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**13. ABSTRACT** *(Maximum 200 words)*

Recent studies from our laboratory have demonstrated that PTHrP is essential for mammary gland development. The current project was designed to explore the hypothesis that PTHrP is an important contributor to epithelial-mesenchymal interactions that are necessary for mammary development. In the first year, we concentrated on defining the temporal-spatial pattern of PTHrP and PTH/PTHrP receptor expression during mammary development. We have learned that during ductal morphogenesis, PTHrP is expressed in mammary epithelial cells and its receptor is expressed in surrounding mesenchymal cells. In addition, it appears that in the post-natal mammary gland, expression of the PTHrP and the PTH/PTHrP receptor genes is most intense in terminal end buds, regions of active proliferation and ductal morphogenesis during puberty. The results from these experiments suggest that PTHrP, produced by mammary epithelial cells, acts on mammary stromal cells and modulates their function. We have generated primary cultures of mouse mammary stromal cells that bind to PTHrP with high affinity and respond to PTHrP with an increase in cAMP. In years two and three, we will use this experimental system to examine the effects of PTHrP on mammary stromal cell function and how these effects impact the proliferation and morphogenesis of mammary epithelial cells.

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Maureen E. Dubar 7/29/98

PI - Signature Date

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## Introduction

Parathyroid hormone-related protein was originally identified as the tumor product responsible for the clinical syndrome of humoral hypercalcemia of malignancy (HHM), a common metabolic complication of many types of cancer (1). It is now clear that PTHrP is a normal product of many tissues, especially in the developing embryo, where it appears to act in a paracrine fashion to regulate organogenesis (2). PTHrP shares a common receptor with parathyroid hormone, the PTH/PTHrP receptor, and recent studies have found that it too has a wide distribution similar to that of PTHrP (3). During fetal life, PTHrP is expressed predominantly in epithelial cells, whereas the PTH/PTHrP receptor is expressed in stromal cells immediately adjacent to the PTHrP expressing cells (4). This type of hand in glove pattern of expression suggests that PTHrP functions as a paracrine factor and may be involved in epithelial-mesenchymal interactions, a process that is central in the regulation of the development of many tissues.

Several recent studies have shown that PTHrP is expressed in a great number of tissues where it appears to play a role in the regulation of cellular proliferation and differentiation during development (2,5). One of these sights is the mammary gland. As mentioned in the original proposal, our laboratory has recently shown that both overexpression and underexpression of PTHrP in the mammary gland leads to severe disruptions in its development. Overexpression of PTHrP in mammary myoepithelial cells, driven by the keratin-14 (K14) promoter, leads to severe defects in ductular proliferation and side branching, the net result being a much simpler, sparser, virgin duct system, as well as reduced lobuloalveolar development during pregnancy and lactation (6). Underexpression of PTHrP or the PTH/PTHrP receptor results in the complete absence of a mammary epithelial duct system and the absence of nipples (7). Previous studies have shown that PTHrP is a normal product of mammary epithelial cells (8-10), and, as will be discussed later, we have shown that the PTH/PTHrP receptor is produced by mammary stromal cells. These results lead us to hypothesize that PTHrP is an important regulator of epithelial-stromal interactions that are necessary for normal mammary development. More specifically, we hypothesized that PTHrP is an epithelial cell product that modulates stromal cell function during mammary gland development. In order to test this hypothesis we proposed a series of three technical objectives that are aimed at elucidating the mechanisms by which PTHrP regulates epithelial-stromal crosstalk in the developing mammary gland. The following sections report our progress over the first year.

## Body

### Technical Objective 1: Temporal-spatial expression of PTHrP and the PTH/PTHrP receptor during mammary gland development

Our first technical objective was to examine the temporal-spatial pattern of PTHrP and the PTH/PTHrP receptor during various stages of mammary development using RNase protection analysis, in situ hybridization, and immunohistochemistry. The nature of the phenotypes of the PTHrP over- and underexpression models suggest that PTHrP is involved in regulating ductal morphogenesis during mammary development. For this reason, we have concentrated our efforts on analyzing PTHrP and PTH/PTHrP receptor expression during embryonic development and during sexual maturation, the periods of mammary development characterized by active ductal morphogenesis.

We initiated these studies by examining the temporal pattern of PTHrP and the PTH/PTHrP receptor expression during preadolescence (3 week old virgin), during puberty, (6 week old virgin) and during early to mid pregnancy (11 days post coitus) by RNase protection analysis. As shown in Fig 1A, both the PTHrP and the PTH/PTHrP receptor are expressed in the mammary gland at each time point examined demonstrating that both PTHrP and its receptor are expressed in the post-natal mammary gland during periods of active ductal morphogenesis. In addition, despite dramatic changes in cellular composition of the mammary gland at these different stages, whole gland levels of PTHrP and the PTH/PTHrP receptor mRNA expression remained relatively constant.

Our prior studies had suggested that, during embryonic mammary development, PTHrP mRNA is expressed in mammary epithelial cells and the PTH/PTHrP receptor is expressed in the mammary mesenchyme (7). In order to determine if this pattern was also present during later stages of ductal morphogenesis, we took advantage of the directional growth of mammary epithelial ducts during puberty. Prior to the initiation of adolescence the mammary ducts occupy only a small portion of one end of the mammary fat pad and, in response to the hormonal stimulation of puberty, they grow towards the opposite end of the mammary fat pad until they completely fill out this stromal component. As a result of this growth pattern, at the initiation of puberty, one can divide the murine mammary gland into a proximal segment that contains both epithelial and stromal components and a distal segment that contains only stroma. A shown in Fig 1B, the proximal component with both epithelial and stromal cells, contains both PTHrP and PTH/PTHrP receptor mRNA, but the distal component, that is the stroma alone, contains only PTH/PTHrP receptor mRNA. These data suggest that PTHrP mRNA is expressed in the mammary epithelium, and that PTH/PTHrP receptor mRNA is expressed within the fat pad stroma.

