OVEREXPRESSON OF OVINE INSULIN-LIKE GROWTH FACTOR-I (IGF-I) IN THE MAMMARY GLANDS OF TRANSGENIC MICE

by

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OVEREXPRESSION OF OVINE INSULIN-LIKE GROWTH FACTOR-I (IGF-I) IN THE MAMMARY GLANDS OF TRANSGENIC MICE

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(ABSTRACT)

Evidence indicates that the mammary gland may contribute to its own development by production of insulin-like growth factor-I (IGF-I). To generate a model for investigation of the effects of enhanced IGF-I synthesis in the mammary gland, six transgenic founder mice were produced by microinjection of a cDNA sequence encoding ovine IGF-I (oIGF-I) under the control of the glucocorticoid-responsive mouse mammary tumor virus (MMTV) promoter. Transgenic lines were established from each MMTV-IGF-I transgenic founder, and four virgin and four lactating mice from each transgenic line were analyzed for transgene expression following administration of dexamethasone or vehicle. Virgin mice from three transgenic lines (15, 26, and 29) expressed oIGF-I mRNA and recombinant IGF-I in the mammary gland, although stimulation with exogenous dexamethasone was required for expression. Lactating mice from four transgenic lines (2, 15, 26, and 29) expressed oIGF-I mRNA in mammary tissue, but only three of those lines (15, 26, and 29) produced detectable recombinant IGF-I in milk and mammary tissue. Recombinant IGF-I was not detected in plasma from any of the transgenic lines. Biological activity of recombinant IGF-I secreted
into milk of lactating transgenic mice was demonstrated in vitro by stimulation of 
$[^3H]$thymidine incorporation into DNA of immortalized bovine mammary epithelial cells. The 
MMTV-IGF-I transgenic mice will provide a model for evaluating the effects of increased 
mammary synthesis of IGF-I on mammary gland development and function.
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INTRODUCTION

Lactation is the culmination of a complex series of events in growth, development, and differentiation of the mammary gland. The milk yield potential and economic value of a dairy heifer about to begin lactation is thus largely a reflection of the mammary gland development that occurred during her first two years of life. At birth, the mammary gland comprises a rudimentary duct system embraced by a comparatively well-developed stroma. Before puberty, the gland grows isometrically with the rest of the body as ducts extend into the expanding stromal tissue. Prior to the first estrous cycle, mammary ducts commence a period of allometric growth, occurring in heifers from about three months until nine months of age and from approximately three weeks until six weeks in mice and rats (Sheffield, 1988). Mammary growth reverts to an isometric pattern following several estrous cycles and remains in a relatively quiescent state until conception. During gestation, the hormonal environment stimulates further elongation of mammary ducts and alveoli begin to fill the fat pad. Lactogenesis is characterized by the differentiation of the alveolar cells to prepare for milk synthesis and secretion in the periparturient period. Mammary cell proliferation continues until the gland is fully developed, which in many species does not occur until the early stages of lactation (Tucker, 1987).

The presence of a number of hormone receptors in the mammary gland indicates that the intricate pattern of growth and development is orchestrated by a large number of hormones and growth factors (Akers, 1985, 1990; Forsyth, 1991). Older studies of
mammogenesis in rodents and lambs defined growth hormone (GH) as a critical component of the hormonal milieu controlling mammary growth and development (Forsyth, 1989). Indeed, GH and estrogen are generally considered to be of primary importance in regulation of ductal growth in peripubertal animals (Topper and Freeman, 1980). However, the mechanism of action of GH has been questioned due to a lack of conclusive evidence demonstrating the presence of receptors for GH in the mammary gland, particularly in ruminants.

Growth hormone is believed to exert many of its growth-promoting effects via insulin-like growth factor-I (IGF-I) (Sara and Hall, 1990). Circulating IGF-I is produced primarily in the liver, but a number of other tissues also produce IGF-I (D'Ercole et al., 1984). Receptors for IGF-I have been detected in mammary epithelial cells from several different species; further, IGF-I has been shown to stimulate DNA synthesis and cell proliferation in mammary tissue in vitro (Akers, 1990). The primary source of IGF-I for the mammary gland and its regulation are not known, and the issue is complicated by the likelihood that mammary development is directed by both autocrine and systemic endocrine actions (Wilde et al., 1990).

Administration of exogenous GH to lactating cows induces an increase in circulating concentrations of IGF-I, and the temporal pattern of the IGF-I response corresponds with its proposed role in mediating GH effects on milk yield (Bauman and Vernon, 1993). In support of a mechanism for GH action directly on the mammary gland, local implants of GH stimulated production of IGF-I mRNA within the mammary glands of rats (Kleinberg et al., 1990), and GH administration induced an increase in immunoreactive IGF-I in bovine
mammary epithelial cells (Glimm et al., 1988). Thus, IGF-I may exert at least a portion of its effects in the mammary gland via an autocrine and (or) paracrine mechanism of action. However, the degree of contribution of locally produced IGF-I relative to systemic IGF-I in the development and function of the mammary gland is not known.

It has been clearly demonstrated for many species that IGF-I is both a mitogen and a metabolic regulator of mammary tissue in vitro (Campbell et al., 1991). It is also evident that IGF-I stimulates growth of isolated mammary epithelial cells (Zhao et al., 1992) and that clonal bovine mammary epithelial cells which synthesize and secrete recombinant IGF-I stimulate epithelial cell proliferation in an autocrine and (or) paracrine fashion (Romagnolo et al., 1992). However, the effects of mammary-specific synthesis of IGF-I in vivo have not been reported. If the mitogenic action of IGF-I persists in mammary tissue in vivo, appropriate temporal and mammary-specific synthesis of IGF-I could stimulate cell proliferation resulting in an increased number of mammary epithelial cells. Because the number of mammary cells is directly related to milk yield (Tucker, 1981), stimulation of mammary cell proliferation during the peripubertal period might increase milk production in the lactating adult. The objective of this study was to generate transgenic mice that contain a cDNA for ovine IGF-I (oIGF-I) under the control of the mouse mammary tumor virus (MMTV) promoter as a model for evaluating the effects of increased mammary synthesis of IGF-I on mammary gland development and function.
REVIEW OF LITERATURE

I. INSULIN-LIKE GROWTH FACTOR-I (IGF-I)

1. Introduction

Insulin-like growth factor-I (IGF-I) is a single-chain 7500 Da peptide that is structurally similar to insulin-like growth factor-II (IGF-II) and to insulin, but the IGFs display distinct receptor binding and biological effects (Sara and Hall, 1990). The IGF-I molecule has an identical 70-amino acid sequence in the bovine, human, and pig, differing from ovine IGF-I at position 66 (alanine is substituted for proline) and from murine IGF-I by 5 amino acids (Wong et al., 1989). Insulin-like growth factor-I functions in the mediation of normal GH-dependent postnatal growth, particularly in skeletal and cartilage tissue, whereas IGF-II is considered to be important in fetal development and is not apparently regulated by GH (Tripathy and Benz, 1994).

The mitogenic activity of IGF-I in stimulating DNA synthesis and cell proliferation has been characterized in a wide variety of cell types (Sara and Hall, 1990). Growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) are competence factors that initiate the cell cycle and stimulate cells to enter the G\textsubscript{i} phase; whereas progression factors such as IGF-I and insulin signal cells in late G\textsubscript{i} to enter the S phase (DeMeyts et al., 1994). In addition, IGF-I has been demonstrated to stimulate cellular differentiation as well as anabolic responses such as amino acid uptake and protein synthesis.
in target cells (Sara and Hall, 1990). Cellular responses to IGF-I are influenced by specific IGF-binding proteins (IGF-BPs) whose postulated functions include facilitating circulatory transport, retarding degradation of IGF-I, and positively or negatively modulating IGF-I activity at the cellular level (Rechler, 1993).

