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GRANT NUMBER DAMD17-96-1-6112

TITLE: Protein Kinases in Mammary Gland Development and Carcinogenesis

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REPORT DATE: October 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

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DTIC QUALITY INSPECTED 2

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE October 1998	3. REPORT TYPE AND DATES COVERED Annual (15 Sep 97 - 14 Sep 98)	
4. TITLE AND SUBTITLE Protein Kinases in Mammary Gland Development and Carcinogenesis		5. FUNDING NUMBERS DAMD17-96-1-6112	
6. AUTHOR(S) Lewis Chodosh, M.D., Ph.D. Heather Perry Gardner			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Pennsylvania Philadelphia, Pennsylvania 19104		8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research Command Fort Detrick, Maryland 21702-5012		10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES			
12a. DISTRIBUTION / AVAILABILITY STATEMENT Distribution authorized to U.S. Government agencies only (proprietary information, Oct 98). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.		12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) In this report, we present evidence that two novel kinases, <i>Hunk</i> (hormonally upregulated <i>neu</i> -tumor associated kinase) and <i>Punc</i> (pregnancy upregulated novel CaM kinase) isolated in a screen to identify kinases involved in mammary gland differentiation and carcinogenesis may be playing a role in both processes. <i>Hunk</i> and <i>Punc</i> are both developmentally regulated with <i>Hunk</i> levels highest early in pregnancy and <i>Punc</i> expression peaking late in pregnancy. In addition, their punctate spatial expression patterns suggest that <i>Hunk</i> and <i>Punc</i> may be markers for mammary epithelial cell subtypes. Consistent with their developmentally regulated expression patterns, we provide initial data suggesting that both kinases, in the context of an MMTV transgenic animal, affect normal differentiation of the mammary gland. Data also suggests that these kinases may be playing a role in carcinogenesis. Both kinases display an oncogene-restricted expression pattern with <i>Punc</i> expressed exclusively in tumor cell lines derived from <i>int-2</i> and <i>c-myc</i> overexpressing glands and <i>Hunk</i> expression restricted to <i>Neu</i> and <i>H-ras</i> transformed cell lines. Initial observations in a panel of human tumor cell lines, reveals overexpression of <i>Hunk</i> in cell lines of different tumor types, including several breast cancer lines. Taken together, we hypothesize that <i>Hunk</i> and <i>Punc</i> may be kinases in signal transduction cascades leading to both the development and possibly the transformation of the mammary epithelium.			
14. SUBJECT TERMS Breast Cancer kinases, transgenic animals, mammary gland development		15. NUMBER OF PAGES 30	
		16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Limited

1 9990216181

FOREWORD

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Stephen Perry Johnson 10/13/98

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INTRODUCTION:

Protein kinases are well defined regulatory molecules involved in almost every cellular process including cell motility, metabolism, proliferation and differentiation. Aberrant expression or mutations in kinases has been shown to drastically alter vital cell functions. In the breast, there are many examples of protein kinases acting as growth factor receptors or mediators of signal transduction cascades leading to differentiation in normal breast tissue. Carcinogenic transformation often results from mutations or aberrant expression of molecules such as c-erbB2/HER2/neu, the EGF receptor, the FGF receptor family, and Met(1-4).¹⁻⁴ The aforementioned kinases are examples of the direct relationship between normal development and carcinogenesis. As such, the protein kinase family is a prime candidate family of molecules for studying the processes of development and carcinogenesis and their relationship to each other.

In the mammary gland, development and carcinogenesis are fundamentally related. For example, a woman's reproductive history is directly related to her risk for developing breast cancer. Having a child early in life (under 30 years old) results in an overall decreased lifetime risk for developing breast cancer when compared to a nulliparous woman and having a child later in life actually increases a woman's lifetime risk for developing breast cancer⁵⁻⁷. Other events which are related to the differentiation of the mammary gland such as early menarche or late menopause also affect a woman's risk for developing breast cancer in her lifetime^{8,9}. Therefore, the mammary gland is an excellent model system for studying the relationship between development and cancer.

PRELIMINARY STUDIES:

The very fact that many tyrosine kinases can function as oncogenes in the breast suggests that phosphorylation events play critical roles in the regulation of cell growth and differentiation in the mammary gland. Therefore, we initiated and carried out a screen designed to both identify protein kinases expressed in the mammary gland and to determine which kinases may be playing a role in development and carcinogenesis based upon their expression patterns¹⁰. As a first step in this study, we first identified protein kinases expressed in the mammary gland by employing degenerate PCR techniques to create a protein kinase catalytic domain library. Degenerate oligonucleotides designed to recognize nucleotide sequences corresponding to highly conserved amino acids in kinase catalytic subdomains VI and IX were used to amplify 200 bp catalytic domain fragments (Fig. 1A)¹¹. To increase the likelihood of amplifying a wide variety of kinases, first strand template cDNA was generated from mammary gland tissue taken at various developmental stages and from mammary epithelial tumor cell lines from different mouse models of breast cancer. To identify protein kinases amplified in the library, 1500 transformants were screened by sequencing and colony lift hybridization. From this screen 41 kinases were

identified including 33 tyrosine (18 RTK, 15 non-RTK) and 8 serine/threonine kinases, 3 of which appear to be novel(12) (Fig. 2)¹².

Based upon sequence analysis within the highly conserved catalytic region spanning subdomains VI through IX, we were able to identify the novel kinases as serine/threonine kinases (Fig. 1B). The subjects of this report, A32 (*Hunk*) and I43 (*Punc*), both contain invariant amino acids common to all protein kinases. In addition, residues conserved between all serine/threonine kinases, as opposed to tyrosine kinases as shown in Fig. 1A, are conserved within A32 and I43¹³. Homology by blast sequence analysis also confirms that both A32 and I43 share homology with known serine/threonine kinases. A32 appears to be a novel member of the SNF1 family and I43 shares close homology with the calcium-calmodulin (CaM) dependent family of serine/threonine kinases.

In order to determine which kinases may be playing a role in development, we screened for kinases with developmentally regulated mRNA expression patterns. Although unregulated mRNA expression does not rule out the possibility that a molecule may be functioning during development, upregulation of a kinase during a distinct developmental timepoint suggests a role during that particular developmental stage. To address steady state mRNA levels, Northern hybridization analysis was used to examine the expression of each kinase during mammary gland development. Mammary glands from mice were harvested at different timepoints during mammary gland development including puberty, adult virgin, early, mid and late pregnancy, lactation and early, mid and late regression. Six duplicate Poly(A)⁺ Northern blots were produced and then hybridized with 200 bp kinase catalytic domain probes made from the fragments isolated in the degenerate PCR screen. Phosphorimager analysis was used to quantitate expression levels for each kinase and to control for dilutional effects of milk protein production during lactation, kinase expression was normalized to actin expression levels. As shown in figure 3, most kinases were regulated at the RNA level during development and kinases could be placed into groups with similar expression patterns. A few kinases such as *Tie1* and *Ron* had unique expression patterns with highest expression in male or prepubescent glands. Regulation during pregnancy was observed fairly often, however, almost every profile was unique in that some kinases came up early in pregnancy (*SK2* and *Abl*), some later (*Hck* and *Src*) and the expression of a couple of kinases peaked during lactation when normalized to actin (*Akt* and *Fes*). Other kinases were downregulated during pregnancy when compared to an age-matched virgin gland (*FgfR1*, *Axl*, and *Jak1*) and a few show highest expression during early regression (*Tyro3*, *Mkl1*, and *Ctk*) suggesting that these kinases may be playing a role in the restructuring of the gland following lactation. When compared to a proliferation marker such as *cyclin A* (data not shown) a few kinases such as *Ebk*, *Tyro 10* and *Hek2* had a pattern that seemed to correlate with proliferation with a peak in puberty, early pregnancy and a slight upregulation in regression. Compared to actin and GAPDH profiles, unregulated expression was also

observed for some kinases (data not shown). Although many changes in expression are subtle (less than 2-3 fold), several kinases show dramatic changes in expression during mammary gland development.

Two of the kinases that show a more dramatic change during postnatal development in the mammary gland were the novel kinases, *Hunk* and *Punc*. Due to the low expression levels of these molecules, we turned to a more sensitive assay, ribonuclease protection to assess changes of *Hunk* and *Punc* during mammary gland development. Antisense probes were generated to the catalytic domain of *Hunk* and to the 3' UTR of *Punc* and then hybridized overnight with total RNA isolated from mammary gland of mice at different developmental timepoints. As shown in figure 4, *Hunk* levels were markedly upregulated early in pregnancy, a period characterized by rapid alveolar proliferation and differentiation. *Punc* was also upregulated in pregnancy, but in contrast to the rapid downregulation of *Hunk* by midpregnancy, expression of *Punc* continued to rise until it peaked late in pregnancy. When normalized to actin, *Punc* expression drops back to basal levels during lactation. This suggests that *Hunk* may be playing a role in early differentiation of the mammary gland during pregnancy and *Punc* may be functioning at a later developmental stage during pregnancy. Since many kinases including *Hunk* and *Punc* show developmentally regulated expression patterns, this screen alone was not sufficient to focus our efforts on kinases which may be playing a role in mammary gland development. As such, we also screened for kinases with interesting spatial expression patterns within the mammary gland and for kinases overexpressed in breast tumor cell lines which may suggest a role in carcinogenesis.

