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AUTHORITY

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AD_____

Award Number: DAMD17-99-1-9349

TITLE: Role of Hunk and Punc in Breast Cancer and Mammary Development

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CONTRACTING ORGANIZATION: University of Pennsylvania
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REPORT DATE: August 2000

TYPE OF REPORT: Annual

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

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Carol B Christian _____

Aug 3, 2001 _____

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2000	Annual (15 Jul 99 - 14 Jul 00)

4. TITLE AND SUBTITLE
Role of Hunk and Pnck in Breast Cancer and Mammary Development

5. FUNDING NUMBERS
DAMD17-99-1-9349

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
This report contains colored photos

12a. DISTRIBUTION / AVAILABILITY STATEMENT
DISTRIBUTION STATEMENT: Distribution authorized to U.S. Government agencies only (proprietary information, Aug 00). 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)***

Major insights into the molecular mechanisms of cancer have been obtained by studies of a family of regulatory molecules known as protein kinases. Many protein kinases serve as relays for signals in the cell that regulate normal growth and cellular function. In addition, several members of this family of molecules have previously been shown to be involved in the development of breast cancer in humans. Indeed, the increased activity of some of these molecules has been shown to correlate with aggressive tumor behavior and poor clinical outcome ibn women with breast cancer. We have cloned two novel protein kinases, *Hunk* and *Pnck*, that are turned on in the breast during specific stages of pregnancy, and that appear to be turned on to different degrees in different subgroups of breast cancer. Our preliminary observations suggest that these genes may represent valuable biological markers in diagnosing cancer, in predicting the biological behavior of breast cancer, or in understanding the causes of breast cancer in humans. As such, we believe that continued study of these molecules may yield novel insights into those cell types in the breast that are most susceptible to carcinogenesis, and into those pathways in the cell that regulate growth.

14. SUBJECT TERMS
Breast Cancer, Protein Kinases, Mammary Development

15. NUMBER OF PAGES
53

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Proprietary Data

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Lewis L Anderson 9/14/00

PI - Signature Date

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1. Chodosh LA*, Gardner HP, Rajan JV, Stairs DB, Marquis ST, and Leder P. Protein kinase expression during murine mammary gland development. *Developmental Biology,* 219:259-276, 2000. (*corresponding author)

2. Gardner HP, Wertheim GB, Ha SI, Copeland NG, Gilbert DJ, Jenkins NA, Marquis ST, and Chodosh LA. Cloning and characterization of *Hunk,* a novel mammalian SNF1-related protein kinase. *Genomics* 63:46-59, 2000.

3. Gardner HP, Rajan JV, Ha SI, Copeland NG, Gilbert DJ, Jenkins NA, Marquis ST and Chodosh LA. Cloning, characterization, and chromosomal localization of *Pnck*, a calcium/calmodulin-dependent protein kinase. *Genomics* 63:279-288, 2000.

INTRODUCTION

Major insights into the molecular mechanisms of cancer have been obtained by studies of a family of regulatory molecules known as protein kinases. Many protein kinases serve as relays for signals in the cell that regulate normal growth and cellular function. In addition, several members of this family of molecules have previously been shown to be involved in the development of breast cancer in humans. Indeed, the increased activity of some of these molecules has been shown to correlate with aggressive tumor behavior and poor clinical outcome ibn women with breast cancer. We have cloned two novel protein kinases, *Hunk* and *Pnck*, that are turned on in the breast during specific stages of pregnancy, and that appear to be turned on to different degrees in different subgroups of breast cancer. Our preliminary observations suggest that these genes may represent valuable biological markers in diagnosing cancer, in predicting the biological behavior of breast cancer, or in understanding the causes of breast cancer in humans. As such, we believe that continued study of these molecules may yield novel insights into those cell types in the breast that are most susceptible to carcinogenesis, and into those pathways in the cell that regulate growth.

BODY

SPECIFIC AIMS

I. Determine the Basis for Differential *Hunk* and *Pnck* Expression in *neu*- and *c-Myc*-initiated Breast Cancers.

Different oncogenes may give rise to different tumor types either because: (1) distinct cell types exist within the breast that are preferentially susceptible to particular types of initiating events (i.e. *neu* vs. *c-myc*); (2) there is one shared target cell type for transformation in the breast that is driven into distinct pathways by the action of different oncogenes.

II. Determine Whether *HUNK* and *PNCK* are Amplified, Overexpressed or Mutated in Human Breast Cancers

Protein kinases function as oncogenes in a variety of human cancers. The finding that *Hunk* and/or *Punc* are overexpressed or mutated in human breast cancers would imply that these molecules play an important role in human carcinogenesis. Therefore, we will examine the status of the human *Hunk* and *Punc* genes in a panel of human breast cancer cell lines, and in a series of primary human breast cancers.

TECHNICAL OBJECTIVES

Technical Objective I: Investigate the mechanism for the differential expression of *Hunk* and *Pnck* in *neu* and *c-myc*-initiated breast cancers.

Task 1: Months 1-12: Generate antiseral specific for Hunk and Pnck .

Task 2: Months 1-24: Determine the basis for differential expression of *Hunk* and *Pnck* in transgenic mice.

Task 3: Months 1-24: Determine the basis for differential expression of *Hunk* and *Pnck* in transgenic breast cancer cell lines.

Technical Objective II: Determine whether *HUNK* and *PNCK* are amplified, overexpressed or mutated in human breast cancers.

Task 1: Months 6-24: Determine whether *HUNK* or *PNCK* are amplified, overexpressed or mutated in human breast cancer cell lines.

Task 2: Months 6-24: Determine whether *HUNK* or *PNCK* are amplified, overexpressed or mutated in primary human breast cancers.

Technical Objective I: Investigate the mechanism for the differential expression of *Hunk* and *Pnck* in *neu* and *c-myc*-initiated breast cancers.

Task 1: Months 1-12: Generate antiseral specific for Hunk and Pnck .

This task has been completed within the anticipated time frame. Understanding the function of *Hunk* and *Pnck* will ultimately require the ability to reliably detect the proteins encoded by these genes and the activities associated with them. To accomplish this goal, we have generated antisera specific for mouse *Hunk* and *Pnck*. For each kinase, glutathione-S-transferase (GST) fusion protein constructs were generated that encode three nonoverlapping regions of each molecule. Recombinant GST fusion proteins were produced in protease-deficient *E. coli*, purified over glutathione-sepharose columns, and cleaved into separate GST and kinase polypeptide domains. Each purified polypeptide was injected into two rabbits. All injections, prebleeds and bleeds were performed by an off-site vendor (Colcalico, Inc) using standard methods. The specificity of affinity-purified antisera was assessed by immunoblotting against cognate polypeptide and against whole-cell lysates, and immunoprecipitation of endogenous *Hunk* or *Pnck* from unlabeled whole-cell lysates followed by immunoblotting using antisera raised against different regions of these molecules. Controls consisted of of preimmune sera, non-cognate GST-fusion proteins, and antisera pre-blocked with specific or nonspecific cognate polypeptides. Our ability to use multiple antisera to detect the same protein, and the ability of cognate GST-fusion polypeptides to specifically block the antigen/antibody binding reaction, provide strong evidence that we have been able to identify the protein products of the *Hunk* and *Pnck* genes.

Task 2: Months 1-24: Determine the basis for differential expression of *Hunk* and *Pnck* in transgenic mice.

Differential expression of *Hunk* and *Pnck* in tumors arising in *neu* and *myc* transgenic mice may be a direct result of the differential induction of these kinases by the overexpression of *neu*- or *c-myc* in the nontransformed mammary epithelium. Alternately, this pattern of differential expression may be found only in the tumors initiated by these transgenes, as would be predicted if the overexpression of *Hunk* (or *Pnck*) in *neu*-induced (or *c-myc*-induced) tumors reflects the selection and outgrowth of a $Hunk^+$ (or $Pnck^+$) epithelial cell subtype that otherwise represents a minor fraction of cells in the mammary epithelium. To distinguish between these possibilities, nontransformed mammary gland tissue has been harvested from MMTV-*neu* and MMTV-*c-myc* transgenic mice prior to the appearance of tumors, and RNA and protein is being prepared from snap-frozen mammary glands. Mammary gland whole mounts and representative H&E sections are also being analyzed to confirm the absence of tumors. *Hunk* and *Pnck* mRNA and protein expression levels will be determined by RNase protection and immunoblotting, respectively, in nontransformed *neu* and *c-myc* transgenic breast tissue, as well as in breast tissue from nontransgenic littermates. In addition, breast tissue will be harvested from virgin animals and from animals during early, mid and late stages of pregnancy. As originally proposed, these studies will be completed during the second year of this project, and will address the basis for the differential expression of *Hunk* and *Pnck* in transgenic mice.

Task 3: Months 1-24: Determine the basis for differential expression of *Hunk* and *Pnck* in transgenic breast cancer cell lines.

The model that a single target cell for transformation can be driven down different pathways by *neu* or *c-myc* to yield tumors with different phenotypes predicts that genes that are preferentially expressed in *neu*-initiated tumors (i.e. *Hunk*) are either up-regulated by the *neu* signal transduction pathway or are down-

regulated by the *c-myc* transduction pathway. To test this hypothesis, the *neu* transgene will be overexpressed in either the nontranformed mammary epithelial cell line, NMuMG, or in the *c-myc* transformed breast cancer cell line, 16MB9A. Conversely, *c-myc* will be overexpressed in either NMuMG or in the *neu*-transformed breast cancer cell line, SMF. To date, the relevant expression constructs required to perform this specific aim are being generated. These will be transfected as described. The observation that *neu*-transfected 16MB9A cells express *Hunk* will suggest that *neu* induces *Hunk* expression. The observation that these cells cease expressing *Pnck* will suggest that *neu* represses *Pnck* expression. Similarly, the observation that *c-myc*-transfected SMF cells express *Pnck* will suggest that *c-myc* induces *Pnck* expression. Comparison of expression patterns between NMuMG, 16MB9A and SMF-transfected cells for each construct will be used to distinguish inductive versus repressive effects, particularly as they relate to dominant interactions of one pathway relative to another. As originally proposed, these studies will be completed during the second year of this project, and will address the basis for differential expression of *Hunk* and *Pnck* in transgenic breast cancer cell lines.

Technical Objective II: Determine whether *HUNK* and *PNCK* are amplified, overexpressed or mutated in human breast cancers.

Task 1: Months 6-24: Determine whether *HUNK* or *PNCK* are amplified, overexpressed or mutated in human breast cancer cell lines.

Protein kinases function as oncogenes in a variety of human cancers. The finding that *HUNK* and/or *PNCK* are overexpressed or mutated in human breast cancers would imply that these molecules play an important role in human carcinogenesis. Therefore, we will examine the status of the human *HUNK* and *PNCK* genes in a panel of human breast cancer cell lines. Genomic DNA and total cellular RNA have been prepared from a panel of 20 human breast cancer cell lines obtained from ATCC and other commercial sources and maintained under similar growth conditions. In order to detect alterations in *HUNK* and *PNCK* expression at the mRNA level, Northern blots will be prepared from these total RNA samples prepared from each of these cell lines. Northern blots will be probed with human *HUNK* and *PNCK* - specific cDNAs. Expression patterns of *HUNK* and *PNCK* in these cell lines will be correlated with *neu* and *c-myc* amplification and overexpression as determined by Southern and Northern hybridization, as well as with estrogen receptor status. To detect DNA amplifications and rearrangements, Southern blots containing genomic DNA harvested from each of these cell lines will be probed with full-length human *HUNK* and *PNCK* cDNAs. As originally proposed, these studies will be completed during the second year of this project, and will address the amplification or overexpression of *HUNK* and *PNCK* in human breast cancer cell lines.

Task 2: Months 6-24: Determine whether *HUNK* or *PNCK* are amplified, overexpressed or mutated in primary human breast cancers.

As an additional step in determining whether these kinases may play a role in the pathogenesis of breast cancer, we will determine whether *HUNK* and *PNCK* are amplified, overexpressed or mutated in primary human breast cancers. A panel of 23 human primary breast cancers and 12 benign breast samples have been obtained from the Mayo Clinic in collaoboration with Dr. Carol Reynolds. RNA has been prepared from these samples. In order to detect alterations in *HUNK* and *PNCK* expression at the mRNA level, RNase protection analysis will be performed on these total RNA samples prepared from each of these cell lines. Northern blots will be probed with control cDNAs. To detect DNA amplifications and

rearrangements, Southern blots containing genomic DNA harvested from each of these samples and will be probed with full-length human *HUNK* and *PNCK* cDNAs. As originally proposed, these studies will be completed during the second year of this project, and will address the amplification or overexpression of *HUNK* and *PNCK* in human primary breast cancers.

KEY RESEARCH ACCOMPLISHMENTS

- Generation of antisera that are specific for the novel serine/threonine kinase, Hunk.
- Generation of antisera that are specific for the novel serine/threonine kinase, Pnck.
- Collection and harvest of critical reagents and materials required for the specific aims of this proposal.

REPORTABLE OUTCOMES

Publication of manuscripts supported by this grant:

1. Chodosh LA*, Gardner HP, Rajan JV, Stairs DB, Marquis ST, and Leder P. Protein kinase expression during murine mammary gland development. *Developmental Biology,* 219:259-276, 2000. (*corresponding author)

2. Gardner HP, Wertheim GB, Ha SI, Copeland NG, Gilbert DJ, Jenkins NA, Marquis ST, and Chodosh LA. Cloning and characterization of *Hunk,* a novel mammalian SNF1-related protein kinase. *Genomics* 63:46-59, 2000.

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CONCLUSIONS

A number of important milestones have been accomplished during the first year of this project. These include the generation of critical reagents, such as specific antibodies, as well as the collection of important cell lines and tumor specimens that are required for the experiments described in this proposal. These will permit us to address each of the remaining specific aims of this project over the next year, as originally anticipated.

REFERENCES

1. Chodosh LA*, Gardner HP, Rajan JV, Stairs DB, Marquis ST, and Leder P. Protein kinase expression during murine mammary gland development. *Developmental Biology,* 219:259-276, 2000. (*corresponding author)

2. Gardner HP, Wertheim GB, Ha SI, Copeland NG, Gilbert DJ, Jenkins NA, Marquis ST, and Chodosh LA. Cloning and characterization of *Hunk,* a novel mammalian SNF1-related protein kinase. *Genomics* 63:46-59, 2000.

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Developmental Biology 219, 259–276 (2000)
doi:10.1006/dbio.2000.9614, available online at http://www.idealibrary.com on IDEAL®

Protein Kinase Expression during Murine Mammary Development

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The susceptibility of the mammary gland to carcinogenesis is influenced by its normal development, particularly during developmental stages such as puberty and pregnancy that are characterized by marked changes in proliferation and differentiation. Protein kinases are important regulators of proliferation and differentiation, as well as of neoplastic transformation, in a wide array of tissues, including the breast. Using a RT-PCR-based cloning strategy, we have identified 41 protein kinases that are expressed in breast cancer cell lines and in the murine mammary gland during development. The expression of each of these kinases was analyzed throughout postnatal mammary gland development as well as in a panel of mammary epithelial cell lines derived from distinct transgenic models of breast cancer. Although the majority of protein kinases isolated in this screen have no currently recognized role in mammary development, most kinases examined were found to exhibit developmental regulation. After kinases were clustered on the basis of similarities in their temporal expression profiles during mammary development, multiple distinct patterns of expression were observed. Analysis of these patterns revealed an ordered set of expression profiles in which successive waves of kinase expression occur during development. Interestingly, several protein kinases whose expression has previously been reported to be restricted to tissues other than the mammary gland were isolated in this screen and found to be expressed in the mammary gland. In aggregate, these findings suggest that the array of kinases participating in the regulation of normal mammary development is considerably broader than currently appreciated. © 2000 Academic Press

Key Words: **mammary gland; protein kinase; development; cell differentiation; carcinogenesis.**

INTRODUCTION

Numerous epidemiologic and animal studies analyzing the impact of reproductive events such as puberty, pregnancy, and parity on early events in carcinogenesis suggest that the developmental state of the breast plays a critical role in the determination of breast cancer risk (Lambe *et al.*, 1994; MacMahon *et al.*, 1970, 1982; Newcomb *et al.*, 1994; Russo and Russo, 1978, 1987). This implies an intrinsic relationship between the process of carcinogenesis and normal pathways of differentiation and development in the breast. Therefore, understanding the mechanisms by which reproductive events influence breast cancer susceptibility will undoubtedly require an improved understanding of normal mammary development, particularly with respect to genes that control mammary proliferation and differentiation.

Protein kinases represent the largest class of genes known to regulate differentiation, development, and carcinogenesis in eukaryotes. Therefore, we have chosen to study members of this family of regulatory proteins as one approach to elucidating the relationship between development and carcinogenesis in the breast. Several protein kinases have been implicated in the development of breast cancer either in humans or in rodent model systems. For instance, the EGF receptor and *ErbB2/HER2* are each amplified and overexpressed in subsets of highly aggressive breast cancers, and these molecules may thereby provide prognostic information relevant to clinical treatment and outcome (Klijn *et al.*, 1993; Slamon *et al.*, 1987, 1989). Furthermore, overexpression of specific protein kinases, or

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0012-1606/00 $35.00

of ligands for protein kinases, in the mammary epithelium of transgenic animals results in neoplastic transformation (Cardiff and Muller, 1993; Guy *et al.*, 1994; Muller *et al.*, 1988, 1990). Finally, by analogy with hematopoiesis, some protein kinases are likely to be expressed in a lineage-restricted manner in the breast and as such may provide insight into biologically meaningful subpopulations of cells (Dymecki *et al.*, 1990; Siliciano *et al.*, 1992; Tsukada *et al.*, 1993). These findings suggest that further analysis of protein kinase function may reveal significant features of the relationship between development and carcinogenesis in the breast, as well as provide insight into how the decision to proliferate or differentiate is made in mammary epithelial cells.

In light of the importance of this class of regulatory molecules, we initiated a systematic study of the role of protein kinases in mammary gland development and carcinogenesis. Examination of 1450 cDNA clones generated using a RT-PCR-based screening strategy identified 41 protein kinases, including 33 tyrosine kinases and 8 serine/threonine kinases, 3 of which were novel. The expression of these kinases was subsequently examined during defined stages in mammary development and in a panel of mammary tumor cell lines derived from distinct transgenic models of breast cancer. Our findings reveal an ordered series of protein kinase expression patterns that occur during each of the stages of postnatal mammary development, suggesting that these molecules may be important regulators of this process.

MATERIALS AND METHODS

Cell Culture

Mammary epithelial cell lines were derived from mammary tumors or hyperplastic lesions that arose in MMTV-*c-myc*, MMTV-*int-2*, MMTV-*neu*/NT, or MMTV-Ha-*ras* transgenic mice and included: the *neu* transgene-initiated mammary tumor-derived cell lines SMF, NAF, NF639, NF11005, and NK-2; the c-*myc* transgene-initiated mammary tumor-derived cell lines 16MB9a, 8Ma1a, MBp6, M158, and M1011; the Ha-*ras* transgene-initiated mammary tumor-derived cell lines AC816, AC236, and AC711; the *int*-2 transgene-initiated hyperplastic cell line HBI2; and the *int*-2 transgene-initiated mammary tumor-derived cell line 1128 (Morrison and Leder, 1994). Additional cell lines were obtained from ATCC and included NIH3T3 cells and the nontransformed murine mammary epithelial cell lines NMuMG and CL-S1. All cells were cultured under identical conditions in DMEM medium supplemented with 10% bovine calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin.

Animals and Tissues

FVB mice were housed under barrier conditions with a 12-h light/dark cycle. The mammary glands from between 10 and 40 age-matched mice were pooled for each developmental point. Mice for pregnancy points were mated at 4–5 weeks of age. Mammary gland harvest consisted in all cases of the No. 3, 4, and 5 mammary glands. The lymph node embedded in the No. 4 mammary gland was removed prior to harvest. Tissues used for RNA preparation were snap frozen on dry ice. Tissues used for *in situ* hybridization analysis were embedded in OCT medium and frozen in a dry ice/isopentane bath. Developmental expression patterns for 13 kinases were confirmed using independent pools of RNA. Analysis of the developmental expression pattern for an additional kinase using these independent pooled samples revealed a similar pregnancy-upregulated expression pattern that differed with respect to the day of pregnancy at which maximal upregulation occurred.

Construction and Analysis of Kinase-Specific cDNA Libraries

Kinase-specific cDNA libraries were constructed using mRNA prepared from the mammary glands of mice at specified stages of development and from a panel of mammary epithelial cell lines. Specifically, total RNA was prepared from the mammary glands of either 5-week-old nulliparous female mice or parous mice that had undergone a single pregnancy followed by 21 days of lactation and 2 days of postlactational regression. Total RNA was also prepared from the mammary epithelial cell lines NMuMG, CL-S1, HBI2, SMF, 16MB9a, AC816, and 1128, described above.

First-strand cDNA was generated from each of these nine sources of RNA using the cDNA Cycle kit according to the manufacturer's directions (Invitrogen). These were amplified using degenerate oligonucleotide primers corresponding to conserved regions in kinase catalytic subdomains VI and IX. The degenerate primers, PTKIa (5′-GGGCCCGGATCCAC(A/C)G(A/G/C/T)GA-(C/T)(C/T)-3′) and PTKIIa (5′-CCCGGGGAATTCCA(A/T)AGG-ACCA(G/C)AC(G/A)TC-3′), have previously been shown to amplify a conserved 200-bp portion of the catalytic domain of a wide variety of tyrosine kinases (Wilks, 1989, 1991; Wilks *et al.*, 1989). Two additional degenerate oligonucleotide primers, BSTKIa (5′-GGGCCCGGATCC(G/A)T(A/G)CAC(A/C)G(A/G/C)GAC(C/T)T-3′) and BSTKIIa (5′-CCCGGGGAATTCC(A/G)(A/T)A(A/G)CTC-CA(G/C)ACATC-3′), that differed from PTKIa and PTKIIa were also designed for this study. Restriction sites, underlined in the primer sequences, were generated at the 5′ (*Apa*I and *Bam*HI) and 3′ (*Xma*I and *Eco*RI) ends of the primer sequences.

Each cDNA source was amplified in three separate PCR reactions using pairwise combinations of the PTKIa/PTKIIa, BSTKIa/BSTKIIa, or BSTKIa/PTKIIa degenerate primers. Following 5-min denaturation at 95°C, samples were annealed at 37°C for 1 min, polymerized at 63°C for 2 min, and denatured at 95°C for 30 s for 40 cycles. The resulting ~200-bp PCR products were purified from low-melting agarose (BMB), ligated into a T-vector (Invitrogen), and transformed in *Escherichia coli*. Following blue/white color selection, approximately 50 transformants were picked from each of the 27 PCR reactions (3 reactions for each of nine cDNA sources) and were subsequently transferred to gridded plates and replica plated. In total, 1450 transformants were analyzed.

Dideoxy sequencing of 100 independent transformants was performed, resulting in the identification of 14 previously described tyrosine kinases. In order to identify and eliminate additional isolates of these kinases from further consideration, filter lifts representing the 1350 remaining transformants were hybridized individually to radiolabeled DNA probes prepared from each of the 14 initially isolated kinases. Hybridization and washing were performed as described under final washing conditions of 0.1× SSC/0.1% SDS at 70°C that were demonstrated to prevent cross-hybridization between kinase cDNA inserts (Marquis *et al.*, 1995). In this manner, 887 transformants containing inserts from the 14 tyrosine kinases that had initially been isolated were identified.

Identifications made by colony hybridization were consistent with those made directly by DNA sequencing.

The remaining 463 transformants were screened by PCR using T7 and SP6 primers to identify those containing cDNA inserts of a length expected for protein kinases. One hundred seventy-two transformants were found to have cDNA inserts between 150 and 300 bp in length and were subjected to further analysis by successive rounds of dideoxy sequencing and colony lift hybridization. This resulted in the identification of 27 additional protein kinases.

Individual clones were sequenced using the Sequenase version 2 dideoxy chain termination kit (U.S. Biochemical Corp.). Putative protein kinases were identified by the DFG consensus located in catalytic subdomain VI. DNA sequence analysis was performed using MacVector 3.5 and the NCBI BLAST server.

RNA Preparation and Analysis

RNA was prepared by homogenization of snap-frozen tissue samples or tissue culture cells in guanidinium isothiocyanate supplemented with 7 μl/ml 2-mercaptoethanol followed by ultracentrifugation through cesium chloride as previously described (Marquis *et al.*, 1995; Rajan *et al.*, 1997). Poly(A)$^+$ RNA was selected using oligo(dT) cellulose (Pharmacia), separated on a 1.0% LE agarose gel, and passively transferred to a Gene Screen membrane (NEN). Northern hybridization was performed as described using ^{32}P-labeled cDNA probes corresponding to catalytic subdomains VI–IX of each protein kinase that were generated by PCR amplification of cloned catalytic domain fragments (Marquis *et al.*, 1995). In all cases calculated transcript sizes were consistent with values reported in the literature.

In Situ Hybridization

In situ hybridization was performed as described (Marquis *et al.*, 1995). Antisense and sense probes were synthesized with the Promega *in vitro* transcription system using ^{35}S-UTP and ^{35}S-CTP from the T7 and SP6 RNA polymerase promoters of a PCR template containing the sequences used for Northern hybridization analysis.

RESULTS

Identification of Protein Kinases Expressed in the Murine Mammary Gland

As an initial step in studying the role of protein kinases in regulating mammary proliferation and differentiation, we designed a screen to identify protein kinases expressed in the mammary gland and in breast cancer cell lines. An RT-PCR cloning strategy was employed that relies on the use of degenerate oligonucleotide primers corresponding to conserved amino acid motifs present within the catalytic domain of protein tyrosine kinases (Wilks, 1989; Wilks *et al.*, 1989). RNA prepared from nine different sources was used as starting material for the generation of kinase-specific cDNA libraries. These sources included mammary glands from 5-week-old nulliparous mice undergoing puberty and parous mice at day 2 of involution, as well as a panel of seven murine mammary epithelial cell lines. Cell lines consisted of two nontransformed mammary epithelial cell lines in addition to five cell lines derived from tumors and hyperplasias that arose in the mammary glands of MMTV-*neu*, MMTV-*c-myc*, MMTV-Ha-*ras*, or MMTV-*int2* transgenic mice (Leder *et al.*, 1986; Muller *et al.*, 1988, 1990; Sinn *et al.*, 1987). Mammary tumors arising in each of these transgenic strains have previously been demonstrated to possess distinct and characteristic histopathologies that have been described as a large basophilic cell adenocarcinoma associated with the *myc* transgene, a small eosinophilic cell papillary carcinoma associated with the Ha-*ras* transgene, a pale intermediate cell nodular carcinoma associated with the *neu* transgene, and a papillary adenocarcinoma associated with the *int*-2 transgene (Cardiff and Muller, 1993; Cardiff *et al.*, 1991; Munn *et al.*, 1995).

First-strand cDNA prepared from each of these sources was independently amplified using the previously described degenerate oligonucleotide primers, PTKI and PTKII, that encode conserved amino acid motifs within catalytic subdomains VIb and IX (Hanks and Quinn, 1991; Wilks, 1991; Wilks *et al.*, 1989). In an effort to isolate a broad array of protein kinases, two additional degenerate oligonucleotide primers, BSTKI and BSTKII, which are also directed against subdomains VIb and IX, but which differ in nucleotide sequence, were designed for use in this screen. Degenerate oligonucleotide primers were used in three pairwise combinations (PTKI/PTKII, BSTKI/BSTKII, and BSTKI/PTKII) to amplify first-strand cDNA from each of the nine sources. The resulting 150- to 300-bp PCR products from each amplification were subcloned into a plasmid vector. Approximately 50 bacterial transformants from each of the 27 PCR reactions were replica plated and screened by a combination of DNA sequencing and colony lift hybridization in order to identify the protein kinase from which each subcloned catalytic domain fragment was derived.

A total of 1450 bacterial transformants were analyzed by this approach. Of these, greater than 70% contained protein kinase cDNA inserts as determined by hybridization and sequencing. Analysis of these clones resulted in the identification of 33 tyrosine kinases and 8 serine/threonine kinases (Table 1). The 19 receptor tyrosine kinases and 14 cytoplasmic tyrosine kinases isolated accounted for all but 33 of the 1056 kinase-containing clones. The remaining clones were derived from 8 serine/threonine kinases, 7 of which were represented by a single clone each, including each of the novel kinases isolated in this screen. Approximately half of the 41 kinases were isolated more than once, and most of these were isolated from more than one tissue or cell line (Table 1 and data not shown). Eight tyrosine kinases, including *Jak2, Fgfr1, EphA2, Met, Igf1r, Hck, Jak1,* and *Neu,* accounted for 830 (79%) of all clones analyzed (Table 1). Conversely, 18 kinases (44%) were represented by a single clone each, suggesting that further screening of cDNA libraries derived from these tissues and cell lines may yield additional kinases. The number of clones isolated for each kinase presumably reflects a combination of mRNA abundance and extent of homology to the oligonucleotide primers used in the amplification reaction.

Three novel protein kinases were identified in this

TABLE 1
Protein Kinases Isolated from Mammary Glands and Mammary Epithelial Cell Lines

Receptor tyrosine kinases		Nonreceptor tyrosine kinases		Serine/threonine kinases	
Axl/Ufo	6	c-Abl	5	c-Akt1	1
EphA2	121	Csk	46	Mlk1	1
EphA7	1	Ctk	1	Plk	26
EphB3	2	c-Fes	24	A-Raf	1
Egfr	1	Fyn	7	SLK	1
Fgfr1	126	Hck	88		
Flt3	1	Jak1	74		
Igf1r	89	Jak2	150		
InsR	1	Lyn	21		
c-Kit	2	Prkmk3	3	Novel kinases	
Met	120	c-Src	23		
MuSK	1	Srm	1	Bstk1	1
Neu	62	Tec	1	Bstk2	1
Ron	10	Tyk2	4	Bstk3	1
Ryk	1				
Tie1	1				
Tie2	27				
Tyro10	2				
Tyro3	1				

Note. Kinases are arranged by family and class. The number of clones isolated for each kinase is shown on the right.

screen, designated *Bstk1, 2,* and *3.* Each of these kinases contains the amino acid motifs characteristic of serine/threonine kinases (Fig. 1). *Bstk1* was isolated from a mammary epithelial cell line derived from a tumor that arose in an MMTV-*neu* transgenic mouse and is most closely related to the *SNF1* family of serine/threonine kinases. A full-length cDNA encoding *Bstk1* has subsequently been isolated (Gardner *et al.*, 2000b), as have partial cDNA sequences (Korobko *et al.*, 1997). *Bstk2* and *Bstk3* were each isolated from the mammary glands of mice undergoing early postlactational regression. *Bstk2* exhibits highest homology to kinases recently identified in *Saccharomyces cerevisiae* and *Arabidopsis thaliana* and analysis of full-length clones encoding this kinase indicates that it represents the first vertebrate member of a new family of mammalian protein kinases (Kurioka *et al.*, 1998; Ligos *et al.*, 1998; Stairs *et al.*, 1998). *Bstk3* is most closely related to calcium/calmodulin-dependent protein kinase I, and full-length isoforms have subsequently been identified in the mouse and rat (Yokokura *et al.*, 1997, and Gardner *et al.*, 2000a).

Expression of Protein Kinases in Mammary Epithelial Cell Lines

As a first step in investigating the range of expression patterns for the 41 protein kinases isolated in this screen, we determined kinase expression profiles in a panel of 18 murine mammary epithelial cell lines (Fig. 2). These included 14 cell lines derived from independent tumors arising in transgenic mice expressing either the *neu, c-myc,* H-*ras,* or *int-2* oncogenes under the control of the MMTV LTR, 1 cell line derived from the hyperplastic mammary epithelium of an MMTV-*int*-2 transgenic mouse, and 3 nontransformed, nontransgenic mammary epithelial cell lines (Fig. 2) (Leder *et al.*, 1986; Morrison and Leder, 1994; Muller *et al.*, 1988, 1990; Sinn *et al.*, 1987). Kinase expression was also investigated in NIH3T3 fibroblasts in order to identify those kinases that might be expressed in a mesenchymal- or epithelial-specific manner. All cell lines were grown under identical conditions and were harvested while actively proliferating.

Of the 41 kinases isolated in this screen, 25 were found to be ubiquitously expressed in the epithelial cell lines examined (Fig. 2 and data not shown). Steady-state mRNA levels for 11 of these ubiquitously expressed kinases exhibited relatively little variation among cell lines. These include *Tyk2, Neu, Ryk, Plk, Csk, Akt1, A-Raf, Prkmk3,* and the insulin receptor. Steady-state mRNA levels for the remaining 14 ubiquitously expressed kinases varied considerably among cell lines. These include the receptor tyrosine kinases *Egfr, Igf1R, Met, Tyro3, EphA2, EphA7/Hek11/Ehk3, and EphB3/Hek2,* as well as the cytoplasmic tyrosine kinases *Jak1, Jak2, c-Abl, c-Src, Lyn, and Tec* and the serine/threonine kinase *SLK* (Fig. 2 and data not shown).