We were next interested in examining the cellular localization of PTHrP and the PTH/PTHrP receptor during mammary development. We planned to initiate these experiments by determining the localization of the PTHrP and the

PTH/PTHrP receptor protein by immunohistochemistry. However, in piloting these experiments using polyclonal antisera to PTHrP on skin sections from normal and PTHrP-knockout mice, we found that this antisera stained both normal and knockout tissue. While we were able to demonstrate this antisera to be specific to an epitope on PTHrP by competition studies, the fact that this antisera stained PTHrP-knockout skin suggests that there is another molecule present in skin that shares a common epitope with PTHrP. Because of these difficulties with the immunohistochemistry we instead focused our efforts on performing in situ hybridization to determine the spatial localization of PTHrP and the PTH/PTHrP receptor expression during mammary development.

In situ hybridization using an antisense probe to PTHrP demonstrated that the PTHrP gene was expressed in epithelial cells during periods of mammary ductal growth (see Fig. 2). During embryonic development, PTHrP expression was very intense in the epithelial cells of the embryonic mammary bud at E12 (Fig. 2A & 2B) and, at E18, when the mammary bud is elongating and initiating ductal branching morphogenesis, PTHrP expression continued to be expressed in mammary epithelial cells (Fig. 2C & 2D). In the post natal mammary gland, PTHrP mRNA expression continued to be localized to epithelial cells. However, overall, expression was less intense, and was restricted to the leading edge of ductal morphogenesis. This is shown in Fig. 2E-I, which reveals that, during puberty, PTHrP expression was restricted to epithelial cells of terminal end buds which are specialized structures present during phases of ductular proliferation and which serve as the sites of cellular proliferation and differentiation (Fig. 2E-2G). Specifically, the PTHrP mRNA signal appeared to localize to the peripheral, or cap cells, of the end buds, a pattern similar to the peripheral location of the PTHrP signal seen during fetal life. PTHrP mRNA was undetectable in epithelial cells of mature ducts during puberty (Fig.2H & 2I). During early pregnancy, there appeared to be a low level of PTHrP expression in the epithelial cells of developing lobuloalveolar units (Fig. 2K & 2L), but, similar to puberty, we could not detect PTHrP mRNA in mature mammary ducts. Therefore, it appears that similar to embryonic life, in the post-natal mammary gland, the major site of PTHrP expression is within epithelial cells.

In contrast to the epithelial pattern of PTHrP mRNA expression, PTH/PTHrP receptor was predominantly expressed in mesenchymal cells immediately surrounding the PTHrP-expressing cells (Fig. 3). In the embryonic mammary gland, at E12, PTH/PTHrP receptor mRNA was expressed throughout the ventral mesenchyme, including the dense mammary mesenchyme (Figs. 3A & 3B). At E18, when the mammary ducts had grown to make contact with the mammary fat pad, PTH/PTHrP receptor mRNA continued to be expressed in stromal cells surrounding the growing mammary ducts as they became surrounded by the developing fatty stroma (Figs. 3C & 3D). During puberty, PTH/PTHrP receptor mRNA expression was expressed at a low level throughout the mammary stroma, but the most intense expression was in stromal cells immediately surrounding terminal end buds (Figs. 3E & 3F). However, in contrast to the pattern of PTHrP mRNA expression, a low level of

PTH/PTHrP receptor mRNA expression was detected in periductal stroma surrounding more mature ducts in the pubescent mammary gland (Figs. 3H &3I). During early to mid pregnancy PTH/PTHrP receptor mRNA also appeared to be expressed at a low level throughout the fat pad stroma within the periductal stroma and surrounding the developing lobuloalveolar units.

Summarizing these data, we have learned that during active ductular branching morphogenesis, PTHrP is expressed in epithelial cells, and its receptor is expressed by surrounding mesenchymal cells. In addition, it appears that in the post natal mammary gland, expression of the PTHrP and the PTH/PTHrP receptor genes is most intense in terminal end buds, regions of active proliferation and ductal morphogenesis during puberty. These experiments have progressed nicely this past year and we have essentially completed this technical objective, which is on time with regards to our original statement of work. A manuscript describing this work has recently been accepted for publication in*Developmental Biology*.

**Technical Objective 2: Effects of PTHrP on Growth Factor Production by Mammary Stromal Cells**

The results from our first technical objective demonstrate that PTHrP and its receptor are expressed in an epithelial-mesenchymal pattern throughout mammary development. This suggests that PTHrP, produced by mammary epithelial cells, may regulate mammary development by regulating mammary stromal cell function. To test this hypothesis, we were interested in examining the effects of PTHrP on growth factor production by mammary stromal cells. We have initiated these experiments by generating primary cultures of mouse mammary stromal cells and have characterized these cells with regards to their cellular composition and their responses to PTHrP. Using a series of antibodies to keratins 8 and 18 (specific to luminal epithelial cells), keratin 14 (present in myoepithelial and some luminal epithelial cells), and vimentin (present in stromal cells) we have established, by immunocytochemistry, that our stromal cell primary cultures are composed of approximately 90-95% stromal cells and 5-10% myoepithelial cells. We have demonstrated by RNase protection analysis that these cells do not produce PTHrP, but do contain the PTH/PTHrP receptor, and therefore appear to represent one "PTHrP-target" cell in the mammary gland (Fig, 4). We have also shown that these cells contain specific binding sites for amino terminal PTHrP (126,000 + 13,000 sites /cell)(Fig. 5), and respond to PTHrP(1-40) with an increase in intracellular cAMP in a time and dose dependent manner (Fig. 6).