In order to define the spatial expression patterns within the mammary gland, the expression of a selection of kinases was assayed by *in situ* hybridization analysis (data not shown)¹⁴. Expression patterns within this select group varied from exclusively epithelial expression, stromal expression, expression in distinct structures within the epithelium (TEBs and developing alveoli) to ubiquitous expression. Although we detected expression in specific structures such as in alveolar buds and not adjacent epithelial duct, the expression pattern was fairly uniform between cells within an expressing structure for every kinase investigated except for the novel kinases, *Hunk* and *Punc* which have highly variable expression levels between adjacent epithelial cells (Fig. 5). Consistent with our RNase protection data, *Hunk* expression is higher at day 7 of pregnancy compared to day 20. The upregulation observed in total RNA by RNase protection appears to be the result of dramatic upregulation with a subset of epithelial cells within the gland. The same is true for *Punc* expression. Highest expression of *Punc* is observed in the mammary gland just prior to parturition with a few cells expressing at very high levels. This strikingly punctate expression pattern was not observed for other kinases examined and suggests that *Hunk* and *Punc* are differentially expressed in distinct epithelial cell subtypes in the breast that are differentially regulated during pregnancy.

Transgenic mouse models of human breast cancer have been generated where commonly amplified and overexpressed genes such as *neu*, *H-ras*, *int-2* and *c-myc* are overexpressed in the murine mammary gland using the MMTV promoter¹⁵⁻¹⁷. Transgenic mice overexpressing these oncogenes develop mammary tumors which have been described to mimic human breast cancers with the same initiating events. Patients with *Neu* amplification and overexpression respond differently to hormonal treatment than do patients with *c-myc* amplification and overexpression indicating that *myc* and *neu* initiated tumors are fundamentally different. Another indication that the biology of the tumors is somehow different is the observation that tumors arising in *Neu* transgenic mice are morphologically and histologically distinct from the *c-myc* initiated tumors^{18, 19}. For example, *Neu* overexpressing tumors have been characterized as being less differentiated than *c-myc* transformed tumors. As such, expression of protein kinases in *c-myc* and *Neu* initiated tumor cell lines may shed some light on the signal transduction pathways activated by or the cell types transformed by these oncogenes.

As a second screen designed to identify kinases potentially involved in breast cancer, we determined the expression of each protein kinase in a panel of mammary epithelial tumor cell lines derived from independent mammary adenocarcinomas arising in transgenic mice expressing either the *neu*, *c-myc*, *H-ras*, or *int-2* oncogenes. Expression patterns of the protein kinases varied from no expression to ubiquitous expression and surprisingly, many kinases were expressed in some tumor cell lines, but not in others (Figure 6A). Of the 41 kinases examined, only two showed an expression pattern that correlated with transformation by a particular oncogene. As shown in Figure 6B, *Hunk* is expressed in 8/8 *neu* and *H-ras* initiated tumors with low levels of expression in a non-transformed mammary tumor cell line, NMuMG. *Punc* also exhibited an oncogene-restricted expression pattern which was opposite of that observed for *Hunk*. *Punc* expression is only detected in tumor cell lines derived from *int-2* and *c-myc* transgenic animals. Consistent with their epithelial expression pattern by *in situ* analysis, neither kinase was expressed in the fibroblast cell line, 3T3, suggesting that both kinases may be epithelial specific. Their mutually exclusive, oncogene-restricted expression patterns suggest that *Hunk* and *Punc* are either downstream of their respective oncogenes or that possibly these kinases are markers for epithelial cell subsets that are preferentially transformed by a particular oncogenes. Experiments designed to address these questions are addressed in objective III.

Taken together, our initial results suggest that we have identified two novel serine/threonine kinases that may be playing a role in mammary gland development and/or carcinogenesis. *Hunk* and *Punc* are both developmentally regulated with *Hunk* levels highest early in pregnancy and *Punc* expression peaking late in pregnancy. These kinases are also expressed in mutually exclusive subsets of mammary epithelial cell lines with defined initiating events which are relevant to human cancers. Tumor cell lines from *H-ras* and *neu* transgenic animals express *Hunk*, whereas *c-myc* and *int-2* initiated tumor cell lines express *Punc*. In

addition, their punctate spatial expression patterns suggest that *Hunk* and *Punc* may be markers for mammary epithelial cell subtypes. As such, we initiated further investigation of the role of *Hunk* and *Punc* in mammary gland development and carcinogenesis. In addition to the data presented above, we summarize below our progress towards completion of the objectives outlined in our proposal and statement of work.

BODY:

Technical Objective I: Isolate and characterize full-length murine cDNA clones for novel protein kinases.

Task 1: Months 1-12: Isolate full-length cDNA clones for novel kinases

In order to obtain full-length cDNA clones for *Hunk* and *Punc*, cDNA libraries were screened, cDNA clones were obtained and sequencing was performed on both the plus and minus cDNA strands. Full-length cDNA clones have been isolated from either a day 14 mouse embryo library (*Hunk*) or a murine brain library (*Punc*). Consistent with the full-length transcript as determined by Northern analysis (Fig.7A), a 5024 nucleotide cDNA has been isolated for *Hunk*. Although no stop codon is observed upstream of the putative translational initiation codon in this clone, several lines of evidence, including high homology in the ORF with a recently isolated human cDNA clone, suggests that we have isolated the entire coding region of *Hunk*. The cDNA clone for *Hunk* is extremely GC-rich at the 5' end, greater than 80% over the first 173 nucleotides. This suggests tight translational control²⁰. There is a putative initiation codon at nucleotide 72 and an inframe stop at nucleotide 2216 resulting in an open reading frame of 714 amino acids and a 3kb 3' untranslated region. *In vitro* translation of *Hunk* results in a protein product consistent with the predicted molecular weight of 79.6 KDa (Fig.7B).

Three full-length *Punc* cDNA clones ranging from 1455 to 1510 nucleotides were isolated from a murine brain cDNA library. These clones differed only in their 5' UTR sequence the significance of which has not been determined. Northern analysis reveals an mRNA transcript of approximately 1.5 kb, consistent with the sizes of the cDNA clones isolated (Fig 7A). Conceptual translation of the 343 amino acid open reading frame predicts a protein product of 38.6 KDa, similar to the protein produce size observed following *in vitro* translation of *Punc* cDNA (Fig. 7B).

Task 2: months 1-12: Characterize structure of protein kinases.

Hunk cDNA codes for a protein of 714 amino acids with a kinase catalytic domain spanning amino acids 58 through 319 (Fig.8). This domain contains all residues conserved between serine/threonine kinases. In addition, a recently described SNF1 homology domain (SNH) is found C-terminal to the catalytic domain²¹. The SNH domain is described to be a region conserved between SNF1 family members, but the function is unknown. The C-terminal region of *Hunk* does not appear to share significant homology with other known molecules and protein analysis software has not been informative in identifying conserved motifs for *Hunk* outside of the kinase and SNH domains.

The kinase catalytic domain of *Punc* stretches from amino acid 13 to 272 and contains all amino acids conserved within serine/threonine kinases²² (Fig. 8). Just C-terminal to the kinase domain is a regulatory domain common to members of the CaM kinase family²³ (amino acids 273-316). This domain is comprised of an N-terminal autoinhibitory domain overlapping with a calcium/calmodulin (CaM) binding domain. In the presence of calcium, calmodulin binds to this regulatory domain and changes the conformation of CaM kinases so that the autoinhibitory domain is no longer able to bind the kinase domain thus relieving inhibition. Alignment of *Punc* with other CaM kinases also reveals lower homology in the unique C-terminal domain with *CaMKI* and *CaMKIV*. This region has been described to be involved in oligomerization and subcellular localization of the protein²³. Homology in this region suggests that *Punc*, *CaMKI* and *CaMKIV* may share similar binding partners or may have similar subcellular localization. Although *CaMKI* appears to be cytoplasmic²⁴, *CaMKIV* is localized to the nucleus and has been implicated in transcriptional regulation²⁵. As such, the subcellular localization of *Punc* may be informative in elucidating its function.

In order to determine the relationship between *Hunk*, *Punc* and other serine/threonine kinases, we used multiple sequence alignment software to align *Hunk* and *Punc* with closely related proteins. Sequence from kinase catalytic subdomains I through XII were used in this analysis. Interestingly, *Hunk* and *Punc* are both related to molecules that are within the same group of serine/threonine kinases, the calcium regulated kinases¹³. This group of the kinase family tree contains members of both the SNF1 family and the calcium/calmodulin dependent (CaM) family of serine/threonine protein kinases. Two more distantly related kinases, *PKC α* and *MAPK2*, were included in the analysis to emphasize the close relationship between the SNF1 and CaM kinase families.

Sequence analysis of *Punc* confirms homology to the CaM kinase family. These calcium/calmodulin dependent protein kinases include smooth muscle myosin light chain kinase (MLCK), DAPK, and the well described CaMKII subfamily, otherwise known as the multifunctional CaM kinases²⁶. *Punc* is most closely related to *CaMKI*, although the homology between *Punc* and *CaMKI* is lower than homology between all four members of the CaMKII subfamily (data not shown). Within the kinase domain, *Punc* is 70% identical to *CaMKI* and

approximately 50% identical to *CaMK II* and *IV*. Homology is also high in the regulatory domain which consists of the autoinhibitory and calmodulin binding regions. Structural analysis also reveals the presence of two unique regions of *Punc* at both the extreme N- and C-terminus.