Biological effects of IGF-I occur through interaction with the IGF-I receptor, a specific cell-surface receptor involving a transmembrane tyrosine kinase that is structurally related to the insulin receptor. Although considered to be mainly involved in mitogenic signaling, the IGF-I receptor binds both IGF-I and -II with similar affinity and insulin with a 500-1,000 times lower affinity, which may account for a portion of the mitogenic actions of insulin at high concentrations (DeMeyts et al., 1994). Insulin-like growth factor-I also binds with a reduced affinity to the IGF-II receptor (LeRoith and Roberts, 1993) and can weakly cross-react with the insulin receptor, explaining in part its insulin-like effects on metabolism.

2. Regulation of IGF-I expression

In recent years, wide-ranging efforts to maximize the productive efficiency of dairy cattle have included the manipulation of endogenous hormone concentrations to enhance mammary growth and development and galactopoiesis. It is well documented that exogenous GH stimulates mammary growth in dairy heifers (Sejrsen et al., 1986; Sandles et al., 1987) and lambs (Johnsson et al., 1986; McFadden et al., 1990) as well as milk production in lactating dairy cattle (Peel and Bauman, 1987). However, the mechanism by which GH exerts its influence on mammogenesis and galactopoiesis remains unclear. The inability to detect
specific receptors in microsomal membrane preparations suggests that GH does not act directly on the bovine mammary gland (Akers, 1985; Keys and Djiane, 1988). Moreover, addition of GH to explants from bovine lactating mammary glands in culture had no stimulatory effect on synthesis of milk components (Gertler et al., 1983). More recently, on the other hand, GH receptor mRNA has been localized to bovine mammary epithelial cells (Glimm et al., 1990) and both epithelial and stromal components of the bovine mammary gland in pregnant animals (Hauser et al., 1990), although Hauser observed that GH receptor mRNA levels were approximately 30-fold lower in mammary tissue compared to liver. Thus regulation of GH receptor expression may occur at a post-transcriptional level, or the receptor number may be too low to allow detection by conventional assays.

Species differences may also exist in regard to responsiveness to GH of the mammary gland, as several recent lines of evidence suggest a direct action of GH on the rat mammary gland. Growth hormone receptor gene expression was detected in poly(A)$^+$ RNA from the mammary gland (Rosato et al., 1994). Furthermore, the GH receptor has been identified in ductal and alveolar epithelial cells of the rat mammary gland by immunocytochemistry (Lincoln et al., 1990) and in whole mammary gland homogenates by a conventional binding assay (Feldman et al., 1993b). A direct action of GH on the rat mammary gland was implied by a stimulation of milk synthesis in treated but not contralateral glands using local GH implants (Flint and Gardner, 1994). Further, local implants of several GH variants stimulated unilateral mammary development in contrast to rat prolactin (PRL) or other PRL variants, suggesting that the effects occurred through the GH receptor and not the PRL receptor.
(Feldman et al., 1993a). It is generally accepted that GH does not interact directly with bovine mammary epithelial cells to yield an effect (Bauman and Vernon, 1993); however, results of these studies do not discount the possibility of a paracrine effect of GH possibly mediated by local production of IGF-I.

According to the original somatomedin hypothesis proposed by Salmon and Daughaday (1957), GH stimulates liver synthesis of IGF-I, which is transported via the circulation to target tissues where it mediates the actions of GH. Increased concentrations of circulating IGF-I in response to exogenous GH may effect such an endocrine action in stimulating milk yield in lactating dairy cows (Cohick et al., 1989; Davis et al., 1987) and in lactating goats (Davis et al., 1989). However, attempts to demonstrate an effect of IGF-I on milk production in vivo have met with limited success. Increasing systemic IGF-I concentrations by jugular infusion of IGF-I did not stimulate milk yield, despite serum IGF-I concentrations being increased to similar levels as in GH-treated animals (Davis et al., 1989).

On the other hand, close-arterial infusion of free IGF-I into the pudic artery of lactating goats for 6 h stimulated milk production in the infused gland relative to the noninfused gland, possibly as a result of association of free IGF-I with plasma IGF-I binding proteins upon recirculation (Prosser et al., 1990). Furthermore, comparison of lactating goats differing in responsiveness to GH treatment showed that increased milk yield corresponded with increased concentrations of IGF-I in milk and mammary tissue, while one animal in which GH treatment did not increase milk yield had unaltered IGF-I concentrations in milk and mammary tissue (Prosser et al., 1991b). Thus, mammary IGF-I may play a key role in the
galactopoietic effects of GH. To explain the observed changes in IGF-I concentrations in milk, it was suggested that transport from serum into milk is largely responsible for the increased milk IGF-I concentrations in lactating cows treated with GH (Prosser et al., 1989a). Interestingly, the transport mechanism appeared to be competitive and saturable, indicating that local mammary production of IGF-I may contribute to IGF-I levels in milk (Prosser et al., 1991a).

Growth hormone may stimulate synthesis of IGF-I in target tissues which then acts locally on cells of the target tissue (Holly and Wass, 1989). The mammary gland may be such a target tissue, as exogenous GH stimulates IGF-I mRNA production within the rat mammary gland (Kleinberg et al., 1990). In addition, GH administration increased the presence of immunoreactive IGF-I in the cytoplasm of mammary epithelial cells from lactating cows (Glimm et al., 1988) and lactating goats (Prosser et al., 1989b). Implants of native IGF-I as well as des-(1-3)-IGF-I, an IGF-I variant lacking 3 amino-terminal residues, were shown to substitute for GH in mammary development in hypophysectomized castrated male rats, although estrogen is required for a full effect (Ruan et al., 1992; Ruan et al., 1995). Similar to other tissues, mammary gland production of IGF-I under control of circulating GH may contribute to the growth and differentiation of mammary epithelial cells, although evidence for the presence of GH receptors on the mammary gland is limited.

Similar to GH, a number of hormones and growth factors can regulate the paracrine synthesis of IGF-I in non-hepatic tissues. For example, estrogen and progesterone induce an increase in IGF-I mRNA levels and IGF-I content in the uterus and ovary (Simmen, 1991).
Further, PDGF and fibroblast growth factor (FGF) can stimulate the production of IGF-I from human fibroblasts. On the other hand, the growth inhibition produced by the synthetic glucocorticoid dexamethasone in rat neuronal and glial cells is associated with a reduction in IGF-I mRNA (Adamo et al., 1988). Thus, the effects of many hormones on growth may be mediated in part by modulation of local IGF-I production. Further investigation is required to determine the mechanism by which these factors stimulate IGF-I synthesis; however, these results serve to highlight the complex regulation of IGF-I production by a variety of determinants including hormonal, nutritional, and developmental factors.

3. Role of IGF-I in mammary growth and function

The mitogenic activity of IGF-I in mammary epithelial cells from a number of species has been well established. Insulin-like growth factor-I enhances DNA synthesis in cultures of normal mammary epithelial cells of the mouse (Imagawa et al., 1986); rat (Ethier et al., 1987); sheep (Winder et al., 1989); and the cow (Shamay et al., 1988; Baumrucker and Stemberger, 1989; McGrath et al., 1991); moreover, IGF-I was demonstrated to stimulate milk protein synthesis in mouse mammary epithelial cells (Prosser et al., 1987). In addition, overexpression of IGF-I in mammary epithelial cells in vitro appears to cause typical mitogenic effects displayed in other tissues in vivo. For example, dexamethasone induction of an oIGF-1 transgene under control of the MMTV promoter in the MD-IGF-I clonal bovine mammary epithelial cell line increased thymidine incorporation and cell proliferation (Romagnolo et al., 1992).
Receptors for IGF-I have been located on mammary epithelial cells in cows (Dehoff et al., 1988; Hadsell et al., 1990), sheep (Winder et al., 1993), and rats (Lavandero et al., 1991). The number of IGF-I receptors is increased during lactogenesis in the bovine (Dehoff et al., 1988; Hadsell et al., 1990). The highest concentrations of IGF-I in mammary secretions have been observed in the last two weeks prepartum and in colostrum immediately following parturition (Einspanier and Schams, 1991). Des-(1-3)-IGF-I is present in colostrum as a larger proportion (~50%) of the total IGF-I fraction in comparison to its low levels (~3%) in milk of normal or GH-treated cows (Shimamoto et al., 1992). Des-(1-3)-IGF-I displays greater biological mitogenic activity than IGF-I in bovine mammary epithelial cells (McGrath et al., 1991), possibly due to lack of binding to IGF-BPs (Ross et al., 1989). Thus, increased unbound IGF-I concentrations in mammary secretions around the time of parturition may stimulate the proliferation or differentiated function of mammary epithelial cells in early lactation. Alternatively, the striking shift in IGF-I form in milk may be significant for growth of the neonate (Baumrucker et al., 1994).