We have begun to investigate the effects of PTHrP on the stromal cell production of three growth factors: hepatoctye growth factor/scatter factor (HGF/SF), insulin like growth factor (IGF-1), and keratinocyte growth factor (KGF). These three growth factors have been previously shown to be produced by fibroblasts and each has been implicated in either regulating ductal branching morphogenesis and/or PTHrP signaling in other systems (11-13). We reported in our original proposal that PTHrP caused an increase in the

steady state levels of HGF/SF mRNA our mammary stromal cell cultures. However, in repeating these experiments we were not able to consistently reproduce this finding. In searching the literature, we discovered that HGF/SF gene has estrogen response elements within its promoter region and that small quantities of estrogen present in fetal bovine serum are sufficient enough to stimulate an increase in HGF/SF mRNA (14,15). Although our experiments were performed in serum free media, the cells were initially grown in medium containing 10% serum for 5 days prior to PTHrP treatment. We have now switched to growing our stromal cells in phenol red-free DMEM and charcoal-stripped serum to avoid possible responses to trace amounts of hormones present in serum. In repeating these experiments under the new culture conditions we were not able to demonstrate an increase in HGF/SF mRNA in mammary stromal cells following treatment with PTHrP (Fig. 7), suggesting that PTHrP does not regulate HGF/SF expression in our mammary stromal cell cultures.

We are currently in the process of examining IGF-1 and KGF expression in our mammary stromal cells in response to PTHrP. Unfortunately, we have been experiencing some technical difficulties with these experiments. We currently do not have a cDNA for mouse KGF so we have not been able to generate a mouse RNase protection probe to examine KGF mRNA in our mouse mammary stromal cells. We have examined the expression of human KGF mRNA in a human dermal fibroblast cell line that has a similar cAMP response to PTHrP as our mammary stromal cells, and have not detected any changes in KGF mRNA in response to PTHrP. We have attempted RNase protections to examine IGF-1 mRNA expression in our mammary stromal cells in response to PTHrP. However, the probe that we are currently using detects several alternatively spliced transcripts for IGF-1 making the RNase protections difficult to interpret. We are in the process of sub-cloning a new RNase protection probe for IGF-1 that will detect only one transcript. While we were in the process of trying to troubleshoot the RNase protections, we have attempted Northern blot analysis to measure both IGF-1 and KGF mRNA levels in our stromal cells. However we found that this technique is not been sensitive enough to detect baseline IGF-1 or KGF mRNA in mammary stromal cells. Although we have been experiencing some technical difficulties with these experiments we are still on time with respect to our original statement of work. We are in the process of troubleshooting the Rnase protection assays and hope to complete these experiments by month 18 of this project.

We have also proposed to examine the expression of HGF/SF, IGF-1 and KGF in our stromal cell cultures in response to PTHrP in the presence of exogenously added estrogen and/or progesterone. Perhaps PTHrP effects the estrogen and progesterone induced production of these growth factors by mammary stromal cells. As outlined in the original statement of work, we plan to begin these experiments at the beginning of year two and we are in the process of generating cultures of mammary stromal cells and treating them with PTHrP and/or estrogen and progesterone.

The second part of this technical objective is to determine if PTHrP affects estrogen and progesterone stimulated growth of mammary epithelial cells. As outlined in the statement of work, we are scheduled to begin these experiments at the beginning of year three.

**Technical Objective 3. Characterization of differentially expressed genes in embryonic mammary buds from normal and PTHrP-null mice**

Under technical objective 3, we proposed performing mRNA differential display on RNA isolated from embryonic mammary buds from normal and PTHrP-null mice in order to identify genes that differentially expressed between normal and PTHrP-knockout mammary buds so that we can gain insight into partner molecules to PTHrP in mammary development. In piloting these experiments using total RNA isolated from mammary stromal cells treated with or without PTHrP, we were finding a large number of false positive clones using the differential display technique. Because we were getting a large number of false positives with a pure population of cultured cells, we realized that performing these experiments on RNA generated from embryonic mammary buds would most likely result in an even larger number of false positives, due to the multiple cell types that would be present. In addition, it has proven to be very difficult to obtain enough RNA from the microdissected embryonic mammary buds to perform differential display. Therefore, we have decided that instead of using mRNA differential display on normal versus knockout embryos, we will instead use the technique of representational difference analysis (RDA) on mammary stromal cells treated with and without PTHrP to define more comprehensively the changes in stromal cell gene expression elicited by PTHrP and then to use the knockout embryos as a screen to identify those changes that are functionally important. This strategy consists of three steps: a) to identify genes that are upregulated in response to PTHrP in cultured mammary stromal cells; b) to determine which of these genes is downregulated in PTHrP and PTH/PTHrP receptor knockout embryos; and c) to determine the functional significance of these molecules to the knockout phenotype by examining their ability to rescue the PTHrP-knockout mammary phenotype in organ culture, and in the collagen gel co-cultures discussed in our original proposal.

The technique of RDA is based conceptually on subtractive cDNA hybridization but utilizes PCR to enhance the sensitivity and to reduce the amount of input RNA required (16). A recent modification to this technique incorporates the addition of terminal repeats to further suppress the amplification of more abundant mRNAs (17). The main advantage of this technique over differential display is its much lower rate of false positives. This is particularly important for this project, for it will significantly lower the numbers of isolated clones that must then be analyzed to ensure the their expression is truly modulated by PTHrP.