Although *Hunk* is most closely related to Snf1 kinase family, it does not fall within one of the subfamilies within this group. Two main branches exist within the Snf1 family. One contains *SNF1* and its plant homologues, *NPK5*, *AKin10*, *BKIN12*, and *Rkin1* as well as the *SNF1* mammalian homolog *AMPK*. *SNF1* itself is required for glucose metabolism derepression in yeast by allowing genes to be expressed in the presence of glucose. Other *SNF1* family members in plants have been implicated in cellular response to stress, either nutritional or environmental, and it has been suggested that they are responding to decreased AMP/ATP levels²⁷. Interestingly, the plant proteins have been shown to be expressed in distinct structures within the plant²⁸. The other main branch of the *SNF1* family contains the newly emerging field of *SNF1* kinases involved in differentiation and cell cycle control, *MARK1*, *MARK2*, *C-TAK1/p78*, *Msk*, and *par-1*. *Mark1* and *Mark2* phosphorylate microtubule-associated proteins resulting in microtubule disruption in the cell²⁹. Interestingly, a close relative of these microtubule-phosphorylating molecules, *C-TAK1/p78* has been implicated in cell cycle regulation. *C-TAK1* has been implicated in cell cycle checkpoint regulation³⁰ and *p78* is downregulated in advanced pancreatic cancers³¹. In addition, *Par-1*, the *C.elegans* homologue of *C-TAK1/p78* is asymmetrically localized in the embryo and is required for establishing polarity in the embryo³². Another *SNF1* family member, *Msk1*, is expressed in the developing myocardium and is downregulated upon differentiation suggesting a role in the development of the mouse myocardium³³. Less closely related to this family is *Wpk4*³⁴, a wheat protein which, by homology appears to define its own branch within this family. Like *Wpk4*, *Hunk* diverges from the *SNF1* family at roughly the same place as the other two main branches. Thus, based upon homology within the kinase domain, *Hunk* appears to define a new class of *SNF1* kinases, the members of which have been implicated in both development and carcinogenesis in higher eukaryotes.

Objective II: Characterize temporal and spatial expression patterns

Task 1: months 6-24: Characterize the temporal expression patterns during mammary gland development

Task 2: months 1-24: Characterize the spatial expression patterns of the novel kinases in the murine mammary gland during development.

The temporal and spatial mRNA expression patterns of *Hunk* and *Punc* during murine mammary gland development are described in detail in the preliminary studies section. Additional expression data is described below.

Although a great deal of regulation is observed for both *Hunk* and *Punc* at the RNA level, information regarding protein regulation including translational control, postranslational modification and subcellular localization may give insight into the biological role of these molecules during mammary gland development. To this end, antisera have been raised against recombinant GST fusion proteins to 3 non-overlapping regions of both *Hunk* and *Punc*. Each antigen has been injected into 2 rabbits and antisera is in the process of being tested for specificity to the antigen it was raised against. Data so far suggests that antisera raised against the middle and C-terminal ends of *Hunk* recognizes recombinant fusion protein in a bacterial lysate reasonably well and the antisera from one rabbit immunized with the N-terminal region of *Hunk* recognizes recombinant protein by Western analysis extremely well. This antisera (UP1015) has been affinity purified and its specificity being evaluated for use by Western blot analysis, immunoprecipitation and immunohistochemistry. Antisera raised against *Punc* recombinant proteins are still in the process of being tested against bacterial lysates. Due to the high homology between *Punc* and other members of the CaM kinase family which are roughly the same size molecular weight proteins, it will be important to prove that antisera generated against *Punc* do not cross react with other CaM kinase family members.

In addition to characterizing *Hunk* and *Punc* expression in the mammary gland, we also investigated their expression patterns during embryogenesis and in adult organs. Levels of *Hunk* and *Punc* in total RNA isolated from embryos during 3 stages of gestation shows an expression pattern similar to that seen in the developing mammary gland (Fig. 10A). Both kinases are upregulated in midgestation as cells are rapidly dividing and differentiating. By embryonic day 18.5, many cells have completed the differentiation process yet continue to divide. Similar to its expression during mammary gland development, *Hunk* expression decreases after much of the differentiation process is complete, and *Punc* levels remain elevated.

Spatial expression of *Hunk* and *Punc* at embryonic day 18.5 is shown in Figure 10B. Both kinases are expressed in distinct structures within the embryo. *Hunk* is expressed at highest levels in the skin and hair follicles, lung, kidney, salivary gland, and in specific regions of the brain. *Punc* RNA is detected in bone, regions of the developing brain, and the outer lining of the intestines.

Figure 11 illustrates the expression of *Hunk* and *Punc* in adult organs. Highest expression of *Hunk* is observed in ovary, lung, and brain with lower levels in breast, uterus, thymus, liver, kidney, and duodenum. As shown in our 1997 annual report, expression of *Hunk* is affected, at least in the uterus and breast, by treatment of animals with estradiol and

progesterone. Therefore, we predict *Hunk* expression in hormonally responsive organs to fluctuate with the estrous cycle and it is possible that expression of *Hunk* is highest in the mammary gland during early pregnancy. *Punc* expression is highest in brain with moderate levels in hormonally responsive tissues such as breast, testis, ovary, and uterus. Expression is also detected in stomach, heart and muscle with lowest levels in thymus, spleen, duodenum, and lung.

To determine the spatial expression of *Hunk* and *Punc* in a select group of tissues, *in situ* hybridization analysis was employed. As shown in figure 12, *Hunk* and *Punc* are expressed in a subset of cells within an expressing tissue. This is consistent with the punctate expression pattern seen for both kinases in the mammary epithelium during development. In the duodenum, *Hunk* RNA is expressed in intestinal crypt cells lining the intestinal wall. These are the most immature cells within the organ and the progenitor stem cells undergo differentiation and maturation in this region. Within the uterus, *Hunk* is expressed in the glandular epithelium. *Punc* expression is also restricted to a subset of cells within expressing tissues. *Punc* expression in the ovary is limited to the granulosa cells surrounding the mature follicle. In the testis, *Punc* is expressed at very high levels in the mature spermatids which reside in the center of the seminiferous tubule. This data is consistent with *Hunk* playing a role during differentiation since expression is detected in more immature cells as they are differentiating. *Punc*, however, has highest expression in cell types that have completed the differentiation process.

Objective III: Determine the functional roles of *Hunk* and *Punc*.

Task1: months 12-48: Determine the role of novel kinases in differentiation.

To test the hypothesis that *Hunk* and *Punc* are involved in the differentiation of the mammary gland during pregnancy as predicted by their expression patterns, transgenic mice were generated to overexpress each kinase in the mammary gland using the hormonally responsive, mammary specific promoter from the mouse mammary tumor virus (MMTV) LTR. This promoter drives transgene expression in the mammary gland and in response to hormonal changes, increases transcription of the transgene during pregnancy and into lactation. *Hunk* is upregulated early in pregnancy and by mid pregnancy is back down to virgin levels. If *Hunk* is playing a developmental role early in pregnancy, then aberrant overexpression of *Hunk* later in pregnancy may affect the ability of the gland to properly differentiate. Although *Punc* expression is upregulated later in pregnancy, endogenous levels drop back to basal expression during lactation, whereas MMTV driven transcription peaks during lactation. This difference in regulation will help us address whether or not *Punc* downregulation is necessary for proper differentiation during lactation. Transgenic mice will be used to study the result of kinase

overexpression on differentiation, proliferation, and carcinogenesis. The development and differentiation of the transgenic mammary gland will be assayed morphologically by analysis of whole mounts, and examination of hematoxylin and eosin (H&E) stained sections to look for changes in ductal morphogenesis during puberty, alveolar formation during pregnancy, programmed cell death during postlactational regression, as well as hyperplasia, dysplasia, and carcinoma. In addition, the expression of defined molecular markers will be used to assay for subtle changes in programs of differentiation and development.

Transgenic constructs were assembled using the MMTV-LTR promoter in conjunction with the Ras leader upstream of the transgene and the SV40 polyadenylation signal sequence was inserted downstream to act as both a splice donor and acceptor sequence to ensure proper processing of the transgene *in vivo*. A plasmid containing the entire coding region of *Hunk*, MMTV-*Hunk* (MHK) was generated by truncating within the 3'UTR. Two constructs for *Punc* were injected into oocytes, one containing the entire cDNA for *Punc* including the endogenous polyadenylation signal (MMTV-*Punc*; MP) and a truncated form of the protein (MMTV-*Punc*/truncated; MPT). Since the regulation of CaM kinase catalytic activity has been well described, we attempted to generate a constitutively active form of *Punc* by truncating the protein prior to the autoinhibitory domain. Truncated forms of *CaMKII* have been shown to be constitutively active. Unfortunately, without a kinase assay, the activity of truncated *Punc* cannot be assessed. All three transgenic constructs were injected into oocytes and after screening potential founder animals for transmission, 5 founder lines were established for MHK, 4 for MP, and 2 for MPT.