Detection of IGF-I expression in many body tissues indicates that indeed, in addition to its endocrine action, IGF-I may exert at least a portion of its mitogenic effects by autocrine and (or) paracrine mechanisms (D'Ercole et al., 1984). Insulin-like growth factor-I mRNA has been localized to stromal cells but not epithelium of the mammary gland (Yee et al., 1989; Hauser et al., 1990), suggesting that IGF-I of stromal origin may influence epithelial cells by a paracrine mechanism of action. Similarly, synthesis of IGF-I was found in bovine mammary tissue explants, but not acini cultures, from lactating and nonlactating cows (Campbell et al.,
1991). In addition, IGF-I mRNA has been found in normal mammary tissue in the pig (Lee et al., 1993) and rat (Marcotty et al., 1994; Murphy et al., 1987). Insulin-like growth factor-I has been immunolocalized to stroma and epithelial cells in mammary tissue from lactating cows, although the source of the IGF-I was not determined (Glimm et al., 1988). Taken together, this evidence indicates that mammary stroma can synthesize IGF-I for a paracrine effect on mammary epithelial cells; however, further investigation is warranted to determine whether expression of IGF-I mRNAs in the mammary gland is temporally regulated (Simmen, 1991).

Thus, IGF-I may be produced by the liver as well as by other tissues, making it difficult to distinguish the effects of circulating IGF-I from those of locally synthesized IGF-I. Hodgkinson et al. (1991) determined the tissue distribution of the recombinant amino terminal methionyl variant of IGF-I following intravenous administration to lactating sheep. Their results indicate that the majority of IGF-I in lactating mammary epithelium is derived from blood; whereas the connective tissue from the mammary gland, which may actively synthesize IGF-I, contained significantly less of the IGF-I variant as a proportion of the total tissue IGF-I.
II. TRANSGENIC ANIMAL MODELS

1. Transgene expression in the mammary gland

In the last decade, transgenic animals have become useful models for elucidating the function of various hormones involved in modulation of mammary growth. The mammary gland is an especially appropriate organ in which to study the functions of proteins involved in tissue remodeling, as the gland undergoes the majority of its development after birth and experiences repeated cycles of growth, differentiation, function, and involution (Forsyth, 1989). For example, expression of transforming growth factor-β (TGF-β) in transgenic mice has resulted in inhibition of mammary development (Pierce et al., 1993) and lactation (Pierce et al., 1993; Jhappan et al., 1993), suggesting a role for TGF-β in the regulation of ductal and alveolar proliferation. Similarly, transgenic mice overexpressing transforming growth factor-α (TGF-α) under regulation of the mouse mammary tumor virus-long terminal repeat (MMTV-LTR) promoter failed to lactate, although this effect was attributed to an inhibition of the normal rise in prolactin receptors following parturition (Sakai et al., 1994). Furthermore, a role for whey acidic protein in modulating growth and differentiation in mammary epithelial cells was identified by the study of transgenic mice (Burdon et al., 1991) and transgenic pigs (Shamay et al., 1992).

Expression of a number of transgenes influencing mammary development and function has been directed to the mammary gland by use of the MMTV-LTR. The MMTV promoter is under transcriptional control of a glucocorticoid response element (GRE) as well
as other regulatory elements within the LTR that serve to confer tissue specificity on expression (Ross et al., 1990; Stewart et al., 1988). Transgenes containing the MMTV promoter are expressed predominantly in the mouse mammary gland and at lower levels in other tissues including the salivary glands, kidneys, and spleen (Choi et al., 1987; Hennighausen et al., 1994). Enhancer sequences present in the LTR of the promoter can direct expression of marker genes to virgin mammary glands of transgenic mice, although hormonal stimulation during lactation induces a dramatic increase in MMTV expression that is mediated by the GRE (Mok et al., 1992). Indeed, administration of dexamethasone to virgin transgenic mice carrying various deletion forms of the MMTV-LTR driving the chloramphenicol acetyltransferase (CAT) reporter gene yielded an increase in CAT activity similar to that seen in lactating females relative to virgin females (Ross et al., 1990).

2. IGF-I transgenic animals

Insulin-like growth factor-I has been implicated in many biological processes including prenatal and postnatal growth, lactation, and reproduction (McGuire et al., 1992). The importance of IGF-I as a mediator of GH action in postnatal growth is illustrated by work with transgenic mice overexpressing human IGF-I under control of the mouse metallothionein-I (mMT) promoter. When mMT-IGF-I mice were crossed with GH-deficient dwarf transgenic mice, IGF-I expression from the transgene was able to restore normal body weight and linear growth (Behringer et al., 1990). Moreover, lines of mice selected for high plasma IGF-I concentrations over seven generations were heavier than control mice, although
body composition was unchanged (Siddiqui et al., 1990). Transgenic mice carrying the nMT-IGF-I construct overexpressed IGF-I in many tissues and exhibited overgrowth of the carcass, brain, pancreas, kidney, and spleen. Total DNA content of the overgrown organs was increased, implying that hyperplasia accounted for the increase in organ size (Mathews et al., 1988). Brem and colleagues have generated transgenic rabbits that also overexpress biologically active human IGF-I under control of regulatory elements from the bovine α<sub>4</sub>-casein gene (Brem et al., 1994). Mammary-specific expression as high as 1 g IGF-I/l of milk has been achieved although mammary effects of the IGF-I overexpression have not been reported. Because the mitogenicity of IGF-I has been clearly demonstrated in mammary tissue in vitro as well as in normal and transgenic mice, such transgenic animals would provide a powerful in vivo approach to studying the role of IGF-I in the mammary gland.
SUMMARY

It has been well established that IGF-I produced in the liver mediates many of the endocrine actions of GH; in addition, IGF-I is locally produced within a variety of tissues and may exert its mitogenic and differentiative effects by an autocrine and (or) paracrine mechanism. The mammogenic and galactopoietic effects of exogenous GH in several species have been largely attributed to the resulting increase in circulating concentrations of IGF-I. On the other hand, stimulation of local synthesis of IGF-I in the mammary gland may enhance mammary gland development and milk production. The objective of this study was to generate transgenic mice producing recombinant IGF-I in the mammary gland as a model for evaluating the effects of increased mammary synthesis of IGF-I on mammary development and function.
MATERIALS AND METHODS

The steps involved in the production and analysis of transgenic mice generated by microinjection have been summarized in Figure 1. Briefly, embryos were collected from superovulated CD-1 donors and microinjected in one of two pronuclei with the MMTV-IGF-I transgene. Following microinjection, embryos were transferred into the oviducts of pseudopregnant recipients that carried the embryos to term. Live pups were analyzed by the polymerase chain reaction (PCR) to identify mice that had integrated the transgene. Transgenic lines were established from each transgenic founder mouse, and mice within each line were evaluated for expression of the transgene. Finally, biological activity of the recombinant protein secreted into milk of transgenic mice was evaluated by comparing the ability of transgenic and control mouse milk to stimulate [3H]thymidine incorporation into DNA of MAC-T cells (Huynh et al., 1991; Zhao et al., 1992).