In contrast, mRNA expression of 11 of the kinases examined was detectable in only a subset of epithelial cell lines. These kinases range from those that are expressed within the majority of cell lines tested, such as *Fgfr1, Fyn, Axl,* and *Mlk1,* to kinases that are expressed in only a small number of these cell lines, such as *Tyro10, c-Fes, c-Kit,* and *Flt3* (Fig. 2 and data not shown). Within this latter group of kinases, *Ron, Srm,* and *Hck* are expressed at detectable

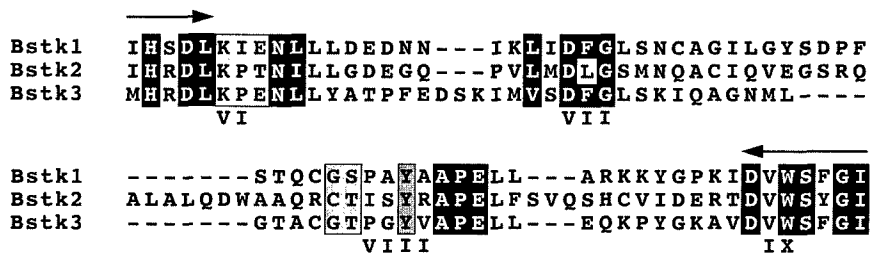

FIG. 1. Amino acid sequence of catalytic subdomains of novel protein kinases. Aligned amino acid sequences for isolated cDNA fragments corresponding to catalytic subdomains VIb–IX of *Bstk1, Bstk2,* and *Bstk3.*

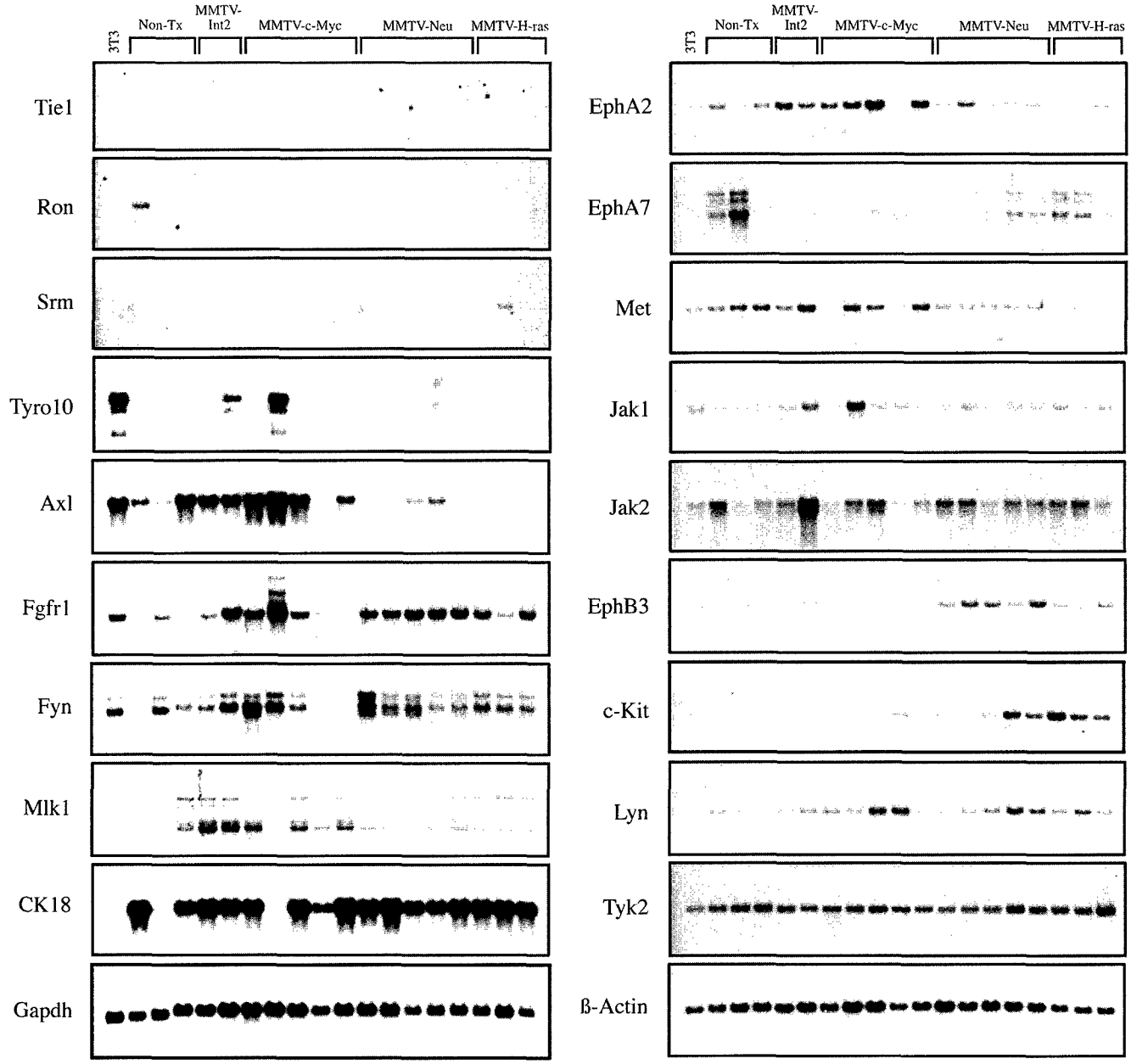

FIG. 2. Protein kinase expression in murine breast cancer cell lines. Transformed cell lines were derived from mammary adenocarcinomas or mammary hyperplasias (HBI2) arising in transgenic animals expressing the *int-2*, c-*myc*, *neu*, or v-Ha-*ras* oncogenes in the mammary gland, as indicated. Northern hybridization analysis of 6 μg of poly(A)$^+$ RNA from actively growing murine cell lines hybridized with cDNA probes specific for each of the kinases indicated is shown. Origins of the cell lines from left to right are as follows: NIH3T3 fibroblast, nontransformed (Non-Tx) (NMuMG, HC11, and CL-S1), MMTV-*int-2* (HBI2 and 1128), MMTV-c-*myc* (8Mala, MBp6, M1011, M158, and 16MB9a), MMTV-*neu* (SMF, NAF, NF639, NF11005, and NK-2), and MMTV-Ha-*ras* (AC816, AC711, and AC236).

levels in only a single mammary epithelial cell line each (Fig. 2 and data not shown). Interestingly, both *EphB3* and *c-Kit* are preferentially expressed in tumor cell lines derived from MMTV-*neu* and MMTV-Ha-*ras* transgenic animals compared to cell lines derived from MMTV-c-*myc* and MMTV-*int-2* transgenic animals. Oncogene-associated patterns of expression have also been observed for *Bstk1* and *Bstk3* (Gardner *et al.*, unpublished results). Similar patterns of expression in this panel of cell lines have previously been reported for protein tyrosine phosphatase ϵ and other molecules (Elson and Leder, 1995; Morrison and Leder, 1994).

Additional patterns of expression that presumably reflect cell-type specificity were observed. For instance, *Mlk1* is expressed in all cell lines except for NIH3T3 fibroblasts and

for MBp6, the sole mammary cell line that does not express the epithelial marker cytokeratin 18. The resulting inference that *Mlk1* expression is epithelial-specific was subsequently confirmed by *in situ* hybridization (Fig. 8). By comparison with *Mlk1*, *Tyro10* exhibited an inverse pattern of expression with steady-state levels of mRNA detectable only in NIH3T3, MBp6, and the int-2-initiated tumor cell line, 1128, suggesting that this kinase is preferentially expressed in stromal compared to epithelial cells. This hypothesis was also confirmed by *in situ* hybridization (Figs. 10E–10H). Similarly, expression in mammary epithelial cell lines was detected neither for *Tie1* nor *Tie2*, each of which has been shown to be expressed in an endothelial-specific manner, nor for *MuSK*, whose expression is restricted to muscle (Ganju *et al.*, 1995; Partanen *et al.*, 1992; Sato *et al.*, 1993; Valenzuela *et al.*, 1995b).

Surprisingly, expression of the receptor tyrosine kinases *EphB3/Hek2* and *EphA7/Hek11/Ehk3* was demonstrated in all of the mammary epithelial cell lines examined, despite the fact that expression of these kinases has been reported to be restricted primarily to the central nervous system (Aasheim *et al.*, 1997; Adams *et al.*, 1999; Bergemann *et al.*, 1998; Fox *et al.*, 1995; Krull *et al.*, 1997; Valenzuela *et al.*, 1995a). Similarly, despite previous reports that expression of each of the nonreceptor tyrosine kinases *Lyn*, *Tec*, and *Hck* is restricted primarily to cells of hematopoietic origin, *Lyn* and *Tec* expression was detected in all 18 mammary epithelial cell lines tested, and *Hck* expression was detected in 2 mammary tumor cell lines (Fig. 2 and data not shown) (Kluppel *et al.*, 1997; Quintrell *et al.*, 1987; Sato *et al.*, 1994; Umemori *et al.*, 1992; Yi *et al.*, 1991; Ziegler *et al.*, 1987). Interestingly, *Lyn* has been shown to specifically bind and phosphorylate *Tec* in hematopoietic cells *in vivo*, suggesting that *Tec* is a downstream effector of *Lyn* (Mano *et al.*, 1994, 1996). Our finding that *Lyn* and *Tec* are also coexpressed in mammary epithelial cells suggests that this signaling pathway may function in mammary epithelial cells as well as in cells of hematopoietic origin.

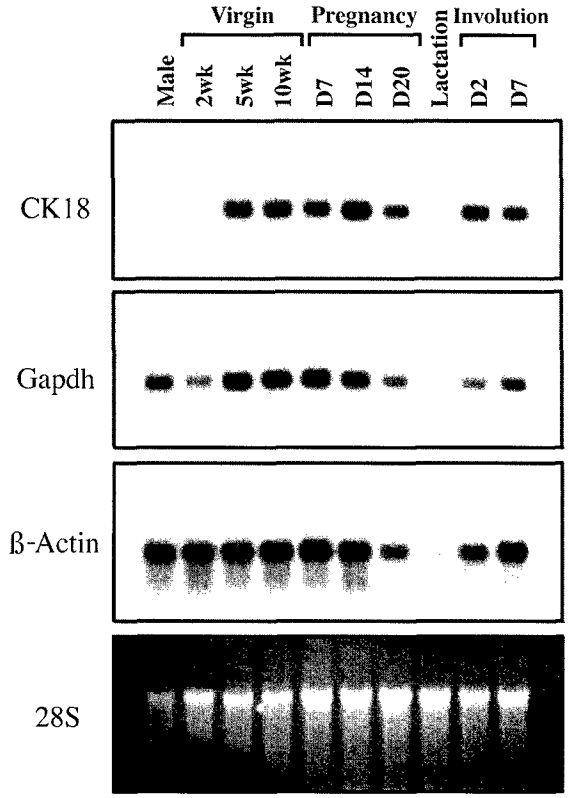

FIG. 3. Expression of control genes during mammary gland development. Northern hybridization analysis of mRNA expression is shown for cytokeratin 18, Gapdh, and β-actin, during postnatal developmental of the murine mammary gland. 3 μg of poly(A)$^+$ RNA isolated from the mammary glands of FVB mice at the indicated time points was hybridized to ^{32}P-labeled cDNA probes for the genes indicated. The smear of poly(A)$^+$ RNA beneath the 28S ribosomal RNA band is shown as a loading control. Note the apparent decrease in gene expression levels during lactation for all three genes, despite the similar amount of poly(A)$^+$ present. Origins of the mammary developmental time points are as follows: adult male; nulliparous females at 2 weeks (prior to puberty), 5 weeks (during puberty), and 10 weeks (following puberty) of age; gravid females at day 7, day 14, and day 20 of pregnancy; day 9 of lactation; and day 2 and day 7 of postlactational regression.

Expression of Protein Kinases during Postnatal Mammary Development

Since the expression of regulatory molecules is frequently controlled at the level of transcription, we analyzed the temporal pattern of expression during postnatal mammary development for each of the protein kinases isolated in this screen. Kinase expression was determined in mammary glands harvested from male FVB mice and from female mice at nine time points corresponding to developmental milestones encompassing puberty (2, 5, and 10 weeks of age), pregnancy (days 7, 14, and 20), lactation (day 9), and postlactational regression (days 2 and 7). Replicate Northern blots containing poly(A)$^+$ mRNA from each of these developmental stages were hybridized with probes prepared from catalytic domain fragments corresponding to each kinase.

As an initial control, Northern blots were hybridized with probes for the genes encoding β-actin, Gapdh, and cytokeratin 18 (Fig. 3). The resulting patterns were consistent with previous observations that steady-state levels of mRNA for many genes appear to decline during lactation and, to a lesser extent, late pregnancy and early postlactational regression (Marquis *et al.*, 1995; Rajan *et al.*, 1997). Since the expression of *β-actin*, *gapdh*, and *cytokeratin 18* does not decrease on a per-cell basis when assayed by *in situ* hybridization (J. Hartman, unpublished results), this phenomenon most likely results from a dilutional effect due to the extraordinary increase in milk protein gene expression that occurs during lactation. Expression levels for each kinase were therefore quantitated by phosphorimager analysis and normalized to *β-actin* in order to correct for these dilutional effects.

A wide range of developmental patterns of gene expression was observed for the kinases surveyed in this study.

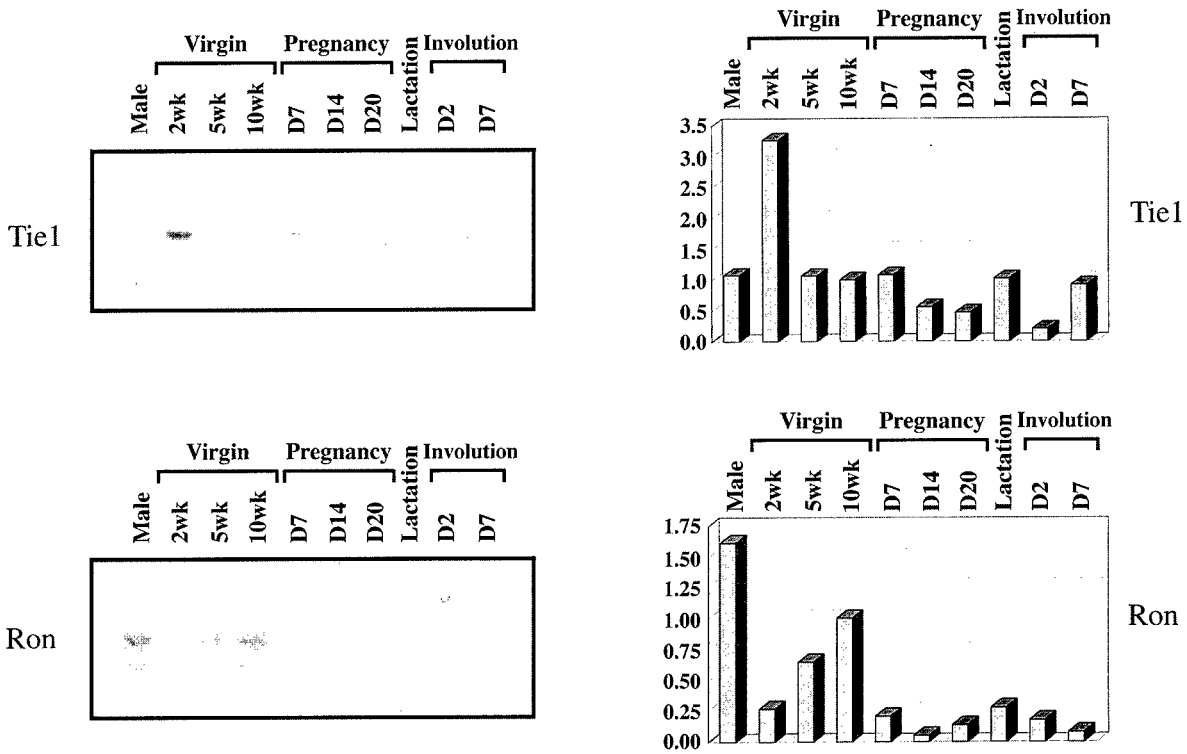

FIG. 4. Expression of protein kinases during ductal morphogenesis. Northern hybridization analysis of *Tie1* and *Ron* expression during postnatal development of the murine mammary gland is shown. 3 μg of poly(A)$^+$ RNA isolated from the mammary glands of FVB mice at the indicated time points was hybridized to ^{32}P-labeled cDNA probes for the genes indicated. Origins of the mammary developmental time points are as in Fig. 3. Phosphorimager analysis of Northern blots is shown for *Tie1* and *Ron*. Protein kinase expression was quantitated and normalized to *β-actin* expression to correct for dilutional effects due to large-scale increases in milk protein gene expression during late pregnancy and lactation. Expression levels normalized to β-actin are shown relative to adult virgin (10 wk).

Based on the assumption that kinases exhibiting distinctive patterns of regulation during particular stages of mammary development may be involved in regulating specific developmental events, we grouped these genes according to similarities in their developmental expression profiles. This approach revealed an ordered set of expression profiles that suggested the coordinated regulation of protein kinases acting at different stages of mammary development. While the observation that a particular kinase is expressed in a developmentally regulated manner does not prove that the kinase plays a role in development, the spatial and temporal patterns of expression for a gene often provide important clues to its biological function. Similarly, the identification of kinases with common developmental expression profiles may identify kinases whose developmental functions are related.

Kinase Expression during Ductal Morphogenesis

Prior to the onset of puberty at 2 weeks of age, the mammary glands of female FVB mice consist of a rudimentary epithelial tree originating from the nipple and penetrating a short distance into a mammary fat pad composed of fibroblasts and adipocytes. Following the onset of puberty at approximately 3 weeks of age, increased levels of ovarian steroids trigger the formation of club-shaped terminal end buds at the ends of growing mammary ducts. These are composed of highly proliferative, relatively undifferentiated epithelial cells that give rise to the differentiated cell types of the mammary tree, and the appearance of these structures marks the onset of the rapid cellular proliferation characteristic of ductal morphogenesis. By 5 weeks of age, over half of the mammary fat pad is filled with epithelial ducts as a consequence of ongoing ductal elongation and branching. The completion of ductal morphogenesis occurs at 10 weeks of age when most terminal end buds have reached the edge of the fat pad and have regressed.

Unlike many strains of mice in which males lack mammary glands, mammary glands are present in male FVB mice and can be studied. This represents one advantage of studying mammary development in this strain of mice. Accordingly, several protein kinases, including c-*Abl, Met, Csk, hck,* c-*Src, Fgfr1, Axl, Jak1, Jak2, Tyro3, Mlk1,* and *Ctk,* exhibited similar levels of expression in the mammary glands of adult male mice and 2-week-old female mice (Figs. 4–9). Presumably, this reflects the fact that both adult males and prepubescent females have low levels of circulating 17β-estradiol and possess only a rudimentary mammary epithelial tree. Conversely, levels of expression for

Tie1, *Ron*, *SLK*, and *Plk* differed in the mammary glands of adult male and 2-week-old female mice, potentially reflecting different levels of androgens, different diets (e.g. chow versus milk), or other age-specific or gender-specific differences between these two groups of mice. Several protein kinases, including *Ron*, *Met*, c-*Abl*, *Axl*, *Jak1*, *Tyro3*, and *Mlk1*, exhibited an increase in expression in the mammary glands of nulliparous mice between 2 and 5 weeks of age concomitant with the onset of ductal morphogenesis (Figs. 4–7). This pattern may reflect increases in circulating steroid hormone levels, increases in cellular proliferation, or increases in epithelial cell content (as reflected by the expression pattern of cytokeratin 18), that occur at the onset of puberty or changes in diet that occur at weaning (Fig. 3). Thus, similarities and differences in diet, hormonal environment, cellular proliferation, cellular differentiation, and epithelial content may account for changes in kinase expression patterns observed at different developmental stages.

The tyrosine kinases *Tie1*, *Ron*, and *Srm* each exhibited unique expression patterns with highest steady-state levels of mRNA occurring during the development of the virgin gland (Fig. 4 and data not shown). The endothelial-specific receptor tyrosine kinases *Tie1* and *Tie2* were each found to be expressed at highest levels just prior to the onset of puberty in 2-week-old female mice (Fig. 4 and data not shown). A similar pattern of developmental expression was observed for the nonreceptor tyrosine kinase, *Srm* (data not shown). Little is currently known regarding the physiological state of the prepubescent mammary gland. However, since *Tie1* and *Tie2* are required for the normal growth and organization of blood vessels, as well as for establishing the structural integrity of the vascular endothelium (Sato *et al.*, 1995), this observation suggests the possibility that changes in endothelial cells or in the vasculature of the mammary gland may precede the rapid ductal growth that begins at puberty.

In contrast to *Tie1* and *Tie2*, expression of the receptor tyrosine kinase *Ron* increases progressively during ductal morphogenesis, is downregulated at the onset of pregnancy, and remains low throughout the remainder of postnatal mammary development (Fig. 4). Since the ligand for *Ron*, Macrophage-Stimulating Protein, is a motility factor that promotes integrin-dependent epithelial cell migration (Wang *et al.*, 1996), it is plausible to hypothesize that *Ron* may contribute to the rapid epithelial migration characteristic of ductal morphogenesis. Consistent with this hypothesis, activation of *Ron* in epithelial cell lines results in enhanced proliferation, migration, and invasion through reconstituted basement membranes (Santoro *et al.*, 1996; Tamagnone and Comoglio, 1997; Wang *et al.*, 1996). Moreover, *Ron* is overexpressed in a subset of human primary breast carcinomas (Maggiora *et al.*, 1998). Together, these observations suggest a potential role for *Ron* in epithelial invasion during both normal and neoplastic mammary development.

Kinase Expression during Pregnancy and Lactation

Early in pregnancy, alveolar epithelial cells proliferate rapidly to form alveolar buds in response to rising levels of estrogens and progesterone. Alveolar cell proliferation occurs primarily during the first half of pregnancy, whereas alveolar differentiation occurs in a graded and progressive manner throughout pregnancy. This culminates in the withdrawal of epithelial cells from the cell cycle late in pregnancy concomitant with their terminal differentiation. Lactation, the final stage of lobuloalveolar development, occurs following parturition in the hormonal setting of high prolactin levels and declining estrogen and progesterone levels. The marked cellular changes that occur in the mammary gland during pregnancy and lactation are reflected on a molecular level by the temporally ordered expression of different milk protein genes (Robinson *et al.*, 1995). Each of the members of this class of genes undergoes a maximal increase in expression at a characteristic time during pregnancy and can be classified as an early, intermediate, or late marker for mammary epithelial differentiation.

Similar to milk protein genes, clustering of protein kinases on the basis of similarities in their developmental expression patterns also yields an ordered temporal set of expression profiles throughout pregnancy and lactation (Figs. 5 and 6). Consistent with the dramatic changes that take place in the mammary gland during these developmental stages, over half of all kinases examined were regulated during lobuloalveolar development. These were grouped into two sets based on whether kinases were upregulated or downregulated during pregnancy.

Seventeen kinases were found to be upregulated during pregnancy, and examination of their temporal expression profiles indicates that successive waves of kinase expression occur at each stage of lobuloalveolar development (Fig. 5). Ten kinases, including *EphA7*, *SLK*, c-*Abl*, *Met*, *Lyn*, c-*Kit*, and *Egfr*, exhibited maximal upregulation during early pregnancy. A smaller number of kinases exhibited maximal upregulation during the remainder of lobuloalveolar development, including mid-pregnancy (*Hck*) as well as late pregnancy and lactation (c-*Akt1* and c-*Fes*).

Expression of the receptor tyrosine kinase *EphA7* in tissues of adult mice has principally been described in the central nervous system. In this study, we detected *EphA7* expression both in mammary epithelial cell lines and in the mammary gland, where it is maximally upregulated during early pregnancy. Interestingly, during fetal development *EphA7* is expressed in bone marrow pro-B and pre-B cells, but not in more mature fetal B-lineage cells or in any B-lineage cells of the adult (Aasheim *et al.*, 1997). In light of the similarities between postnatal mammary development and the embryonic development of other organs, it is possible that *EphA7* may play a lineage-specific or differentiation-dependent role in the mammary gland during early pregnancy.

The receptor tyrosine kinase *Met* has previously been implicated in mammary development by virtue of its ability to stimulate branching morphogenesis and lumen for-

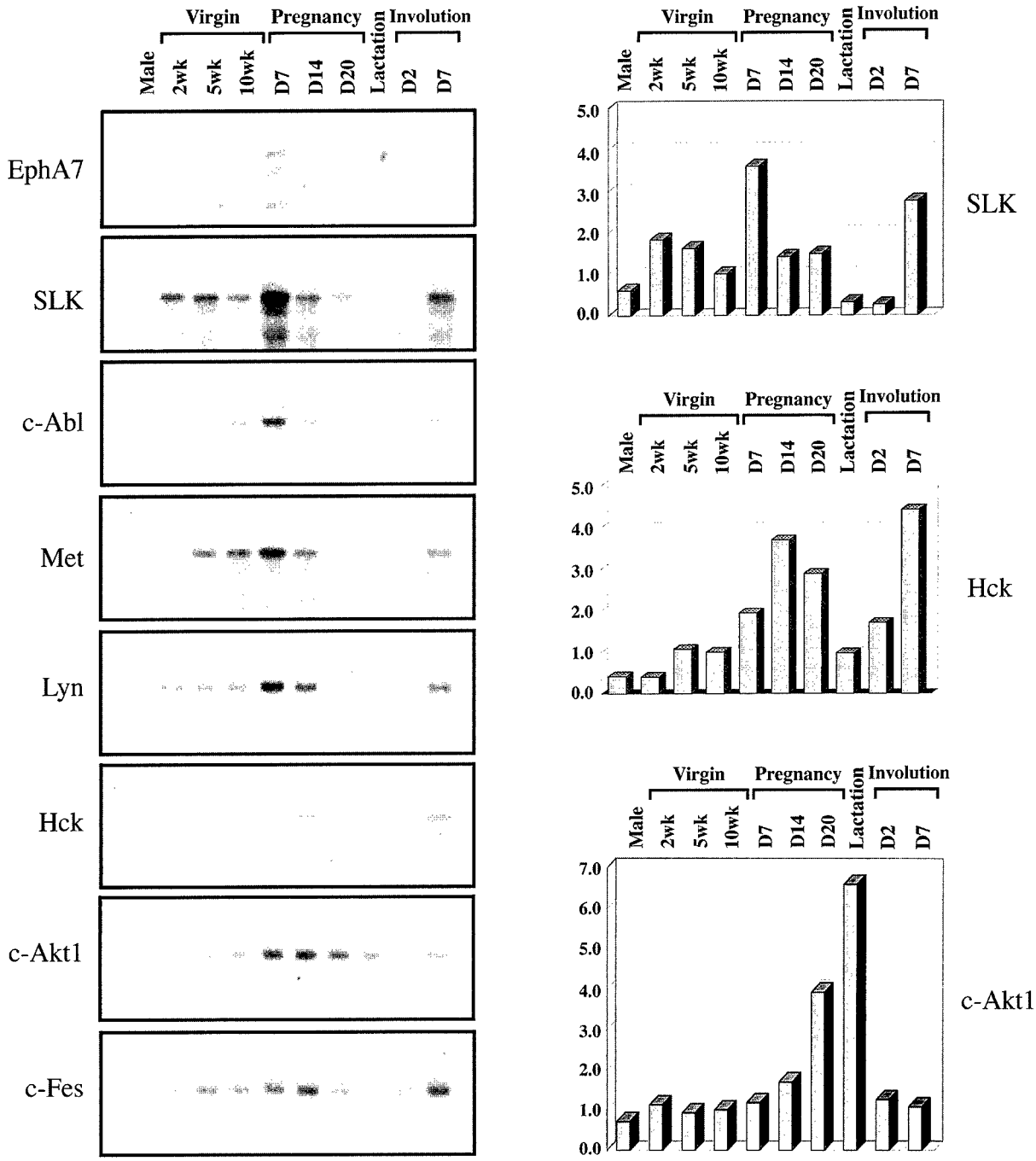

FIG. 5. Expression of protein kinases upregulated during pregnancy. Northern hybridization analysis of protein kinase expression during postnatal development of the murine mammary gland is shown for a selection of kinases that are upregulated during pregnancy. 3 μg of poly(A)$^+$ RNA isolated from the mammary glands of FVB mice at the indicated time points was hybridized to ^{32}P-labeled cDNA probes for the genes indicated. Northern blots are arranged with kinases exhibiting upregulation early in pregnancy at the top and kinases exhibiting upregulation late in pregnancy at the bottom. Origins of the mammary developmental time points are as in Fig. 3. Phosphorimager analyses of selected Northern blots are shown on the right. Expression levels normalized to β-actin are shown relative to adult virgin (10 wk).

mation in mammary epithelial cells (Niemann *et al.*, 1998; Tsarfaty *et al.*, 1992). In addition, some studies have suggested that c-*Met* is overexpressed in a subset of human breast cancers, and the mammary glands of transgenic mice expressing the *tpr-met* oncogene develop hyperplastic alveolar nodules and carcinomas (Jin *et al.*, 1997; Liang *et al.*, 1996). Nevertheless, a role for Met in mammary development has not been directly demonstrated.

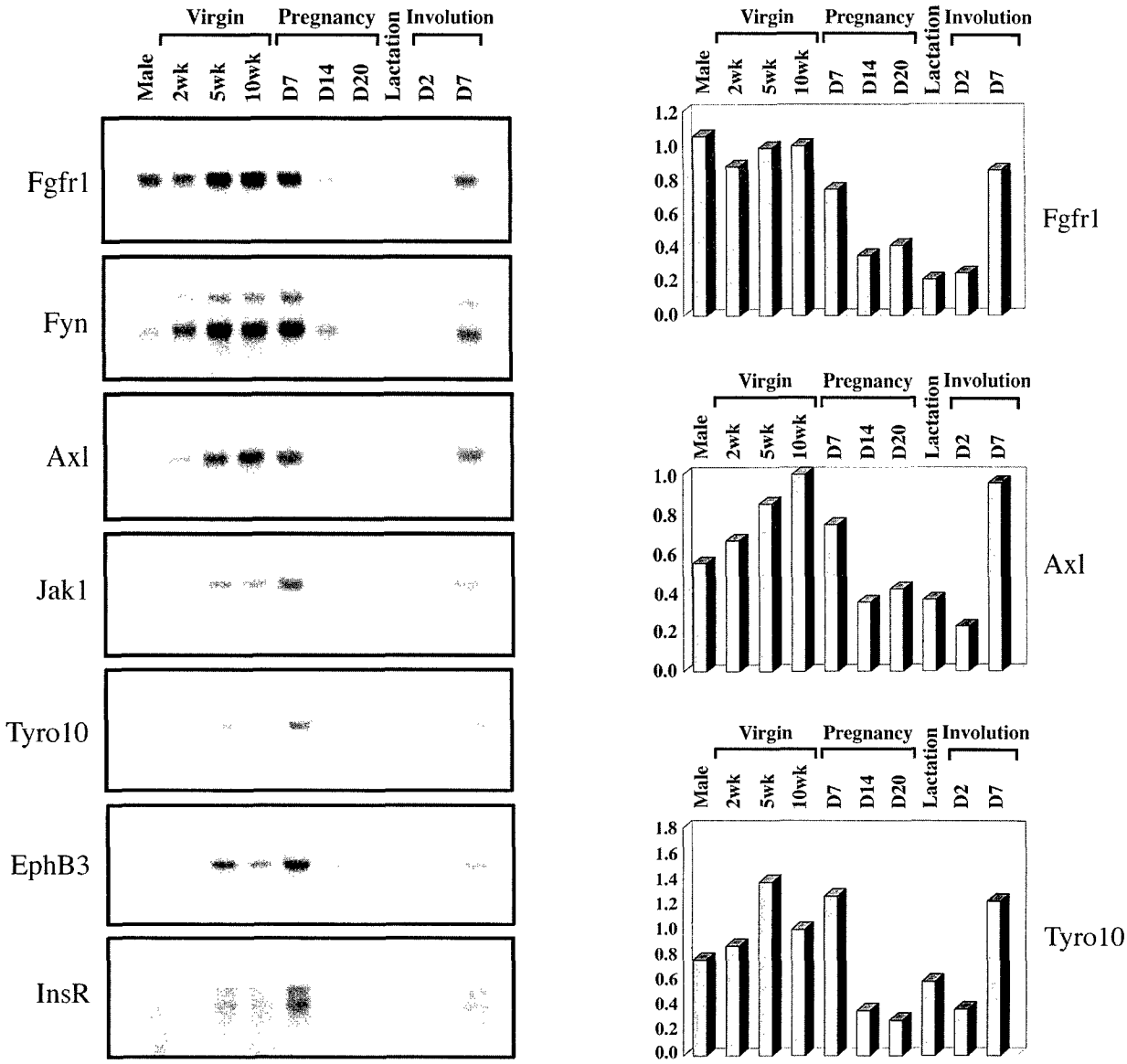

FIG. 6. Expression of protein kinases downregulated during pregnancy. Northern hybridization analysis of protein kinase expression during postnatal development of the murine mammary gland is shown for a selection of kinases that are downregulated during pregnancy. 3 μg of poly(A)$^+$ RNA isolated from the mammary glands of FVB mice at the indicated time points was hybridized to ^{32}P-labeled cDNA probes for the genes indicated. Origins of the mammary developmental time points are as in Fig. 3. Phosphorimager analyses of selected Northern blots are shown on the right. Expression levels normalized to β-actin are shown relative to adult virgin (10 wk).