Of the five founder lines generated for MHK, MHK1 passes at a low frequency (10%), MHK5 passes only to males (Y linked) and the other 3 lines pass at roughly Mendelian transmission rates (except MHK2 >50%). In order to determine the relative transgene expression levels in the five MHK lines, RNA was isolated from mammary glands of animals during virgin development and Northern analysis was used to determine the levels of transgene expression using a *Hunk* specific probe (Fig. 13A). Since endogenous *Hunk* was not detected by Northern analysis, RNase protection, a more sensitive assay, was used in conjunction with phosphorimager analysis to determine the levels of transgene expression relative to endogenous *Hunk* expression. Although expression varied slightly from one animal to another within a line, MHK3 females express the transgene at levels approximately 200 fold higher than endogenous and MHK2 roughly 50 fold higher. Transgene expression was equivalent to endogenous *Hunk* expression in MHK4 animals and no expression was detected in either the MHK1 or MHK5 lines. Based upon transgene expression levels, MHK3 is the most likely line to have a phenotype, therefore we will use this line to investigate the role of *Hunk* in mammary gland development.

Mammary gland specificity of the transgene was investigated in MHK3 animals. Organs were harvested from a virgin MHK3 animal and transgene expression was determined by RNase

protection analysis using an antisense riboprobe spanning junction between the 3' end of *Hunk* and the 5' end of the SV40 polyA sequence. With this probe, we are able to distinguish endogenous transcript from transgene. As shown in figure 14, highest expression of the transgene is detected in the mammary glands of female mice. Consistent with our results, MMTV driven expression has also been described in spleen, salivary gland and thymus^{15, 17, 35}. In addition, we detect transgene expression in the lung. This expression profile was observed in a second independent animal from the MHK3 line and in a female from the MHK2 line. Quantitation of transgene expression (Fig. 14B) demonstrates the tissue specificity of the MMTV promoter in the MHK3 line.

MHK3 transgenic females were sacrificed at different stages of development including puberty, pregnancy, lactation and post-lactational regression. Whole mounts and H&E stained sections were used to screen for abnormal mammary gland development. As shown in figure 15A, the most dramatic developmental abnormality in the MHK3 animals occurs during lactation. Preliminary evidence suggests that exogenous overexpression of *Hunk* prevents terminal differentiation of the mammary gland during lactation. During pregnancy, the mammary gland differentiates and changes from an epithelial duct network infiltrating a fat pad to a completely epithelial structure comprised of lobular alveoli milk-producing cells. In MHK3 transgenic animals, adipose tissue persists in the gland well into lactation. Although female animals from this line are able to lactate, their litters are often smaller than normal. Presumably, pups die due to lack of adequate food supply. In an attempt to quantitate the differentiation phenotype in the MHK3 animals at the molecular level, Northern analysis will be used to assess the levels of different differentiation markers during pregnancy and lactation. Our preliminary data suggests that overexpression of *Hunk* prevents the mammary epithelium from completely differentiating into milk-secreting alveolar cells as determined by the persistence of adipose tissue in the lactating glands of transgene positive animals.

Four founder lines have been established for MP and two for MPT. RNase protection was used to determine transgene expression in these animals. No expression was detected in any of the MP lines presumably because the presence of the endogenous polyadenylation signal in the cDNA prevents proper processing of the transgene transcript. It has been shown that splicing is required for efficient processing, stabilization and transport of mRNA species from the nucleus. In a second attempt to overexpress wild-type *Punc* in the mammary gland, a construct has been injected into oocytes that lacks the endogenous *Punc* polyadenylation signal (MPK). Transgene positive pups are breeding in an attempt to establish MPK founder lines. Since the MPT construct is truncated at the C-terminal end, it does not contain the endogenous polyA signal and as shown in Figure 13B, high levels of transgene expression are obtained in the MPT1 line. Unfortunately, expression is not detected in virgin females from the MPT2 line (data not shown). A third potential founder animal, MPT3, did not breed and therefore this line could not

be established. However, when the transgene positive male was sacrificed at 10 months of age, strikingly abnormal ductal morphogenesis was observed in the mammary glands. The epithelial tree in male animals does not normally develop past the rudimentary tree seen in the left panel of Figure 15B. As shown in the right panel, the mammary gland from the MPT3 male mouse contains an epithelial tree similar to what might be seen in a female gland during mid puberty, although no terminal end buds were observed in this male. RNase protection analysis revealed transgene expression was observed in this animal at levels approximately 3 times higher than endogenous (data not shown). This suggests that overexpression of *Punc* may affect differentiation of the mammary gland and experiments are underway to further investigate this hypothesis.

Task 2: months 12-48: Define the role of the novel kinases in proliferation.

Clones stably transfected with *Hunk* and *Punc* are being analyzed to determine the level of transgene overexpression and the effect of overexpression on proliferation in tissue culture cells. Endogenous *Punc* RNA is upregulated in confluent cells (Fig. 16), suggesting that it may be playing a role in proliferation, however expression is not affected when cells are forced into Go by serum starvation (data not shown). In combination with the analysis of transgenic animals, these experiments will be important in determining whether or not *Hunk* and *Punc* directly affect cellular proliferation.

Task 3: months 18-48: Determine the role of Hunk and Punc in carcinogenesis.

As shown in figure 6B, *Hunk* and *Punc* are expressed in a mutually exclusive set of murine epithelial tumor cell lines with defined initiating events. There are at least two possible explanations for this oncogene-restricted expression pattern. The most obvious is that *Hunk* and *Punc* directly upregulated by *Neu* and *c-myc* respectively. The second is that *Hunk* and *Punc* are markers for subsets of epithelial cells that are preferentially transformed by their respective oncogenes. In a previous report, several genes were described to be expressed exclusively in *neu* and *H-ras* initiated tumors³⁶. The hypothesis proposed was that the oncogene restricted expression was a result of preferential transformation of a particular cell type by a particular oncogene and that gene expression was not a result of transformation, *per se*, but rather reflects the endogenous expression of genes in a particular subtype of epithelial cell, i.e. those genes expressed in *neu* and *H-ras* transformed cell lines are markers for epithelial cells preferentially transformed by *neu* and *H-ras*. Consistent with this hypothesis, overexpression of *neu* and *H-ras* in *c-myc* tumor lines was not sufficient to induce genes expressed in the *neu* and *H-ras* transformed lines. Conversely, overexpression of *c-myc* in *neu* and *H-ras* lines did not turn off

expression of these genes. The conclusion of this study was genes expressed in *neu* and *H-ras* lines were markers for a subtype of epithelial cell preferentially transformed by *neu* and *H-ras*. This hypothesis is consistent with the spatial expression pattern of *Hunk* and *Punc* which suggests that these kinases may be markers for epithelial cell subtypes.

To address the hypothesis that that differential expression *Hunk* and *Punc* in the tumor cell lines may be a direct result of differential induction of these kinases by the overexpression of *neu* and *c-myc* in the nontransformed mammary epithelium, MMTV-*neu* and MMTV-*c-myc* transgenic mice were obtained. Analysis of transgene expression by Northern analysis and RNase protection showed a 30 fold increase in *c-myc* expression above endogenous, however upregulation of *neu* was not detected in mammary glands from the transgenic mice. The expression of *Hunk* and *Punc* was not affected in either transgenic line suggesting that *Punc* is not directly induced by *c-myc*. Since *neu* overexpression was not detected, the possibility that *Hunk* is induced by *neu* cannot be ruled out by this experiment. To more clearly address this question, *neu c-myc* under the control of the CMV promoter has been transfected into cells with varying levels of endogenous kinase expression. The effect of oncogene overexpression on kinase induction will be analyzed in these cell lines.

To address the hypothesis that *Hunk* and *Punc* are markers for epithelial cell subtypes that are preferentially transformed by different oncogenes, MMTV-*c-myc* and MMTV-*neu* animals were allowed to get tumors and kinase expression will be determined in the primary adenocarcinomas from these mice. If the cell type hypothesis is correct, we would expect *Hunk* overexpression in primary tumors arising in MMTV-*neu* animals and no *Hunk* expression in MMTV-*c-myc* derived tumors. Conversely, *Punc* expression is expected to be restricted to *c-myc* transformed epithelium. Analysis of these tumors is in progress.

In order to determine the role of *Hunk* and *Punc* in human cancer, we cloned the human cDNAs for *Hunk* and *Punc* by screening a human fetal brain cDNA library. The full coding region of *Hunk* has been isolated and amino acid conservation in the coding region between mouse and human is greater than 90% identical. The entire coding region of *Punc* has been more elusive. We have isolated all but the extreme 5' end of human *Punc* and efforts are underway to obtain a full-length clone. Regardless, partial cDNA sequence has allowed us to generate human specific probes to investigate the role of *Hunk* and *Punc* in human cancers.

Based upon expression of *Hunk* and *Punc* in the murine tumor cell lines, we hypothesize that these kinases may also be differentially regulated in human tumor cell lines. To address this question, RNA and DNA was harvested from 60 human tumor cell lines representing different types of human cancers. Southern blot analysis will be used to determine if *Hunk* and *Punc* are amplified in the tumor cell lines. Northern blot and RNase protection analysis is currently underway to determine if *Hunk* and *Punc* are overexpressed in human tumor cell lines. As shown in figure 17, *Hunk* is overexpressed in a number of cell lines from a variety of tumor

types. This data suggests that *Hunk* may be playing a role in human carcinogenesis. It will be interesting to investigate the *Neu* and *c-myc* status of the mammary tumor cell lines to determine if the correlation observed in the mouse cell lines is consistent in human cell lines. *Punc* expression in these tumor cell lines is still being examined.