Construct preparation. A 3.7 kb fragment composed of a 0.7 kb cDNA encoding an ovine exon-2 preproIGF-I (Wong et al., 1989) under control of the MMTV-LTR promoter was prepared for microinjection to generate MMTV-IGF-I transgenic mice. An oIGF-I cDNA was used in the transgene because the biological activity of the recombinant IGF-I protein had been characterized previously in clonal bovine mammary epithelial cells containing the MMTV-IGF-I construct, and the construct was readily available. The construct was isolated from the expression vector pMMTV1GF-I (Romagnolo et al., 1992) following digestion with the restriction endonucleases Bgl II and Nde I (USB, Cleveland, OH). The
Figure 1. Steps involved in the generation and analysis of transgenic mice.
fragment was excised from a 0.8% low melting point agarose gel and purified using the GeneClean procedure (BIO 101 Inc., La Jolla, CA) followed by an additional precipitation with 0.3 M Na acetate and ethanol to remove salts. The MMTV-IGF-I construct was diluted in TE buffer (10 mM Tris-HCl, pH 7.5; 0.25 mM EDTA) and DNA concentration and purity was evaluated by absorbance readings at 260 nm and 280 nm. A DNA solution of 3.45 ng/ml (~850 copies/pl) was prepared and filtered through a 0.45 mm spin filter prior to use.

**Superovulation.** Female CD-1 mice (Charles River Laboratories, Wilmington, Massachusetts) 3 to 4 wk of age were superovulated with intraperitoneal injections of 5.0 IU Pregnant Mare's Serum Gonadotropin (PMSG) (Diosynth Inc., Chicago, IL) followed by 2.5 IU human Chorionic Gonadotropin (hCG) (SIGMA, St. Louis, MO) 47 h later and placed with intact CD-1 males. Also at this time, mature CD-1 females were placed with vasectomized males to generate pseudopregnant recipients.

**Embryo recovery and microinjection.** Approximately 20 h after hCG administration, superovulated females displaying a copulatory plug were sacrificed by cervical dislocation. Oviducts were excised and placed in a culture dish containing M2 media (Quinn et al., 1982), and the ampulla of each oviduct was ruptured with a 25 gauge hypodermic needle to release embryos from the oviduct. The embryos were collected and washed briefly in M2 containing 100 μg/ml hyaluronidase (800 U/mg) (SIGMA, St. Louis, MO) to remove cumulus cells. Embryos were maintained in M2 at 37° C on a slide warmer. For microinjection, approximately 50 embryos were placed in a 100 μl drop of M2 covered with mineral oil in a culture dish and held on the heated stage of an inverted microscope (Zeiss,
model ICM 405; Eastern Microscopes, Raleigh, NC). Microinjections were performed under Hoffman modulation optics at 200x using a Leitz micromanipulator and an Eppendorf (Eppendorf model 5242; Eastern Microscopes, Raleigh, NC) automatic microinjector. One-cell embryos were microinjected in 1 of the 2 pronuclei with 1 to 3 pl of the DNA solution. Following microinjection, embryos were washed and held in M2 at 37°C on a warm plate until transfer to recipients.

**Embryo transfers.** Approximately 1 h following microinjection, viable embryos were selected on the basis of the presence of a distinct outline and an apparent perivitelline space and transferred into pseudopregnant recipient CD-1 mice according to Polites and Pinkert (1994) with slight modifications. Briefly, embryos were separated into groups within a culture dish for each transfer. Each group of embryos was loaded in 1 to 2 µl of M2 media into a flame-polished capillary tube using a mouthpiece for fine suction control. A pseudopregnant female was anesthetized with 0.4 mg/g body weight of sodium pentobarbital (Anpro Pharmaceutical, Arcadia, CA) and placed in ventral recumbency. The surgical incision site was swabbed with 70% alcohol and a skin incision was made approximately 1 cm to the left of the spine directly posterior to the last rib. The ovarian fat pad was located through the intact body wall, and a second smaller incision was made through the body wall to expose the fat pad. The ovarian fat pad was grasped with blunt forceps and pulled out through the incisions to clearly expose the oviduct. The oviduct was held in place by gripping the fat pad with a serafine clamp. A drop of epinephrine (1:1000; Vedco Inc., St. Joseph, MO) was applied to the ovarian bursa to reduce bleeding before tearing the bursa to expose
the ostium of the oviduct. Embryos in the transfer pipette were then discharged through the ostium into the oviduct by gentle pressure through the mouthpiece until an air bubble was expelled behind the embryos, indicating that transfer was complete. The reproductive tract was replaced in the abdomen and both incisions were closed with a wound clip. Recipients were observed 19 to 20 d later for the presence of offspring (Bronson and McLaren, 1970).

**DNA isolation and PCR analysis.** Tail tissue was biopsied from 21-day-old pups at weaning and immediately frozen in liquid nitrogen. Isolation of DNA was performed according to an adaptation of the procedure developed by Marmur (1961). Briefly, tissue samples were incubated overnight at 55 °C in 840 μl of lysis solution (50 mM Tris-HCl, pH 8.0; 0.15 M NaCl, 1 M Na$_2$CO$_3$, 10 mM EDTA, 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol, and 100 μg/ml proteinase K). The samples were extracted with 250 μl of phenol and chloroform (1:1) by mixing for 15 s on a "Mini Bead-Beater" (Biospec Products, Bartlesville, OK) and centrifuging for 10 min at 11,000 × g. Next, DNA was precipitated from the supernatant by the addition of 600 μl of isopropyl alcohol followed by centrifugation at 11,000 × g. The DNA pellet was washed in 80% ethanol and dried at 37 °C before resuspension in 250 μl of TE buffer (10 mM Tris-HCl, pH 8.0; 1mM EDTA). The DNA samples were extracted with 50 μl of chloroform and isoamyl alcohol (25:1) by mixing. This was followed by centrifugation at 11,000 × g for 10 min. Finally, DNA was precipitated from the supernatant with 500 μl of 95% ethanol in the presence of 25 μl of 3 M Na acetate, followed by a wash of the pellets with 80% ethanol. The pellets were dried and dissolved in 150 μl of TE buffer and stored at -20°C. Concentration of the DNA was measured by an
absorbance reading at 260 nm.

Thirty nanograms of DNA in 1 μl of solution served as the template for PCR analysis in 25 μl reaction volumes [1X Taq polymerase buffer, 0.2 mM dNTP's, 0.4 μM oligonucleotide primers, 1.5 mM MgCl₂, 0.625 U Taq polymerase] (Promega Corp., Madison, WI). The oligonucleotide primers 3c (5'-GATGCCAGTCACATCTCCTCGC-3'; exon 3, nucleotides 190-212) and 4a (5'-GAGCCTTGGGATGTCGGTG-3'; exon 4, nucleotides 383-403) in the reaction amplified a 329-bp sequence in the ovine IGF-I cDNA (Ohlsen et al., 1993). Initial denaturation of the samples was performed at 98°C for 2 min followed by 40 cycles of annealing (56°C for 45 sec), elongation (72°C for 45 sec), and denaturation (94.5°C for 45 sec). Control mouse DNA was used as a negative control, and a dilution series of MMTV-IGF-I plasmid standards was used as a positive control. Amplification products from mouse tail DNA and plasmid DNA were visualized concurrently on 1% agarose gels containing 0.5 mg/ml ethidium bromide.

**Development of transgenic lines.** Transgenic lines of MMTV-IGF-I mice were developed to maintain transgenic mice possessing the copy number and integration site characteristics of the hemizygous transgenic founder (F₀) mice (Figure 2). Transgenic founders that were identified by PCR analysis were mated to control CD-1 mice, and the transgenic offspring were designated as the F₁ generation. Subsequent generations within each transgenic line were produced and identified as described in Figure 2.

**Induction of transgene expression in virgin mice.** Four transgenic F₁ females from each of five transgenic lines (2, 15, 26, 29, and 30) and four transgenic F₂ females from one
Figure 2. Development of transgenic lines of hemizygous MMTV-IGF-I mice.