Unlike other kinases analyzed in this study, both c-*Akt1* and c-*Fes* were maximally upregulated in the mammary gland during lactation. Akt1 has recently been shown to provide survival signals in response to a variety of growth factors and cytokines, and the Akt pathway is suppressed by the *PTEN* tumor suppressor gene. The further finding that germ-line mutations in *PTEN* predispose women to breast cancer suggests that Akt1 may be a prosurvival signal in the mammary epithelium as well (Li *et al.*, 1997, 1998; Liaw *et al.*, 1997; Stambolic *et al.*, 1998; Steck *et al.*, 1997). Consistent with this hypothesis, *Akt1* is expressed at high levels in human breast cancer cell lines. Similarly, while c-*Fes* expression has not previously been reported in the mammary gland, this kinase has been implicated in the induction of terminal myeloid differentiation and in promoting the survival of differentiating myeloid cells (Ferrari *et al.*, 1994; Manfredini *et al.*, 1997; Yu *et al.*, 1989). The high levels of *Akt1* and c-*Fes* expression observed in the mammary gland during lactation suggest that these kinases may play a role in propagating survival signals in terminally differentiated cells. As such, the rapid downregulation of *Akt1* and c-*Fes* expression at parturition may contribute to

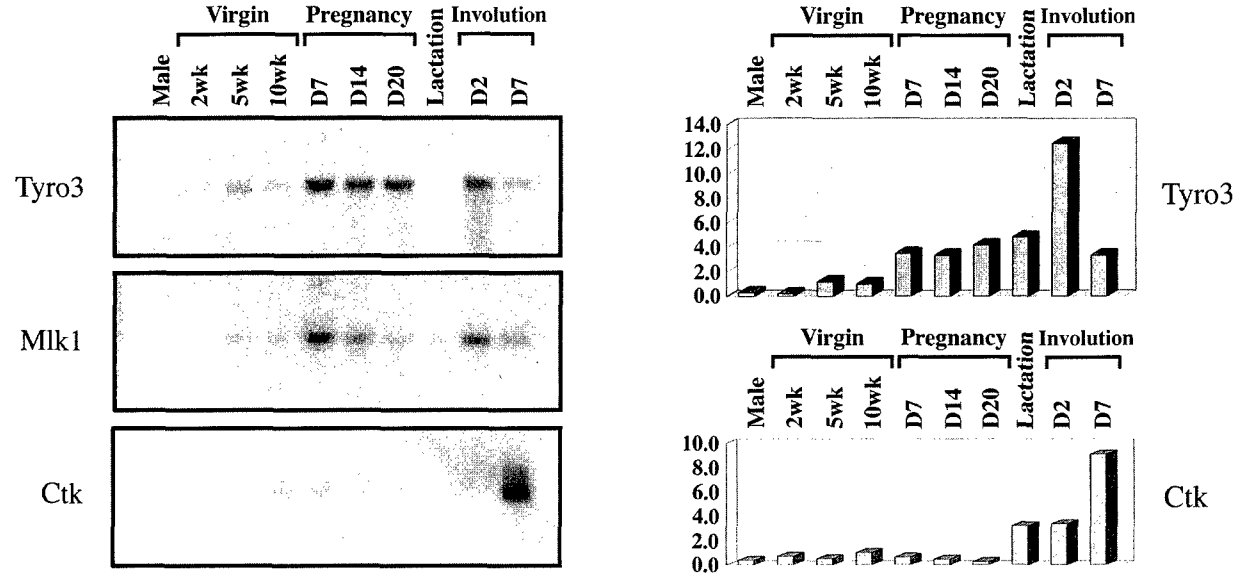

FIG. 7. Expression of protein kinases during postlactational involution. Northern hybridization analysis of protein kinase expression during postnatal development of the murine mammary gland is shown for protein kinases that are upregulated during involution. 3 μg of poly(A)$^+$ RNA isolated from the mammary glands of FVB mice at the indicated time points was hybridized to ^{32}P-labeled cDNA probes for the genes indicated. Origins of the mammary developmental time points are as in Fig. 3. Phosphorimager analyses of Northern blots for *Tyro3* and *Ctk* are shown on the right. Expression levels normalized to β-actin are shown relative to adult virgin (10 wk).

the onset of large-scale apoptosis at day 2 of postlactational involution. Such a model is consistent with the hypothesis that terminally differentiated cells are dependent on survival signals from hormones and growth factors to prevent death (Wyllie *et al.*, 1992).

Ten protein kinases were found to be downregulated in the mammary gland during pregnancy. In most cases, no marked changes in expression were observed until day 14 of pregnancy. However, for each kinase downregulation persisted throughout late pregnancy, lactation, and early postlactational regression (Fig. 6). The function of most of the downregulated kinases in mammary development is unknown. The majority of downregulated tyrosine kinases are growth factor receptors. These include *Fgfr1*, *Axl*, and the insulin receptor, each of which appears to mediate mitogenic responses in mammary epithelial cells. Overexpression of *Fgfr1* and of the insulin receptor has been described in subsets of human breast cancers (Adnane *et al.*, 1991; Ugolini *et al.*, 1999; Webster *et al.*, 1996). Conversely, downregulation of mitogenic growth factor pathways during mid- and late pregnancy may be required for the withdrawal of epithelial cells from the cell cycle that accompanies terminal differentiation. This hypothesis is consistent with the finding that downregulation of Fgfr-mediated signaling is required for the terminal differentiation of myogenic cells during avian development (Itoh *et al.*, 1996).

Alternately, changes in the expression of some receptor tyrosine kinases, such as the insulin receptor, may reflect the marked metabolic changes that occur in mammary gland during pregnancy.

Kinase Expression during Postlactational Involution

Immediately following weaning, secretory alveoli rapidly involute as the majority of mammary epithelial cells die in the apoptotic process of postlactational regression or involution. Cell death begins within 2 days following weaning, and by day 7 of postlactational involution, intensive remodeling of the mammary epithelium, stroma, and extracellular matrix is well underway.

Three protein kinases identified in this screen were found to be upregulated in the involuting mammary gland (Fig. 7). Expression of the receptor tyrosine kinase *Tyro3* is dramatically upregulated in the mammary gland at day 2 of involution, yet returns to preregression levels by day 7. Since *Tyro3* has been proposed to play a role in the growth and remodeling of the central nervous system, it is possible that it plays an analogous role in the mammary gland (Lai *et al.*, 1994; Stitt *et al.*, 1995).

Like *Tyro3*, *Mlk1* is also maximally upregulated in the mammary gland at day 2 of involution (Fig. 7). The developmental expression profiles of *Mlk1* and *Tyro3* share additional similarities as each kinase undergoes a modest initial upregulation in the mammary glands of nulliparous mice between 2 and 5 weeks of age and is further upregulated at the onset of pregnancy.

The developmental pattern of *Mlk1* expression was further investigated by *in situ* hybridization (Fig. 8). This revealed that *Mlk1* is expressed in the mammary gland in an epithelial-specific manner, as was predicted based on the similarity of its expression pattern in cell lines to that of cytokeratin 18. During puberty, *Mlk1* is preferentially ex-

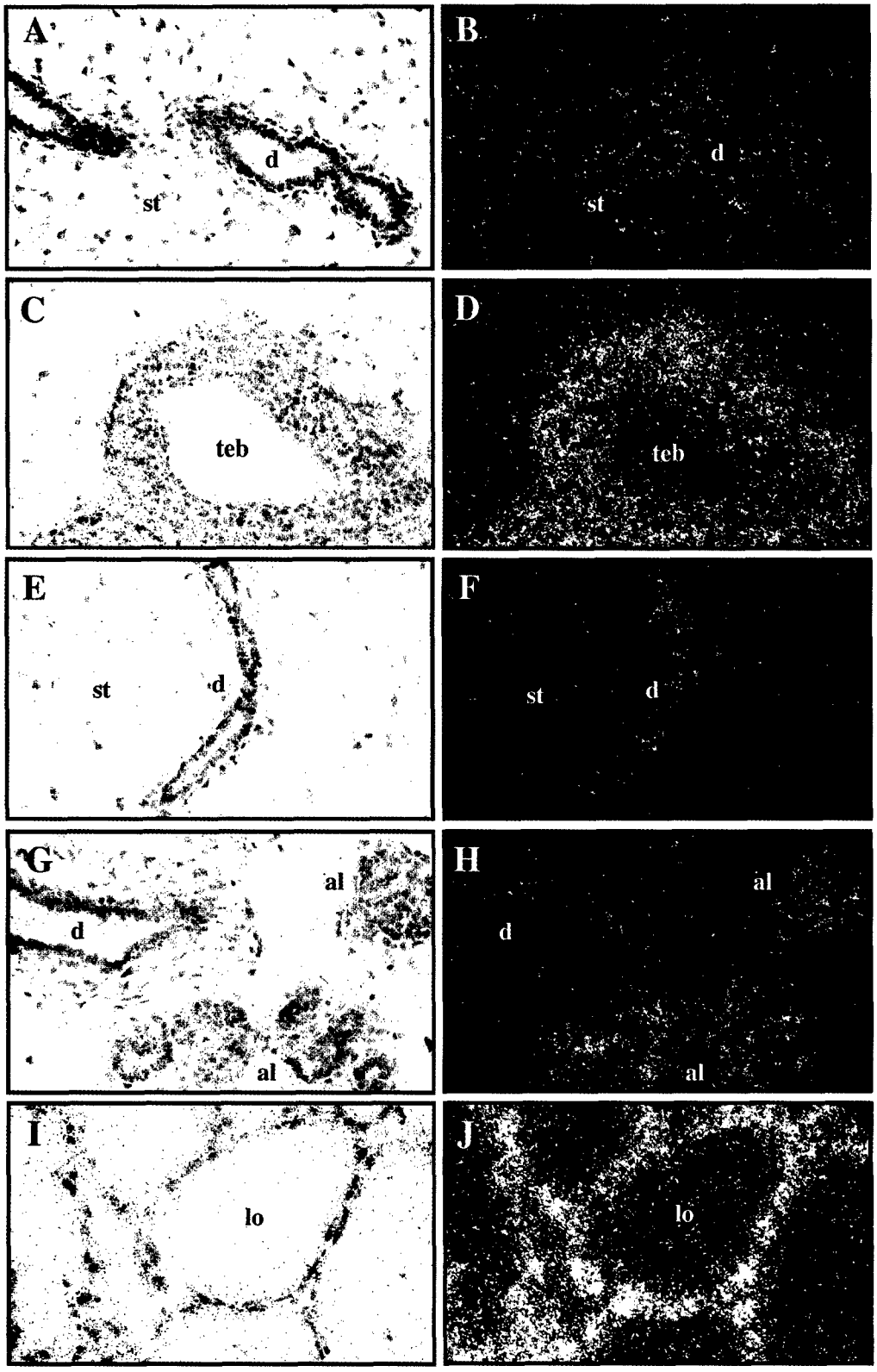

FIG. 8. Spatial expression of *Mlk1* during mammary development. *In situ* hybridization analysis of *Mlk1* expression during postnatal mammary development is shown. Bright-field (left) and dark-field (right) photomicrographs of mammary gland sections from female mice at 6 weeks of age (A–D), 16 weeks of age (E and F), day 7 of pregnancy (G and H), and day 2 of postlactational involution (I and J) hybridized with an ^{35}S-labeled *Mlk1*-specific antisense probe. No signal over background was detected in serial sections hybridized with a sense *Mlk1* probe. Exposure times were identical for all dark-field photomicrographs to illustrate changes in *Mlk1* expression during pregnancy. al, alveoli; d, duct; lo, secretory lobule; st, adipose stroma; teb, terminal end bud. Original magnification 500×.

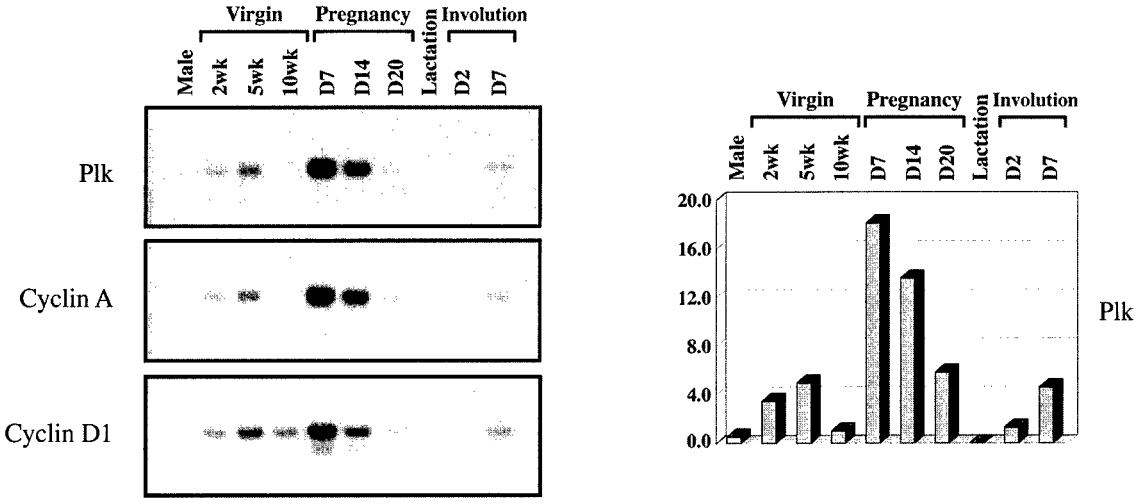

FIG. 9. Expression of protein kinases as a function of proliferation. Northern hybridization analysis of protein kinase and cyclin expression during postnatal development of the murine mammary gland is shown. 3 μg of poly(A)$^+$ RNA isolated from the mammary glands of FVB mice at the indicated time points was hybridized to ^{32}P-labeled cDNA probes for the genes indicated. Origins of the mammary developmental time points are as in Fig. 3. Phosphorimager analysis of the Northern blot for *Plk* is shown on the right. Expression levels normalized to β-actin are shown relative to adult virgin (10 wk).

pressed in epithelial cells of terminal end buds compared to ducts (Fig. 8, cf. 8B and 8D). This finding may explain the upregulation of *Mlk1* observed in the mammary glands of 5-week-old mice. *In situ* hybridization further revealed that the modest upregulation of *Mlk1* expression that occurs during pregnancy is due to preferential induction of *Mlk1* expression in developing alveoli compared to ducts, and also confirmed the dramatic upregulation of this kinase in involuting alveoli at day 2 of regression. The preferential expression of *Mlk1* in specific structures within the epithelial compartment, such as terminal end buds and developing alveoli, demonstrates that the expression of this kinase is regulated spatially as well as temporally during mammary development.

In contrast to *Tyro3* and *Mlk1*, expression of the *Csk*-related cytoplasmic tyrosine kinase *Ctk* remains low throughout virgin development and pregnancy (Fig. 7). Induction of *Ctk* expression is initially observed during lactation, with maximal upregulation occurring at day 7 of involution. *Ctk* expression has previously been described only in the brain (Brinkley *et al.*, 1995). Together, the developmental patterns of expression observed for *Tyro3*, *Mlk1*, and *Ctk* suggest that these kinases may play a role in the dramatic changes that occur in the mammary gland during involution.

Proliferation-Dependent Patterns of Kinase Expression

An intriguing pattern of developmental expression was observed for the mammalian polo-like kinase, *Plk*. *Plk* expression is maximally upregulated in the mammary gland at day 7 of pregnancy, with a progressive decline in expression observed thereafter (Fig. 9). No expression was detected during lactation. Smaller increases in *Plk* expression were noted in the mammary glands of 5-week-old nulliparous animals. The observation that the upregulation of *Plk* expression coincides with peak alveolar proliferation rates during early pregnancy, as well as previous observations that *Plk* expression is cell cycle-regulated (Lee *et al.*, 1995), suggested that the developmental pattern of expression of this kinase reflects proliferative events in the mammary gland. This hypothesis is supported by the marked similarities in the expression profiles of *Plk*, cyclin A, and cyclin D1 (Fig. 9). As such, these findings strongly suggest that the temporal profile of *Plk* expression reflects increases in mitotic activity that occur in the mammary gland during puberty and early pregnancy. Consistent with this hypothesis, the developmental expression of *Plk* in the mammary gland is spatially restricted to proliferating cellular compartments, particularly in terminal end buds during puberty and alveolar buds during pregnancy (data not shown).

Spatial Regulation of Kinase Expression

In addition to the diverse temporal patterns of expression observed for the kinases analyzed in this study, diverse spatial patterns of expression were also observed. Similar to *Mlk1* and *Plk*, the serine/threonine kinase *SLK* is upregulated in the mammary epithelium at day 7 of pregnancy (Figs. 5, 7–9, and 10A–10D, and data not shown). However, whereas *Mlk1* and *Plk* are preferentially expressed in alveoli compared to ducts at this stage of development, *SLK* upregulation occurs in both ducts and alveoli. This observation suggests that upregulation of *SLK* expression may occur in response to signals that are distributed throughout

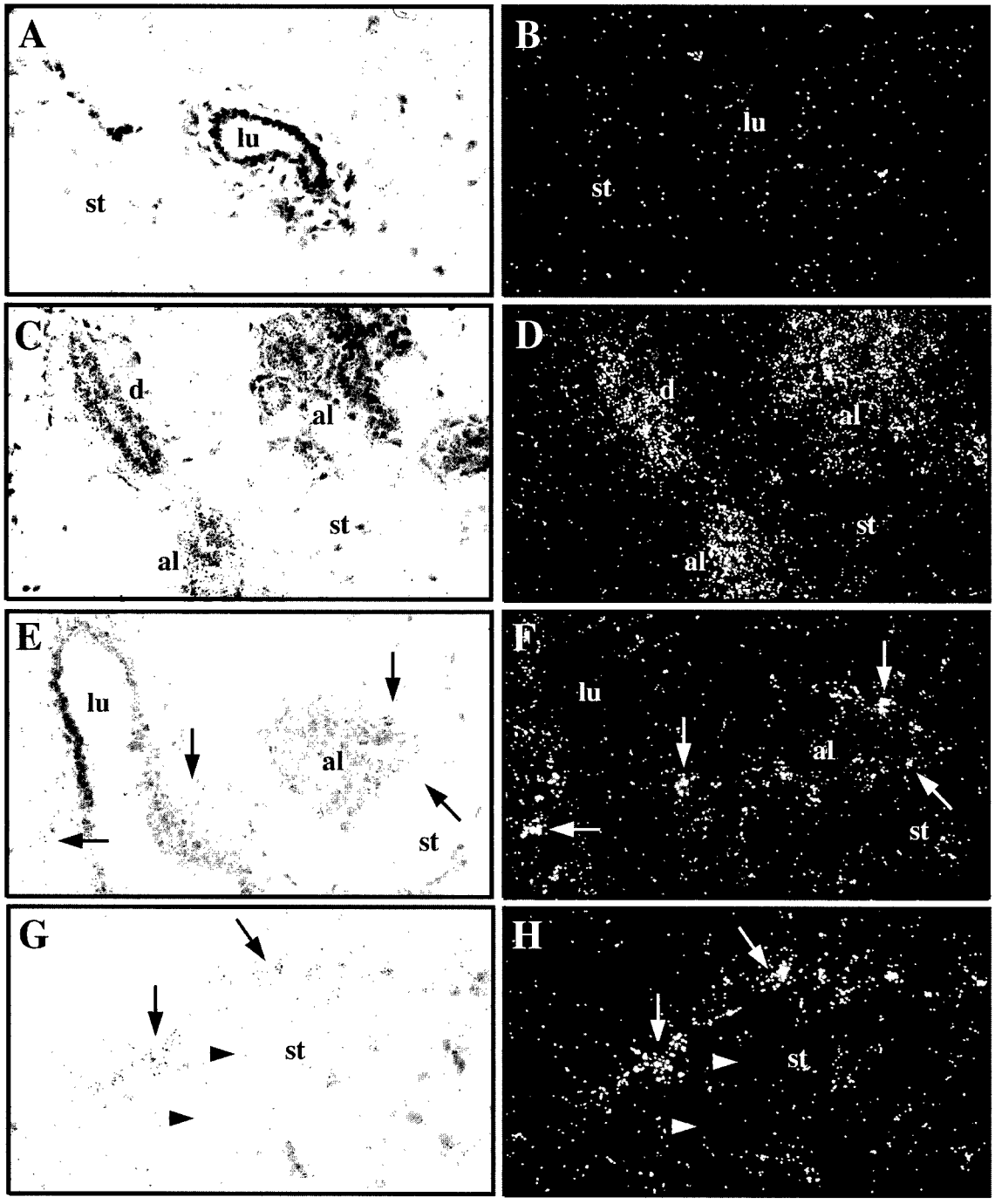

FIG. 10. Spatial expression of *SLK* and *Tyro3* during mammary development. *In situ* hybridization analysis of *SLK* (A–D) *and Tyro3* (E–H) expression during postnatal mammary development. Bright-field (left) and dark-field (right) photomicrographs of mammary gland sections from nulliparous female mice at 16 weeks of age (A and B) or day 7 of pregnancy (C–H) hybridized with ^{35}S-labeled antisense probes specific for *SLK* or *Tyro3*. Stromal cells at the edge of the mammary fat pad are shown in panels G and H. No signal over background was detected in serial sections hybridized with sense *SLK* or *Tyro3* control probes. Exposure times were identical for dark-field photomicrographs for *SLK* to illustrate changes in expression during pregnancy. al, alveoli; d, duct; lu, lumen of epithelial duct; st, adipose stroma. Arrows indicate cells expressing *Tyro3*. Cells without detectable *Tyro3* expression are indicated by arrowheads. Original magnification 500×.

the epithelium, rather than to changes specific to a subset of epithelial cells within a particular compartment.

Unlike the majority of kinases analyzed in this study, expression of the receptor tyrosine kinase *Tyro10* was restricted to the stroma of the mammary gland (Figs. 10E–10H). This cell type-specificity was predicted based on

our initial finding that *Tyro10* is preferentially expressed in cell lines that are negative for expression of the epithelial marker cytokeratin 18 (Fig. 2). Within the mammary gland, *Tyro10* is expressed in regions immediately surrounding epithelial structures (Fig. 10E and 10F) as well as in regions at the periphery of the mammary fat pad (Fig. 10G and 10H). Interestingly, *Tyro10* expression is strikingly heterogeneous in stromal cells, suggesting that the expression of this kinase may be restricted to a specific stromal cell type.

DISCUSSION

We have isolated 33 tyrosine kinases and 8 serine/threonine kinases that are expressed during the postnatal development of the murine mammary gland. Since transcription is one of the key steps at which gene expression is regulated, we chose to examine the mRNA expression of each of these kinases in a panel of transgenic mammary epithelial cell lines and in the mammary gland during multiple stages of development. Although protein kinases are typically regulated at the posttranslational level, the majority of kinases analyzed in this study were also found to be developmentally regulated at the mRNA level. Kinases were subsequently clustered into groups based on similarities in these expression patterns as a first step in drawing inferences about developmental processes in which they might be involved. In this manner, the panel of protein kinases identified in this study was used to produce a temporal map of developmental gene expression for the mammary gland.

While the temporal patterns of gene expression observed for the protein kinases surveyed in this study were diverse, application of a clustering approach revealed an ordered set of expression profiles in which successive waves of kinase expression occur during development. This finding suggests that a coordinated program of protein kinase expression exists that may play a role in regulating the cascade of events constituting mammary development.

A wide range of kinases was isolated in this study, including members of multiple receptor tyrosine kinase, cytoplasmic tyrosine kinase, and serine/threonine kinase subfamilies. A subset of the kinases that were identified in mammary gland samples was not expressed in the mammary epithelial cell lines tested, presumably as a result of their expression in a nonepithelial cell type. In contrast, virtually all of the kinases identified in mammary tumor cell lines were also found to be expressed in the mammary gland during development. This observation suggests that the expression of kinases detected in mammary epithelial cell lines is not merely a consequence of malignant transformation, nor of culture conditions, but rather suggests that these molecules may play a role in the normal physiology of the mammary gland. Of note, the kinase expression patterns observed in the panel of mammary epithelial cell lines tested were markedly heterogeneous, with dissimilar expression patterns observed even for kinases that displayed similar expression profiles during development. We conclude that kinase expression patterns in mammary gland samples and in epithelial cell lines reflect distinct aspects of mammary biology.

In recent cDNA microarray experiments in the yeast *S. cerevisiae*, more than 60% of characterized genes that were found to be regulated in a cell cycle-dependent manner were already known to have functions related to the cell cycle (Cho *et al.*, 1998). Thus, while the finding that expression of a particular kinase is developmentally regulated does not prove that the kinase plays a role in development, the spatial and temporal patterns of expression for a gene may provide important clues to its biological role. Kinases exhibiting distinctive patterns of regulation during mammary development may, in fact, be involved in controlling or mediating developmental events. Similarly, the identification of kinases that share developmental expression profiles may also identify kinases whose developmental functions are related.

The vast majority of protein kinases isolated in this screen currently have no recognized role in mammary development. Since poorly characterized genes whose expressions fluctuate in parallel may not only be regulated by parallel pathways, but also may function in parallel pathways, RNA expression patterns may provide a straightforward means of gaining insight into roles played in mammary development. As such, extrapolation may yield insights into the role in mammary development played by kinases whose functions have been elucidated in other tissues. Conversely, insight into the function of kinases lacking previously described roles in murine development may be gained by extrapolation from patterns of kinase expression during mammary development.

The expression of a number of kinases isolated in our study has previously been reported to be restricted to tissues other than the mammary gland. Such kinases include *EphB3, EphA7, Ctk, Lyn, Hck,* and *Tec.* Each of these kinases is also expressed in the mammary epithelial cell lines tested. Our observation that the majority of these kinases are developmentally regulated in the mammary gland suggests that these molecules have additional unrecognized functions. It will be of great interest to determine whether the functions of these kinases in mammary development are analogous to their functions in other tissues.

RT-PCR screens designed to identify protein kinases expressed in mammary carcinomas or in the mammary gland have previously been reported by three groups: Lehtola *et al.* isolated 10 protein kinases from the human breast cancer cell line MCF-7; Cance *et al.* isolated 25 protein kinases from a human breast carcinoma and from the human breast cancer cell line 600PEI; and Andres *et al.* isolated 24 protein kinases from murine mammary glands (Andres *et al.*, 1995; Cance *et al.*, 1993; Lehtola *et al.*, 1992). In total, these screens resulted in the identification of 43 protein kinases, 21 of which were also identified in the present study. In addition to these previously isolated kinases, our screen has resulted in the identification of an additional 20 kinases expressed in the mammary gland,

bringing the total number of kinases identified in this manner to 63.

In aggregate, beyond providing clues to the regulation of different stages of postnatal mammary development by specific protein kinases, approaches similar to those taken here should prove useful in identifying sequences of events, as well as coherent regulatory patterns, in mammary development. Moreover, by analogy with hematopoiesis, certain kinases may be expressed in the mammary gland in a lineage-restricted manner and may thereby serve as useful markers for epithelial or stromal cell subtypes. As such, it is likely that the composite spatial and temporal pattern of kinase expression in the mammary gland at any given developmental stage can provide important information regarding its physiological state and should provide insight into the molecules that regulate different stages of postnatal mammary development. Ultimately, the finding that most kinases expressed in the mammary gland are developmentally regulated suggests that the array of kinases participating in the regulation of mammary development is considerably broader than currently appreciated.

ACKNOWLEDGMENTS

The authors thank Celina D'Cruz, Stephen Master, Gerald Wertheim, and Eunkyung Kauh for helpful discussions and for critically reading the manuscript. This research was supported by the Elsa U. Pardee Foundation, American Cancer Society RPG-99-259-01-DDC, NIH Grants CA83849, CA71513 and CA78410 from the National Cancer Institute, the Charles E. Culpeper Foundation, and U.S. Army Breast Cancer Research Program Grants DAMD17-96-1-6112 (H.P.G.), DAMD17-98-1-8235 (D.B.S.), DAMD17-98-1-8226, DAMD-99-1-9463, and DAMD-99-1-9349. L.A.C. is a Charles E. Culpeper Medical Scholar.

REFERENCES

Aasheim, H. C., Terstappen, L. W., and Logtenberg, T. (1997). Regulated expression of the Eph-related receptor tyrosine kinase Hek11 in early human B lymphopoiesis. *Blood* **90,** 3613–3622.

Adams, R. H., Wilkinson, G. A., Weiss, C., Diella, F., Gale, N. W., Deutsch, U., Risau, W., and Klein, R. (1999). Roles of ephrinB ligands and EphB receptors in cardiovascular development: Demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. *Genes Dev.* **13,** 295–306.

Adnane, J., Gaudray, P., Dionne, C. A., Crumley, G., Jaye, M., Schlessinger, J., Jeanteur, P., Birnbaum, D., and Theillet, C. (1991). BEK and FLG, two receptors to members of the FGF family, are amplified in subsets of human breast cancers. *Oncogene* **6,** 659–663.

Andres, A.-C., Zuercher, G., Djonov, V., Flueck, M., and Ziemiecki, A. (1995). Protein tyrosine kinase expression during the estrous cycle and carcinogenesis of the mammary gland. *Int. J. Cancer* **63,** 288–296.

Bergemann, A. D., Zhang, L., Chiang, M. K., Brambilla, R., Klein, R., and Flanagan, J. G. (1998). Ephrin-B3, a ligand for the receptor EphB3, expressed at the midline of the developing neural tube. *Oncogene* **16,** 471–480.

Brinkley, P. M., Class, K., Bolen, J. B., and Penhallow, R. C. (1995). Structure and developmental regulation of the murine ctk gene. *Gene* **163,** 179–184.

Cance, W. G., Craven, R. J., Weiner, T. M., and Liu, E. T. (1993). Novel protein kinases expressed in human breast cancer. *Int. J. Cancer* **54,** 571–577.

Cardiff, R. D., and Muller, W. J. (1993). Transgenic mouse models of mammary tumorigenesis. *Cancer Surv.* **16,** 97–113.

Cardiff, R. D., Sinn, E., Muller, W., and Leder, P. (1991). Transgenic oncogene mice. Tumor phenotype predicts genotype. *Am. J. Pathol.* **139,** 495–501.

Cho, R. J., Campbell, M. J., Winzeler, E. A., Steinmetz, L., Conway, A., Wodicka, L., Wolfsberg, T. G., Gabrielian, A. E., Landsman, D., Lockhart, D. J., and Davis, R. W. (1998). A genome-wide transcriptional analysis of the mitotic cell cycle. *Mol. Cell* **2,** 65–73.

Dymecki, S. M., Niederhuber, J. E., and Desiderio, S. V. (1990). Specific expression of a tyrosine kinase gene, blk, in B lymphoid cells. *Science* **247,** 332–336.

Elson, A., and Leder, P. (1995). Protein-tyrosine phosphatase epsilon. An isoform specifically expressed in mouse mammary tumors initiated by v-Ha-ras or neu. *J. Biol. Chem.* **270,** 26116–26122.

Ferrari, S., Manfredini, R., Tagliafico, E., Grande, A., Barbieri, D., Balestri, R., Pizzanelli, M., Zucchini, P., Citro, G., Zupi, G., *et al.* (1994). Antiapoptotic effect of c-fes protooncogene during granulocytic differentiation. *Leukemia* **8,** S91–94.

Fox, G. M., Holst, P. L., Chute, H. T., Lindberg, R. A., Janssen, A. M., Basu, R., and Welcher, A. A. (1995). cDNA cloning and tissue distribution of five human EPH-like receptor protein-tyrosine kinases. *Oncogene* **10,** 897–905.

Ganju, P., Walls, E., Brennan, J., and Reith, A. D. (1995). Cloning and developmental expression of Nsk2, a novel receptor tyrosine kinase implicated in skeletal myogenesis. *Oncogene* **11,** 281–290.

Gardner, H., Rajan, J., Copeland, N., Gilbert, D., Jenkins, N., and Chodosh, L. (2000a). Cloning, characterization, and chromosomal localization of *Pnck*, a calcium/calmodulin-dependent protein kinase. *Genomics*, in press.

Gardner, H. P., Wertheim, G. W., Ha, S. I., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Marquis, S. T., and Chodosh, L. A. (2000b). Cloning and characterization of *Hunk*, a novel mammalian SNF1-related protein kinase. *Genomics*, in press.