Even with these changes to this technical objective, we are still on schedule with regards to our statement of work. We are currently in the process of generating poly A+ RNA from mammary stromal cells treated with or without PTHrP and are planning on performing subtractive hybridization using the PCR Select kit from Clontech within the next two months. We plan on completing the subtractive hybridization step in the first half of year two and we will then begin screening the cDNA clones by Northern blot analysis and RNase protection analysis. We will then perform in situ hybridization on PTHrP-null and normal embryonic mammary buds on candidate clones that we feel are interesting biologically and we will perform functional assays in year three as planned.

## Conclusions

Our major findings in the first year of this project have been defining the temporal-spatial pattern of PTHrP and the PTH/PTHrP receptor expression during various stages of mammary development. Specifically, our studies have demonstrated that during active ductal morphogenesis, PTHrP is expressed in epithelial cells and its receptor is expressed in the surrounding mesenchymal cells. In addition, it appears that in the post-natal mammary gland, expression of the PTHrP and the PTH/PTHrP receptor genes is most intense in terminal end buds, regions of active proliferation and ductal morphogenesis during puberty. We have also shown that cultured mammary stromal cells bind amino terminal PTHrP with high affinity and generate a cAMP response to PTHrP. These results underscore the concept that PTHrP, produced by epithelial cells, acts on stromal cells. The mammary stroma is an important contributor to the transformed phenotype and the possibility that PTHrP effects stromal cell function is truly exciting since it might also influence the growth and/or survival of breast carcinoma cells. Therefore, we are concentrating our efforts in years two and three on defining the effects of PTHrP on stromal cell function and how these effects impact the proliferation and morphogenesis of mammary epithelial cells.

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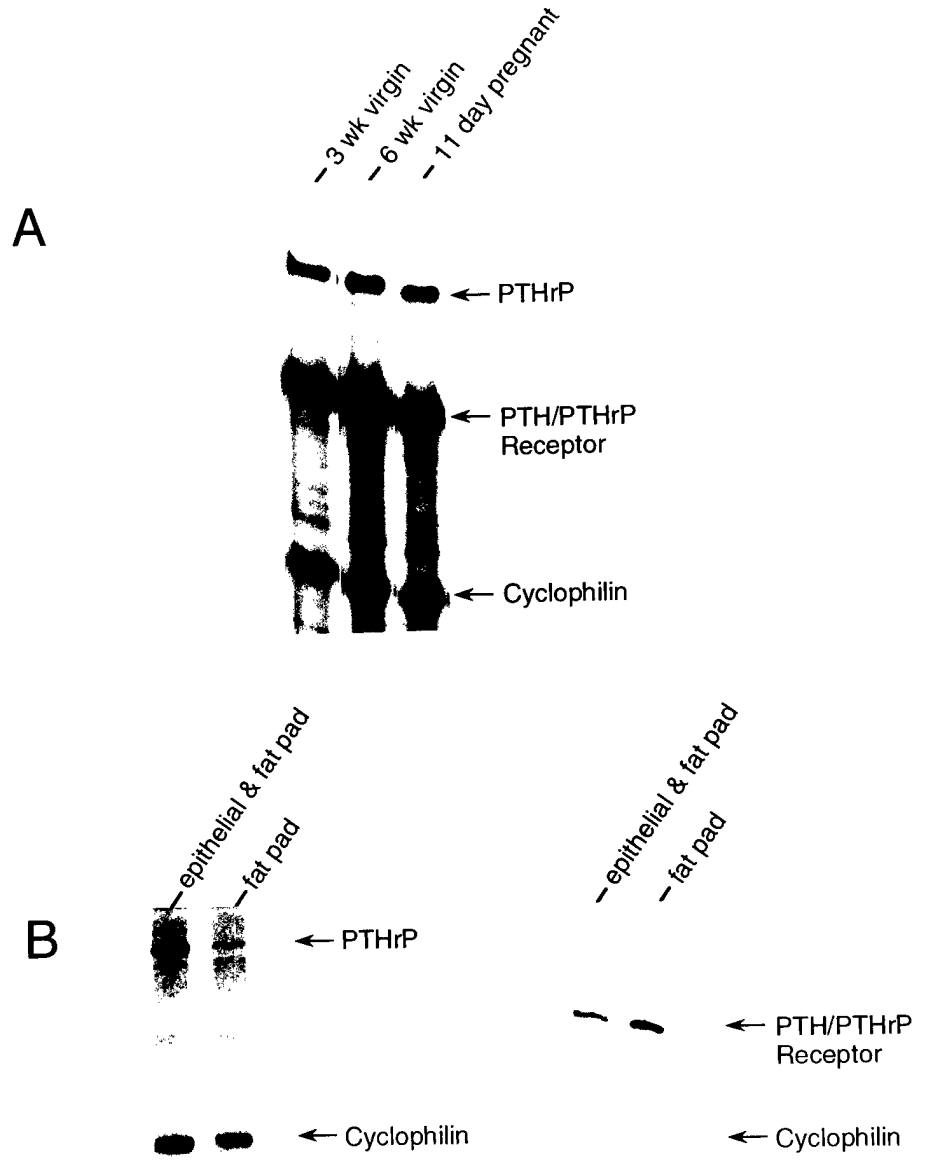


**Figure 1.** (A) Analysis of PTHrP and PTH/PTHrP receptor RNA in the mammary gland during preadolescence, sexual maturation, and pregnancy. 50 μg of total cellular RNA prepared from mammary tissue from preadolescent (3-week-old virgin), adolescent (6-week-old virgin), and pregnant (11 days post coitus) mice was assayed for PTHrP and PTH/PTHrP receptor expression by RNase protection analysis. The murine cyclophilin RNA was included as a loading control. Note that both PTHrP and the PTH/PTHrP receptor are expressed in the mammary gland at each time point. (B) Analysis of PTHrP and PTH/PTHrP receptor mRNA expression in proximal and distal segments of preadolescent mammary glands. Mammary glands from 3 week-old virgin mice were dissected and separated into proximal and distal segments. Whole mount analysis confirmed that the proximal segment contained both epithelial and stromal components, while the distal segment contained only stroma (data not shown). 50 μg of total cellular RNA prepared from either the proximal component (epithelial + fat pad) or the distal component (fat pad) was assayed for PTHrP and PTH/PTHrP receptor expression by RNase protection analysis. Note that the proximal component with both epithelial and stromal cells, contains both PTHrP and the PTH/PTHrP receptor mRNA, but the distal component, with stromal cells alone, contains only the PTH/PTHrP receptor.