Transgenic founder mice (F₀) were mated to control CD-1 mice. Transgenic offspring were designated as the F₁ generation, with 29-1 indicating the first pup analyzed from F₀ 29, which tested positive for presence of the transgene. The F₁ animals were mated to control CD-1 mice to produce transgenic mice of the F₂ generation, with 29-1-13 indicating the 13th pup analyzed from 29-1, which was transgenic.
transgenic line (10) as well as controls were subjected to steroid priming of mammary gland development prior to the induction of expression of the transgene. Females of the F₂ generation were used for line 10 because the transgenic founder had died before the experiment and a sufficient number of F₁ females was not available. At 28 to 30 d of age, females were injected subcutaneously with 0.1 ml of a solution containing estrogen (10 µg/ml) and progesterone (10 mg/ml) in saline, gum arabic (1 mg/ml), and ethanol (1%) once daily for a period of 9 days.

To induce expression of the IGF-I transgene under control of the glucocorticoid-responsive MMTV promoter, 2 transgenic females from each of the 6 transgenic lines and 2 control females were injected with 50 µg dexamethasone (SIGMA, St. Louis, MO). In addition, two transgenic females from each transgenic line and two control females received injections of vehicle. Twelve hours after the final estrogen and progesterone injection, 50 µg of dexamethasone dissolved in 0.1 ml of saline containing ethanol (1%) and gum arabic (1 mg/ml) was injected intraperitoneally every 12 h for a total of 5 injections, with controls receiving vehicle injections on the same time schedule.

**Induction of transgene expression in lactating mice.** Transgene expression was induced in lactating transgenic mice by administration of exogenous dexamethasone. Four transgenic F₁ females from each of 3 lines (2, 15, and 30), 4 transgenic F₂ females from the remaining 3 lines (10, 26, and 29), and 4 control females were mated at 42 d of age to mature CD-1 males. Females of the F₂ generation from 3 transgenic lines were used because a sufficient number of F₁ females was not available, either as a result of death of the transgenic
founder or recent establishment of the transgenic line. Females littered at approximately 62
d of age. Beginning on d 12 of lactation, 2 transgenic females from each line and 2 control
females were injected intraperitoneally with 50 μg of dexamethasone dissolved in 0.1 ml of
saline containing ethanol (1%) and gum arabic (1 mg/ml) every 12 h for a total of 5 injections.
Two transgenic females from each line and two control females received injections of vehicle.
On d 15 of lactation, 9 to 10 h following the last injection, females were separated from their
pups for 4 to 6 h to allow accumulation of milk in the mammary gland.

Collection of milk, blood, and mammary tissue samples. For collection of milk
samples, lactating females were anesthetized with Metofane (methoxyfluoromethane;
Pitmann-Moore, Washington Crossing, NJ) and injected intramuscularly with 5.0 IU of
oxytocin (Vedco Inc., St. Joseph, MO) to stimulate milk letdown. Milk was collected as
described by Kokkalis et al. (1986) using flame-polished capillary tubes (1.5 mm i.d.) inserted
into a hand-held chamber containing a 1.8 ml microfuge tube, while the receiving chamber
was operating at 100 mm Hg vacuum. Five hundred to 1000 μl of milk was collected within
a 15-min period and stored at -20°C. Prior to extraction of IGF-BPs, chilled milk samples
were centrifuged at 1,000 × g for 5 min and the defatted milk fraction was collected.

Blood samples were collected from virgin mice 12 to 13 h after dexamethasone
treatment was completed and from lactating mice following milk collection. Mice were
anesthetized with Metofane, and a 1-ml blood sample was drawn by cardiac puncture using
a tuberculin syringe rinsed with heparin (1500 IU/ml in saline; SIGMA, St. Louis, MO).
Blood samples were mixed with 25 μl of heparin in a microcentrifuge tube at room
temperature. Within 2 h, blood samples were centrifuged at 1,000 × g for 5 min and the plasma fraction was collected and immediately frozen at -20°C.

After blood samples were obtained, mice were sacrificed by cervical dislocation. Mammary tissue was removed with flame-sterilized forceps, and lymph nodes were dissected out from the fourth inguinal mammary glands. Samples of approximately 100 mg were weighed and snap-frozen in liquid nitrogen within 5 min of animal sacrifice and stored at -80°C.

**RNA isolation.** Total RNA was isolated from mammary tissue from all except five virgin females by a guanidinium thiocyanate isolation procedure (Puissant and Houdebine, 1990). Briefly, a tissue sample of 150 to 250 mg frozen in liquid nitrogen was crushed in a wax paper envelope between chilled metal plates in a vise. The crushed tissue was placed in a 2-ml microfuge tube containing 800 μl of lysing solution (4 M guanidinium thiocyanate; 25 mM sodium citrate, pH 7.0; 0.5% sarkosyl; 0.1 M mercaptoethanol) and the samples were vortexed vigorously. Eighty microliters of 2 M Na acetate (pH 4.0), 500 μl phenol, and 100 μl chloroform were added in succession, with vortexing after each addition. Samples were centrifuged at 8,000 × g for 10 min, and RNA was precipitated from the supernatant by addition of an equal volume of isopropanol. The RNA pellet was washed in 80% ethanol and partially dried prior to resuspension in 100 μl diethylpyrocarbonate (DEPC)-treated water. To remove contaminating DNA, LiCl (4 M) was added and the sample was vortexed vigorously and centrifuged. The resulting pellet was dissolved in 500 μl TE-SDS and extracted with 250 μl chloroform by centrifugation. The RNA was precipitated with 50 μl
3M Na acetate (pH 6.0) and an equal volume of isopropanol (500 µl) followed by vortexing and centrifugation. The pellet was washed in ethanol and dissolved in 50 µl DEPC-treated water followed by storage at -80°C. The concentration and purity of the RNA sample was measured by absorbance readings at 260 nm and 280 nm.

Total RNA was isolated from the remaining mammary tissue samples from five virgin animals and from all lactating tissue samples using TRI Reagent, a mono-phase solution containing phenol and guanidine thiocyanate (Molecular Research Center, Inc., Cincinnati, OH; Chomczynski, 1993). Briefly, a mammary tissue sample frozen in liquid nitrogen was crushed in a wax paper envelope between chilled metal plates in a vise, and 75 mg of crushed tissue was weighed into a glass-Teflon pestle containing 1 ml of TRI Reagent. The sample was homogenized, transferred with a pipette to a 1.7 ml microfuge tube, and stored at room temperature for 5 min. After 5 min had elapsed, samples were stored at 4 °C until all of the samples had been homogenized. The homogenates were centrifuged at 11,000 × g for 10 min to remove insoluble polysaccharides, extracellular membranes, and high molecular weight DNA. Two hundred microliters of chloroform was added to the supernatant before vortexing and incubation at room temperature for 15 min. Samples were centrifuged and the upper aqueous phase of approximately 600 µl was mixed with 60 µl of isopropanol, stored at room temperature for 5 min, and centrifuged to remove contaminating DNA. The supernatant was mixed with 440 µl of isopropanol, incubated at room temperature for 5 min, and centrifuged to yield an RNA pellet. The pellet was washed with ethanol, dried briefly, and dissolved in DEPC-treated water by incubating for 15 min at 55°C. The concentration and purity of the
RNA sample was measured by absorbance readings at 260 nm and 280 nm, and the integrity of the isolated RNA was evaluated by ethidium bromide staining of agarose-formaldehyde gels. The freedom from DNA contamination of the RNA samples was confirmed by absence of amplification products from 100 ng RNA in a PCR as described previously.

**DNase treatment of RNA samples.** Polymerase chain reaction analysis of total RNA samples isolated using guanidinium thiocyanate indicated the presence of contaminating amounts of DNA which could yield a false positive signal for the presence of IGF-I mRNA. To eliminate DNA contamination, RNA samples were treated with RNase-free DNase (Promega Corp., Madison, WI). Briefly, RNA samples were digested with 2 U DNase in the presence of 10 mM MgCl₂ and 50 mM Tris (pH 7.5) for 1 h at 37°C. Samples were then incubated with 0.15 U of proteinase K (5 mg/ml) at 55°C for 1 h. Seventy-five microliters of water and 150 μl of phenol and chloroform (1:1) were mixed with the samples before vortexing and centrifugation for 10 min at 8,000 × g. Ribonucleic acid was precipitated with 10 μl of 3 M Na acetate and 100 μl of isopropanol by centrifugation at 11,000 × g for 45 min. The RNA pellet was washed with ethanol, partially dried and dissolved in water prior to storage at -80°C. The concentration and purity of the RNA was measured by absorbance readings at 260 nm and 280 nm.

