 LacZ cells forn thick-walled spheroidal cysts enclosing a widely patent lumen (A), as previosly shown for untransfected cells. In marked contrast, TAC-2 Wnt-1 cells form short branched tubules (B). The three-dimensional structures illustrated in A and B are representative of the vast majority of colonies formed by TAC-2 LacZ and TAC-2 Wnt-1 cells, respectively. Magnification = 180x.

Figure 5

Schematic representation of int-3 deletion mutants (A) and immunoblot analysis on TAC-2 cell lysates using anti-HA antibodies (B). TAC-2 cells programmed to express either ΔNT, ΔCDC, ΔCT, and ΔNTΔCT were grown in the presence or absence of sodium butyrate. The int-3 deletion proteins are epitope tagged and deletion proteins of appropriate molecular weight can be detected in the respective TAC-2 cell lines, and the level of protein expression can be increased by sodium butyrate treatment.

Figure 6

TAC-2 cell ductal morphogenesis assay with int-3 mutants. TAC-2 cells programmed to express ΔNT (A,B), ΔCDC (C,D), ΔCT (E,F), and ΔNTΔCT (G,H) were grown in collagen gels either in the absence of exogenous growth factor (A,C,E,G), or in the presence of HGF (B,D,F,H). HGF induces branching morphogenesis of TAC-2 ΔNT cells (B), TAC-2 ΔCDC cells (D) and TAC-2 ΔNTΔCT cells (H) TAC-2 ΔCT cells fail to undergo branching morphogenesis when grown in the presence of either HGF (F).

Figure 7

TAC-2 cell ductal morphogenesis assay. TAC-2 cells programmed to express LacZ/ctr (A,B,C), LacZ/Wnt-1 (D,E,F), int-3/ctr (G,H,I), or int-3/Wnt-1 (J,K,L) were grown in collagen gels either in the absence of exogenous growth factor (A,D,G,J), in the presence of HGF (20 ng/ml)(B,E,H,K), or in the presence of TGF-β2 (50 pg/ml)(C,F,I,L). HGF and TGF-β2 induce branching morphogenesis of TAC-2 LacZ/ctr cells (compare A to B or C). Wnt-1 expression in TAC-2 cells induces modest branching morphogenesis of TAC-2 LacZ/Wnt-1 cells in the absence of either HGF or TGF-β2 (D), and robust branching is observed when TAC-2 LacZ/Wnt-1 cells are grown in the presence of either HGF (E) or TGF-β2 (F). TAC-2 cells programmed to express int-3/ctr fail to undergo branching morphogenesis when grown in the presence of either HGF (H) or TGF-β2 (I). TAC-2 cells programmed to express both int-3 and Wnt-1 undergo branching morphogenesis in the absence of exogenous growth factor (J), and form a robust branching network when grown in the presence of HGF (K) or TGF-β2 (L) in a similar manner when compared to TAC-2 cells programmed to express Wnt-1 solely (D,E,F).