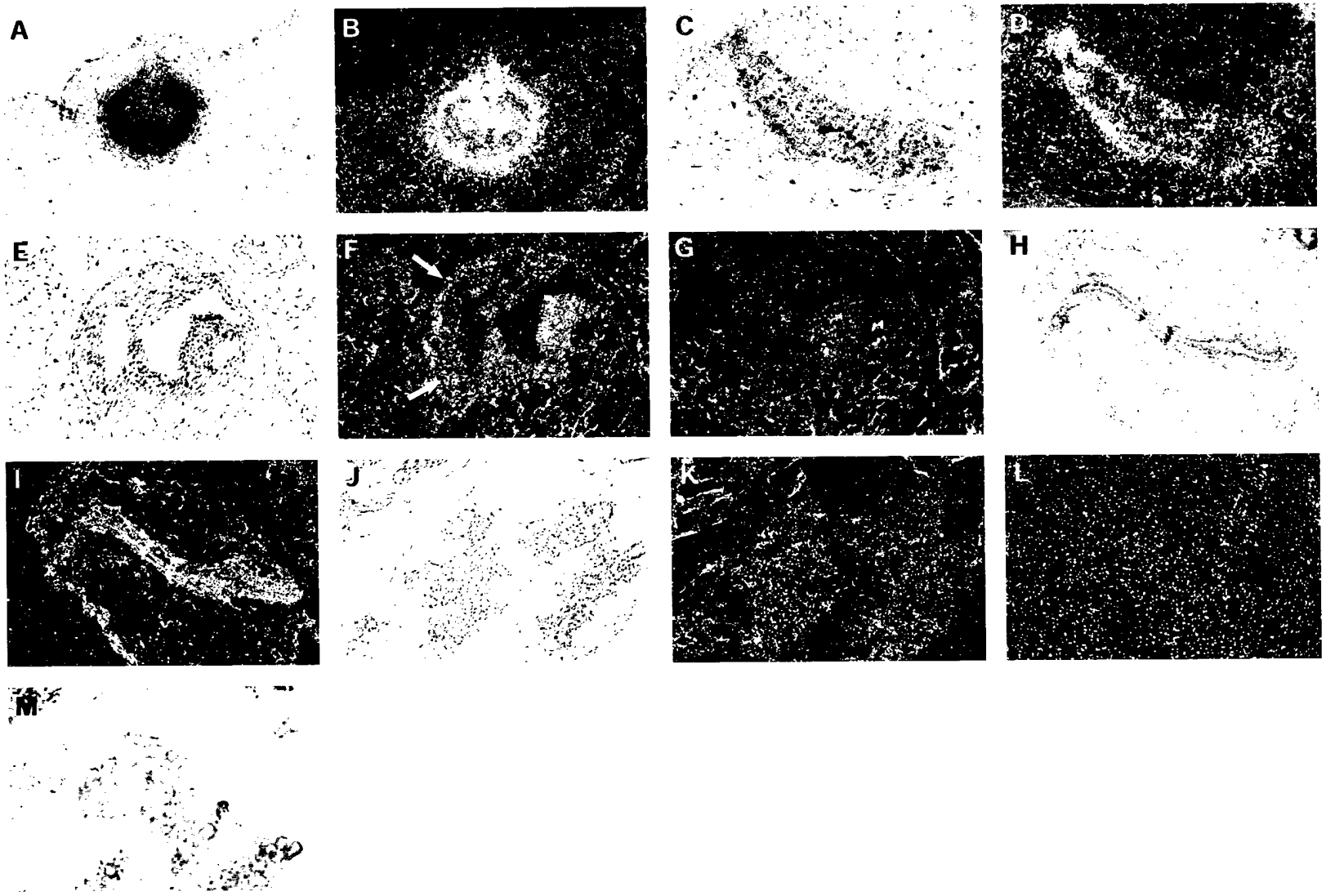


**Figure 2. Localization of PTHrP mRNA expression in mammary glands of embryonic, adolescent, and pregnant mice.** A-D. In situ hybridization for PTHrP mRNA in embryonic mammary rudiments at E12 (A&B) and E18 (C&D). A&C represent brightfield images and B&D represent darkfield images, respectively, of the same sections hybridized with antisense probe. E-I. In situ hybridization for PTHrP mRNA in the adolescent mammary gland. E&F represent brightfield and darkfield images, respectively, of a section through an end bud of mammary gland from an adolescent (4-week-old virgin) mouse hybridized with an antisense probe. G represents a darkfield image of an adjacent section hybridized with a sense probe as a control. H & I represent brightfield and darkfield images, respectively, of a section through a mature duct of an adolescent mammary gland hybridized with an antisense probe. J-M. In situ hybridization for PTHrP mRNA in the pregnant mammary gland. J&K represent brightfield and darkfield images, respectively, through a developing lobuloalveolar unit of a mammary gland from a pregnant (11 days post coitus) mouse hybridized with an antisense probe. L&M represent brightfield and darkfield images, respectively, of a section through a developing lobuloalveolar unit of a mammary gland from a pregnant mouse hybridized with a sense probe as a control.