Guy, C. T., Muthuswamy, S. K., Cardiff, R. D., Soriano, P., and Muller, W. J. (1994). Activation of the c-Src tyrosine kinase is required for the induction of mammary tumors in transgenic mice. *Genes Dev.* **8,** 23–32.

Hanks, S., and Quinn, A. (1991). Protein kinase catalytic domain sequence database: Identification of conserved features of primary structure and classification of family members. *Methods Enzymol.* **200,** 38–79.

Itoh, N., Mima, T., and Mikawa, T. (1996). Loss of fibroblast growth factor receptors is necessary for terminal differentiation of embryonic limb muscle. *Development* **122,** 291–300.

Jin, L., Fuchs, A., Schnitt, S. J., Yao, Y., Joseph, A., Lamszus, K., Park, M., Goldberg, I. D., and Rosen, E. M. (1997). Expression of scatter factor and c-met receptor in benign and malignant breast tissue. *Cancer* **79,** 749–760.

Klijn, J., Berns, E., and Foekens, J. (1993). Prognostic factors and response to therapy in breast cancer. *In* "Breast Cancer" (I. Fentiman and J. Taylor-Papadimitriou, Eds.), Vol. 18, pp. 165–198. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Kluppel, M., Donoviel, D. B., Brunkow, M. E., Motro, B., and Bernstein, A. (1997). Embryonic and adult expression patterns of the Tec tyrosine kinase gene suggest a role in megakaryocyto-

poiesis, blood vessel development, and melanogenesis. *Cell Growth Differ.* **8,** 1249–1256.

Korobko, I. V., Kabishev, A. A., and Kiselev, S. L. (1997). [Identification of the new protein kinase specifically transcribed in mouse tumors with high metastatic potential]. *Doklady Akad. Nauk* **354,** 554–556.

Krull, C. E., Lansford, R., Gale, N. W., Collazo, A., Marcelle, C., Yancopoulos, G. D., Fraser, S. E., and Bronner-Fraser, M. (1997). Interactions of Eph-related receptors and ligands confer rostrocaudal pattern to trunk neural crest migration. *Curr. Biol.* **7,** 571–580.

Kurioka, K., Nakagawa, K., Denda, K., Miyazawa, K., and Kitamura, N. (1998). Molecular cloning and characterization of a novel protein serine/threonine kinase highly expressed in mouse embryo. *Biochim. Biophys. Acta* **1443,** 275–284.

Lai, C., Gore, M., and Lemke, G. (1994). Structure, expression, and activity of Tyro 3, a neural adhesion-related receptor tyrosine kinase. *Oncogene* **9,** 2567–2578.

Lambe, M., Hsieh, C.-C., Tricholpoulos, D., Ekbom, A., Pavia, M., and Adami, H.-O. (1994). Transient increase in the risk of breast cancer after giving birth. *N. Engl. J. Med.* **331,** 5–9.

Leder, A., Pattengale, P. K., Kuo, A., Stewart, T. A., and Leder, P. (1986). Consequences of widespread deregulation of the c-myc gene in transgenic mice: Multiple neoplasms and normal development. *Cell* **45,** 485–495.

Lee, K. S., Yuan, Y.-L. O., Kuriyama, R., and Erikson, R. L. (1995). Plk is an M-phase-specific protein kinase and interacts with a kinesin-like protein, CHO1/MKLP-1. *Mol. Cell. Biol.* **15,** 7143–7151.

Lehtola, L., Partanen, J., Sistonen, L., Korhonen, J., Warri, A., Harkonen, P., Clarke, R., and Alitalo, K. (1992). Analysis of tyrosine kinase mRNAs including four FGF receptor mRNAs expressed in MCF-7 breast cancer cells. *Int. J. Cancer* **50,** 598–603.

Li, J., Simpson, L., Takahashi, M., Miliaresis, C., Myers, M. P., Tonks, N., and Parsons, R. (1998). The PTEN/MMAC1 tumor suppressor induces cell death that is rescued by the AKT/protein kinase B oncogene. *Cancer Res.* **58,** 5667–5672.

Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, S. H., Giovanella, B. C., Ittmann, M., Tycko, B., Hibshoosh, H., Wigler, M. H., and Parsons, R. (1997). PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer [see comments]. *Science* **275,** 1943–1947.

Liang, T. J., Reid, A. E., Xavier, R., Cardiff, R. D., and Wang, T. C. (1996). Transgenic expression of tpr-met oncogene leads to development of mammary hyperplasia and tumors. *J. Clin. Invest.* **97,** 2872–2877.

Liaw, D., Marsh, D. J., Li, J., Dahia, P. L., Wang, S. I., Zheng, Z., Bose, S., Call, K. M., Tsou, H. C., Peacocke, M., Eng, C., and Parsons, R. (1997). Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. *Nat. Genet.* **16,** 64–67.

Ligos, J. M., Gerwin, N., Fernandez, P., Gutierrez-Ramos, J. C., and Bernad, A. (1998). Cloning, expression analysis, and functional characterization of PKL12, a member of a new subfamily of Ser/Thr kinases. *Biochem. Biophys. Res. Commun.* **249,** 380–384.

MacMahon, B., Cole, P., Lin, T. M., Lowe, C. R., Mirra, A. P., Ravnihar, B., Salber, E. J., Valaoras, V. G., and Yuasa, S. (1970). Age at first birth and breast cancer risk. *Bull. WHO* **43,** 209–221.

MacMahon, B., Trichopoulos, D., Brown, J., Andersen, A. P., Aoki, K., Cole, P., DeWaard, F., Kaureniemi, T., Morgan, R. W., Purde, M., Ravnihar, B., Stormby, N., Westlund, K., and Woo, N.-C. (1982). Age at menarche, probability of ovulation and breast cancer risk. *Int. J. Cancer* **29,** 13–16.

Maggiora, P., Marchio, S., Stella, M. C., Giai, M., Belfiore, A., De Bortoli, M., Di Renzo, M. F., Costantino, A., Sismondi, P., and Comoglio, P. M. (1998). Overexpression of the RON gene in human breast carcinoma. *Oncogene* **16,** 2927–2933.

Manfredini, R., Balestri, R., Tagliafico, E., Trevisan, F., Pizzanelli, M., Grande, A., Barbieri, D., Zucchini, P., Citro, G., Franceschi, C., and Ferrari, S. (1997). Antisense inhibition of c-fes proto-oncogene blocks PMA-induced macrophage differentiation in HL60 and in FDC-P1/MAC-11 cells. *Blood* **89,** 135–145.

Mano, H., Sato, K., Yazaki, Y., and Hirai, H. (1994). Tec protein-tyrosine kinase directly associates with Lyn protein-tyrosine kinase through its N-terminal unique domain. *Oncogene* **9,** 3205–3211.

Mano, H., Yamashita, Y., Miyazato, A., Miura, Y., and Ozawa, K. (1996). Tec protein-tyrosine kinase is an effector molecule of Lyn protein-tyrosine kinase. *FASEB J.* **10,** 637–642.

Marquis, S. T., Rajan, J. V., Wynshaw-Boris, A., Xu, J., Yin, G.-Y., Abel, K. J., Weber, B. L., and Chodosh, L. A. (1995). The developmental pattern of *Brca1* expression implies a role in differentiation of the breast and other tissues. *Nat. Genet.* **11,** 17–26.

Morrison, B., and Leder, P. (1994). neu and ras initiate murine mammary tumors that share genetic markers generally absent in c-myc and int-2-initiated tumors. *Oncogene* **9,** 3417–3426.

Muller, W. J., Lee, F. S., Dickson, C., Peters, G., Pattengale, P., and Leder, P. (1990). The int-2 gene product acts as an epithelial growth factor in transgenic mice. *EMBO J.* **9,** 907–913.

Muller, W. J., Sinn, E., Pattengale, P. K., Wallace, R., and Leder, P. (1988). Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. *Cell* **54,** 105–115.

Munn, R., Webster, M., Muller, W., and Cardiff, R. (1995). Histopathology of transgenic mouse mammary tumors (a short atlas). *Semin. Cancer Biol.* **6,** 153–158.

Newcomb, P., Storer, B., Longnecker, M., Mittendorf, R., Greenberg, E., Clapp, R., Burke, K., Willett, W., and MacMahon, B. (1994). Lactation and a reduced risk of premenopausal breast cancer. *N. Engl. J. Med.* **330,** 81–87.

Niemann, C., Brinkmann, V., Spitzer, E., Hartmann, G., Sachs, M., Naundorf, H., and Birchmeier, W. (1998). Reconstitution of mammary gland development in vitro: Requirement of c-met and c-erbB2 signaling for branching and alveolar morphogenesis. *J. Cell Biol.* **143,** 533–545.

Partanen, J., Armstrong, E., Makela, T. P., Korhonen, J., Sandberg, M., Renkonen, R., Knuutila, S., Huebner, K., and Alitalo, K. (1992). A novel endothelial cell surface receptor tyrosine kinase with extracellular epidermal growth factor homology domains. *Mol. Cell. Biol.* **12,** 1698–1707.

Quintrell, N., Lebo, R., Varmus, H., Bishop, J. M., Pettenati, M. J., Le Beau, M. M., Diaz, M. O., and Rowley, J. D. (1987). Identification of a human gene (HCK) that encodes a protein-tyrosine kinase and is expressed in hemopoietic cells. *Mol. Cell. Biol.* **7,** 2267–2275.

Rajan, J. V., Marquis, S. T., Gardner, H. P., and Chodosh, L. A. (1997). Developmental expression of Brca2 colocalizes with Brca1 and is associated with differentiation in multiple tissues. *Dev. Biol.* **184,** 385–401.

Robinson, G. W., McKnight, R. A., Smith, G. H., and Hennighausen, L. (1995). Mammary epithelial cells undergo secretory differentiation in cycling virgins but require pregnancy for the establishment of terminal differentiation. *Development* **121,** 2079–2090.

Russo, I. H., and Russo, J. (1978). Developmental stage of the rat mammary gland as determinant of its susceptibility to 7,12-dimethylben(a)anthracene. *J. Natl. Cancer Inst.* **61,** 1439–1449.

Russo, J., and Russo, I. H. (1987). Biological and molecular bases of mammary carcinogenesis. *Lab. Invest.* **57,** 112–137.

Santoro, M. M., Collesi, C., Grisendi, S., Gaudino, G., and Comoglio, P. M. (1996). Constitutive activation of the RON gene promotes invasive growth but not transformation. *Mol. Cell. Biol.* **16,** 7072–7083.

Sato, K., Mano, H., Ariyama, T., Inazawa, J., Yazaki, Y., and Hirai, H. (1994). Molecular cloning and analysis of the human Tec protein-tyrosine kinase. *Leukemia* **8,** 1663–1672.

Sato, T. N., Qin, Y., Kozak, C. A., and Audus, K. L. (1993). Tie-1 and tie-2 define another class of putative receptor tyrosine kinase genes expressed in early embryonic vascular system. *Proc. Natl. Acad. Sci. USA* **90,** 9355–9358. [Published erratum appears in *Proc. Natl. Acad. Sci. USA,* 1993, **15,** 12056]

Sato, T. N., Tozawa, Y., Deutsch, U., Wolburg-Buchholz, K., Fujiwara, Y., Gendron-Maguire, M., Gridley, T., Wolburg, H., Risau, W., and Qin, Y. (1995). Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* **376,** 70–74.

Siliciano, J. D., Morrow, T. A., and Desiderio, S. V. (1992). itk, a T-cell-specific tyrosine kinase gene inducible by interleukin 2. *Proc. Natl. Acad. Sci. USA* **89,** 11194–11198.

Sinn, E., Muller, W., Pattengale, P., Tepler, I., Wallace, R., and Leder, P. (1987). Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: Synergistic action of oncogenes in vivo. *Cell* **49,** 465–475.

Slamon, D. J., Clark, G. M., and Wong, S. G. (1987). Human breast cancer: Correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. *Science* **235,** 177–182.

Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A., *et al.* (1989). Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* **244,** 707–712.

Stairs, D. B., Gardner, H. P., Ha, S. I., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and Chodosh, L. A. (1998). Cloning and characterization of Krct, a member of a novel subfamily of serine–threonine kinases. *Hum. Mol. Genet.* **7,** 2157–2166.

Stambolic, V., Suzuki, A., de la Pompa, J. L., Brothers, G. M., Mirtsos, C., Sasaki, T., Ruland, J., Penninger, J. M., Siderovski, D. P., and Mak, T. W. (1998). Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* **95,** 29–39.

Steck, P. A., Pershouse, M. A., Jasser, S. A., Yung, W. K., Lin, H., Ligon, A. H., Langford, L. A., Baumgard, M. L., Hattier, T., Davis, T., Frye, C., Hu, R., Swedlund, B., Teng, D. H., and Tavtigian, S. V. (1997). Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat. Genet.* **15,** 356–362.

Stitt, T. N., Conn, G., Gore, M., Lai, C., Bruno, J., Radziejewski, C., Mattsson, K., Fisher, J., Gies, D. R., Jones, P. F., *et al.* (1995). The anticoagulation factor protein S and its relative, Gas6, are ligands for the Tyro 3/Axl family of receptor tyrosine kinases. *Cell* **80,** 661–670.

Tamagnone, L., and Comoglio, P. M. (1997). Control of invasive growth by hepatocyte growth factor (HGF) and related scatter factors. *Cytokine Growth Factor Rev.* **8,** 129–142.

Tsarfaty, I., Resau, J. H., Rulong, S., Keydar, I., Faletto, D. L., and Vande Woude, G. F. (1992). The met proto-oncogene receptor and lumen formation. *Science* **257,** 1258–1261.

Tsukada, S., Saffran, D. C., Rawlings, D. J., Parolini, O., Allen, R. C., Klisak, I., Sparkes, R. S., Kubagawa, H., Mohandas, T., Quan, S., *et al.* (1993). Deficient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. *Cell* **72,** 279–290.

Ugolini, F., Adelaide, J., Charafe-Jauffret, E., Nguyen, C., Jacquemier, J., Jordan, B., Birnbaum, D., and Pebusque, M. J. (1999). Differential expression assay of chromosome arm 8p genes identifies Frizzled-related (FRP1/FRZB) and fibroblast growth factor receptor 1 (FGFR1) as candidate breast cancer genes. *Oncogene* **18,** 1903–1910.

Umemori, H., Wanaka, A., Kato, H., Takeuchi, M., Tohyama, M., and Yamamoto, T. (1992). Specific expressions of Fyn and Lyn, lymphocyte antigen receptor-associated tyrosine kinases, in the central nervous system. *Brain Res. Mol. Brain Res.* **16,** 303–310.

Valenzuela, D. M., Rojas, E., Griffiths, J. A., Compton, D. L., Gisser, M., Ip, N. Y., Goldfarb, M., and Yancopoulos, G. D. (1995a). Identification of full-length and truncated forms of Ehk-3, a novel member of the Eph receptor tyrosine kinase family. *Oncogene* **10,** 1573–1580.

Valenzuela, D. M., Stitt, T. N., DiStefano, P. S., Rojas, E., Mattsson, K., Compton, D. L., Nunez, L., Park, J. S., Stark, J. L., Gies, D. R., *et al.* (1995b). Receptor tyrosine kinase specific for the skeletal muscle lineage: Expression in embryonic muscle, at the neuromuscular junction, and after injury. *Neuron* **15,** 573–584.

Wang, M. H., Dlugosz, A. A., Sun, Y., Suda, T., Skeel, A., and Leonard, E. J. (1996). Macrophage-stimulating protein induces proliferation and migration of murine keratinocytes. *Exp. Cell Res.* **226,** 39–46.

Webster, N. J., Resnik, J. L., Reichart, D. B., Strauss, B., Haas, M., and Seely, B. L. (1996). Repression of the insulin receptor promoter by the tumor suppressor gene product p53: A possible mechanism for receptor overexpression in breast cancer. *Cancer Res.* **56,** 2781–2788.

Wilks, A. F. (1989). Two putative protein-tyrosine kinases identified by application of the polymerase chain reaction. *Proc. Natl. Acad. Sci. USA* **86,** 1603–1607.

Wilks, A. F. (1991). Cloning members of protein-tyrosine kinase family using polymerase chain reaction. *Methods Enzymol.* **200,** 533–546.

Wilks, A. F., Kurban, R. R., Hovens, C. M., and Ralph, S. J. (1989). The application of the polymerase chain reaction to cloning members of the protein tyrosine kinase family. *Gene* **85,** 67–74.

Wyllie, A. H., Arends, M. J., Morris, R. G., Walker, S. W., and Evan, G. (1992). The apoptosis endonuclease and its regulation. *Semin. Immunol.* **4,** 389–397.

Yi, T. L., Bolen, J. B., and Ihle, J. N. (1991). Hematopoietic cells express two forms of lyn kinase differing by 21 amino acids in the amino terminus. *Mol. Cell. Biol.* **11,** 2391–2398.

Yokokura, H., Terada, O., Naito, Y., and Hidaka, H. (1997). Isolation and comparison of rat cDNAs encoding Ca^{2+}/calmodulin-dependent protein kinase I isoforms. *Biochim. Biophys. Acta* **1338,** 8–12.

Yu, G., Smithgall, T. E., and Glazer, R. I. (1989). K562 leukemia cells transfected with the human c-fes gene acquire the ability to undergo myeloid differentiation. *J. Biol. Chem.* **264,** 10276–10281.

Ziegler, S. F., Marth, J. D., Lewis, D. B., and Perlmutter, R. M. (1987). Novel protein-tyrosine kinase gene (hck) preferentially expressed in cells of hematopoietic origin. *Mol. Cell. Biol.* **7,** 2276–2285.

Received for publication November 22, 1999
Revised December 30, 1999
Accepted December 30, 1999

Genomics 63, 46–59 (2000)
doi:10.1006/geno.1999.6078, available online at http://www.idealibrary.com on IDEAL®

Cloning and Characterization of *Hunk*, a Novel Mammalian SNF1-Related Protein Kinase

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Received September 24, 1999; accepted November 23, 1999

We previously identified a novel protein kinase, *Hunk*, by means of a degenerate PCR screen designed to isolate kinases expressed in the murine mammary gland. We now describe the molecular cloning, chromosomal localization, and activity of this kinase and characterize its spatial and temporal pattern of expression in the mouse. We have isolated a 5.0-kb full-length cDNA clone that contains the 714-amino-acid open reading frame encoding Hunk. Analysis of this cDNA reveals that Hunk is most closely related to the SNF1 family of serine/threonine kinases and contains a newly described SNF1 homology domain. Accordingly, antisera specific for Hunk detect an 80-kDa polypeptide with associated phosphotransferase activity. *Hunk* is located on distal mouse chromosome 16 in a region of conserved synteny with human chromosome 21q22. During fetal development and in the adult mouse, *Hunk* mRNA expression is developmentally regulated and tissue-specific. Moreover, *in situ* hybridization analysis reveals that *Hunk* expression is restricted to subsets of cells within a variety of organs in the adult mouse. These findings suggest a role for Hunk in murine development. © 2000 Academic Press

INTRODUCTION

Major insights into the molecular mechanisms of differentiation, development, and carcinogenesis have been obtained through studies of protein kinases in a wide range of biological systems. The finding that aberrantly regulated or aberrantly functioning protein kinases can disrupt normal developmental processes or promote carcinogenesis illustrates the fact that phosphorylation events play critical roles in the regulation of cell growth and differentiation. In addition, some protein kinases are expressed in a lineage-specific manner and are thereby useful markers for defining cellular subtypes (Dymecki *et al.*, 1990; Mischak *et al.*, 1991; Rawlings and Witte, 1994; Schnurch and Risau, 1993; Siliciano *et al.*, 1992; Valenzuela *et al.*, 1995).

The key role played by serine/threonine kinases in regulating diverse cellular processes is exemplified by studies of SNF1-related kinases. Several members of the SNF1 family of kinases function in signal transduction pathways involved in the cellular response to nutritional or environmental stresses (Hardie *et al.*, 1994). The *Saccharomyces cerevisiae* protein kinase, SNF1, and its mammalian counterpart, AMP-activated protein kinase (AMPK), function in highly conserved signal transduction pathways that promote energy conservation. SNF1 family members have also been implicated in a variety of developmental processes including the regulation of cellular proliferation and differentiation. These include *Msk* in murine cardiac development, SNRK in adipocyte differentiation, C-TAK1 in cell cycle control, and the *Caenorhabditis elegans* SNF-1 related kinase, PAR-1, in the establishment of embryonic polarity (Becker *et al.*, 1996; Guo and Kemphues, 1995; Peng *et al.*, 1998; Ruiz *et al.*, 1994). In fact, SNF-1 itself has been found to mediate cell cycle arrest in response to starvation (Thompson-Jaeger *et al.*, 1991). Thus, members of the SNF1 kinase family have been demonstrated to regulate a variety of important cellular processes.

In light of the importance of protein kinases in development and carcinogenesis, we previously performed a degenerate PCR-based screen aimed at identifying protein kinases expressed in the murine mammary gland during development and in mammary epithelial cell lines derived from different transgenic mouse models of breast cancer (Chodosh *et al.*, 1999, submitted for publication; Gardner *et al.*, in press; Stairs *et al.*, 1998). In the course of these studies, we identified a cDNA encoding a catalytic domain fragment from a novel protein kinase, *Hunk*. In this report, we show that *Hunk* encodes an 80-kDa polypeptide

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0888-7543/00 $35.00

associated with phosphotransferase activity. *Hunk* is evolutionarily conserved, being most closely related to the SNF1 family of serine/threonine kinases, and is located on mouse chromosome 16 in a region of conserved synteny with human chromosome 21q22. *Hunk* expression in the mouse is developmentally regulated and tissue-specific. Interestingly, within several tissues in the adult mouse, *Hunk* is expressed in a heterogeneous pattern, suggesting that the expression of this kinase is restricted to particular subtypes of cells within a variety of tissues.

MATERIALS AND METHODS

Cloning of a full-length Hunk cDNA. Poly(A)$^+$ RNA isolated from the MMTV-*Ha-ras* transgenic mammary epithelial tumor cell line, AC816 (Morrison and Leder, 1994), or from FVB mouse embryos harvested at day 14 of gestation was used to generate independent cDNA libraries using either the Uni-ZAP (AC816) or the Zap Express (day 14 embryo) lambda phage vector (Stratagene) according to the manufacturer's instructions. A total of 5×10^5 plaques from each library were screened by standard methods using a [α-^{32}P]dCTP-labeled random-primed cDNA probe (BMB Random Prime). The catalytic domain fragment corresponding to nucleotides 618 to 824 of *Hunk* was used to screen two independently generated AC816 cDNA libraries. The day 14 mouse embryo cDNA library was subsequently screened using cDNA fragments corresponding to nucleotides 132 to 500 and 276 to 793 of *Hunk*. Hybridization was performed at a concentration of 10^6 cpm/ml in 48% formamide, 10% dextran sulfate, 4.8× SSC, 20 mM Tris (pH 7.5), 1× Denhardt's solution, 20 μg/ml salmon sperm DNA, and 0.1% SDS at 42°C overnight. Following hybridization, blots were washed in 2× SSC/0.1% SDS at room temperature (RT) for 30 min (×2), followed by 0.2× SSC/0.1% SDS at 50°C for 20 min (×2), and subjected to autoradiography (Kodak XAR-5). Positive phage clones were plaque purified, and plasmids were liberated by *in vivo* excision according to the manufacturer's instructions (Stratagene). From each library, the independent clone with the largest insert size was completely sequenced by automated sequencing using an ABI Prism 377 DNA sequencer. The full-length *Hunk* cDNA sequence has been deposited with the GenBank database (Accession No. AF167987).

Sequence analysis. Sequence analysis including predicted open reading frames and calculation of predicted molecular weights was performed using MacVector (Oxford Molecular Group). Pairwise and multiple sequence alignments of kinase catalytic domains were performed using the ClustalW alignment program BLOSUM series with an open gap penalty of 10, an extend gap penalty of 0.05, and a delay divergent of 40%. Multiple sequence alignment and phylogenetic calculations were performed using the ClustalX multisequence alignment program with the same parameters as above. DendroMaker 4.0 was used to draw an unrooted phylogenetic tree.

Tissue preparation. FVB mouse embryos were harvested at specified time points following timed matings. Day 0.5 postcoitus was defined as noon of the day on which a vaginal plug was observed. Tissues used for RNA preparation and protein extracts were harvested from 15- to 16-week-old virgin mice and snap frozen on dry ice. Tissues used for *in situ* hybridization analysis were embedded in OCT compound.

Northern analysis. RNA was prepared by homogenization of snap-frozen tissue samples or tissue culture cells in guanidinium isothiocyanate supplemented with 7 μl/ml of 2-mercaptoethanol followed by ultracentrifugation through cesium chloride as previously described (Marquis *et al.,* 1995; Rajan *et al.,* 1997). Poly(A)$^+$ RNA was selected using oligo(dT) cellulose (Pharmacia), separated on a 0.7% LE agarose gel, and passively transferred to a GeneScreen membrane (NEN). Northern hybridization was performed as described using a ^{32}P-labeled cDNA probe encompassing nucleotides 1149 to 3849 of *Hunk* generated by random-primed labeling (BMB) (Marquis *et al.,* 1995). Hybridization was carried out as detailed above for cDNA library screening.

In vitro transcription/translation. In vitro transcription and translation were performed on 1 μg of plasmid DNA using rabbit reticulocyte lysates in the presence of either [^{35}S]Met or unlabeled methionine according to the manufacturer's instructions (TNT kit, Promega). Completed reactions were electrophoresed on a 10% SDS–PAGE gel and were subjected either to autoradiography or to immunoblotting as described below.

Generation of anti-Hunk antisera. GST–Hunk recombinant fusion proteins containing amino-teminal (amino acids 32–213) or carboxyl-terminal (amino acids 556–714) regions of Hunk were expressed in BL21 bacterial cells and purified using glutathione–Sepharose beads according to the manufacterer's instructions (Pharmacia). Following removal of the GST portion by cleavage with Prescission Protease (Pharmacia), the liberated carboxyl-terminal Hunk polypeptide was further purified by isolation on a 15% SDS–PAGE gel. Purified Hunk polypeptides were injected into rabbits (Cocalico Biologicals) in cleavage buffer (amino-terminal) or embedded in acrylamide gel slices (carboxyl-terminal). Antisera were affinity-purified on cyanogen bromide-coupled Sepharose columns crosslinked with their respective antigens according to the manufacturer's instructions (Pharmacia). Bound antibodies were then eluted sequentially with 100 mM glycine, pH 2.5, and 100 mM triethylamine, pH 11.5, and neutralized with 1/10 vol of 1.0 M Tris (pH 7.5) (Harlow and Lane, 1999).

Immunoblotting analysis. Protein extracts were generated by lysing tissue culture cells or homogenizing murine mammary glands in EBC buffer composed of 50 mM Tris (pH 7.9), 120 mM NaCl, and 0.5% NP-40 supplemented with 1 mM β-glycerol phosphate, 50 mM NaF, 20 μg/ml aprotinin, 100 μg/ml Pefabloc (BMB), and 10 μg/ml leupeptin. Equivalent amounts of each extract were electrophoresed on 10% SDS–PAGE gels and transferred overnight onto nitrocellulose membranes. Following visualization by Ponceau staining to verify equal protein loading and even transfer, membranes were incubated with blocking solution consisting of 4% dry milk, 0.05% Tween 20, and 1× phosphate-buffered saline (PBS) at RT. Primary antibody incubation with affinity-purified antisera was performed at RT for 1 h at a final concentration of approximately 2 μg/ml in blocking solution. Following three RT washes in blocking solution, blots were incubated with a 1:10,000 dilution of a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch) for 30 min at RT. Following three washes in blocking solution and two washes in 1× PBS, blots were developed using the ECL Plus system according to the manufacturer's instructions (Amersham Pharmacia) followed by exposure to film (Kodak XAR-5).

Immunoprecipitation of Hunk. Protein was extracted from tissue culture cells by lysis in EBC buffer for 15 min at 4°C. From each extract, 500 μg of protein in 250 μl of EBC was precleared with 40 μl of 1:1 Protein A-Sepharose:PBS for 3 h at 4°C. Precleared lysates were incubated overnight at 4°C with affinity-purified antisera raised against the amino-terminus of Hunk (3 μg), the carboxyl-terminus of Hunk (0.1 μg), or polypeptides unrelated to Hunk (0.1 or 3 μg). Immune complexes were precipitated by incubating with 40 μl of 1:1 Protein A-Sepharose:PBS for 3 h at 4°C. Complexes were washed twice with PBS, washed once with EBC, and electrophoresed on a 10% SDS–PAGE gel. Following transfer onto nitrocellulose membranes immunoblotting was performed as described above.

Kinase assay. Protein was extracted from snap-frozen lactating murine mammary glands and from 8Ma1a cells (Morrison and Leder, 1994) by dounce homogenization in EBC buffer containing protease inhibitors. Extracts containing 820 μg protein in 1 ml EBC were precleared with 40 μl 1:1 Protein A-Sepharose:PBS (Pharmacia) for 1 h at 4°C. One-quarter of the precleared lysate was incubated at 4°C overnight with 1.2 μg/ml of affinity-purified antisera raised against the amino-terminus of Hunk. Immune complexes were precipitated with 40 μl of 1:1 Protein A-Sepharose:PBS. *In vitro*

kinase activity of the resulting immunoprecipitates was assayed under final reaction conditions consisting of 20 mM Tris (pH 7.5), 5 mM $MgCl_2$, 100 μM dATP, 0.5 μCi/μl [γ-^{32}P]ATP, and 0.15 μg/μl histone H1 for 45 min at 37°C. Reactions were electrophoresed on a 15% SDS–PAGE gel and were subjected to autoradiography.

RNase protection analysis. Ribonuclease protection analysis was performed as described (Marquis *et al.,* 1995). Body-labeled antisense riboprobes were generated using linearized plasmids containing nucleotides 276 to 500 of the *Hunk* cDNA and 1142 to 1241 of *β-actin* (GenBank Accession No. X03672) using [α-^{32}P]UTP and the Promega *in vitro* transcription system with T7 polymerase. The β-actin antisense riboprobe was added to each reaction as an internal control. Probes were hybridized with RNA samples at 58°C overnight in 50% formamide/100 mM Pipes (pH 6.7). Hybridized samples were digested with RNase A and T1, purified, electrophoresed on a 6% denaturing polyacrylamide gel, and subjected to autoradiography.

In situ hybridization. In situ hybridization was performed as described (Marquis *et al.,* 1995). Antisense and sense probes were synthesized with the Promega *in vitro* transcription system using ^{35}S-UTP and ^{35}S-CTP from the T7 and SP6 RNA polymerase promoters of a PCR template containing sequences corresponding to nucleotides 276 to 793 of *Hunk,* a region demonstrated to recognize both mRNA transcripts. Exposure times were 6 weeks in all cases.

Interspecific mouse backcross mapping. Interspecific backcross progeny were generated by mating (C57BL/6J × *Mus spretus*)F_1 females and C57BL/6J males as described (Copeland and Jenkins, 1991). A total of 205 N_2 mice were used to map the *Hunk* locus (see below for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern transfer, and hybridization were performed essentially as described (Jenkins *et al.,* 1982). All blots were prepared with Hybond-N^+ nylon membrane (Amersham). A 520-bp *Eco*RI fragment corresponding to nucleotides 276 to 793 of the *Hunk* cDNA was labeled with [α-^{32}P]dCTP using a nick-translation labeling kit (Boehringer Mannheim). Washing was performed at a final stringency of 1.0× SSCP/0.1% SDS at 65°C. A major fragment of 6.9 kb was detected in *Sac*I-digested C57BL/6J DNA, and a major fragment of 5.8 kb was detected in *Sac*I-digested *M. spretus* DNA. The presence or absence of the 5.8-kb *Sac*I *M. spretus*-specific fragment was followed in backcross mice.

A description of the probes and RFLPs for the loci linked to *Hunk* including *App, Tiam1,* and *Erg* has been reported previously (Fan *et al.,* 1996). Recombination distances were calculated using Map Manager, version 2.6.5. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

RESULTS

We previously performed a screen designed to detect protein kinases expressed in the murine mammary gland and in breast cancer cell lines to identify regulatory molecules potentially involved in mammary development and carcinogenesis. RT-PCR with degenerate oligonucleotide primers was employed to amplify catalytic subdomains of protein kinases expressed in six murine breast cancer cell lines or in the murine mammary gland during various stages of development (Chodosh *et al.,* 1999, submitted for publication; Gardner *et al.,* in press; Stairs *et al.,* 1998). Individual PCR products were subcloned and screened by a combination of DNA sequencing and colony-lift hybridization. Examination of approximately 1500 cDNA clones from this screen resulted in the identification of 41 protein kinases including 33 tyrosine kinases and 8 serine/threonine kinases, 3 of which were novel.

