AUTOCRINE MECHANISMS OF ACTION OF INSULIN-LIKE GROWTH FACTOR-I (IGF-I) AND HORMONAL REGULATION OF EXPRESSION OF IGF-BINDING PROTEINS IN MAMMARY EPITHELIAL CELLS

by

DONATO ROMAGNOLO

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APPROVED:

R. M. Akers, Chairman

E. A. Wong

F. C. Gwazdauskas

J. L. Johnson

J. D. Turner

W. E. Vinson, Department Head

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(ABSTRACT)

Limited information is available concerning the molecular and cellular mechanisms that regulate expression of insulin-like growth factor-I (IGF-I) and IGF-binding proteins (IGFBPs) genes in mammary epithelial cells. To test the hypothesis that IGF-I affects growth of bovine mammary epithelial cells through an autocrine and/or paracrine pathway, several cell lines were developed expressing an ovine exon-2 containing IGF-I cDNA under the control of the mouse mammary tumor virus-long terminal repeat (pMMTV-IGF-I), early simian virus (pSV40-IGF-I), and herpes simplex thymidine kinase (pTK-IGF-I) promoters. Stably transfected clones were generated by cotransfection of clonal MAC-T cells with the IGF-I expression vectors and a plasmid conferring resistance to hygromycin-B (HYG-B), using a calcium phosphate precipitation procedure. Induction of the MMTV-LTR with the glucocorticoid

dexamethasone (DEX) was required for enhanced expression of IGF-I in MD-IGF-I (MD=Mammary Derived) cells, whereas SV40-IGF-I cells constitutively expressed the highest levels of IGF-I, followed by TK-IGF-I cells. Activity of the MMTV promoter in MD-IGF-I cells was coordinately regulated by lactogenic hormones and extracellular matrix. Acute secretion of DEX-induced recombinant IGF-I by MD-IGF-I cells stimulated cell proliferation through an autocrine/paracrine pathway and triggered the expression of IGFBP-3. Neither acute nor constitutive expression of IGF-I affected expression of type 1 IGF receptor mRNAs, but down-regulated cell surface receptor levels, in the order SV40-> TK- > MD-IGF-I. Secretion of IGF-I-induced IGFBP-3 potentiated the mitogenic actions of IGF-I as evidenced by enhancement of [^{3}H]thymidine uptake into DNA of parental MAC-T cells. This study provides evidence that local production of IGF-I can stimulate cell proliferation of bovine mammary epithelial cells through an autocrine/paracrine mode of action. We suggest that secretion of IGF-I-induced IGFBP-3 by bovine mammary epithelial cells enhances cell responsivenes to IGF-I, but does not prevent down-regulation of the IGF-I receptor in cells constitutively expressing IGF-I.

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INTRODUCTION

The primary function of the mammary gland is the production of milk for the nourishment of the newborn. Therefore, the purpose of mammary development is to assure that mammary tissue develops into a milk-producing gland to satisfy the nutritional needs of the neonate (Knight and Peaker, 1982). At birth, the mammary gland is a rudimental branching duct system, which develops isometrically with the rest of the body. With the onset of puberty, ovarian hormones induce cycles of allometric mammary cell proliferation, which allow further branching of the duct system and fat deposition (Forsyth, 1989). With pregnancy, the mammary gland enters a phase of sustained proliferation of the secretory cells that continues during early lactation in some species and leads to full lobulo-alveolar development. This process is termed mammogenesis and is largely responsible for most of the volumetric development of the mammary tissues. However, during the periparturient period cell proliferation diminishes and lactogenesis occurs during which time the mammary gland acquires the ability to synthesize and secrete milk. Finally, involution of the gland begins during mid to late lactation and/or with onset of the dry period (in dairy cattle) and the epithelial cells become non-secretory (Tucker, 1987).

The presence of cell surface receptors for a variety of steroid and peptide hormones suggests that mammary functions are synergistically controlled by a large number of hormones (Akers, 1985; Howlett and Bissell, 1990) and growth factors (Akers, 1990; Forsyth, 1991). Insulin-like growth factor-I (IGF-I) is a growth factor known to stimulate secretory cell proliferation of normal murine (Imagawa et al., 1986), rat (Deeks and Nandi, 1988), bovine (Shamay et al., 1988), and malignant breast epithelium (Lippman and Dickson, 1989). IGF-I is primarily produced in the liver and is believed to mediate growth hormone (GH) actions on target cells through an endocrine mode of action (McGuire et al., 1992). In addition, experimental evidence suggests that mammary development may be controlled in an autocrine or paracrine manner (Wilde et al., 1990). In fact, a molecular basis for endocrine versus local action of IGF-I has recently been suggested (LeRoith and Roberts, 1991). Although the levels of IGF-I mRNA in the rat mammary gland were very low (Murphy et al., 1987a), treatment in vivo with GH triggered a significant increase in immunoreactive IGF-I in the cytoplasm of bovine mammary epithelial cells (Glimm et al., 1988). However, it is not known whether locally produced IGF-I contributes to the development of the mammary gland in a coordinate fashion through an autocrine and/or paracrine pathway, and whether

stimulatory action of IGF-I may be coordinately regulated during different stages of development (Sara and Hall, 1990; Hodgkinson et al., 1991).

Moreover, it has become increasingly clear that the mitogenic actions of IGF-I are mediated by binding of the IGF-I peptide to IGF-binding proteins (IGFBPs) (Ooi, 1990). Consequently, to better understand the role of IGF-I in mammary function it is essential to consider how circulating and cell-derived IGFBPs mediate the mitogenic actions of IGF-I.

Because the physiological significance of IGF-I is functionally determined at the molecular and cellular levels, objective of this study was to test the hypothesis that secretion of IGF-I originating from an ovine exon 2-encoded preproIGF-I under the control of the mouse mammary tumor virus-long terminal repeat (MMTV-LTR) would stimulate growth of bovine mammary epithelial MD-IGF-I (MD=Mammary Derived) cells through an autocrine and/or paracrine pathway. Using the MD-IGF-I cells, we investigated regulation of activity of the mammary gland-specific MMTV-LTR promoter by lactogenic hormones and extracellular matrix and the effects of secretion of IGF-I-induced IGFBPs on cell responsiveness to IGF-I. In addition, we investigated regulation of expression of the type-1 IGF receptor in transfected mammary epithelial cells

in which expression of the IGF-I cDNA was under the control of the constitutively-active herpes simplex thymidine kinase (TK) and early simian virus (SV40) promoters. Presentation of the data generated in this study will be preceded by a review of the molecular and cellular mechanisms that regulate expression of IGFs and IGF receptors and an overview of the prominent structural features and physiological roles of IGFBPs.

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

1. Molecular biology of IGF-I

The term IGF was adopted in 1987 in substitution of the more general "somatomedin" previously introduced to classify uncharacterized growth factors that mediated the somatotropic action of GH (Salmon and Daughaday, 1957). Purification of serum somatomedin fractions exerting insulin-like growth actions led to the discovery of the two peptides, insulin-like growth factor-I and II (IGF-I, IGF-II) (Sara and Hall, 1990). IGF-I is generally regarded as an essential postnatal growth GH-dependent factor, whereas IGF-II appears to be more important in fetal development and is GH-independent (Sussenbach et al., 1992). Both peptides are single-chain molecules of 70 and 67 amino acids, respectively, which contain three disulfide bonds. IGF-I and IGF-II share significant amino acid similarity (~62%) and like proinsulin, both contain A and B domains. However, unlike proinsulin, the IGFs contain a full-length C domain connecting the A and B regions, and a short D domain at the carboxyl terminus. In human and rat, variant carboxy-terminal E peptides are generated by alternate splicing of IGF-I mRNAs (Roberts et al., 1987), but they are subsequently removed by processing of the preproIGF-I.

The primary sequence of IGF-I is identical between bovine, human, and porcine, whereas ovine IGF-I differs by one amino acid at position 66 (alanine is substituted for proline) (Wong et al., 1989). In addition, a truncated form of IGF-I, designated Des(1-3)IGF-I lacking three amino acids (gly-pro-glu) at the amino terminus has been purified from bovine colostrum (Francis et al., 1986). It is believed that the Des(1-3)IGF-I is the product of post-translational modifications as no mRNA transcripts encoding such a variant have been characterized to date. Moreover, the possibility that the truncation might be the result of splicing of IGF-I transcripts is quite unlikely as no putative intron-exon boundaries have been identified in this region of the IGF-I gene (Sara and Hall, 1990).

Identification of the structural organization of the IGF-I gene began with the isolation of a human IGF-I cDNA (Jansen et al., 1983). In spite of the relatively small size of the mature IGF-I peptide, the IGF-I gene has a very complex structure and spans at least 80 Kb in both human (chromosome 12) and bovine (chromosome 5) genomes. Both the human and rat IGF-I genes contain 6 exons, whereas exon 5 has not been identified for the ovine IGF-I gene. Nevertheless, in the ovine IGF-I gene an additional exon (W) has been characterized in the region 5′ of exon 1 (Wong et al., 1989). The presence of multiple transcription

initiation sites along with alternate splicing events and differential polyadenylation sites all lead to the generation of several IGF-I mRNA transcripts in human (Rotwein et al., 1986; Daughaday and Rotwein, 1989; Jansen et al., 1991), mouse (Bell et al. 1986), rat (Roberts et al., 1987; Shimatsu and Rotwein, 1987), bovine (Fotsis et al., 1989), and ovine (Ohlsen et al., 1993). In humans, transcription of the IGF-I gene yields primarily three IGF-I mRNAs of 1.1, 1.3, and 7.6 Kb (Sussenbach et al., 1992).

Historically, the liver was thought to be the primary, if not the only, site of IGF-I production. Recent characterization of hepatic and non-hepatic IGF-I mRNA transcripts with different 5′ untranslated regions derived from initiation sites in exon 1 and 2 suggests that exon 2 transcripts are primarily expressed in the liver, as compared to exon 1 transcripts that can be detected in several tissues (Sussenbach et al., 1992). These results show the intricate regulation of IGF-I expression, and support the existence of a molecular basis for endocrine versus autocrine/paracrine regulation of IGF-I gene expression. In particular, LeRoith and Roberts (1991) have suggested that exon 2 transcripts, perhaps, encode the predominant endocrine form of IGF-I. This conclusion is substantiated by experimental evidence of decreased levels

of exon 2-derived transcripts in the liver of hypophysectomized rats (Lowe et al., 1987). In contrast, exon 1-derived transcripts were expressed in a variety of tissues, and their expression was not affected by treatment with GH (Murphy et al., 1987b). Similarly, it has been reported that ovine IGF-I mRNAs from exon 1 were most abundant in various non-hepatic fetal and adult tissues, whereas exon 2 transcripts exhibited the highest expression in adult liver (Ohlsen et al., 1993).

Based on these data it has been proposed that production of the endocrine vs autocrine/paracrine form of IGF-I results from differential exon usage (Adamo et al., 1991). This assumption is supported by collateral studies which have demonstrated that particular structural features of the amino terminus of eukaryotic signal peptides may be responsible for determining cleavage by signal peptidases (Nothwehr and Gordon, 1990). In human and rat (LeRoith and Roberts, 1991), exon-1 generated IGF-I mRNAs encode a 48-amino acid signal peptide (49 in ovine and bovine), as compared to exon 2-containing IGF-I mRNAs which encode primarily a 32-amino acid signal peptide (33 in ovine and bovine). The smaller signal peptide variant is proposed to be cleaved by signal peptidases thereby generating a proIGF-I lacking three amino acids at the N-terminus termed Des(1-3)IGF-I. The truncated Des(1-3)IGF-I variant of the

mature IGF-I peptide displays enhanced biological action since the molecule has reduced affinity for binding proteins. Conversely, exon 2-derived IGF-I mRNA transcripts would presumably not be cleaved by signal peptidases and would generate a full-length mature IGF-I peptide. This hypothesis, however, has not been corroborated with experimental evidence. This matter has been further complicated by early (Rotwein, 1986) and recent (Adamo et al., 1991) reports which also indicated heterogeneity at the 3′ end of IGF-I mRNAs, and the existence in rats of exon 1-derived mRNA species encoding a third distinct leader peptide of 22 amino acids. The true physiological significance, if any, of these IGF-I mRNA variants is unknown. Investigation of whether these different IGF-I transcripts are translated with different efficiencies may provide a broader understanding of the biological significance of regulation of start site usage.

2. Regulation of expression and cellular actions of IGF-I

Regulation of expression of IGF-I is exerted at the levels of transcription, RNA processing, and translation. These levels of regulation appear to be activated in a developmental and tissue-specific fashion, so that synthesis of the endocrine versus autocrine/paracrine forms of IGF-I may be independently regulated. Growth hormone

which is a primary stimulator of IGF-I production by the liver increased IGF-I expression levels in human fetal tissues (D'Ercole et al., 1980; Han et al., 1987). The stimulatory effect of GH is exerted at the transcriptional level (Doglio et al., 1987; Bichell et al., 1992) and is functionally mediated by activation of various IGF-I promoters, whose activity may be developmentally controlled (Sussenbach et al., 1992). More recently (Adamo et al., 1993), distinct promoters with differential transcriptional activity have been identified within exon 1 and 2 of the rat IGF-I gene. The characterization of these promoters now offers the opportunity to investigate cis- and trans-acting factors responsible for regulation of IGF-I expression.

In rats, the lack of GH receptors in the early stages of embryonic or fetal development was associated with minimal production of IGF-I and reduced cell proliferation. In late gestation, IGF-I expression in the fetus was stimulated and increased significantly with the onset of postnatal development to reach a maximum level around puberty. Specifically, IGF-I mRNAs originated from initiation sites within exon 1 appeared earlier than those resulting from initiation sites within exon 2 (Philipps et al., 1988).

Low levels of circulating IGF-I are characteristic of patients with liver diseases (Clemmons et al., 1985) or malnutrition (Emler and Schalch, 1987). Moreover, reduced

serum concentrations of IGF-I and lower IGF-I mRNA levels in liver and skeletal muscle were reported in protein-restricted growing rats (VandeHaar et al., 1991). Effects of catabolic states such as fasting and diabetes are thought to be mediated by decreased use of all initiation start sites (Adamo et al., 1991). Perturbations in circulating IGF-I are also typical of patients with clinical GH deficiences, such as Laron dwarfism and in the African pygmy in which high GH levels are associated with low concentrations of serum IGF-I due to a lack of functional liver GH receptors (Rosenbloom et al., 1991).

The inductive effect of GH on IGF-I production is under the control of the hypothalamic GH-releasing hormone (GHRH) released by the hypothalamus (Shibasaki et al., 1986). High levels of circulating IGF-I provide a negative feedback to the hypothalamus, which inhibits the release of GH from the pituitary gland (Ceda et al., 1987). This functional negative feedback loop provides a mechanism for fine tuning of circulating concentrations of GH and IGF-I (Hill, 1989).

The lactational effects of GH appear to be partially mediated by increased production of IGF-I by mammary tissue (Simmen, 1991). Moreover, cloning of the bovine GH receptor has allowed detection of GH mRNA receptor expression in mammary tissue of pregnant heifers (Hauser et al., 1990). In the same study, expression of IGF-I mRNA was limited to

stromal components of mammary tissue, which indicated a possible paracrine mechanism for GH action in the mammary gland. While these findings are supported by recent experimental observations suggesting a stimulatory role of GH on production of glandular IGF-I mRNA (Kleinberg et al., 1990), they are in contrast with previous studies which demonstrated no GH binding to microsomal membranes (Akers, 1985; Keys and Djiane, 1988) or mammary explants (Gertler et al., 1983; Shamay et al., 1988). Therefore, it is possible that in the mammary gland regulation of expression of the GH-receptor may be exerted at the post-transcriptional level. Nevertheless, it remains to be demonstrated whether expression of GH and IGF-I mRNAs by mammary epithelial cells and/or fibroblasts is temporally regulated (Simmen et al., 1989).

In addition to GH, other hormones control the local production of IGF-I. Both fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) increased the production of IGF-I by cultured human fibroblasts (Clemmons and Shaw, 1983), whereas the glucocorticoid dexamethasone inhibited IGF-I expression by rat neuronal cells (Adamo et al., 1988). In addition, the sex steroids estrogen and progesterone are potent stimulators of IGF-I synthesis in uterus (Simmen et al. 1990) and granulosa cells (Oliver et al., 1989). These data suggest that many of the

growth-promoting actions exerted by several known mammogenic hormones and growth factors may be mediated at the local tissue level by changes in expression of IGF-I. Unfortunately, knowledge of the mechanisms through which these factors exert their stimulatory effects on IGF-I expression in various species is lacking.

The primary biological action of IGF-I is stimulation of cell proliferation. It has been shown that IGF-I stimulates DNA synthesis and cell growth of mammary tissues from peripubertal and pregnant rodents (Imagawa et al., 1986), and ruminants (Shamay et al., 1988; Waksman et al., 1991). IGF-I acts as a progression factor in control of the cell cycle following initiation by competence factors, such as PDGF and FGF (Lu et al., 1989). Likely, IGF-I derives its mitogenic effects by stimulating transcription of enzymes involved in DNA synthesis, such as thymidine kinase (Yang and Pardee, 1986). In addition, IGF-I exerts some anabolic activities in that it stimulates terminal differentiation of a variety of cell types and may alter carbohydrate metabolism (Sara and Hall, 1990). Interestingly, the development of terminal end buds and formation of alveolar structures was stimulated by IGF-I and the analog Des(1-3)IGF-I in mammary tissue from male rats (Ruan et al., 1992).

3. IGF-I receptor and signal transduction

The biological actions of IGF-I are mediated by the presence of cell surface receptors. Two IGF transmembrane receptors have been characterized: the type 1 (IGF-IR) and type II (IGF-IIR) IGF receptors. The IGF-IR is a glycoprotein structurally similar to the insulin receptor and encompasses two cysteine-rich extracellular domains (α-subunits) with a molecular weight of approximately 135-KDa and two transmembrane tyrosine kinase domains (β-subunits) with a molecular weight of approximately 98-KDa. Expression studies of IGF-IR in human have revealed that two primary transcripts of 11- and 7-Kb were expressed in human placenta (Ullrich et al., 1986), whereas a single transcript of 11-Kb was detected in rat granulosa cells (Werner et al., 1989). Recent cloning and structural analysis of the IGF-IR promoter allowed detailed investigation of the regulation of IGF-IR expression (Werner et al., 1990, 1992; Cooke et al., 1991).

The IGF-IR is structurally and functionally similar to the insulin receptor and is assembled through a complex series of modifications. Synthesis of the IGF-IR starts from the production of a very large amino acid receptor precursor (~152-KDa) (NH_2-α-β-COOH), which is glycosylated, dimerized (NH_2-α-β-COOH)$_2$ and proteolytically processed to generate a mature (β-α-α-β) IGF-IR molecule. The α-and

β-domains are joined by disulfide bonds. Although the IGF-IR binds specifically to IGF-I, cross-reactivity with IGF-II and with high concentrations of insulin has been repeatedly reported (Sara and Hall, 1990).

Binding of IGF-I to the IGF-IR is likely mediated by the cysteine rich domains of the α-subunits (LeRoith et al., 1991). The biochemical events that induce the activation of the intracellular signal transduction pathway are initiated by binding of the ligand to the extracellular regions of the IGF-IR, which causes autophosphorylation of tyrosine residues of the β-subunits (Morgan et al., 1986). However, the intracellular postreceptor mechanism that follows this transmembrane reaction is still unknown.

Early studies (Gammeltoft et al., 1985; Roth et al., 1986) demonstrated that IGF-IR variants exist in both human and rat brain membranes. Specifically, it was shown by these investigators that neural-derived IGF-IR molecules had a lower molecular weight due to differences in glycosylation of the α-subunits, which is proposed to allow alterations in the orientation of the binding site. Specifically, it has been suggested by Sara and Carlsson-Skwirut (1988) that existence of IGF-IR variants in neuron cells may be functionally associated with the predominant presence in the central nervous system of truncated forms of IGF-I.

The type 2 IGF-R (IGF-IIR) is structurally simpler than the IGF-IR and is composed of a single-chain polypeptide with a molecular weight of ~250-KDa. The extracellular portion of the human IGF-IIR is composed of 15 repeated sequences of cysteine residues and hydrophobic domains. The IGF-IIR extends from the extracellular space through the membrane into the cytoplasm as a single polypeptide (Morgan et al., 1987). Interestingly, the human IGF-IIR shares a great deal of similarity (~80%) with the bovine 215-KDa mannose 6-phosphate (M-6-P) receptor (Lobel et al., 1987). Therefore, the IGF-II peptide cross-reacts with the M-6-P receptor, whereas the IGF-IIR diplays low affinity for IGF-I and no binding to insulin (Rechler and Nissley, 1985).

Presence of the IGF-IR and IGF-IIR in a variety of tissues has been reported (Rechler and Nissley, 1985). Specifically, it has been shown that expression of the IGF-IR is developmentally regulated in rats (Werner et al., 1989). These authors showed steady state levels of expression of IGF-IR mRNAs in a variety of tissues during fetal development. However, expression of IGF-IR decreased dramatically during postnatal development in the order liver > lung > heart > kidney > stomach. Conversely, expression of IGF-I mRNAs was controlled in a diametrically opposed manner and increased significantly postnatally in

liver and brain tissues. The high expression levels of IGF-IR during prenatal stages suggest an important role in fetal growth and development (Tollefsen et al., 1989). Nonetheless, the mechanisms responsible for the divergent regulation of transcription of IGF-I and IGF-IR are not known. It has been shown that both IGF-I and insulin down-regulated IGF-IR in human fibroblasts (Rosenfeld and Dollar, 1982), and human lymphocytes (Rosenfeld and Hintz, 1980; Kosmakos and Roth, 1980). It is possible that reduction in expression of IGF-IR observed during postnatal development may be a reflection of increased concentrations of IGF-I, which subsequently induce down-regulation of the IGF-IR. The molecular and cellular mechanisms responsible for this differential regulation remain to be determined.

Kosmakos and Roth (1980) suggested that down-regulation of insulin receptors could be exerted through inhibition of receptor biosynthesis and/or accelerated loss of insulin receptors. Specifically, these authors demonstrated that chronic increases of insulin induced significant down-regulation of the insulin receptor in human lymphocytes. In contrast, Lowe et al., (1989) reported that binding of IGF-I and expression of the IGF-IR was increased in various tissues in fasting rats. These authors suggested a correlation between membrane receptor concentration and receptor gene expression and proposed that upregulation of

the IGF-IR may represent a mechanism to maximize response to IGF-I at times of nutrient shortage. It is not known whether these mechanisms of regulation also apply to the IGF-IIR. However, levels of IGF-IIR are not downregulated by circulating IGF-II and treatment with insulin upregulates the number of IGF-IIR in adipocytes (Ota et al., 1984).

II. IGF-I BINDING PROTEINS (IGFBPs)

The identification of serum protein fractions with high affinity for IGF-I and IGF-II (Elgin et al., 1987; Szabo et al., 1988) suggested that soluble proteins present in serum and extracellular fluids were likely involved in mediation of the mitogenic actions of both IGFs. Progress in understanding the physiological significance of presence of IGFBPs in the circulation and in local tissues has come from cloning of cDNAs for all of the IGFBPs characterized to date, and through the use of western and ligand blotting techniques (Hossenlopp et al., 1986). Although IGFBPs mediate both the endocrine and autocrine/paracrine mitogenic effects of IGF-I, a clear function for each of the six IGFBPs has not yet been identified (Clemmons, 1992).

1. Structural analysis of IGFBPs

To date, six IGFBPs have been characterized and are referred to as IGFBP-1, 2, 3, 4, 5, and 6. Complementary and genomic DNA clones are available for IGFBP-1, 2, and 3, whereas only cDNA clones have been isolated for IGFBP-4, 5, and 6 (Rechler and Brown, 1992).

The human IGFBP-1, 2, and 3 genes are 5.2-, 32-, and 8.9-Kb in length and include 4 exons, except for IGFBP-3 for which a fifth exon has been identified at the 3′ end. Transcription of these IGFBPs yields mRNA fragments of 1.5-(human), 1.4- (rat), and 2.5-Kb (human and rat), respectively for IGFBP-1 (Cubbage et al., 1990), IGFBP-2 (Brown and Rechler, 1990), and IGFBP-3 (Wood et al., 1988; Shimasaki et al., 1989).

In humans, all of the IGFBPs contain highly conserved regions at the $-NH_2$ and -COOH termini including 18 cysteine residues except for IGFBP-6 (16 in human and 14 in rat). IGFBP-1 is a protein with a molecular weight of ~28-KDa and is composed of three distinct domains that can be characterized based on the abundance of cysteine residues (Brewer et al., 1988). These authors reported that while regions 1 and 3 are highly conserved and contain 11 and 7 cysteine residues, respectively, region 2 contains no cysteines and is not highly conserved. The biological activity of IGFBP-1 is probably mediated by the formation

of multimeric units linked by disulfide bonds (Busby et al., 1989).

Analysis of the primary sequence of IGFBP-2 reveals that this protein (31-KDa) is highly conserved and is 91% similar between human (Brown et al., 1989). Similar to IGFBP-1, IGFBP-2 contains the Arg-Gly-Asp sequence at the carboxyl terminus, which is believed to mediate attachment of the IGFBPs to the cell surface. In contrast, IGFBP-3 (40-43-KDa) does not have such a sequence, but conserves the 18 cysteine residues (Wood et al., 1988) and hosts three sites for potential N-glycosylation. Uniquely, IGFBP-3 has a higher affinity for cell membrane adherence than other IGFBPs, but the molecular mechanisms responsible for this peculiar characteristic have not been elucidated. The IGFBP-4 and 6 also contain potential glycosylation sites. IGFBP-5 has a lower affinity (10-fold) for IGF-I than IGF-II (Bautista et al., 1991).

2. Physiological properties of IGFBPs

While the physiological properties of IGFBP-1, 2, and 3 have been the target of extensive investigations during the last few years, the question as to whether these three IGFBPs possess a unique role in mediating the mitogenic actions of IGF-I is still unanswered (Baxter, 1991).

Moreover, virtually no information is available concerning the function of the three most recently characterized IGFBP-4, 5, 6 (Moser et al., 1992; Conover et al., 1993) and consequently this discussion will focus on examining the possible functions of IGFBP-1, IGFBP-2, and IGFBP-3.

It has been shown (Ooi, 1990; McGuire et al., 1992b) that both IGFBP-1 and IGFBP-2 inhibit the mitogenic actions of IGF-I and that the levels of IGFBP-1, but not IGFBP-2, are inversely related to concentrations of circulating insulin. Increased levels of IGFBP-1 and 2 are observed with fasting and may represent an attempt to hamper growth functions stimulated by IGF-I, in favor of more important metabolic pathways. Conversely, levels of plasma IGFBP-1 are rapidly reduced following a meal (Busby et al., 1988). This reduction is probably mediated by suppression of IGFBP-1 gene transcription (Clemmons, 1991).

The great majority (80%) of the IGF-I in circulation is part of the 150-KDa complex, which comprises an acid-labile subunit (88-KDa) and the 40-43 KDa IGFBP-3. Therefore, the 150-KDa complex present in serum acts as a storage source of IGF-I and IGFBP-3 and increases the half-life of IGF-I when compared with free or truncated IGF-I (Drakenberg et al., 1990; Clemmons, 1991). Because of the high affinity of IGF-I for IGFBP-3, there is very little free IGF-I in circulation. IGFBP-3 is regarded as GH-dependent, as it is

found in lower concentrations in hypophysectomized rats. In contrast, concentrations of IGFBP-1 and IGFBP-2 were increased (Clemmons, 1990). Conversely, IGF-I infusions restored high levels of IGFBP-3, but not the 150-KDa complex. Only the administration of GH stimulated the production of the acid labile subunit suggesting that formation of the 150-KDa complex is under the coordinate control of both GH and IGF-I. In lactating dairy cows GH induced a significant reduction in plasma IGFBP-2, but stimulated plasma levels of IGFBP-3 (Cohick et al., 1992).

The mechanisms that regulate distribution of IGF-I between vascular and extravascular compartments are not well understood. It has been shown that IGFBP-1 and 2 can be transported across capillaries (Clemmons, 1991). It has been speculated (Clemmons, 1992) that IGF-I could be transferred from IGFBP-3 onto IGFBP-1 and/or IGFBP-2. Moreover, the recent discovery that an IGFBP-3 protease is produced by the placenta in pregnant human and rats and is released into circulation may provide a mechanism to reduce the levels of IGFBP-3 and increase the amount of IGF-I bound to IGFBP-1 and IGFBP-2 (Davenport et al., 1990).

While one of the prominent roles of IGFBPs present in the circulation is transport of IGFs to peripheral tisssues, local production of IGFBPs has also been reported for a variety of cell types. Local secretion of IGFBPs may

represent a mechanism to modulate the mitogenic actions of IGF-I. Specifically, secretion of IGFBP-1 was inhibited by high concentrations of local IGF-I in hepatoma, breast carcinoma, and renal tubular epithelial cells, whereas secretion of IGFBP-2 was stimulated by cAMP, but inhibited by IGF-I in MDBK cells (Clemmons, 1991).

A clear role for IGFBP-1 and IGFBP-2 in cell proliferation has not been defined. Clemmons (1991) reported that these binding proteins can be present in monomeric or multimeric forms and that the monomeric form has a lower affinity than the multimeric one for the sequence recognition (Arg-Gly-Asp) receptors. This author suggested that this may be a mechanism by which IGFBP-2 can stimulate or inhibit cell proliferation. Finally, phosphorylation of IGFBP-1 has been recently reported, but the physiological significance of this modification is not entirely understood (Frost and Tseng, 1991). Interestingly, it has been shown that IGFBP-2 at high concentrations may act synergistically with IGF-II and stimulate cell proliferation of sheep thyroid cells, whereas at low concentrations IGFBP-2 suppressed cell growth (Wang et al., 1991).

The role of IGFBP-3 as a negative or positive mediator of the mitogenic actions of IGF-I has been the subject of several investigations. Early studies (DeMellow and Baxter,

1988) indicated that IGFBP-3 inhibited cell proliferation of skin fibroblasts. Conversely, others (Blum et al., 1989) reported that experimental conditions that allowed adherence of IGFBP-3 to the cell surface were favorable to stimulation by IGF-I. Recently, Conover and Powell, (1991) reported IGFBP-3 potentiated the effects of IGF-I-induced cell proliferation by preventing downregulation of the IGF-I receptor. Ironically, most recently (Cohen et al., 1993) it has been demonstrated that overexpression of recombinant human IGFBP-3 by Balb/c fibroblasts inhibited cell proliferation through an autocrine/paracrine mode of action. Further investigations of the biological functions of IGFBP-3 on cell responsiveness to IGF-I seem to be warranted. Nonetheless, there is virtually no information concerning the effects of local production of IGF-I on expression of IGFBPs and cell responsiveness in mammary epithelial cells.

SUMMARY

It is well accepted that IGF-I mediates GH actions by stimulating cell proliferation through an endocrine pathway. In addition, a large body of data suggests that IGF-I can also exert its mitogenic stimulus through autocrine/paracrine modes of action. While it has been shown that expression of IGF-I by the mammary gland was lower compared with other tissues, efforts to understand whether local production of IGF-I may stimulate cell proliferation through autocrine and/or paracrine pathways may provide new insights to bring about novel strategies to enhance mammary development and milk production in dairy animals. This project, therefore, focused on the development of an in vitro model with the primary objectives of investigating 1) the effects of secretion of recombinant IGF-I on cell proliferation of bovine mammary epithelial cells, 2) regulation of expression of IGF-I linked to the mammary specific MMTV-LTR promoter by lactogenic hormones and extracellular matrix, 3) roles of IGF-I-induced IGFBPs in mediating cell responsiveness to IGF-I, and 4) regulation of expression of IGFBP-3 and the type 1 IGF receptor in the presence of constitutive expression of IGF-I driven by the TK and SV40 promoters.

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CHAPTER 1

OVEREXPRESSION OF OVINE INSULIN-LIKE GROWTH FACTOR-I STIMULATES AUTONOMOUS AUTOCRINE GROWTH IN BOVINE MAMMARY DERIVED (MD-IGF-I) EPITHELIAL CELLS

INTRODUCTION

Insulin-like growth factor I (IGF-I) plays an important role in mediating growth and development of a number of tissues (Simmen, 1991). The liver largely contributes to the pool of circulating IGF-I, which exerts an endocrine effect on peripheral target cells. Although intracellularly produced growth factors may associate with the plasma membrane in a "juxtacrine" pathway (Brachmann et al., 1989) or via a strict "intracrine" mode of action (Fleming et al., 1989), a molecular basis for an autocrine or paracrine function of IGF-I has been postulated (Wilde et al., 1990).

Levels of IGF-I mRNA in the mammary gland of adult rats were the lowest when compared to other tissues. Nevertheless, it is thought that the endocrine as well as autocrine or paracrine pathways likely contribute to the development of the mammary gland in a coordinate fashion during different stages of development (Sara, 1990;

Hodgkinson, et al., 1991). Certainly, there are several demonstrations that exogenous IGF-I stimulates proliferation of mammary tissue from peripubertal and pregnant rodents (Imagawa et al., 1986) and ruminants (Shamay et al., 1988; Baumrucker and Stemberger, 1989; Waksman et al., 1991). Also, both primary (McGrath et al., 1991) and clonal (Freed and Herington, 1989; Katzenellenbogen and Norman, 1990; Zhao et al., 1992) mammary epithelial cells respond to IGF-I. However, it is not known to what degree mammary tissue contributes to its own development by autocrine and/or paracrine production of IGF-I.

As endogenous expression of IGF-I in the mammary gland is relatively low, mammary epithelial cells represent an excellent system for inducing overexpression of IGF-I to investigate presence of autocrine and/or paracrine pathways as well as intracellular routing and post-translational modification of IGF-I peptides (LeRoith and Roberts, 1991). Because the physiological significance of IGF-I is functionally determined at the molecular and cellular levels, we tested the hypothesis of whether growth of transformed mammary epithelial cells MD-IGF-I was stimulated by synthesis and secretion of IGF-I originating from an ovine exon 2-encoded preproIGF-I (oIGF-I) cDNA.

MATERIALS AND METHODS

MMTV-LTR plasmids. A 0.7 kb fragment encoding an ovine exon-2 (previously exon 1A) (Wong et al., 1989) preproIGF-I cDNA (IGF-I) containing a 33 amino acid signal peptide was isolated from clone A21 by a BglII restriction digest and purified from a low melting agarose gel using the geneclean system (BIO 101 Inc. La Jolla, CA). The ends of the fragment were filled in using T4 DNA polymerase to generate blunt ends. The blunted oIGF-I insert was cloned into the SmaI site of the 7.6-Kb expression vector pMSG (Pharmacia, LKB Biotechnology Inc., Piscataway, NJ). Thus, the ovine IGF-I cDNA was placed under the control of the MMTV-LTR promoter contained in the expression vector pMSG denoted pMMTVIGF-I. To evaluate the activity of the MMTV promoter, bovine mammary epithelial MAC-T cells (Huynh et al., 1991) were transiently transfected with plasmid pMSG-CAT (Pharmacia).

Development of Stable MD-IGF-I Transformants. MAC-T cells were plated at a density of $2x10^5$ in 60 mm tissue culture plates and co-transfected, using the calcium phosphate precipitation procedure, with the construct pMMTVIGF-I and a plasmid containing a cassette for resistance to hygromycin-B (HYG-B) (Blochlinger and

Diggelmann, 1984). Cells were glycerol shocked for 2 min at 37° C. After incubation for 48 h at 37°C, cells were placed in selective medium containing 200 μg/ml of HYG-B (Sigma). Resistant cells were selected for 14 d and cloned by limiting dilution using 96 well plates. For Southern blot analysis, genomic DNA was extracted (Sambrook et al., 1989), digested with EcoRI and run on 1% agarose gels. The Southern blot was probed with a 0.7-Kb ovine IGF-I cDNA labeled by nick translation.

Northern Analysis. MD-IGF-I clones were plated in 100mm tissue culture plates in DMEM, 10% fetal calf serum (FCS) with 200 μg/ml of HYG-B. When cells were at 85% confluency, media were removed and cells were washed with Dulbecco's Phosphate Buffered Saline (DPBS). Then, fresh DMEM media containing 0.1 μM DEX to induce the MMTV-LTR promoter was added. Cells were trypsinized after 24 h and total RNA was extracted using a guanidinium thiocyanate isolation procedure (Puissant and Houdebine, 1990) and separated on a 1% agarose gel containing 2.2 M formaldehyde. Northern blots (Nitroplus, MSI Inc., Westborough, MA) were hybridized overnight at 42°C and washed at high stringency according to the manufacturer's instruction (MSI Inc., Westborough, MA)

Growth of MD-IGF-I Cells and Detection of IGF-I in Media. A series of experiments were designed to monitor the mitogenic activity of the MD-IGF-I cell line. For all growth experiments, MD-IGF-I and control MAC-T cells were plated at a density of $2x10^4$ cells in 24 well tissue culture plates in DMEM with 10% FCS. After 24 h, media were removed and cells were washed with PBS. In a first experiment, test media were DMEM without FCS but with or without 200 μg/ml HYG-B and with 0.0, 0.1, and 2.5 μM DEX. Cells were collected 24, 48, and 72 h following induction with DEX and total DNA/well was measured (LaBarca and Paigen, 1980). Six replicates were collected for each treatment and samples were assayed in duplicate. To test the sensitivity over time of the MD-IGF-I cells to induction with DEX, cells were induced with 0.1 and 2.5 μM DEX up to 240 h. At the end of each treatment, cells were harvested for determination of total DNA.

Growth response of MD-IGF-I cells to exogenous human IGF-I (100 ng/ml)(Boehringer Mannheim, Indianapolis, IN) was ascertained in presence or absence of 0.1 μM DEX. Cells were harvested after 72 h and total DNA was used as an indicator of mitogenic activity.

To confirm the secretion of biologically active IGF-I in conditioned media of MD-IGF-I cells, $5x10^5$ MD-IGF-I and MAC-T cells were plated in 100 mm tissue culture plates for

72 h in presence and absence of 0.1 μM DEX. At the end of the conditioning period, media from each treatment were collected and used to culture MAC-T or MD-IGF-I cells seeded in 24 well culture plates. After 16 h, cells were pulsed for 2 h with [^{3}H]-thymidine (ICN Biomedicals Inc., Costa Mesa, CA) and incorporation was measured as previously described (Zhao et al., 1992). Presence of IGF-I in the media was measured by a previously validated radioimmunoassay procedure (Herring and McFadden, 1990). Media were subjected to methanol-formic acid extraction to correct for possible false positive results due to IGF-I binding proteins. After extraction, amounts of IGF-I associated with binding proteins in control serum samples was 8.0% which confirmed that minimal amounts of binding proteins remained in the samples tested by RIA. The RIA intra-assay coefficient of variation in control serum samples was less than 10%.

Statistics. Data are presented as mean $\pm$ SEM. Effects of DEX levels and time on growth of MAC-T and MD-IGF-I cells were tested using the analysis of variance procedure in SAS (SAS Institute Inc., Cary, NC).

RESULTS

An exon-2 containing ovine IGF-I cDNA, encoding a 33 amino acid leader peptide, was cloned into expression vector pMSG. The resulting plasmid, designated pMMTV-IGF-I contained the ovine IGF-I cDNA under the control of the MMTV-LTR promoter (Figure 1). Expression vector pMMTV-IGF-I contained a transcription initiation site 134 base pairs upstream and a SV40 polyadenylation site 822 base pairs downstream of the cloned oIGF-I cDNA. Thus, the predicted length of the oIGF-I transcript from pMMTV-IGF-I was approximately 1.65-Kb. The MMTV-LTR promoter was shown to be active and inducible by DEX in bovine mammary epithelial cell line MAC-T, following transient transfection studies with plasmid pMSG-CAT (data not shown).