**Acid-ethanol extraction and radioimmunoassay (RIA).** Extraction of IGF-I from defatted milk, plasma, and mammary tissue was performed using an acid-ethanol cryoprecipitation method as described by Breier et al. (1991). Two hundred microliters of defatted milk or plasma was mixed with 800 μl of extraction buffer (87.5% ethanol, 12.5%
HCl) at a ratio of 1:4. One hundred milligrams of frozen crushed mammary tissue was weighed into a glass-Teflon pestle containing 1,000 µl of extraction buffer and homogenized. Lesser amounts of tissue (40, 45 or 50 mg) were homogenized for mammary tissue samples from four virgin mice. The homogenate was transferred to a test tube. Additional steps for IGF-I extraction followed the procedure described by Breier et al. (1991). The supernatant was frozen at -20°C until analysis of IGF-I by RIA as previously described (Shimamoto et al., 1992). Recombinant IGF-I and total IGF-I in extracts were measured with a mouse anti-human IGF-I monoclonal primary antibody (Kerr, 1989) and with a rabbit anti-human IGF-I polyclonal primary antibody (GroPep, Ltd., Adelaide, Australia), respectively. The lower limit of detection for assays using the monoclonal antibody was approximately 10 ng/ml.

**Reverse transcription and PCR analysis.** To detect the presence of mRNA for ovine IGF-I in mammary tissue, 1 µg of total RNA was used as a template for the reverse transcription and amplification of a partial cDNA. Three hundred nanograms of the oligonucleotide primer 6a (5'-CTGCACCTCCCTCCTGCTTG-3', exon 6, nucleotides 426-445) was hybridized to 1 µg of total RNA at 95°C for 5 min followed by cooling for 15 min at 37°C (Ohlsen et al., 1993). Reverse transcription was performed in 1X first strand buffer (50 mM Tris-HCl, pH 8.3; 75 mM KCl; 3 mM MgCl₂), 200 units of Moloney-Mouse Leukemia Virus (M-MLV) reverse transcriptase (GIBCO-BRL, Gaithersburg, MD), 2.0 mM dNTP's, 10 mM DTT, and 40 units of rRNAsin (Promega Corp., Madison, WI) at 37°C for 1 h. To remove RNA remaining at the end of the incubation period, samples were incubated with 1 µg of RNase A for 15 min at 37°C followed by mixing with 0.1 N NaOH and 0.03
M EDTA for 15 min at 37°C. Samples were neutralized with 0.3 M Tris-HCl (pH 7.4) and fractionated on Sephadex-G50 spin columns to remove excess oligonucleotide primers. The samples were precipitated with ethanol in the presence of 2.5 M ammonium acetate. The reverse transcription product was dissolved in 10 μl TE buffer and 5 μl was used in each PCR.

**Southern analysis of copy number.** To determine the transgene copy number in the 6 transgenic founders, 10 μg of genomic mouse DNA was digested with 10 U of the restriction endonuclease Bam HI (USB, Cleveland, OH) in the presence of 1X buffer (10 mM Tris-HCl, pH 7.5; 7 mM MgCl₂; 150 mM KCl; 7 mM 2-mercaptoethanol; and 100 μg/ml BSA) and 0.5 μl BSA for 1 h; subsequently, 10 U of Bam HI was added again and the samples were incubated for another hour. The digested sample was mixed with gel loading dye (SIGMA, St. Louis, MO) and electrophoresed through a 0.8% agarose gel. The DNA fragments were transferred to nitrocellulose membranes (MSI, Westborough, MA) and copy number was assessed by Southern blotting. Blots were hybridized with the 0.7 kb ovine IGF-I cDNA³²P-labeled by random priming and washed according to manufacturer instructions, followed by exposure to Kodak XAR-5 film at -80°C.

**Biological activity of transgenic mouse milk.** The biological activity of transgenic and control mouse milk was compared for the ability to stimulate [³H]thymidine incorporation in the MAC-T bovine mammary epithelial cell line (Huynh et al., 1991). Defatted milk from one F₂ female from the 29 transgenic line and a pooled sample from control CD-1 mice were extracted to remove IGF-BPs followed by lyophilization and resuspension to the same volume
in DMEM. The MAC-T cells were serum-starved for 72 h and cultured with milk extracts for 16 h. To obtain a dose-response effect of milk extract concentration on [\(^3\)H]thymidine incorporation into DNA, extracts of control and transgenic mouse milk were added in concentrations of 0.5, 1, 2, 4, 8, and 16 μl per well. Cells were then pulsed for 2 h with 1 μCi of [\(^3\)H] thymidine per well. At the end of the incubation, cells were harvested for measurement of [\(^3\)H]thymidine incorporation (Zhao et al., 1992).

**Statistical analysis.** Statistical analysis was performed using the GLM procedure in SAS (SAS, 1985). The statistical model included transgene presence, milk extract concentration, and transgene presence × milk extract concentration. A set of four linear contrasts were designed to determine effects of the addition of milk extracts from transgenic and control mice on [\(^3\)H]thymidine incorporation into DNA of MAC-T cells. The first contrast compared the means of [\(^3\)H]thymidine incorporation into DNA of MAC-T cells for transgenic and control mouse milk extract. The second contrast determined the overall linear effect of milk extract concentration on [\(^3\)H]thymidine incorporation within each treatment group. The third contrast compared the slope of regression lines for [\(^3\)H]thymidine incorporation for transgenic and control mouse milk extracts. The final contrast compared the difference in [\(^3\)H]thymidine incorporation stimulated by transgenic and mouse milk extracts within each concentration.
RESULTS

Polymerase chain reaction analysis of live pups born from microinjected embryos identified six transgenic MMTV-IGF-I founder mice, yielding an integration rate (number transgenic/number of live pups) of 2.3% (Table 1). Transgenic founder mice were designated as 2, 10, 15, 26, 29, and 30 (Figure 3); the 2 female transgenic founder mice were identified as 10 and 29. Transgenic mouse lines were established from each of the 6 transgenic founder animals as shown in Figure 2.

To determine the presence or absence of recombinant IGF-I in mammary tissue from transgenic virgin females, acid-extracted tissue homogenates were analyzed by RIA using a mouse anti-human IGF-I monoclonal antibody that detected ovine and bovine but not mouse IGF-I (Kerr, 1989). Recombinant IGF-I was detected in the mammary glands of dexamethasone-treated animals from transgenic lines 15, 26, and 29, with a range from 11.8 ± 0.8 ng in line 15 to 21.9 ± 0.5 ng IGF-I/100 mg of tissue in line 29 (Table 2). The recombinant protein was not detected in vehicle-treated animals from those lines or in vehicle- or dexamethasone-treated animals from transgenic lines 2, 10, and 30. Transgene expression in dexamethasone-treated females from lines 26 and 29 was confirmed by RT-PCR analysis of total RNA from the mammary gland (Figure 4); total RNA from transgenic line 15 was not available for analysis. Transgene expression was induced by dexamethasone, as oIGF-I mRNA or recombinant IGF-I was detected in each of the two virgin animals from transgenic lines 15, 26, and 29 that received dexamethasone but not in transgenic females that received
**Table 1: Mouse Embryo Microinjection, Transfer, and Founder Generation Data**

<table>
<thead>
<tr>
<th>Production Stage</th>
<th>Number</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryos injected</td>
<td>3528</td>
<td></td>
</tr>
<tr>
<td>Embryos survived</td>
<td>2176</td>
<td>(61.7)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Embryos transferred</td>
<td>2106</td>
<td></td>
</tr>
<tr>
<td>Number of recipients</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>Embryos per recipient</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>Number pregnant</td>
<td>42</td>
<td>(73.7)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Number of live pups</td>
<td>256</td>
<td></td>
</tr>
<tr>
<td>Number transgenic</td>
<td>6</td>
<td>(2.3)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Number transgenic females</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Embryos survived/embryos injected.  
<sup>b</sup>Number pregnant/number of recipients.  
<sup>c</sup>Number transgenic/number of live pups.
Figure 3. PCR analysis of $F_0$ transgenic mice. A CD-1 mouse is a control. Six mice (2, 10, 15, 26, 29, and 30) were positive for the ovine IGF-I cDNA.
Table 2: Recombinant IGF-I in Mammary Tissue from Virgin MMTV-IGF-I Mice