One of these novel putative serine/threonine kinases, originally referred to as *Bstk1,* was first identified as a 207-bp RT-PCR product isolated from a mammary epithelial cell line derived from an adenocarcinoma arising in an MMTV-*neu* transgenic mouse (Chodosh *et al.,* 1999, submitted for publication). The cDNA encoding *Bstk1* was subsequently found to be upregulated in the mammary gland during early pregnancy and following treatment with 17β-estradiol and progesterone. In addition, *Bstk1* was found to be preferentially expressed in mammary tumor cell lines derived from MMTV-*neu* as compared with MMTV-*c-myc* transgenic mice (Gardner *et al.,* submitted for publication). Based upon this expression pattern, *Bstk1* was renamed *Hunk,* for hormonally upregulated, *neu*-tumor-associated kinase.

Isolation of cDNA Clones Encoding Hunk

To isolate the full-length mRNA transcript from which *Bstk1* was derived, the initial 207-bp RT-PCR product was used to screen a cDNA library prepared from the *H-ras* transformed mammary epithelial cell line, AC816 (Morrison and Leder, 1994). A cDNA probe derived from the 5′ end of the longest clone isolated, G3, was subsequently used to screen a day 14 murine embryonic cDNA library. Six additional nonchimeric cDNA clones ranging in length from 4.4 to 5.0 kb were isolated from this library; each of the clones possessed a poly(A) tail and a restriction pattern similar to that of G3 (data not shown). Dideoxy sequencing of the 5′ and 3′ termini of these clones in addition to restriction mapping revealed that all seven cDNA clones were contiguous. The longest cDNA clones isolated from each library, G3 and E8, were completely sequenced on both strands (Fig. 1). Comparison of the 5024-nt sequence of clone E8 with that of clone G3 revealed that clone E8 contains an additional 40 nucleotides at its 5′ end and that the length of a poly(T) tract in the 3′-untranslated region (UTR) of the two clones differs by a single nucleotide. There were no additional differences between these two clones.

The 5024-nucleotide sequence of clone E8, hereafter referred to as *Hunk,* contains the entire 207-bp RT-PCR fragment, *Bstk1,* from positions 618 to 824 (Fig. 1). *Hunk* possesses an open reading frame (ORF) 2142 nucleotides in length beginning with a putative initiation codon at nucleotide 72. Comparison of the nucleotide sequence surrounding this site with the Kozak (1987, 1991) consensus sequence, $GCC^{A}/_{G}CCAUGG$, reveals matches at positions −4, −3, and −2. The nucleotide sequence of the 5′-UTR and the first 100 nt of the *Hunk* ORF are extremely GC-rich (>80%). Other genes bearing such GC-rich sequences have been found to be subject to translational control (Kozak, 1991). The 3′-UTR of *Hunk* is 2.8 kb in length and lacks a canonical AATAAA polyadenylation signal, containing instead the relatively uncommon signal, AATACA, 18 nucleotides upstream of the poly(A) tract (Bishop *et al.,*

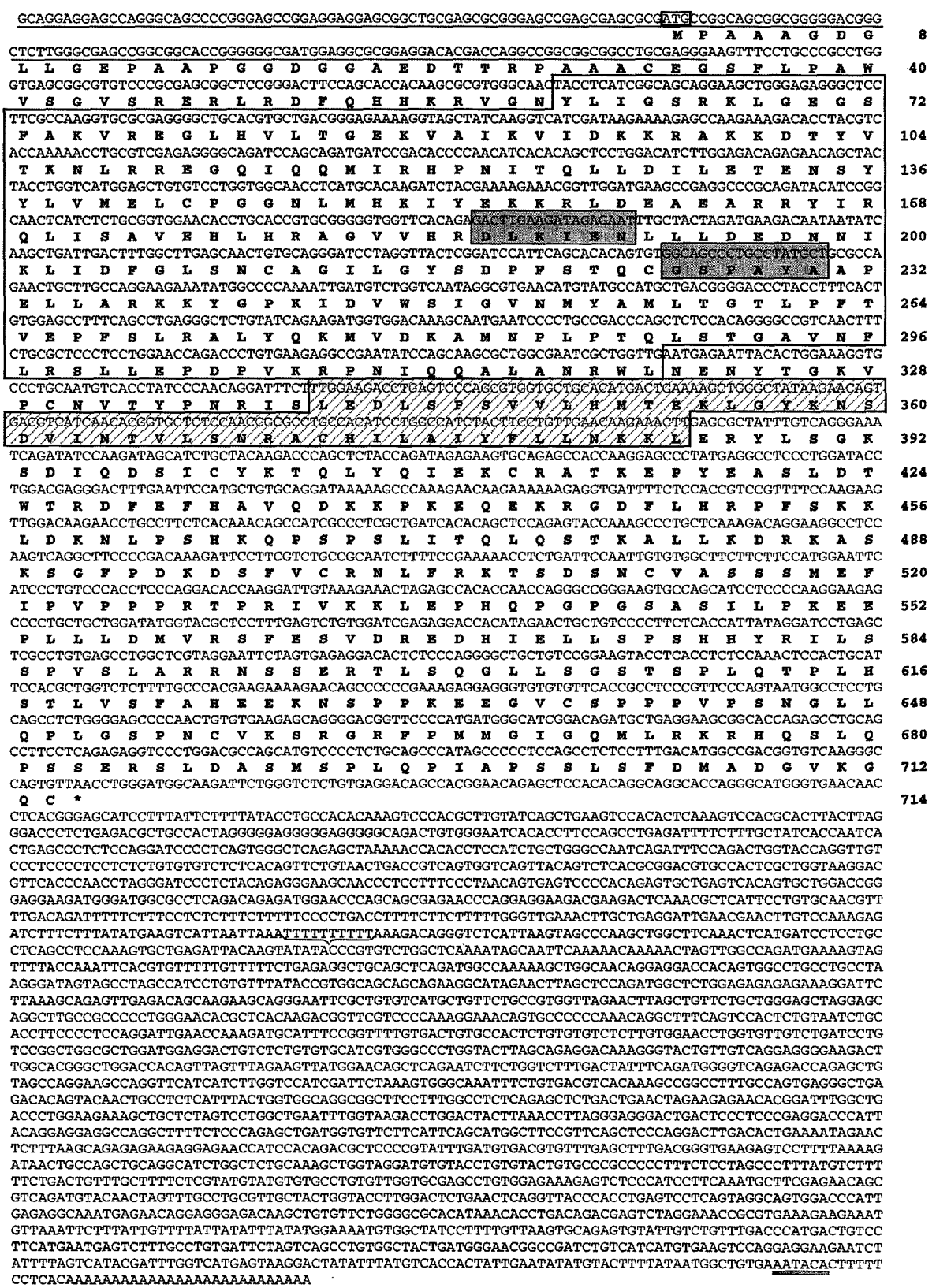

FIG. 1. Nucleotide and deduced amino acid sequence of *Hunk*. The composite nucleic acid sequence and conceptual translation of full-length *Hunk* cDNA are shown. Nucleotide coordinates are shown on the left. Amino acid coordinates are shown in boldface type on the right. A light shaded box indicates the kinase catalytic domain. Dark shaded boxes denote amino acid motifs characteristic of serine/threonine kinases. The SNF1 homology region, SNH, is denoted by a hatched box. The GC-rich region in the 5′-UTR and the putative polyadenylation sequence in the 3′-UTR are underlined by thin and thick lines, respectively. An asterisk denotes the stop codon. A bracket in the 3′ UTR denotes the poly(T) tract, which differs in length between the two independent cDNA clones (clone E8 is shown here).

1986; Herve *et al.,* 1995; Myohanen *et al.,* 1991, 1994; Parthasarathy *et al.,* 1997; Tokishita *et al.,* 1997).

While this work was in progress, a 588-nucleotide portion of the catalytic domain of *Hunk* was independently isolated by another group and shown to recognize an mRNA approximately 4 kb in length (Korobko *et al.,* 1997). Subsequently, this same group deposited a 5026-nt full-length sequence in GenBank (Accession No. AF055919) that is 10 nucleotides shorter at the 5′ end and 98% identical to *Hunk.* No additional information is available regarding the cloning, localization, function, or *in vivo* expression of this molecule.

The conceptual ORF of *Hunk* comprises 714 amino acids and encodes a polypeptide of predicted molecular mass 79.6 kDa. This polypeptide can be divided into an amino-terminal domain of 60 amino acids, a 260-amino-acid kinase catalytic domain, and a 394-amino-acid carboxyl-terminal domain. The carboxyl-terminal domain of Hunk contains a 46-amino-acid conserved motif located 18 amino acids C-terminal to the catalytic domain that is homologous to the previously described SNF1 homology region or SNH (Becker *et al.,* 1996). The 330 amino acids carboxyl-terminal to the SNH lack homology to other known proteins.

The putative catalytic domain of Hunk contains each of the invariant amino acid motifs characteristic of all protein kinases as well as sequences specific to serine/threonine kinases (Hanks and Quinn, 1991; Hanks *et al.,* 1988). In particular, the DLKPEN motif in subdomain VIB of the *Hunk* cDNA predicts serine/threonine kinase specificity (ten Dijke *et al.,* 1994). *Hunk* also contains the serine/threonine consensus sequence, G(T/S)XX(Y/F)X, in subdomain VIII N-terminal to the APE motif conserved among all protein kinases. In addition, several amino acids in subdomains I, VII, VIII, IX, X, and XI that are conserved among tyrosine kinases are absent from the *Hunk* ORF. Thus, primary sequence analysis strongly suggests that *Hunk* encodes a functional serine/threonine kinase.

To determine whether the length of the cDNA clone encoding Hunk is consistent with the size of the *Hunk* mRNA message, Northern hybridization was performed on poly(A)$^+$ RNA isolated from a *Hunk*-expressing mammary epithelial cell line (Fig. 2A). This analysis revealed a predominant mRNA transcript 5.1 kb in length, as well as a less abundant transcript approximately 5.6 kb in length, suggesting that clone E8 may correspond to the shorter *Hunk* mRNA transcript.

The finding that all six cDNA clones isolated from a cDNA library generated from mRNA containing both 5.1- and 5.6-kb *Hunk* mRNA species contain poly(A) tails and are colinear suggests that the 5.6-kb transcript may contain additional 5′ or 3′ sequence relative to our longest cDNA clone. Consistent with this supposition is the observation that insertions or deletions relative to our *Hunk* cDNA sequence were not detected using using multiple PCR primer pairs to perform RT-PCR on first-strand cDNA prepared from RNA containing both transcripts (data not shown). The failure to identify cDNA clones containing additional 5′ sequence may be related to the GC-rich nature of the 5′ UTR of *Hunk* and the tendency of reverse transcriptase to terminate prematurely in such regions. Alternately, the difference in size between the 5.1- and the 5.6-kb transcripts may be due to utilization of an alternate downstream polyadenylation site during mRNA processing.

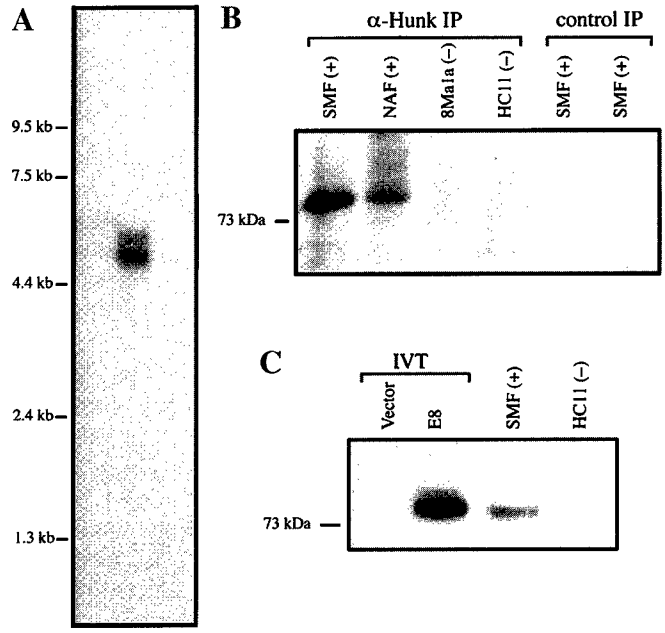

FIG. 2. Expression, identification, and coding potential of *Hunk.* (**A**) Northern hybridization analysis of 1 μg poly(A)$^+$ RNA from NAF mammary epithelial cells hybridized with a cDNA probe specific for Hunk. The relative migration of RNA size markers is indicated. (**B**) Immunoprecipitation of Hunk. Antisera raised against the amino-terminus of Hunk (α-Hunk IP) or against polypeptides unrelated to Hunk (control IP) were used to immunoprecipitate protein from lysates prepared from cells that either express (+) or do not express (−) *Hunk* mRNA. Immunoprecipitated protein was immunoblotted with antisera raised against the carboxyl-terminus of Hunk. (**C**) Immunoblotting analysis of Hunk protein using antisera raised against the carboxyl-terminus of Hunk. IVT reactions were performed in rabbit reticulocyte lysates in the presence of unlabeled methionine using either plasmid control (vector) or full-length Hunk cDNA (E8) as a template. IVT reaction products were resolved by SDS–PAGE along with lysates from *Hunk*-expressing (+) and non-expressing (−) cell lines. Note that the *in vitro* translated product detected with anti-Hunk antisera comigrates with the endogenous form of Hunk protein. The relative migration of the closest molecular weight marker is indicated.

To confirm the coding potential of the *Hunk* cDNA, *in vitro* transcription and translation of clone E8 were performed in the presence of [^{35}S]Met. This yielded an 80-kDa labeled polypeptide species, consistent with the 79.6-kDa predicted size of Hunk (data not shown), suggesting that the predicted initiation codon at nucleotide 72 is capable of functioning as a translation initiation site.

Detection of Hunk in Mammalian Cells

To detect the polypeptide encoded by the *Hunk* locus, anti-Hunk antisera were raised against recom-

binant proteins encoding amino-teminal (amino acids 32–213) and carboxyl-terminal (amino acids 556–714) regions of Hunk. Each of the antisera raised against the amino- and carboxyl-termini of Hunk identifies a polypeptide of approximately 80 kDa present in extracts from mammary epithelial cells that express *Hunk* mRNA, but not in extracts from mammary epithelial cells that do not (Fig. 2C; and data not shown). To demonstrate that this 80-kDa polypeptide corresponds to Hunk, protein extracts prepared from two mammary epithelial cell lines that express *Hunk* mRNA and from two mammary epithelial cell lines that do not express *Hunk* mRNA were subjected to immunoprecipitation/immunoblotting protocols (Fig. 2B). Immunoprecipitation of Hunk using antisera raised against the amino-terminus of Hunk, followed by immunoblotting with antisera raised against the carboxyl-terminus of Hunk, identified an 80-kDa polypeptide only in extracts prepared from cells that express *Hunk* mRNA (Fig. 2B). Similarly, immunoprecipitation of Hunk using antisera raised against the carboxyl-terminus of Hunk, followed by immunoblotting with antisera raised against the amino-terminus of Hunk, also identified an 80-kDa polypeptide only in extracts prepared from cells that express *Hunk* mRNA (data not shown). The 80-kDa polypeptide was not detected when immunoblotting was performed on immunoprecipitates prepared from *Hunk*-expressing cells when immunoprecipitation was carried out using either of two control affinity-purified antisera (Fig. 2B; and data not shown). We conclude that this 80-kDa polypeptide represents the endogenous Hunk gene product in these mammary epithelial cell lines.

To prove that clone E8 encodes the predominant form of Hunk found in mammary epithelial cells, we determined whether the *in vitro* translated product of clone E8 comigrates with endogenous Hunk. Immunoblotting of protein extracts prepared from the *Hunk* mRNA-expressing mammary epithelial cell line SMF and from rabbit reticulocyte lysates programmed with sense RNA prepared by *in vitro* transcription of clone E8 identified comigrating 80-kDa polypeptides (Fig. 2C). No band was detected in reticulocyte lysates programmed with an empty vector or in whole-cell lysates from a cell line that does not express *Hunk* mRNA. The observation that the 80-kDa polypeptide identified by anti-Hunk antisera comigrates with the polypeptide obtained following *in vitro* transcription and translation of clone E8 strongly suggests that clone E8 contains the entire ORF encoding the predominant form of *Hunk* found in mammary epithelial cells. Nevertheless, due to the absence of in-frame stop codons upstream of the putative translation initiation codon, the possibility that additional 5′ coding sequence exists cannot be excluded.

Predicted Structure and Homology to Previously Isolated Protein Kinases

Multiple sequence alignment was used to determine the homology between the kinase catalytic domain of Hunk and other previously isolated protein kinases (Fig. 3A). This analysis revealed that Hunk displays highest homology to the *S. cerevisiae* SNF1 family of serine/threonine kinases. The SNF1 family of protein kinases is composed of at least two subfamilies. The first subfamily includes SNF1 and its plant homologues including NPK5, AKin10, BKIN12, and Rkin1 as well as the mammalian SNF1 functional homologue, AMPK (Alderson *et al.,* 1991; Carling *et al.,* 1994; Le Guen *et al.,* 1992; Muranaka *et al.,* 1994). More recently, additional mammalian SNF1-related kinases have been identified that define a second subfamily. These include C-TAK1/p78, MARK1, MARK2/Emk, SNRK, and Msk, as well as the *C. elegans* kinase, PAR-1 (Becker *et al.,* 1996; Drewes *et al.,* 1997; Peng *et al.,* 1997, 1998; Ruiz *et al.,* 1994). Less closely related to either subfamily are Wpk4, Melk, and KIN1, SNF1-related kinases found in wheat, mice, and *Schizosaccharomyces pombe,* respectively (Heyer *et al.,* 1997; Levin and Bishop, 1990; Sano and Youssefian, 1994). Similar to these more distantly related SNF1 kinases, Hunk does not appear to belong to a previously defined SNF1 subfamily. Thus, based upon homology within the kinase domain, *Hunk* appears to represent a new branch of the SNF1 family tree.

Outside of a conserved kinase catalytic domain, SNF1-related protein kinases contain a region of homology referred to as the SNH or SNF1 homology domain (Becker *et al.,* 1996). Although amino acids in this motif are conserved, the functional significance of the SNH domain is unknown. Multiple sequence alignment confirms the presence of the SNH in all SNF1 family members shown in Fig. 3A and permits refinement of the conserved features of this domain (Fig. 3B). This analysis reveals that the SNH is anchored approximately 20 amino acids carboxyl-terminal to the kinase domain, spans approximately 45 amino acids, and extends further toward the amino terminus than previously reported. Our consensus identifies amino acids exhibiting greater than 70% conservation among the SNF1 family members shown as well as residues that are specific for particular SNF1 kinase subfamilies.

Although most conserved residues are shared among all family members, some residues are relatively specific for a particular subfamily. For example, the consensus amino acid at position 32 of the SNH is glutamine in subfamily I SNF1 kinases and tyrosine in subfamily II kinases. Subclass-specific residues are also found at positions 37 (A versus V) and 45 (K/R versus N). More distantly related SNF1 family members such as Wpk4 and KIN1 also have SNH domains, though the degree of homology is lower and in some cases the spacing is not conserved. Outside of its kinase and SNH domains, Hunk displays no detectable homol-

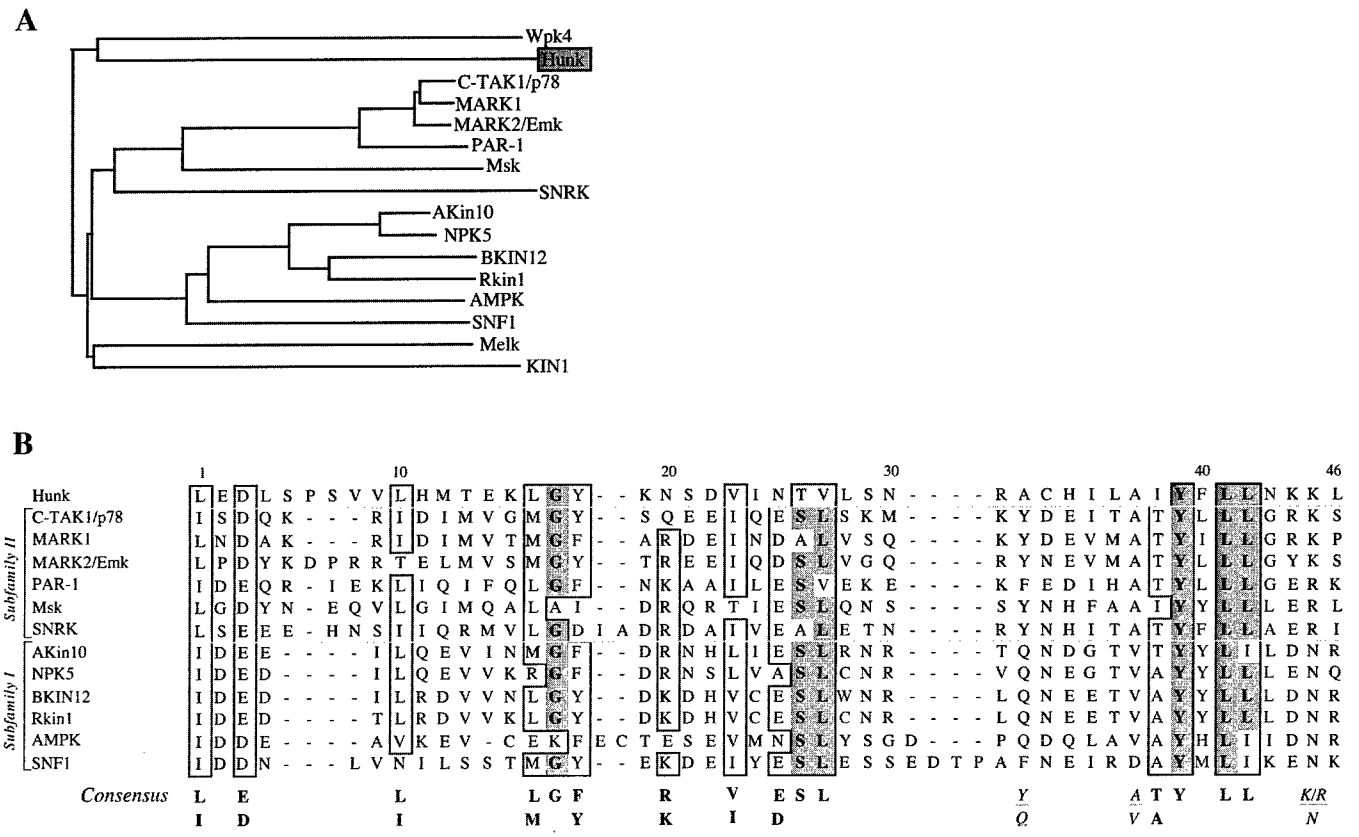

FIG. 3. Hunk represents a member of a novel subfamily of SNF1-related serine/threonine kinases. (**A**) Phylogenetic tree illustrating the relationship of Hunk kinase catalytic subdomains I–XI to other SNF1 family members. Analysis of results was performed using the ClustalX multisequence alignment program, and results were depicted using DendroMaker 4.0. (**B**) Amino acid alignment of SNF1 family members demonstrating conserved residues in the SNF homology domain. Positions at which an amino acid occurs with greater than 70% frequency are indicated in boldface type with dark shading. Positions at which similar amino acids occur with greater than 70% frequency are shown with light shading. A consensus sequence for all conserved residues is shown in boldface type at the bottom. Residues conserved within subfamilies are shown on the consensus line in regular type and separated by a gray line. A gray line also separates members of the two SNF1 subfamilies as denoted on the left side. Gaps (−) were introduced to maximize the alignment. Numbering is shown on top and is relative to Hunk spacing. Database accession numbers used are as follows: 80944 (Wpk4); 3089349 (C-TAK1); Z83868 (MARK1); Z83869 (MARK2); U22183 (PAR-1); U11494 (Msk); X89383 (SNRK); JC1446 (AKin10); A56009 (NPK5); S24578 (BKIN12); A41361 (Rkin1); Z29486 (AMPK); A26030 (SNF1); X95351 (Melk); and A38903 (*S. pombe* KIN1).

ogy to other members of the SNF1 family or to other known molecules.

Chromosomal Localization

The mouse chromosomal location of *Hunk* was determined by interspecific backcross analysis using progeny derived from matings of [(C57BL/6J × *M. spretus*)F_1 × C57BL/6J] mice (Fig. 4). This interspecific backcross mapping panel has been typed for over 2800 loci that are well distributed among all the autosomes as well as the X chromosome (Copeland and Jenkins, 1991). C57BL/6J and *M. spretus* DNA samples were digested with several enzymes and analyzed by Southern blot hybridization for informative restriction fragment length polymorphisms (RFLPs) using a mouse *Hunk* cDNA probe. The 5.8-kb *Sac*I *M. spretus* RFLP (see Materials and Methods) was used to follow the segregation of the *Hunk* locus in backcross mice. The mapping results indicated that *Hunk* is located in the distal region of mouse chromosome 16 linked to *App, Tiam1,* and *Erg*. Although 104 mice were analyzed for every marker and are shown in the segregation analysis (Fig. 4), up to 152 mice were typed for some pairs of markers. Each locus was analyzed in pairwise combinations for recombination frequencies using the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the most likely gene order are: centromere–*App*–4/123–*Hunk*–0/130–*Tiam1*–4/152–*Erg*. The recombination frequencies (expressed as genetic distances in centimorgans ± the standard error) are –*App*–3.3 ± 1.6 (*Hunk, Tiam1*)–2.6 ± 1.3–*Erg*. No recombinants were detected between *Hunk* and *Tiam1* in 130 animals typed in common, suggesting that the two loci are within 2.3 cM of each other (upper 95% confidence limit).

We have compared our interspecific map of chromosome 16 with a composite mouse linkage map that reports the map location of many uncloned mouse mutations (http://www.informatics.jax.org/). *Hunk* mapped in a region of the composite map that lacks mouse mutations (data not shown).

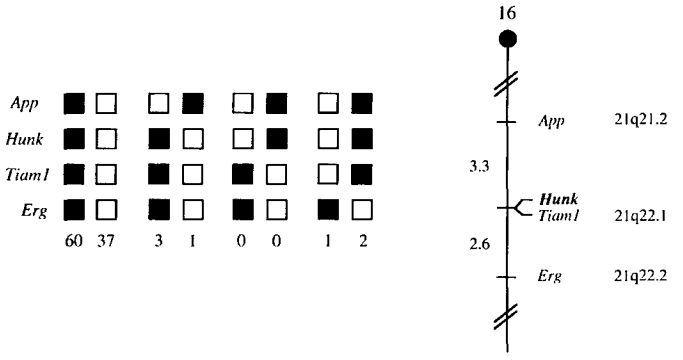

FIG. 4. *Hunk* maps in the distal region of mouse chromosome 16. *Hunk* was mapped to mouse chromosome 16 by interspecific backcross analysis. The segregation patterns of *Hunk* and flanking genes in 104 backcross animals that were typed for all loci are shown at the top of the figure. For individual pairs of loci, more than 104 animals were typed (see text). Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J × *M. spretus*)F_1 parent. The shaded boxes represent the presence of a C57BL/6J allele, and white boxes represent the presence of a *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. A partial chromosome 16 linkage map showing the location of *Hunk* in relation to linked genes is shown at the right. Recombination distances between loci in centimorgans are shown to the left of the chromosome, and the positions of loci in human chromosomes, where known, are shown to the right. References for the human map positions of loci cited in this study can be obtained from the GDB (Genome Data Base).

Hunk Encodes a Functional Protein Kinase

To address whether *Hunk* encodes a functional kinase, transgenic mice were engineered to overexpress Hunk in the mammary gland using the mouse mammary tumor virus LTR to direct *Hunk* expression (Gardner *et al.,* submitted for publication). Affinity-purified amino-terminal and carboxyl-terminal anti-Hunk antisera were used in immunoblotting experiments to detect Hunk in protein extracts prepared from the mammary glands of wildtype mice or MMTV-*Hunk* transgenic mice harvested at day 9 of lactation (Fig. 5A). Consistent with the degree of overexpression estimated from steady-state mRNA levels (data not shown), substantially higher levels of Hunk were detected in extracts prepared from transgenic compared with wildtype mammary glands. No Hunk protein was detected in extracts prepared from a mammary epithelial cell line previously shown not to express *Hunk* mRNA.

To demonstrate that Hunk protein levels are correlated with kinase activity, *in vitro* kinase assays were performed. Affinity-purified anti-Hunk antisera were used to immunoprecipitate Hunk from protein extracts prepared from the mammary glands of wildtype mice, transgenic mice overexpressing Hunk, or a mammary epithelial cell line that does not express *Hunk* mRNA. The resulting immunoprecipitates were incubated with [γ-^{32}P]ATP and either histone H1 or myelin basic protein as substrates (Fig. 5B; and data not shown). Hunk immunoprecipitates were able to phosphorylate both histone H1 and MBP *in vitro*. As predicted based on the relative quantities of Hunk immunoprecipitated from transgenic and wildtype mammary glands, Hunk-associated phosphotransferase activity was substantially greater in immunoprecipitates prepared from transgenic compared to wildtype mammary glands. No activity was observed in immunoprecipitates prepared from a cell line known not to express *Hunk* mRNA. These findings demonstrate that anti-Hunk antisera coimmunoprecipitate Hunk and a phosphotransferase, strongly suggesting that *Hunk* encodes a functional protein kinase.

Analysis of Hunk mRNA Expression

To begin to analyze the biological role played by *Hunk,* the spatial and temporal pattern of mRNA expression of this gene was determined both during fetal development and in adult tissues in the mouse. Northern hybridization analysis was performed on RNA isolated from FVB embryos at embryonic days E6.5, E13.5, and E18.5 using a unique *Hunk* cDNA probe. *Hunk* expression was not detected at E6.5, was dramatically up-regulated at E13.5, and was subsequently down-regulated at E18.5 (Fig. 6A). Similar to results obtained in mammary epithelial cells, analysis of embryonic mRNA revealed *Hunk* mRNA transcripts approximately 5.1 and 5.6 kb in length. Unlike expression in the mammary epithelial cell line, however, the 5.6-kb Hunk mRNA transcript was more abundant than the 5.1-kb transcript at E13.5, whereas the abundance of the two transcripts was equivalent at E18.5. This pattern suggests that *Hunk* transcripts may be regulated in both a developmental stage-specific and a tissue-specific manner.

To determine the spatial localization of *Hunk* mRNA expression during fetal development, ^{35}S-labeled antisense probes were used to perform *in situ* hybridization on E13.5 and E18.5 embryos (Figs. 6B–6K). These studies revealed intense organ-specific expression of *Hunk* mRNA at E13.5 in the brain, skin, and developing bone, as well as more diffuse expression throughout the embryo. Expression of *Hunk* was more restricted at

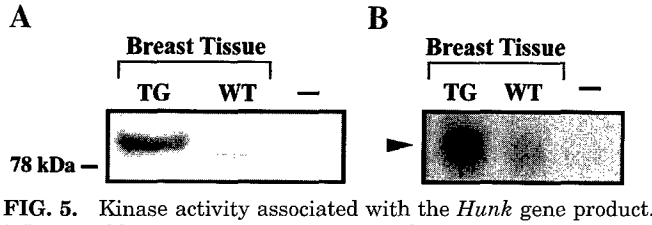

FIG. 5. Kinase activity associated with the *Hunk* gene product. (**A**) Immunoblotting using amino-terminal anti-Hunk antisera to analyze Hunk protein expression. 50 μg of protein extract prepared from mammary glands harvested from either MMTV-Hunk transgenic (TG) or wildtype (WT) mice, or 100 μg of protein extract prepared from HC11 cells, a mammary epithelial cell line that does not express *Hunk* mRNA (—), was analyzed by immunoblotting using amino-terminal anti-Hunk antisera. The relative migration of the 78-kDa marker is indicated. (**B**) *In vitro* kinase assay of Hunk immunoprecipitates. Histone H1 was used as an *in vitro* kinase substrate for Hunk protein immunoprecipitated from extracts containing 205 μg of protein as in Fig. 2B. An arrowhead indicates the relative migration of histone H1.