CONCLUSIONS

In this report, we present evidence that two novel kinases identified in a screen for kinases involved in mammary gland differentiation and carcinogenesis may be playing a role in both processes. *Hunk* and *Punc* are both regulated during mammary gland development and their spatial expression patterns suggest that they may be markers for epithelial cell subtypes within the mammary gland. There are several different types of cells in the mammary gland - stromal cells including adipocytes, and epithelial cells including both luminal and myoepithelial cells. Although the idea that multiple subsets of mammary epithelial cells exist, there are no molecular markers to distinguish between possible epithelial subsets. Our data suggests that *Hunk* and *Punc* may serve as markers for as yet unidentified epithelial cell subtypes. Consistent with their developmentally regulated expression patterns, we provide initial data suggesting that both kinases, in the context of an MMTV transgenic animal, affect normal differentiation of the mammary gland. Data also suggests that these kinases may be playing a role in carcinogenesis. Both kinases display an oncogene-restricted expression pattern with *Punc* expressed exclusively in tumor cell lines derived from *int-2* and *c-myc* overexpressing glands and *Hunk* expression restricted to *Neu* and *H-ras* transformed cell lines. Initial observations in a panel of human tumor cell lines, reveals overexpression of *Hunk* in cell lines of different tumor types, including several breast cancer lines. Taken together, we hypothesize that *Hunk* and *Punc* may be kinases in signal transduction cascades leading to both the development and possibly the transformation of the mammary epithelium.

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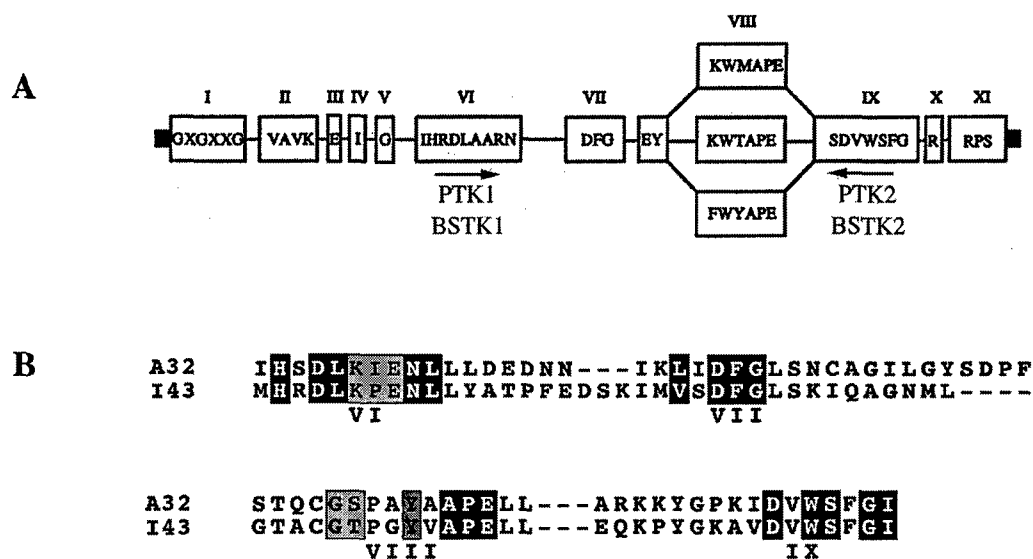


Figure 1: Kinase subdomain sequences. A) Degenerate oligonucleotide primers were designed to recognize nucleotide sequences coding for invariant amino acids in tyrosine kinase subdomains VI and IX. B) Aligned novel serine/threonine kinase catalytic domain sequences (A32/*Hunk*; I43/*Punc*) with invariant amino acids common to all protein kinases darkly shaded. Grey boxes surround amino acids distinguishing serine/threonine from tyrosine kinases as shown in panel (A).

Receptor Tyrosine Kinases		Nonreceptor Tyrosine Kinases		Serine/threonine Kinases	
	# Clones		# Clones		# Clones
Axl/UFO	6	Abl	5	Akt	1
Ebk	1	Csk	46	MPK10	*
EGFR	1	Ctk	1	Plk	26
FGFR1	126	Eck	121	Raf	1
Flk2	1	Fes	24	SK2	1
Hek2	2	Fyn	7		
IGF1R	89	Hck	88		
Insulin R	1	Jak1	74		
Kit	2	Jak2	150		
Met	120	Lyn	21	Novel Kinases	
Neu	62	MapKK3b	3	Hunk	1
Nsk2	1	Src	23	Krc	1
Ron	10	Srm	1	Punc	1
Ryk	1	Tec1	1		
Tie1	1	Tyk2	4		
Tie2	27				
Tyro10	2				
Tyro3	1				

Figure 2: Protein kinases identified. Degenerate PCR was used to generate a kinase catalytic domain library. 1500 transformants were screened to yield 41 kinases; 18 receptor tyrosine kinases, 15 nonreceptor tyrosine kinases, and 8 serine/threonine kinases including 3 novel kinases. Some kinases were identified multiple times, others only once.

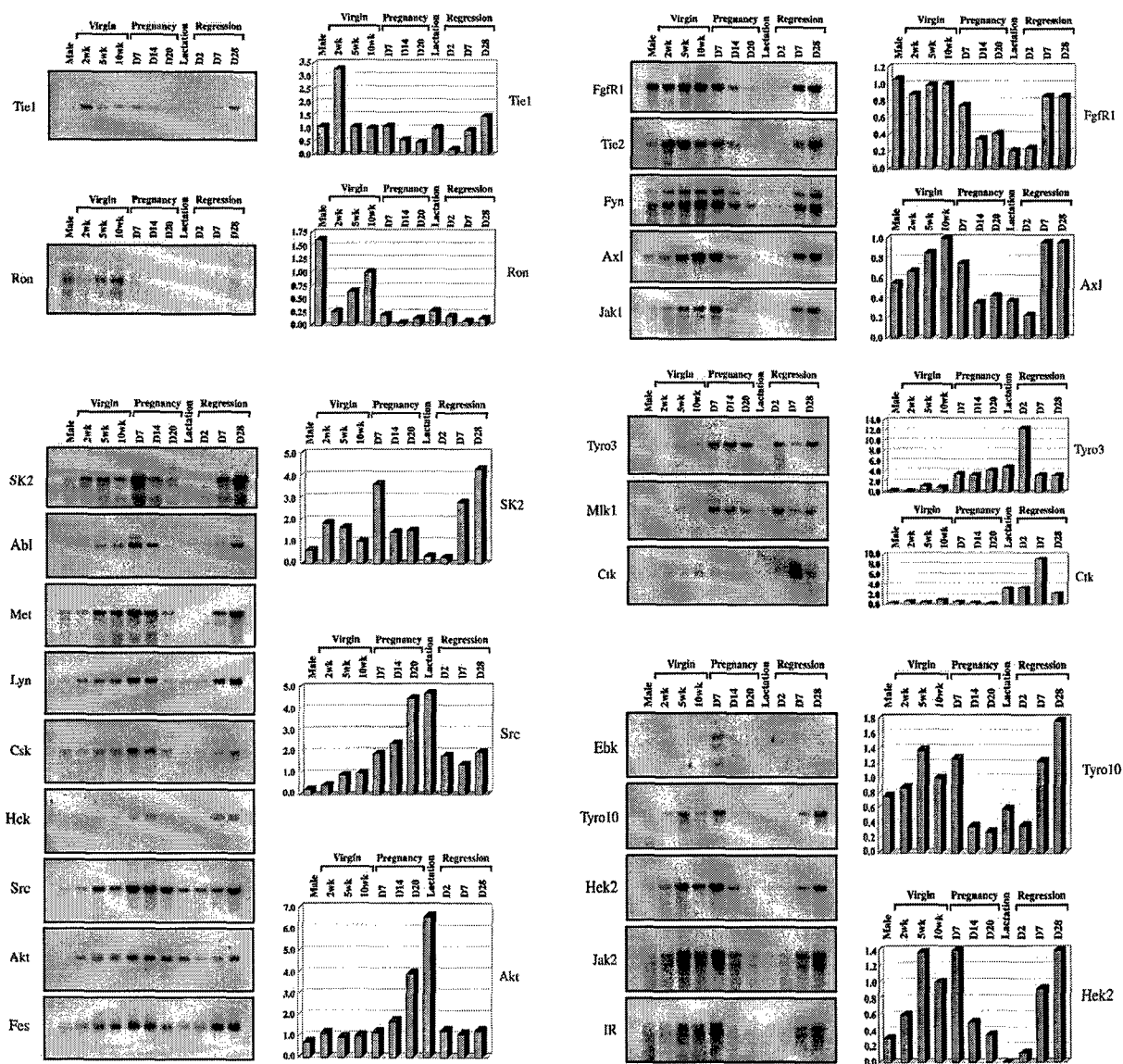


Figure 3: Kinase expression during mammary gland development. Kinase catalytic domain subunit fragments were hybridized to Northern blots containing 3 μ g poly(A)⁺ RNA per lane. Kinases with similar expression profiles were placed into groups with representative graphs alongside. Expression levels were quantitated by phosphorimager analysis. Values were first normalized to actin to correct for dilutional effects of milk protein production and then the 10 week virgin point was set equal to 1.0.