Figure 1

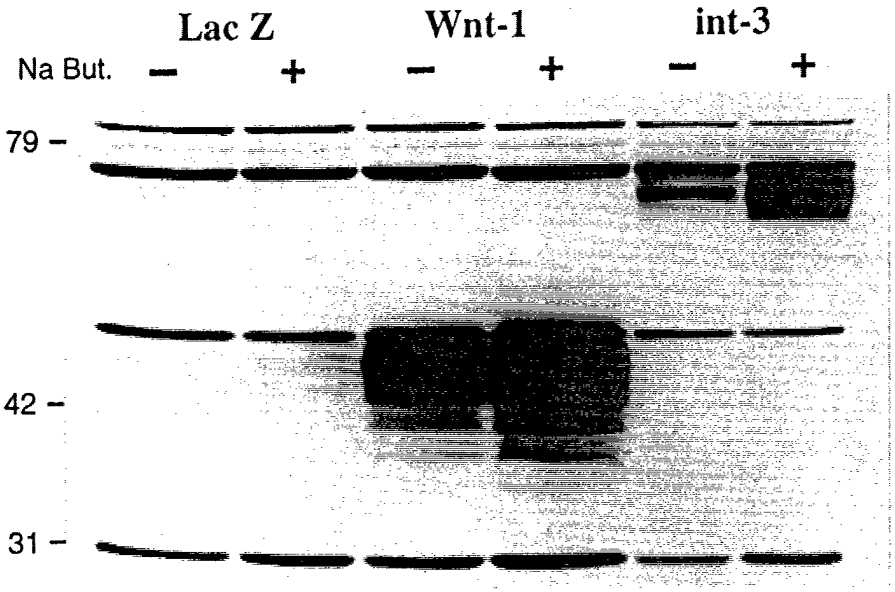

Figure 2

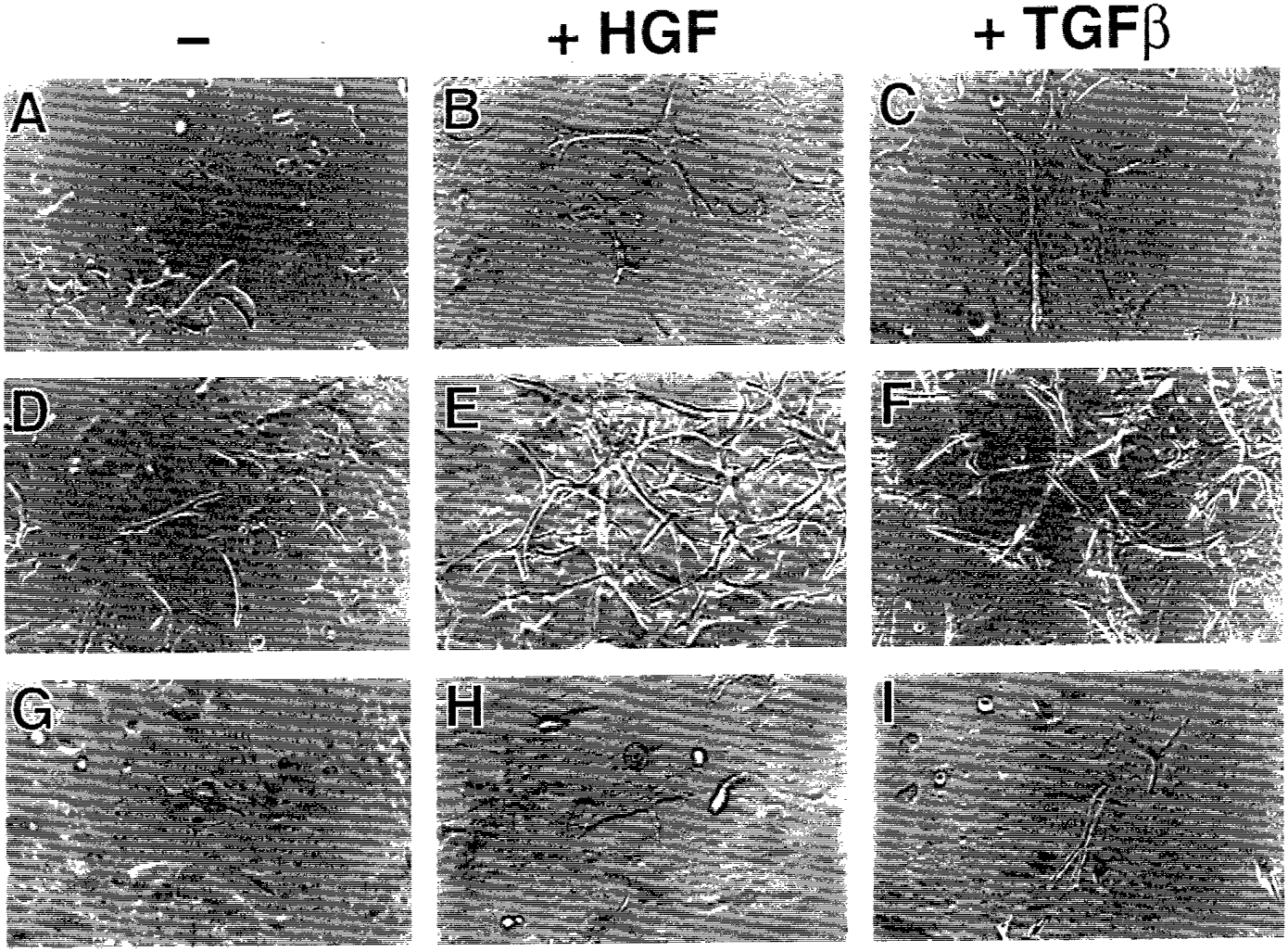

Figure 3

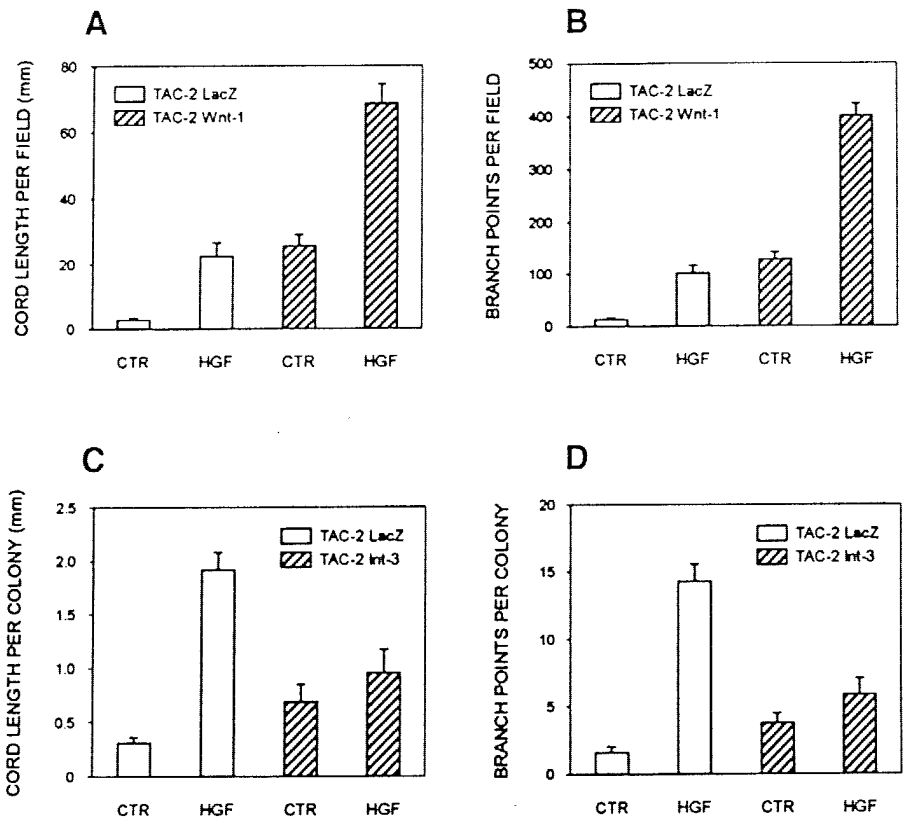
A
CORD LENGTH PER FIELD (mm)
0
20
40
60
80
TAC-2 LacZ
TAC-2 Wnt-1
CTR
HGF
CTR
HGF
B
BRANCH POINTS PER FIELD
0
100
200
300
400
500
TAC-2 LacZ
TAC-2 Wnt-1
CTR
HGF
CTR
HGF
C
CORD LENGTH PER COLONY (mm)
0
0.5
1.0
1.5
2.0
2.5
TAC-2 LacZ
TAC-2 Int-3