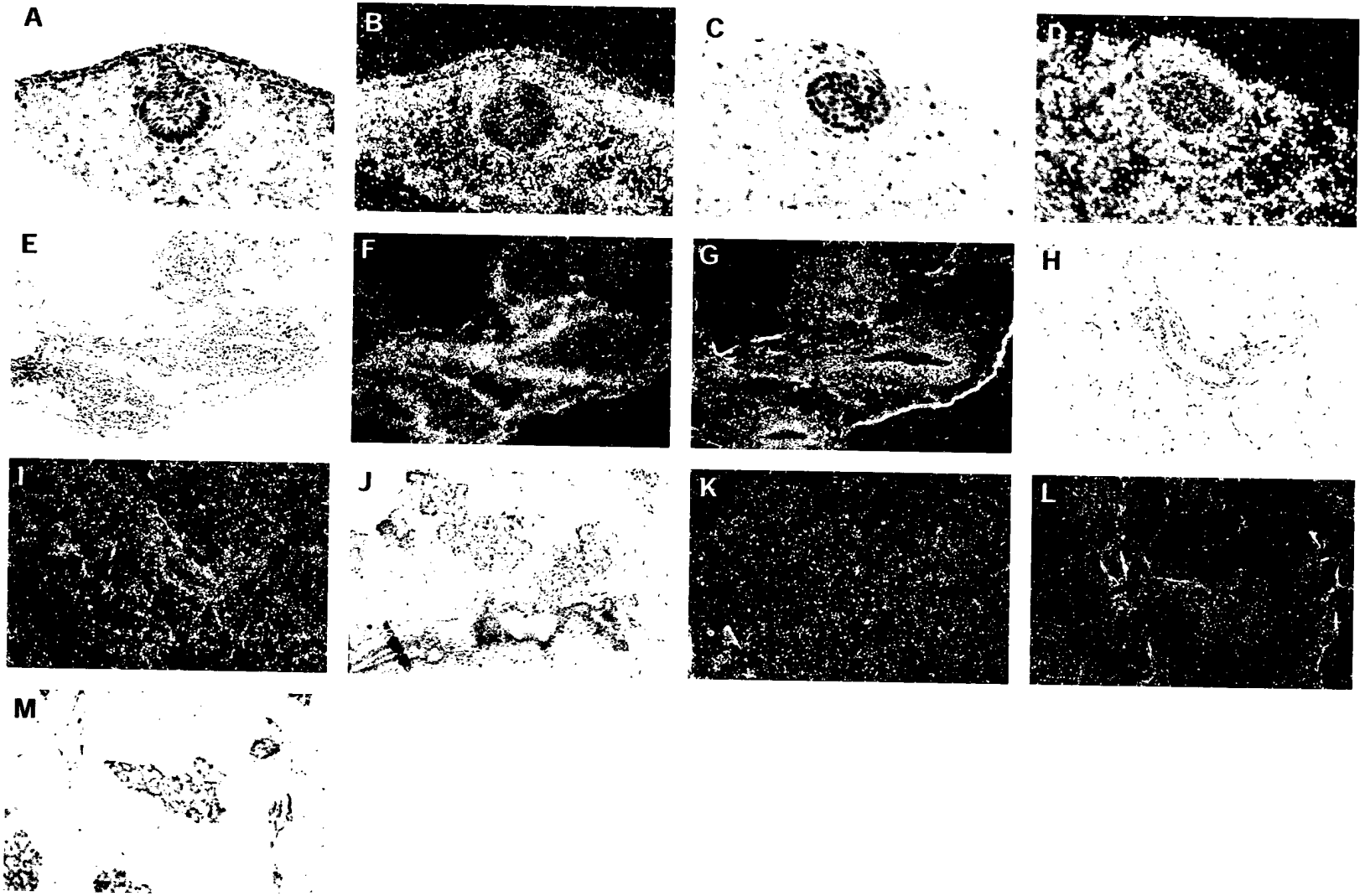


**Figure 3. Localization of PTH/PTHrP receptor mRNA during mammary development.** In situ hybridization for PTH/PTHrP receptor mRNA in embryonic mammary rudiments at E12 (A&B) and E18 (C&D). A&C represent brightfield images and B&D represent darkfield images of the same sections hybridized with antisense probe. E-I. In situ hybridization for PTH/PTHrP receptor mRNA in the adolescent mammary gland. E&F represent brightfield and darkfield images, respectively, of a section through an end bud of a mammary gland from an adolescent (4-week-old virgin) mouse hybridized with an antisense probe. G represents a darkfield image of an adjacent section hybridized with a sense probe as a control. H&I represent brightfield and darkfield images, respectively, of a section through a mature duct of a mammary gland from an adolescent (4 week old virgin) mouse hybridized with an antisense probe. J-M. In situ hybridization for PTH/PTHrP receptor mRNA in the pregnant mammary gland. J&K represent brightfield and darkfield images, respectively, of a section through a developing lobuloalveolar unit of a mammary gland from a pregnant (11 days post coitus) mouse hybridized with an antisense probe. L&M represent brightfield and darkfield images, respectively, of a section through a developing lobuloalveolar unit of a mammary gland from a pregnant mouse hybridized with a sense probe as a control.