Stable MAC-T transformants containing pMMTV-IGF-I were isolated in DMEM plus hygromycin-B after cotransformation with a plasmid conferring hygromycin-B resistance. The presence of integrated copies of pMMTV-IGF-I was confirmed by Southern blotting (data not shown). Stable transformants contained single or multiple copies of pMMTV-IGF-I. One clone, MD-IGF-I, contained approximately 30 copies of pMMTV-IGF-I integrated in a tandem array and was used for further analysis. Northern blot of total RNA extracted from

MAC-T and MD-IGF-I cells indicated that an RNA species of approximately 1.8 kb hybridized strongly when the MD-IGF-I cells were induced with DEX (Figure 2A). The length of this transcript is in agreement with the predicted size of processed mRNAs transcribed from pMMTV-IGF-I. Densitometric analysis of autoradiograms showed a 40-fold increase in the intensity of hybridizing RNA species as compared to MAC-T cells (Figure 2B). Even in the absence of DEX the MD-IGF-I cells produced detectable quantities of this RNA species.

To investigate the mitogenic action of endogenously produced recombinant IGF-I, cells were plated in selective media in the absence of fetal calf serum (FCS), but in the presence of 0.0, 0.1, and 2.5 μM DEX. Total DNA from MAC-T and MD-IGF-I cells is depicted in Figure 3. MD-IGF-I cells (Panel B) had the highest growth response in association with induction of the MMTV-LTR promoter and by 48 h total DNA was 47 to 54% higher than parental MAC-T cells (Panel A). By 72 h, total DNA from MD-IGF-I cells treated with 0.1 and 2.5 μM DEX was 80% higher than uninduced MD-IGF-I cells, but it was 2.4-and 2.5-fold higher ($P<.01$) as compared to DNA from DEX-treated MAC-T cells. Induction of MD-IGF-I cells with 0.1 μM DEX up to 240 h (Panel C) provided further evidence of the responsiveness of MD-IGF-I cells to glucocorticoid stimulation ($P<.01$) and their capacity for continued growth. MD-IGF-I cells responded to

exogenous IGF-I (100 ng/ml) with a 2-fold increase ($P<.01$) in total DNA in DMEM alone (Figure 4). More importantly, treatment with 0.1 μM DEX caused a mitogenic response comparable to the one promoted by 100 ng/ml of exogenous IGF-I.

Because binding proteins can interfere with RIAs for IGF-I, conditioned media from MAC-T and MD-IGF-I cells were extracted to confirm secretion of IGF-I into the media. Average media IGF-I concentrations from two independent experiments are summarized in Table 1. IGF-I in conditioned media from MAC-T cells ranged from 0.75 to 1.45 ng/ml (11.3 to 21.7 ng/10^6 cells). Average concentration of IGF-I for MD-IGF-I cells ranged from 1.85 to 7.10 ng/ml (27.7 to 106.5 ng/10^6 cells) in the absence and presence of DEX.

To investigate whether the endogenously produced recombinant IGF-I stimulated proliferation of MAC-T and MD-IGF-I cells, conditioned media from both cell types were used to study effects on [^{3}H]-thymidine incorporation (Figure 5). Panel A demonstrates that conditioned media from MD-IGF-I cells stimulated ($P<.01$) labeling of MAC-T cells to a greater extent than conditioned media from MAC-T cells, particularly when MD-IGF-I cells were induced with DEX. Similarly, conditioned media from MD-IGF-I or MAC-T cells cultured with DEX markedly stimulated thymidine uptake into MD-IGF-I (Panel B). Response of MD-IGF-I cells

to media from MAC-T cells incubated with DEX is due to induction of MMTV-IGF-I by residual DEX. While addition of FCS increased thymidine incorporation by both cell types (Panel C), the somewhat reduced uptake with conditioned media, compared with fresh media, likely reflected some exhaustion of nutrients since conditioned samples were not supplemented with fresh media prior to testing.

DISCUSSION

The mitogenic effect of IGF-I is well documented for a large number of cell types. Among them, bovine mammary epithelial cells have been shown to respond to exogenous IGF-I (Shamay et al., 1988; Baumrucker and Stemberger, 1989; Zhao et al., 1992). As expression of IGF-I by the mammary gland in adult rats (Murphy et al., 1987) and pigs (Tavakkol et al., 1988) was very low as compared to other tissues, it has been suggested that local production of IGF-I may limit growth of mammary epithelial cells, so that the pool of circulating IGF-I is relatively more important. On the other hand, efforts to stimulate local mammary tissue production of IGF-I might offer a mechanism for enhanced mammary development and milk production.

To investigate whether IGF-I can stimulate growth of

bovine mammary epithelial cells by an autocrine and/or paracrine mode of action, MAC-T cells were transformed with a construct containing an ovine IGF-I cDNA under control of the MMTV-LTR promoter. The MMTV-LTR has been previously used in mammalian systems to induce expression of cloned cDNAs in the presence of glucocorticoids (Doppler et al., 1991;, Buetti and Diggelmann, 1981). Presence of the transgene in MD-IGF-I cells increased steady state levels of IGF-I mRNA when the MMTV promoter was induced with DEX. Northern blot analysis of total RNA from MAC-T cells confirmed that endogenous production of IGF-I mRNA was very low, although production of IGF-I mRNA by bovine mammary tissue has been previously reported (Campbell et al., 1991; Hauser et al., 1990).

Because cell proliferation and thymidine incorporation by MD-IGF-I cells induced with DEX were comparably higher than MAC-T cells, it is suggested that the oIGF-I transgene supported enhanced mitogenic activity. Although DEX stimulated activity of the MMTV-LTR promoter, no difference in total DNA was observed between 0.1 and 2.5 μM DEX. MAC-T cells, on the other hand, did not show a growth response to addition of DEX. In addition, analysis of the growth response of MD-IGF-I cells for up to 10 d provided further evidence of their accelerated mitogenic activity in comparison with parental MAC-T cells.

Although it could be argued that faster growth of MD-IGF-I cells was due to an intracellular mechanism associated with IGF-I production, the fact that IGF-I was found in conditioned media of MD-IGF-I cells from two independent experiments suggested that this cell line secreted recombinant oIGF-I. Rates of IGF-I production (Table 1) are similar to those reported for expression of recombinant human IGF-I in CHO and mouse L cells (Bovenberg et al., 1990).

While dexamethasone caused a reduction in steady state concentration of endogenous IGF-I mRNA in several tissues (Adamo et al., 1988; Luo and Murphy, 1989), recent reports (Luo et al., 1990; Price et al., 1992) have suggested that glucocorticoids reduced IGF-I bioactivity not by causing a reduction of serum IGF-I, but, rather, by sequestering IGF-I due to stimulated local production of IGF-I binding proteins (IGFBPs). Although we did not measure whether changes in free IGF-I were concomitant with variation in production of IGFBPs, it is possible that the DEX treatment may have affected production of IGFBPs by MD-IGF-I cells thereby influencing the availability of IGF-I (Ross et al., 1989; Blum et al., 1989).

Autonomous growth by transformed phenotypes has been previously utilized to investigate presence of autocrine and/or paracrine pathways for effects of human basic

fibroblast growth factor in hamster kidney derived cells (Neufeld et al., 1988) and insulin-like growth factor II in MCF-7 cells (Cullen et al., 1992). Similarly, introduction of the oIGF-I gene in MAC-T cells triggered autonomous growth due to secretion of recombinant IGF-I. Fundamental prerequisites for autocrine and/or paracrine stimulation are endogenous production of a growth factor, its secretion, corresponding presence of cell membrane receptors, and subsequent stimulation of cell growth (Sara, 1990; Heldin and Westermark, 1989). Accordingly, our data suggest that the autocrine and/or paracrine pathway for IGF-I was active in these mammary epithelial MD-IGF-I cells. In a related study Zhao et al., (1992) illustrated that the parental MAC-T cells exhibit a proliferative response to exogenous IGF-I and express IGF-I receptors.

In this study we used an exon 2-encoded preproIGF-I form of IGF-I encoding a 33 amino acid leader peptide. Consequently, one could ask the question whether the IGF-I secreted in the media represented the intact form of IGF-I or the truncated Des(1-3)IGF-I form, which has been shown to be biologically more active (Ross et al., 1989; Carlsson-Skwirut et al., 1989). We plan to test this hypothesis by purification and sequence analysis of the IGF-I protein secreted by the MD-IGF-I cell line. We propose that the MD-IGF-I cell line may be a suitable

system to study translational and post-translational modifications leading to production of various IGF-I peptides.

SUMMARY

To test the hypothesis of whether insulin-like growth factor-I affects growth of bovine mammary epithelial cells through an autocrine and/or paracrine pathway, a cell line (MD-IGF-I) was originated from MAC-T cells by co-transfection with a construct containing the cDNA for an ovine exon-2 encoded prepro IGF-I (oIGF-I) under control of the MMTV-LTR promoter. Clone MD-IGF-I contained multiple copies of the plasmid integrated into the genome, expressed the highest level of IGF-I mRNA, and secreted radioimmunoreactive IGF-I. The mitogenic activity of MD-IGF-I cells was stimulated 80% with DEX. Total DNA of MD-IGF-I cells was 2.5-fold higher than parental MAC-T cells in the presence of DEX. Conditioned media from MD-IGF-I cells, induced with DEX, stimulated [^{3}H]thymidine incorporation into DNA of MAC-T cells and uninduced MD-IGF-I cells. These data provide evidence that IGF-I was secreted by MD-IGF-I cells and stimulated growth of mammary epithelial cells. The MD-IGF-I cell line may be a suitable system to study translational and post-translational modifications of IGF-I peptides.

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TABLE 1. RIA IGF-I OF CONDITIONED MEDIA FROM PARENTAL MAC-T AND MD-IGF-I CELLS AFTER 72 H.

Treatment	ng IGF-I/ml		ng IGF-I/10^6cells	
	MAC-T	MD-IGF-I	MAC-T	MD-IGF-I
DMEM	1.45	1.85	21.7	27.7
DMEM+DEX	0.75	7.10	11.3	106.5

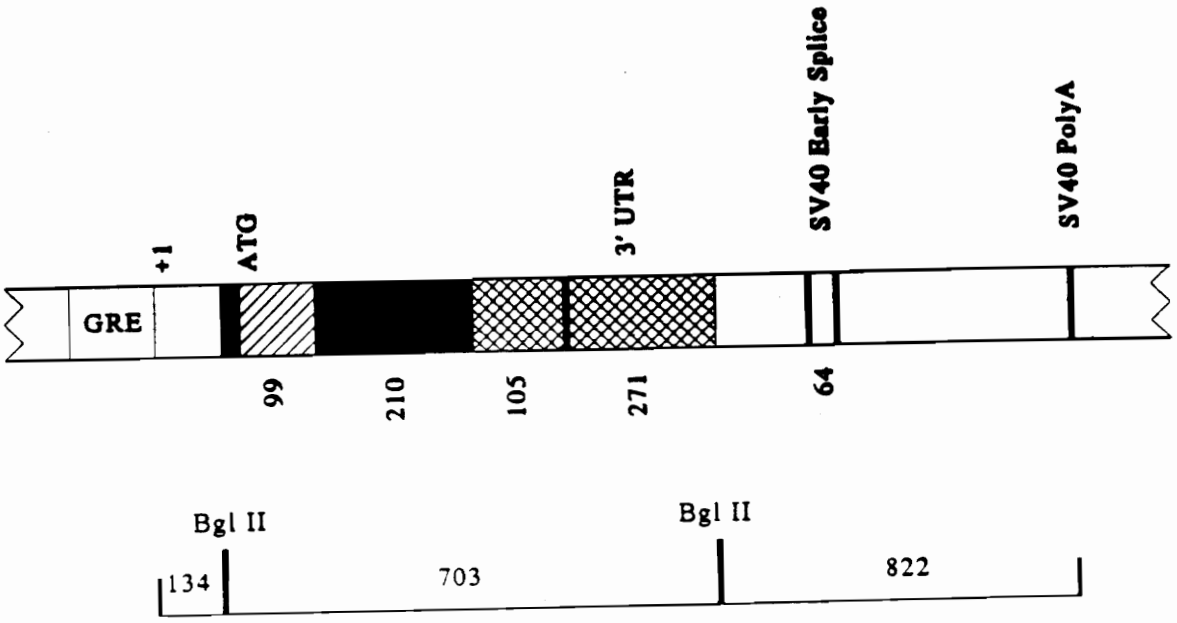

Figure 1. Cloning of the oIGF-I cDNA into the expression vector pMSG. Expression of the oIGF-I cDNA was under the control of the MMTV-LTR promoter. The approximate sites of initiation of transcription (+1), start codon (ATG), leader sequence (hatched box), mature peptide (black box), E-peptide (105 bp), 3'UTR (271 bp), the SV40 early splice and SV40 polyA sites are indicated. The lower panel shows the predicted length of mRNA transcripts from the pMMTV-IGF-I cDNA construct.

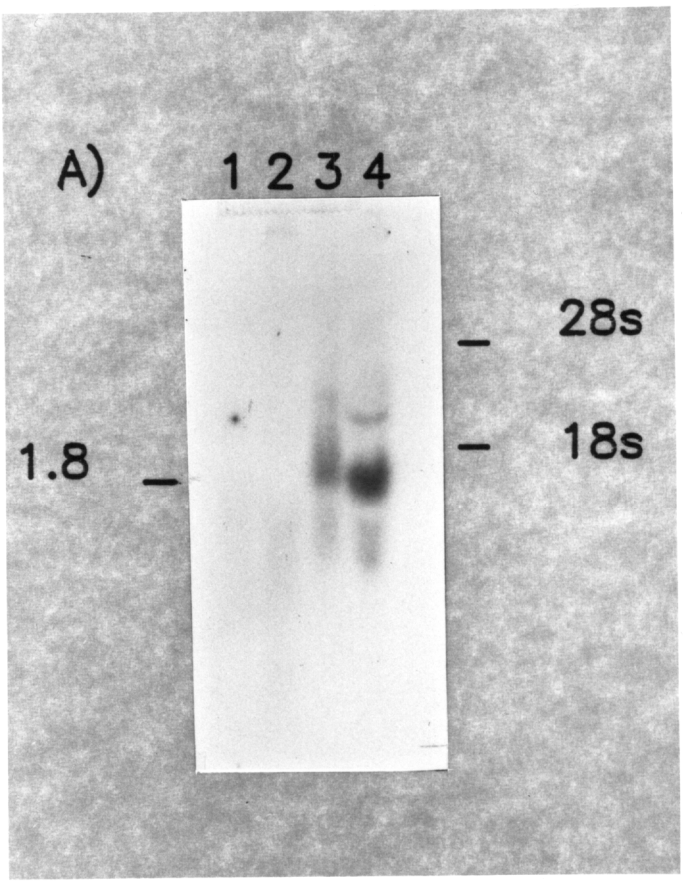

Figure 2A. Northern analysis of total RNA from parental MAC-T and MD-IGF-I cells. Cells were incubated for 24 h in the presence (0.1 μM) or absence of dexamethasone (DEX). Total RNA was extracted and separated (15 μg) on a 1% agarose gel in 2.2 M formaldehyde and transferred to nitrocellulose membrane. Blots were probed with a [^{32}P]-labeled IGF-I cDNA probe and washed at high stringency. RNA samples were from: MAC-T cells minus DEX (lane 1) and 0.1 μM DEX (lane 2); MD-IGF-I cells minus DEX (lane 3) and with 0.1 μM DEX (lane 4). Migration of the 28s and 18s ribosomal RNAs is indicated.

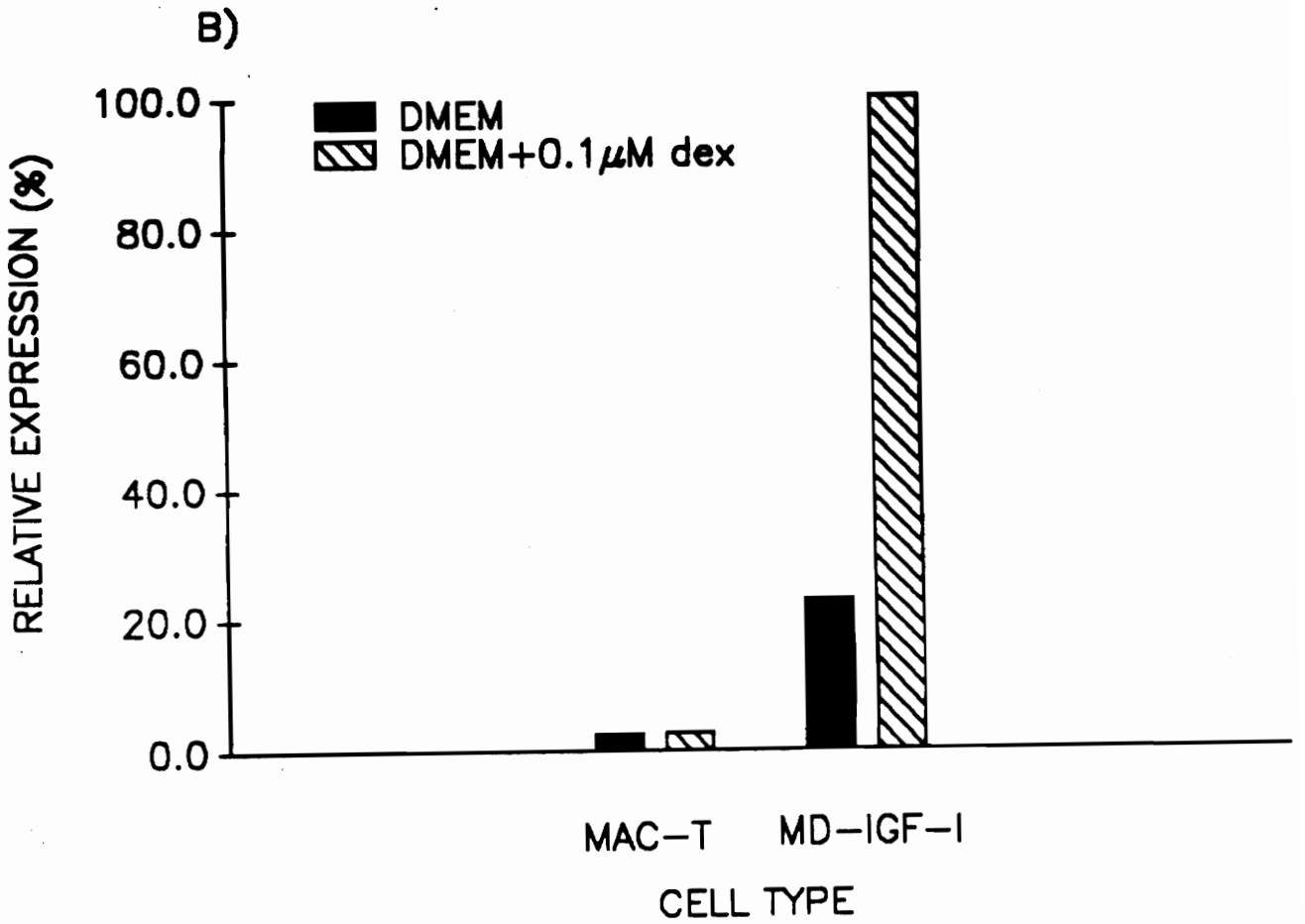

Figure 2B. Northern analysis of total RNA. Relative expression of IGF-I mRNA was estimated by densitometric analysis of Northern blots. Values are expressed as percentage of maximal intensity.

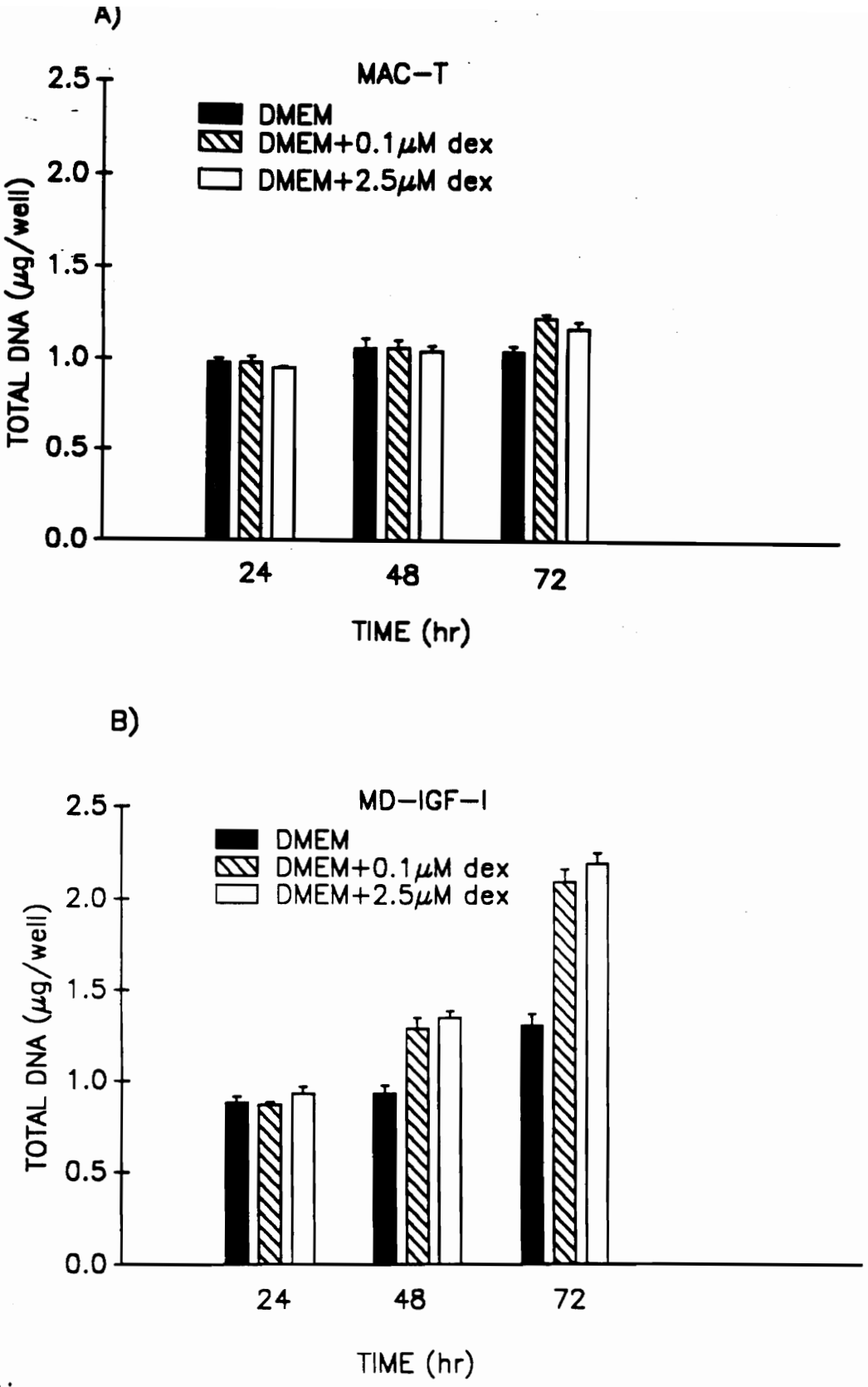

Figure 3 A,B. Effect of dexamethasone on growth of MAC-T and MD-IGF-I cells. In Panels A and B, MAC-T and MD-IGF-I cells were cultured in DMEM (solid bars), DMEM with 0.1 μM dexamethasone (DEX) (hatched bars), and DMEM with 2.5 μM DEX (open bars) for 24, 48, and 72 h.

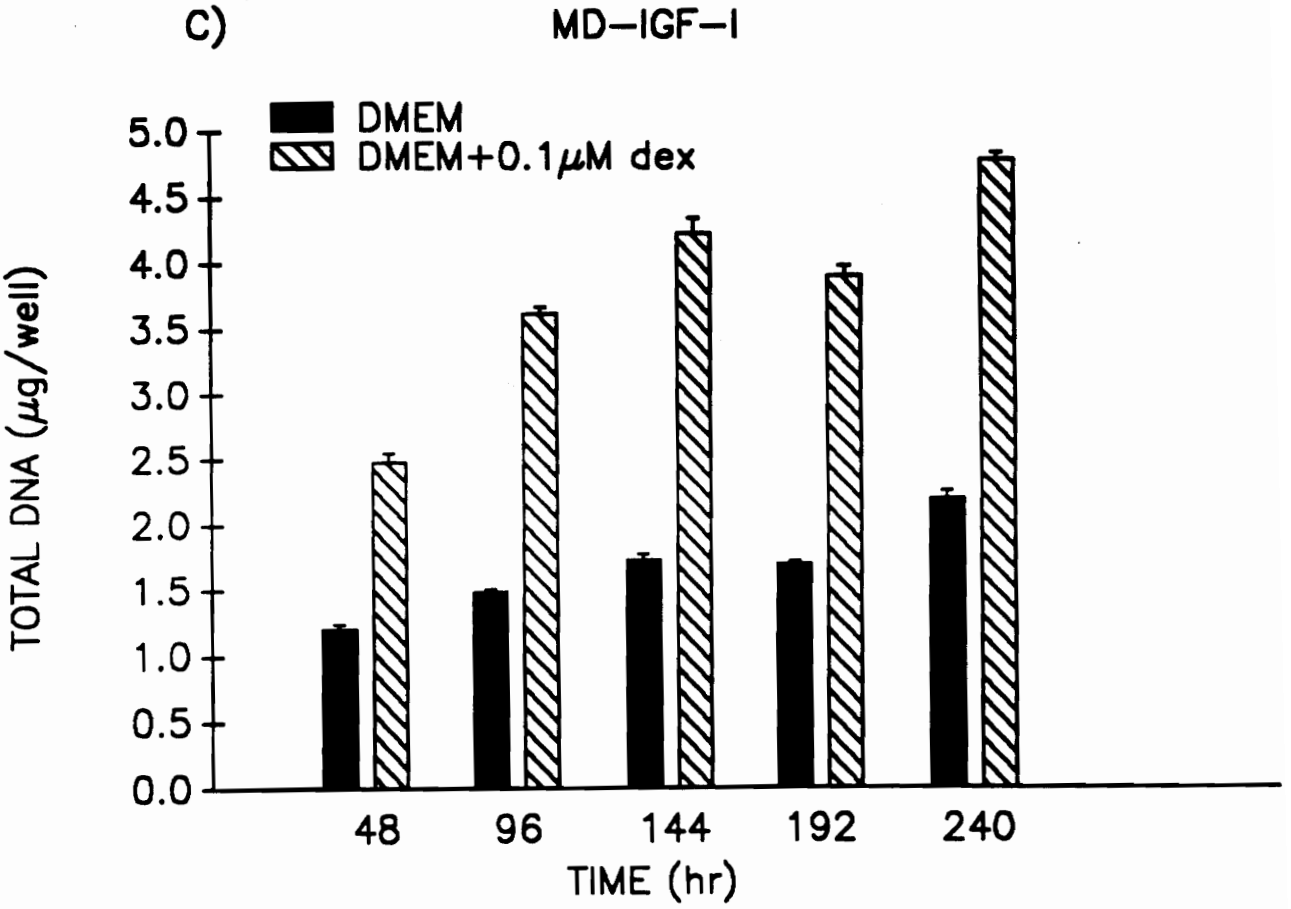

Figure 3C. Effect of dexamethasone on growth of MD-IGF-I cells. MD-IGF-I cells were cultured up to 240 h in DMEM (solid bars) and DMEM plus 0.1 μM (DEX) (hatched bars). At the end of each incubation, cells were harvested and total DNA (μg/well) determined. Bars represent means $\pm$ SEM of values obtained from six samples assayed in duplicate.

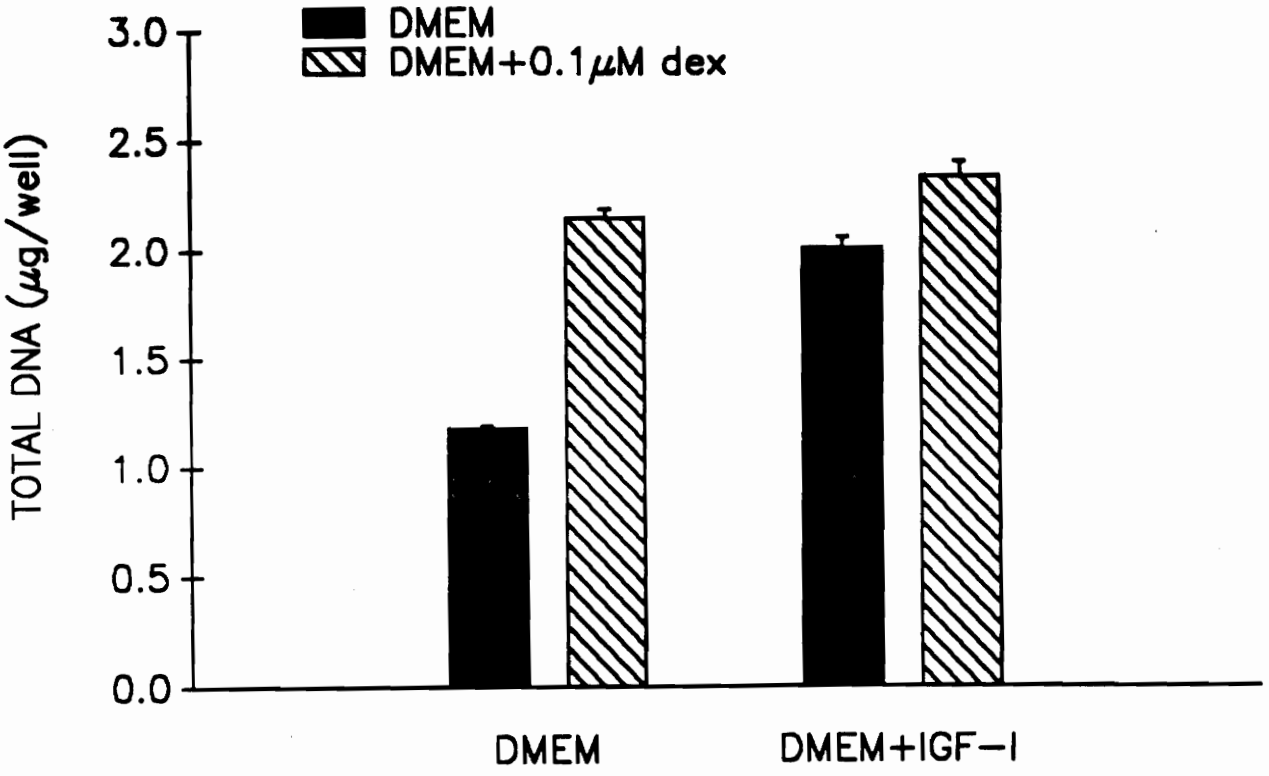

Figure 4. Response of bovine mammary epithelial cells MD-IGF-I to exogenous IGF-I. MD-IGF-I cells were cultured for 72 h in DMEM (solid bars) and DMEM with 0.1 μM dexamethasone (DEX) (hatched bars) in the presence (100 ng/ml) or absence of IGF-I. At the end of the incubation period, cells were harvested and total DNA (μg/well) determined. Bars represent means ± SEM of values from four samples assayed in duplicate.

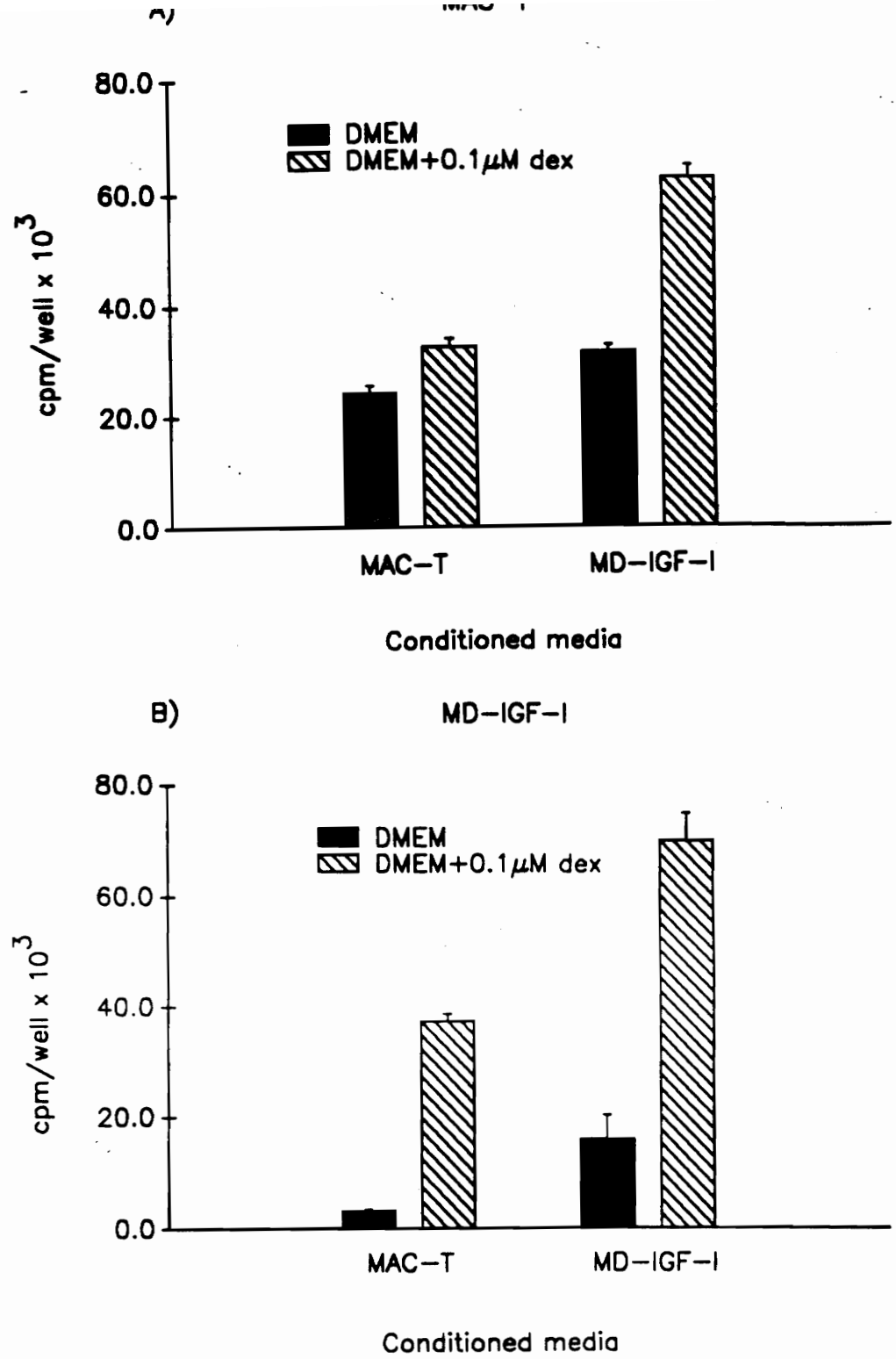

Figure 5 A,B. [^{3}H]Thymidine incorporation into DNA of MAC-T and MD-IGF-I cells. MAC-T and MD-IGF-I cells were cultured for 72 h in DMEM (solid bars) and DMEM with 0.1 μM dexamethasone (DEX) (hatched bars). At the end of the incubation period, conditioned media were harvested and used to culture MAC-T (panel A) and MD-IGF-I (panel B) cells. [^{3}H]-thymidine incorporation was determined after 16 h with a 2-h pulse.

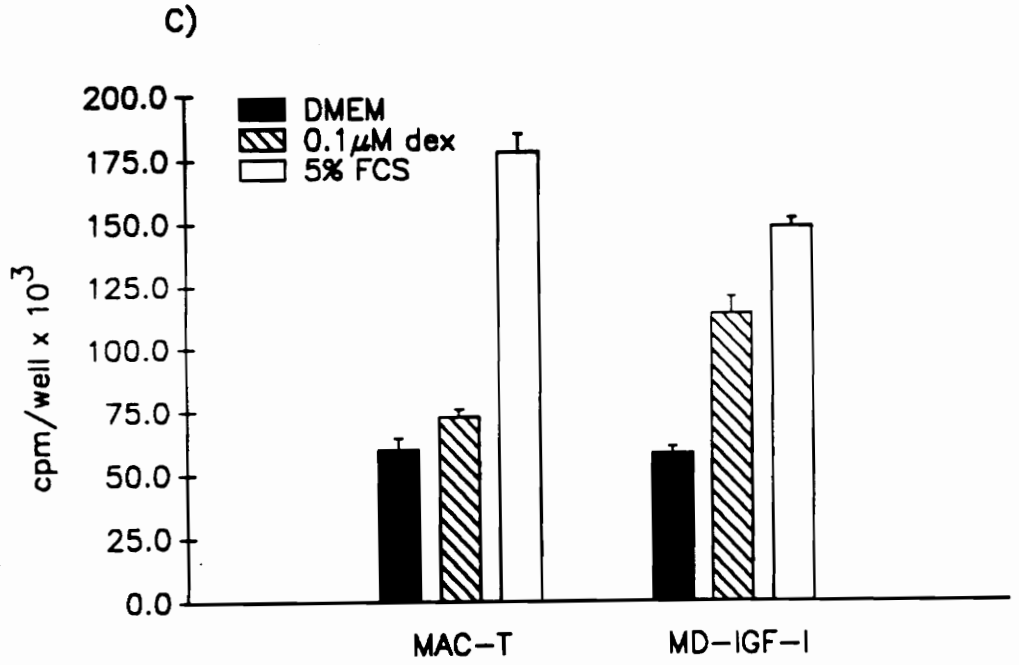

Figure 5C. Thymidine incorporation into DNA of MAC-T and MD-IGF-I cells. Cells were cultured in DMEM, DMEM plus 0.1 μM dexamethasone, and DMEM plus 5% FCS. Bars represent means $\pm$ SEM of four values.

CHAPTER 2

LACTOGENIC HORMONES AND EXTRACELLULAR MATRIX REGULATE EXPRESSION OF IGF-I LINKED TO MMTV-LTR IN MAMMARY EPITHELIAL CELLS

INTRODUCTION

Growth and subsequent differentiation of epithelial cells during gestation and early lactation are required for maximum development of the mammary gland. While cellular growth is controlled by a variety of hormones (Akers, 1985) and growth factors (Forsyth, 1991), differentiation to a lobulo-alveolar structure requires contemporary presence of lactogenic hormones (Howlett and Bissell, 1990), interaction between cells and extracellular matrix components (Streuli et al., 1991), and is characterized by high expression levels of milk proteins (Eisenstein and Rosen, 1988).

Studies on expression of milk protein genes have provided insights into regulatory elements that may be important in targeting expression of foreign proteins to the mammary gland (Salmons and Gunzburg, 1987). Similarly to milk casein genes, the mouse mammary tumor virus-long

terminal repeat (MMTV-LTR) is primarily expressed in the mammary gland during pregnancy and lactation. Moreover, activity of the hormone responsive element (HRE) of the MMTV-LTR is modulated by sex steroids and glucocorticoids (Chalepakis et al., 1988).

To study autocrine/paracrine effects of endogenous production of recombinant insulin-like growth factor-I (IGF-I) in mammary tissues, a bovine mammary epithelial cell line MD-IGF-I (Romagnolo et al., 1992) was generated by transfection of a bovine mammary cell line (Huynh et al., 1991) with a construct containing an ovine exon-2 encoded prepro IGF-I cDNA (Wong et al., 1989) under control of the MMTV-LTR promoter. Dexamethasone (DEX), representative of glucocorticoids known to stimulate secretion of specific milk proteins by bovine tissue in culture (Goodman et al., 1983), was shown to enhance mRNA synthesis for the transgene, secretion of IGF-I, and growth of the cells. These findings suggested that regulation of expression of the transgene might be closely associated with the physiological processes which drive normal mammary epithelial cell proliferation and differentiation. Indeed, the growth and differentiation status of the mammary gland in vivo may play a significant role in controlling the transcriptional activity of the LTR promoter (Gunzburg and Salmons, 1992).