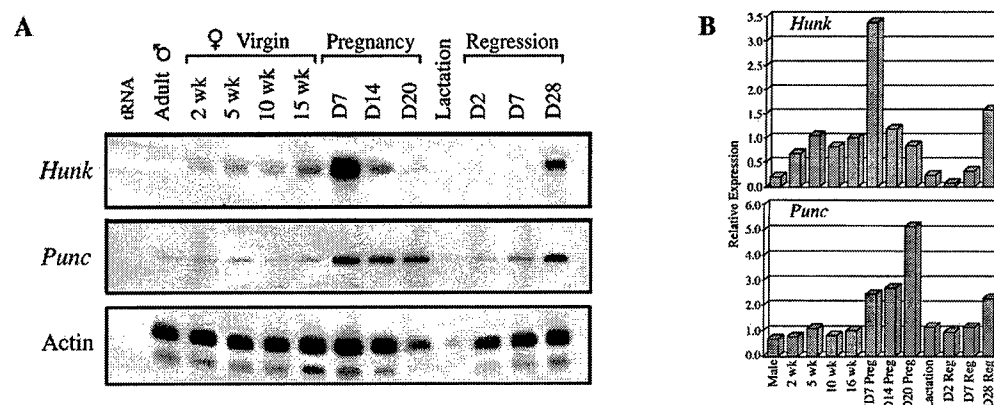


Figure 4: Differential expression of *Hunk* and *Punc* during mammary gland development. A) RNase protection analysis performed on RNA isolated from the mammary glands of mice at the indicated stages of virgin development, pregnancy, lactation, and regression. B) Phosphorimager quantitation of *Hunk* and *Punc* expression in (A) normalized to actin. Expression in 15 week adult virgins is defined as 1.0. Note that *Hunk* expression is induced early in pregnancy, whereas *Punc* expression peaks late in pregnancy.

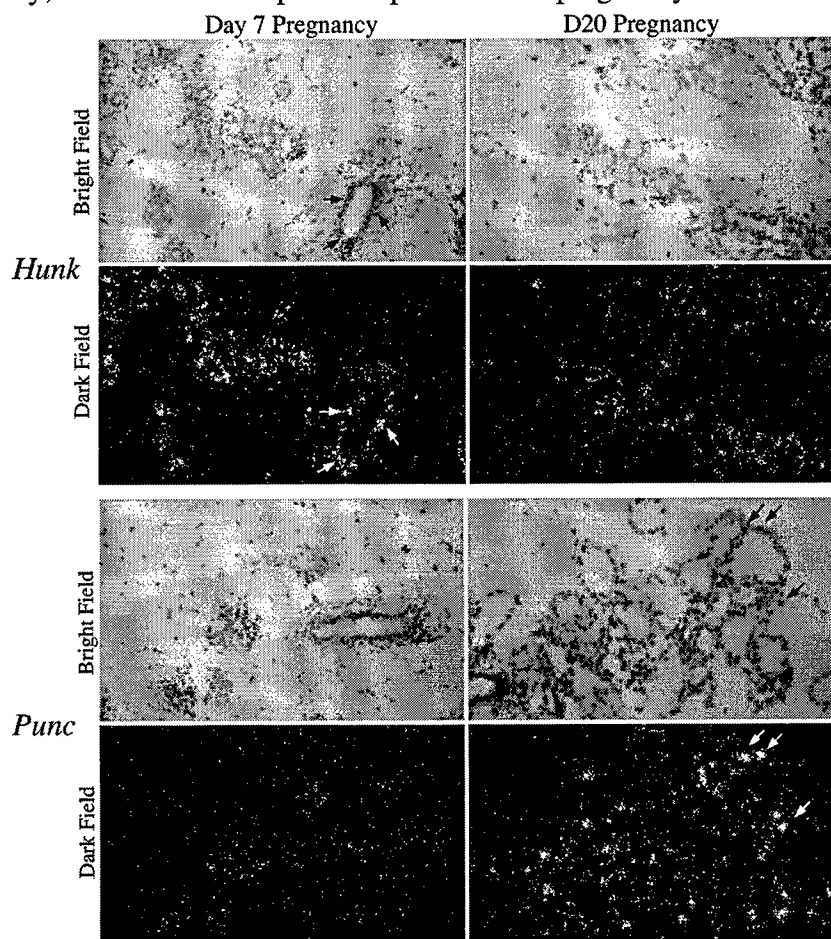


Figure 5: *Hunk* and *Punc* are differentially expressed in distinct subsets of mammary epithelial cells during pregnancy. *In situ* hybridization analysis of *Hunk* and *Punc* expression in the mammary gland at day 7 and day 20 of pregnancy. Dark field observation reveals that *Hunk* and *Punc* are each expressed in punctate patterns that define temporally and spatially distinct subsets of mammary epithelial cells. The number of *Hunk* expressing cells peaks at day 7 of pregnancy, whereas the number of *Punc* expressing cells peaks at day 20 of pregnancy.

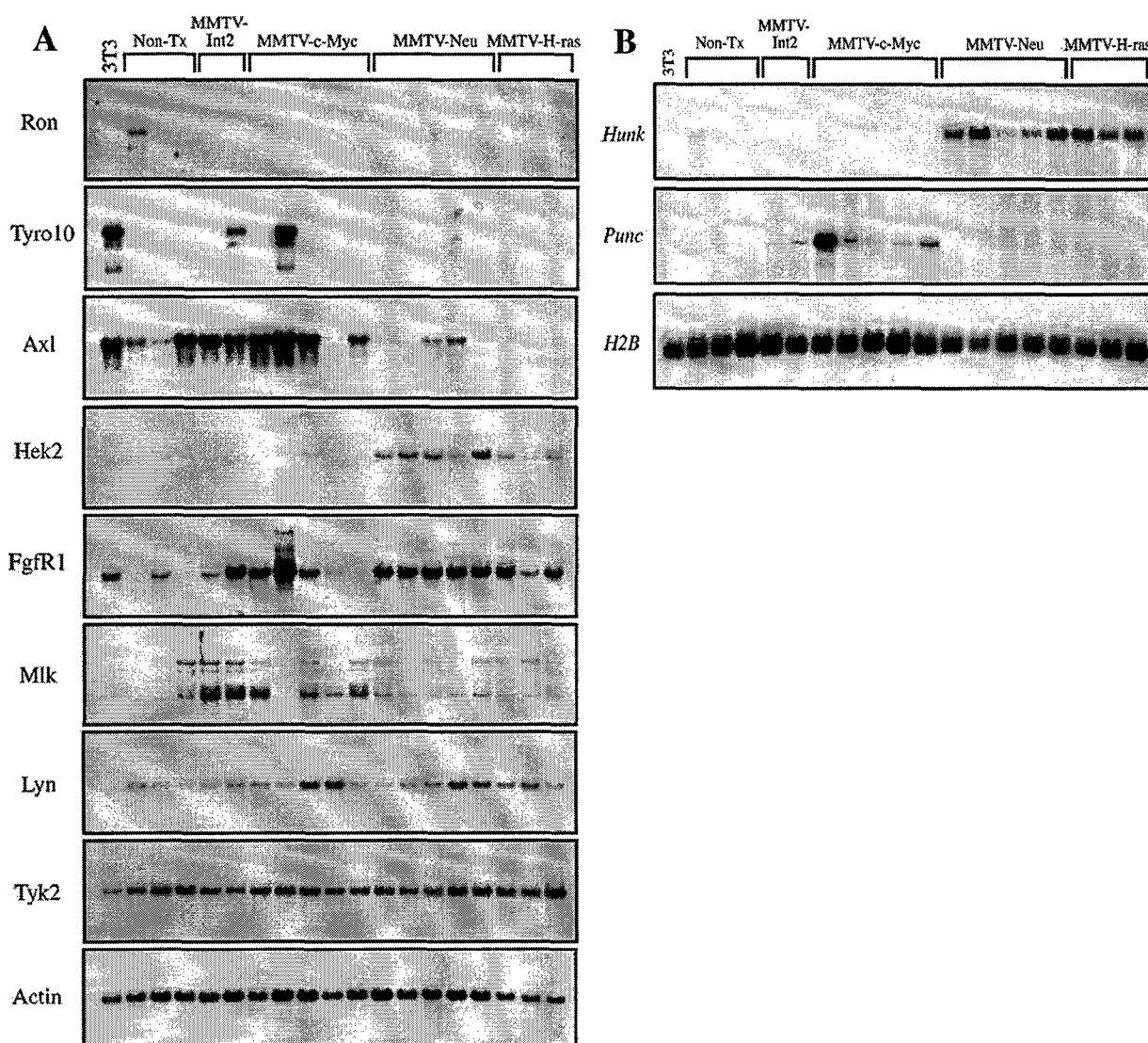


Figure 6: Protein kinase expression in mammary tumor cell lines. Northern hybridization analysis of kinase expression in a panel of either fibroblast (3T3), nontransformed cell lines (Non-Tx) or cell lines derived from breast cancers arising in transgenic animals overexpressing either the *int2*, *c-myc*, *neu*, or *Ha-ras* oncogenes. A) Representative Northern blots of protein kinases demonstrating the range of expression patterns observed in the kinase screen. B) Oncogene-restricted expression pattern of *Hunk* and *Punc* in tumor cell lines

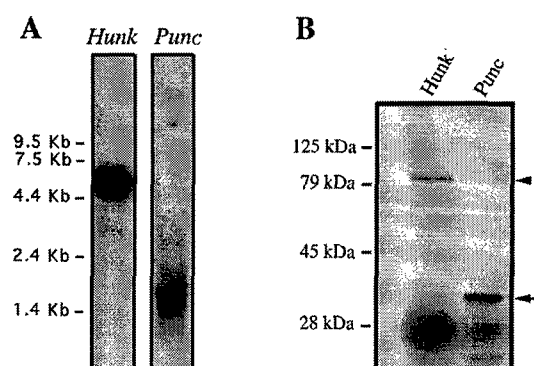


Figure 7: Northern/IVT analysis of cDNAs. A) Poly(A)⁺ blots containing RNA isolated either from a murine mammary epithelial cell line (*Hunk*) or murine brain (*Punc*) were hybridized with cDNA probes specific for Hunk or Punc. B) *Hunk* and *Punc* cDNA was transcribed and translated *in vitro*. Protein product run on a denaturing gel reveal bands of the predicted molecular weight for each kinase.