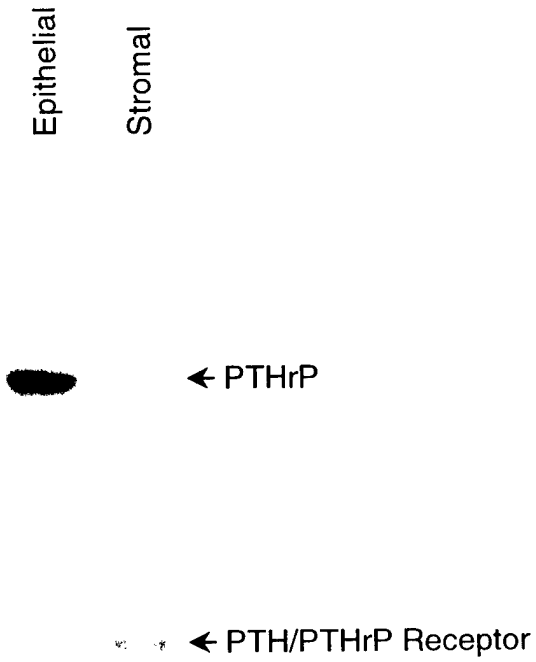


**Figure 4. Analysis of PTHrP and PTH/PTHrP receptor expression in freshly isolated mammary epithelial cells and mammary stromal cells in culture.** 50 μg of total cellular RNA from freshly isolated mammary organoids and cultured mammary stromal cells were assayed for both PTHrP and PTH/PTHrP receptor expression by RNase protection analysis. Note that mammary stromal cells in culture express the PTH/PTHrP receptor but not PTHrP mRNA, whereas mammary epithelial cells express PTHrP mRNA but not PTH/PTHrP receptor mRNA.

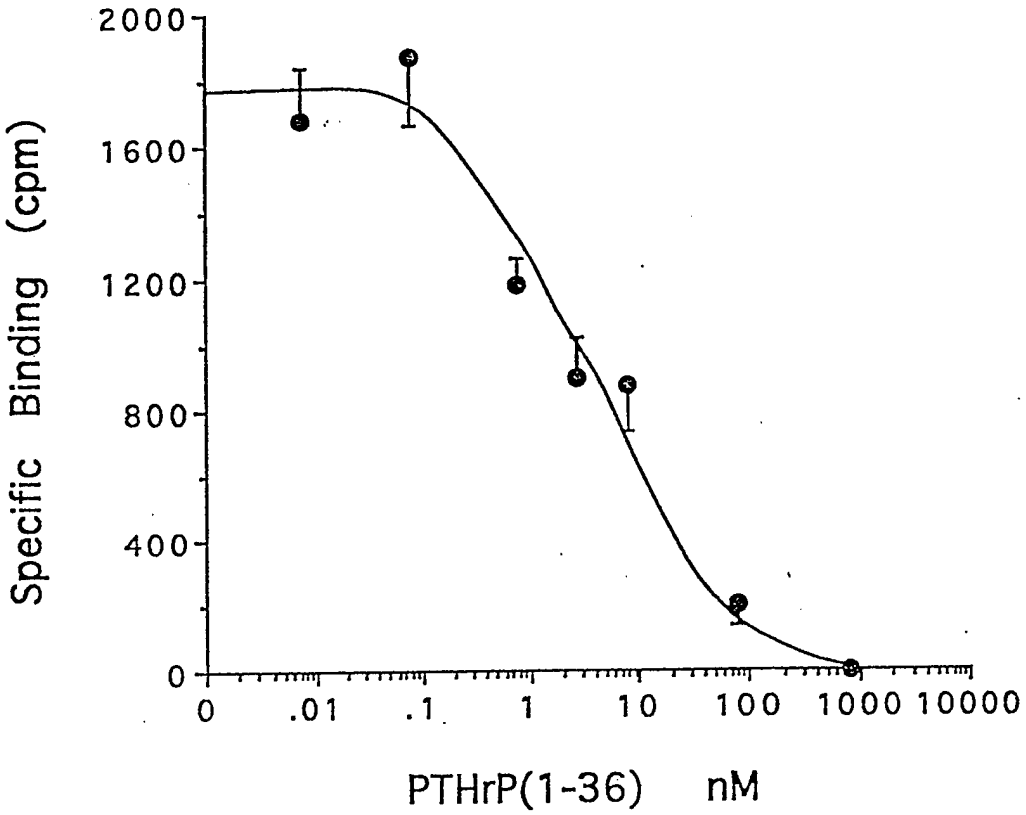


**Figure 5. Binding of PTHrP(1-36) to mammary stromal cells in culture.** Receptor binding assays were performed with $^{125}$I-labeled PTHrP (1-36) amide as a ligand for 4 hours at 4°C with increasing concentrations of unlabeled PTHrP(1-36) as competitor. The data represent the mean ±SE. Shown is a representative of three independent experiments.