In this paper, we report on the effects of several known mammary-active hormones and growth factors on the proliferative response of MD-IGF-I cells. Furthermore, we present evidence that expression of the MMTV-LTR is enhanced by lactogenic hormones and that culture of the cells on extracellular matrix derived from Engelbreth-Holm-Swarm tumor (EHS) augments this response. We also demonstrate that culture of MD-IGF-I cells on EHS matrix stimulates the formation of multicellular spherical alveolar-like structures and significantly increases the secretion of recombinant IGF-I.

MATERIALS AND METHODS

MMTV-LTR-CAT transient transfection. Hormonal regulation of the MMTV promoter was studied by transient transfection of clonal bovine mammary epithelial MAC-T cells (Huynh et al., 1991), with plasmid pMSG-CAT, (Pharmacia, LKB Biotechnology Inc. Piscataway, NJ) using a liposome-mediated system (GIBCO BRL, Gaithersburg, MD). A plasmid (pRSVCAT) containing the promoter of the Rous Sarcoma Virus (RSV) was used as positive control. Cells ($6x10^5$) were seeded in 60 mm plates in Dulbecco's Modified Eagle Media (DMEM) with 10% fetal calf serum (FCS) and

transfected at approximately 85% confluency for 4 h, at 37°C. After 18 h, cells were washed with Phosphate Buffered Saline (PBS), and then cultured for 24 h in DMEM plus 10% FCS containing equimolar concentrations (0.1 μM) of 17β-estradiol (E_2), progesterone (P_4), dexamethasone (DEX), or hydrocortisone (F) (Sigma, St. Louis, MO). At the end of the incubation period, cells were washed with PBS and incubated 5 min in TEN buffer (40 mM Tris-HCl, pH 7.5, 1 mM EDTA, 15 mM NaCl). Cells were harvested with a rubber policeman and resuspended in buffer A (15 mM Tris pH 8.0; 60 mM KCl, 15 mM NaCl, 2 mM EDTA, 0.15 mM spermine, 1 mM dithiothreitol (DTT), 0.4 mM phenylmethylsulfonyl fluoride (PMSF)) (Pothier et al., 1992) and subjected to three freeze-thaw cycles. Samples were incubated at 60°C for 10 min and centrifuged at 12,000xg for 5 min. Extracts were assayed for chloramphenicol acetyltransferase (CAT) activity in the presence of n-butyryl Coenzyme A (Promega, Madison, WI, USA) and ^{14}C-chloramphenicol (ICN, Irvine, CA, USA) as described previously (Doppler et al., 1991).

Cell Culture. The growth response of MD-IGF-I cells to various protein mitogens and steroids (all from Sigma unless noted otherwise) was investigated by plating the cells at a density of $2x10^4$ in 24-well tissue culture plates in DMEM containing 10% FCS. After 24 h, media were

removed and cells washed with PBS. For all growth experiments, test medium was serum-free DMEM, supplemented with one of the following growth factors or hormones: 100 ng/ml insulin (I), 100 ng/ml mouse epidermal growth factor (EGF), 1 μg/ml bovine growth hormone (GH; NHPP, lot #b18), 100 ng/ml human insulin-like growth factor-I (IGF-I) (Boehringer Mannheim, Indianapolis, IN, USA), 1 μg/ml bovine prolactin (PRL; NHPP, lot # b6), with or without 0.1 μM DEX. Three separate experiments were performed to study the response of MD-IGF-I cells to increasing concentrations of IGF-I (0.1 to 100 ng/ml), I (1 to 1000 ng/ml), and transforming growth factor-α and -β (TGF-α and -β) (1 to 100 ng/ml)(GIBCO BRL) in the presence or absence of 0.1 μM DEX.

The growth response of MD-IGF-I cells to selected steroids was studied by plating the cells in DMEM supplemented with 0.1 μM of one of the following steroids: 17β-estradiol, progesterone, hydrocortisone, corticosterone, aldosterone, and testosterone, in the absence or presence of 0.1 μM DEX.

Modulation of IGF-I secretion and corresponding proliferation of MD-IGF-I cells, in response to induction of the MMTV-LTR promoter by lactogenic hormones, was ascertained by culturing the cells for 192 h, in DMEM or DMEM supplemented with 0.1 μM DEX, 1.0 μg/ml I, or 1.0

μg/ml PRL, or combinations of the three hormones. In this instance, to ensure secretion of readily quantifiable amounts of IGF-I, cells were plated at a starting density of $1x10^5$ cells/well. Media were changed every 48 h and concentrations of IGF-I measured by a previously validated radioimmunoassay procedure (Herring and McFadden, 1990).

For all growth experiments, cells were harvested at the end of the incubation periods, and total DNA (μg/well) was used as the indicator of cell growth (Labarca and Paigen, 1980). Six replicates were performed for each treatment and samples assayed in duplicate.

To investigate whether expression of IGF-I driven by the MMTV-LTR promoter, in stably transfected MD-IGF-I cells, was coordinately regulated by lactogenic hormones and substratum, MAC-T and MD-IGF-I cells were seeded on plastic or extracellular matrix. Reconstituted basement membrane gel derived from the Engelbreth-Holm-Swarm (EHS) tumor was a gift of H. K. Kleinman (National Institute of Health, Bethesda, MD, USA) and was prepared as described previously (Kleinman et al., 1987). Briefly, the EHS matrix was thawed at 4^oC and 200 μl-aliquots were pipetted into 24-well tissue culture plates. After gelation of the matrix at 37^oC, $1x10^5$ cells/well were seeded in DMEM with 10% FCS. Cells were kept on EHS for four days with a media change after 48 h. Then, cells were washed with PBS and cultured

in fresh media containing DMEM, DMEM supplemented with I (1 μg/ml), I and DEX (0.1 μM), or I, DEX, and PRL (1 μg/ml). After 72 h, conditioned media were harvested and stored at -20°C for analysis of IGF-I concentrations. The EHS matrix was dissociated at 37°C for 2 h using Dispase (Collaborative Biomedical Products, Bedford, MA, USA). Cells were harvested by low speed centrifugation and aliquots saved for estimation of total DNA.

Statistical Analysis. Data are presented as means±SEM. Effects of protein mitogens and steroids on growth of MD-IGF-I cells and of lactogenic hormones and ECM on growth and secretion of recombinant IGF-I were evaluated by one-way analysis of variance procedure in SAS (SAS Institute Inc., Cary, NC). Specific means comparisons utilized Duncan's multiple range test with $P<.05$ selected as a level of significance.

RESULTS

The induction of the mouse mammary tumor virus-long terminal repeat (MMTV-LTR) promoter with dexamethasone (DEX), in MAC-T cells transiently transfected with plasmid pMSG-CAT, demonstrated that the MMTV-LTR promoter was

inducible by glucocorticoids in clonal bovine mammary epithelial cells (Fig. 1). In contrast, 17β-estradiol and progesterone did not affect the activity of the LTR element. As expected, control MAC-T cells did not show CAT activity.

The mitogenic actions of several known mammary-active protein hormones and growth factors on MD-IGF-I cells are depicted in Figure 2. Exogenous I and IGF-I, both at the concentration of 100 ng/ml, stimulated a 1.4- and 1.6-fold increase ($P<.01$) in total DNA, compared with DMEM treated MD-IGF-I cells (Figure 2A). Conversely, EGF and PRL did not affect the growth rates of MD-IGF-I cells, and the combination of EGF and IGF-I, did not enhance cell proliferation, compared with IGF-I alone. Neither did the combination of I with GH and PRL induce greater growth than I alone. On the other hand, the addition of 0.1 μM DEX to culture media triggered a 1.8-fold increase in total DNA ($P<.01$), due to the stimulation of the MMTV-IGF-I construct, which largely masked, however, the effects of exogenous hormones (Figure 2B).

In further experiments, MD-IGF-I cells responded positively (1.4-fold) to exogenous IGF-I starting at concentrations of 10 ng/ml and no further cell growth stimulation was evident with 100 ng/ml (Figure 3A). A similar cell growth response (1.8-fold) was measured in the

presence of I but at concentrations of 1000 ng/ml (Figure 3B).

Effects of TGF-α and -β on cell growth of MD-IGF-I cells are summarized in Figure 4. TGF-α had a stimulatory effect, ($P<.01$) at the concentration of 20 ng/ml, and total DNA was increased 1.5-fold at 80 ng/ml. In contrast, TGF-β decreased ($P<.01$) basal cell proliferation at concentrations equal to or greater than 10 ng/ml.

Investigation of the responsiveness of MD-IGF-I cells to various steroids in the absence of DEX showed that 17β-estradiol, progesterone, and testosterone, did not stimulate cell growth (Figure 5). Conversely, hydrocortisone, corticosterone, and aldosterone triggered a 1.7-, 1.5-, and 1.3-fold increase in cell proliferation compared with DMEM ($P<.01$). Not surprisingly, total DNA in the presence of 0.1 μM DEX was on average 1.7-fold higher ($P<.01$) than that of control DMEM treated cells.

To provide direct evidence that the faster growth rate of MD-IGF-I cells induced with DEX was in fact mediated by secretion of recombinant IGF-I, MD-IGF-I and MAC-T cells were cultured for 192 h, in the presence or absence of lactogenic hormones. Table 1 shows that secretion of IGF-I by MD-IGF-I cells was significantly stimulated by DEX, and ranged from 4.26 ng/ml, at 48 h, to 3.30 ng/ml, at 192 h. In contrast, I and PRL, alone or in combination, compared

with DMEM had no significant effect on secretion of IGF-I at any time period.

Compared with DMEM, I and DEX, alone or in combination, stimulated a 1.7- to 2.0-fold increase in total DNA (Figure 6A), whereas PRL did not influence cell proliferation. To account for these differences in cell number, concentrations of IGF-I were normalized for total DNA (μg/well), measured at the end of the incubation period, and expressed as pg IGF-I/μg DNA (Figure 6B). These data demonstrated that the DEX-enhanced secretion of IGF-I (348.9 pg/μg DNA) by MD-IGF-I cells, compared with DMEM (11.8 pg/μg DNA) was not biased by differences in cell number at the end of the incubation period, but rather was the consequence of increased IGF-I expression driven by the MMTV-LTR. Also, although DEX and I both effectively stimulated proliferation of MD-IGF-I cells, I had no appreciable capacity to alter secretion of IGF-I with or without DEX. Conversely, secretion of IGF-I by MAC-T cells was not affected by any hormone treatment, at any time period, and ranged from 5.0 pg/μg DNA at 48 h, to 10 pg/μg DNA at 192 h (data not shown).

To test for effects of cell-substratum interactions on activity of the MMTV-LTR, MAC-T and MD-IGF-I cells were seeded on plastic or EHS in the presence or absence of lactogenic hormones (Table 2). Little or no IGF-I was

detected in media, when control MAC-T and MD-IGF-I cells were cultured on plastic or EHS in DMEM alone. The addition of 0.1 μM DEX or the combination of DEX with 1 μg/ml PRL, however, caused a significant ($P<.01$) 25.5- and 13.9-fold increase, respectively, in IGF-I (278.2 and 151.1 pg/μg DNA) secreted into media conditioned by MD-IGF-I cells compared with DMEM (10.9 pg/μg DNA). More interestingly, secretion of IGF-I by MD-IGF-I cells was stimulated an additional 2.5-and 3.5-fold (529.8 and 689.1 pg/μg DNA) on EHS in the presence of DEX or DEX plus PRL, respectively, and 63.2-fold (689.1 vs 10.9 pg/μg DNA), compared with MD-IGF-I cells cultured in DMEM on plastic.

The stimulatory role of EHS and lactogenic hormones on the MMTV-LTR in MD-IGF-I cells is depicted in Figure 7. Not unexpectedly, both cell types were stimulated to proliferate in the presence of I on either plastic (Figure 7A) or EHS (Figure 7B). However, proliferation associated with the addition of DEX in MD-IGF-I cells was largely masked by the addition of I. The induction with I plus DEX, or with I plus DEX and PRL, caused a sharp 8.6- and 5.9-fold increase in IGF-I (225.0 and 156.0 pg IGF-I/μg DNA) compared with MD-IGF-I (26.3 pg/μg DNA) in the presence of I alone for cells cultured on plastic (Figure 7C). The induction was even greater for cells cultured on EHS, i.e. a 29.0- to 26.0-fold increase in IGF-I (592 and

531 pg/μg DNA) compared with MD-IGF-I cells with I alone (20.6 pg/μg DNA) (Figure 7D). MAC-T cells secreted minimal amounts of IGF-I irrespective of hormonal treatment or substratum (data not shown).

When plated on plastic, MD-IGF-I cells assumed a monolayer organization with a generally polygonal shape and centrally located nuclei (Figure 8A). Conversely, after 24 h on EHS, complexes of cells oriented into sheets or raised clumps were visible. The cells appeared to be associated in areas of EHS loosely detached from the surface of the culture dish, such that when viewed from underneath, the gels had multiple rounded patches of bare plastic without cells or a two-dimensional "swiss cheese-like" appearance. After 48 h in culture, rounded, spherical groups of cells were also routinely observed among patches of cells between areas of bare plastic (Figure 8B).

DISCUSSION

The mouse mammary tumor virus-long terminal repeat has been previously used to study gene expression regulation by glucocorticoids in mammalian systems (Ross and Solter, 1985; Stewart et al., 1988). Although the MMTV is primarily expressed in the mammary gland in response to

glucocorticoids, other placental and pituitary hormones are important in controlling the transcriptional activity of the HRE in vivo (Bolander, 1991). In this study, the MD-IGF-I cell line was used as an in vitro model to study regulation of the MMTV-LTR driving expression of IGF-I, in response to various hormonal and substratum stimuli. Transient transfection of MAC-T cells indicated that the MMTV-LTR was indeed inducible by glucocorticoids (Figure 1). In contrast, estrogen and progesterone showed no stimulatory effects on the activity of the MMTV-LTR.

While EGF was required to enhance the mitogenic effects of IGF-I in mouse mammary epithelial cells (Imagawa et al., 1986), in this study, neither EGF alone or in combination with IGF-I, stimulated additional cell growth as compared to IGF-I or DEX treated cells. The failure of EGF to stimulate cell proliferation may be in accordance with previous observations suggesting that EGF may be more important in supporting the development of the lobulo-alveolar differentiated phenotype in the presence of other growth factors and extracellular matrix in rodents (McGrath et al., 1985). Expression of mRNA transcripts for EGF and TGF-α vary with respect to stage of development of the rodent mammary gland. Messenger RNA for both EGF and TGF-α were detected in mammary tissue from virgin and pregnant mice, but only EGF mRNA was expressed during

lactation (Snedeker et al., 1991).

Increased EGF receptor levels in uterine membranes following administration of 17β-estradiol to immature female rats has been previously reported (Mukku and Stancel, 1985). This was also correlated with increased tissue DNA synthesis (Stancel et al., 1990). In this study, however, pretreatment of the MD-IGF-I cells for 96 h with 0.1 or 1.0 μM progesterone and 0.1 or 1.0 μM 17β-estradiol, did not enhance growth of these cells in response to subsequent treatment with EGF, I, or progesterone (data not shown). Therefore, it is possible that the lack of growth stimulation by EGF, progesterone, or 17β-estradiol, may reflect some uncoupling of the sex steroid and EGF receptor pathway, as previously reported for the developing mouse mammary gland (Snedeker et al., 1991).

Essentially, little is known about the role of EGF or TGF-α in mammary development in ruminants. Collier and McGrath (1988) showed that infusion of EGF or EGF plus IGF-I into the teat canal increased udder growth in beef cows, but infusion of EGF into teats of pregnant ewes was without apparent effect on udder development or subsequent milk production. Finally, a TGF-α transcript has been detected in mammary tissue from pregnant cows after amplification with polymerase chain reaction, but no mRNA for EGF was detected (J.C. Byatt, personal comunication).

Neither growth hormone nor PRL were capable of stimulating cell proliferation of MD-IGF-I cells cultured on plastic (Figure 2). The failure of GH to stimulate a mitogenic response can be explained by the known absence of cell receptors for GH in bovine mammary epithelial cells, as measured by ligand binding analysis (Akers, 1985). As PRL did not stimulate the growth of MD-IGF-I cells, this suggests that the MMTV-LTR promoter may not be inducible by PRL alone in bovine mammary epithelial cells. Therefore, the data presented in this study support previous observations that effects of PRL on the LTR element of the MMTV may be indirect, and largely a function of the differentiation status of mammary epithelial cells (Munoz and Bolander, 1989).

Proliferation of MD-IGF-I cells was stimulated by exogenous IGF-I at concentrations above 1 ng/ml, with concentrations of 10 or 100 ng/ml giving similar responses (Figure 3). This was in agreement with data previously reported for baby hamster kidney fibroblasts (BHK-21) (Blum et al., 1989) and clonal MAC-T bovine mammary epithelial cells (Zhao et al., 1992) which were used to generate the MD-IGF-I cells. Addition of DEX triggered a similar growth response, due to induction of the MMTV-LTR, equivalent to that seen in the presence of 10 ng/ml of exogenous IGF-I. In support, we have previously reported that cell

proliferation of the parent bovine mammary epithelial MAC-T cells was not stimulated by DEX (Romagnolo et al., 1992).

MD-IGF-I cells were sensitive to increasing levels of I (Figure 3), although concentrations greater than approximately 2 ng/ml are clearly supraphysiological for cattle (Barnes et al., 1985; Denbow et al., 1986). Likely, this effect was due to cross-reaction of I with the type I receptor for IGF-I (Forsyth, 1989).

Growth of MD-IGF-I cells was stimulated by exogenous TGF-α, thus suggesting a role for this growth factor as an autocrine mediator of cell growth in the mammary gland. Nevertheless, this raised the question of why MD-IGF-I cells responded to TGF-α, but were not sensitive to EGF. Because TGF-α activity was coupled to the presence of functional EGF receptors (Heldin and Westermark, 1989), it is possible that EGF receptors were present in MD-IGF-I cells, but the proliferative stimulus of EGF in bovine mammary epithelial cells may be mediated by other factors. In contrast, TGF-β did not stimulate cell growth, and at higher levels showed an inhibitory effect. A negative modulatory role of TGF-β on cell growth has been previously reported in MCF-7 human breast cancer cells (Knabbe et al., 1987) and in mouse mammary glands (Daniel et al., 1989).

Characterization studies of the HRE of the MMTV-LTR promoter (Ham et al., 1988) have shown that four inverted

repeats of the sequence 5′-TGTTCT-3′ located between -202 and -59 base pairs upstream of the initiation of transcription were targets for binding by sex steroid, mineralaldocorticoid, and glucocorticoid receptors. In this study, in accordance with the lack of stimulation of CAT activity in transiently transfected MAC-T cells, estrogen and progesterone showed no effects on cell proliferation of MD-IGF-I cells. However, a growth response by MD-IGF-I cells was measured in response to hydrocortisone, corticosterone, and dexamethasone (Figure 5). Therefore, one could ask the question of whether functional receptors were present for estrogen and progesterone, or if activity of the MMTV-LTR was regulated by trans-acting factors in a cell type specific manner (Cato et al., 1988, Langer and Ostrowski, 1988). Irrespective of possible effects of estradiol or progesterone on activity of the MMTV-LTR in these cells, it is possible that these steroids could stimulate cell proliferation by other mechanisms. For example, estradiol stimulated proliferation of MCF-7 breast cancer cells, but the effects were much more apparent when cultures were in media without phenol red. In support, it has been shown that the estrogen-like activity of phenol red could mask the effects of estradiol (Lippman and Dickson, 1989). In this regard, we (Woodward et al., 1992) have previously shown, however, that neither estradiol nor

progesterone affected growth of the MAC-T cells used in generation of the MD-IGF-I cells, regardless of the presence or absence of phenol red, serum, or pretreatment with progesterone prior to addition of estradiol. Thus, it was not surprising that the MD-IGF-I cells also, did not exhibit a proliferative response to these steroids, at least independent of MMTV-mediated effects.

Most recently, a role for tissue-specific enzymes in modulating interactions between steroids and specific receptors has been suggested (Stewart and Sheppard, 1992). This could explain the failure of 17β-estradiol in inducing transcription at the MMTV-LTR promoter in human mammary tumor T47D cells (Cato et al., 1987) as well as in our MD-IGF-I cells. Similarly, no effects on MMTV binding protein levels was seen in mouse mammary gland explant cultures in response to estradiol (Bolander, 1991).

The ability of the MD-IGF-I cells to proliferate at a faster rate when induced with DEX, was depicted in Figures 2, 5, and 6. Analysis of these data supported the observation that, while I and DEX, alone or in combination, markedly stimulated cell proliferation, only cells cultured with DEX exhibited stimulated secretion of IGF-I. Therefore, it was possible to conclude that the increased proliferation of MD-IGF-I cells, induced with DEX, was a direct consequence of the enhanced expression of IGF-I,

whose secretion was stimulated in a cell number-independent fashion (Fig. 6). These data are in accordance with observations previously reported (Romagnolo et al., 1992).

Direct confirmation that the induction of the MMTV-LTR with DEX was responsible for the enhanced secretion of IGF-I by MD-IGF-I cells is summarized in Table 1. In addition, combination of the lactogenic hormones and extracellular matrix derived from the Engelbreth-Holm-Swarm tumor, further stimulated secretion of IGF-I. These findings were in accordance with studies on the coordinate role of EHS and lactogenic hormones on expression of casein genes in primary mouse mammary tumor cells in vitro (Chou et al., 1989). Similarly, our data suggest that expression of the MMTV in MD-IGF-I cells may be controlled by similar regulatory mechanisms that drive expression of casein gene(s) in mammary epithelial cells during proliferation and differentiation. Finally, it is reasonable to conclude that the enhanced secretion of IGF-I in the presence of DEX, stimulated growth of MD-IGF-I cells on the EHS matrix (Figure 7). This is also supported by evidence for stimulated growth of both MAC-T and MD-IGF-I cells cultured on EHS in the presence of I.

The morphological changes of MD-IGF-I cells, cultured on EHS, served to strengthen our belief that the MD-IGF-I and parent MAC-T cells exhibited morphological characteristics

which support their use as models to study mammary epithelial cell development. Specifically, these cells displayed morphogenic responses on EHS gel cultures, which essentially mimicked the developmental patterns previously described for parent MAC-T cells (Huynh et al., 1991), mammary organoids derived from calves (Shamay and Gertler, 1986), or pregnant cows (Akers, 1990). Moreover, development of the cells on EHS was virtually identical with histological data for cultures of primary murine mammary cells (Barcellos-Hoff et al., 1989; Aggeler et al., 1991).

As previously shown for milk protein regulatory elements, this study provided evidence that activity of the MMTV-LTR was coordinately regulated by lactogenic hormones and extracellular matrix, and may be used as a regulatory element to target expression of foreign genes to the mammary gland. It is suggested that the cell line MD-IGF-I may prove a useful in vitro system to investigate mammary epithelial specific regulatory mechanisms important in controlling activity of the MMTV-LTR promoter.

SUMMARY

The cell line MD-IGF-I, containing an ovine IGF-I cDNA driven by the mouse mammary tumor virus-long terminal repeat (MMTV-LTR) promoter, was used to study expression of IGF-I in bovine mammary epithelial cells in response to various hormonal and substratum stimuli. Acute sensitivity of the MMTV-LTR promoter to glucocorticoids and sex steroids was ascertained by transient transfection of parental MAC-T cells with an MMTV-CAT construct. Specifically, CAT activity was induced by glucocorticoids, but not by 17β-estradiol or progesterone. Induction of MD-IGF-I cells with dexamethasone (DEX) alone triggered a 29.5-fold increase in secretion of recombinant IGF-I (348.9 vs 11.8 pg/μg DNA), and stimulated a 1.7-fold increase in total DNA within 72 h. Growth of MD-IGF-I cells was enhanced by exogenous IGF-I, insulin (I), and TGF-α. In contrast, TGF-β inhibited cell proliferation, while epidermal growth factor, prolactin (PRL), estrogen, progesterone, and testosterone had no effect. Extracellular matrix from the Engelbreth-Holm-Swarm (EHS) tumor, in the presence of DEX, prolactin (PRL), and I stimulated a 29.4-fold increase in secretion of IGF-I (591.9 pg/μg DNA), compared with cells in absence of hormones (20.1 pg/μg DNA). EHS and DEX plus PRL triggered a 63.2-fold increase

in IGF-I secretion (689.1 pg/μg DNA), compared with MD-IGF-I cells cultured on plastic (10.9 pg/μg DNA), in the absence of hormones. These data indicate that the MMTV-LTR is regulated by both lactogenic hormones and extracellular matrix in MD-IGF-I cells and that the MMTV-LTR may be a useful regulatory element for targeting expression of foreign proteins to bovine mammary epithelial cells.

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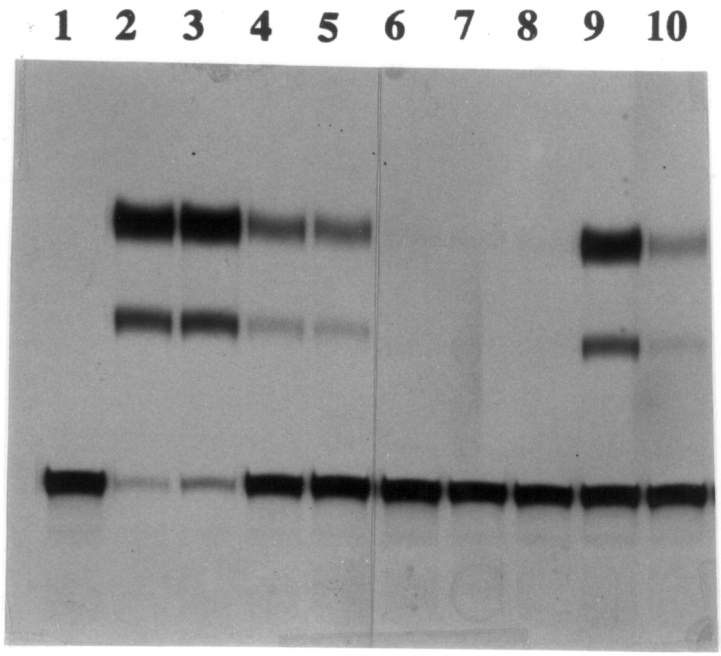

Figure 1. MMTV-LTR promoter activity in transiently transfected MAC-T cells. MAC-T cells were transiently transfected at 85% confluency and cell extracts prepared as described in Materials and Methods. Autoradiogram represents CAT activity of: MAC-T cell extracts (lane 1); positive controls (0.1, 0.05, 0.01, 0.005 units of purified CAT enzyme) (lanes 2-5); MAC-T cells transfected with pMSGCAT not induced (lane 6), or induced with 17β-estradiol (lane 7), progesterone (lane 8), or dexamethasone (lane 10), and MAC-T cells transfected with pRSVCAT (lane 9).

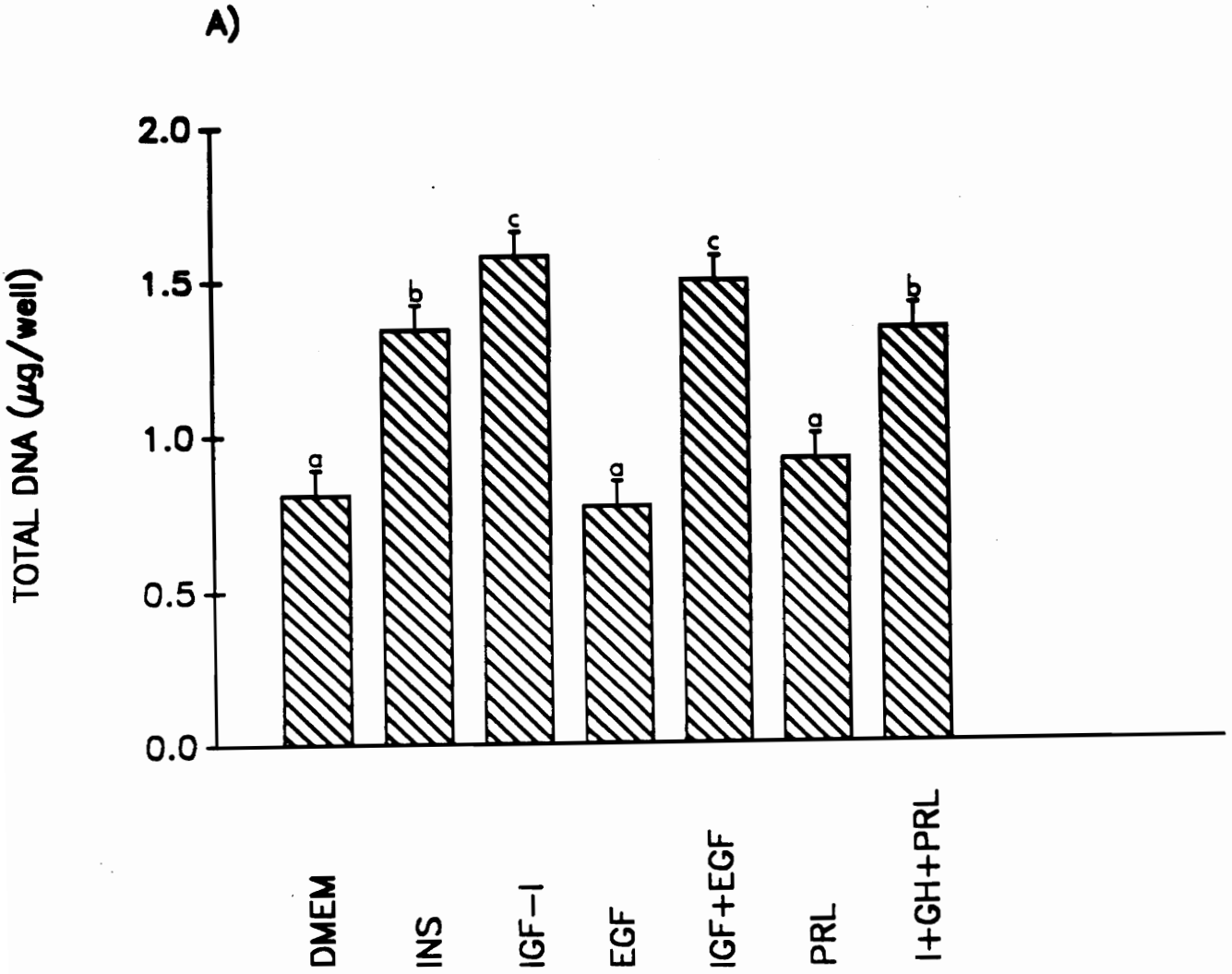

Figure 2A. Growth response of MD-IGF-I cells to various protein mitogens. MD-IGF-I cells were seeded in 24-well plates and cultured in DMEM in the presence of insulin (I) (100 ng/ml), IGF-I (100 ng/ml), EGF (100 ng/ml), IGF-I plus EGF (100 ng/ml), prolactin (PRL) (1 μg/ml), insulin, growth hormone (GH)(1 μg/ml), and prolactin. Bars are total DNA (μg/well) and represent the mean±SEM values from six samples, assayed in duplicate. Bars marked with different symbols were significantly ($P<.05$) different by Duncan's multiple comparison test.

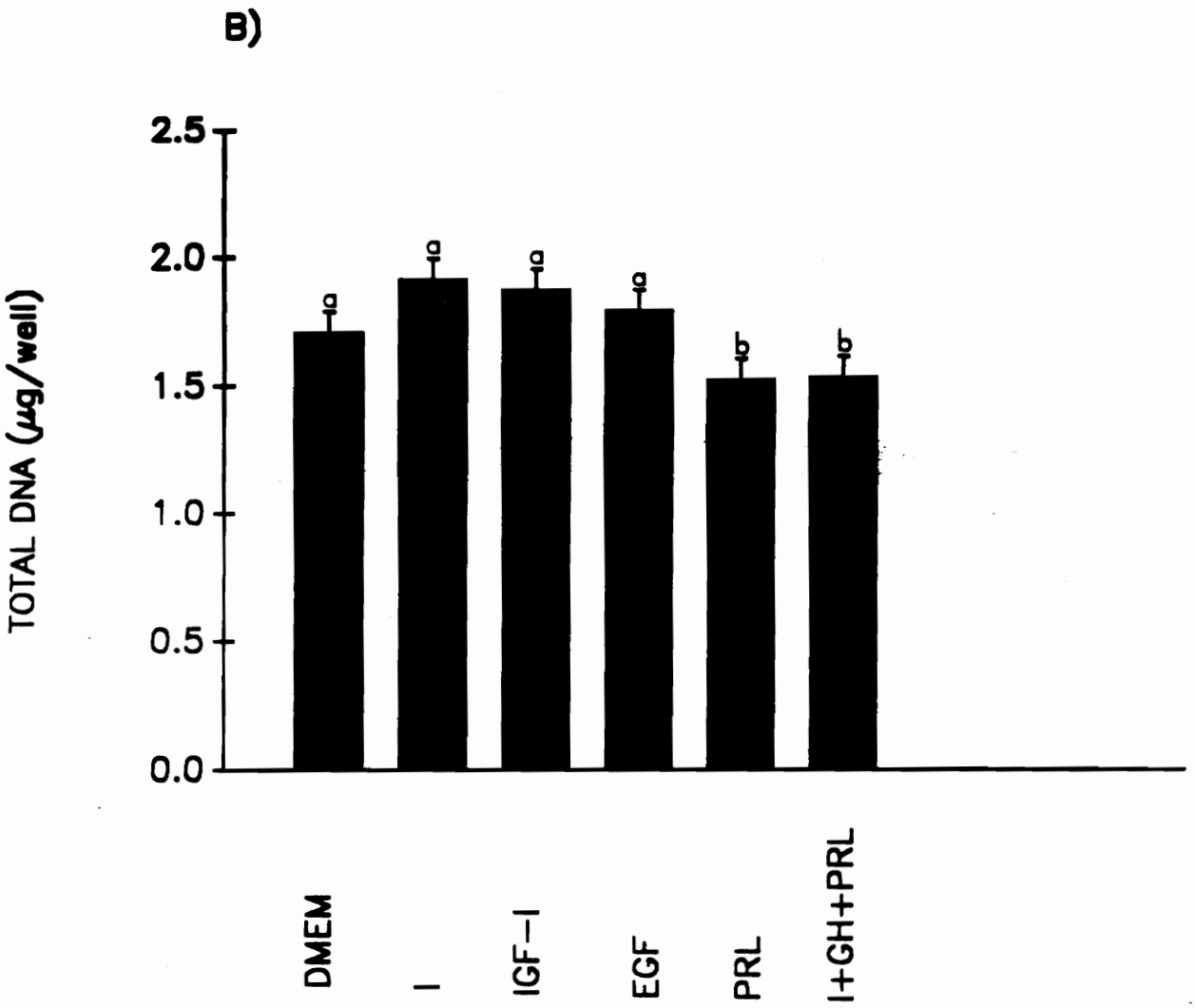

Figure 2B. Growth response of MD-IGF-I cells to various proteins mitogens in the presence of 0.1 μM dexamethasone.

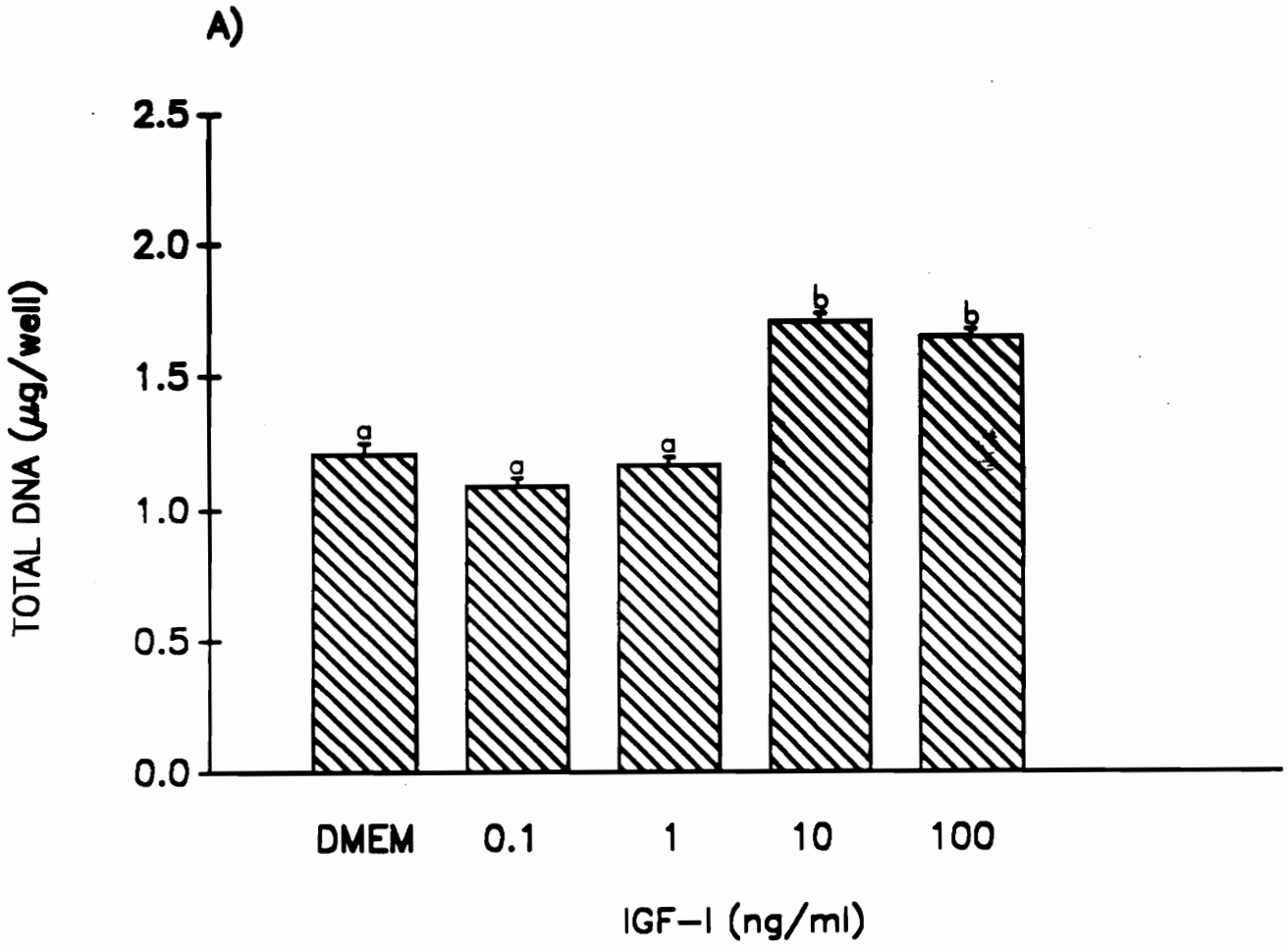

Figure 3A. Effects of various concentrations of IGF-I on growth of MD-IGF-I cells. MD-IGF-I cells were seeded in 24-well plates and cultured in DMEM in the presence of increasing concentrations of IGF-I (0.1 to 100 ng/ml) for 72 h. Bars are total DNA (μg/well) and represent the mean$\pm$SEM values from six samples, assayed in duplicate. Bars with different symbols were significantly ($P<.05$) different by Duncan's multiple comparison test.