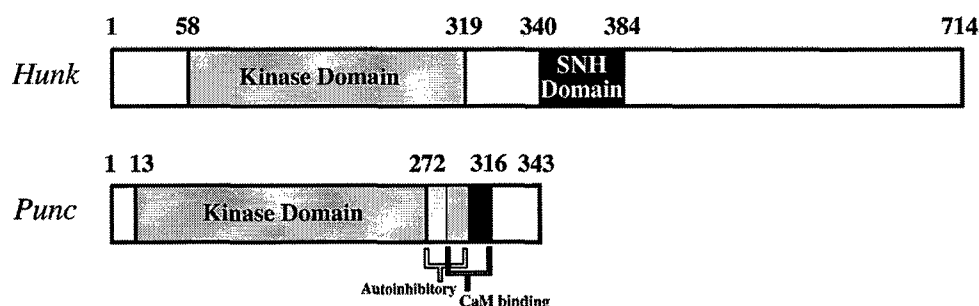


Figure 8: Schematic diagram of Hunk and Punc. Amino acid numbers correspond to defined motifs as shown.

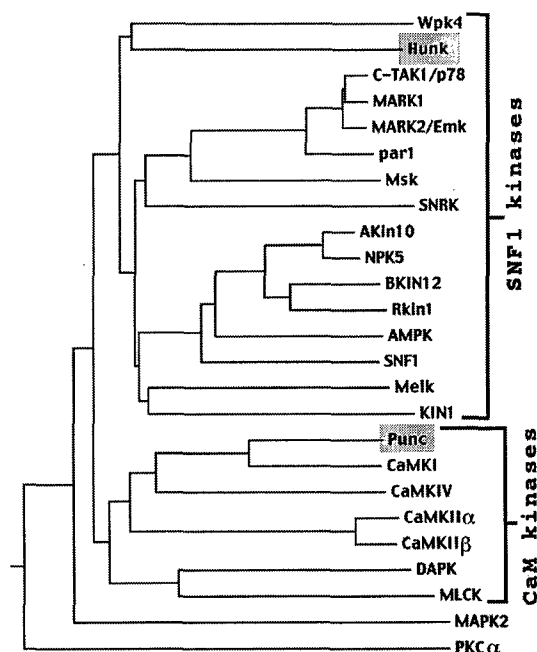


Figure 9: Phylogenetic analysis of kinase catalytic domain sequences. Kinase catalytic domain sequences were analyzed using ClustalW sequence analysis software. Relationships between kinases are relative to branch distances.

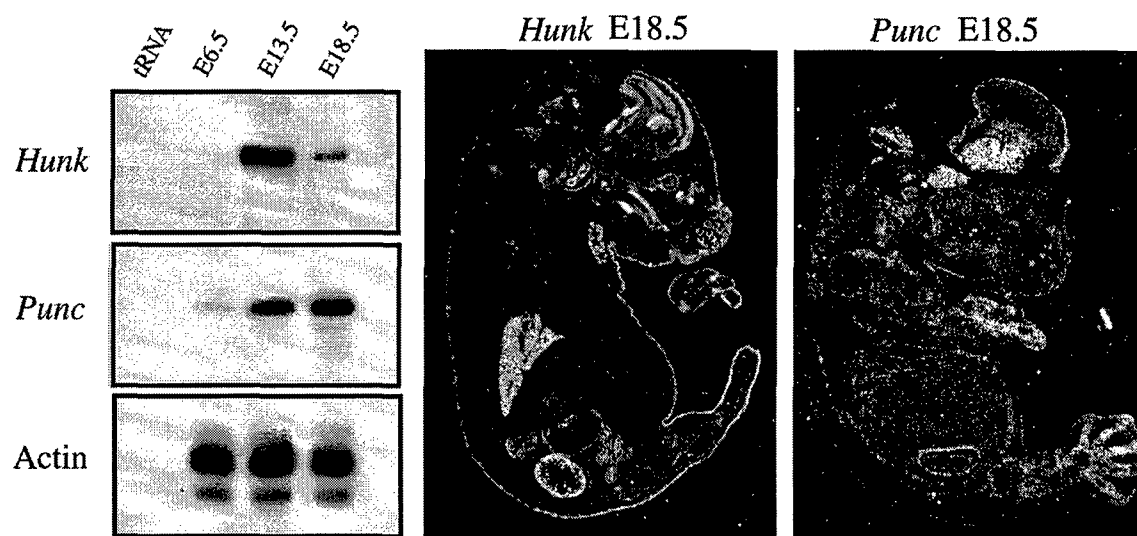


Figure 10: RNA expression during embryogenesis. A) RNase protection analysis of total RNA from mouse embryos. Actin is shown as a loading control. B) *In situ* hybridization analysis of *Hunk* and *Punc* expression in frozen day 18.5 embryo sections. Note the tissue specific expression of *Hunk* and *Punc*.

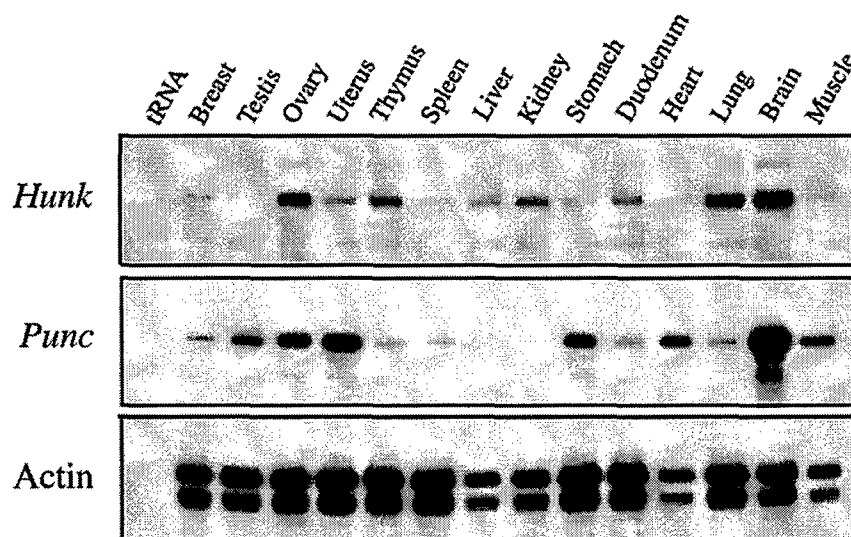


Figure 11: Tissue distribution of *Hunk* and *Punc* mRNA expression. RNase protection analysis demonstrating expression of *Hunk* and *Punc* in a panel of murine tissues from adult virgin female animals (males for testis). Actin is shown as a positive control.

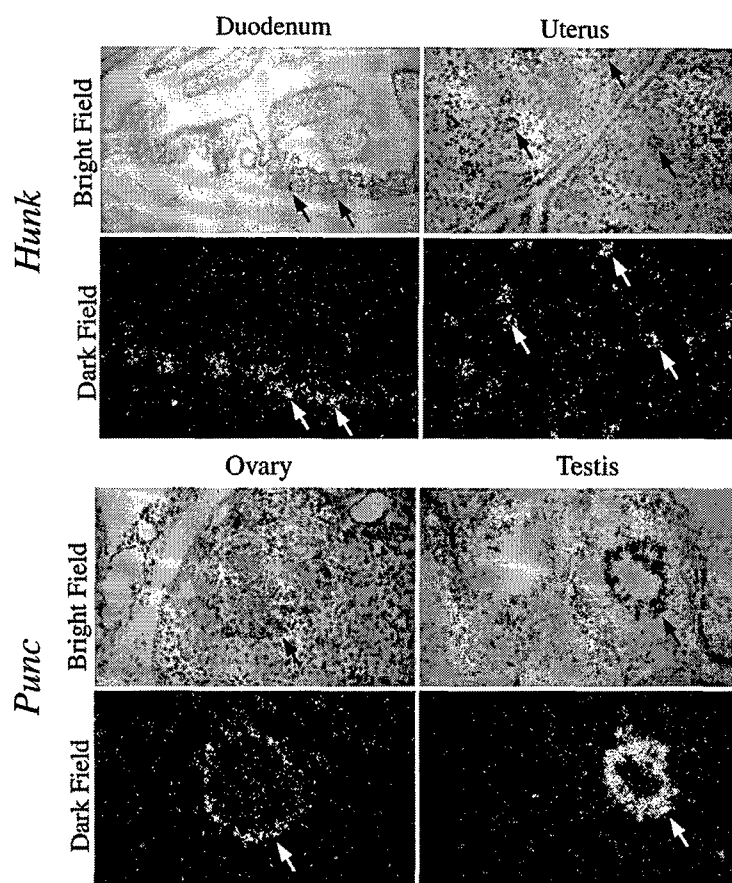


Figure 12: Cell type specific expression. *In situ* hybridization analysis demonstrating A) expression of *Hunk* in duodenum and uterus and B) expression of *Punc* in ovary and testis. Note that *Hunk* and *Punc* are expressed only in a subset of cells in each organ.