CTR
HGF
CTR
HGF
D
BRANCH POINTS PER COLONY
0
5
10
15
20
TAC-2 LacZ
TAC-2 Int-3
CTR
HGF
CTR
HGF

Figure 4

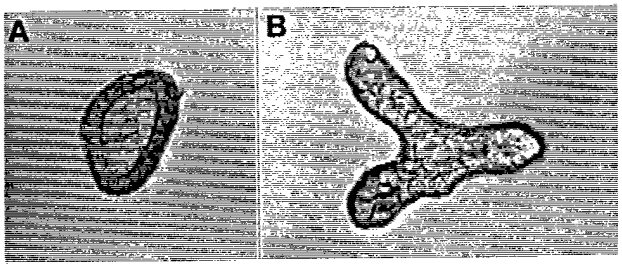

Figure 5

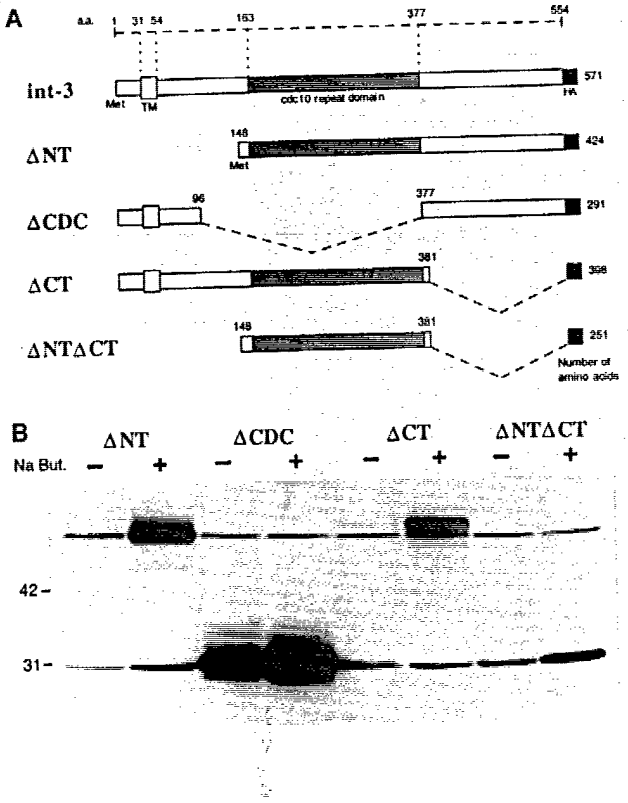
A
aa.
1
31
54
163
377
554
int-3
Met
TM
cdc10 repeat domain
HA
571
ΔNT
148
Met
424
ΔCDC
96
377
291
ΔCT
381
398
ΔNTΔCT
149
381
251
Number of amino acids
B
ΔNT
ΔCDC
ΔCT
ΔNTΔCT
Na But.
42-
31-