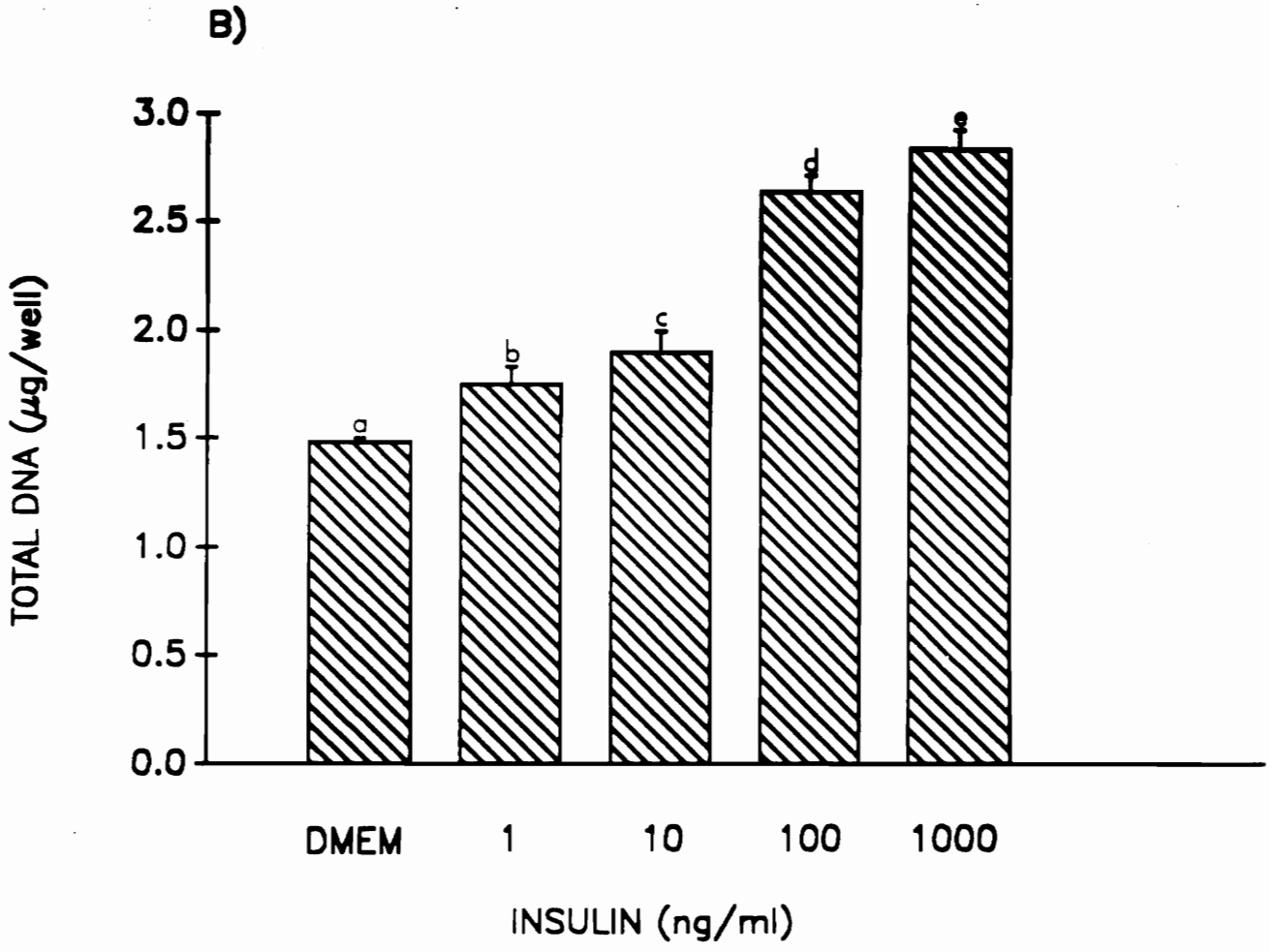

Figure 3B. Effects of various concentrations of insulin on growth of MD-IGF-I cells.

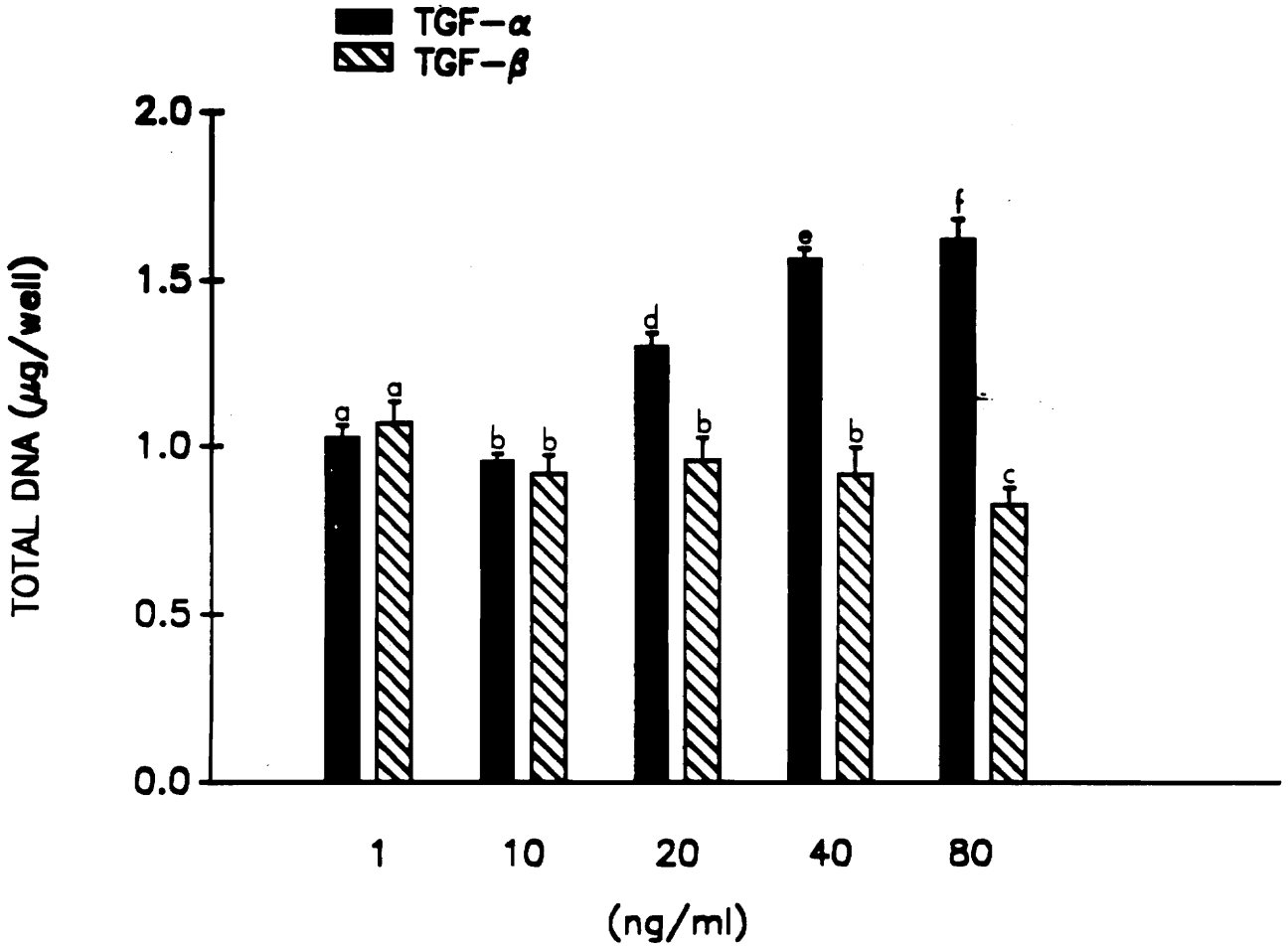

Figure 4. Growth response of MD-IGF-I cells to TGF-α and TGF-β. MD-IGF-I cells were seeded in 24-well plates and cultured in DMEM and in the presence of increasing concentrations of TGF-α and TGF-β for 72 h. Bars are total DNA (μg/well) and represent the mean$\pm$SEM values from six samples, assayed in duplicate. Bars with different symbols were significantly ($P<.05$) different by Duncan's multiple comparison test.

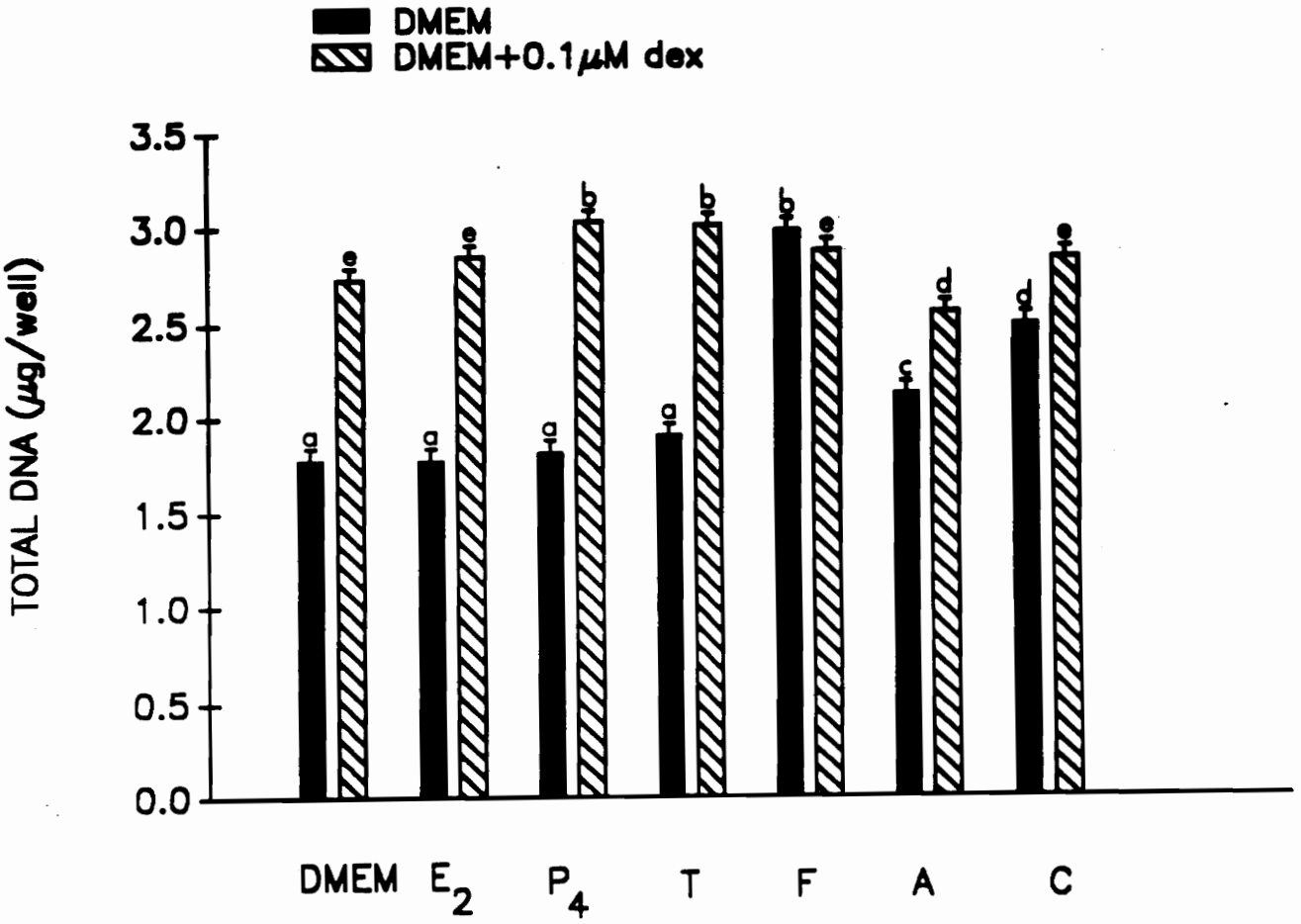

Figure 5. Effects of various steroids on growth of MD-IGF-I cells. MD-IGF-I cells were seeded in 24-well plates and cultured in DMEM and in the presence of equimolar concentrations (0.1 μM) of one of the following steroids: 17β-estradiol (E_2), progesterone (P_4), testosterone (T), hydrocortisone (F), aldosterone (A), corticosterone (C) for 72 h. Bars are total DNA (μg/well) and represent the means$\pm$SEM values from six samples, assayed in duplicate. Bars with different symbols were significantly ($P<.05$) different by Duncan's multiple comparison test.

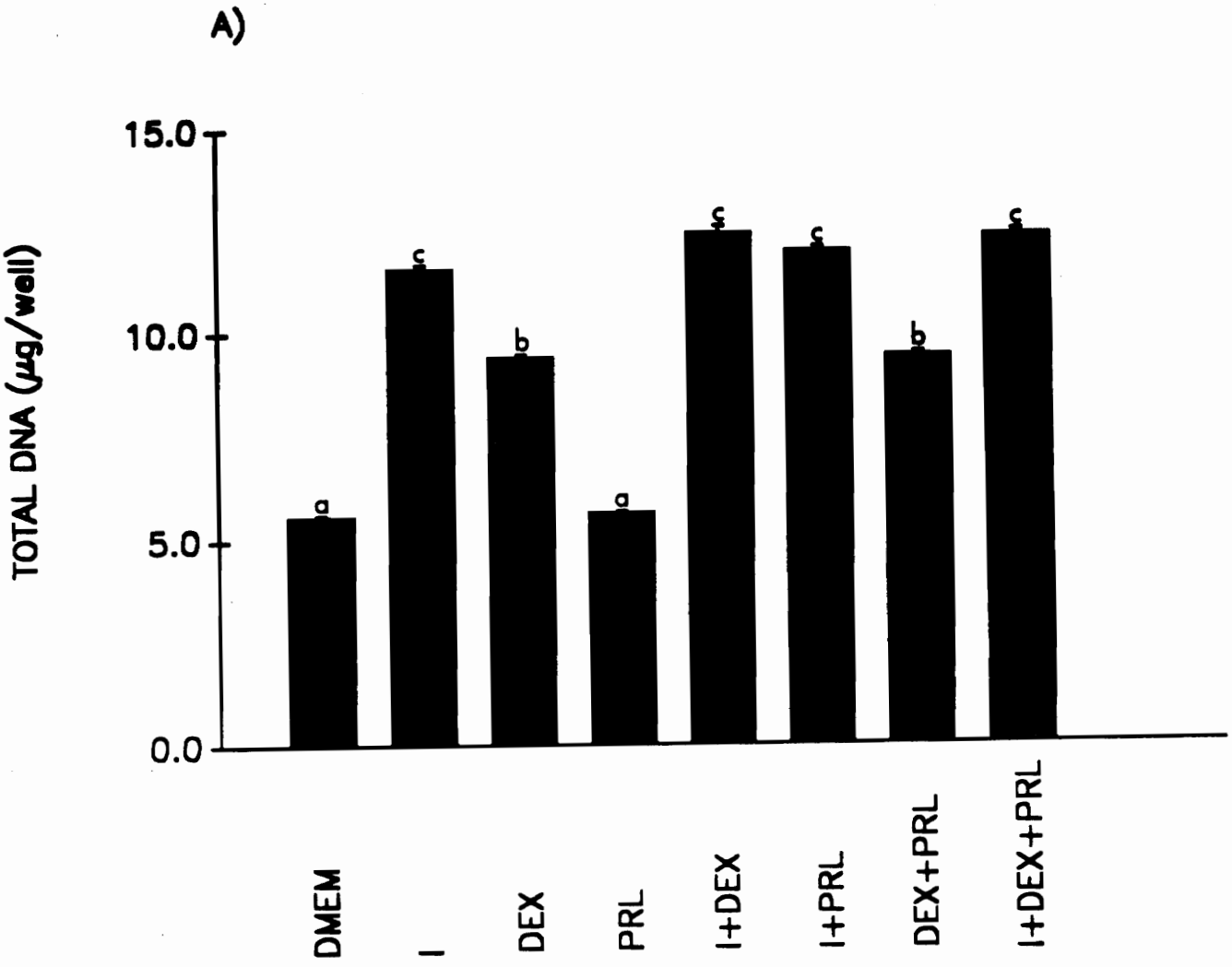

Figure 6A. Effects of lactogenic hormones on growth of MD-IGF-I cells. Cells were seeded in 24-well plates and cultured in DMEM in the presence of 0.1 μM dexamethasone (DEX), 1.0 μg/ml insulin (I), 1.0 μg/ml prolactin (PRL), or their combinations for 192 h, with a media change every 48 h. Bars are total DNA (μg/well) at the end of the incubation period, and represent the mean$\pm$SEM of six wells assayed in duplicate. Bars with different symbols were significantly ($P<.05$) different by Duncan's multiple comparison test.

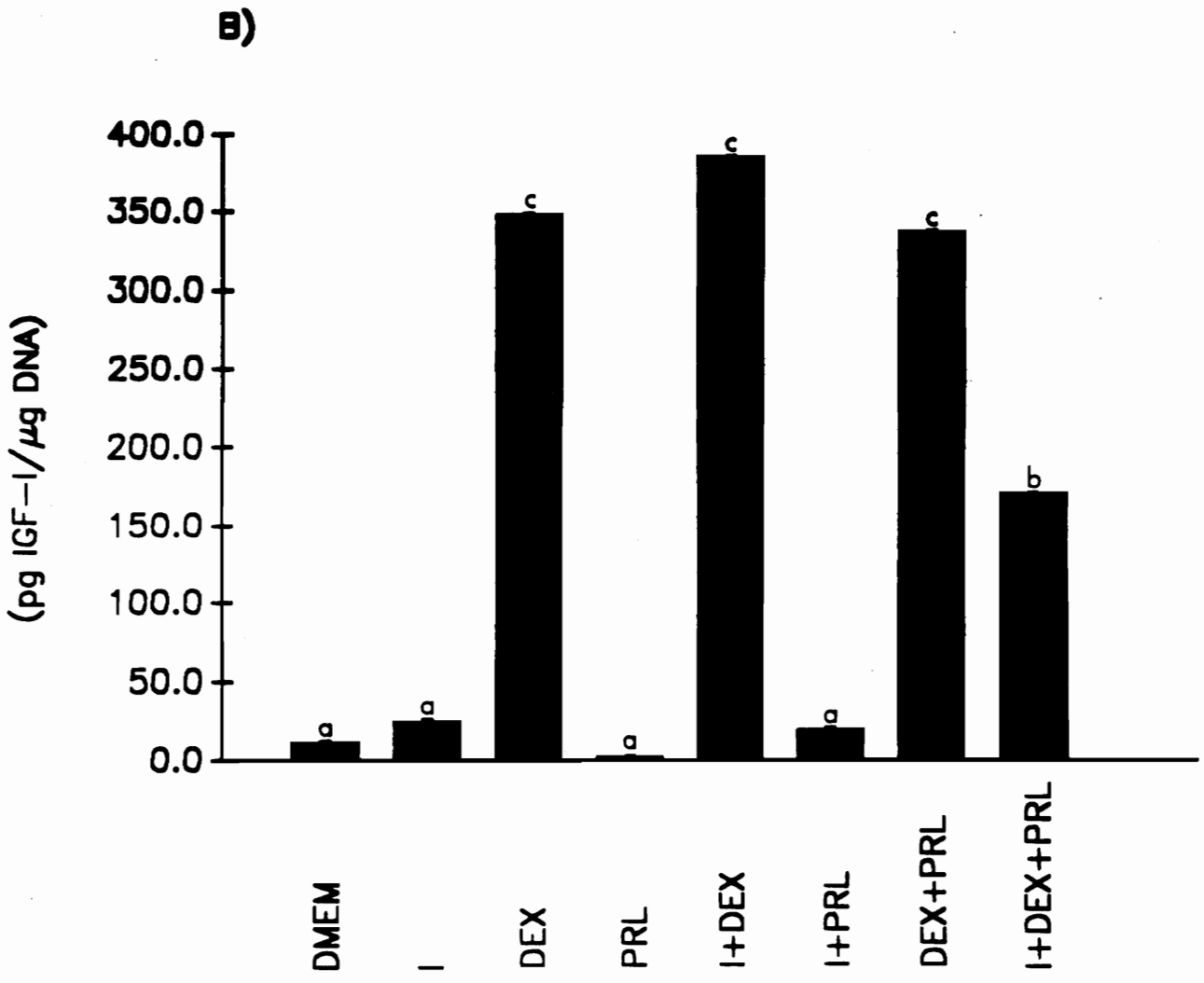

Figure 6B. Effects of lactogenic hormones on secretion of IGF-I by MD-IGF-I cells.

TABLE 1. EFFECTS OF LACTOGENIC HORMONES ON SECRETION OF IGF-I BY MD-IGF-I CELLS

Treatment[1]	Time (h)				pooled mean SEM
	48	96	144	192	
	IGF-I (ng/ml) in medium				
DMEM	0.43	0.30	0.32	0.10	0.03
I	0.53	0.25	0.16	0.29	0.04
DEX	4.26[a]	4.70[a]	3.75[a]	3.30[a]	0.12
PRL	0.37	0.08	0.10	0.09	0.02
I + DEX	4.83[a]	6.10[b]	5.11[b]	4.82[b]	0.12
I + PRL	0.54	0.52	0.33	0.24	0.03
DEX + PRL	4.81[a]	5.50[b]	4.10[a]	3.20[a]	0.13
I + DEX + PRL	4.39[a]	6.60[b]	5.26[b]	2.80[a]	0.09

[1]Data represent the mean of three samples assayed in duplicate. Values within a column with different superscripts were significantly ($P<.05$) different by Duncan's multiple comparison test.

TABLE 2. CELL SUBSTRATUM REGULATION OF MMTV-LTR IGF-I EXPRESSION

Treatment[1]	Plastic				EHS			
	MAC-T		MD-IGF-I		MAC-T		MD-IGF-I	
	IGF-I (pg/μg DNA) in medium							
	mean	SE	mean	SE	mean	SE	mean	SE
DMEM	n.d.	-	10.9[a]	0.9	20.7[a]	0.8	47.3[b]	5.5
DMEM+DEX	n.d.	-	278.2[d]	5.1	21.2[a]	1.6	689.1[f]	9.9
DMEM+DEX+PRL	n.d.	-	151.1[c]	8.4	64.4[b]	5.7	529.8[e]	8.3

[1]Values are pg IGF-I/μg DNA and represent the mean±SEM from two separate experiments. Values within a column with different superscripts were significantly (P<.05) different by Duncan's multiple comparison test.

[2]Not detectable.

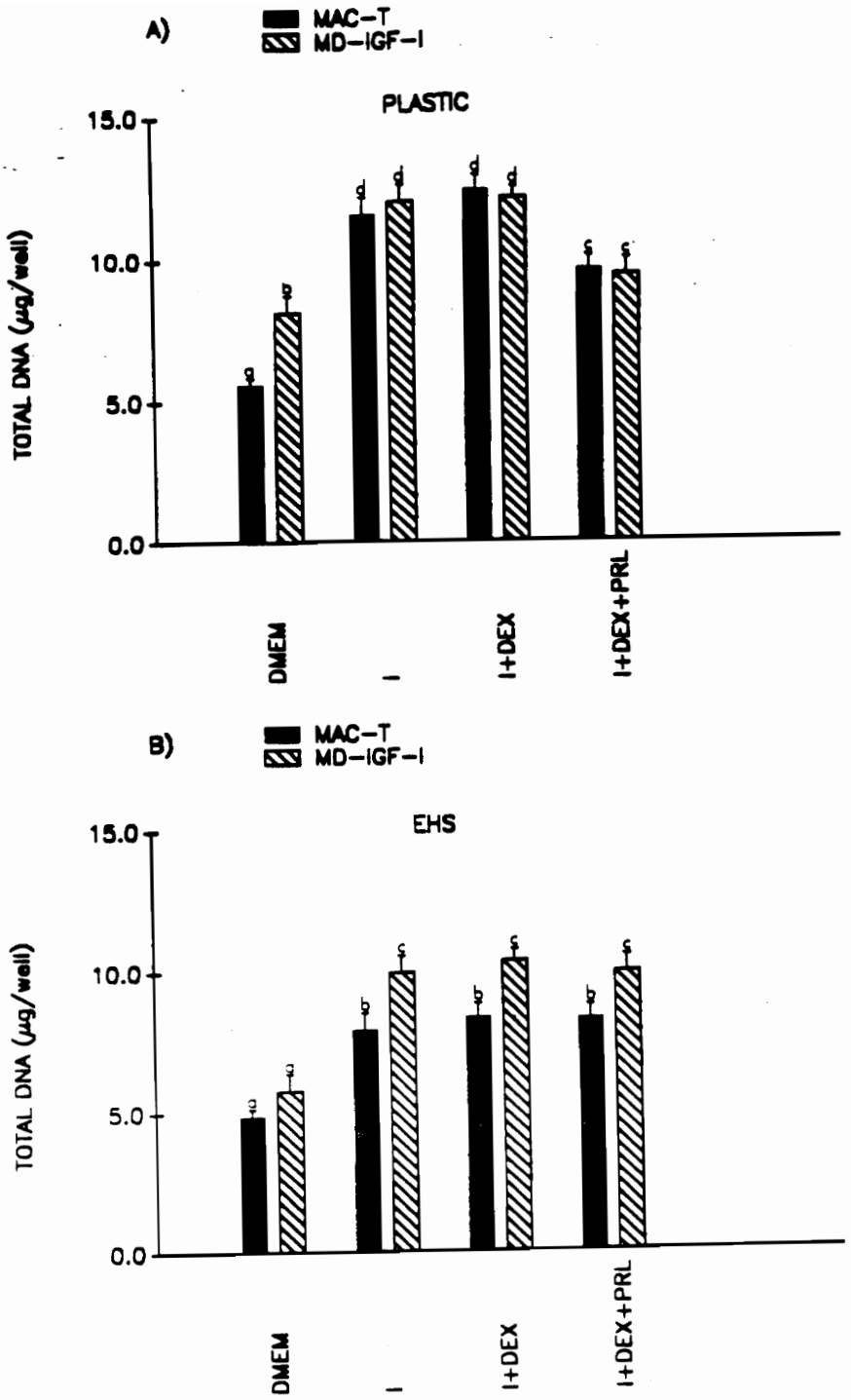

Figure 7A, B. Hormonal induction of MMTV-LTR IGF-I expression in MD-IGF-I cells. Cells were seeded on 60 mm plates on plastic or extracellular matrix derived from the Engelbreth-Holm-Swarm tumor (EHS) and cultured for 4 days in DMEM supplemented with 10% FCS with a media change after 48 h. Thereafter, cells were cultured in serum-free DMEM or DMEM supplemented with 1 μg/ml insulin (I), I and 0.1 μM dexamethasone (DEX), or I, DEX, and 1 μg/ml prolactin (PRL). Bars are total DNA (μg/well) and represent the mean$\pm$SEM from two separate experiments. Bars with different symbols were significantly different ($P<.05$) by Duncan's multiple comparison test.

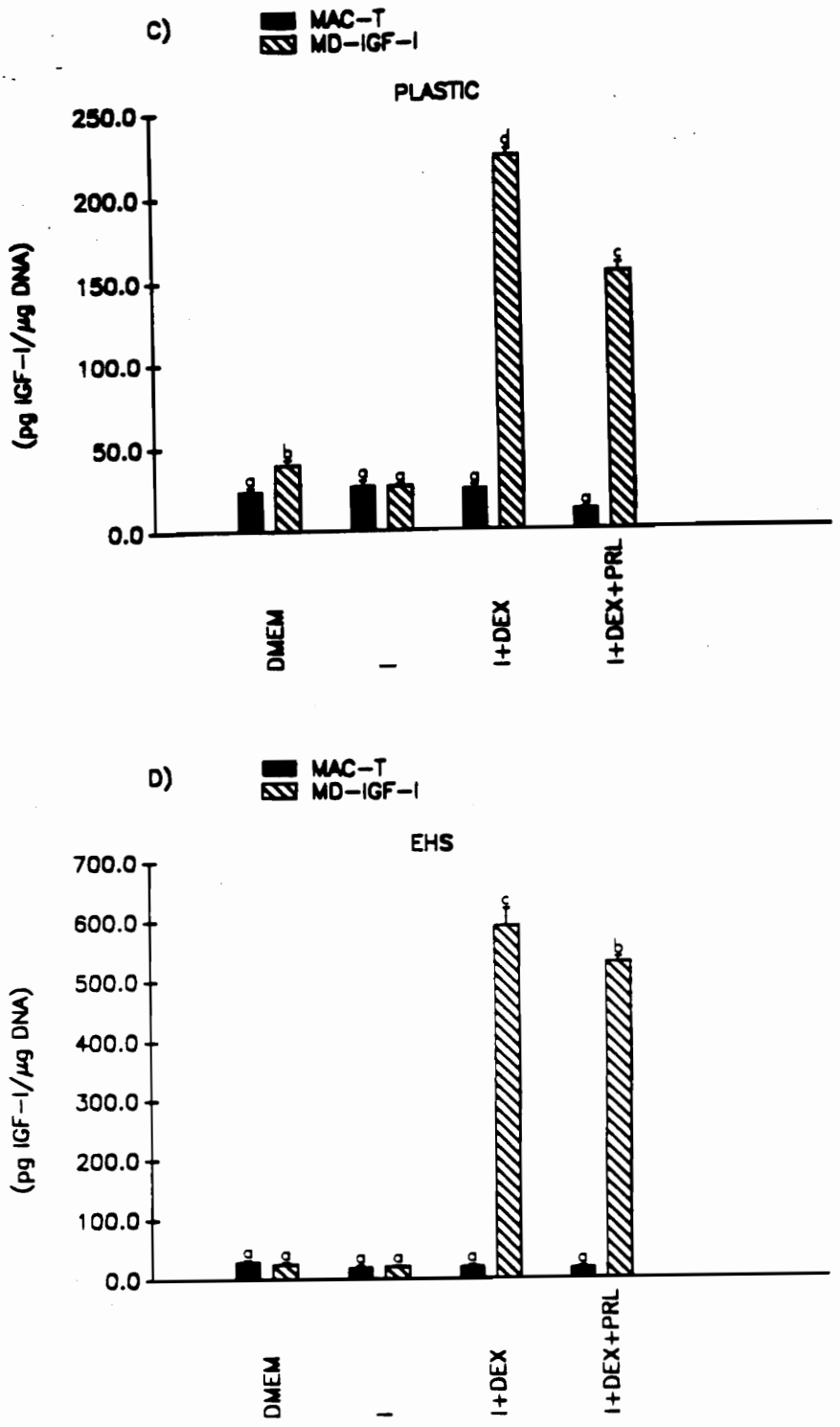

Figure 7C, D. Hormonal induction of MMTV-LTR IGF-I expression in MD-IGF-I cells. Bars are pg IGF-I/μg DNA and represent the mean$\pm$SEM from two separate experiments. Bars with different symbols were significantly different ($P<.05$) by Duncan's multiple comparison test.

Figure 8A. Microscopic appearance of MD-IGF-I cells. In each of the examples, cells were cultured in DMEM supplemented with 10% FCS and in the presence of 1 μg/ml insulin, 0.1 μM dexamethasone, and 1 μg/ml prolactin. Fig. 8A illustrates cells 48 h following seeding onto a plastic culture dish. The cells are attached, flattened, and assume a generally polygonal shape (200x).

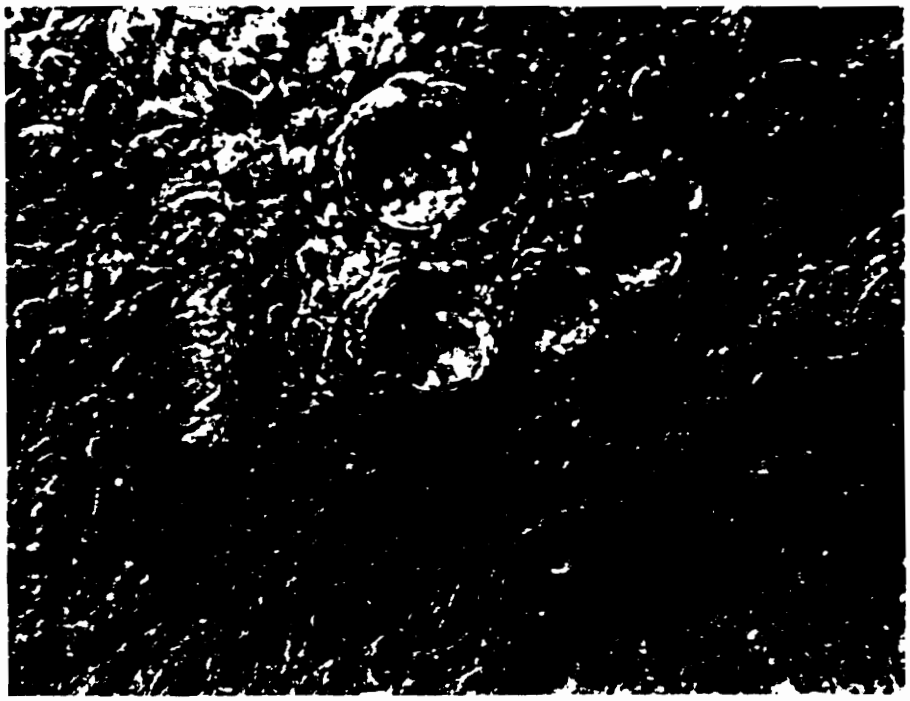

Figure 8B. Microscopic appearance of MD-IGF-I cells 48 h after plating onto Engelbreth-Holm-Swarm matrix. Several spherical clusters are evident within the monolayer of cells (100x).

CHAPTER 3

IGF-I-INDUCED IGFBP-3 POTENTIATES THE MITOGENIC ACTIONS OF IGF-I IN MAMMARY EPITHELIAL MD-IGF-I CELLS

INTRODUCTION

The role of insulin-like growth factor-I (IGF-I) as a mediator of growth hormone action and as a local autocrine/paracrine stimulator of cell growth for a variety of cell types is widely accepted (LeRoith and Roberts, 1991). In addition, the notion that the local mitogenic effects of IGF-I are modulated by interactions of the IGF-I peptide with its binding proteins (IGFBPs) is also well documented (Elgin et al., 1987; Ooi, 1990; Clemmons et al., 1992). Experimental evidence supports the concept that IGFBPs may play a dual role in mediating IGF-I actions: first, IGFBPs serve as a reservoir to potentiate the mitogenic effects of IGF-I by continuous release of the growth factor (Blum et al., 1989). Alternatively, IGFBPs may exert an inhibitory effect on IGF-I actions as previously indicated for human skin fibroblasts (DeMellow and Baxter, 1988), granulosa cells (Adashi et al., 1992), and MCF-7 cells (McGuire et al., 1992).

To date six IGFBPs have been cloned and their cDNAs sequenced (Sheikh et al., 1992). Studies of local cell growth regulation by IGFBPs in response to various hormonal stimuli, have shown that insulin decreased IGFBP-1 production by liver explants (Lewitt and Baxter, 1990). Conversely, the glucocorticoid dexamethasone stimulated local production of IGFBP-1 and IGFBP-2 by fetal liver in rats, whereas IGFBP-3 was decreased (Price et al., 1992). Furthermore, secretion of IGFBP-3 by human and bovine fibroblasts (Bale and Conover, 1992) was triggered by IGF-I, but only in the case of bovine fibroblasts was a significant increase in IGFBP-3 mRNA measured in response to stimulation with IGF-I. Therefore, these studies suggest that regulation of expression of IGFBPs may be hormonally controlled in a species-, tissue-, and cell-specific fashion.

Limited information is available concerning the molecular and cellular mechanisms that regulate expression of IGFBPs in mammary epithelial cells. Exogenous IGF-I stimulated secretion of both IGFBP-2 and IGFBP-3 in normal bovine mammary epithelial cells (McGrath et al., 1991). Nevertheless, it is unclear whether these locally produced IGFBPs alter the growth and development of the mammary gland through a negative and/or positive pathway.

To investigate whether endogenously produced IGF-I

affects growth of mammary epithelial cells through an autocrine/paracrine pathway, we have previously developed the cell line MD-IGF-I (Romagnolo et al., 1992) by transfecting clonal MAC-T cells (Huynh et al., 1991) with an IGF-I construct expressing an ovine exon-2 containing IGF-I cDNA (Ohlsen et al., 1993). The IGF-I cDNA was placed under the control of the glucocorticoid-inducible mouse mammary tumor virus-long terminal repeat (MMTV-LTR). In these cells, stimulation with the glucocorticoid dexamethasone (DEX) was required for high expression levels of IGF-I from the LTR promoter. Here, we report on the autocrine mechanisms of action of the recombinant IGF-I and hormonal regulation of expression of IGFBP-2 and -3 in parental MAC-T and transformed MD-IGF-I cells.

MATERIALS AND METHODS

IGF-I Constructs and Transfection. Details concerning the cloning strategy of a 0.7-kb ovine exon-2 prepro IGF-I cDNA downstream of the MMTV-LTR promoter and induction of MMTV-LTR directed IGF-I transgene expression by dexamethasone (DEX) (Sigma, St. Louis, MO) are described in detail elsewhere (Romagnolo et al., 1992). Briefly, expression of IGF-I by parental MAC-T and IGF-I transfected

cells was studied by culturing the cells in 100-mm tissue culture plates in serum-free Dulbecco's Modified Eagle Medium (DMEM) in the presence or absence of 0.1 μM DEX. After 72 h, cells were trypsinized and total RNA was extracted using a guanidinium thiocyanate procedure (Puissant and Houdebine, 1990). The RNA was separated on a 2.2 M formaldehyde-1% agarose gel and Northern blots were hybridized overnight, at 42^{o} C, with an IGF-I cDNA probe labeled by nick translation.

Changes in expression of IGFBP-3 were monitored by hybridizing Northern blots of total RNA (10 μg) with a rat IGFBP-3 probe, prepared by nick translation of a 440 bp XbaI-HindIII cDNA fragment isolated from plasmid rIGFBP-3 (Smith et al., 1990).

IGF-I radioimmunoassay and ligand blotting. Detection of media IGF-I was performed by radioimmunoassay, as previously described (Shimamoto et al., 1992). Briefly, 100 μl of media conditioned by parental MAC-T and transfected MD-IGF-I cells in the presence or absence of 0.1 μM DEX was extracted in 2 M formic acid/methanol (1:7, v/v) at 4^{o} C for 2 h. After samples were centrifuged and organic solvents removed by rotary vacuum evaporation, pellets were resuspended in RIA buffer (30 mM sodium phosphate, 10 mM EDTA, 0.02% protamine sulfate, 0.05% Tween-20, pH 8.0) and

duplicate aliquots included for assay.

Secretion of IGFBPs into conditioned media was studied by Western ligand blotting (Hossenlopp et al., 1986). Medium samples (200 μl) were concentrated by lyophilization and reconstituted in water. Then, samples were dissolved in non-reducing SDS-polyacrylamide gel (PAGE) sample buffer and separated overnight at constant current on a 12.5% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane, which was then incubated with [^{125}I]-IGF-I. Finally, autoradiography of the blots was carried out at -70^{o} C for 7-14 days with intensifying screens.

Cell proliferation and hormonal regulation of IGFBPs expression. A series of experiments were designed to study the apparent effects of secretion of recombinant IGF-I on cell proliferation and expression of IGFBPs by parental MAC-T and transfected MD-IGF-I cells in serum-free conditions. Cells were plated in DMEM or DMEM supplemented with 0.1 μM DEX, 15 ng/ml human IGF-I (IGF-I) (Boehringer Mannheim, Indianapolis, IN), DEX plus a monoclonal antibody against IGF-I (MAb) (lot 3D1/2/1, diluted 1:4000; obtained from the NHPP, Baltimore, MD) and IGF-I plus MAb (1:4000). Media were harvested after 72 h and assayed for presence of IGF-I by radioimmunoassay and IGFBPs by ligand blotting. Cell pellets were saved for extraction of total RNA for

Northern blot analysis of IGF-I and IGFBP-3 expression.

To investigate the modulatory role of IGF-I on expression of IGFBPs, transformed MD-IGF-I cells were cultured for 72 and 144 h in DMEM, in the presence or absence of 0.1 μM DEX or 100 ng/ml IGF-I. After 72 h, cells were either maintained on the same treatment for an additional 72 h, or alternatively, cells previously treated with DEX or IGF-I were switched to DMEM. At the end of the incubation periods, cells were harvested for immediate extraction of total RNA for Northern blot analysis of IGFBP-3, and conditioned media collected for analysis of IGF-I and IGFBPs by radioimmunoassay and ligand blotting, respectively.