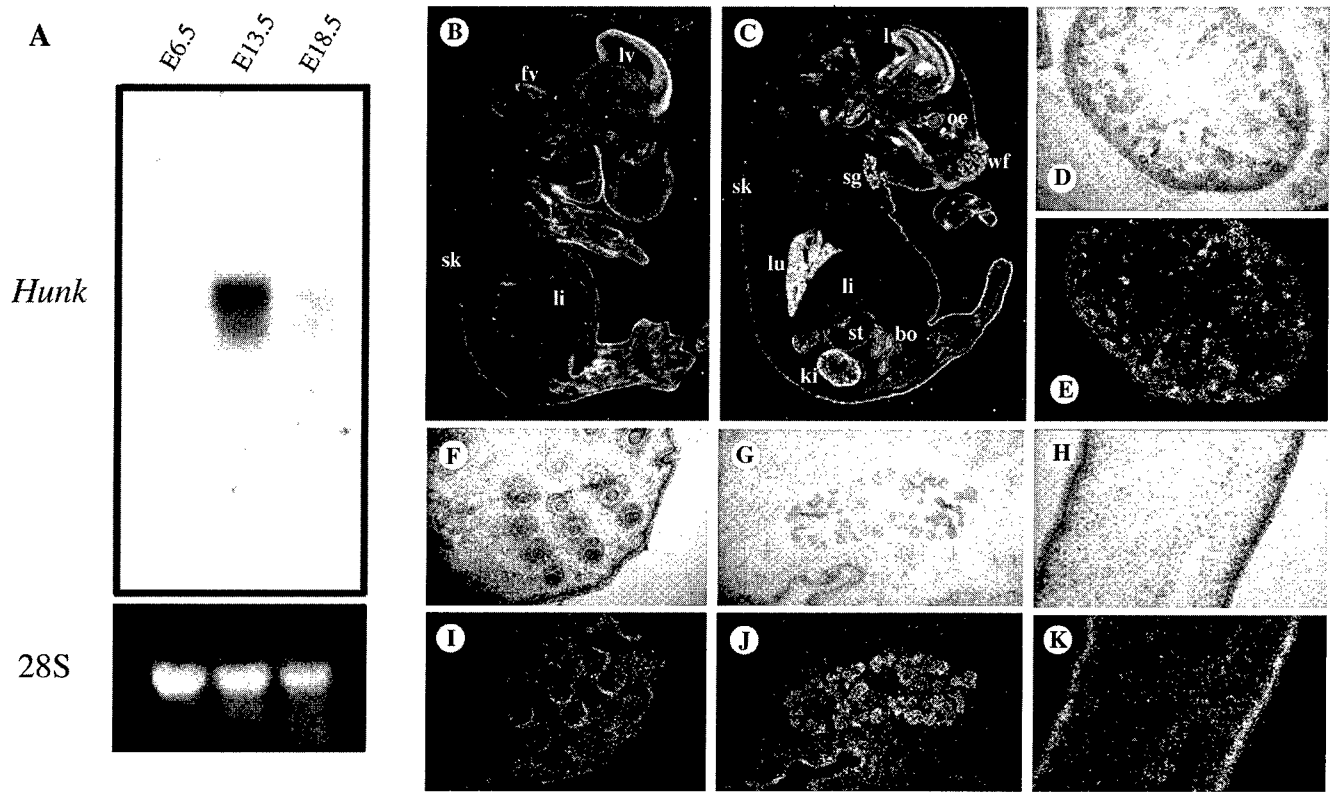

FIG. 6. Expression of *Hunk* during murine embryogenesis. (**A**) Northern hybridization analysis of 2 μg of poly(A)$^+$ RNA from day E6.5, E13.5, and E18.5 embryos hybridized with a cDNA probe specific for *Hunk*. The 28S ribosomal RNA band is shown as a loading control. (**B–K**) *In situ* hybridization analysis of *Hunk* mRNA expression . Bright-field (**D, F, G, H**) and dark-field (**B, C, E, I, J, K**) photomicrographs of E13.5 (**B**) and E18.5 (**C–K**) FVB embryo sections hybridized with an ^{35}S-lableled *Hunk* antisense cDNA probe. Tissues shown are kidney (**D, E**), whisker hair follicles (**F, I**), submandibular gland (**G, J**), and skin (**H, K**). No signal over background was detected in serial sections hybridized with sense *Hunk* probes. bo, bowel; fv, fourth ventricle; ki, kidney; li, liver; lu, lung; lv, lateral ventricle; oe, olfactory epithelium; sg, submandibular gland; sk, skin; st, stomach; wf, whisker hair follicle. Magnification: 8× (**B, C**); 20× (**D–K**). Exposure times were optimized for each panel.

E18.5, with particularly prominent hybridization in the brain, lung, salivary gland, olfactory epithelium, skin, whisker hair follicles, and kidney. Thus, *Hunk* expression during fetal development occurs in a developmentally regulated and tissue-specific manner.

The distribution of *Hunk* expression in tissues of the adult mouse was analyzed by RNase protection (Fig. 7A). High levels of *Hunk* expression were detected in ovary, thymus, lung, and brain, with modest levels of expression in breast, uterus, liver, kidney, and duodenum. *Hunk* mRNA expression was very low or undetectable in heart, skeletal muscle, testis, spleen, and stomach.

The spatial pattern of *Hunk* expression was determined in murine tissues by *in situ* hybridization (Figs. 7B–7M). Interestingly, this analysis revealed that *Hunk* is expressed in only a subset of cells within each expressing organ. In the duodenum, *Hunk* is expressed in a subset of epithelial cells located in duodenal crypts, whereas little or no expression is observed in more differentiated epithelial cells of the duodenum or in the mesenchymal compartment of this tissue (Figs. 7B and 7C). Heterogeneity is also observed among the crypt cells themselves, whereby cells expressing *Hunk* mRNA at high levels are located adjacent to cells expressing *Hunk* at substantially lower levels. Heterogeneous expression patterns are also observed in other tissues. For instance, *Hunk* mRNA expression in the uterus is restricted to isolated epithelial cells located in mesometrial glands (Figs. 7D and 7E). Similarly, *Hunk* expression in the prostate is found within only a subset of ductal epithelial cells (Figs. 7F and 7G). *Hunk* expression in the ovary is found principally in the stroma, with little or no expression detected in developing follicles or corpora lutea (Figs. 7H and 7I). *Hunk* expression in the thymus is limited primarily to the thymic medulla with lower levels of expression in the thymic capsule (Figs. 7J and 7K). High-power examination revealed that, as in other tissues, expression in the thymic medulla is markedly heterogeneous (Fig. 7L). *Hunk* is expressed throughout the brain, with particularly high levels in the cortex, dentate gyrus, and CA1–3 region of the hippocampus (Fig. 7M). High-power examination also revealed marked heterogeneity in *Hunk* expression among different cell types in the cerebral cortex (data not shown). Thus, *Hunk* is expressed in a variety of tissues of the adult mouse, and expression within these tissues is generally restricted to a subset of cells within a particular compartment or compartments.

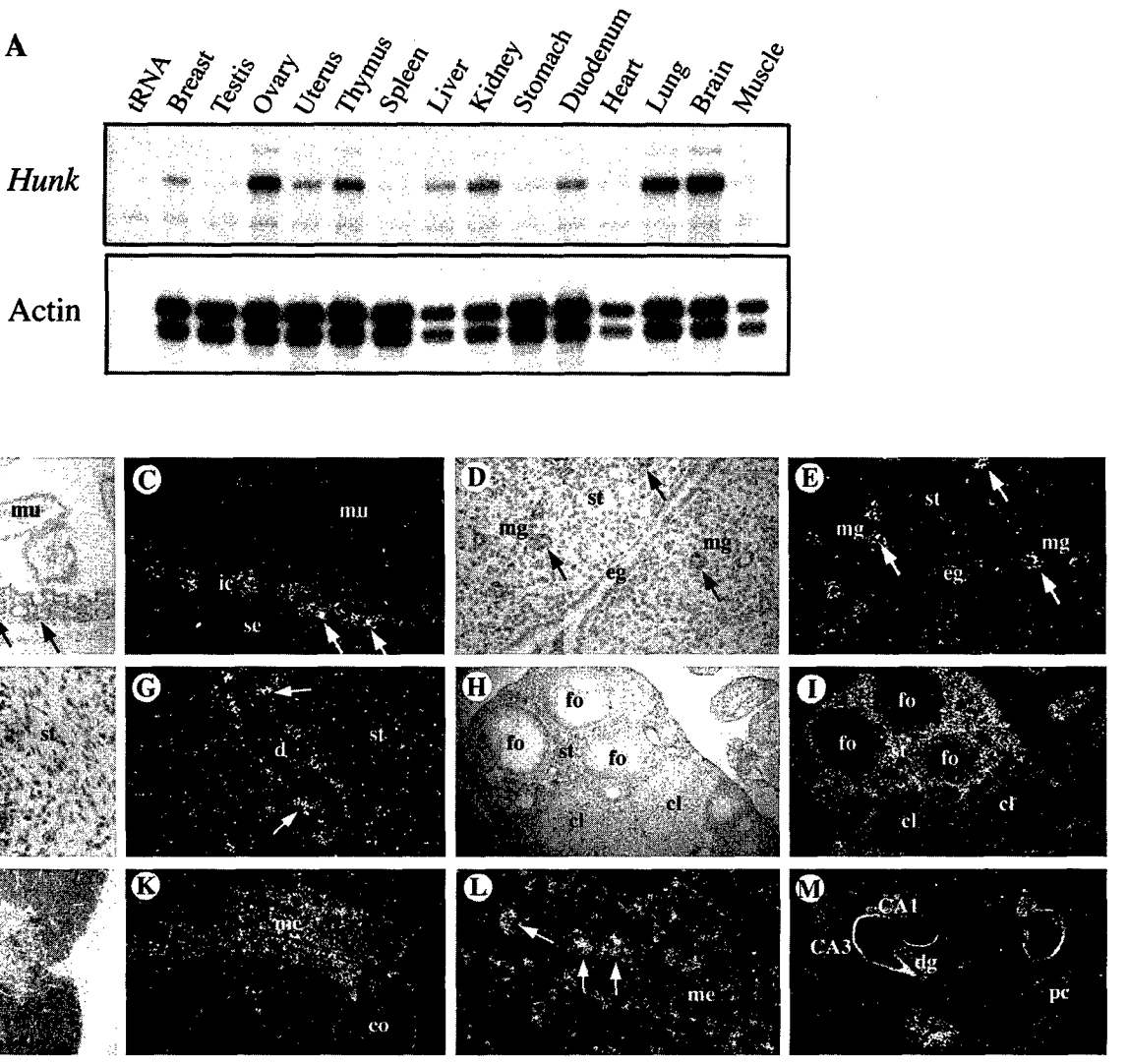

FIG. 7. Tissue-specific expression of *Hunk* in adult tissues. (**A**) RNase protection analysis of *Hunk* mRNA expression in tissues of the adult mouse. 30 μg of RNA isolated from the indicated murine tissues was hybridized with antisense RNA probes specific for *Hunk* and for *β-actin*. (**B–M**) Spatial localization of *Hunk* expression in tissues of the adult mouse. Bright-field (**B, D, F, H, J**) and dark-field (**C, E, G, I, K, L, M**) photomicrographs of *in situ* hybridization analysis performed on sections of duodenum (**B, C**), uterus (**D, E**), prostate (**F, G**), ovary (**H, I**), thymus (**J–L**), and brain (**M**), hybridized with a ^{35}S-labeled *Hunk* antisense probe. No signal over background was detected in serial sections hybridized with a sense *Hunk* probe. Arrows indicate cells expressing *Hunk* at high levels. CA1 and CA3, regions of the hippocampus; cl, corpus luteum; co, cortex; d, epithelial duct; dg, dentate gyrus; eg, endometrial gland; fo, follicle; ic, intestinal crypt; me, medulla; mg, mesometrial gland; mu, mucosa; pc, parietal cortex; se, serosa; st, stroma. Magnification: 10× (**M**), 90× (**H–K**), 180× (**B, C**), 300× (**D, E**) or 500× (**F, G, L, M**).

DISCUSSION

We initially identified the novel serine/threonine kinase, *Hunk,* in a screen designed to isolate protein kinases involved in mammary development and carcinogenesis. We have now described the cloning, chromosomal localization, and activity of this kinase and have characterized its expression in the mouse. *Hunk* is located on distal mouse chromosome 16, is transcribed as 5.1- and 5.6-kb mRNA species, and encodes an 80-kDa protein containing each of the amino acid motifs characteristic of serine/threonine kinases. Consistent with this, antisera that specifically immunoprecipitate Hunk coimmunoprecipitate phosphotransferase activity, and overexpression of Hunk in mammary epithelial cells increases the level of this phosphotransferase activity. *Hunk* expression in the mouse is developmentally regulated and tissue-specific both during fetal development and in the adult. Interestingly, within multiple tissues *Hunk* expression is restricted to subsets of cells within specific cellular compartments. These data suggest a role for Hunk in developmental processes in multiple tissues.

Several lines of evidence suggest that the *Hunk* cDNA sequence obtained represents the full-length *Hunk* ORF. First, Northern hybridization analysis of poly(A)$^+$ RNA isolated from mammary epithelial cell lines using a *Hunk*-specific cDNA probe identifies a predominant mRNA species 5.1 kb in length, consistent with the 5025-nt cDNA sequence obtained for clone E8. Second, *in vitro* transcription and translation

of clone E8 yield a polypeptide that is detected by anti-Hunk antisera, that comigrates with endogenous Hunk, and whose size is consistent with that predicted for the *Hunk* ORF. Third, comparison of the sequence of clone E8 with a recently isolated human *Hunk* cDNA clone reveals a high level of homology within the predicted ORF and a lower level of homology 5′ of the predicted initiation codon and 3′ of the predicted termination codon (H. P. Gardner *et al.,* in preparation). Finally, the observation that anti-Hunk antisera appear to recognize a single polypeptide species in lysates from cells known to express both transcripts provides evidence that we have isolated the entire ORF and that the 5.6-kb *Hunk* mRNA contains additional 5′ or 3′ untranslated sequence. Taken together, these findings suggest that the cDNA clones isolated represent a full-length *Hunk* transcript and contain the complete coding region.

Within the kinase catalytic domain, Hunk is most closely related to the SNF1 family of protein kinases, although Hunk appears to define a new branch on the SNF1 family tree. SNF1 is composed of a heterotrimeric complex that is activated by glucose starvation and is required for the expression of genes in response to nutritional stress (Carlson *et al.,* 1981; Celenza *et al.,* 1989; Ciriacy, 1977; Fields and Song, 1989; Wilson *et al.,* 1996; Yang *et al.,* 1992, 1994; Zimmermann *et al.,* 1977). Like SNF1, the mammalian SNF1-related kinase, AMPK, is involved in the cellular response to environmental stresses, particularly those that elevate cellular AMP:ATP ratios. Once activated, AMPK functions to decrease energy-requiring anabolic pathways such as sterol and fatty acid synthesis while upregulating energy-producing catabolic pathways such as fatty acid oxidation (Moore *et al.,* 1991; Ponticos *et al.,* 1998). AMPK complements the *snf1* mutation in yeast and phosphorylates some of the same targets as SNF1 (Hardie, 1999; Hardie *et al.,* 1997, 1999; Woods *et al.,* 1996). Like SNF1, AMPK is a heterotrimer composed of α, β, and γ subunits that are homologous to the subunits of SNF1 (Hardie, 1999). Thus, AMPK and SNF1 are closely related both functionally and structurally, demonstrating that the regulatory pathways in which they operate have been highly conserved during evolution.

Other SNF1 family members in plants, including Rkin1, BKIN12, AKin10, NPK5, and Wpk4, have been implicated in nutritional and environmental stress responses (Alderson *et al.,* 1991; Muranaka *et al.,* 1994; Sano and Youssefian, 1994; Wilson *et al.,* 1996). Like Hunk, several plant SNF1 family members are expressed in a tissue-specific manner. For example, AKIN10 is expressed in roots, shoots, and leaves, whereas RKIN1 is detected in developing endosperms but not in shoots (Alderson *et al.,* 1991; Le Guen *et al.,* 1992).

More recently, SNF1-related kinases have been identified in mammals and have been implicated in development processes, particularly in the regulation of cellular proliferation and differentiation. For instance, C-TAK1/p78 appears to be involved in cell cycle regulation based on its ability to phosphorylate and inactivate Cdc25c (Peng *et al.,* 1997, 1998). Since Cdc25c controls entry into mitosis by activating cdc2, inactivation of Cdc25c by C-TAK1 would be predicted to regulate proliferation negatively. Consistent with this model, C-TAK1/p78 is down-regulated in adenocarcinomas of the pancreas (Parsa, 1988).

Perhaps the most compelling evidence that SNF1 kinases are involved in development is the observation that mutations in the *C. elegans* SNF1-related kinase PAR-1 result in an inability to establish polarity in the developing embryo (Guo and Kemphues, 1995). Specifically, *par-1* mutations disrupt P granule localization, asymmetric cell divisions, blastomere fates, and mitotic spindle orientation during early embryogenesis. In an analogous manner, the mammalian PAR-1 homologue MARK2/Emk is asymmetrically localized in epithelial cells in vertebrates, and expression of a dominant negative form of MARK2 disrupts both cell polarity and epithelial cell–cell contacts (Bohm *et al.,* 1997). In addition, overexpression of either MARK2 or its close family member MARK1 results in hyperphosphorylation of microtubule-associated proteins, disruption of the microtubule array, and cell death (Drewes *et al.,* 1997).

Additional SNF1-related molecules, such as Msk and SNRK, have been implicated in vertebrate differentiation and development on the basis of their temporal and spatial patterns of expression (Becker *et al.,* 1996; Ruiz *et al.,* 1994). For example, *Msk* is expressed in presumptive myocardial cells during embryogenesis and is down-regulated following primitive heart tube formation, whereas SNRK is up-regulated during adipocyte differentiation. In a similar manner, our analysis of *Hunk* mRNA expression patterns suggests the possibility of a developmental role for Hunk in specific tissues. *Hunk* is expressed at high levels in the embryo during midgestation as cells are rapidly proliferating and differentiating and is down-regulated in the embryo prior to parturition. During fetal development, *Hunk* mRNA is expressed in a tissue-specific manner and is restricted to particular compartments within expressing tissues. Similarly, *Hunk* is also expressed in a tissue-specific manner in the adult mouse, and its expression is restricted to subsets of cells within these tissues. In aggregate, these data indicate that SNF1 family members participate in a wide range of developmental processes in higher eukaryotes and suggest that Hunk may also play an important role in one or more of these processes.

Outside the catalytic domain, a region of homology exists between SNF1 family members previously described as the SNH, or SNF1 homology region (Becker *et al.,* 1996). Since the distance between the catalytic domain and the SNH is conserved and since many kinases contain autoregulatory domains, it is plausible that the SNH domain functions to regulate kinase ac-

tivity (Yokokura *et al.,* 1995). Consistent with this speculation is the presence of weak homology between the SNH domain of SNF1 kinases and the autoinhibitory domain of the closely related family of calcium–calmodulin regulated kinases (data not shown). This homology does not extend into the adjacent calmodulin binding region, consistent with the observation that SNF1 kinases are not regulated by calcium. Regardless, the presence of the SNH domain in all SNF1 kinases raises the possibility that members of this family of molecules may be regulated by a common mechanism.

The distal portion of mouse chromosome 16 shares a region of conserved synteny with human chromosome 21q (summarized in Fig. 4). In particular, *Tiam1* has been mapped to 21q22.1. Mutations or segmental trisomy in this region of human chromosome 21 are associated with Alzheimer disease and Down syndrome, respectively. The close linkage between *Tiam1* and *Hunk* in the mouse suggests that the human homologue of *Hunk* will map to 21q22, as well. In fact, BLAST alignment of *Hunk* to sequences in GenBank reveals homology to human genomic DNA sequences cloned from 21q22.1 (gi4835629). This indicates that the human homologue of *Hunk* lies within a region of chromosome 21q22 believed to contribute to several of the phenotypic features characteristic of Down syndrome (Delabar *et al.,* 1993; Korenberg *et al.,* 1994; Rahmani *et al.,* 1989). In this regard, it is interesting to note that *Hunk* is expressed at high levels throughout the brain during murine fetal development as well as in the adult, with particularly high levels being found in the hippocampus, dentate gyrus, and cortex. Whether increased Hunk expression in the brain is related to the pathophysiology of Alzheimer disease or Down syndrome is unknown.

ACKNOWLEDGMENTS

The authors thank Christopher J. Sarkisian and Douglas B. Stairs for providing control antisera and technical expertise, members of the Chodosh laboratory for helpful discussions and for critically reading the manuscript, and Deborah B. Householder and Jayant V. Rajan for excellent technical assistance. This research was supported by the Elsa U. Pardee Foundation (L.A.C.), NIH Grants CA83849, CA71513, and CA78410 (L.A.C.) from the National Cancer Institute and the National Institute of Diabetes and Digestive and Kidney Diseases, RPG-99-259-01-DDC from the American Cancer Society (L.A.C.), the Dolores Zohrab Liebmann Fund (G.B.W.W.), the Charles E. Culpeper Foundation (L.A.C.), U.S. Army Breast Cancer Research Program Grants DAMD17-96-1-6112 (H.P.G.), DAMD17-99-1-9463, DAMD17-99-1-9349, and DAMD17-98-1-8226 (L.A.C.), and the National Cancer Institute, DHHS, under contract with ABL (N.A.J.). L.A.C. is a Charles E. Culpeper Medical Scholar.

REFERENCES

Alderson, A., Sabelli, P., Dickinson, J., Cole, D., Richardson, M., Kreis, M., Shewry, P., and Halford, N. (1991). Complementation of snf1, a mutation affecting global regulation of carbon metabolism in yeast, by a plant protein kinase cDNA. *Proc. Natl. Acad. Sci. USA* **88:** 8602–8605.

Becker, W., Heukelbach, J., Kentrup, H., and Joost, H. G. (1996). Molecular cloning and characterization of a novel mammalian protein kinase harboring a homology domain that defines a subfamily of serine/threonine kinases. *Eur. J. Biochem.* **235:** 736–743.

Bishop, D. F., Calhoun, D. H., Bernstein, H. S., Hantzopoulos, P., Quinn, M., and Desnick, R. J. (1986). Human alpha-galactosidase A: Nucleotide sequence of a cDNA clone encoding the mature enzyme. *Proc. Natl. Acad. Sci. USA* **83:** 4859–4863.

Bohm, H., Brinkmann, V., Drab, M., Henske, A., and Kurzchalia, T. V. (1997). Mammalian homologues of *C. elegans* PAR-1 are asymmetrically localized in epithelial cells and may influence their polarity. *Curr. Biol.* **7:** 603–606.

Carling, D., Aguan, K., Woods, A., Verhoeven, A. J., Beri, R. K., Brennan, C. H., Sidebottom, C., Davison, M. D., and Scott, J. (1994). Mammalian AMP-activated protein kinase is homologous to yeast and plant protein kinases involved in the regulation of carbon metabolism. *J. Biol. Chem.* **269:** 11442–11448.

Carlson, M., Osmond, B., and Botstein, D. (1981). Mutants of yeast defective in sucrose utilization. *Genetics* **98:** 25–40.

Celenza, J. L., Eng, F. J., and Carlson, M. (1989). Molecular analysis of the SNF4 gene of *Saccharomyces cerevisiae:* Evidence for physical association of the SNF4 protein with the SNF1 protein kinase. *Mol. Cell. Biol.* **9:** 5045–5054.

Chodosh, L. A., D'Cruz, C. M., Gardner, H. P., Ha, S. I., Marquis, S. T., Rajan, J. V., Stairs, D. B., Wang, J. Y., and Wang, M. (1999). Mammary gland development, reproductive history, and breast cancer risk. *Cancer Res.* **59:** 1765–1771S.

Chodosh, L. A., Gardner, H. P., Rajan, J. V., Stairs, D. B., Marquis, S. T., and Leder, P. Protein kinase expression during murine mammary gland development. Submitted for publication.

Ciriacy, M. (1977). Isolation and characterization of yeast mutants defective in intermediary carbon metabolism and in carbon catabolite repression. *Mol. Gen. Genet.* **154:** 213–220.

Copeland, N. G., and Jenkins, N. A. (1991). Development and applications of a molecular genetic linkage map of the mouse genome. *Trends Genet.* **7:** 113–118.

Delabar, J. M., Theophile, D., Rahmani, Z., Chettouh, Z., Blouin, J. L., Prieur, M., Noel, B., and Sinet, P. M. (1993). Molecular mapping of twenty-four features of Down syndrome on chromosome 21. *Eur. J. Hum. Genet.* **1:** 114–124.

Drewes, G., Ebneth, A., Preuss, U., Mandelkow, E. M., and Mandelkow, E. (1997). MARK, a novel family of protein kinases that phosphorylate microtubule-associated proteins and trigger microtubule disruption. *Cell* **89:** 297–308.

Dymecki, S. M., Niederhuber, J. E., and Desiderio, S. V. (1990). Specific expression of a tyrosine kinase gene, blk, in B lymphoid cells. *Science* **247:** 332–336.

Fan, C. M., Kuwana, E., Bulfone, A., Fletcher, C. F., Copeland, N. G., Jenkins, N. A., Crews, S., Martinez, S., Puelles, L., Rubenstein, L. R., and Tessier-Lavigne, M. (1996). Expression patterns of two murine homologs of *Drosophila* single-minded suggest possible roles in embryonic patterning and in the pathogenesis of Down syndrome. *Mol. Cell. Neurosci.* **7:** 519.

Fields, S., and Song, O. (1989). A novel genetic system to detect protein–protein interactions. *Nature* **340:** 245–246.

Gardner, H. P., Belka, G. K., Wertheim, G. B. W., Hartman, J. L., Ha, S. I., Gimmoty, P. A., Marquis, S. T., and Chodosh, L. A. Developmental role of the SNF1-related kinase, *Hunk,* in pregnancy-induced changes in the mammary gland. Submitted for publication.

Gardner, H. P., Rajan, J. V., Ha, S. I., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Marquis, S. T., and Chodosh, L. A. Cloning, characterization, and chromosomal localization of *Pnck,* a Ca^{2+}/calmodulin-dependent protein kinase. *Genomics,* in press.

Guo, S., and Kemphues, K. (1995). par-1, a gene required for establishing polarity in C. elegans embryos, encodes a putative ser/thr kinase that is asymmetrically distributed. *Cell* **81:** 611–620.

Hanks, S., and Quinn, A. (1991). Protein kinase catalytic domain sequence database: Identification of conserved features of primary structure and classification of family members. *Methods Enzymol.* **200:** 38–79.

Hanks, S. K., Quinn, A. M., and Hunter, T. (1988). The protein kinase family: Conserved features and deduced phylogeny of the catalytic domains. *Science* **241:** 42–52.

Hardie, D. G. (1999). Roles of the AMP-activated/SNF1 protein kinase family in the response to cellular stress. *Biochem. Soc. Symp.* **64:** 13–27.

Hardie, D. G., Carling, D., and Halford, N. (1994). Roles of the Snf1/Rkin1/AMP-activated protein kinase family in the response to environmental and nutritional stress. *Semin. Cell Biol.* **5:** 409–416.

Hardie, D. G., Corton, J., Ching, Y. P., Davies, S. P., and Hawley, S. (1997). Regulation of lipid metabolism by the AMP-activated protein kinase. *Biochem. Soc. Trans.* **25:** 1229–1231.

Hardie, D. G., Salt, I. P., Hawley, S. A., and Davies, S. P. (1999). AMP-activated protein kinase: An ultrasensitive system for monitoring cellular energy charge. *Biochem. J.* **338:** 717–722.

Harlow, E., and Lane, D. (1999). "Using Antibodies: A Laboratory Manual," Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Herve, D., Rogard, M., and Levi-Strauss, M. (1995). Molecular analysis of the multiple Golf alpha subunit mRNAs in the rat brain. *Brain Res. Mol. Brain Res.* **32:** 125–134.

Heyer, B. S., Warsowe, J., Solter, D., Knowles, B. B., and Ackerman, S. L. (1997). New member of the Snf1/AMPK kinase family, Melk, is expressed in the mouse egg and preimplantation embryo. *Mol. Reprod. Dev.* **47:** 148–156.

Jenkins, N. A., Copeland, N. G., Taylor, B. A., and Lee, B. K. (1982). Organization, distribution, and stability of endogenous ecotropic murine leukemia virus DNA sequences in chromosomes of *Mus musculus*. *J. Virol.* **43:** 26–36.

Korenberg, J. R., Chen, X. N., Schipper, R., Sun, Z., Gonsky, R., Gerwehr, S., Carpenter, N., Daumer, C., Dignan, P., Disteche, C., *et al.* (1994). Down syndrome phenotypes: The consequences of chromosomal imbalance. *Proc. Natl. Acad. Sci. USA* **91:** 4997–5001.

Korobko, I. V., Kabishev, A. A., and Kiselev, S. L. (1997). [Identification of the new protein kinase specifically transcribed in mouse tumors with high metastatic potential]. *Dokl. Akad. Nauk.* **354:** 554–556.

Kozak, M. (1987). An analysis of 5′-noncoding sequences from 699 vertebrate messanger RNAs. *Nucleic Acids Res.* **15:** 8125–8132.

Kozak, M. (1991). An analysis of vertebrate mRNA sequences: Intimations of translational control. *J. Cell Biol.* **115:** 887–903.

Le Guen, L., Thomas, M., Bianchi, M., Halford, N. G., and Kreis, M. (1992). Structure and expression of a gene from *Arabidopsis thaliana* encoding a protein related to SNF1 protein kinase. *Gene* **120:** 249–254.

Levin, D. E., and Bishop, J. M. (1990). A putative protein kinase gene (kin1+) is important for growth polarity in *Schizosaccharomyces pombe*. *Proc. Natl. Acad. Sci. USA* **87:** 8272–8276.

Marquis, S. T., Rajan, J. V., Wynshaw-Boris, A., Xu, J., Yin, G.-Y., Abel, K. J., Weber, B. L., and Chodosh, L. A. (1995). The developmental pattern of *Brca1* expression implies a role in differentiation of the breast and other tissues. *Nat. Genet.* **11:** 17–26.

Mischak, H., Kolch, W., Goodnight, J., Davidson, W. F., Rapp, U., Rose-John, S., and Mushinski, J. F. (1991). Expression of protein kinase C genes in hemopoietic cells is cell-type- and B cell-differentiation stage specific. *J. Immunol.* **147:** 3981–3987.

Moore, F., Weekes, J., and Hardie, D. G. (1991). Evidence that AMP triggers phosphorylation as well as direct allosteric activation of rat liver AMP-activated protein kinase. A sensitive mechanism to protect the cell against ATP depletion. *Eur. J. Biochem.* **199:** 691–697.

Morrison, B., and Leder, P. (1994). neu and ras initiate murine mammary tumors that share genetic markers generally absent in c-myc and int-2-initiated tumors. *Oncogene* **9:** 3417–3426.

Muranaka, T., Banno, H., and Machida, Y. (1994). Characterization of tobacco protein kinase NPK5, a homolog of *Saccharomyces cerevisiae* SNF1 that constitutively activates expression of the glucose-repressible SUC2 gene for a secreted invertase of *S. cerevisiae*. *Mol. Cell. Biol.* **14:** 2958–2965.

Myohanen, S., Kauppinen, L., Wahlfors, J., Alhonen, L., and Janne, J. (1991). Human spermidine synthase gene: Structure and chromosomal localization. *DNA Cell Biol.* **10:** 467–474.

Myohanen, S., Wahlfors, J., Alhonen, L., and Janne, J. (1994). Nucleotide sequence of mouse spermidine synthase cDNA. *DNA Seq.* **4:** 343–346.

Parsa, I. (1988). Loss of a M_r 78,000 marker in chemically induced transplantable carcinomas and primary carcinoma of human pancreas. *Cancer Res.* **48:** 2265–2272.

Parthasarathy, L., Parthasarathy, R., and Vadnal, R. (1997). Molecular characterization of coding and untranslated regions of rat cortex lithium-sensitive myo-inositol monophosphatase cDNA. *Gene* **191:** 81–87.

Peng, C. Y., Graves, P. R., Ogg, S., Thoma, R. S., Byrnes, M. J., 3rd, Wu, Z., Stephenson, M. T., and Piwnica-Worms, H. (1998). C-TAK1 protein kinase phosphorylates human Cdc25C on serine 216 and promotes 14-3-3 protein binding. *Cell Growth Differ.* **9:** 197–208.

Peng, C. Y., Graves, P. R., Thoma, R. S., Wu, Z., Shaw, A. S., and Piwnica-Worms, H. (1997). Mitotic and G2 checkpoint control: Regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. *Science* **277:** 1501–1505.

Ponticos, M., Lu, Q. L., Morgan, J. E., Hardie, D. G., Partridge, T. A., and Carling, D. (1998). Dual regulation of the AMP-activated protein kinase provides a novel mechanism for the control of creatine kinase in skeletal muscle. *EMBO J.* **17:** 1688–1699.

Rahmani, Z., Blouin, J. L., Creau-Goldberg, N., Watkins, P. C., Mattei, J. F., Poissonnier, M., Prieur, M., Chettouh, Z., Nicole, A., Aurias, A., *et al.* (1989). Critical role of the D21S55 region on chromosome 21 in the pathogenesis of Down syndrome. *Proc. Natl. Acad. Sci. USA* **86:** 5958–5962.

Rajan, J. V., Marquis, S. T., Gardner, H. P., and Chodosh, L. A. (1997). Developmental expression of Brca2 colocalizes with Brca1 and is associated with differentiation in multiple tissues. *Dev. Biol.* **184:** 385–401.

Rawlings, D. J., and Witte, O. N. (1994). Bruton's tyrosine kinase is a key regulator in B-cell development. *Immunol. Rev.* **138:** 105–119.