<table>
<thead>
<tr>
<th>Mouse Line</th>
<th>IGF-I (ng/100 mg)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- dex</td>
</tr>
<tr>
<td>2</td>
<td>ND</td>
</tr>
<tr>
<td>10</td>
<td>ND</td>
</tr>
<tr>
<td>15</td>
<td>ND</td>
</tr>
<tr>
<td>26</td>
<td>ND</td>
</tr>
<tr>
<td>29</td>
<td>ND</td>
</tr>
<tr>
<td>30</td>
<td>ND</td>
</tr>
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</table>

*Expression was not detected in control mice receiving vehicle or dexamethasone (dex) (ND = not detected). Values are presented as the mean ± SD for tissue from two animals within each treatment.
Figure 4. RT-PCR analysis of total RNA in mammary tissue from dexamethasone-treated virgin transgenic mice. Ovine liver is used as a positive control. Exogenous dexamethasone induced expression of oIGF-I mRNA in virgin mice from lines 26 and 29 but not from line 2.
vehicle. Recombinant IGF-I was not detected in plasma from transgenic virgin females. Total IGF-I was also measured using a rabbit anti-human IGF-I polyclonal primary antibody. These data confirmed the presence of native mouse IGF-I as well as increased total IGF-I in milk of transgenic mice (see below).

During lactation, transgene expression in the mammary gland was evident in females from 4 transgenic lines (2, 15, 26, and 29) (Figure 5). Analysis of total RNA by RT-PCR indicated the presence of oIGF-I mRNA in mammary tissue from both vehicle- and dexamethasone-treated females of those lines, although a relatively faint band indicated a low level of expression in line 2. Measurement of recombinant IGF-I in milk extracts and mammary tissue confirmed RT-PCR results for transgene expression in transgenic lines 15, 26, and 29. Recombinant IGF-I was secreted into milk at concentrations up to approximately 1 µg/ml, but levels did not appear to be increased by dexamethasone (Table 3). Similarly, recombinant IGF-I was detected in mammary tissue homogenates from transgenic lines 15, 26, and 29 with no apparent effect of dexamethasone treatment (Table 4). However, levels of recombinant IGF-I detected in mammary tissue from lactating mice may be variably influenced by milk remaining in the tissue sample at the time of collection. Recombinant IGF-I was not detected in milk extracts or mammary tissue homogenates from line 2. Thus, oIGF-I mRNA in mammary tissue from line 2 may not be translated; alternatively, levels of protein may have been below the assay limit of detection. Recombinant IGF-I was not detected in plasma from lactating transgenic animals.

The number of copies of the MMTV-IGF-I transgene was estimated in the six founder
Figure 5. RT-PCR analysis of total RNA in mammary tissue from lactating transgenic mice treated with dexamethasone (dex) or vehicle as described previously. Ovine liver is used as a positive control. Expression of mRNA for oIGF-I is evident in lactating mice from lines 2, 15, 26, and 29.
Table 3: Recombinant IGF-I in Milk from Lactating MMTV-IGF-I Mice

<table>
<thead>
<tr>
<th>Mouse Line</th>
<th>IGF-I (ng/ml)*</th>
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<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>- dex</td>
<td>+ dex</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>716 ± 42</td>
<td>1170 ± 716</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>405 ± 314</td>
<td>301 ± 19</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>1213 ± 0.6</td>
<td>1337 ± 428</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

*Expression was not detected in control mice receiving vehicle or dexamethasone (dex) (ND = not detected). Values are presented as the mean ± SD for milk from two animals within each treatment.
Table 4: Recombinant IGF-I in Mammary Tissue from Lactating MMTV-IGF-I Mice

<table>
<thead>
<tr>
<th>Mouse Line</th>
<th>IGF-I (ng/100mg)*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>- dex</td>
</tr>
<tr>
<td>2</td>
<td>ND</td>
</tr>
<tr>
<td>10</td>
<td>ND</td>
</tr>
<tr>
<td>15</td>
<td>26.9 ± 8.8</td>
</tr>
<tr>
<td>26</td>
<td>14.2 ± 6.3</td>
</tr>
<tr>
<td>29</td>
<td>35.3 ± 3.8</td>
</tr>
<tr>
<td>30</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Expression was not detected in control mice receiving vehicle or dexamethasone (dex) (ND = not detected). Values are presented as the mean ± SD for tissue from two animals within each treatment. Values may be variably influenced by milk remaining in the tissue sample at time of collection.
transgenic mice and in randomly selected offspring by Southern blotting analysis. Mice from each of the six lines displayed from one to five copies of the transgene. Breeding data for lines with multiple copies indicated that the transgenes are integrated on a single chromosome, and the transgenic founders were apparently not mosaic.

To compare the biological activity of milk from transgenic mice and control mice, milk extracts from a mouse of line 29 and from control mice were cultured with MAC-T cells. The enhanced biological activity of transgenic mouse milk relative to control mouse milk was demonstrated by a greater stimulation of $[^3H]$thymidine incorporation in MAC-T cells (P<.05) (Figure 6).
Figure 6. Effect of transgenic and control mouse milk extracts on $[^3H]$thymidine incorporation into DNA of MAC-T cells. Transgenic mouse milk extract stimulated $[^3H]$thymidine incorporation more than control mouse milk extract at concentrations equal to or greater than 2 μl/well ($P<.05$).
DISCUSSION

Enhancement of mammary epithelial cell proliferation offers the potential to increase efficiency of milk production, as the number of alveolar cells is directly related to milk yield (Tucker, 1987). It has been clearly demonstrated for many species that IGF-I is both a mitogen and a metabolic regulator of mammary tissue in vitro (Campbell et al., 1991). In addition, transgenic mice overexpressing IGF-I in many tissues exhibit a growth response as measured by increased weight of selected organs (Mathews et al., 1988). Insulin-like growth factor-I expression and synthesis has been detected in the mammary gland; however, the physiological significance of locally produced IGF-I relative to circulating IGF-I is unknown. If the mitogenic actions of IGF-I persist in mammary tissue in vivo, enhanced IGF-I synthesis in the mammary gland could stimulate cell proliferation resulting in greater capacity for milk production.

To study the role of IGF-I in mammary development, transgenic mice were generated that produce IGF-I in the mammary gland under control of the MMTV promoter. Expression of oIGF-I mRNA in the mammary gland was evident in four of the six transgenic lines generated (2, 15, 26, and 29), but not in transgenic lines 10 and 30. The presence of mRNA does not ensure that the corresponding functional protein product will be synthesized, however. For example, efficient translation of certain mRNAs may require specific trans-acting factors that are only present in specific tissues. On the other hand, protein products may require post-translational modifications such as proteolytic cleavage to become
functionally active (Bieberich et al., 1994). The importance of detection of the functional recombinant IGF-I in addition to oIGF-I mRNA is illustrated by analysis of protein products in the mammary glands of mice from the six transgenic lines. Recombinant IGF-I was detected in virgin and lactating mice from lines 15, 26, and 29, but not in a fourth line (2) shown to express oIGF-I mRNA during lactation. In line 2, translation of the oIGF-I mRNA may have not occurred; alternatively, levels of the recombinant protein may have been below the assay limit of detection.