Changes in total DNA for MAC-T and MD-IGF-I cells, and expression of IGFBPs in response to insulin and various IGF-I analogs were monitored by culturing the cells in DMEM or DMEM with increasing concentrations of insulin (I) (Sigma), IGF-I, Des(1-3)IGF-I (Gropep, Adelaide, Australia), and LongR^3IGF-I (Gropep) for 72 h. At the end of the incubation period, cells were harvested for measurement of total DNA (μg/well) (LaBarca and Paigen, 1980), and expression of IGFBP-3 by Northern and ligand blot analysis. Acute effects of I, IGF-I, and the IGF-I analogs on proliferation of MAC-T and MD-IGF-I cells were determined as described previously (Zhao et al., 1992).

Briefly, cells were serum starved for 72 h and exposed to hormones or growth factors for 18 h. Incorporation of[^{3}H]thymidine was evaluated during the last 2 h of culture.

Regulation of expression of IGFBPs by lactogenic hormones in MD-IGF-I cells was studied by plating the cells in serum-free DMEM, or DMEM supplemented with 1 μg/ml I, 0.1 μM DEX, 1 μg/ml bovine prolactin (PRL) (NHPP, Baltimore, MD) or in DMEM containing two and three-way combinations of the hormones. Cells were cultured for 192 h, with a media change every 48 h. At the end of the incubation period, cells were trypsinized and total DNA (μg/well) was used as an indicator of cell growth. Cell aliquots were saved for extraction of total RNA for further Northern analysis of IGFBP-3, and conditioned media analyzed for secretion of recombinant IGF-I and IGFBPs, by radioimmunoassay and ligand blotting, respectively.

Finally, to examine whether growth of transfected MD-IGF-I cells was stimulated by secretion of recombinant IGF-I through an autocrine loop in the presence of IGF-I-induced IGFBPs, parental MAC-T and transfected MD-IGF-I cells were cultured for 72 and 144 h in serum-free DMEM in the presence or absence of 0.1 μM DEX. Thereafter, test MAC-T cells were serum starved for 72 h, then cultured in conditioned media (CM) from both cell types, CM plus 10

ng/ml IGF-I, and CM supplemented with 10 ng/ml Des(1-3)IGF-I up to 16 h, and thereafter pulsed for 2 h with [^{3}H]thymidine. At the end of the incubation period, cells were harvested and [^{3}H]thymidine incorporation measured.

Statistical Analysis. Data are presented as mean±SEM. Effects of DEX on cell proliferation, IGF-I secretion, and [^{3}H]thymidine uptake, and influences of I and IGF-I analogs on cell growth of parental MAC-T and MD-IGF-I cells were evaluated by the analysis of variance procedure in SAS (SAS Institute Inc., Cary, NC, USA). Specific comparisons utilized Duncan's multiple range test with $P<.05$ selected as a level of significance.

RESULTS

Expression of IGF-I and IGFBP-3. Expression of IGF-I by parental MAC-T and transfected MD-IGF-I cells was investigated by culturing the cells in DMEM in the presence or absence of 0.1 μM DEX. Northern blot analysis of total RNA (Figure 1) showed that MAC-T cells expressed no detectable IGF-I mRNA species, whereas the presence of DEX

was required for high level expression of IGF-I from the MMTV-LTR contained in MD-IGF-I cells (Figure 1A). Probing of the same membrane with a nick-translated IGFBP-3 cDNA (Figure 1B) revealed that both the secretion of DEX-induced recombinant IGF-I, or the addition of exogenous IGF-I triggered the expression of IGFBP-3 mRNA. Likewise, secretion of IGFBP-3 (40-42 KDa) into conditioned media (Figure 1C) was also induced by IGF-I. However, the addition of DEX or IGF-I had no effect, as measured by Northern and ligand blotting, on expression of IGFBP-3 by MAC-T cells, which produced primarily IGFBP-2 (29-32 KDa). More importantly, the addition of a monoclonal antibody against IGF-I inhibited 14.0-fold the stimulatory effects of exogenous IGF-I on secretion of IGFBP-3.

Similarly, the induction of MD-IGF-I cells for 72 h with IGF-I (100 ng/ml) triggered the expression of IGFBP-3 mRNA (Figure 2A). This effect was detectable up to 144 h, but substitution of the IGF-I-containing media with basal DMEM, reduced the levels of IGFBP-3 mRNA. Interestingly, switching to DMEM after 72 h decreased mRNA levels for IGFBP-3 but had not effects on concentrations of IGFBP-3 in media. Consistent with these observations, ligand blot analysis (Figure 2B) of conditioned media from the same experiment confirmed the stimulatory role of both DEX-induced IGF-I and exogenous IGF-I on secretion of

IGFBP-3 from MD-IGF-I cells.

[^{3}H]thymidine incorporation and cell proliferation of MAC-T and MD-IGF-I cells. Increasing amounts of I, IGF-I, Des(1-3)IGF-I, and LongR^{3}IGF-I stimulated in a dose-dependent fashion [^{3}H]thymidine incorporation into DNA of MAC-T and MD-IGF-I cells. However, in all instances, [^{3}H]thymidine uptake by MD-IGF-I cells was higher than parental MAC-T cells. This difference in label incorporation was not due to differences in cell number at time of plating (data not shown) but reflected the ability of the MD-IGF-I cells to proliferate at a faster rate compared with parental MAC-T cells. The potency of the IGF-I analogs was in the order LongR^{3}IGF-I > Des(1-3)IGF-I > IGF-I (Figure 3). Specifically, [^{3}H]thymidine incorporation by MD-IGF-I cells was induced 1.8-fold (226 cpmx10^3/well) by LongR^{3}IGF-I at the concentration of 20 ng/ml and 1.6-fold (198 cpmx10^3/well) by Des(1-3) and IGF-I at concentrations of 80 ng/ml compared with control DMEM (126 cpmx10^3/well). [^{3}H]thymidine incorporation by MAC-T cells was also stimulated 2.1- and 1.9-fold by Des(1-3) (187 cpmx10^3/well) and LongR^{3}IGF-I (163 cpmx10^3/well) at concentrations of 80 ng/ml compared with control DMEM (86 cpmx10^3/well).

Insulin stimulated in a dose-dependent fashion cell

proliferation of both MAC-T and MD-IGF-I cells (Figure 4A) and at concentrations of 400 ng/ml total DNA was increased 1.5-(2.1 μg/well) and 1.5-fold (2.0 μg/well), respectively, compared with DMEM (1.3 μg/well). Moreover, both the Des(1-3)-and LongR^{3}IGF-I analogs, at concentrations of 40 ng/ml, stimulated a 1.7- (2.3 μg DNA/well) and 1.5-fold (2.1 μg/well) increase ($P<.01$) in cell proliferation of parental MAC-T (Figure 4B) and transfected MD-IGF-I (Figure 4C) cells, compared with control DMEM (1.3 μg/well). However, further addition of 0.1 μM DEX to the IGF-I analogs did not enhance additional growth response of MD-IGF-I cells (data not shown).

Ligand blot analysis. Interestingly, ligand blot analysis of conditioned media indicated that treatment of MAC-T cells with the IGF-I analogs induced the secretion of IGFBP-2 (29-32 KDa), but not IGFBP-3, starting at concentrations of 1.0 ng/ml. Again LongR^{3}IGF-I was more potent than Des(1-3) and IGF-I (Figure 5). Also, production of IGFBP-2 was stimulated by I starting at concentrations of 10 ng/ml. In contrast, in addition to IGFBP-2 and a 24-KDa IGFBP, MD-IGF-I cells responded to stimulation with insulin and IGF-I analogs with enhanced secretion of IGFBP-3 (Figure 5C and 5D). These stimulatory effects of the IGF-I analogs on IGFBP-3 mRNAs and secretion of IGFBP-3

in MD-IGF-I cells is also depicted in Figure 6. Both the LongR^{3}IGF-I and Des(1-3)IGF-I, at concentrations of 40 ng/ml, significantly increased IGFBP-3 mRNA levels, compared with native IGF-I or DMEM, but had no effects on steady-state levels of IGFBP-2 (data not shown). However, the levels of IGFBP-3 in media were not different (Figure 6B).

Effects of lactogenic hormones on expression of IGFBPs. MD-IGF-I cells were cultured in DMEM in the presence or absence of DEX, I, PRL, or the combinations of the three hormones. Table 1 indicates that secretion of IGF-I was stimulated 28.0-fold ($P<.01$) by DEX (349.9 pg/μg DNA) compared with DMEM (12.5 pg/μg DNA). Although there was no effect of I and PRL alone or in combination, secretion of IGF-I by MD-IGF-I cells in the presence of I plus DEX plus PRL was 1.5-fold lower (225.4 pg/μg DNA) than DEX (349.9 pg/μg DNA) or DEX plus pRL (338.3 pg/μg DNA). In the same study, total DNA measured at the end of the incubation period (192 h) was enhanced 1.7-fold by DEX (9.4 μg/well), 2.1-fold by I (11.6 μg/well), and 2.2-fold by I plus DEX (12.4 μg/well) ($P<.01$), whereas PRL (5.7 μg/well) had no effect on cell proliferation compared with DMEM (5.6 μg/well).

Results of the Northern blot analysis of total RNA

obtained at the end of the incubation period (192 h) (Table 1) revealed that DEX-induced IGF-I, I, and DEX plus I stimulated expression of IGFBP-3 (Figure 7). In contrast, no IGFBP-3 transcripts were detected when MD-IGF-I cells were cultured in DMEM (Figure 7A). These observations were confirmed by ligand blot analysis of conditioned media (Figure 7B). Normalization of the relative densitometric peak areas for IGFBP-3 with the corresponding DNA values (Figure 7C) indicated that secretion of IGFBP-3 (40-42 KDa) by MD-IGF-I cells cultured in DMEM was minimal. However, the presence of DEX or I increased ($P<.01$) 26.5- and 18.3-fold the secretion of IGFBP-3, respectively (Figure 7C). The combination of I plus DEX had no additional effect on secretion of IGFBP-3 compared with DEX alone, whereas PRL reduced 1.7-and 2.0-fold the stimulatory effects of I and DEX, respectively. These data confirmed that stimulation of IGFBP-3 production was not biased by differences in cell number, but reflected an independent stimulatory effect of both recombinant IGF-I and I on IGFBP-3 expression.

Biological activity of recombinant IGF-I and cell-derived IGFBPs. MAC-T and IGF-I transfected cells were cultured in serum-free DMEM in the absence or presence of DEX to produce conditioned media. These samples were used

to study the effects on [^{3}H]thymidine incorporation into DNA of MAC-T cells, in the absence or presence of exogenous IGFs. Data in Figure 8 indicate that conditioned medium from MD-IGF-I cells induced with DEX stimulated a 2.8- and 1.5-fold increase (14.9 cpmx10^3/well) in labeling of MAC-T cells, compared with medium from MAC-T (5.4 cpmx10^3/well) or uninduced MD-IGF-I (9.9 cpmx10^3/well) cells, respectively (Figure 8A). Moreover, the addition of IGF-I (Figure 8B) or Des(1-3)IGF-I (Figure 8C) to CM from MD-IGF-I cells stimulated an additional 3.1- and 4.9-fold increase (45.8 and 72.5 cpmx10^3/well), respectively, in [^{3}H]thymidine incorporation, compared with conditioned media from MD-IGF-I cells induced with DEX (14.9 cpmx10^3/well). Conversely, label uptake in the presence of CM from MAC-T cells plus IGF-I was stimulated only 1.5-fold (8.5 vs 5.4 cpmx10^3/well), whereas the addition of Des(1-3)IGF-I triggered an additonal 4.7-fold increase (40.4 vs 8.5 cpmx10^3/well). Taken together, these data provided evidence that parental MAC-T cells expressed primarily IGFBP-2, whereas the secretion of recombinant IGF-I or the addition of exogenous IGF-I, enhanced the expression of IGFBP-3 by transfected MD-IGF-I cells. Stimulation of parental MAC-T cells with IGF-I did not induce IGFBP-3 expression. Secretion of IGF-I-induced IGFBP-3 was associated with autonomous cell proliferation

by MD-IGF-I cells and enhanced [^{3}H]thymidine incorporation into DNA of parental MAC-T cells.

DISCUSSION

It has been shown in previous studies that IGF-I stimulated IGFBP-3 mRNA levels in cultured bovine fibroblasts (Bale and Conover, 1992) and secretion of IGFBP-3 by primary bovine mammary cells (McGrath et al., 1991). However, while cell-associated IGFBP-3 potentiated cell-membrane binding of IGF-I in bovine fibroblasts (Conover, 1992), contrasting evidence that overexpression of recombinant IGFBP-3 in Balb/c fibroblasts inhibits cell growth has been recently documented (Cohen et al., 1993). Therefore, the question as to whether production of IGF-I-induced IGFBP-3 positively or negatively affects cell proliferation of bovine mammary epithelial cells (Clemmons, 1992; Rechler and Brown, 1992) has been the focus of this investigation.

Our experimental approach has been that of transfecting an ovine IGF-I cDNA (Huynh et al., 1991) under the control of the glucocorticoid-inducible MMTV-LTR into MAC-T cells. Clonal MAC-T cells provided a unique model in that these cells express neither IGF-I nor IGFBP-3, but proliferate in

response to the addition of exogenous IGF-I (Romagnolo et al., 1993). Therefore, using the MD-IGF-I cell line (Romagnolo et al., 1992) we examined first, the extent to which acute production of IGF-I stimulates autonomous cell growth; second, how lactogenic hormones in concert with IGF-I control the autocrine/paracrine expression of IGFBP-3; and third, whether expression of IGF-I-induced IGFBP-3 participates in a functionally active autocrine loop to modulate local mitogenic actions of IGF-I in mammary epithelial cells.

Compared to other experimental models (Camacho-Hubner et al., 1991a; Camacho, 1991b; McGrath et al., 1991), the transfected MD-IGF-I cell line provided us with a system to assess directly the effects of autocrine IGF-I-induced IGFBP-3 on cell growth. While IGFBP-3 is the most important vehicle for circulating IGF-I, local tissue production of IGFBPs is probably more critical in defining the mitogenic actions of IGF-I (Davenport et al., 1992). We have shown previously (Romagnolo et al., 1992) and in this study, that expression of IGF-I by parental MAC-T cells was very low. Moreover, MAC-T cells were not capable of autonomous growth in serum-free conditions. Conversely, secretion of recombinant IGF-I by MD-IGF-I cells was coupled with autonomous cell proliferation (Table 1) and CM from these cells enhanced [^{3}H]thymidine uptake into DNA of parental

MAC-T cells (Figure 8).

Here, we provided experimental evidence that the presence of the IGF-I transgene in MD-IGF-I cells was a prerequisite for enhanced expression of IGFBP-3 in response to secretion of recombinant IGF-I (Figures 1, 2, 5, 6, and 7). Consistent with these observations, the addition of a monoclonal antibody against IGF-I or the replacement of IGF-I-based media with DMEM inhibited the IGF-I-induced effects on IGFBP-3 secretion, and confirmed the stimulatory role of IGF-I on expression of IGFBP-3 in transfected MD-IGF-I cells. In agreement with previous results (Bale and Conover, 1992), our data provided further evidence of the stability of the IGFBP-3 transcripts, as IGFBP-3 mRNAs were detectable up to 144 h (Figure 2) following induction with IGF-I, or 192 h in the presence of I or DEX (Figure 7).

Limited information is available concerning the control of IGFBP-3 expression in mammary epithelial cells. In this study, I and various IGF-I analogs stimulated DNA synthesis, in a dose-dependent fashion, of both parental MAC-T and transfected MD-IGF-I cells. Parental MAC-T cells, however, produced primarily IGFBP-2, not IGFBP-3, in response to increasing amounts of I and IGF-I analogs (Figure 5). Specifically, the IGF-I analog LongR^{3}IGF-I was more potent than Des(1-3)IGF-I and native IGF-I in

triggering secretion of IGFBP-2 by MAC-T cells. Likely, the stimulatory effects of I on secretion of IGFBP-2 and IGFBP-3 by MAC-T and MD-IGF-I cells, respectively, were exerted through activation of intracellular pathways mediated by the type-1 IGF-I receptor (Rosenfeld, 1987; Grimes and Hammond, 1992).

The role of IGF-I as a stimulator of expression of IGFBP-3 in transgenic mice has been previously reported (Camacho-Hubner et al., 1991a). Accordingly, administration of growth hormone to lactating cows increased serum levels of IGFBP-3, but decreased serum concentrations of IGFBP-2 (Cohick et al., 1992). Our data support the conclusion that expression of the IGF-I transgene in MD-IGF-I cells was a prerequisite for enhanced expression of IGFBP-3. The possibility exists that expression of recombinant IGF-I in MD-IGF-I cells may activate intracellular pathways, which may enhance the expression of IGFBP-3 at the expense of IGFBP-2. While the mechanisms responsible for these changes are yet to be defined, it is unlikely that this phenomenon is mediated by cell surface IGF-I receptors since the addition of exogenous IGFs did not induce an increase in expression of IGFBP-3 in parental MAC-T cells (Figure 1 and 5).

It has been shown that expression of IGFBP-2 by cultured myoblasts was regulated in a differentiation-dependent

manner (Ernst et al., 1992). Likewise, expression of IGFBP-2 in mammary epithelial cells may be developmentally regulated. Perhaps, this could explain why the expression of IGFBP-2 by MAC-T cells, as opposed to IGFBP-3, was enhanced by stimulation with I and IGF-I analogs. This relationship could be tested by culturing MAC-T and MD-IGF-I cells in conditions known to alter growth and differentiation of mammary epithelial cells (Liang et al., 1987; Romagnolo et al., 1993).

Previous studies (Martin and Baxter, 1988; Conover, 1991) have shown that both synthesis and secretion of IGFBP-3 by cultured fibroblasts was stimulated by IGF-I, but not I or IGF-I analogs with reduced binding affinity for the homologous binding proteins. Similarly, secretion of IGFBP-2 by mammary epithelial cells from non-lactating heifers cultured on collagen substrate was augmented by IGF-I analogs, but not I (McGrath et al., 1991). Therefore, in contrast to what has been previously suggested for fibroblasts (Martin and Baxter, 1988) and MDBK epithelial cells (Cohick and Clemmons, 1991), our findings suggest that stimulation of IGFBP-2 and IGFBP-3 production by I or IGF-I analogs in normal or transfected mammary epithelial cells, respectively, does not require binding to IGFBPs (Clemmons, 1992). These effects were probably mediated by the type-1 IGF-I receptor as I alone stimulated DNA

synthesis and IGFBP production of both parental MAC-T and transfected MD-IGF-I cells.

The development of the mammary gland is the under control of a number of growth factors and lactogenic hormones which in concert play a fundamental role in determining the growth and differentiative status of mammary epithelial cells (Akers, 1985). In our study, the addition of PRL, at levels of 1.0 μg/ml, alone or in combination with I and DEX, had no effect on cell proliferation of MD-IGF-I cells. However, the addition of PRL to cultures with I or DEX reduced significantly the expression of IGFBP-3, as measured by ligand blot analysis (Figure 7C). These effects are in accordance with previous reports (Fielder et al., 1992) which demonstrated a dose-dependent inhibitory effect for PRL on stimulation of IGFBP-3 by IGF-I in murine cells. Conversely, others (Guyette et al., 1979) have shown that PRL stimulated expression of casein genes at the transcriptional and post-transcriptional levels in mammary gland organ cultures from Sprague Dawley rats. Therefore, only studies aimed at investigating the molecular mechanisms whereby PRL regulates expression of IGF-I-induced IGFBP-3 could provide an explanation for the reduced secretion of IGFBP-3 by MD-IGF-I cells stimulated with PRL. However, as the secretion of IGF-I-induced IGFBP-3 was associated with

autonomous cell proliferation of MD-IGF-I cells, then it is possible that the presence of PRL may favor the expression of specialized differentiated functions, such as casein expression, at the expense of secretion of IGFBP-3 involved in cell-proliferating pathways.

The overexpression of IGFBP-3 in response to secretion of recombinant IGF-I did not cause loss of the proliferating phenotype characteristic of MD-IGF-I cells stimulated with DEX, or MAC-T cells cultured in media conditioned by MD-IGF-I cells. Our current studies do not support a negative role for IGFBP-3 on cell proliferation of transfected mammary epithelial cells. At least three mechanisms could be taken into consideration to explain the permissive role of IGFBP-3 on IGF-I action. First, IGFBP-3 may act in MD-IGF-I cells as a reservoir for IGF-I (Blum et al., 1989). Thus, the mitogenic stimulus of IGF-I in the presence of IGFBP-3 could be perpetuated by the continuous release of low amounts of IGF-I compared with the absence of IGFBP-3. Second, it is possible that secreted IGFBP-3 may sequester the IGF-I, thereby increasing the number of IGF-I molecules at the cell surface capable of interacting with the IGF-I receptor. Third, the gradual release of IGF-I from the IGF-I-BP-3 ligand complex may sustain faster cell proliferation of MD-IGF-I cells by preventing down-regulation of the IGF-I receptor by a high

concentration of free IGF-I. This assumption is supported by the experimental evidence that addition of exogenous IGFBP-3 blocked downregulation of the IGF-I receptor (Conover and Powell, 1991). Our preliminary data also suggest that promoters which stimulate constitutive expression of IGF-I in mammary cells reduce the number of IGF-I receptors per cell, compared with MAC-T or MD-IGF-I cells (Romagnolo et al., unpublished observations).

However, others (Baxter and Martin, 1989; Conover et al., 1990) have demonstrated that the inhibition of cell proliferation by exogenous IGFBP-3 was dose-dependent. In this study we did not quantitate the actual amounts of IGFBP-2 or IGFBP-3 secreted into the media. Consequently, it could be argued that production of IGFBP-3 by MD-IGF-I cells may have not been sufficient to hamper significantly cell proliferation. Nevertheless, we have shown that presence of IGFBP-3 in media conditioned by MD-IGF-I cells (Figure 7) not only was associated with autonomous growth of MD-IGF-I cells (Table 1), but also the presence of IGFBP-3 in conditioned media from MD-IGF-I cells augmented [^{3}H]thymidine uptake by parental MAC-T cells induced with exogenous IGF-I (Figure 8). In agreement with previous observations (DeMellow and Baxter, 1988; Ross et al., 1989), IGFBP-2-containing media attenuated the mitogenic actions of exogenous IGF-I (Figure 8B).

Previous reports attribute a dual role to IGFBP-3 depending on whether IGFBP-3 is pre-incubated or co-incubated with IGF-I (DeMellow and Baxter, 1988). In our system, however, IGFBP-3 was endogenously produced by MD-IGF-I cells in response to IGF-I. Therefore, use of this cell system may be particularly advantageous as it does not require the addition of exogenous IGFBP-3, which compared with endogenously produced IGFBP-3, does not not fully reproduce the same biological effects on IGF-I.

In this study, IGFBP-3 apparently potentiated the proliferating stimulus of exogenous IGF-I. This conclusion is strengthened by the experimental evidence that conditioned media from MAC-T and MD-IGF-I cells sustained similar rates of thymidine incorporation in parental MAC-T cells when the Des(1-3)IGF-I analog was substituted for the native IGF-I (Figure 8C). This increase in isotope incorporation was somewhat expected if one considers that the Des(1-3)IGF-I analog possesses the same affinity for the type-1 IGF-I receptor, but exhibits lower affinity for IGFBP-2 and IGFBP-3 than its native counterpart (Collier et al., 1989). More importantly, from our data we suggest that in MD-IGF-I cells, endogenously produced IGF-I-induced IGFBP-3, compared with IGFBP-2, acted as a positive component of an autocrine loop to potentiate the mitogenic stimulus of IGF-I. Perhaps, this effect was mediated

through increased reactivity of the type-1 IGF-I receptor upon binding of IGFBP-3 to the cell surface (Conover, 1992). Alternatively, increased IGFBP-3 in MD-IGF-I cells might serve to counteract the inhibitory effects of IGFBP-2. Failure of mature IGF-I to stimulate thymidine incorporation when added to CM from parental cells containing predominantly IGFBP-2, supports this conclusion. Moreover, growth stimulation by IGF-I in osteoblastic cells (Ernst and Rodan, 1990) and estrogen receptor-negative breast carcinoma cells (Shao et al., 1992; Figueroa and Yee, 1992) were positively correlated with presence of IGFBP-3 in culture media. Similarly, increased levels of IGFBP-3 mRNA by primary breast tumors from Sprague-Dawley rats have been recently reported (Manni et al., 1992).

In summary, we have shown that production of IGF-I by transfected mammary epithelial cells triggered the autocrine expression of IGFBP-3, which in turn potentiated the mitogenic action of IGF-I. This effect was peculiar to the transfected MD-IGF-I cells, which expressed an IGF-I cDNA under the control of the MMTV-LTR promoter. Parental MAC-T cells, conversely, expressed primarily IGFBP-2 which attenuated the mitogenic actions of IGF-I. MD-IGF-I cells exhibited the phenotypic behavior of fibroblasts (Conover, 1991) and breast cancer cells (Camacho-Hubner et al., 1991b), in that they expressed through an autocrine loop

IGF-I-induced IGFBP-3. We propose that the local induction of IGFBP-3 by acute secretion of IGF-I in bovine mammary epithelial cells may represent a positive functional pathway to mediate the mitogenic action of IGF-I. The mechanisms by which IGFBP-3 exerts its potentiating role are subjects of ongoing investigations.

SUMMARY

Limited information is available concerning the molecular and cellular mechanisms that regulate expression of insulin-like growth factor binding proteins (IGFBPs) in bovine mammary epithelial cells. In this study, we report on the autocrine mechanisms of action of insulin-like growth factor-I (IGF-I) and hormonal regulation of expression of IGFBPs in bovine mammary epithelial MD-IGF-I cells (Mol. Endocrinol. 1992, 6:1774-1780). Expression of recombinant IGF-I was under the control of the glucocorticoid-inducible mouse mammary tumor virus-long terminal repeat (MMTV-LTR). Levels of IGFBP-3 mRNA and secretion of IGFBP-3 by MD-IGF-I cells into media were stimulated by IGF-I, insulin (I), and IGF-I analogs (LongR^3IGF-I > Des(1-3)IGF-I), but not by prolactin. Conversely, parental MAC-T cells secreted no IGF-I and produced primarily IGFBP-2 (29-32 KDa) in response to stimulation with I, DEX, or IGF-I analogs, as measured by ligand blotting. Secretion of recombinant IGF-I caused a 26.5-fold increase in secretion of IGFBP-3 by transfected MD-IGF-I cells, which was associated with a 1.7-fold increase in total DNA (9.4 vs 5.6 μg DNA/well). Conditioned media (CM) from MD-IGF-I cells induced with DEX stimulated a 2.8- and 1.5-fold increase (14.9 cpmx10^3/well) in

[^{3}H]thymidine incorporation into DNA of parental MAC-T cells, compared with media from uninduced MAC-T (5.4 cpmx 10^3/well) and MD-IGF-I (9.9 cpmx10^3/well) cells. Moreover, the addition of exogenous IGF-I to CM from MD-IGF-I cells triggered an additional 3.0-fold increase in [^{3}H]thymidine incorporation (45.8 vs 14.9 cpmx10^3/well), but only a 1.6-fold increase (13.2 vs 8.2 cpmx10^3/well) in the presence of IGFBP-2-containing media conditioned by MAC-T cells. Conversely, the addition of Des(1-3)-IGF-I to CM from both MAC-T and MD-IGF-I cells respectively, stimulated a significant 10.2- (55.6 vs 5.4 cpmx10^3/well) and 6.9-fold (68.6 vs 9.9 cpmx10^3/well) increase in [^{3}H]thymidine incorporation into DNA of MAC-T cells. We suggest that expression of IGF-I-induced IGFBP-3 may be an important component of an autocrine loop to modulate the local mitogenic actions of IGF-I in bovine mammary epithelial cells.

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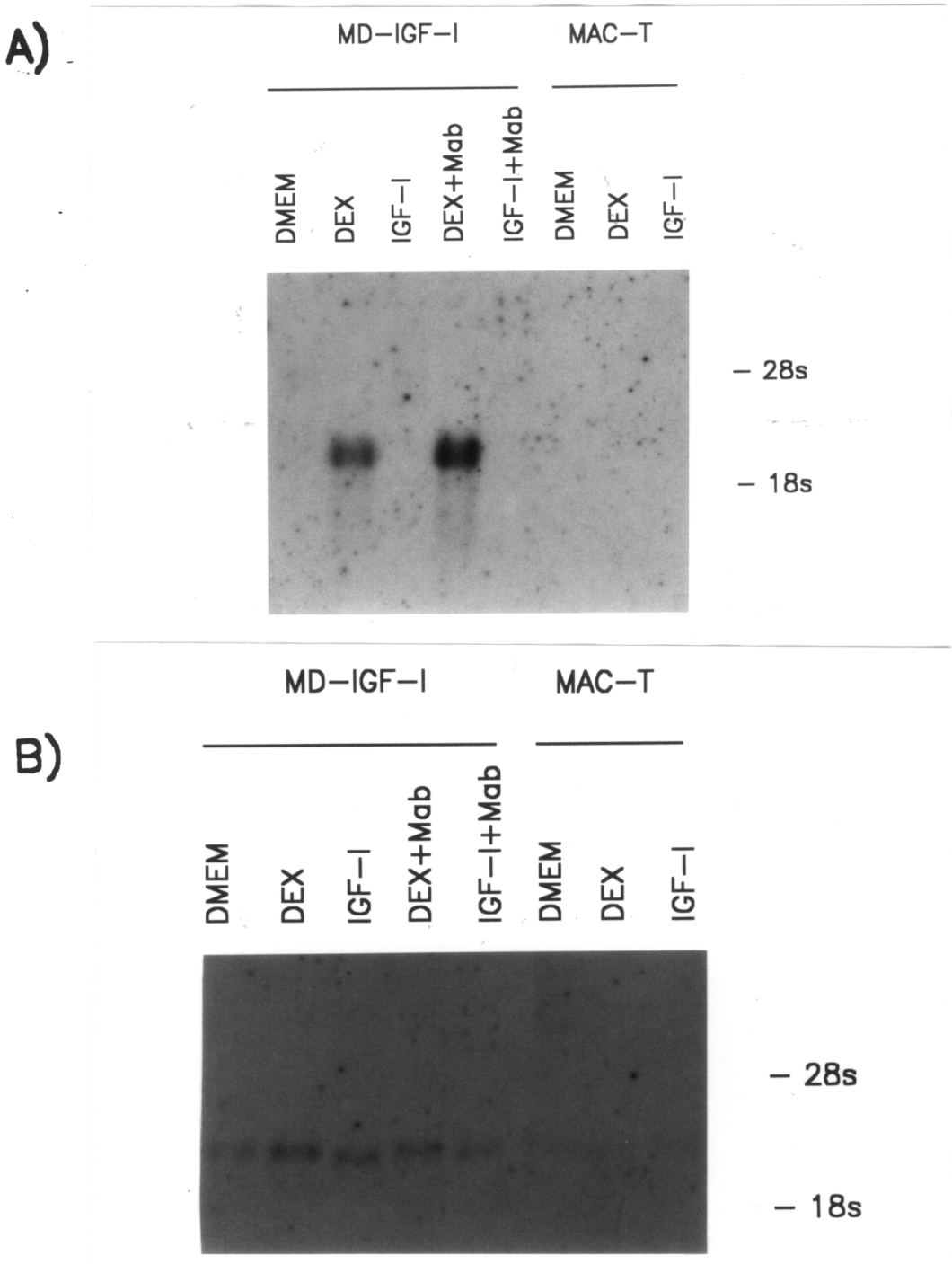


Figure 1A, B. Effects of IGF-I on expression of IGFBPs by parental MAC-T and MD-IGF-I cells. Cells were cultured in DMEM or in DMEM with 0.1 μM DEX, 15 ng/ml IGF-I, DEX plus a monoclonal antibody against IGF-I (Mab) (dilution 1:4000), and IGF-I plus Mab. Cells were harvested after 72 h and total RNA extracted for Northern analysis of IGF-I (A) and IGFBP-3 (B). Migration of the 28s and 18s ribosomal RNAs are indicated.

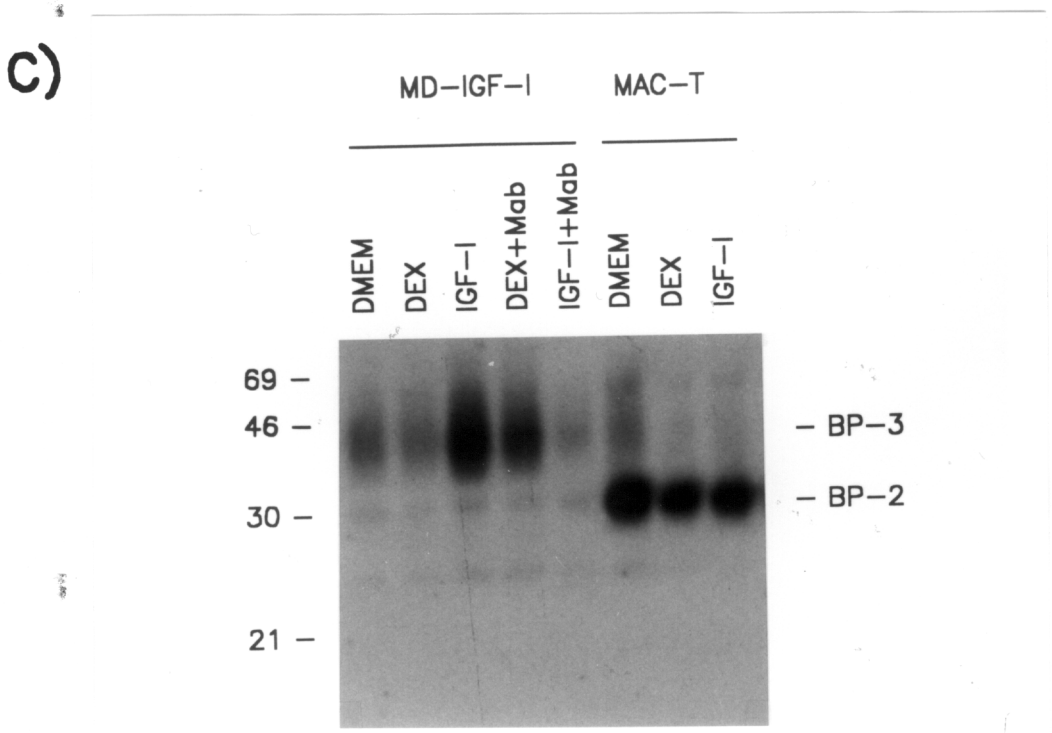


Figure 1C. Effects of IGF-I on expression of IGFBPs by parental MAC-T and MD-IGF-I cells. Conditioned media was assayed for presence of IGFBPs by ligand blot analysis. Migrations of the molecular weight markers (KDa) are indicated.

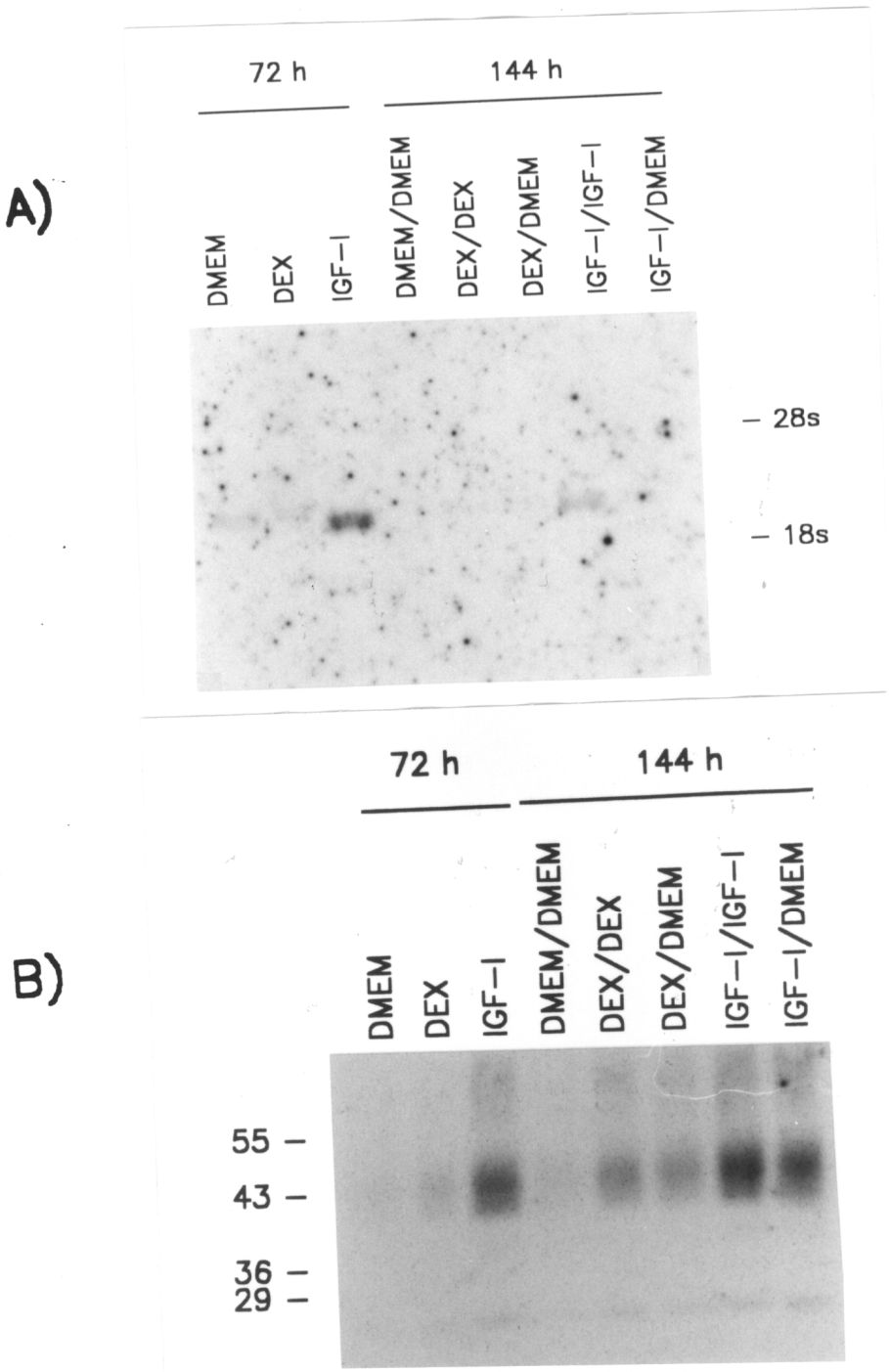


Figure 2. Induction of IGFBP-3 expression by IGF-I in MD-IGF-I cells. MD-IGF-I cells were cultured for 72 and 144 h in DMEM in the presence or absence of 0.1 μM DEX or 100 ng/ml human IGF-I. After the first 72 h period, cells were either maintained on the same treatment for an additional 72 h, or alternatively, switched to DMEM. At the end of the incubation periods, cells were harvested for immediate extraction of total RNA for Northern analysis of IGFBP-3 (A), and conditioned media collected for analysis of IGFBPs by ligand blotting (B). Migration of the 28s and 18s ribosomal RNA and molecular weight markers (KDa) are indicated.