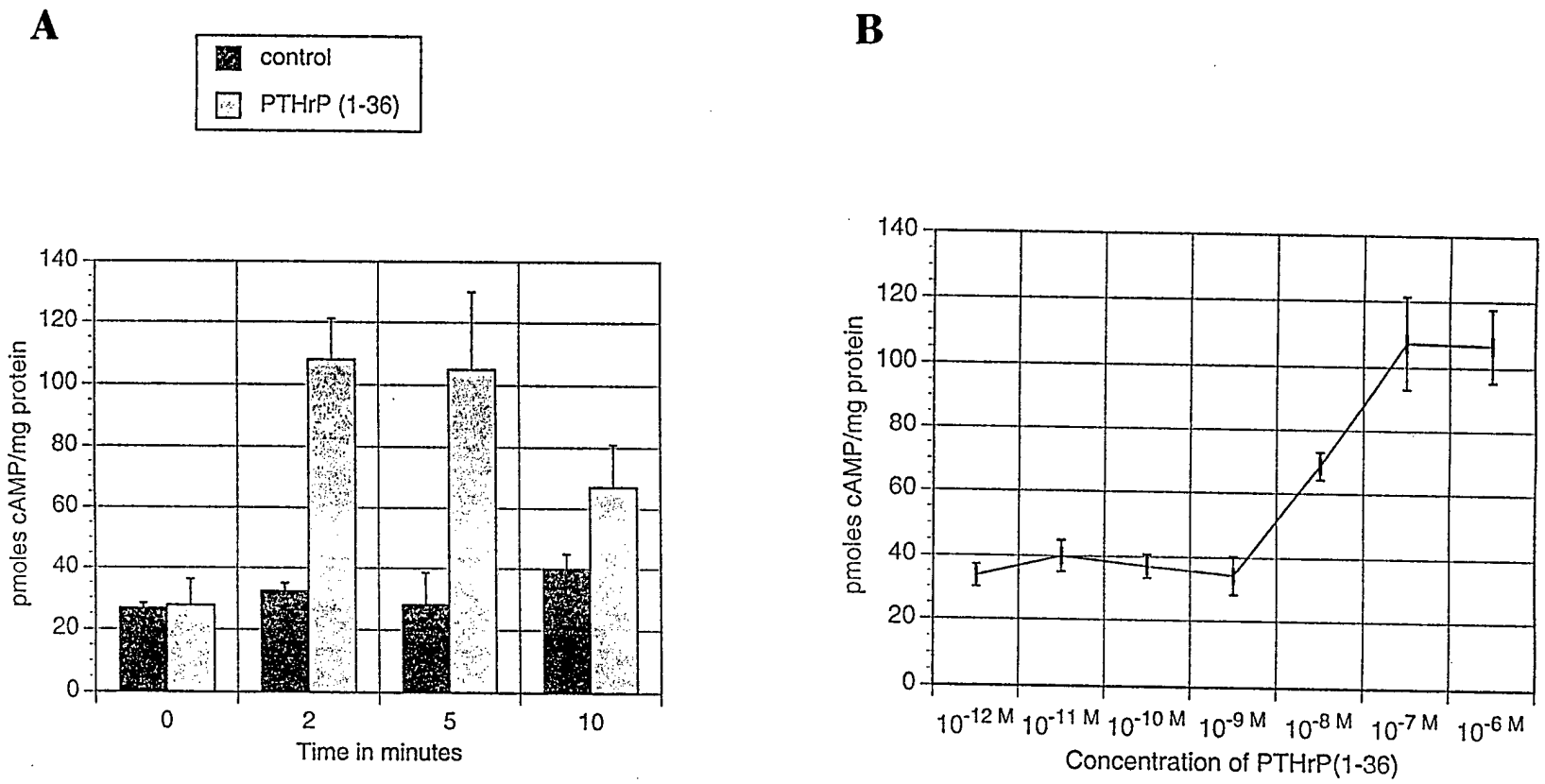


**Figure 6. cAMP response of mammary stromal cells in culture in response to PTHrP(1-36).** A. Time course of cAMP accumulation in primary cultures of mouse mammary stromal cells following treatment with PTHrP(1-36). Mammary stromal cells were incubated for the indicated times at 37 °C in serum free-medium with or without $10^{-7}$ M PTHrP(1-36), and intracellular cAMP was measured by radioimmunoassay. Each point represents the mean ±SE for duplicate samples from three separate experiments. B. Dose-dependent effects of PTHrP(1-36) on cAMP accumulation in mammary stromal cells. Mammary stromal cells were treated with varying concentrations of PTHrP(1-36) for 2 minutes in serum-free medium, and intracellular cAMP was measured by radioimmunoassay. Each point represents the mean ±SE of three experiments each run in duplicate.

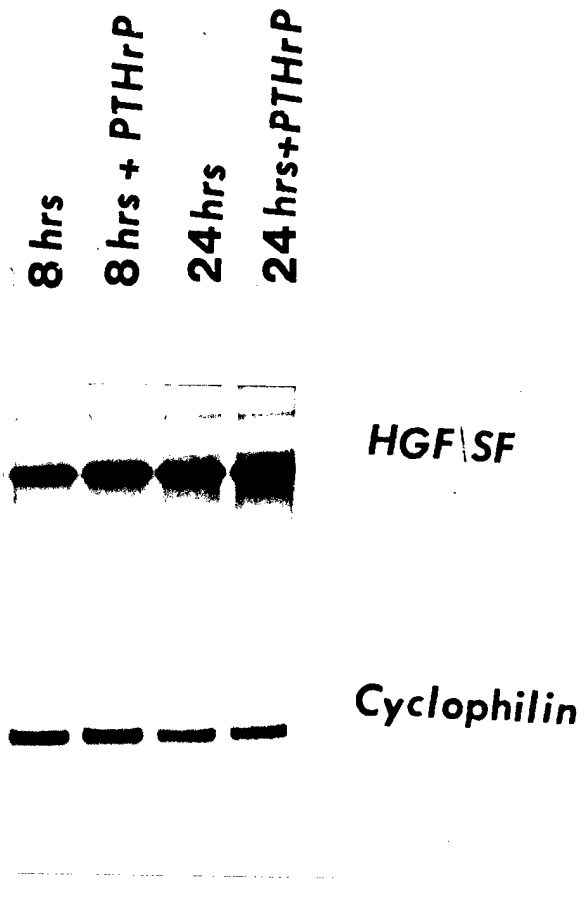


**Figure 7. Effects of PTHrP on the expression of HGF/SF mRNA in mammary stromal cells.** Mammary stromal cells were grown in phenol red free media containing 10% charcoal stripped serum for 5 days. The cells were then treated with either serum free media or serum free media containing $10^{-7}$ M PTHrP for 8 or 24 hours. 20 µg of total cellular RNA was then assayed for HGF/SF mRNA by RNase protection. Note that HGF/SF mRNA levels do not significantly change in response to PTHrP in mammary stromal cells.

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