Figure 6

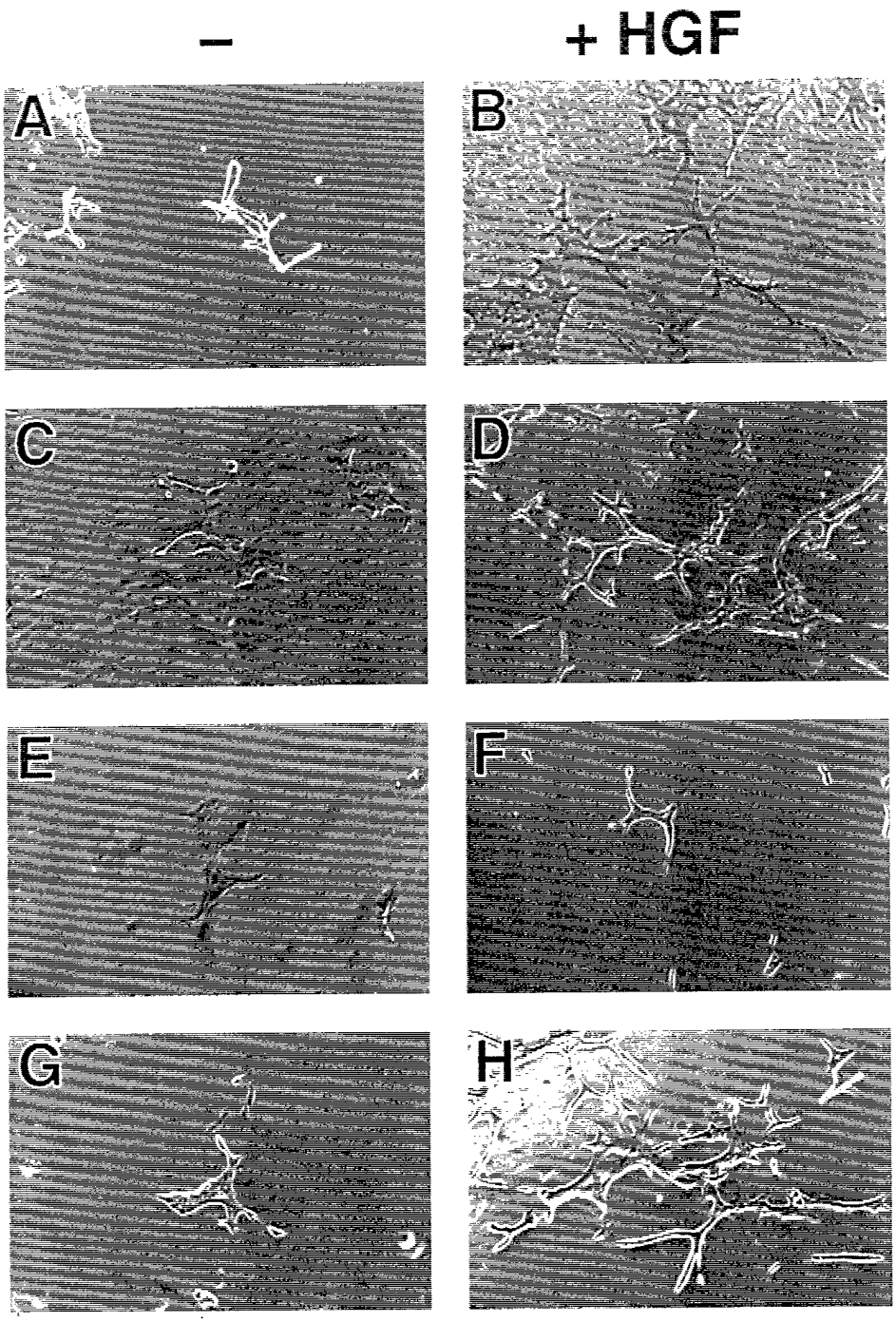

Figure 7