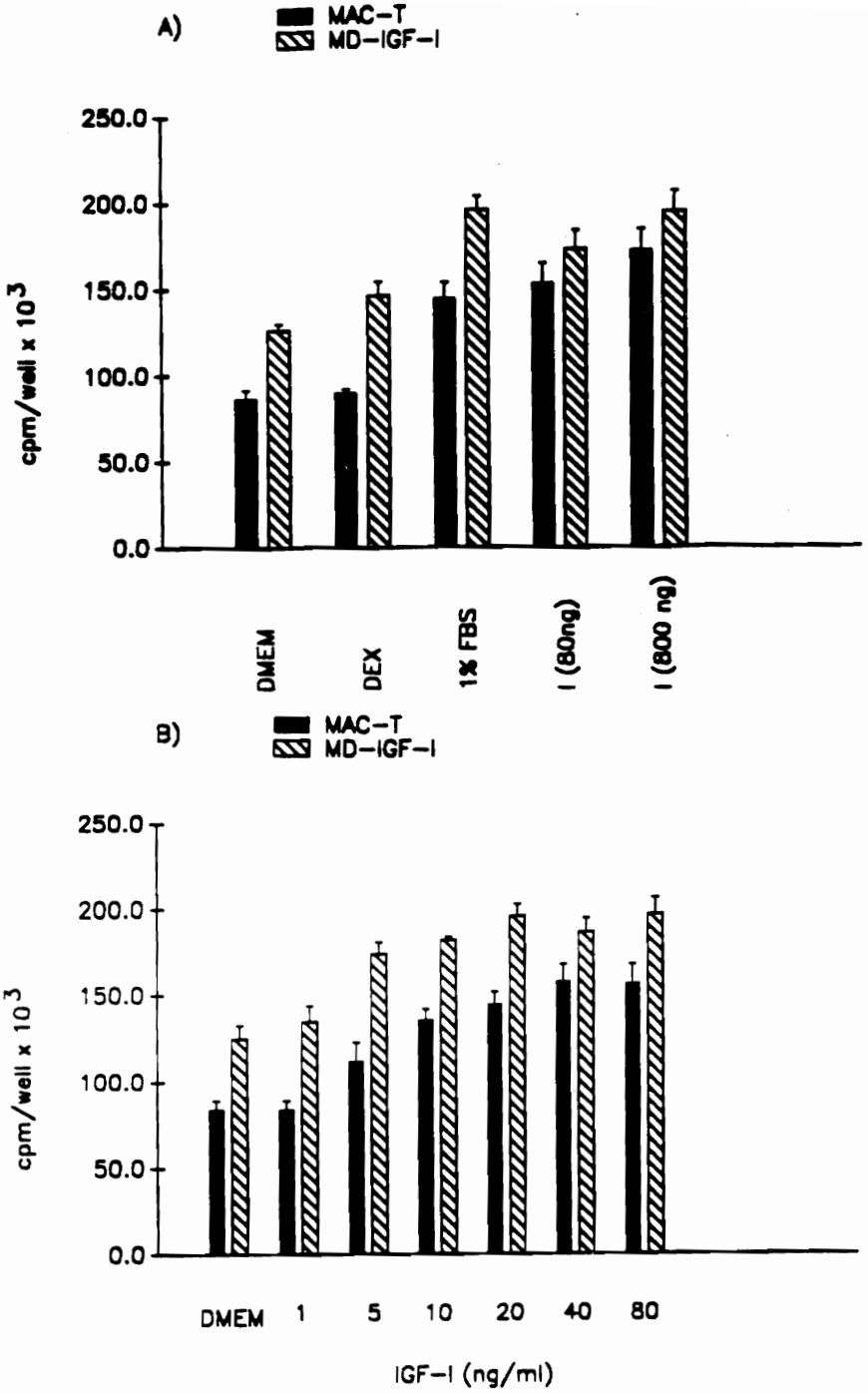
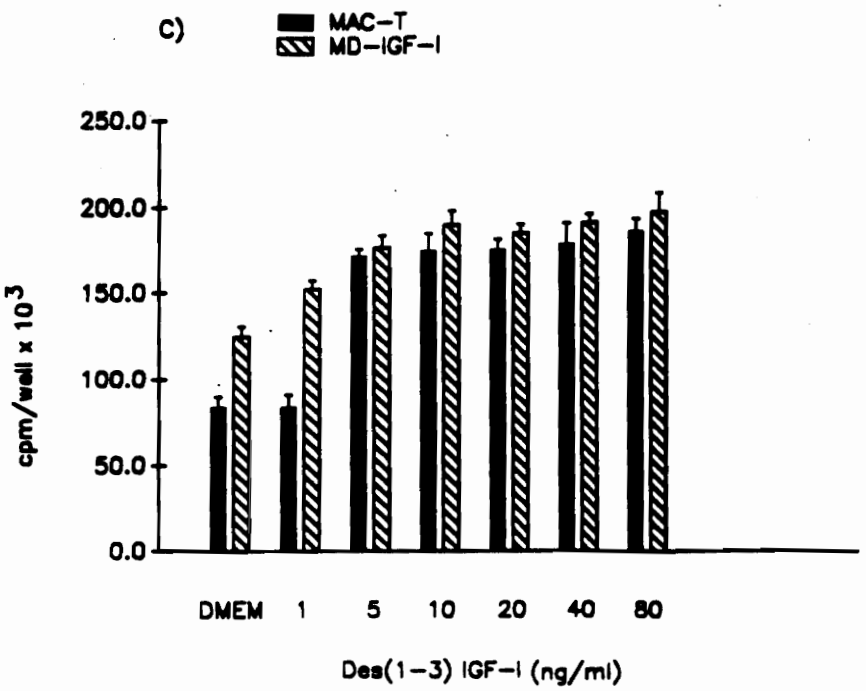


Figure 3A,B. Effects of IGF-I analogs on [^{3}H]thymidine incorporation into DNA of parental MAC-T and MD-IGF-I cells. Cells were cultured for 72 h in DMEM or DMEM with 0.1 μM DEX, 1% FCS, 80 and 800 ng/ml insulin (I) (Fig. 3A), and increasing amounts (ng/ml) of IGF-I (Fig. 3B). At the end of the incubation period, cells were pulsed for 2 h with [^{3}H]thymidine and incorporation of label was measured. Bars are cpmx10^3/well and represent the mean$\pm$SEM from eight samples assayed in duplicate.



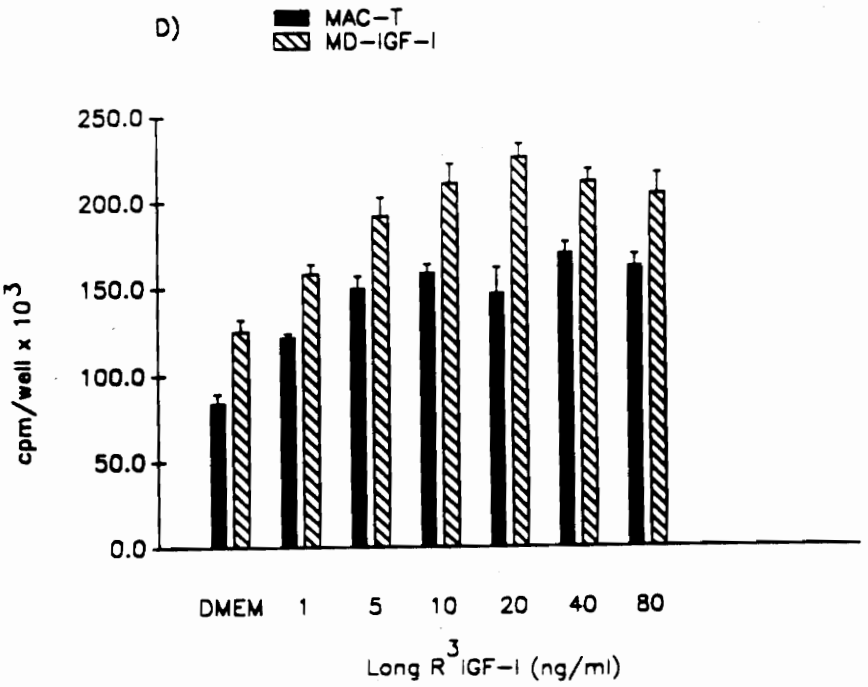


Figure 3C,D. Effects of Des(1-3)IGF-I (C) and LongR^3IGF-I (D) analogs on [^{3}H]thymidine incorporation into DNA of parental MAC-T and MD-IGF-I cells.

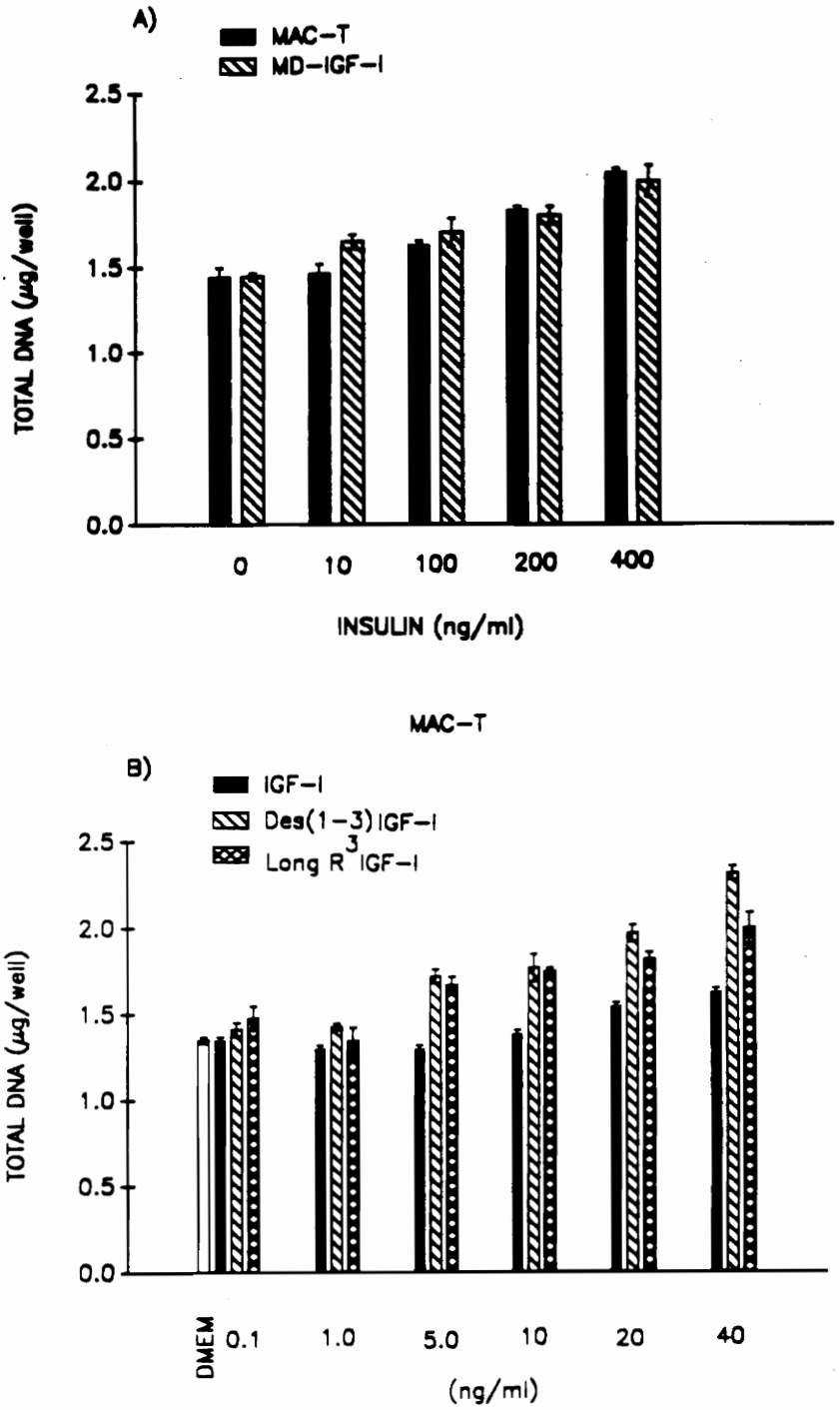


Figure 4A,B. Effects of IGF-I analogs on total DNA of parental MAC-T and MD-IGF-I cells. Cells were cultured for 72 h in Dulbecco's Modified Eagle Medium (DMEM) or DMEM with increasing amounts (ng/ml) of insulin (A), and IGF-I, Des(1-3)IGF-I and LongR^3IGF-I. (B) Bars are total DNA (μg/well) and represent the mean$\pm$SEM values from six samples assayed in duplicate.

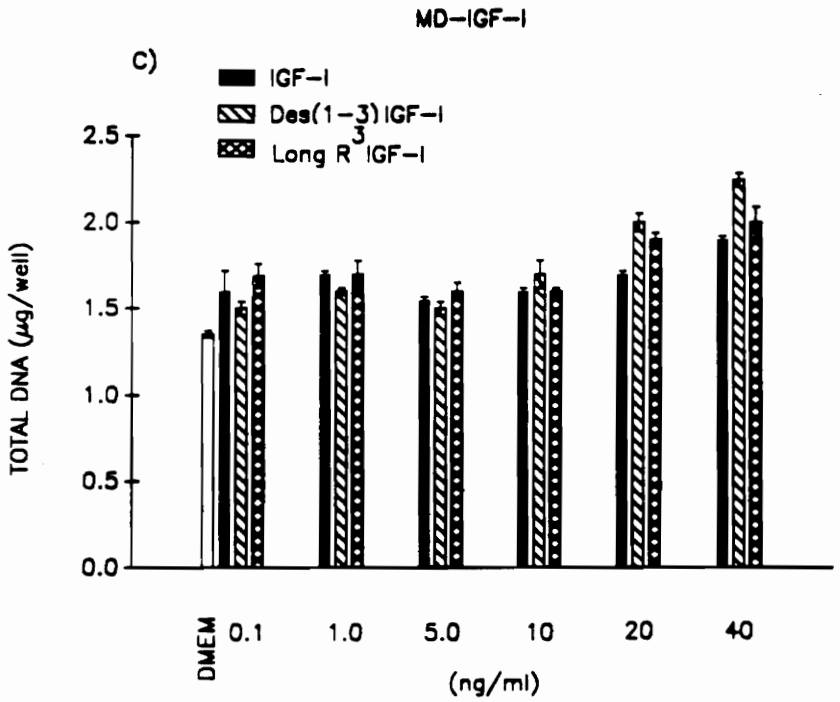


Figure 4C. Effects of IGF-I, Des(1-3)IGF-I, and LongR^3IGF-I on total DNA of parental MAC-T and MD-IGF-I cells.

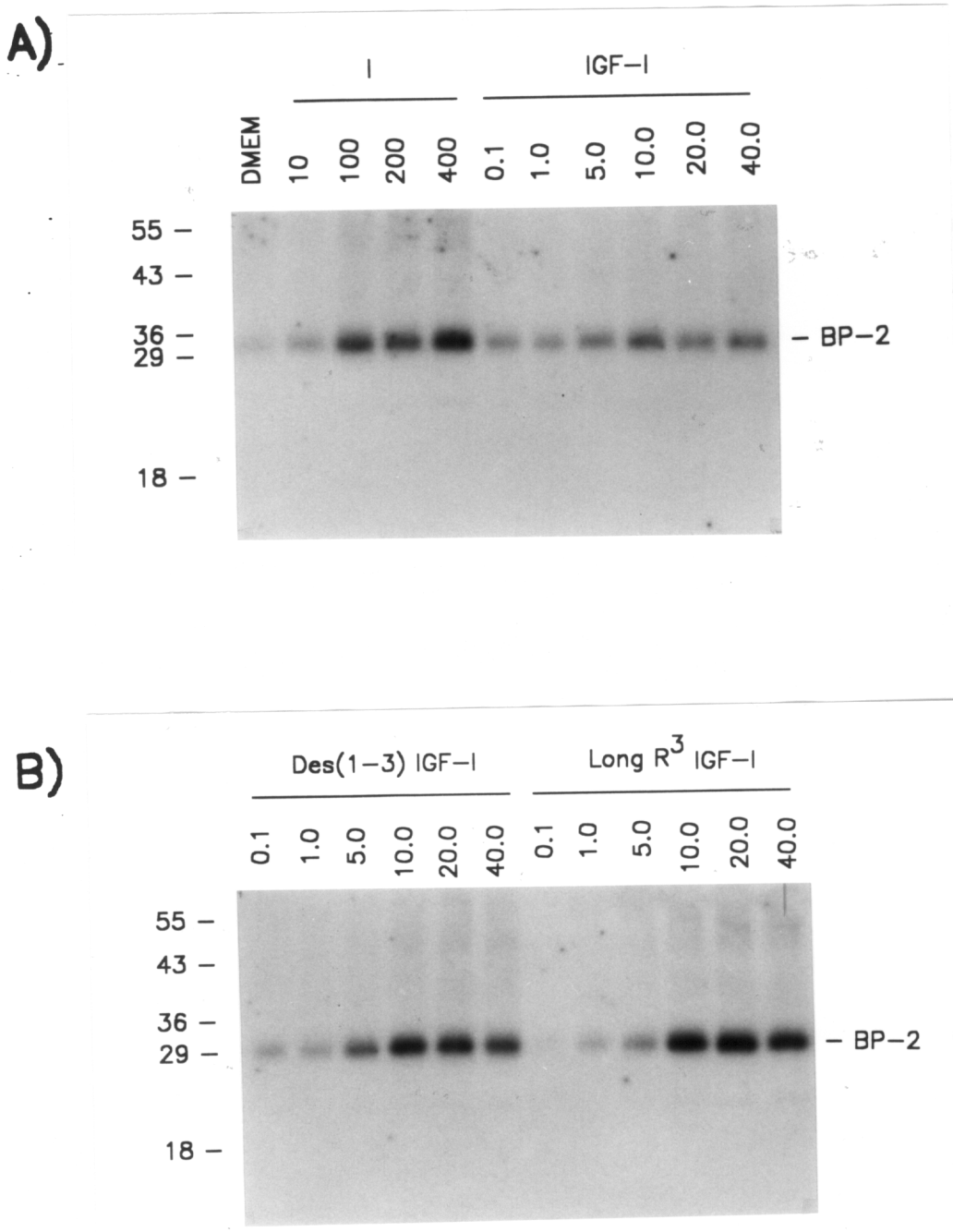
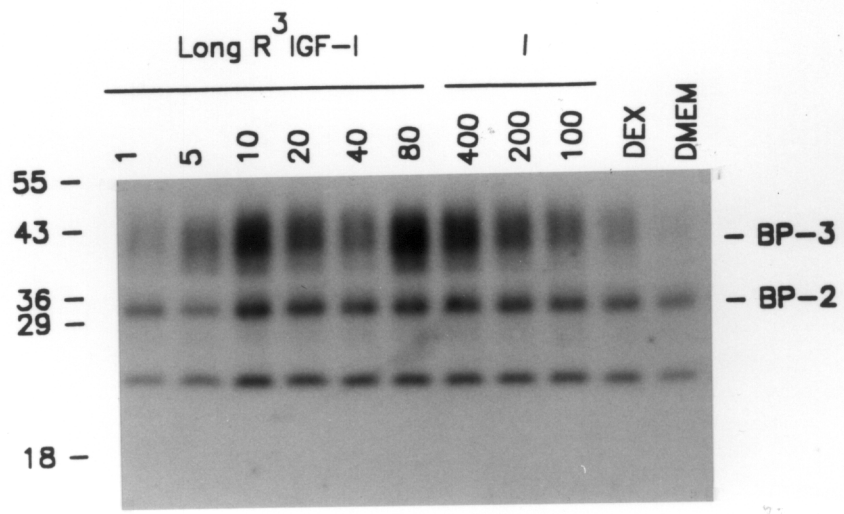


Figure 5A,B. Effects of IGF-I analogs on production of IGFBPs by parental MAC-T cells. Cells were cultured for 72 h in DMEM in the presence of increasing amounts of insulin (I), IGF-I, Des(1-3)IGF-I, and LongR^3IGF-I. At the end of the incubation period, conditioned media was collected for determination of IGFBPs by ligand blot analysis. Migrations of the molecular weight markers (KDa) are indicated. Concentrations are in ng/ml.

C)



D)

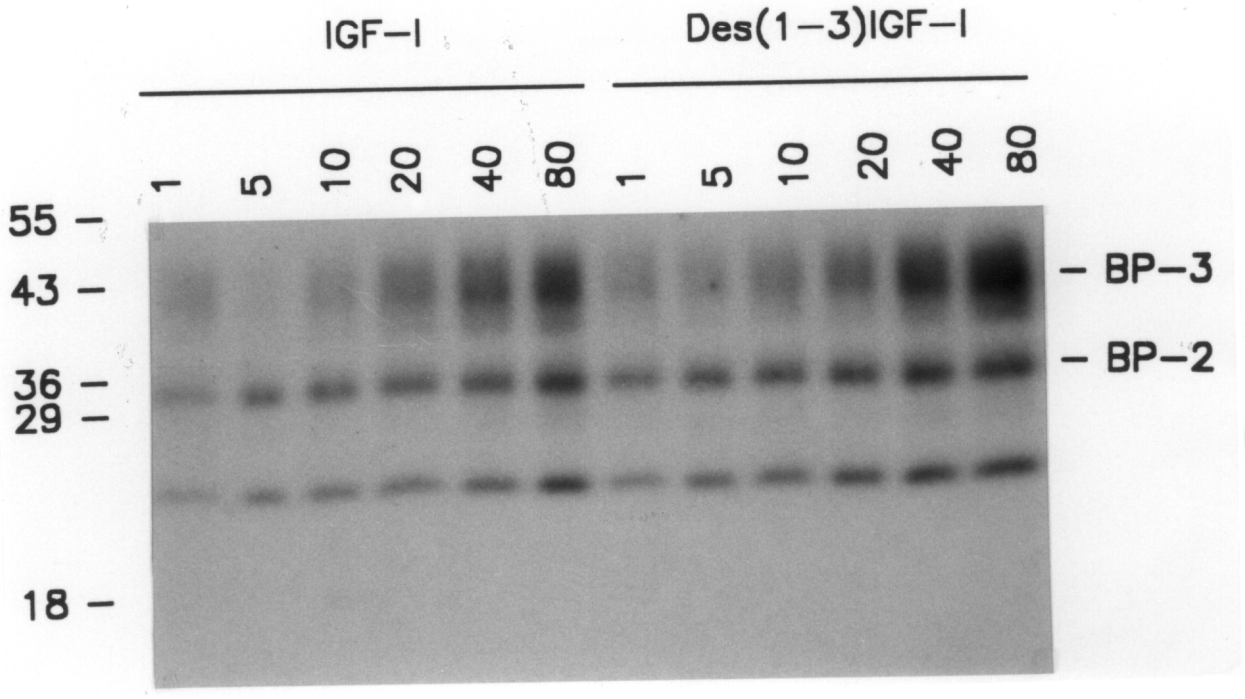


Figure 5C,D. Effects of IGF-I analogs on production of IGFBPs by MD-IGF-I cells. Control medium for MD-IGF-I cells was DMEM plus 0.1 μM DEX.

A)

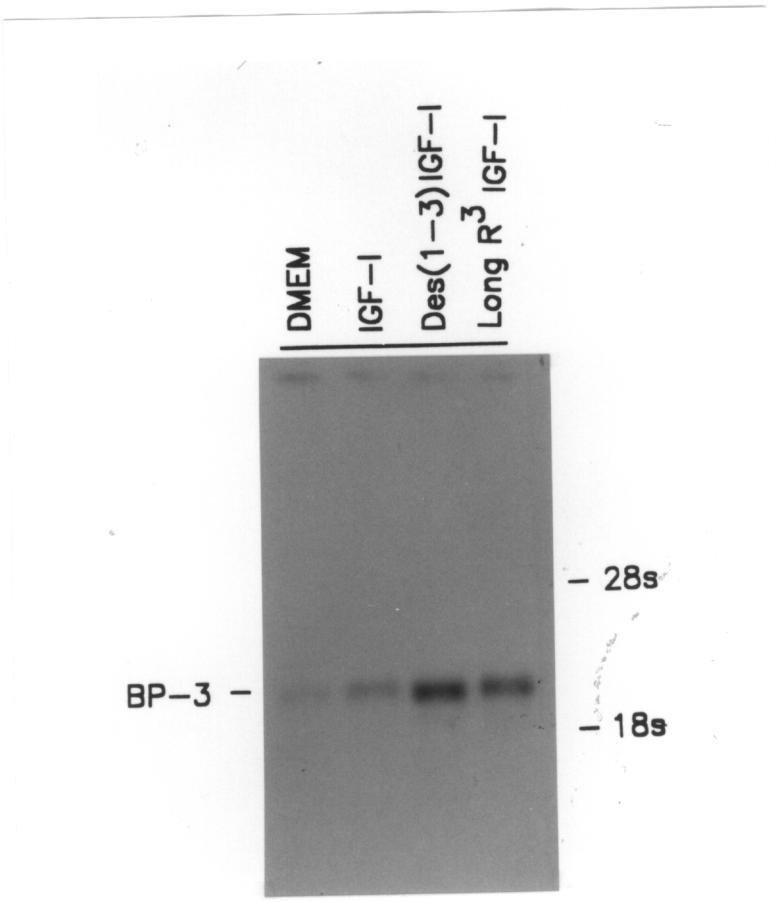


Figure 6A. Effects of IGF-I analogs on expression of IGFBP-3 in MD-IGF-I cells. Transfected MD-IGF-I cells were incubated for 72 h in DMEM or DMEM with 40 ng/ml of IGF-I, Des(1-3), LongR^3IGF-I. At the end of the incubation period, cells were harvested for Northern analysis of IGFBP-3 mRNA.

B)

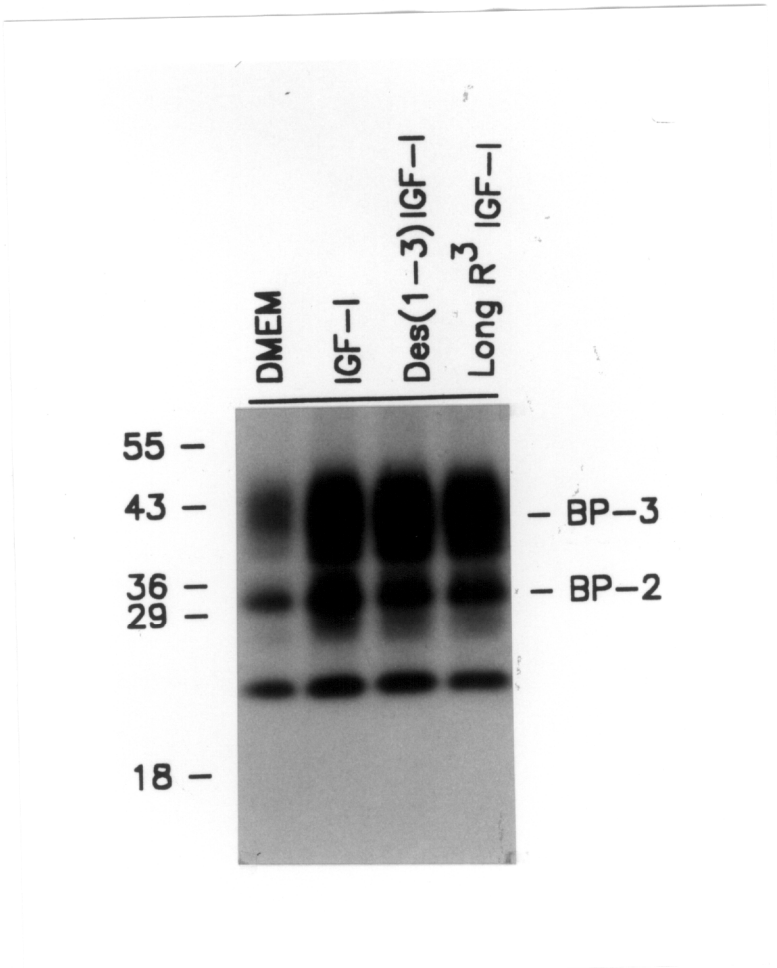


Figure 6B. Effects of IGF-I analogs on secretion of IGFBPs by MD-IGF-I cells. IGFBPs in conditioned media were determined by ligand blot analysis.

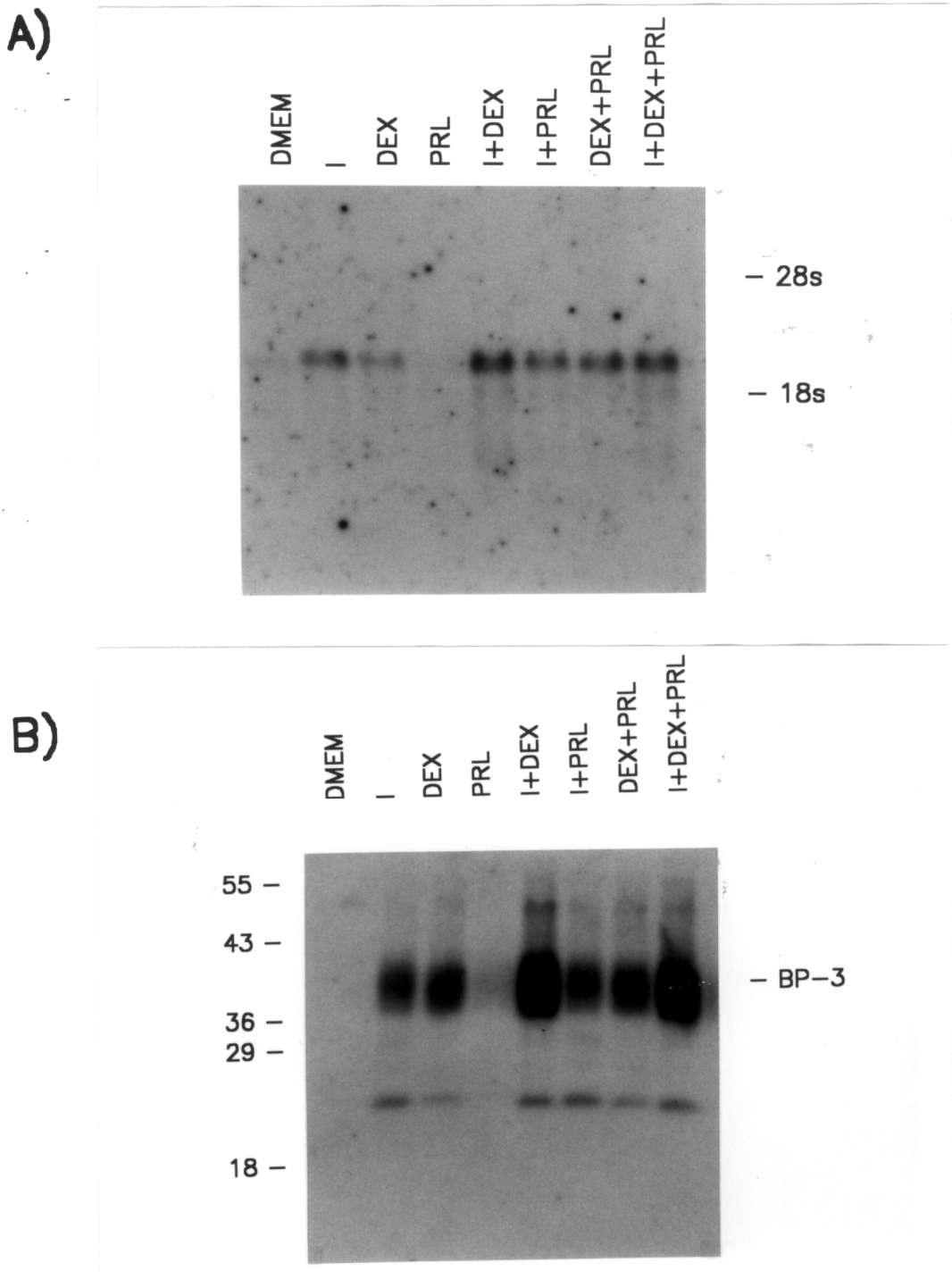


Figure 7A,B. Effects of lactogenic hormones on expression of IGFBP-3 in MD-IGF-I cells. MD-IGF-I cells were cultured for 192 h in DMEM or DMEM with 1 μg/ml insulin (I), 0.1 μM DEX, 1 μg/ml prolactin (PRL), or in DMEM containing combinations of the three hormones. At the end of the incubation period, total RNA was extracted from cells for analysis of IGFBP-3 mRNA levels (A) and secretion of IGFBPs into conditioned media assayed by ligand blot analysis (B).

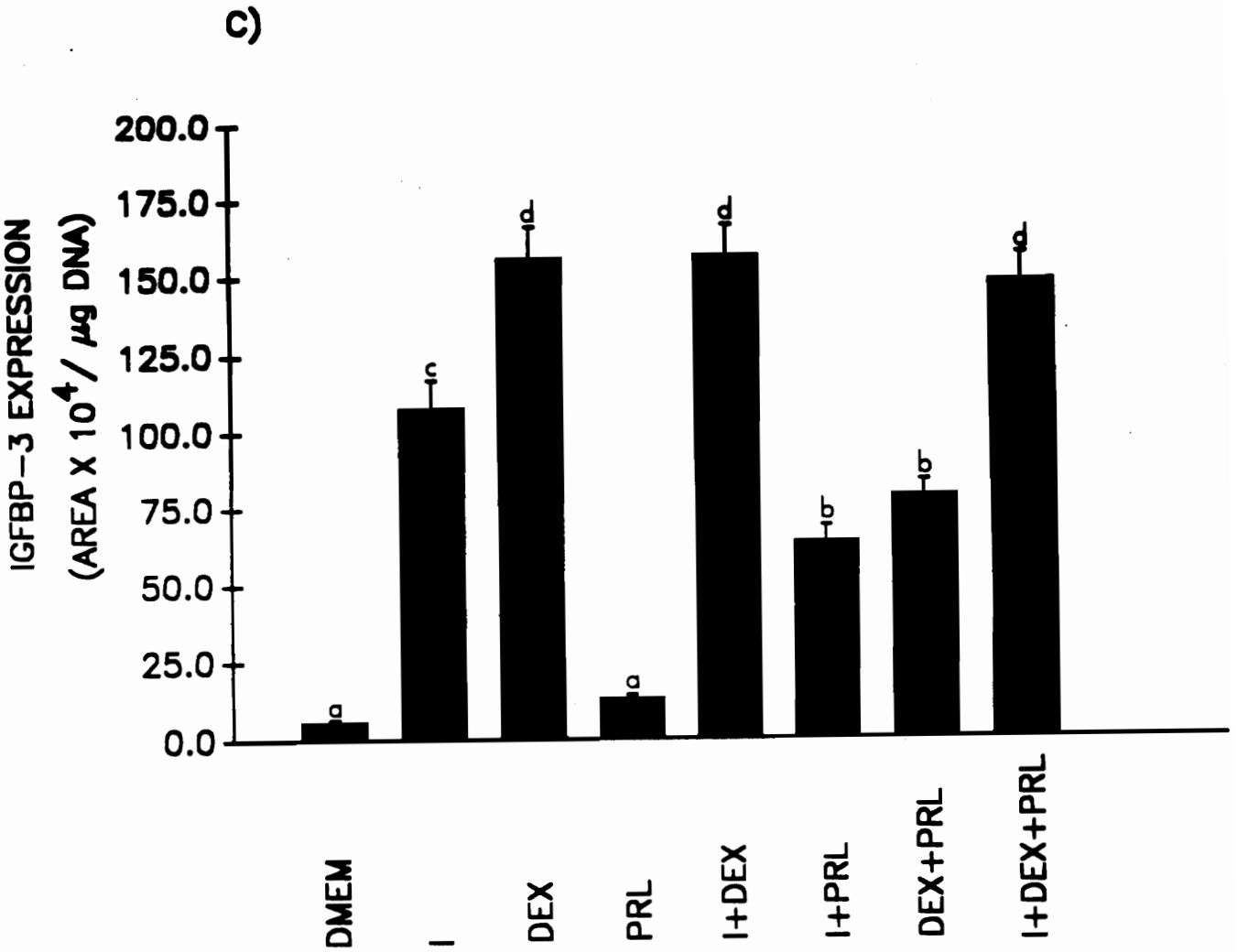


Figure 7C. Effects of lactogenic hormones on expression of IGFBP-3 by MD-IGF-I cells. Secretion of IGFBP-3 was quantitated by densitometric analysis of ligand blots from three separate experiments. Bars are relative IGFBP-3 area$x10^4/\mu g$ DNA and represent the mean±SEM of three samples assayed in duplicate. Bars labeled with different symbols were significantly ($P<.05$) different by Duncan's multiple comparison test.

TABLE 1. EFFECTS OF LACTOGENIC HORMONES ON SECRETION OF RECOMBINANT IGF-I AND CELL PROLIFERATION IN MD-IGF-I CELLS*

Treatment	DNA		IGF-I			
	(μg/well)	SE	(ng/ml)	SE	(pg/μg DNA)	SE
DMEM	5.6	.03	0.1	.01	12.5	.01
I	11.6^{b}	.09	0.3	.02	15.1	.06
DEX	9.4^{a}	.04	3.3^{a}	.02	349.9^{b}	.09
PRL	5.7	.02	0.1	.01	15.9	.02
I+DEX	12.4^{b}	.16	4.8^{b}	.19	387.1^{b}	.20
I+PRL	12.0^{b}	.08	0.2	.04	19.9	.05
DEX+PRL	9.5^{a}	.05	3.2^{a}	.02	338.3^{b}	.09
I+DEX+PRL	12.4^{b}	.10	2.8^{a}	.02	225.4^{a}	.03

*Transformed bovine mammary epithelial MD-IGF-I cells were cultured for 192 h in DMEM or DMEM with 1 μg/ml insulin (I), 0.1 μM dexamethasone (DEX), 1 μg/ml prolactin, or in DMEM containing combinations of the three hormones. At the end of the incubation period cells were harvested for determination of total DNA and conditioned media assayed for IGF-I by radioimmunoassay as described in Materials and Methods. Values are the mean$\pm$SEM of three samples assayed in duplicate from two separate experiments. Values within a column with different superscripts were significantly ($P<.05$) different by Duncan's multiple comparison test.

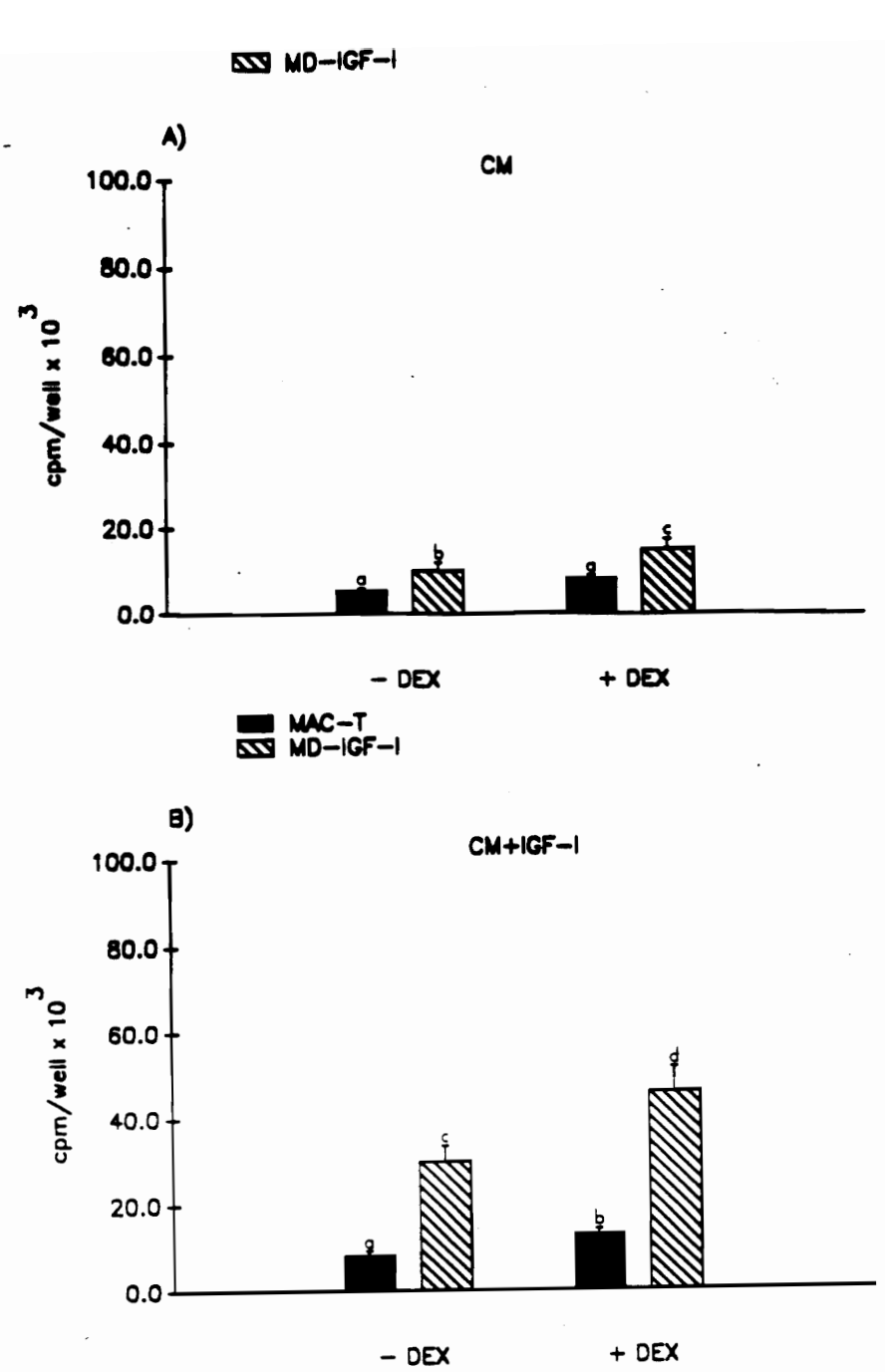


Figure 8A,B. [^{3}H]thymidine incorporation into DNA of MAC-T cells in response to secretion of recombinant IGF-I and IGFBP-3. Transformed MD-IGF-I cells were cultured for 72 h in DMEM in the absence (-) or presence (+) of 0.1 μM DEX. At the end of the incubation period, conditioned media (CM) were harvested and used to culture parental MAC-T cells in CM (A), CM plus 10 ng/ml IGF-I (B). Conditioned media were added at 9:1 ratio of CM:fresh DMEM (control) or DMEM supplemented with IGF-I in a total volume of 1 ml. [^{3}H]thymidine incorporation into 72h serum-starved MAC-T cells was measured after 16 h, with a 2-h pulse. Bars are cpm x 10^3/well and represent the mean$\pm$SEM from six samples assayed in duplicate. Bars with different symbols were significantly ($P<.05$) different by Duncan's multiple comparison test.