Ruiz, J., Conlon, F., and Robertson, E. (1994). Identification of novel protein kinases expressed in the myocardium of the developing mouse heart. *Mech. Dev.* **48:** 153–164.

Sano, H., and Youssefian, S. (1994). Light and nutritional regulation of transcripts encoding a wheat protein kinase homolog is mediated by cytokinins. *Proc. Natl. Acad. Sci. USA* **91:** 2582–2586.

Schnurch, H., and Risau, W. (1993). Expression of tie-2, a member of a novel family of receptor tyrosine kinases, in the endothelial cell lineage. *Development* **119:** 957–968.

Siliciano, J. D., Morrow, T. A., and Desiderio, S. V. (1992). itk, a T-cell-specific tyrosine kinase gene inducible by interleukin 2. *Proc. Natl. Acad. Sci. USA* **89:** 11194–11198.

Stairs, D. B., Gardner, H. P., Ha, S. I., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and Chodosh, L. A. (1998). Cloning and characterization of Krct, a member of a novel subfamily of serine-threonine kinases. *Hum. Mol. Genet.* **7:** 2157–2166.

ten Dijke, P., Franzen, P., Yamashita, H., Ichijo, H., Heldin, C., and Miyazono, K. (1994). Serine-threonine kinase receptors. *Progr. Growth Factor Res.* **5:** 55–72.

Thompson-Jaeger, S., Francois, J., Gaughran, J. P., and Tatchell, K. (1991). Deletion of SNF1 affects the nutrient response of yeast and

resembles mutations which activate the adenylate cyclase pathway. *Genetics* **129:** 697–706.

Tokishita, S., Shiga, Y., Kimura, S., Ohta, T., Kobayashi, M., Hanazato, T., and Yamagata, H. (1997). Cloning and analysis of a cDNA encoding a two-domain hemoglobin chain from the water flea *Daphnia magna*. *Gene* **189:** 73–78.

Valenzuela, D. M., Stitt, T. N., DiStefano, P. S., Rojas, E., Mattsson, K., Compton, D. L., Nunez, L., Park, J. S., Stark, J. L., Gies, D. R., *et al.* (1995). Receptor tyrosine kinase specific for the skeletal muscle lineage: Expression in embryonic muscle, at the neuromuscular junction, and after injury. *Neuron* **15:** 573–584.

Wilson, W., Hawley, S., and Hardie, D. (1996). Glucose repression/derepression in budding yeast: SNF1 protein kinase is activated by phosphorylation under derepressing conditions, and this correlates with a high AMP:ATP ratio. *Curr. Biol.* **6:** 1426–1434.

Woods, A., Cheung, P. C., Smith, F. C., Davison, M. D., Scott, J., Beri, R. K., and Carling, D. (1996). Characterization of AMP-activated protein kinase beta and gamma subunits. Assembly of the heterotrimeric complex in vitro. *J. Biol. Chem.* **271:** 10282–10290.

Yang, X., Hubbard, E. J., and Carlson, M. (1992). A protein kinase substrate identified by the two-hybrid system. *Science* **257:** 680–682.

Yang, X., Jiang, R., and Carlson, M. (1994). A family of proteins containing a conserved domain that mediates interaction with the yeast SNF1 protein kinase complex. *EMBO J.* **13:** 5878–5886.

Yokokura, H., Picciotto, M. R., Nairn, A. C., and Hidaka, H. (1995). The regulatory region of calcium/calmodulin-dependent protein kinase I contains closely associated autoinhibitory and calmodulin-binding domains. *J. Biol. Chem.* **270:** 23851–23859.

Zimmermann, F., Kaufmann, I., Rasenberger, H., and Haussman, P. (1977). Genetics of carbon catabolite repression in *Saccharomyces cerevisiae:* Genes involved in the derepression process. *Mol. Gen. Genet.* **154:** 95–103.

Genomics 63, 279–288 (2000)
doi:10.1006/geno.1999.6091, available online at http://www.idealibrary.com on IDEAL®

Cloning, Characterization, and Chromosomal Localization of *Pnck*, a Ca^{2+}/Calmodulin-Dependent Protein Kinase

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Received September 20, 1999; accepted November 23, 1999

Calcium is an important second messenger in eukaryotic cells. Many of the effects of calcium are mediated via its interaction with calmodulin and the subsequent activation of Ca^{2+}/calmodulin-dependent (CaM) kinases. CaM kinases are involved in a wide variety of cellular processes including muscle contraction, neurotransmitter release, cell cycle control, and transcriptional regulation. While CaMKII has been implicated in learning and memory, the biological role of the other multifunctional CaM kinases, CaMKI and CaMKIV, is largely unknown. In the course of a degenerate RT-PCR protein kinase screen, we identified a novel serine/threonine kinase, *Pnck*. In this report, we describe the cloning, chromosomal localization, and expression of *Pnck*, which encodes a 38-kDa protein kinase whose catalytic domain shares 45–70% identity with members of the CaM kinase family. The gene for *Pnck* localizes to mouse chromosome X, in a region of conserved synteny with human chromosome Xq28 that is associated with multiple distinct mental retardation syndromes. *Pnck* is upregulated during intermediate and late stages of murine fetal development with highest levels of expression in developing brain, bone, and gut. *Pnck* is also expressed in a tissue-specific manner in adult mice with highest levels of expression detected in brain, uterus, ovary, and testis. Interestingly, *Pnck* expression in these tissues is restricted to particular compartments and appears to be further restricted to subsets of cells within those compartments. The chromosomal localization of *Pnck*, along with its tissue-specific and restricted pattern of spatial expression during development, suggests that *Pnck* may be involved in a variety of developmental processes including development of the central nervous system.

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. AF181984.

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INTRODUCTION

Many protein kinases function as intermediates in signal transduction pathways that control complex processes such as differentiation, development, and carcinogenesis (Birchmeier *et al.,* 1993; Bolen *et al.,* 1992; Rawlings and Witte, 1994). Accordingly, studies of protein kinases in a wide range of biological systems have led to a more comprehensive understanding of the regulation of cell growth and differentiation (Bolen, 1993; Fantl *et al.,* 1993; Hardie, 1990). Not surprisingly, several members of the protein kinase family have been shown to be involved in the pathogenesis of cancer both in humans and in rodent model systems (Cardiff and Muller, 1993; Dickson *et al.,* 1992; Guy *et al.,* 1992, 1994; Slamon *et al.,* 1989). In light of these findings, we performed a screen designed to identify and study the role of protein kinases in mammary development and carcinogenesis (Chodosh *et al.,* 2000, in press; Gardner *et al.,* in press; Stairs *et al.,* 1998). In the course of these studies, we identified a novel serine/threonine kinase, *Pnck* (pregnancy-upregulated nonubiquitous CaM kinase), that is related to the Ca^{2+}/calmodulin-dependent (CaM) family of protein kinases.

Ca^{2+} is an important intracellular second-messenger molecule in eukaryotic signal transduction pathways. Many of the effects of Ca^{2+} are mediated through its interaction with the Ca^{2+}-binding protein, calmodulin. The Ca^{2+}/calmodulin complex is, in turn, required for maximal activation of CaM-dependent protein kinases, which ultimately regulate cellular processes as diverse as neurotransmitter release, metabolism, and gene transcription (Fukunaga and Miyamoto, 1999; Lukas *et al.,* 1998; Matthews *et al.,* 1994; Nairn and Picciotto, 1994; Polishchuk *et al.,* 1995; Schulman, 1993; Sheng *et al.,* 1991). In addition to their regulation by CaM, CaM kinases share structural and functional homology both in the kinase catalytic domain and in a regulatory region composed of composite autoinhibitory and CaM-

0888-7543/00 $35.00

binding domains (Hanks and Quinn, 1991; Hanks *et al.,* 1988; Haribabu *et al.,* 1995; Knighton *et al.,* 1992; Picciotto *et al.,* 1996; Yokokura *et al.,* 1995).

Despite these similarities, significant differences exist between CaM kinase family members. For instance, this family includes members with high substrate specificity, such as myosin light-chain kinase (MLCK) and phosphorylase kinase, as well as members with broader substrate specificities collectively referred to as the multifunctional CaM kinases, such as CaMKI, CaMKIV, and members of the CaMKII subfamily (Braun and Schulman, 1995; Cawley *et al.,* 1993; Herring *et al.,* 1990; Matthews *et al.,* 1994; Schulman, 1993). Other properties that differ among CaM kinase family members include their subcellular localization, regulation by autophosphorylation, and regulation by other proteins. In addition, CaM kinases have unique amino- and carboxyl-terminal domains that contribute to kinase-specific differences in subcellular localization, subunit interactions, and other protein–protein interactions.

Much of the information available regarding the multifunctional CaM kinases is derived from studies conducted in the brain, in part because each of these kinases is expressed at high levels in this organ. However, CaMKII is the only multifunctional CaM kinase whose biological function has been defined (Hanley *et al.,* 1987; Jensen *et al.,* 1991b; Lin *et al.,* 1987; Picciotto *et al.,* 1993; Tobimatsu and Fujisawa, 1989; Tobimatsu *et al.,* 1988). The CaMKII holoenzyme is an oligomeric complex composed of combinations of independently encoded, highly homologous α, β, γ, and δ subunits. Mice with targeted disruption of CaMKIIα are deficient in long-term potentiation and exhibit specific defects in learning and memory (Silva *et al.,* 1992a, b). Unlike the α subunit of CaMKII whose expression is restricted to the brain, CaMKI, CaMKIV, and the δ and γ subunits of CaMKII have a broader tissue distribution and therefore presumably have as yet unrecognized functions in other tissues (Hanley *et al.,* 1987; Lin *et al.,* 1987; Naito *et al.,* 1997; Picciotto *et al.,* 1993, 1995; Tobimatsu and Fujisawa, 1989; Tobimatsu *et al.,* 1988).

In this report, we describe the cloning, chromosomal localization, and initial characterization of *Pnck,* a member of the CaM kinase family of protein serine/threonine kinases. We have isolated cDNA clones for *Pnck* that encode a 38-kDa polypeptide. The gene for *Pnck* localizes to mouse chromosome X in a region of conserved synteny with human chromosome Xq28 that has been implicated in distinct mental retardation syndromes (Lubs *et al.,* 1999). *Pnck* expression is developmentally regulated and tissue-specific during murine fetal development with high levels of expression in developing brain, bone, and gut. *Pnck* expression is also tissue-specific in adult mice with highest levels of expression in the hippocampus and dentate gyrus of the brain. Interestingly, within expressing tissues, *Pnck* expression is restricted to subsets of cells within particular compartments. These data suggest a role for *Pnck* in the development of the central nervous system and other tissues.

MATERIALS AND METHODS

Cloning of a full-length Pnck cDNA. The original catalytic domain fragment, *Bstk3,* was isolated from first-strand cDNA derived from mammary glands of mice at day 2 of postlactational involution using the degenerate oligonucleotide primers BSTKIa (5′-GGGCCCGGATCC(G/A)T(A/G)CAC(A/C)G(A/G/C)GAC(C/T)T-3′) and PTKIIa (5′-CCCGGGGAATTCCA(A/T)AGGACCA(G/C)AC(G/A)TC-3′) (Chodosh *et al.,* in press; Wilks, 1989, 1991; Wilks *et al.,* 1989). This original fragment, corresponding to nucleotides 501 to 704 of full-length *Pnck,* was used to screen 5×10^5 lambda phage plaques from an oligo(dT)-primed murine brain cDNA library according to standard protocols (CPMB). Primary screening yielded a total of 73 clones of varying hybridization intensity that were positive on duplicate filters. Ten clones with medium to high hybridization intensity were plaque purified, and plasmids were liberated by *in vivo* excision according to the manufacturer's instructions (Stratagene). Sequence analysis of 5 of these clones revealed a high level of homology to CaMKI. The remaining 5 clones were found to encode portions of *Pnck* as determined by overlapping sequence identity to one another and to *Bstk3.* Two clones were not studied further since one clone was chimeric and a second clone contained only partial *Pnck* sequence. Three nonchimeric clones, U7, V1, and Q3, were completely sequenced by automated sequencing using an ABI Prism 377 DNA sequencer. Nucleotide sequence alignment revealed no differences between the three clones outside of their respective 5′-untranslated region (UTR) sequences. The full-length *Pnck* cDNA sequence corresponding to the clone with the longest 5′-UTR, U7, has been deposited with the GenBank database (Accession No. AF181984).

Sequence analysis. Sequence analysis, including prediction of open reading frames, calculation of predicted molecular weights, multiple sequence alignment, and phylogenetic analysis, was performed using MacVector (Oxford Molecular Group), ClustalW, ClustalX, and DendroMaker 4.0. Pairwise and multiple sequence alignments of kinase catalytic domains I–XI were performed using the ClustalW alignment program. Calculations were made using the BLOSUM series with an open gap penalty of 10, an extended gap penalty of 0.05, and a delay divergent of 40%. Phylogenetic calculations with the same parameters were performed using the ClustalX multisequence alignment program. An unrooted phylogenetic tree was drawn using DendroMaker 4.0.

Tissue preparation. FVB mouse embryos were harvested at specified time points following timed matings. Day 0.5 postcoital was defined as noon of the day on which a vaginal plug was observed. Tissues used for RNA preparation were harvested from 15- to 16-week-old virgin mice and snap-frozen on dry ice. Tissues used for *in situ* hybridization analysis were embedded in OCT compound.

RNA analysis. RNA was prepared by homogenization of snap-frozen tissue samples in guanidinium isothiocyanate supplemented with 7 μl/ml 2-mercaptoethanol followed by ultracentrifugation through cesium chloride as previously described (Marquis *et al.,* 1995; Rajan *et al.,* 1997). Poly(A)$^+$ RNA was selected using oligo(dT) cellulose (Pharmacia), separated on a 1% LE agarose gel, and passively transferred to a Gene Screen membrane (NEN). Northern hybridization was performed as described using a ^{32}P-labeled cDNA probe encompassing nucleotides 1135 to 1509 of *Pnck* generated by random-primed labeling (BMB) (Marquis *et al.,* 1995).

Southern hybridization analysis was performed on a zoo-blot (Clontech) hybridized with a ^{32}P-labeled cDNA probe corresponding to nucleotides 1321 to 1509 from the 3′-UTR of *Pnck.* Hybridization and washes were performed according to the manufacturer's directions (Clontech). A single band was detected in genomic DNA from both mouse and rat, confirming that, under these conditions, this *Pnck*-specific 3′-UTR probe recognizes a single locus.

Ribonuclease protection analysis was performed as described (Marquis *et al.,* 1995). Body-labeled antisense riboprobes were generated using linearized plasmids containing nucleotides 1321 to 1509 of *Pnck* and 1142 to 1241 of *β-actin* (GenBank Accession No. X03672) using [α-^{32}P]UTP and the Promega *in vitro* transcription system with T7 polymerase. A β-actin antisense riboprobe was added to each reaction as an internal control. Probes were hybridized with RNA samples at 58°C overnight in 50% formamide/100 mM Pipes (pH 6.7). Hybridized samples were digested with RNase A and T1, purified, electrophoresed on a 6% denaturing polyacrylamide gel, and subjected to autoradiography.

In vitro transcription and translation. *In vitro* transcription and translation were performed on 1 μg of plasmid DNA using rabbit reticulocyte lysates in the presence of [^{35}S]methionine according to the manufacturer's instructions (TNT kit, Promega). Completed reactions were electrophoresed on a 10% SDS–PAGE gel and were subjected to autoradiography.

Interspecific mouse backcross mapping. Interspecific backcross progeny were generated by mating (C57BL/6J × *M. spretus*)F_1 females and C57BL/6J males as described (Copeland and Jenkins, 1991). A total of 205 N_2 mice were used to map the *Pnck* locus (see text for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer, and hybridization were performed essentially as described (Jenkins *et al.,* 1982). All blots were prepared with Hybond-N^+ nylon membrane (Amersham). The probe, a 375-bp fragment corresponding to nucleotides 1135 to 1509 of mouse *Pnck* cDNA, was labeled with [α-^{32}P]dCTP using a nick-translation labeling kit (Boehringer Mannheim); washing was performed at a final stringency of 1.0× SSCP, 0.1% SDS at 65°C. A fragment of 13.0 kb was detected in *Pst*I-digested C57BL/6J DNA, and a fragment of 5.1 kb was detected in *Pst*I-digested *M. spretus* DNA. The presence or absence of the 5.1-kb *Pst*I *M. spretus*-specific fragment was followed in backcross mice.

A description of the probes and RFLPs for the loci linked to *Pnck* including *Tnfsf5, Il1rak,* and *Ar* has been reported previously (Centanni *et al.,* 1998). Recombination distances were calculated using Map Manager, version 2.6.5. Gene order was determined by minimizing the number of recombination events required to account for the allele distribution patterns.

In situ hybridization. *In situ* hybridization was performed as described (Marquis *et al.,* 1995). Antisense and sense probes were synthesized with the Promega *in vitro* transcription system using ^{35}S-UTP and ^{35}S-CTP from the T7 and SP6 RNA polymerase promoters of a PCR template containing sequences corresponding to nucleotides 1135 to 1509 of *Pnck.*

RESULTS

To isolate regulatory molecules potentially involved in development and carcinogenesis, a degenerate RT-PCR-based screen was performed to identify protein kinases expressed in murine breast cancer cell lines and in the mammary gland during development (Chodosh *et al.,* 1999, in press; Gardner *et al.,* in press; Stairs *et al.,* 1998). This screen resulted in the identification of 41 protein kinases including 33 tyrosine kinases and 8 serine/threonine kinases, 3 of which were novel.

One of these novel kinases, originally called *Bstk3,* is the subject of this study. *Bstk3* was subsequently found to be upregulated in the mammary gland during pregnancy and to be expressed in a punctate pattern in multiple tissues. Therefore, *Bstk3* was renamed *Pnck,* for pregnancy-upregulated, nonubiquitous CaM kinase, to reflect this unique temporal and spatial expression pattern.

Isolation of cDNA Clones Encoding Pnck

Of approximately 1500 clones examined in the context of a screen for expressed protein kinases, a single clone corresponding to *Bstk3* was isolated from the mammary glands of mice undergoing early postlactational regression. To isolate the full-length mRNA transcript from which *Bstk3* was derived, this initial 204-bp RT-PCR product was used to screen a murine brain cDNA library. Three cDNA clones ranging from 1455 to 1554 nucleotides in length were isolated by these means. All three clones were completely sequenced and were found to differ only in their respective 5′-UTRs (Fig. 1B). The sequence of each cDNA clone contains the entire 204-bp RT-PCR fragment, *Bstk3,* as well as a 1029-nucleotide open reading frame (ORF) and a 420-bp 3′-UTR possessing a polyadenylation signal and poly(A) tract (Fig. 1A).

Inspection of the nucleotide sequence surrounding the putative initiation codon at nucleotide 105 of the longest clone, U7, reveals matches with the Kozak translational initiation consensus sequence at positions −1, −3, −5, and −6 (Kozak, 1987, 1991). Conceptual translation of the *Pnck* ORF yields a 343-amino-acid polypeptide of predicted molecular mass 38.6 kDa. The coding sequence for Pnck can be divided into a 14-amino-acid unique amino-terminal segment, a 256-amino-acid kinase catalytic domain, a 41-amino-acid regulatory domain, and a 32-amino-acid unique carboxyl-terminal region. The Pnck kinase catalytic domain contains all of the amino acid motifs conserved among serine/threonine kinases.

To determine whether the lengths of the cDNA clones encoding *Pnck* are consistent with the size of the *Pnck* mRNA transcript, Northern hybridization was performed. Due to potential cross-hybridization between *Pnck* and homologous CaM kinase family members, Southern hybridization was used to confirm the specificity of a probe generated from the 3′-UTR of *Pnck* (data not shown). This *Pnck*-specific probe was used for Northern hybridization analysis performed on poly(A)$^+$ RNA isolated from adult murine brain. Consistent with the lengths of the isolated *Pnck* cDNA clones, this analysis revealed an mRNA transcript approximately 1.6 kb in length (Fig. 2A).

To confirm the coding potential of the *Pnck* ORF, *in vitro* transcription and translation were performed in the presence of [^{35}S]methionine using each of the three *Pnck* cDNA clones as template. In each case, incubation of plasmid DNA with reticulocyte lysate yielded a single labeled polypeptide species of approximately 38 kDa, consistent with the predicted *Pnck* ORF (Fig. 2B). This demonstrates that the predicted initiation codon is capable of functioning as a translation initiation site. Since clone U7 contains multiple in-frame termination codons upstream of this putative initiation codon, these findings suggest that we have isolated the entire *Pnck* coding sequence. However, since the alternate 5′-UTR sequence present in clone V1 does not contain

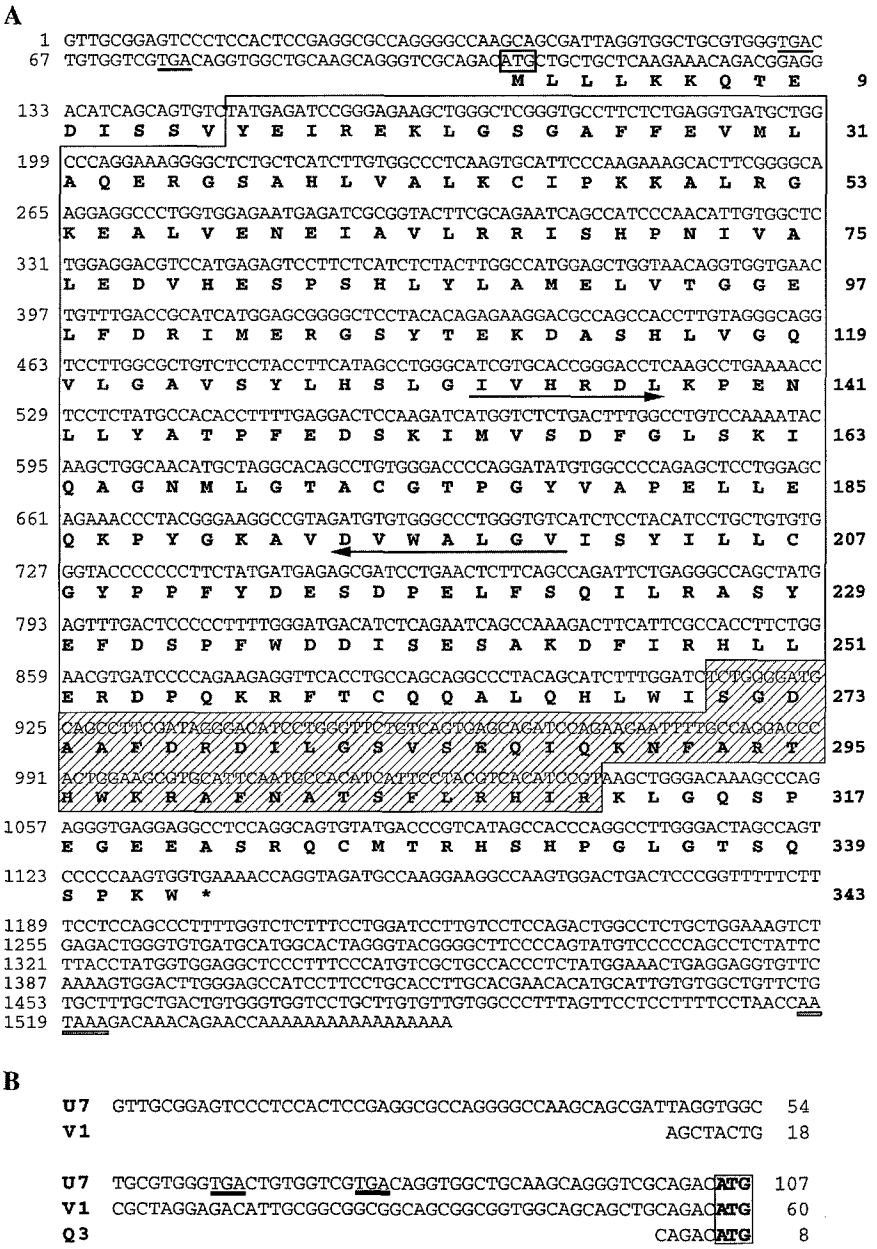

FIG. 1. Nucleotide and deduced amino acid sequence of *Pnck*. (**A**) Composite nucleic acid sequence and conceptual translation of full-length *Pnck* cDNA. Nucleotide coordinates are shown on the left. Amino acid coordinates are shown in boldface type on the right. A shaded box indicates the kinase catalytic domain, and a hatched box denotes the putative regulatory region. The in-frame upstream termination codons in the 5′-UTR and the putative polyadenylation sequence in the 3′-UTR are underlined by thin and thick lines, respectively. The putative initiation codon is boxed, and an asterisk denotes the stop codon. Arrows underline the regions corresponding to the degenerate oligonucleotides used to clone *Bstk3* initially. (**B**) 5′-UTR sequences of three full-length cDNA clones encoding *Pnck*. Nucleotide coordinates relative to each clone are shown to the right. Upstream in-frame termination codons are underlined, and the putative initiation codons are boxed.

an upstream termination codon, we cannot exclude the possibility that alternate polypeptides encoded by the *Pnck* locus that have distinct amino-terminal sequences exist.

Homology to Related Protein Kinases

Multiple sequence alignment of Pnck kinase catalytic subdomains I–XI was used to determine the relationship between Pnck and other CaM kinases (Fig. 3) (Hanks and Quinn, 1991; Hanks *et al.*, 1988). Pnck lies within the group of multifunctional CaM kinases and is most similar to CaMKI. Within the kinase domain, Pnck is 70% identical to CaMKI, 50% identical to CaMKIV, and approximately 45% identical to members of the CaMKII subfamily at the amino acid level. Pnck is also homologous to members of the CaM kinase family in the regulatory domain, although the extent of similarity is lower than that found in the catalytic domain. While Pnck is most closely related to CaMKI in both the catalytic and

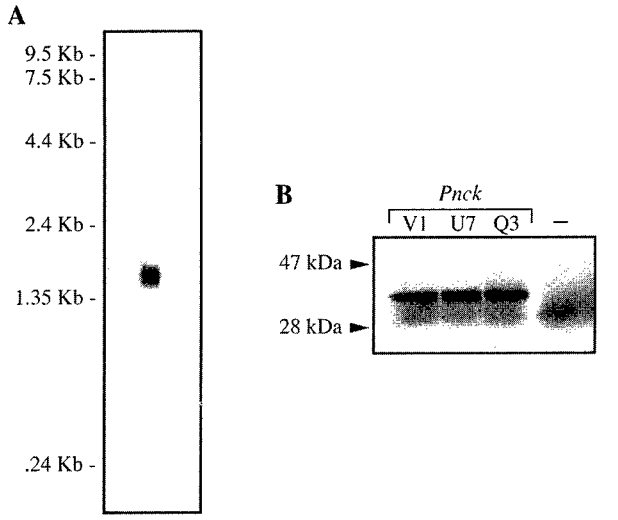

FIG. 2. Expression and coding potential of *Pnck*. (**A**) Northern hybridization analysis of 4 μg poly(A)$^+$ RNA isolated from adult murine brain hybridized with a 3′-UTR probe specific for *Pnck*. The relative migration of RNA size markers is indicated. (**B**) *In vitro* transcription/translation reactions performed using rabbit reticulocyte lysates in the presence of ^{35}S-labeled methionine and 1 μg of template consisting of a full-length *Pnck* cDNA clone (V1, U7, or Q3) or a cDNA plasmid encoding an unrelated kinase (–) as a negative control. IVT reactions were resolved on a 10% SDS–PAGE gel and subjected to autoradiography. The relative migration of molecular weight markers is indicated.

the regulatory domains, the amino acid identity between Pnck and CaMKI is significantly lower than that between CaMKII subfamily members, most of which exhibit greater than 90% amino acid identity within their catalytic and regulatory domains. Moreover, outside of these conserved functional domains, the amino- and carboxyl-terminal regions of Pnck bear no significant similarity to CaMKI, CaMKIV, or other CaM kinase family members.

Chromosomal Localization

The chromosomal location of murine *Pnck* was determined by interspecific backcross analysis using progeny derived from matings of [(C57BL/6J × *Mus spretus*)F_1 ×

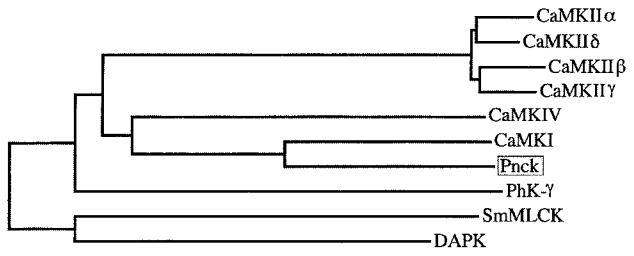

FIG. 3. Homology between Pnck and CaM kinase family members. Phylogenetic tree illustrating the relationship of Pnck kinase catalytic subdomains I–XI to other CaM kinase family members. Analysis and depiction of results were performed using the ClustalX multisequence alignment program and DendroMaker 4.0. Evolutionary relationships are proportional to horizontal branch distances. Database accession numbers used are A30355 (CaMKIIα); A34366 (CaMKIIδ); A26464 (CaMKIIβ); A31908 (CaMKIIγ); M64757 (CaMKIV); L26288 (CaMKI); X07320 (PhK-γ); A41674 (SmMLCK); and X76104 (DAPK).

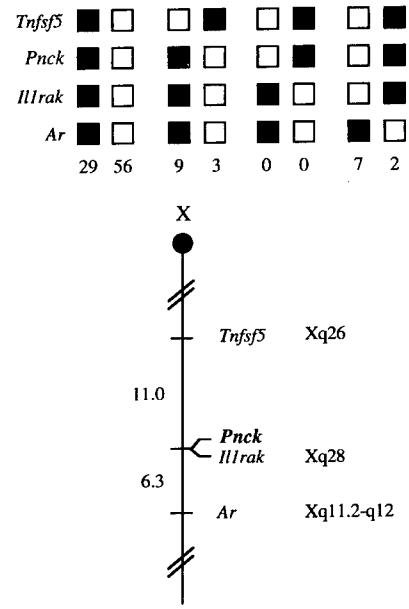

FIG. 4. *Pnck* maps in the central region of the mouse X chromosome. *Pnck* was placed on the mouse X chromosome by interspecific backcross analysis. The segregation patterns of *Pnck* and flanking genes in 106 backcross animals that were typed for all loci are shown at the top of the figure. For individual pairs of loci, more than 106 animals were typed (see text). Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J × *M. spretus*)F_1 parent. The shaded boxes represent the presence of a C57BL/6J allele, and white boxes represent the presence of a *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. A partial X chromosome linkage map showing the location of *Pnck* in relation to linked genes is shown at the bottom of the figure. Recombination distances between loci in centimorgans are shown to the left of the chromosome, and the positions of loci in human chromosomes are shown to the right. References for the human map positions of loci cited in this study can be obtained from GDB (Genome Data Base).

C57BL/6J] mice. This interspecific backcross mapping panel has been typed for over 2800 loci that are well distributed among all the autosomes as well as the X chromosome (Copeland and Jenkins, 1991). C57BL/6J and *M. spretus* DNAs were digested with several enzymes and analyzed by Southern blot hybridization for informative restriction fragment length polymorphisms (RFLPs) using a cDNA probe specific for the 3′-UTR of *Pnck*. The 5.1-kb *Pst*I *M. spretus* RFLP (see Materials and Methods) was used to follow the segregation of the *Pnck* locus in backcross mice. The mapping results indicated that *Pnck* is located in the central region of the mouse X chromosome linked to *Tnfsf5, Il1rak,* and *Ar*. Although 106 mice were analyzed for every marker and are shown in the segregation analysis (Fig. 4), up to 142 mice were typed for some pairs of markers. Each locus was analyzed in pairwise combinations for recombination frequencies using the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the most likely gene order are centromere–*Tnfsf5*–15/137–*Pnck*–0/134–*Il1rak*–9/142–*Ar*. The recombination frequencies expressed as genetic distances in centimorgans ± the standard error are –*Tnfsf5*–11.0 ±

2.7–(*Pnck, Il1rak*)–6.3 ± 2.0–*Ar*. No recombinants were detected between *Pnck* and *Il1rak* in 134 animals typed in common, suggesting that the two loci are within 2.2 cM of each other (upper 95% confidence limit).

We have compared our interspecific map of the X chromosome with a composite mouse linkage map that reports the map location of many uncloned mouse mutations (provided from Mouse Genome Database, http://www.informatics.jax.org/). *Pnck* maps to a region of the composite map that lacks uncloned mouse mutations (data not shown).

The central region of the mouse X chromosome shares a region of conserved homology with the long arm of the human X chromosome (summarized in Fig. 4). In particular, *Il1rak* has been mapped to Xq28. The close linkage between *Il1rak* and *Pnck* in mouse suggests that the human homologue of *Pnck* will map to Xq28, as well.