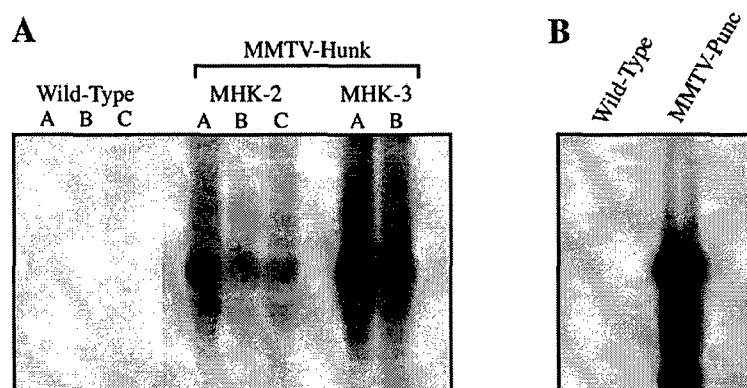


Figure 13: Transgene expression in mammary glands. A) Northern blot analysis of *Hunk* transgene expression in MHK virgin female animals. B) RNase protection analysis of *Punc* transgene expression in the mammary gland from an MPT1 virgin female animal.

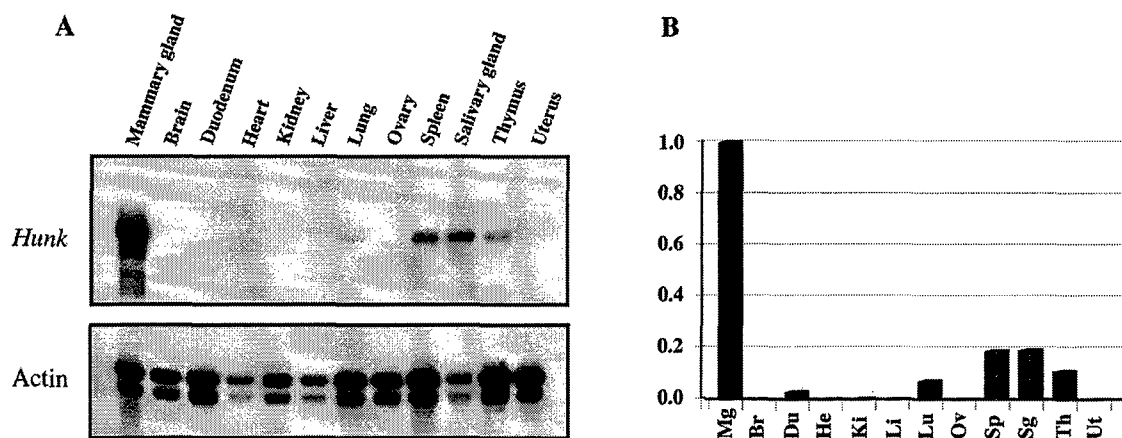


Figure 14: Tissue specific transgene expression. A) RNase protection analysis demonstrating tissue specific expression of *Hunk* in an MHK virgin female. B) Phosphorimager quantitation analysis of (A) with mammary gland set equal to 1.0.

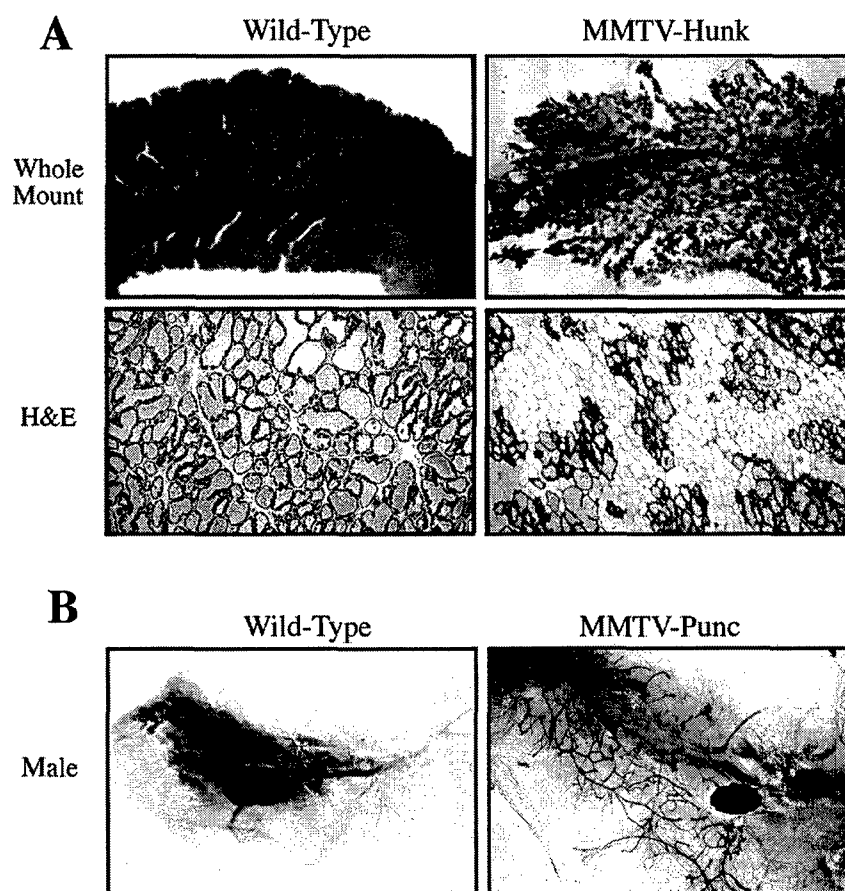


Figure 15: Developmental defects in *Hunk* and *Punc* overexpressing transgenic mice. A) Whole mount comparison of lactation mammary glands from wild-type and MHK3 transgenic. Note the decreased alveolar development in the transgenic gland. H&E staining shows the persistence of adipose tissue during lactation in the transgenic gland. B) Abnormal development in an MPT3 transgenic male gland.

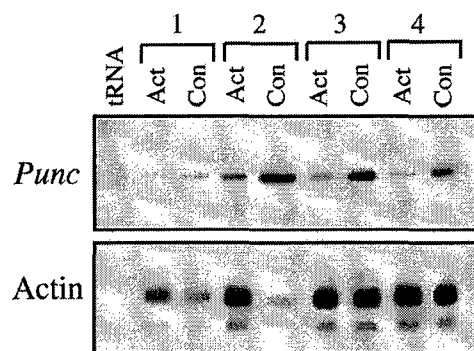


Figure 16: Increased *Punc* expression in confluent cells. RNase protection demonstrating the upregulation of *Punc* in confluent cells versus actively growing cells. Actin expression is decreased in confluent cells.

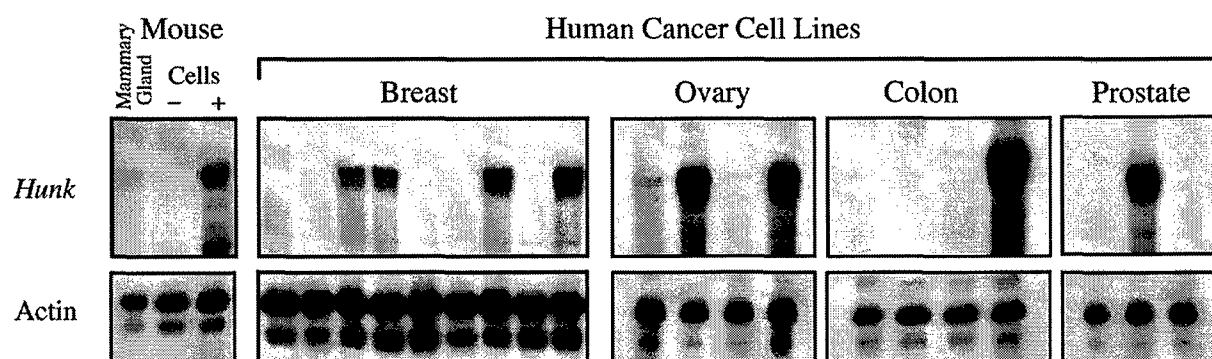


Figure 17: Overexpression of *Hunk* in a subset of human tumor cell lines. RNase protection analysis demonstrated the heterogenous expression of *Hunk* in a panel of human tumor cell lines. *Hunk* is overexpressed in a subset of tumor cell lines from a variety of human cancers.



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SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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