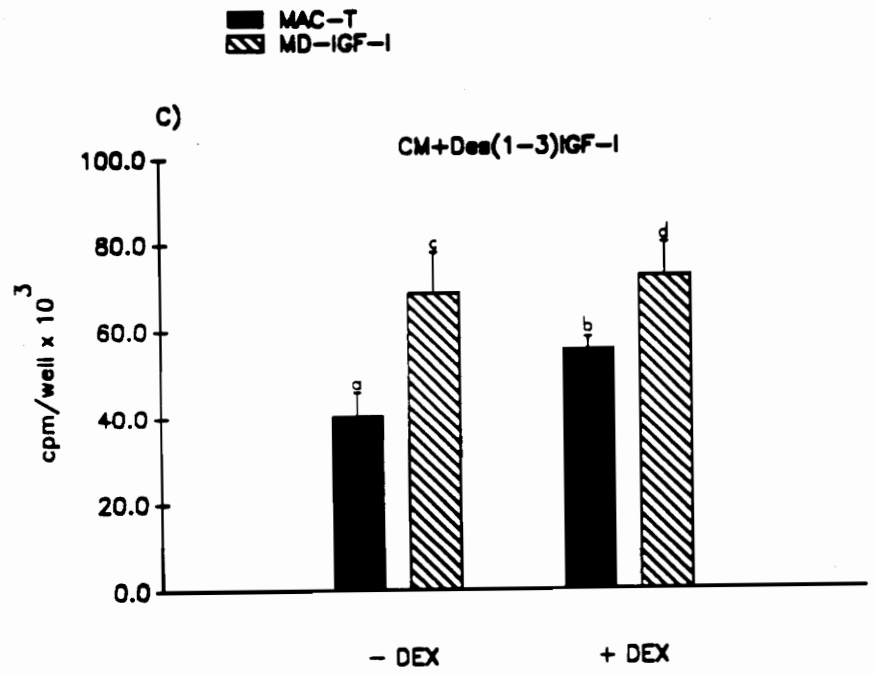


Figure 8C. [^{3}H]thymidine incorporation into DNA of MAC-T cells. Test MAC-T cells were cultured in media conditioned (CM) by MAC-T and MD-IGF-I cells in the absence (-) or presence (+) of 0.1 μM DEX supplemented with 10 ng/ml Des(1-3)IGF-I. Bars with different symbols were significantly ($P<.05$) different by Duncan's multiple comparison test.

CHAPTER 4

REGULATION OF EXPRESSION OF IGF-I-INDUCED IGFBP-3 AND IGF-I RECEPTOR BY CHRONIC vs ACUTE EXPRESSION OF RECOMBINANT IGF-I IN TRANSFECTED MAMMARY EPITHELIAL CELLS

INTRODUCTION

Experimental evidence that insulin-like growth factor-I binding proteins (IGFBPs) markedly potentiated cell proliferation of a variety of cell types (Elgin et al., 1987) suggested a role for IGFBPs as important mediators of cell responsiveness to IGF-I. Of the six IGFBPs characterized to date (Sheick et al., 1992), the growth hormone-dependent IGFBP-3 is the predominant circulating IGF-I binding protein and is a component of the 150-KDa IGF-binding protein complex (Wood et al., 1988). Moreover, local secretion of IGFBP-3 by a variety of cell types (Martin and Baxter, 1988; Schmid et al., 1989; Smith et al., 1990; Conover et al., 1989) suggested the existence of local autocrine/paracrine pathways through which IGFBP-3 might influence the mitogenic actions of IGF-I (Blum et al., 1989).

Cell-derived IGFBP-3 might enhance the mitogenic actions of IGF-I by blocking down-regulation of the IGF-I receptor, thereby buffering sudden changes in concentration of ambient IGF-I (Conover and Powell, 1991). These effects could be exerted through association of IGFBP-3 with the cell surface, which in turn may limit access of IGF-I to target cell receptors (Conover et al., 1990). Nevertheless, the physiological significance of locally-secreted IGFBP-3 in controlling IGF-I-mediated cell proliferation is not completely understood (Rechler and Brown, 1992). In human skin fibroblasts, IGFBP-3 both inhibited and potentiated IGF-I stimulated DNA synthesis (DeMellow and Baxter, 1988), whereas overexpression of human IGFBP-3 in transfected Balb/c fibroblasts inhibited cellular proliferation (Cohen et al., 1993). In addition, experimental evidence (Lewitt and Baxter, 1990; Price et al., 1992; Bale and Conover, 1992) suggests that regulation of expression of cell-derived IGFBPs may be controlled through cell-, tissue-, and species-specific mechanisms.

We have previously shown that bovine mammary epithelial cells (MD-IGF-I) (Romagnolo et al., 1992) containing an IGF-I cDNA under the control of the MMTV-LTR promoter proliferated in response to secretion of recombinant IGF-I. Moreover, secretion of IGF-I-induced IGFBP-3 apparently potentiated the mitogenic effects of IGF-I. Induction of

expression of IGFBP-3 by these cells was a type-1 IGF-I receptor-dependent process as the treatment with insulin mimicked IGF-I-induced expression of IGFBP-3 (Romagnolo et al., 1993, submitted).

The present study was undertaken to investigate the effects of chronic versus acute expression of IGF-I on production of IGF-I-induced IGFBP-3 and regulation of expression of type-1 IGF-I receptor. We provide evidence that constitutive expression of recombinant IGF-I under the control of the herpes simplex thymidine kinase (TK) and early simian virus 40 (SV40) promoters, in transfected bovine mammary epithelial MAC-T cells, was associated with marked secretion of recombinant IGF-I and IGF-I-induced IGFBP-3. However, overexpression of IGFBP-3 did not prevent down-regulation of the type-1 IGF-receptor in TK-and SV40-IGF-I cells constitutively expressing IGF-I, but potentiated the mitogenic effects of recombinant IGF-I on parental MAC-T cells.

MATERIALS AND METHODS

Construction of IGF-I cDNA Expression Vectors and Development of Stable IGF-I-expressing Cell Lines. A 0.7-Kb fragment encoding an exon-2 containing IGF-I preproIGF-I

cDNA (Ohlsen et al., 1993) was isolated from plasmid A21 by a BglII restriction digest and purified from a low-melting agarose gel using the Geneclean system (BIO 101, Inc., La Jolla, CA). The 0.7-Kb IGF-I fragment (oIGF-I) was cloned into the BglII sites of expression vectors pXT1 (Stratagene, La Jolla, CA) containing the Herpes Simplex Thymidine Kinase promoter (TK) and pSG5 (Stratagene) containing the early SV40 promoter (Figure 1). Thus, the oIGF-I was under the control of the TK- (pTK-IGF-I) and SV40 (pSV40-IGF-I) promoters.

MAC-T cells (Huynh et al., 1991) were plated at a density of 2 x 10^5 in 60-mm tissue culture plates and cotransfected, using the calcium phosphate procedure, with the constructs pTK-IGF-I and pSV40-IGF-I and a plasmid containing a cassette for resistance to hygromycin-B (HYG-B) (Blochlinger and Diggelman, 1984). After incubation for 48 h at 37^{o} C, cells were placed in selective medium containing 200 μg/ml HYG-B (Sigma, St. Louis, MO). Resistant cells were selected after 14 days, and single cell-colonies cloned by the limiting dilution procedure using 96-well plates. Integration of the IGF-I contructs was assessed by Southern analysis (Sambrook et al., 1989). Genomic DNA was digested with the restriction endonuclease EcoRI, and fractionated on 1% agarose gels. Southern blots were probed with a 0.7-Kb oIGF-I cDNA labeled by nick

translation. Details concerning the cloning strategy of the oIGF-I cDNA downstream of the mouse mammary tumor virus-long terminal repeat (pMMTV-IGF-I) used to generate the cell line MD-IGF-I (MD=Mammary Derived) are described elsewhere (Romagnolo et al., 1992).

Northern Blot Analysis. Parental MAC-T and transfected MD-, TK-, and, SV40-IGF-I cells were plated in 100-mm tissue culture plates in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FCS with 200 μg/ml HYG-B. When cells were at 85% confluency, media were removed, and cells washed with Dulbecco's Phosphate Buffered Saline (PBS). Then, cells were cultured in fresh DMEM in the absence or presence of 0.1 μM dexamethasone (DEX). Cells were trypsinized after 72 h, and total RNA was extracted using a guanidinium thiocyanate isolation procedure (Puissant and Houdebine, 1990) and separated on a 1% agarose gel containing 2.2 M formaldehyde. Northern blots (Nitroplus, MSI, Inc., Westborough, MA) were hybridized overnight with a labeled 0.7-Kb oIGF-I fragment at 42 C and washed at high stringency according to the manufacturer's instruction (MSI).

Changes in expression of IGFBP-3 were monitored by hybridizing Northern blots of total RNA (10 μg) with an IGFBP-3 cDNA probe prepared by nick translation of a 440-bp

XbaI-HindIII cDNA fragment isolated from plasmid rIGFBP-3 (Smith et al., 1990). Expression of IGF-I receptor (IGF-IR) in parental MAC-T and IGF-I transfected cells cultured in the presence or absence of 0.1 μM DEX or in response to treatment with IGF-I and IGF-I analogs Des(1-3)IGF-I (Gropep, Adelaide, Australia) and LongR^{3}IGF-I (Gropep) was ascertained by Northern analysis of total RNA (30 μg) with an EcoRI-HindIII fragment of the rat IGF-IR cDNA cloned in plasmid pGEM3 (Werner et al., 1989).

IGF-I Radioimmunoassay and Western Ligand Blotting. Detection of media IGF-I was performed by radioimmunoassay, as previously described (Shimamoto et al., 1992). Briefly, 100 μl of media conditioned by parental MAC-T and transfected IGF-I cells, in the presene or absence of 0.1 μM DEX, was extracted in 2 M formic acid/methanol (1:7), v/v at 4^{o} C, for 2 h. After samples were centrifuged and organic solvents removed by rotary vacuum evaporation, pellets were resuspended in RIA buffer (30 mM sodium phosphate, 10 mM EDTA, 0.02% protamine sulfate, 0.05% Tween-20, pH 8.0) for assay.

Secretion of IGFBPs into conditioned media by MAC-T cells and IGF-I transfected cells in response to treatment with 0.1 μM DEX or IGF-I, Des(1-3)IGF-I, and LongR^{3}IGF-I was studied by western ligand blotting (Hossenlopp et al.,

1986). Medium samples (200 μl) were concentrated by lyophilization and reconstituted in water. Then, samples were dissolved in non-reducing SDS-PAGE buffer and separated at constant current on a 12.5% SDS-PAGE. Proteins were transferred to nitrocellulose membranes which were incubated with [^{125}I]-IGF-I. Ligand blots were exposed at -70^{o} C for 7-14 days. Relative secretion of IGFBPs by MAC-T and transfected IGF-I cells was estimated by densitometric scanning of western ligand blots. Values were expressed as arbitrary densitometric units (ADU) normalized with total DNA (μg/well).

Mitogenic Activity of Recombinant IGF-I. Parental MAC-T and IGF-I transfected cells were cultured for 96 and 144 h in DMEM, DMEM plus 0.1 or 1.0 μM DEX, DMEM plus 0.1 or 1.0 μg/ml insulin (I), and DMEM with 5% FCS. At the end of the incubation periods, cells were harvested, and total DNA (μg/well) was determined (LaBarca and Paigen, 1980).

To assess the mitogenic activity of recombinant IGF-I in concomitance with altered secretion of IGFBPs, parental MAC-T and IGF-I transformed cells were cultured in DMEM in the absence or presence of 0.1 μM DEX. At the end of the incubation period, conditioned media (CM) were harvested and used to culture MAC-T cells in CM, CM plus 10 ng/ml IGF-I, or CM plus 10 ng/ml Des (1-3)IGF-I. [^{3}H]thymidine

incorporation (ICN Biomedicals, Inc., Costa Mesa, CA) into MAC-T cells was measured after 16 h, with a 2-h pulse, as previously described (Zhao et al., 1992).

IGF-I Receptor Binding Experiments. Receptor binding studies were carried out as previously described (Zhao et al., 1992). Parental MAC-T and IGF-I transfected cells were plated at a density of 1.5×10^5 in 24-well tissue culture plates. After 24 h, media were removed and cells cultured at 4^{o} C in 1 ml receptor buffer for 72 h containing 0.5 ng/ml[^{125}I]-labeled LongR^{3}IGF-I and increasing amounts of unlabeled LongR^{3}IGF-I, 100 ng/ml IGF-I or 100 ng/ml insulin. At the end of the incubation period, media were removed and cells rinsed once with PBS (Phosphate Buffered Saline) receptor buffer and twice with PBS. Then, cells were incubated overnight in 0.3 N NaOH and aliquots counted. Dissociation constants (Kd) and binding capacity (B_o) were derived by Scatchard analysis (Scatchard, 1949) as previously described (Munson and Rodbard, 1980).

RESULTS

IGF-I cDNA expression vectors and transfection of MAC-T cells. Parental MAC-T cells were cotransfected with IGF-I expression vectors containing an IGF-I cDNA under the control of the mouse mammary tumor virus long-terminal repeat (pMMTV-LTR) (Romagnolo et al., 1992), herpes simplex thymidine kinase (pTK) (Boutler and Wagner, 1987), and early simian virus (pSV40) (Green et al., 1988) promoters (Figure 1) and a plasmid conferring resistance to the antibiotic HYG-B. Following selection of transfected cells in media containing HYG-B, integration of the IGF-I constructs was ascertained by Southern blot analysis of genomic DNA. Stable transformants contained single or multiple copies of the IGF-I constructs. For further studies, clones were selected based on expression levels of IGF-I and their ability to proliferate autonomously in serum-free media. For this purpose total DNA was used as an indicator of cell proliferation (data not shown).

Expression of recombinant IGF-I and IGFBP-3. Expression of IGF-I mRNA by MAC-T and transfected MD-, SV40-, and TK-IGF-I cells was investigated by Northern blot analysis of total RNA (15 μg) extracted from cells cultured in the presence or absence of 0.1 μM DEX (Figure 2). Parental

MAC-T cells expressed no detectable IGF-I mRNA species, irrespective of the presence or absence of DEX. However, induction with DEX of the glucocorticoid-sensitive MMTV-LTR contained in MD-IGF-I cells was required to enhance expression of IGF-I mRNA. In contrast, the SV40- and TK-promoters were constitutively active and expressed the highest levels of IGF-I transcripts. The length of the transcripts was in accordance with the predicted size of mRNAs transcribed from pMMTV-, pSV40- and pTK-IGF-I. However, for cells containing the pSV40-IGF-I construct transcripts of various molecular weights were detected by Northern blotting. Likely, they represented unprocessed mRNA transcripts.

Expression of IGFBP-3 by parental MAC-T and IGF-I transfected cells was studied by hybridizing Northern blots of total RNA (15 μg) with a nick-translated rat IGFBP-3 cDNA probe (Figure 3). No IGFBP-3 mRNA species were detectable for MAC-T cells in the presence or absence of DEX, or MD-IGF-I cells cultured in control DMEM. However, IGFBP-3 transcripts (~2.5-Kb) for MD-IGF-I cells were stimulated in the presence of DEX-induced expression of IGF-I. Conversely, clones SV40- and TK-IGF-I constitutively expressed high levels of IGFBP-3 mRNA in the presence or absence of DEX.

Secretion of recombinant IGF-I and western ligand blot analysis of conditioned media. Secretion of IGF-I by MAC-T and transfected IGF-I cells was investigated by plating the cells in DMEM, in the presence or absence of 0.1 μM DEX. The IGF-I values shown in Table 1 indicate that conditioned media from MAC-T cells contained little IGF-I (on average 0.50 ng/ml), whose levels were not influenced by DEX. In contrast, secretion of IGF-I by the cell line MD-IGF-I was stimulated by DEX ($P<.01$), and was 14.8 ng/ml at 48 h and 10.3 ng/ml at 192. As expected, secretion of IGF-I by TK-and SV40-IGF-I cells was constitutive. Specifically, the cell line SV40-IGF-I secreted the highest levels ($P<.01$) of IGF-I at 96 h (38.6 ng/ml), and remained relatively high (32.1 ng/ml) up to 192 h. Concentrations of IGF-I in media conditioned by TK-IGF-I were relatively lower, and ranged from 2.4 ng/ml at 48 h to 4.31 ng/ml at 192 h.

To assess the relative IGF-I secretory ability of the various IGF-I cell lines, conditioned media from MAC-T and IGF-I transfected cells were collected after 72 h following induction with or without 0.1 μM DEX. The average IGF-I concentrations from two independent experiments are summarized in Table 2. Levels of IGF-I in conditioned media from MAC-T cells were very low, and ranged from 9.0 to 7.5 ng IGF-I/10^6 cells. Conversely, secretion of IGF-I by MD-IGF-I cells ranged from 27.2 to 99.5 ng/10^6 cells),

respectively in the absence or presence of DEX. These IGF-I concentrations were comparable with those measured for TK-IGF-I (31.6-65.0 ng/10^6 cells). Compared with parental MAC-T cells, the activity of the SV40 promoter in SV40-IGF-I cells resulted in markedly enhanced secretion of IGF-I (64.0-fold), with levels ranging from 578.4 to 419.0 ng/10^6 cells.

Ligand blotting analysis of conditioned media from MAC-T and IGF-I transfected cells is depicted in Figure 4. Parental MAC-T cells secreted primarily an IGFBP species of 29-32 KDa, identified by western immunobloting as IGFBP-2 (data not shown), which was not affected by DEX (Figure 4A). Conversely, SV40- and TK-cells secreted primarily an IGFBP species of 40-42 KDa, identified as IGFBP-3, whose levels were also not affected by treatment with DEX. Consistent with the results of the Northern analysis shown in Figure 3, secretion of IGFBP-3 by MD-IGF-I cells containing the MMTV-LTR IGF-I transgene was stimulated in the presence of DEX-induced secretion of IGF-I. In addition, lower levels of IGFBP-3 were secreted by MD-IGF-I cells even in the absence of DEX, which is also consistent with the secretion of low levels of IGF-I in the absence of stimulation of the MMTV-LTR promoter.

Densitometric analysis of the ligand blots was performed to quantitate the relative expression of IGFBP-3 by MAC-T

and IGF-I transfected cells (Figure 4B). Production of IGFBP-3 was expressed as arbitrary densitometric units (ADU) corrected for total DNA (ADU/μg DNA). Expression of IGFBP-3 by MAC-T cells was very low, and ranged from 0.8 to 0.7 ADU, in the absence or presence of DEX, respectively. As expected, production of IGFBP-3 by MD-IGF-I cells was greatly enhanced (7.6-fold) by secretion of DEX-induced IGF-I (0.6-4.8 ADU). This was comparable with the levels observed for TK-IGF-I cells (5.6-5.7 ADU). Conversely, compared with MAC-T cells, expression of IGFBP-3 by SV40-IGF-I cells resulted in a 17.8- (13.2 ADU) and 14.4-fold (10.4 ADU) increase in production of IGFBP-3, in the absence or presence of DEX, respectively.

Mitogenic actions of secreted IGF-I and exogenous insulin. To examine the biological activity of endogenously produced recombinant IGF-I secreted by the various IGF-I clones in association with altered secretion of IGFBPs, cells were cultured for 96 h in the presence of two levels of DEX (0.1 vs 1.0 μM), and 5% FCS (Figure 5). When cultured in DMEM, total DNA (μg/well) measured at the end of the incubation period was similar for all cell types, in spite of the high constitutive expression of IGF-I by SV40- and TK-IGF-I cells. Moreover, the addition of 0.1 μM or 1.0 μM DEX equally induced a 1.5-fold increase

in total DNA of MD-IGF-I cells, but as expected had no effect on cell proliferation of SV40-and TK-IGF-I cells. In contrast, total DNA of TK- and SV40-IGF-I cells cultured in 5% FCS was increased 4.1- and 3.6-fold (13.5 and 10.6 μg/well) compared with DMEM (3.3 and 3.0 μg DNA/well), whereas total DNA of MAC-T and MD-IGF-I cells was stimulated only 2.0- (6.2 vs 3.0 μg/well) and 2.2-fold (6.5 vs 3.0 μg/well) by the addition of 5% FCS.

Cell responsiveness of parental MAC-T and IGF-I cells to insulin (I) was investigated by culturing the cells for 72 and 144 h in DMEM, DMEM with two levels of I (0.1 and 1.0 μg/ml), and DMEM with 5% FCS (Figure 6). These experiments indicated that treatment with I (1.0 μg/ml) augmented total DNA of MAC-T cells by 1.9- (4.3 μg/well) and 2.4-fold (8.6 μg/well) at 72 and 144 h, respectively, compared with DMEM (2.3 vs 3.6 μg DNA/well). Similarly, MD-IGF-I cells responded to the addition of I (1.0 μg/ml) with a 2.1- (4.9 μg/well) and 2.5-fold (9.4 μg/well) increase in total DNA at 72 and 144 h, compared with DMEM (2.4 and 3.8 μg DNA/well). TK-IGF-I cells responded also to 1.0 μg/ml I with a 2.0- (4.9 vs 2.3 μg/well) and 2.1-fold (5.1 vs 2.3 μg/well) increase in total DNA at 72 and 144 h, respectively. Moreover, the addition of 5% FCS stimulated a 4.3- (10.2 vs 2.3 μg/well) and 6.9-fold (15.8 vs 2.3 μg/well) increase in total DNA of TK-IGF-I cells,

respectively, at 72 and 144 h. Conversely, SV40-IGF-I cells were refractory to stimulation with I, and only treatment of these cells with 5% FCS induced a significant ($P<.01$) 3.5-fold increase (9.5 vs 2.7 μg/well) in total DNA at 72 h, and a 4.9-fold (14.3 vs 2.9 μg/well) increase at 144 h, compared with control DMEM.

Therefore, these experiments suggested that cell growth of MD-IGF-I cells was stimulated by secretion of IGF-I resulting from inducible expression of IGF-I mRNA from the MMTV-LTR promoter. In contrast, constitutive overexpression of IGF-I by the SV40-IGF-I cells was associated with loss of the proliferating phenotype characteristic of parental MAC-T cells incubated with IGF-I or insulin. Intermediate expression of IGF-I by TK-IGF-I cells resulted in a minimal proliferative response to insulin.

Effects of Secreted IGFBPs on Bioactivity of IGF-I. To test whether secretion of IGF-I-induced IGFBP-3 by transfected cells, compared with IGFBP-2 secreted by parental MAC-T cells, potentiated cell responsiveness to IGF-I, cells were cultured for 72 h, in the presence or absence of 0.1 μM DEX. Thereafter, test MAC-T cells were cultured for 16 h in conditioned media (CM), CM supplemented with exogenous IGF-I, or CM plus Des(1-3)IGF-I, and pulsed for 2 h with [^{3}H]thymidine. The

results of these experiments are depicted in Figure 7. [^{3}H]thymidine incorporation into DNA of MAC-T cells cultured in CM from MD-IGF-I cells induced with DEX was stimulated 1.8-fold (15.0 cpmx10^3/well) compared with CM from parental MAC-T cells (8.0 cpmx10^3/well) (Figure 7A). Conversely, CM from TK-and SV40-IGF-I cells induced with DEX sustained higher [^{3}H]thymidine uptakes than control media from MAC-T cells, and ranged from 58.9 cpmx10^3/well (7.2-fold) to 47.8 cpmx10^3/well (5.8-fold).

The further addition of exogenous IGF-I (Figure 7B) to CM from MAC-T cells triggered a modest 1.6-fold increase in [^{3}H]thymidine incorporation into DNA of test MAC-T cells (13.2 vs 8.2 cpmx10^3/well), compared with a sharp 3.0-fold increase ($P<.01$) stimulated by CM from MD-IGF-I cells cultured with (45.8 cpmx10^3/well) or without (29.9 cpmx10^3/well) DEX. Conversely, the addition of IGF-I to CM from SV40-and TK-IGF-I cells had no effect on [^{3}H]thymidine incorporation by parental MAC-T cells. When the IGF-I analog Des(1-3), which has equal affinity for the type 1 IGF-I receptor as its native counterpart but a lower affinity for IGFBPs, was substituted for IGF-I (Figure 7C) a significant 6.8- and 4.9-fold increase in [^{3}H]thymidine incorporation was stimulated by CM from parental MAC-T and MD-IGF-I cells, respectively. In contrast, the Des(1-3)IGF-I analog did not induce any additional

[^{3}H]thymidine incorporation when added to CM from either SV40- or TK-IGF-I cells.

Taken together, these data suggested that IGFBP-3 containing media from MD-, SV40-, and TK-IGF-I cells potentiated the mitogenic activity of exogenous IGF-I, as compared to media from parental MAC-T cells containing IGFBP-2. Nevertheless, when the Des(1-3)IGF-I analog was added to CM from both MAC-T and MD-IGF-I cells to remove interactions with IGFBPs, both CMs equally stimulated [^{3}H]thymidine incorporation into DNA of MAC-T cells.

Expression of type 1 IGF-I receptor (IGF-IR) and binding studies. Effects of secretion of recombinant IGF-I and IGF-I-induced IGFBP-3 on expression of IGF receptor (IGF-IR) in transfected IGF-I clones were examined by culturing the cells in DMEM in the presence or absence of 0.1 μM DEX. Changes in expression of IGF-IR were investigated by Northern blot analysis (Figure 8) of total RNA (30 μg). Two mRNA species of 6.0- and 12.0-Kb (Figure 8A) hybridized with a rat IGF-IR cDNA probe. This pattern of hybridization was similar for parental MAC-T and IGF-I transfected cells and levels of the IGF-IR transcripts were not influenced by treatment with DEX. Moreover, there was no indication of downregulation of the IGF-I receptor transcripts due to overexpression of IGF-I by MD-, SV40-,

and TK-IGF-I cells. Neither did the addition of 40 ng/ml of exogenous IGF-I or the IGF-I analogs Des(1-3)IGF-I and LongR^{3}IGF-I induce downregulation of the IGF-IR transcripts in MD-IGF-I cells (Figure 8B). In contrast, Northern blot analysis of total RNA from MD-IGF-I cells hybridized with an IGFBP-3 cDNA probe revealed that native IGF-I and IGF-I analogs stimulated transcription of IGFBP-3 in the order Des(1-3)IGF-I > LongR^{3}IGF-I > IGF-I, which was associated with augmented secretion of IGFBP-3 into conditioned media, compared with control DMEM (data not shown). Therefore, these data suggested that secretion of recombinant IGF-I or the addition of native or IGF-I analogs did not influence transcription of the IGF-IR, but affected significantly the expression of IGFBP-3.

To examine whether constitutive versus acute overexpression of IGF-I-induced IGFBP-3 influenced levels of IGF-IR at the cell-surface, parental MAC-T, MD-, SV40-, and TK-IGF-I cells were incubated with 0.5 ng/ml [^{125}I]-LongR^{3}IGF-I and increasing amounts of unlabeled IGF-I and LongR^{3}IGF-I. Investigation of time-dependent uptake of [^{125}I]-labeled IGF-I revealed that specific binding increased over time and reached a plateau at 72 h at 4^{o} C. Therefore, all the subsequent binding studies were performed for 72 h at 4^{o} C.

The data summarized in Figure 9 depict competitive

displacement curves for MAC-T and IGF-I transfected cells. In Figure 9A values are specific binding expressed as percentage of total radioactivity in the presence of increasing amounts of unlabelled LongR^3IGF-I. Nonspecific binding was estimated as the amount of bound radioactivity in the presence of 128 ng/ml of unlabeled LongR^3IGF-I. In the absence of unlabeled hormone, total binding was the same for parental MAC-T and MD-IGF-I cells, and the addition of increasing amounts of unlabeled LongR^3IGF-I equally displaced radiolabeled hormone, with no apparent differences in binding affinity. In contrast, total specific binding for TK- and SV40-IGF-I cells was 1.9- and 5.9-fold lower compared with that of MAC-T and MD-IGF-I cells. For SV40-IGF-I cells there was virtually no decrease in binding due to competition with increasing amounts of unlabeled LongR^3IGF-I. Moreover, while there were no differences across cell types in specific bound activity in the presence of 100 ng/ml IGF-I or LongR^3IGF-I, the addition of 100 ng/ml insulin (Figure 9B) further demonstrated that SV40- and TK-IGF-I cells exhibited lower binding capacity (3.2- and 1.6-fold) than parental MAC-T and MD-IGF-I cells.

Analysis of the dissociation constants (Kd) and binding capacities (Bo) (Table 3) by Scatchard analysis revealed that parental MAC-T cells exhibited the highest binding

capacity (21.5 x 10^{-12}M/10^5 cells) followed by MD- (19.3 x 10^{-12}M/10^5 cells), and TK-IGF-I cells (11.3 x 10^{-12}M/10^5 cells). This contrasted with a lower value of 3.6 x 10^{-12}M/10^5 cells observed for SV40-IGF-I cells. The Kd for SV40- and TK-IGF-I cells ranged from 6.0 to 7.7 x 10^{-9}M/l, respectively. More interestingly, in spite of the marked differences in receptor occupancy, both MAC-T and SV40-IGF-I cells exhibited similar Kd (5.5 vs 6.0 x 10^{-9}M/l), which suggested that reductions in receptor occupancy for SV40-and TK-IGF-I cells were not affected by changes in receptor binding affinity. These observations were also supported by estimation of similar correlation coefficients (r) for each of the straight lines, which provided a measure of closeness-of-fit of the linear regression models used to estimate the dissociation constants.

DISCUSSION

We have previously reported on the development of the cell line MD-IGF-I (Romagnolo et al., 1992) containing an ovine IGF-I cDNA under the control of the glucocorticoid-inducible MMTV-LTR promoter. The cell line MD-IGF-I proved a useful system to investigate stimulation

of cell proliferation by endogenously produced recombinant IGF-I through an autocrine/paracrine pathway (Romagnolo et al., 1993). In these cells, secretion of recombinant IGF-I induced, through an autocrine loop, expression of IGFBP-3, which in turn potentiated the mitogenic actions of IGF-I (Romagnolo et al., submitted).

In this study, we have shown that parental MAC-T cells expressed the lowest levels of IGF-I and secreted primarily IGFBP-2 into conditioned media. Expression of IGFBP-2 by MAC-T cells was not perturbated by treatment with DEX (Figure 4) or IGF-I (data not shown). Conversely, secretion of recombinant IGF-I by transfected cells stimulated a marked increase of IGFBP-3 in conditioned media. Specifically, expression of IGFBP-3 was hormonally regulated in MD-IGF-I cells by the secretion of DEX-induced IGF-I, whereas cell lines SV40- and TK-IGF-I constitutively expressed the highest levels IGF-I and IGFBP-3, irrespective of treatment with DEX. These effects were reproducible in MD-IGF-I cells by treatment with I, IGF-I, and the IGF-I analogs Des(1-3)IGF-I and LongR^{3}IGF-I (Romagnolo et al., submitted). Therefore, we concluded that changes in IGFBP-3 expression observed in transfected cells were mediated by a type-1 IGF receptor-dependent process. These data are in disagreement with previous reports (Conover, 1991) in which stimulation of human fibroblasts

through a receptor-independent process.

We also demonstrated in this study that enhanced production of IGFBP-3 was peculiar of the IGF-I-transfected cells. Our results were consistent with those previously reported for normal and SV40-transformed human fibroblasts (Conover, 1991), in which IGF-I increased the production of a 38-42-KDa IGFBP-3. Similarly, Martin and Baxter (1988) reported an increase in expression of IGFBP-3 by human skin fibroblasts stimulated with IGF-I. However, it is important to note that enhanced expression of IGFBP-3 in our transfected IGF-I cells required the endogenous synthesis of IGF-I, since exogenous IGF-I did not induce IGFBP-3 production by parental MAC-T cells.

Experimental evidence supports the concept that IGFBP-3 potentiates the autocrine/paracrine mitogenic actions of IGF-I (Baxter and Martin, 1989), likely by inducing changes in IGFBP-3 and type-1 IGF receptor responsiveness (Conover, 1992). Nevertheless, others have proposed a negative role for IGFBP-3 on IGF-I actions (Cohen et al., 1993; Conover et al., 1990;). Although cell proliferation of MD-IGF-I cells was stimulated by secretion of recombinant IGF-I, constitutive overexpression of IGF-I by both SV40- and TK-IGF-I cells was associated with lack of autonomous cell growth. Loss of cell proliferation was not reverted by secretion of IGF-I-induced IGFBP-3. In contrast, treatment

secretion of IGF-I-induced IGFBP-3. In contrast, treatment with 5% FCS supported faster cell growth of these cells, compared with parental MAC-T and MD-IGF-I cells. It is not known whether FCS enhanced directly the mitogenic actions of recombinant IGF-I on TK-and SV40-IGF-I cells. Aside from possible effects of other growth factors, binding proteins present in FCS may have enhanced cell proliferation of these cells, perhaps, by sequestering recombinant IGF-I secreted into conditioned media, thus preventing down-regulation of the IGF-IR. This observation is supported by the notion that the IGF-binding protein complex is a better mitogen than free IGF-I (Blum et al., 1989).

The presence of IGFBP-3 in CM from SV40- and TK-IGF-I cells induced the highest [^{3}H]thymidine uptakes by test MAC-T cells, compared with CM from parental MAC-T and MD-IGF-I cells. Furthermore, addition of exogenous IGF-I to CM from MD-IGF-I cells resulted in even greater [^{3}H]thymidine incorporation into DNA of MAC-T cells. The observation that stimulation of [^{3}H]thymidine uptake by IGF-I was somewhat reduced by CM from MAC-T cells suggested that presence of IGFBP-2 attenuated cellular responsiveness to IGF-I. This conclusion was supported by the observation that the addition of the IGF-I analog Des(1-3), which has a lower affinity for IGFBPs than its native counterpart, to

conditioned media from MAC-T cells did stimulated [^{3}H]thymidine uptake by MAC-T cells. The potentiating effect of IGFBP-3 on IGF-I actions in our bovine mammary epithelial cells is supported by earlier reports (DeMellow and Baxter, 1988) which have demonstrated that IGFBP-3 enhanced cell responsiveness to IGF-I in human skin fibroblasts, likely through association of IGFBP-3 with the cell surface (Conover et al., 1990).

The hypothesis that constitutive expression of IGF-IR in TK- and SV40-IGF-I cells downregulated expression of the IGF-IR was supported by the early observation that both TK- and SV40-IGF-I cells were relatively refractory to stimulation with I, compared with parental MAC-T and MD-IGF-I cells. We tested this concept first by Northern analysis of total RNA extracted from parental MAC-T and IGF-I cells cultured in the absence or presence of DEX. We found that parental MAC-T and IGF-I transfected cells expressed primarily two IGF-IR mRNAs of 6.0- and 12.0-Kb. This pattern of hybridization was similar to that found for human RNA (Ullrich et al., 1986). In transfected cells, overexpression of IGF-I did not cause downregulation of the IGF-IR mRNAs. Neither did the treatment of MD-IGF-I cells with IGF-I or the IGF-I analogs Des(1-3) and LongR^{3}IGF-I reduce expression of IGF-IR mRNAs. Therefore, these data suggested that in IGF-I transfected cells regulation of

expression of IGF-IR was exerted at the posttranscriptional level. It is possible that decrease in receptor number may be due to a more rapid recycling of the IGF-IR molecules at the cell surface.

Positive confirmation of constitutive downregulation of the IGF-IR at the cell-surface in TK-and SV40-IGF-I cells was provided by IGF-IR binding studies. The IGF-I analog LongR^{3}IGF-I, along with the native counterpart, was utilized because the LongR^{3}IGFI analog binds with equal affinity to the IGF-IR, but displays a lower affinity for IGFBPs than native IGF-I. Therefore, the mitogenic effects of the LongR^{3}IGF-I would not be confounded by interactions with IGFBPs, and would allow a more accurate interpretation of the results from the IGF-I receptor binding studies.

Increasing amounts of unlabeled LongR^{3}IGF-I diplaced in a dose-dependent manner [^{125}I]LongR^{3}IGF-I bound to cell surface IGF-IR. These data were in agreement with previous IGF-I binding studies with parental MAC-T cells (Zhao et al., 1991). A lower affinity component was not saturated by excess amounts of cold LongR^{3}IGF-I, which was therefore considered binding to nonspecific proteins. Cell-surface IGF-IR availability was significantly lower in TK- and SV40-IGF-I cells, compared with parental MAC-T and MD-IGF-I cells. These data suggested that first, in SV40- and TK-IGF-I cells constitutive expression of recombinant IGF-I

stimulated down-regulation of IGF-IR at the cell surface, and secondly that a new steady-state concentration of IGF-IR was brought about in these cells as a result of increased concentrations of local IGF-I. These results were consistent with those of binding studies of cultured lymphocytes (Kosmakos and Roth, 1980) in which chronic stimulation with insulin decreased the total number of insulin receptors. Similarly, exposure of human (Rosenfeld and Dollar, 1982) and bovine fibroblasts (Conover and Powell, 1991) to IGF-I triggered a rapid decrease in IGF-I binding capacity. Therefore, the results of our binding studies supported the conclusion that lack of growth stimulation of TK- and SV40-IGF-I cells in response to constitutive secretion of recombinant IGF-I or stimulation with exogenous insulin was due to loss of binding capacity. While previous reports suggested a role for IGFBP-3 in preventing IGF-IR down-regulation and cell desensitization in cultured bovine fibroblasts (Conover and Powell, 1991), in this study, overexpression of IGFBP-3 by SV40- and TK-IGF-I cells did not prevent down-regulation of the IGF-IR induced by constitutive expression of IGF-I. It is therefore conceivable to hypothesize that down-regulation of the IGF-IR by constitutive expression of IGF-I or sudden increases in local concentrations of IGF-I may enable cell-derived IGFBP-3 from exerting its potentiating effects

in bovine mammary epithelial cells.

In summary, IGF-I-induced IGFBP-3 appears to potentiate the mitogenic actions of IGF-I in bovine mammary epithelial cells. This effect is exerted through an autocrine loop in which IGFBP-3 may act as a reservoir for IGF-I. The potentiating effects of IGFBP-3, however, are hampered under conditions of chronic expression of IGF-I which induce downregulation of the IGF-IR receptor. Generation of the IGF-I transfected cell lines proved a useful strategy to investigate effects of IGF-I-induced IGFBP-3 on cell proliferation and IGF-IR receptor binding in bovine mammary epithelial cells.