The remaining two transgenic lines (10 and 30) displayed no evidence of recombinant IGF-I synthesis, confirming the observed lack of oIGF-I mRNA expression in those lines. The absence of transgene expression in several transgenic lines is not surprising, considering the variability in expression levels often obtained from different lines carrying the same construct (Bishop and Smith, 1989). For example, constructs containing cDNAs encoding the protein of interest are generally expressed at low frequencies, possibly due to effects of introns on transcriptional efficiency (Brinster et al., 1988). In addition, variability in transgene expression has been attributed to the influence of the chromosomal regions at or near the insertion site of the transgene as well as the number or arrangement of transgene copies in the inserted array (Bishop and Smith, 1989; Clark et al., 1994; Palmiter et al., 1991). On the other hand, a direct relationship between expression level and transgene copy number has been achieved with the use of promoter regions that confer position-independent expression on the transgene, such as the β-globin or the chicken lysozyme genes (Clark et al., 1994). However, this is not true for all transgenes; indeed, a high copy number may result in low
expression and may negatively influence the genetic stability of the transgenic locus and the flanking DNA sequences. It has been suggested that two to five copies are generally ideal (Tinkle et al., 1994). Copy number analysis of the MMTV-IGF-I mice revealed the presence of one to four copies in the six transgenic lines, and expression levels were not related to copy number.

Analysis of the effects of transgene expression in multiple transgenic lines is critical because the site of integration may influence the pattern of transgene expression (Bieberich et al., 1994). For example, if the expression patterns in two transgenic lines do not parallel one another, a third line must be analyzed to delineate a consistent pattern of expression that is unrelated to the site of integration. Analysis of a minimum of two transgenic lines is also necessary to confirm any distinct phenotype that results from transgene expression. Therefore, the three expressing MMTV-IGF-I transgenic lines have been selected as models for study of the effects of overexpression of IGF-I on mammary gland development and function. Although the data for these transgenic lines are currently limited, gross morphological changes in the mammary gland due to transgene expression were not apparent in virgin or lactating mice.

Transgene expression under control of the MMTV promoter likely occurs in other tissues such as the salivary gland, kidney, and spleen (Mok et al., 1992), but screening of other tissues remains to be accomplished in the MMTV-IGF-I mice. However, recombinant IGF-I present in milk and mammary tissue of the three expressing lines is likely derived from mammary synthesis rather than transfer from other tissues to the mammary gland via the
circulation, based on the absence of detectable recombinant IGF-I in plasma. Moreover, any adverse health effects due to transgene expression were not readily apparent; neither was growth performance of progeny observed to be different from control pups.

Regulation of expression by the MMTV-LTR restricts transgene expression mainly to mid-pregnancy and lactation in transgenic mice, although expression is detectable at relatively low levels throughout life. Activation of the MMTV promoter occurs primarily at the GRE in response to glucocorticoids, and additional hormonal regulation is provided through interaction with the hormone response element (HRE) (Mok et al., 1992). Expression in the MMTV-IGF-I virgin mice of the three expressing lines was detected only in response to exogenous dexamethasone, a synthetic glucocorticoid; whereas the hormonal changes associated with lactation were likely responsible for induction of transgene expression in the absence of dexamethasone treatment. The usefulness of the MMTV promoter for studies of hormonal effects on the mammary gland in transgenic mice is enhanced by the ability to activate the promoter with exogenous dexamethasone. In these transgenic mice, for example, induction of transgene expression with dexamethasone in virgin mice will permit evaluation of the effects of IGF-I overexpression on the prepubertal mammary gland. An increased level of glucocorticoids is not without its negative effects, however, as dexamethasone has been associated with reduced concentrations of IGF-I mRNA in several tissues (Adamo et al., 1988). Moreover, it has been suggested that glucocorticoids may reduce IGF-I bioactivity by stimulating production of IGF-BPs which modulate IGF-I actions (Price et al., 1992).
A critical prerequisite for the study of hormone function in transgenic animals is the demonstration of normal biological activity of the recombinant protein. In a clonal bovine mammary epithelial cell line transfected with the MMTV-IGF-I construct, recombinant IGF-I produced from the ovine IGF-I cDNA stimulated cell proliferation by an autocrine and (or) paracrine mode of action (Romagnolo et al., 1992). To confirm the mitogenic activity of recombinant IGF-I secreted into milk by transgenic mice, the milk extracts from one F$_2$ transgenic female from line 29 and from control mice were cultured with MAC-T cells, and [$^{3}$H]thymidine incorporation was used as an index of proliferative activity. The MAC-T cells have been shown to express IGF-I receptors and proliferate in response to exogenous IGF-I (Zhao et al., 1992). The milk sample from the transgenic mouse stimulated cell proliferation to a greater degree than the milk extract from control mice. It is suggested that the recombinant IGF-I present in the transgenic mouse milk sample as detected by RIA is the source of the proliferative activity, although further characterization of the response is warranted.

The mitogenic effects of IGF-I are influenced by its association with the IGF-BPs, which have been postulated to prolong the half-life of circulating IGF-I, limit IGF-I availability to tissues, and to modulate the biological activity of IGF-I at the cellular level (Conover et al., 1994). The factors that regulate the synthesis of IGF-BPs are not clear, although expression of IGF-BP-3 appears to be directly dependent upon IGF-I (Clemmons, 1991). Insulin-like growth factor-I increased serum concentrations of IGF-BP-3 in transgenic mice (Camacho-Hubner et al., 1991), and factors that reduce serum IGF-I concentrations
such as energy-deficient diets also lower IGF-BP-3 levels (Clemmons, 1991). It has been demonstrated that mammary tissue synthesizes IGF-I (Campbell et al., 1991) as well as secreting both IGF-BP-2 and IGF-BP-3 (Campbell et al., 1991; McGrath et al., 1991), implying that IGF-I synthesis and bioactivity is regulated locally within the mammary gland. Conover et al. (1994) suggested that the actions of IGF-I in the cell microenvironment may be partially determined by the balance between the inhibitory effects of soluble IGF-BP-3 and the potentiating effects of cell-associated IGF-BP-3. Specifically, soluble IGF-BP-3 may serve to maintain the type I IGF receptor concentration by sequestering IGF-I and preventing receptor binding, whereas cell-associated IGF-BP-3 may enhance the cellular response to receptor binding of IGF-I. Observations by Ross et al. (1989) and Romagnolo et al. (1994) support an inhibitory role for IGF-BP-2 in binding available IGF-I and preventing association with cell receptors (Clemmons, 1991). For example, overexpression of recombinant IGF-I in the MD-IGF-I clonal bovine mammary epithelial cell line induced the autocrine secretion of IGF-BP-3 which enhanced the mitogenic effects of IGF-I, while IGF-BP-2 production by the parental MAC-T cells inhibited the actions of IGF-I (Romagnolo et al., 1994). Taken together, these results suggest that overexpression of recombinant IGF-I in the MMTV-IGF-I transgenic mice might be accompanied by alterations in IGF-BP concentrations in the mammary gland to modulate the biological effects of IGF-I.

In summary, biologically active recombinant IGF-I that has been shown to stimulate mammary cell proliferation in vitro is produced in the mammary glands of transgenic mice. Because expression of the MMTV-IGF-I transgene does not appear to adversely affect
animal health, the effects of overexpression of IGF-I in the mammary gland may be studied in an otherwise physiologically normal animal. Appropriate temporal increases in IGF-I synthesis in the mouse mammary gland might stimulate cell proliferation similar to effects in vitro, leading to enhancement of mammary development and function. In virgin MMTV-IGF-I mice, expression of the transgene may be induced by exogenous dexamethasone, allowing evaluation of the role of IGF-I in peripubertal growth and development of the mammary gland. During pregnancy and lactation, increased endogenous glucocorticoid concentrations will allow observation of the effects of recombinant IGF-I production on mammary growth and function; in addition, the possibility of changes in milk yield or composition resulting from recombinant IGF-I synthesis in the mammary gland remains to be examined. Increased IGF-I synthesis in mammary epithelial cell lines has been accompanied by alterations in IGF-BPs (Romagnolo et al., 1994); corresponding changes in IGF-BP concentrations that may occur in vivo will be evaluated in the MMTV-IGF-I transgenic mice. The generation of transgenic mice overexpressing IGF-I in the mammary gland will provide a powerful in vivo strategy for studying the role of IGF-I in mammary gland development and function.
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