Analysis of Pnck mRNA Expression

To begin to determine the biological role of *Pnck,* the developmental expression pattern of *Pnck* mRNA was analyzed during murine embryogenesis. Northern hybridization analysis was performed on poly(A)$^+$ RNA isolated from embryos during early, mid-, and late gestation using a *Pnck*-specific probe (Fig. 5A). Compared to mRNA expression levels in early embryogenesis, steady-state *Pnck* mRNA levels are markedly upregulated in the embryo during midgestation and remain elevated through embryonic day 18.5.

To investigate the spatial pattern of *Pnck* expression during fetal development, *in situ* hybridization analysis was performed on embryonic sections at day 14.5 of gestation using an ^{35}S-labeled *Pnck*-specific antisense probe (Fig. 5B). This analysis revealed tissue-specific expression of *Pnck* in the embryo at midgestation with highest levels of expression detected in developing bone, the outer lining of the stomach, and the developing central nervous system, including periventricular regions and the trigeminal ganglion.

The expression profile of *Pnck* in tissues of the adult mouse was determined by RNase protection analysis (Fig. 6A). As in the embryo, *Pnck* expression in the adult mouse is highest in brain. In addition, moderate to low levels of *Pnck* expression are detected in hormonally responsive tissues such as uterus, ovary, testis, and mammary gland, as well as in other tissues such as stomach, heart, and skeletal muscle. Lower, but detectable, levels of *Pnck* expression were observed in thymus, spleen, duodenum, and lung.

Finally, the spatial expression pattern of *Pnck* in adult murine tissues was determined by *in situ* hybridization analysis (Fig. 6B–M). Interestingly, within expressing tissues *Pnck* mRNA was detected in only a subset of cells. In the brain, *Pnck* expression is highest in the dentate gyrus and CA1–3 regions of the hippocampus (Figs. 6B, 6C, 6F, and 6G). *Pnck* is also expressed at relatively high levels in the cortex and is markedly

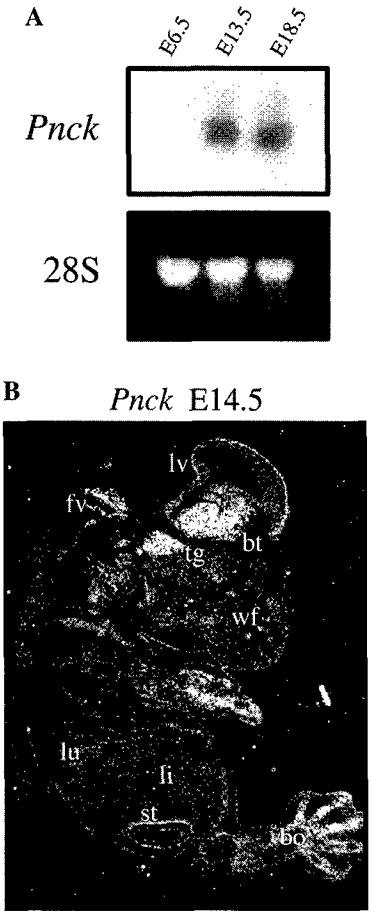

FIG. 5. Expression of *Pnck* during murine embryogenesis. (**A**) Northern hybridization analysis of 3 μg of poly(A)$^+$ RNA isolated from day E6.5, E13.5, and E18.5 embryos hybridized with a ^{32}P-labeled DNA probe specific for the 3′-UTR of *Pnck*. (**B**) *In situ* hybridization analysis of *Pnck* mRNA expression in the murine embryo. Sections of embryos at day 14.5 of gestation were hybridized with an ^{35}S-labeled *Pnck* antisense RNA probe. No signal over background was detected in serial sections hybridized with a sense *Pnck* probe. bo, bone; bt, basal telencephalon; fv, fourth ventricle; li, liver; lu, lung; lv, lateral ventricle; st, stomach; tg, trigeminal ganglion; wf, whisker hair follicle. Magnification: 10×. Exposure time was 6 weeks.

heterogeneous with highly expressing cells found adjacent to nonexpressing cells (Figs. 6J and 6K). *Pnck* is expressed throughout the ovary, but is preferentially localized in the thecal cell layers immediately surrounding the corpora lutea (Figs. 6H and 6I). In the testis, *Pnck* is expressed at high levels in mature spermatids residing at the center of seminiferous tubules and, to a lesser extent, in cells located adjacent to the basement membrane (Figs. 6D and 6E). Finally, in the dorsolateral prostate, *Pnck* mRNA is detected in a stromal layer of cells immediately surrounding the prostatic epithelial ducts. As in other tissues, *Pnck* expression in this compartment is spatially heterogeneous (Figs. 6L and 6M).

DISCUSSION

We have described the cloning, chromosomal localization, and developmental expression pattern of *Pnck,*

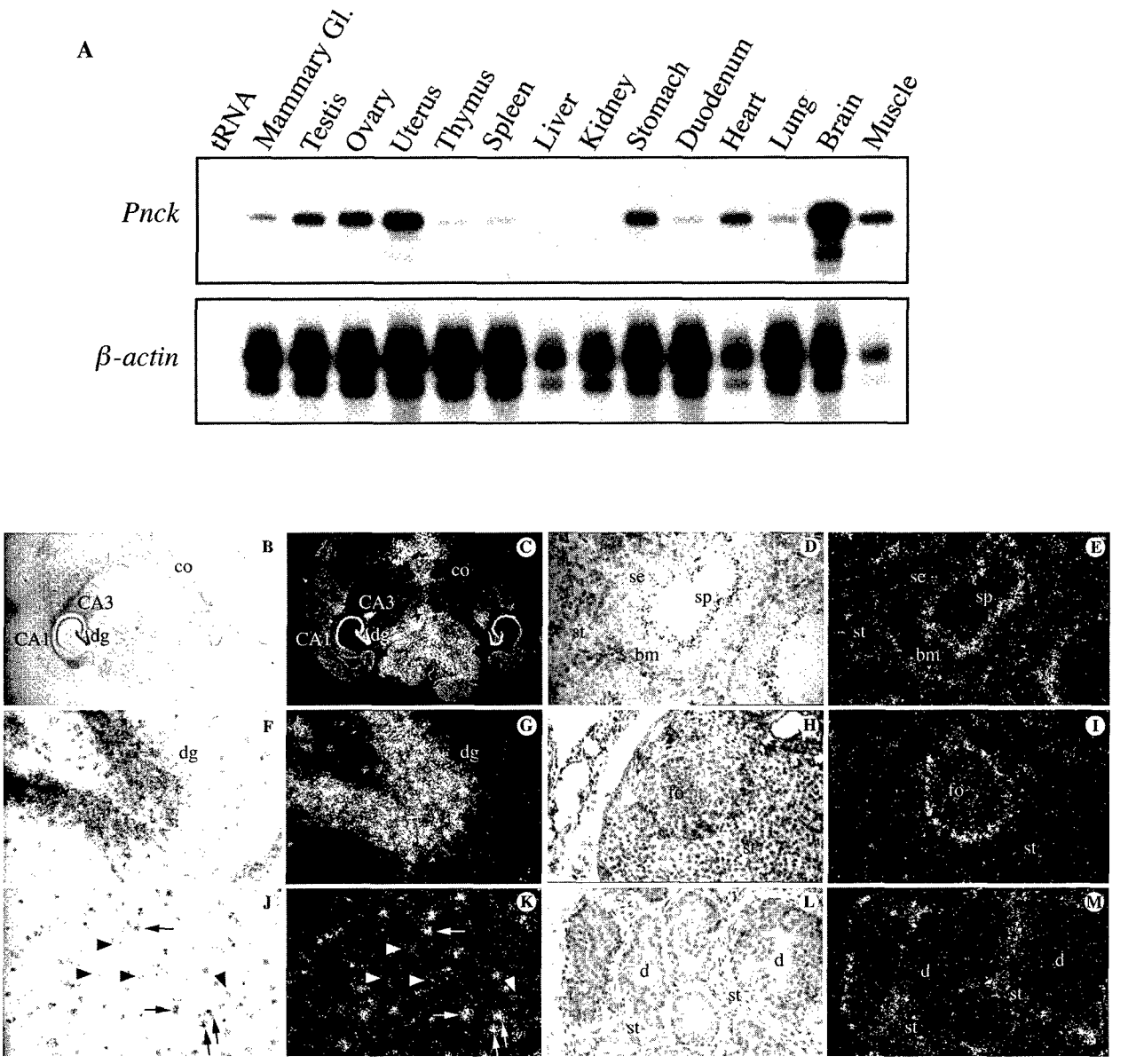

FIG. 6. Expression of *Pnck* in adult tissues. (**A**) RNase protection analysis of *Pnck* mRNA expression in tissues of the adult mouse. RNase protection analysis was performed using 30 μg of RNA isolated from the indicated murine tissues using antisense RNA probes specific for *Pnck* as well as for *β-actin* as an internal control. tRNA was used as a negative control for nonspecific hybridization. (**B–M**) Spatial localization of *Pnck* expression in tissues of the adult mouse. Bright-field (**B, D, F, H, J, L**) and dark-field (**C, E, G, I, K, M**) photomicrographs of *in situ* hybridization analysis performed on sections of brain (**B, C, F, G, J, K**), testis (**D, E**), ovary (**H, I**), and prostate (**L, M**), hybridized with an ^{35}S-labeled *Pnck* antisense probe. No signal over background was detected in serial sections hybridized with a sense *Pnck* probe. Arrows and arrowheads indicate *Pnck* expressing and *Pnck* nonexpressing cells, respectively. bm, basement membrane; CA1 and CA3, regions of the hippocampus; co, cortex; d, duct; dg, dentate gyrus; fo, follicle; se, seminiferous tubule; sp, spermatids; st, stroma. Magnifications: 10× (**B, C**), 300× (**D–M**). Exposure times were 6–7 weeks.

a new member of the CaM kinase family of serine/threonine kinases that is most closely related to CaMKI. *Pnck* expression during embryogenesis is developmentally regulated and tissue-specific with highest levels of expression detected in the developing brain, bone, and gut. Interestingly, *Pnck* expression in adult animals is both tissue-specific and markedly heterogeneous. *Pnck* expression is restricted to specific compartments within several tissues. Moreover, within these compartments, *Pnck* expression is heterogeneous with expressing cells found adjacent to non-expressing cells. The *Pnck* locus maps to the mouse X chromosome in a region of conserved synteny with human chromosome Xq28, a region associated with several mental retardation syndromes. In aggregate, our data suggest that *Pnck* may be involved in a variety of developmental processes.

Within the catalytic and regulatory domains conserved among all CaM kinases, Pnck is most closely related to the multifunctional CaM kinases, CaMKI, CaMKIV, and members of the CaMKII subfamily. CaMKI is a monomeric kinase that is expressed in

multiple tissues and is reported to phosphorylate several substrates including synapsin, the cystic fibrosis transmembrane conductance regulator, and transcription factors such as the cyclic AMP response element-binding protein, CREB and ATF-1 (Lukas *et al.,* 1998; Nairn and Piciotto, 1994; Nastluk and Nairn, 1996; Sheng *et al.,* 1991). CaMKIV is located in the nucleus and has been proposed to mediate CaM-induced changes in gene expression (Jensen *et al.,* 1991a; Sun *et al.,* 1996). In contrast to CaMKI and IV, which function as monomers, CaMKII forms 300- to 600-kDa multimers composed of different combinations of α, β, γ, and δ subunits (Schulman, 1993). While the α and β subunits are expressed predominantly in brain, the γ and δ CaMKII subunits are expressed ubiquitously (Hanley *et al.,* 1987; Lin *et al.,* 1987; Tobimatsu and Fujisawa, 1989; Tobimatsu *et al.,* 1988).

Functional analysis of CaM kinase mutants as well as crystal structure information has been used to define amino acids involved in the regulation of this family of molecules (Goldberg *et al.,* 1996; Haribabu *et al.,* 1995; Yokokura *et al.,* 1995). Carboxyl-terminal to their catalytic domain, CaM kinases possess a regulatory region that is composed of an autoinhibitory domain and a CaM-binding domain. Current evidence suggests that CaM binding disrupts an interaction between the autoinhibitory domain and the kinase catalytic domain, thereby resulting in kinase activation. In contrast to other CaM kinases, the activities of CaMKI and CaMKIV are dependent upon phosphorylation by a CaM-dependent kinase kinase, CaMKK (Haribabu *et al.,* 1995; Tokumitsu *et al.,* 1994, 1995). Since Pnck may be regulated in a manner similar to that of other CaM kinases, particularly CaMKI, structural homologies between Pnck and other CaM kinases may help elucidate the mechanisms by which Pnck activity is regulated.

The homology between Pnck and CaMKI raises the issue of whether Pnck should be classified as a CaMKI family member. Currently, the only widely recognized CaM kinase subfamily is that of CaMKII. Primary amino acid sequences of CaMKII subfamily members are typically greater than 90% identical in the catalytic and regulatory domains and actually function together in a multiprotein complex. In contrast, while the 70% amino acid identity in the catalytic domain between Pnck and CaMKI is greater than that between Pnck and other CaM kinases, the similarity between Pnck and CaMKI is significantly less than the approximately 90% identity observed between CaMKII family members. Moreover, there is currently no evidence to suggest that CaMKI family members function as subunits in a manner analogous to CaMKII subfamily members. As such, while the primary amino acid sequence of Pnck is most similar to that of CaMKI, it is unclear at present whether this kinase should be classified as a CaMKI family member.

While this work was in progress, the rat homologue of *Pnck* was described and shown to be expressed as two isoforms, tentatively named *CaMKIβ1* and *CaMKIβ2* (Naito *et al.,* 1997). Similar to the clones isolated for *Pnck, CaMKIβ1* and *CaMKIβ2* differ in their 5′-UTR regions and are homologous to *Pnck* clones V1 and U7, respectively. However, unlike the full-length clones isolated for *Pnck,* CaMKIβ1 contains a unique carboxyl-terminal coding region that appears to result from an alternative splicing event. Whether this form exists in the mouse remains to be determined. Northern hybridization analysis using a probe encompassing portions of the highly conserved kinase domain and regulatory region of *CaMKIβ* isoforms detected a 1.8-kb band exclusively in brain, whereas a 4.0-kb band was detected in all other tissues. RT-PCR analysis detected approximately equal levels of *CaMKIβ1* in all tissues examined in the rat, including tissues that we find to express low or undetectable levels of *Pnck* in the mouse as determined by RNase protection analysis using a probe specific for the 3′-UTR of *Pnck.* Insofar as the tissue-specific expression pattern of *Pnck* has been confirmed by *in situ* hybridization analysis, it is possible that cross-hybridization of the *CaMKIβ* probe used for Northern hybridization with other CaM kinases or the nonlinear nature of RT-PCR may underlie the discrepancy between the tissue-specific pattern of *Pnck* expression and the ubiquitous expression pattern reported for its rat homologue, *CaMKIβ.*

Several CaM kinases are expressed at high levels in the brain including CaMKI, CaMKII, and CaMKIV (Lukas *et al.,* 1998; Miyano *et al.,* 1992; Picciotto *et al.,* 1995). In fact, it has been estimated that CaMKII accounts for approximately 2% of all protein found in the hippocampus (Hanson and Schulman, 1992). It is therefore perhaps not surprising that *Pnck* is also expressed at high levels in the murine brain both in the adult and during embryogenesis. In the adult brain, *Pnck* is expressed at highest levels in the hippocampus and dentate gyrus, two areas of the brain involved in learning and memory.

While expression of CaMKI, CaMKIV, and isoforms of CaMKII has been reported in tissues other than the brain, a physiological role for these enzymes in other tissues has not been described. Similar to these multifunctional kinases, *Pnck* is expressed in a variety of tissues other than the brain. Moreover, in most tissues examined, *Pnck* is expressed in a spatially heterogeneous manner with expression restricted to a subset of cells. Although little is known about the role of CaM or CaM kinases in development, evidence suggests that both CaM and CaM kinases may play such a role. Point mutations in the *Drosophila* calmodulin gene result in defects in development with phenotypes ranging from pupal lethality to ectopic wing vein formation and melanotic scales on the cuticle (Nelson *et al.,* 1997). Additionally, CaMKIV has been implicated in T cell development based upon its regulation in the thymus during T cell development (Krebs *et al.,* 1997). The observation that *Pnck* expression is developmentally regulated and spatially restricted to distinct compartments of the

ovary, testis, prostate, and brain suggests that *Pnck* may play a biological role in these tissues. As such, the elucidation of signaling pathways in which *Pnck* is involved may shed light on the broader physiological role played by CaM kinases.

We have mapped the murine gene encoding *Pnck* to within 2.2 cM of *Il1rak* in the central region of the X chromosome. The observation that *Il1rak,* as well as markers that lie within 2.2 cM on either side of *Il1rak,* have been mapped to human chromosome Xq28 strongly suggests that the human homologue of *Pnck* will map to Xq28 as well. Chromosome Xq28 is one of the most densely mapped regions of the human chromosome, and several distinct mental retardation syndromes including Fragile X and X-linked mental retardation (XLMR) have been mapped to this region (Knight *et al.,* 1993; Lubs *et al.,* 1999). Interestingly, the only biological role described for any of the multifunctional CaM kinases is that of CaMKII in learning and memory. This suggests that CaM kinases may play an important role in signal transduction pathways controlling cognitive function (Silva *et al.,* 1992a, b; Soderling, 1993). In addition to mental retardation, many of these syndromes include phenotypes such as short stature, cleft palate, altered hand or digit size, and sterility (Lubs *et al.,* 1999). Given that *Pnck* is expressed at high levels in the brain, developing bone, ovary, and testis, it will be interesting to determine whether *Pnck* plays a role in one or more of these Xq28-linked syndromes.

ACKNOWLEDGMENTS

The authors thank Douglas B. Stairs for providing control expression plasmids, Deborah B. Householder for excellent technical assistance, and members of the Chodosh laboratory for helpful discussions and for critically reading the manuscript. This research was supported by the Elsa U. Pardee Foundation (L.A.C.), American Cancer Society RPG-99-259-01-DDC (L.A.C.), NIH Grants CA83849, CA71513, and CA78410 from the National Cancer Institute (L.A.C.), the Charles E. Culpeper Foundation (L.A.C.), and U.S. Army Breast Cancer Research Program Grants DAMD17-96-1-6112 (H.P.G.), DAMD17-98-1-8226 (L.A.C.), DAMD-99-1-9463 (L.A.C.), and DAMD-99-1-9349 (L.A.C.), and by the National Cancer Institute, DHHS, under contract with ABL (N.A.J.). L.A.C. is a Charles E. Culpeper Medical Scholar.

REFERENCES

Birchmeier, C., Sonnenberg, E., Weidner, K. M., and Walter, B. (1993). Tyrosine kinase receptors in the control of epithelial growth and morphogenesis during development. *BioEssays* **15:** 185–190.

Bolen, J. B. (1993). Nonreceptor tyrosine protein kinases. *Oncogene* **8:** 2025–2031.

Bolen, J. B., Rowley, R. B., Spana, C., and Tsygankov, A. Y. (1992). The Src family of tyrosine protein kinases in hemopoietic signal transduction. *FASEB J.* **6:** 3403–3409.

Braun, A. P., and Schulman, H. (1995). The multifunctional calcium/calmodulin-dependent protein kinase: From form to function. *Annu. Rev. Physiol.* **57:** 417–445.

Cardiff, R. D., and Muller, W. J. (1993). Transgenic mouse models of mammary tumorigenesis. *Cancer Surv.* **16:** 97–113.

Cawley, K. C., Akita, C. G., Angelos, K. L., and Walsh, D. A. (1993). Characterization of the gene for rat phosphorylase kinase catalytic subunit. *J. Biol. Chem.* **268:** 1194–1200.

Centanni, J. M., de Miguel, M., Gopalan, G., Gilbert, D. J., Copeland, N. G., Jenkins, N. A., and Donovan, P. J. (1998). Interleukin-1 receptor-associated kinase gene Il1rak maps to the mouse X chromosome. *Mamm. Genome* **9:** 340–341.

Chodosh, L. A., D'Cruz, C. M., Gardner, H. P., Ha, S. I., Marquis, S. T., Rajan, J. V., Stairs, D. B., Wang, J. Y., and Wang, M. (1999). Mammary gland development, reproductive history, and breast cancer risk. *Cancer Res.* **59:** 1765–1771S.

Chodosh, L. A., Gardner, H. P., Rajan, J. V., Stairs, D. B., Marquis, S. T., and Leder, P. A. Protein kinase expression during murine mammary development. *Dev. Biol.,* in press.

Copeland, N. G., and Jenkins, N. A. (1991). Development and applications of a molecular genetic linkage map of the mouse genome. *Trends Genet.* **7:** 113–118.

Dickson, R. B., Salomon, D. S., and Lippman, M. E. (1992). Tyrosine kinase receptor–nuclear protooncogene interactions in breast cancer. *Cancer Treatment Res.* **61:** 249–273.

Fantl, W. J., Johnson, D. E., and Williams, L. T. (1993). Signalling by receptor tyrosine kinases. *Annu. Rev. Biochem.* **62:** 453–481.

Fukunaga, K., and Miyamoto, E. (1999). Current studies on a working model of CaM kinase II in hippocampal long-term potentiation and memory. *Jpn. J. Pharmacol.* **79:** 7–15.

Gardner, H. P., Wertheim, G. B. W., Ha, S. I., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Marquis, S. T., and Chodosh, L. A. Cloning and characterization of *Hunk,* a novel mammalian SNF1-related protein kinase. *Genomics,* in press.

Goldberg, J., Nairn, A. C., and Kuriyan, J. (1996). Structural basis for the autoinhibition of calcium/calmodulin-dependent protein kinase I. *Cell* **84:** 875–887.

Guy, C. T., Muthuswamy, S. K., Cardiff, R. D., Soriano, P., and Muller, W. J. (1994). Activation of the c-Src tyrosine kinase is required for the induction of mammary tumors in transgenic mice. *Genes Dev.* **8:** 23–32.

Guy, C. T., Webster, M. A., Schaller, M., Parsons, T. J., Cardiff, R. D., and Muller, W. J. (1992). Expression of the c-neu proto-oncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc. Natl. Acad. Sci. USA* **89:** 10578–10582.

Hanks, S., and Quinn, A. (1991). Protein kinase catalytic domain sequence database: Identification of conserved features of primary structure and classification of family members. *Methods Enzymol.* **200:** 38–79.

Hanks, S. K., Quinn, A. M., and Hunter, T. (1988). The protein kinase family: Conserved features and deduced phylogeny of the catalytic domains. *Science* **241:** 42–52.

Hanley, R. M., Means, A. R., Ono, T., Kemp, B. E., Burgin, K. E., Waxham, N., and Kelly, P. T. (1987). Functional analysis of a complementary DNA for the 50-kilodalton subunit of calmodulin kinase II. *Science* **237:** 293–297.

Hanson, P. I., and Schulman, H. (1992). Neuronal Ca^{2+}/calmodulin-dependent protein kinases. *Annu. Rev. Biochem.* **61:** 559–601.

Hardie, D. G. (1990). Roles of protein kinases and phosphatases in signal transduction. *Symp. Soc. Exp. Biol.* **44:** 241–255.

Haribabu, B., Hook, S. S., Selbert, M. A., Goldstein, E. G., Tomhave, E. D., Edelman, A. M., Snyderman, R., and Means, A. R. (1995). Human calcium-calmodulin dependent protein kinase I: cDNA cloning, domain structure and activation by phosphorylation at threonine-177 by calcium-calmodulin dependent protein kinase I kinase. *EMBO J.* **14:** 3679–3686.

Herring, B. P., Stull, J. T., and Gallagher, P. J. (1990). Domain characterization of rabbit skeletal muscle myosin light chain kinase. *J. Biol. Chem.* **265:** 1724–1730.

Jenkins, N. A., Copeland, N. G., Taylor, B. A., and Lee, B. K. (1982). Organization, distribution, and stability of endogenous ecotropic

murine leukemia virus DNA sequences in chromosomes of *Mus musculus*. *J. Virol.* **43:** 26.

Jensen, K. F., Ohmstede, C. A., Fisher, R. S., Olin, J. K., and Sahyoun, N. (1991a). Acquisition and loss of a neuronal Ca^{2+}/calmodulin-dependent protein kinase during neuronal differentiation. *Proc. Natl. Acad. Sci. USA* **88:** 4050–4053.

Jensen, K. F., Ohmstede, C. A., Fisher, R. S., and Sahyoun, N. (1991b). Nuclear and axonal localization of Ca^{2+}/calmodulin-dependent protein kinase type Gr in rat cerebellar cortex. *Proc. Natl. Acad. Sci. USA* **88:** 2850–2853.

Knight, S. J., Flannery, A. V., Hirst, M. C., Campbell, L., Christodoulou, Z., Phelps, S. R., Pointon, J., Middleton-Price, H. R., Barnicoat, A., Pembrey, M. E., *et al.* (1993). Trinucleotide repeat amplification and hypermethylation of a CpG island in FRAXE mental retardation. *Cell* **74:** 127–134.

Knighton, D. R., Pearson, R. B., Sowadski, J. M., Means, A. R., Ten Eyck, L. F., Taylor, S. S., and Kemp, B. E. (1992). Structural basis of the intrasteric regulation of myosin light chain kinases. *Science* **258:** 130–135.

Kozak, M. (1987). An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res.* **15:** 8125–8132.

Kozak, M. (1991). An analysis of vertebrate mRNA sequences: Intimations of translational control. *J. Cell Biol.* **115:** 887–903.

Krebs, J., Wilson, A., and Kisielow, P. (1997). Calmodulin-dependent protein kinase IV during T-cell development. *Biochem. Biophys. Res. Commun.* **241:** 383–389.

Lin, C. R., Kapiloff, M. S., Durgerian, S., Tatemoto, K., Russo, A. F., Hanson, P., Schulman, H., and Rosenfeld, M. G. (1987). Molecular cloning of a brain-specific calcium/calmodulin-dependent protein kinase. *Proc. Natl. Acad. Sci. USA* **84:** 5962–5966.

Lubs, H., Chiurazzi, P., Arena, J., Schwartz, C., Tranebjaerg, L., and Neri, G. (1999). XLMR genes: Update 1998. *Am. J. Med. Genet.* **83:** 237–247.

Lukas, T., Mirzoeva, S., and Watterson, D. (1998). Calmoduin-regulated protein kinase. *In* "Calmodulin and Signal Transduction" (L. Van Eldik and D. Watterson, Eds.), pp. 65–168, Academic Press, San Diego.

Marquis, S. T., Rajan, J. V., Wynshaw-Boris, A., Xu, J., Yin, G.-Y., Abel, K. J., Weber, B. L., and Chodosh, L. A. (1995). The developmental pattern of *Brca1* expression implies a role in differentiation of the breast and other tissues. *Nat. Genet.* **11:** 17–26.

Matthews, R. P., Guthrie, C. R., Wailes, L. M., Zhao, X., Means, A. R., and McKnight, G. S. (1994). Calcium/calmodulin-dependent protein kinase types II and IV differentially regulate CREB-dependent gene expression. *Mol. Cell. Biol.* **14:** 6107–6116.

Miyano, O., Kameshita, I., and Fujisawa, H. (1992). Purification and characterization of a brain-specific multifunctional calmodulin-dependent protein kinase from rat cerebellum. *J. Biol. Chem.* **267:** 1198–1203.

Nairn, A., and Picciotto, M. (1994). Calcium/calmodulin-dependent protein kinases. *Semin. Cancer Biol.* **5:** 295–303.

Naito, Y., Watanabe, Y., Yokokura, H., Sugita, R., Nishio, M., and Hidaka, H. (1997). Isoform-specific activation and structural diversity of calmodulin kinase I. *J. Biol. Chem.* **272:** 32704–32708.

Nastluk, K., and Nairn, A. (1996). Structure, regulation, and function of calcium/calmodulin-dependent protein kinase I. *Adv. Pharmacol.* **36:** 251–275.

Nelson, H. B., Heiman, R. G., Bolduc, C., Kovalick, G. E., Whitley, P., Stern, M., and Beckingham, K. (1997). Calmodulin point mutations affect *Drosophila* development and behavior. *Genetics* **147:** 1783–1798.

Picciotto, M., Czernick, A., and Nairn, A. (1993). Calcium/calmodulin-dependent protein kinase I. cDNA cloning and identification of autophosphorylation site. *J. Biol. Chem.* **268:** 26512–26521.

Picciotto, M. R., Nastiuk, K. L., and Nairn, A. C. (1996). Structure, regulation, and function of calcium/calmodulin-dependent protein kinase I. *Adv. Pharmacol.* **36:** 251–275.

Picciotto, M. R., Zoli, M., Bertuzzi, G., and Nairn, A. C. (1995). Immunochemical localization of calcium/calmodulin-dependent protein kinase I. *Synapse* **20:** 75–84.

Polishchuk, S. V., Brandt, N. R., Meyer, H. E., Varsanyi, M., and Heilmeyer, L. M., Jr. (1995). Does phosphorylase kinase control glycogen biosynthesis in skeletal muscle? *FEBS Lett.* **362:** 271–275.

Rajan, J. V., Marquis, S. T., Gardner, H. P., and Chodosh, L. A. (1997). Developmental expression of Brca2 colocalizes with Brca1 and is associated with differentiation in multiple tissues. *Dev. Biol.* **184:** 385–401.

Rawlings, D. J., and Witte, O. N. (1994). Bruton's tyrosine kinase is a key regulator in B-cell development. *Immunol. Rev.* **138:** 105–119.

Schulman, H. (1993). The multifunctional Ca^{2+}/calmodulin-dependent protein kinases. *Curr. Opin. Cell Biol.* **5:** 247–253.

Sheng, M., Thompson, M., and Greenberg, M. (1991). CREB: A calcium-regulated transcription factor phosphorylated by calmodulin-dependent kinases. *Science* **252:** 1427–1430.

Silva, A. J., Paylor, R., Wehner, J. M., and Tonegawa, S. (1992a). Impaired spatial learning in alpha-calcium-calmodulin kinase II mutant mice. *Science* **257:** 206–211.

Silva, A. J., Stevens, C. F., Tonegawa, S., and Wang, Y. (1992b). Deficient hippocampal long-term potentiation in alpha-calcium-calmodulin kinase II mutant mice. *Science* **257:** 201–206.

Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A., *et al.* (1989). Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* **244:** 707–712.

Soderling, T. R. (1993). Calcium/calmodulin-dependent protein kinase II: Role in learning and memory. *Mol. Cell. Biochem.* **127–128:** 93–101.

Stairs, D. B., Gardner, H. P., Ha, S. I., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and Chodosh, L. A. (1998). Cloning and characterization of Krct, a member of a novel subfamily of serine/threonine kinases. *Hum. Mol. Genet.* **7:** 2157–2166.

Sun, P., Lou, L., and Maurer, R. (1996). Regulation of activating transcription factor-1 and the cAMP response element-binding protein by Ca^{2+}/calmodulin-dependent protein kinases type I, II and IV. *J. Biol. Chem.* **271:** 3066–3073.

Tobimatsu, T., and Fujisawa, H. (1989). Tissue-specific expression of four types of rat calmodulin-dependent protein kinase II mRNAs. *J. Biol. Chem.* **264:** 17907–17912.

Tobimatsu, T., Kameshita, I., and Fujisawa, H. (1988). Molecular cloning of the cDNA encoding the third polypeptide (gamma) of brain calmodulin-dependent protein kinase II. *J. Biol. Chem.* **263:** 16082–16086.

Tokumitsu, H., Brickey, D. A., Glod, J., Hidaka, H., Sikela, J., and Soderling, T. R. (1994). Activation mechanisms for Ca^{2+}/calmodulin-dependent protein kinase IV. Identification of a brain CaM-kinase IV kinase. *J. Biol. Chem.* **269:** 28640–28647.

Tokumitsu, H., Enslen, H., and Soderling, T. R. (1995). Characterization of a Ca^{2+}/calmodulin-dependent protein kinase cascade. Molecular cloning and expression of calcium/calmodulin-dependent protein kinase kinase. *J. Biol. Chem.* **270:** 19320–19324.

Wilks, A. F. (1989). Two putative protein-tyrosine kinases identified by application of the polymerase chain reaction. *Proc. Natl. Acad. Sci. USA* **86:** 1603–1607.

Wilks, A. F. (1991). Cloning members of protein-tyrosine kinase family using polymerase chain reaction. *Methods Enzymol.* **200:** 533–546.

Wilks, A. F., Kurban, R. R., Hovens, C. M., and Ralph, S. J. (1989). The application of the polymerase chain reaction to cloning members of the protein tyrosine kinase family. *Gene* **85:** 67–74.

Yokokura, H., Picciotto, M. R., Nairn, A. C., and Hidaka, H. (1995). The regulatory region of calcium/calmodulin-dependent protein kinase I contains closely associated autoinhibitory and calmodulin-binding domains. *J. Biol. Chem.* **270:** 23851–23859.

DEPARTMENT OF THE ARMY
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 21 Feb 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

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2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

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