SUMMARY

This study was undertaken to investigate the effects of chronic versus acute expression of IGF-I-induced IGFBP-3 on cellular responsiveness to IGF-I and regulation of expression of the type 1 IGF-receptor (IGF-IR) in transfected bovine mammary epithelial cells. Clonal MAC-T cells were transfected with expression vectors containing an ovine 0.7-Kb exon-2 containing IGF-I cDNA under the control of the constitutively-active herpes simplex thymidine kinase (TK) and early simian virus 40 (SV40) promoters, and glucocorticoid-inducible mouse mammary tumor virus-long terminal repeat (MD-IGF-I) (Mol. Endocriol. 1992, 6:1774-1780). Constitutive expression of IGF-I in TK- and SV40-IGF-I cells sustained chronic secretion of both IGF-I and IGF-I-induced IGFBP-3. Chronic secretion of IGF-I triggered loss of cell responsiveness to IGF-I and insulin in TK- and SV40-IGF-I cells and corresponded to downregulation of the IGF-IR at the cell surface. Loss of binding capacity by these cells was not a reflection of changes in IGF-I binding affinity and was not reverted by secretion of IGF-I-induced IGFBP-3. In contrast, induction of MD-IGF-I cells with the glucocorticoid dexamethasone was required to enhance secretion of IGF-I-induced IGFBP-3, whose secretion was associated with autonomous

proliferation. The presence of IGFBP-3 in conditioned media from MD-, TK-, and SV40-IGF-I cells, as compared to IGFBP-2 in conditioned media from parental MAC-T cells, was associated with enhanced cellular responsiveness of test MAC-T cells to IGF-I, but not Des(1-3)IGF-I. Here, we propose that cell derived IGF-I-induced IGFBP-3 is an important component of an autocrine loop to modulate the mitogenic effects of IGF-I in bovine mammary epithelial cells expressing IGF-I.

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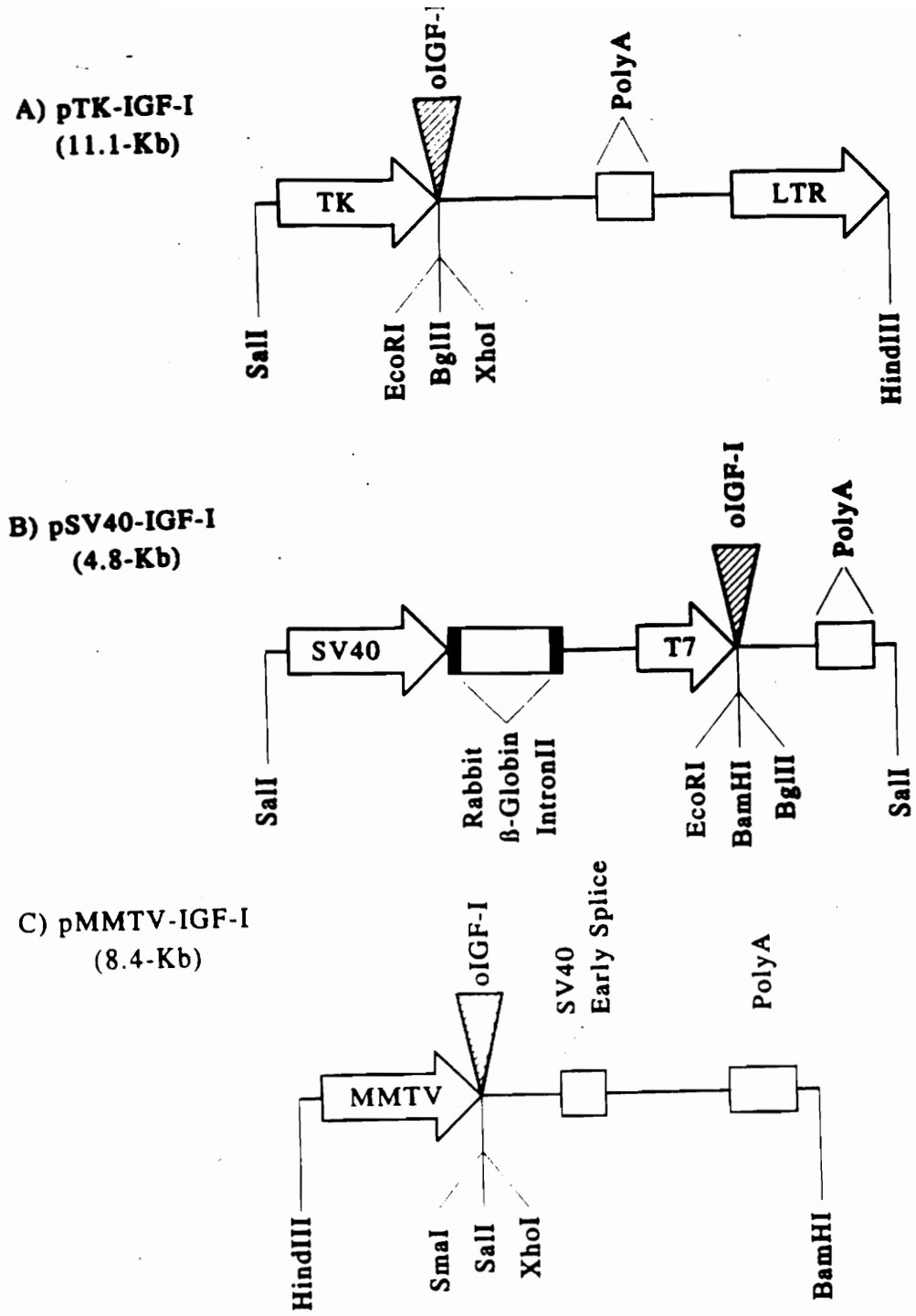

Figure 1. Construction of IGF-I cDNA expression vectors. A 0.7-Kb fragment encoding an exon-2 containing IGF-I preproIGF-I cDNA was cloned into the BglII sites of expression vectors pXT1 (A) containing the herpes simplex thymidine kinase and pSG5 containing the early simian virus 40 (B) promoters. Details concerning the cloning strategy of the IGF-I cDNA downstream of the MMTV-LTR (C) promoter are described elsewhere (Romagnolo et al., 1992). Clonal MAC-T cells were cotransfected by calcium phosphate precipitation with the IGF-I cDNA constructs and a plasmid conferring resistance to hygromycin-B (pHYG-B).

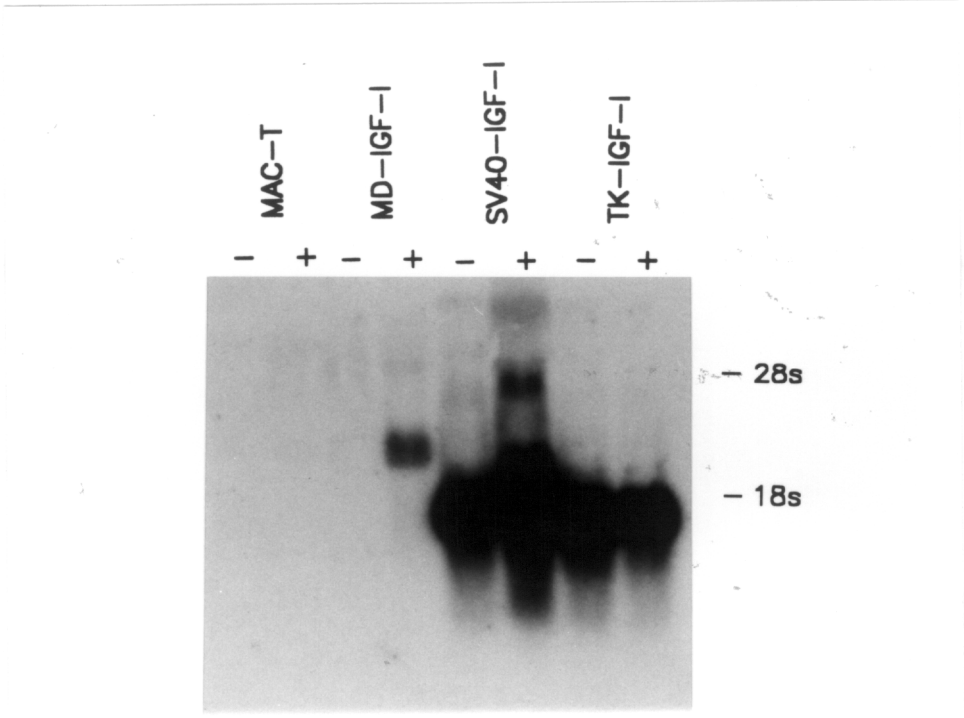

Figure 2. Expression of IGF-I in parental MAC-T and IGF-I transfected cells. Cells were cultured in DMEM in the absence (-) or presence (+) of 0.1 μM DEX. At the end of the incubation period (72 h), total RNA (15 μg) was extracted using a guanidinium thiocyanate procedure and fractionated on a 1% agarose gel. Northern blots were hybridized with a ^{32}P-labeled IGF-I cDNA probe. Migrations of the 28s and 18s ribosomal RNAs are indicated.

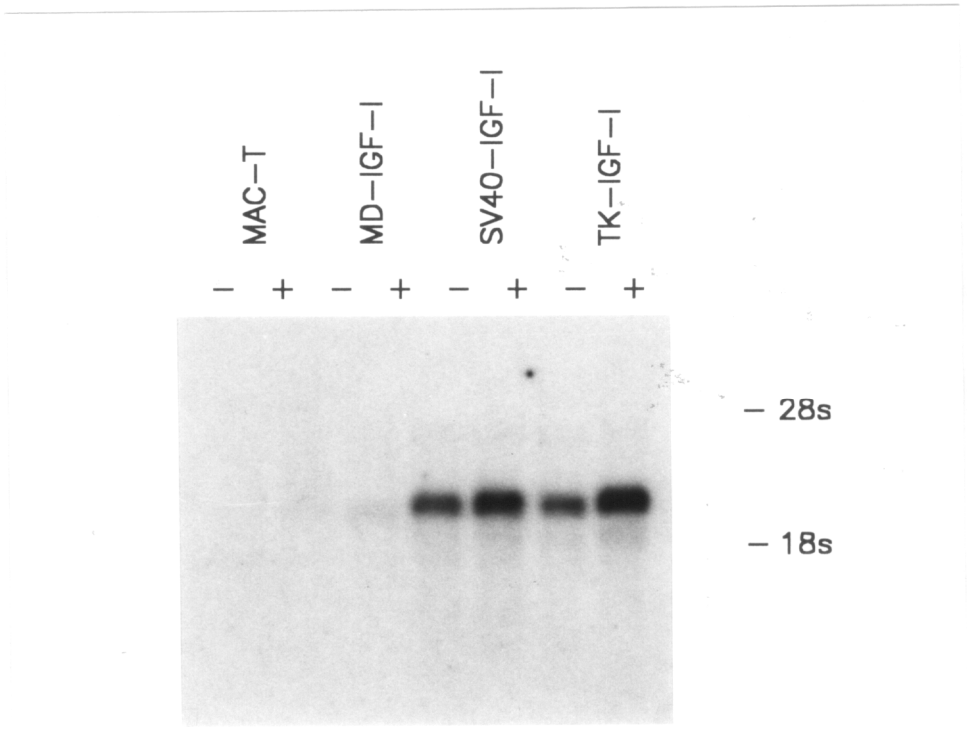

Figure 3. Expression of IGFBP-3 in MAC-T and IGF-I transfected cells. Parental MAC-T cells were cultured for 72 h in the absence (-) or presence (+) of 0.1 μM DEX. Total RNA (15 μg) was separated on a 1% agarose gel and transferred to nitrocellulose membrane. Northern blots were probed with a ^{32}P-labeled rat IGFBP-3 cDNA probe. Migrations of the 28s and 18s ribosomal RNAs are indicated.

TABLE 1. RIA IGF-I OF CONDITIONED MEDIUM FROM PARENTAL MAC-T AND TRANSFECTED IGF-I CELLS AT DIFFERENT TIME INTERVALS

Time	Treatment*	ng IGF-I/ml			
		MAC-T	MD-IGF-I	SV40-IGF-I	TK-IGF-I
48h	DMEM	0.3	2.0^{b}	33.1^{c}	2.2^{c}
	DEX	0.2	14.8^{d}	31.3^{c}	2.4^{c}
96h	DMEM	0.7	1.8^{b}	38.6^{d}	1.7^{b}
	DEX	0.3	9.3^{c}	27.9^{b}	1.2^{a}
144h	DMEM	0.7	2.1^{b}	34.7^{c}	0.9^{a}
	DEX	0.4	8.6^{c}	28.4^{b}	2.4^{c}
192h	DMEM	0.7	0.5^{a}	32.1^{c}	2.1^{c}
	DEX	0.5	10.3^{c}	24.1^{a}	4.3^{d}

*Cells were cultured in DMEM or DMEM plus 0.1 μM DEX for up to 192 h. Media was collected at 48h intervals for estimation of IGF-I by RIA (C.V. < 10%). Values are means obtained from six wells at the end of each incubation period such that four samples/time/treatment were assayed. Values within a column with different superscripts were significantly ($P<.05$) different by Duncan's multiple comparison test.

TABLE 2. EFFECTS OF DEX ON SECRETION OF IGF-I BY PARENTAL MAC-T AND IGF-I TRANSFECTED CELLS

Cell Type*	ng IGF-I/10^6 cells			
	DMEM	SEM	DEX	SEM
MAC-T	9.0^a	0.4	7.5^a	0.2
MD-IGF-I	27.2^b	1.3	99.5^c	9.9
SV40-IGF-I	578.4^c	15.7	419.0^d	10.6
TK-IGF-I	31.6^b	0.6	65.0^b	8.3

*Cells were cultured for 72 h in DMEM or DMEM plus 0.1 μM DEX. At the end of the incubation period conditioned media were collected and IGF-I concentrations estimated by RIA. Values are the mean$\pm$SEM of three samples assayed in duplicate from two separate experiments. Values within a column with different superscripts were significantly ($P<.05$) different by Duncan's multiple comparison test.

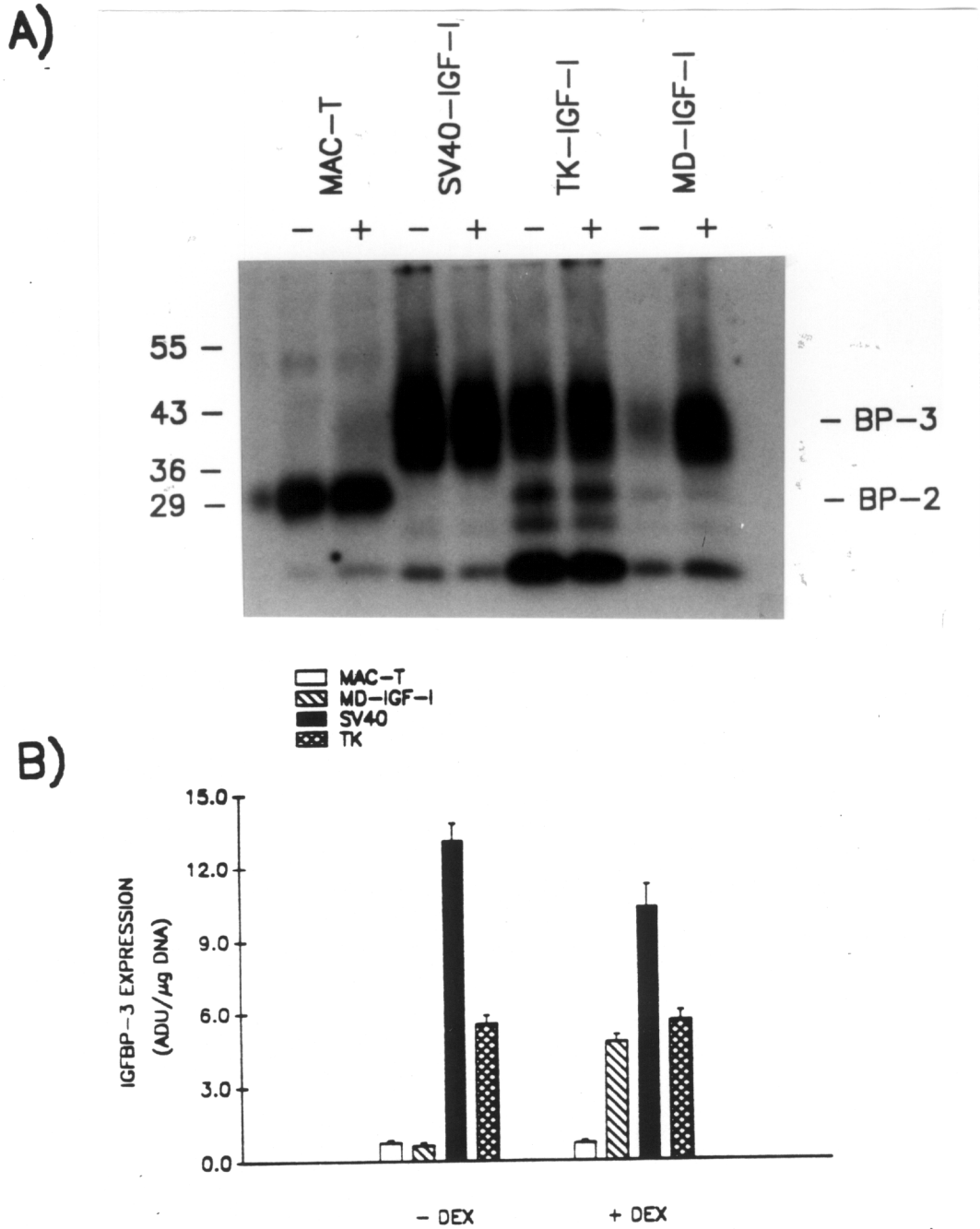

Figure 4. A) Western ligand blot analysis of IGF-binding proteins secreted into conditioned media by parental MAC-T and IGF-I transfected cells. Cells were cultured for 72 h in the absence (-) or presence (+) of 0.1 μM DEX. Conditioned media were collected at the end of the incubation period for ligand blot analysis. Proteins in media were separated by SDS-PAGE and transferred to nitrocellulose membranes. Blots were probed with a[^{125}]I-IGF-I-labeled probe. Migrations of the molecular weight markers (KDa) are indicated. B) Relative secretion of IGFBP-3 by MAC-T and IGF-I transfected cells was estimated by densitometric scanning of western blots. Values are expressed as arbitrary densitometric units (ADU) and normalized with total DNA (μg/well).

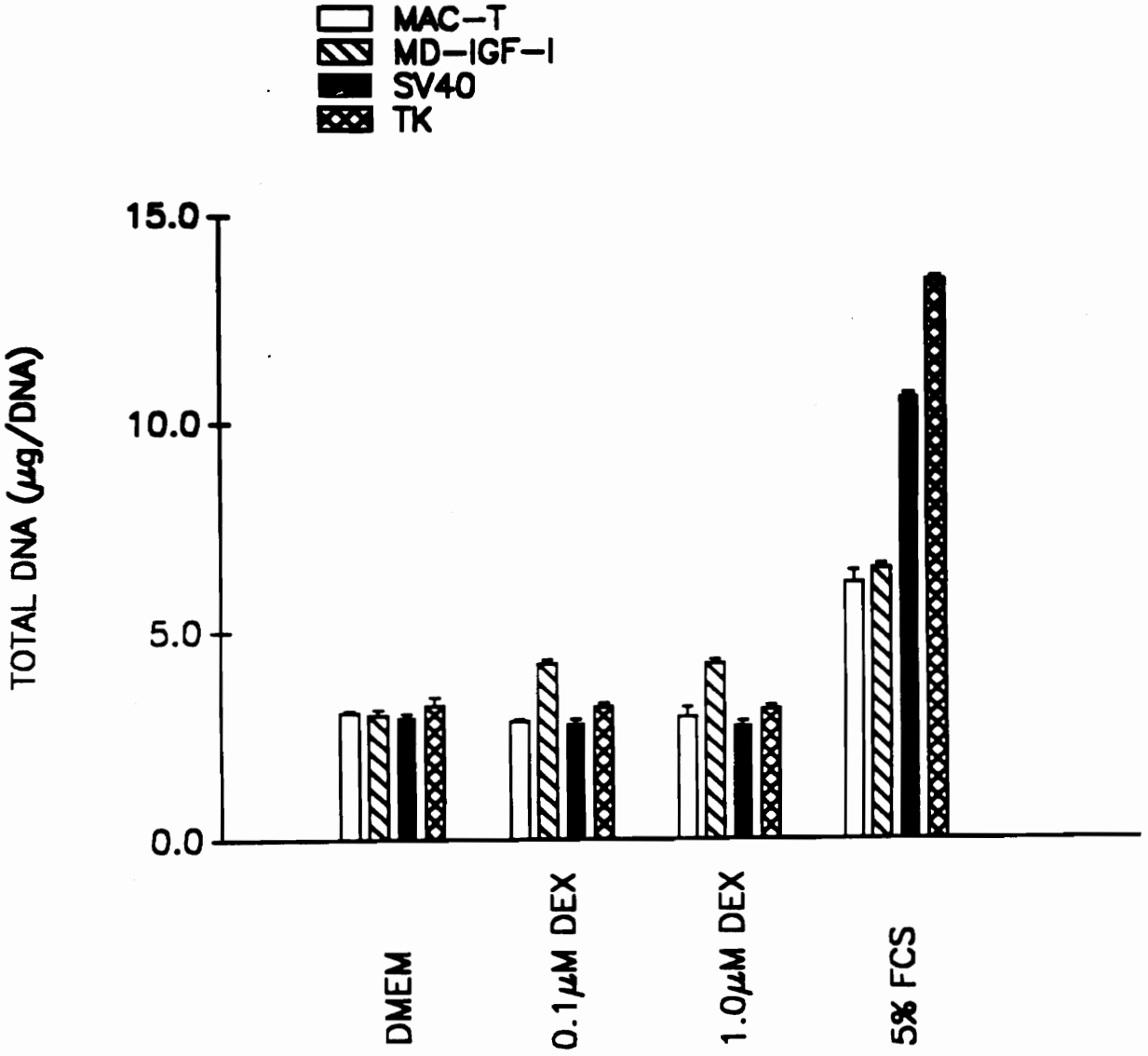

Figure 5. Effects of DEX on growth of parental MAC-T and IGF-I transfected cells. MAC-T and IGF-I transfected cells were cultured for 96 h in DMEM, DMEM with 0.1 or 1.0 μM DEX, and DMEM plus 5% FCS. At the end of the incubation period cells were harvested and total DNA (μg/well) was determined. Bars represent the mean$\pm$SEM values from six samples, assayed in duplicate.

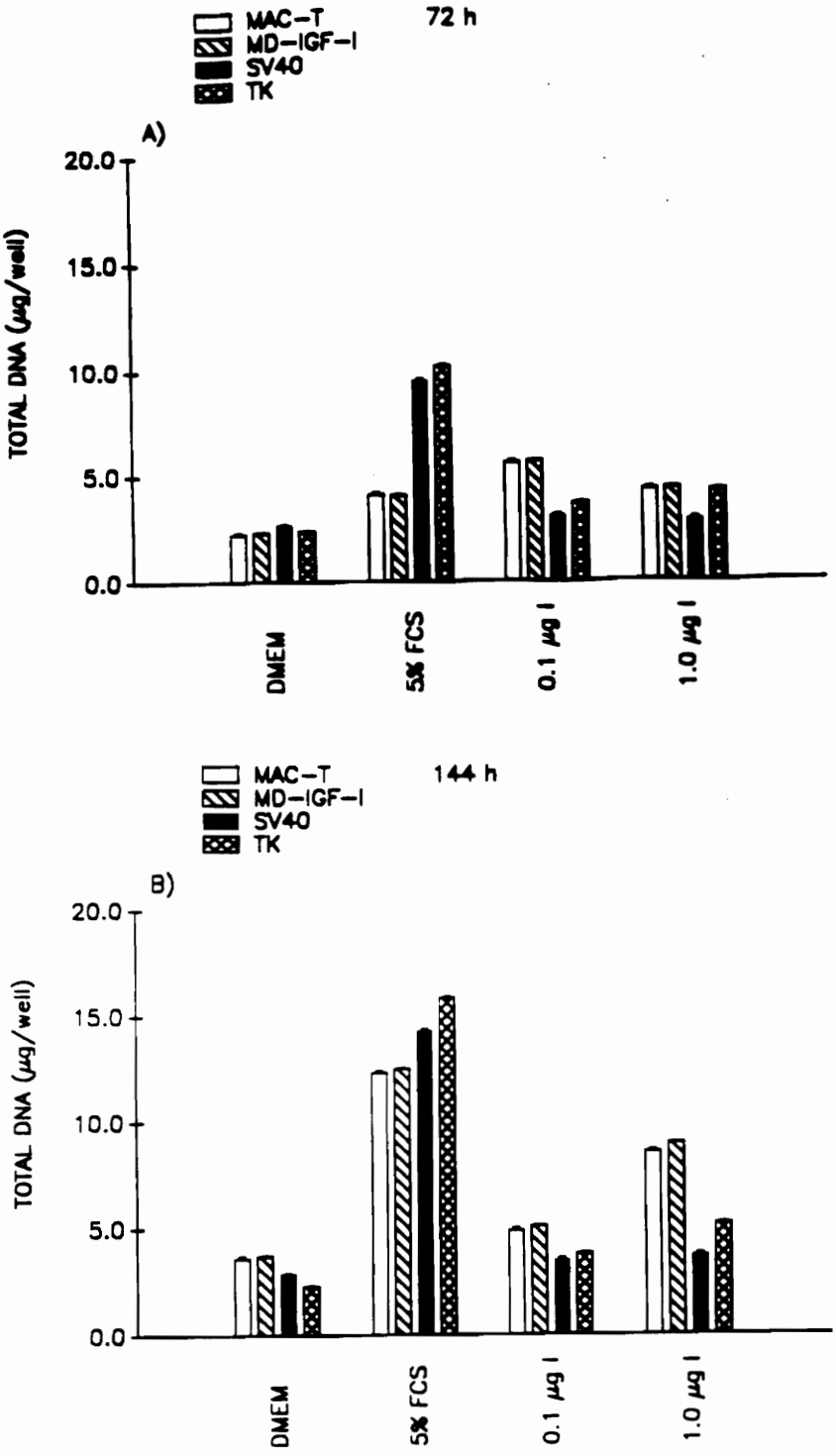

Figure 6. Effects of insulin on growth of parental MAC-T and IGF-I transfected cells. Cells were cultured for 72 and 144 h in DMEM, DMEM plus 5% FCS, and DMEM with 0.1 or 1.0 µg/ml insulin (I). At the end of the incubation periods cells were harvested and total DNA (µg/well) was determined. Bars represent the mean±SEM values from six samples, assayed in duplicate.

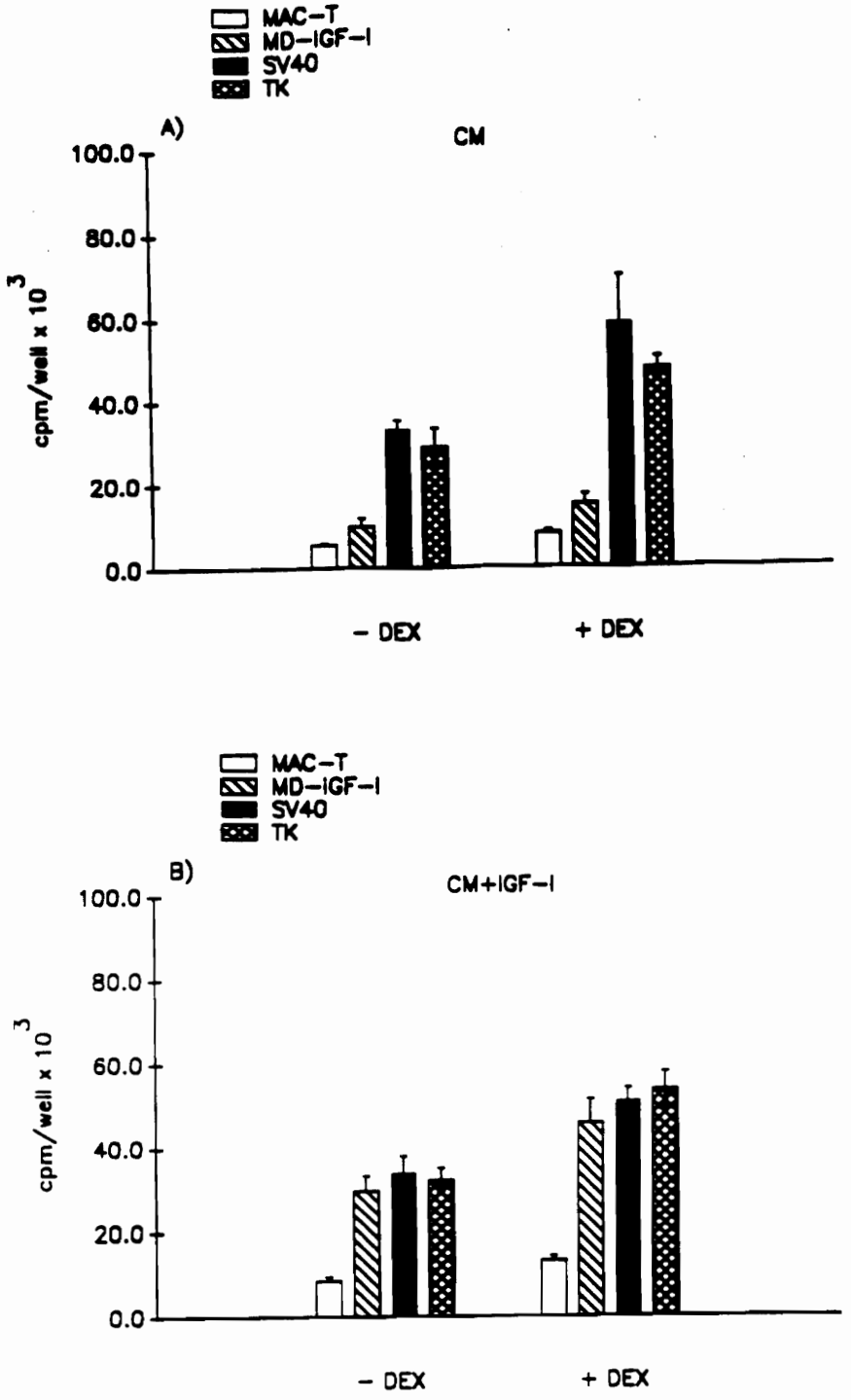

Figure 7A,B. [^{3}H]thymidine incorporation into DNA of MAC-T cells. Cells were cultured for 72h in DMEM in the absence or presence of 0.1 μM dexamethasone (DEX). At the end of the incubation period, conditioned media (CM) were harvested and used to culture test MAC-T cells in A) CM and B) CM plus 10 ng/ml IGF-I. [^{3}H]thymidine incorporation into MAC-T cells was measured after 16 h, with a 2-h pulse. Bars are the mean$\pm$SEM from six wells assayed in duplicate.

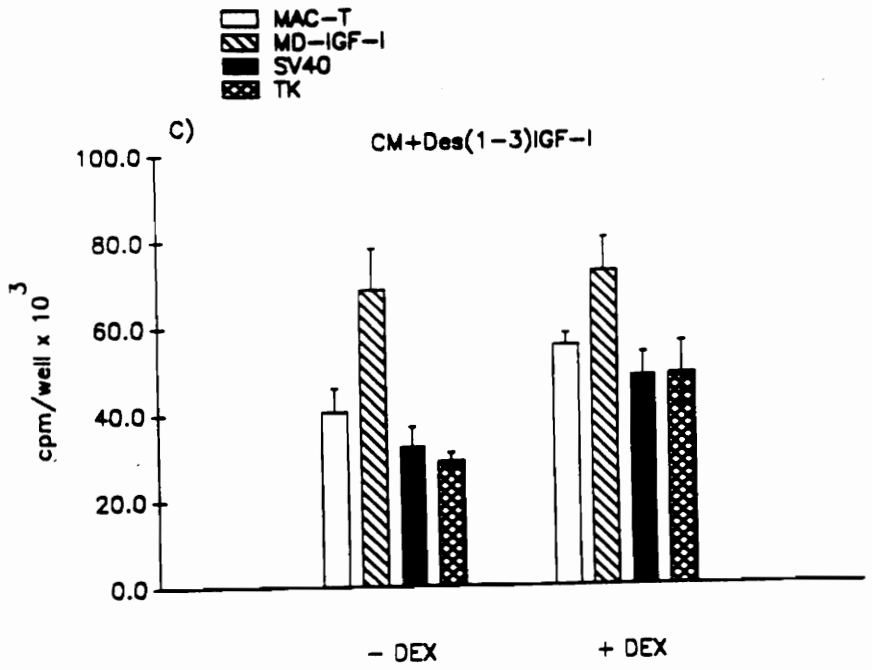

Figure 7C. [^{3}H]thymidine incorporation into DNA of MAC-T cells cultured in CM plus 10 ng/ml Des(1-3)IGF-I.

A)

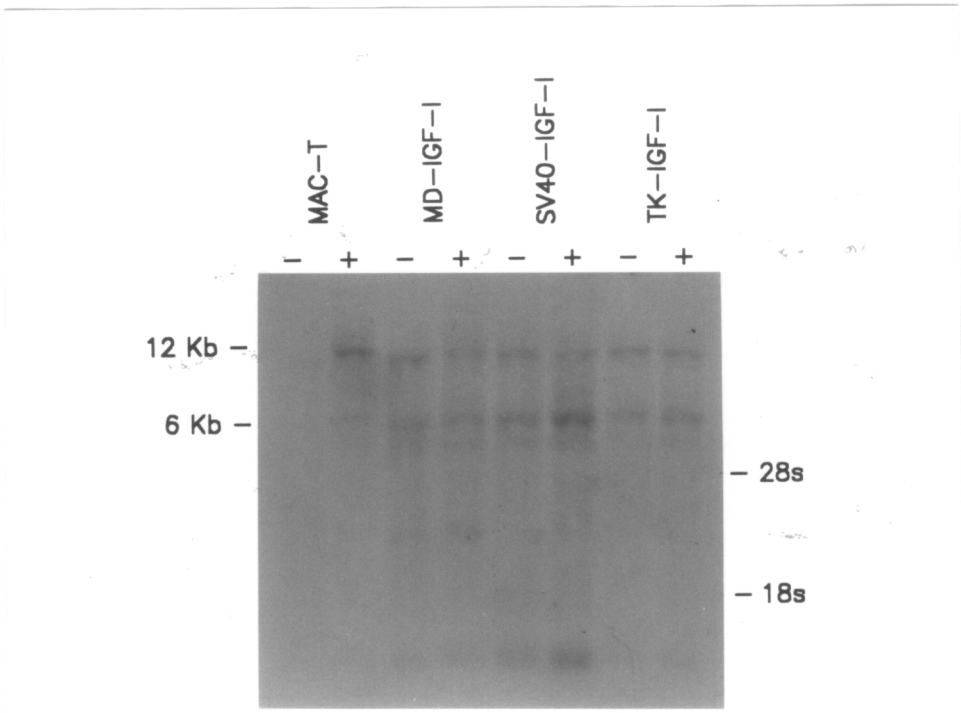

Figure 8A. Expression of IGF-I receptor in parental MAC-T and IGF-I transfected cells, A) Cells were cultured in DMEM in the absence (-) or presence (+) of 0.1 μM DEX for 72 h. At the end of the incubation period total RNA was extracted using a guanidinium thiocyanate procedure and separated on a 1% agarose gel. Northern blots were probed with a rat IGF-IR cDNA probe cloned in plasmid pGEM3 (Werner et al., 1989). Migrations of the 28s and 18s ribosomal RNAs are indicated.

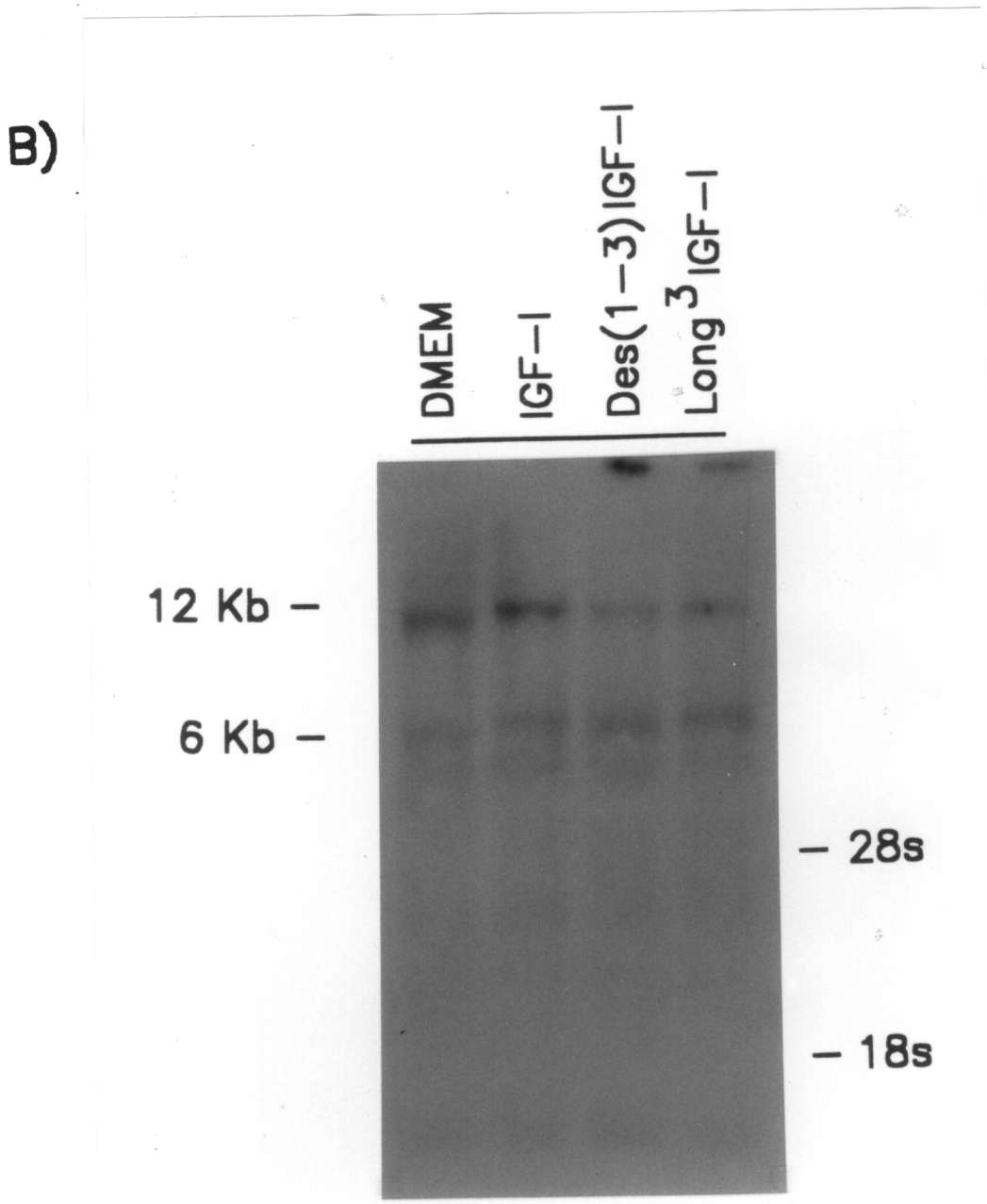

Figure 8B. Effects of IGF-I analogs on expression of IGF-IR in MD-IGF-I cells. Cells were cultured for 72 h in DMEM, DMEM plus 40 ng/ml IGF-I, DMEM plus 40 ng/ml Des(1-3)IGF-I, and DMEM plus 40 ng/ml Long^{3}IGF-I. Expression of IGF-IR was assessed by Northern blot analysis of total RNA (30 μg).

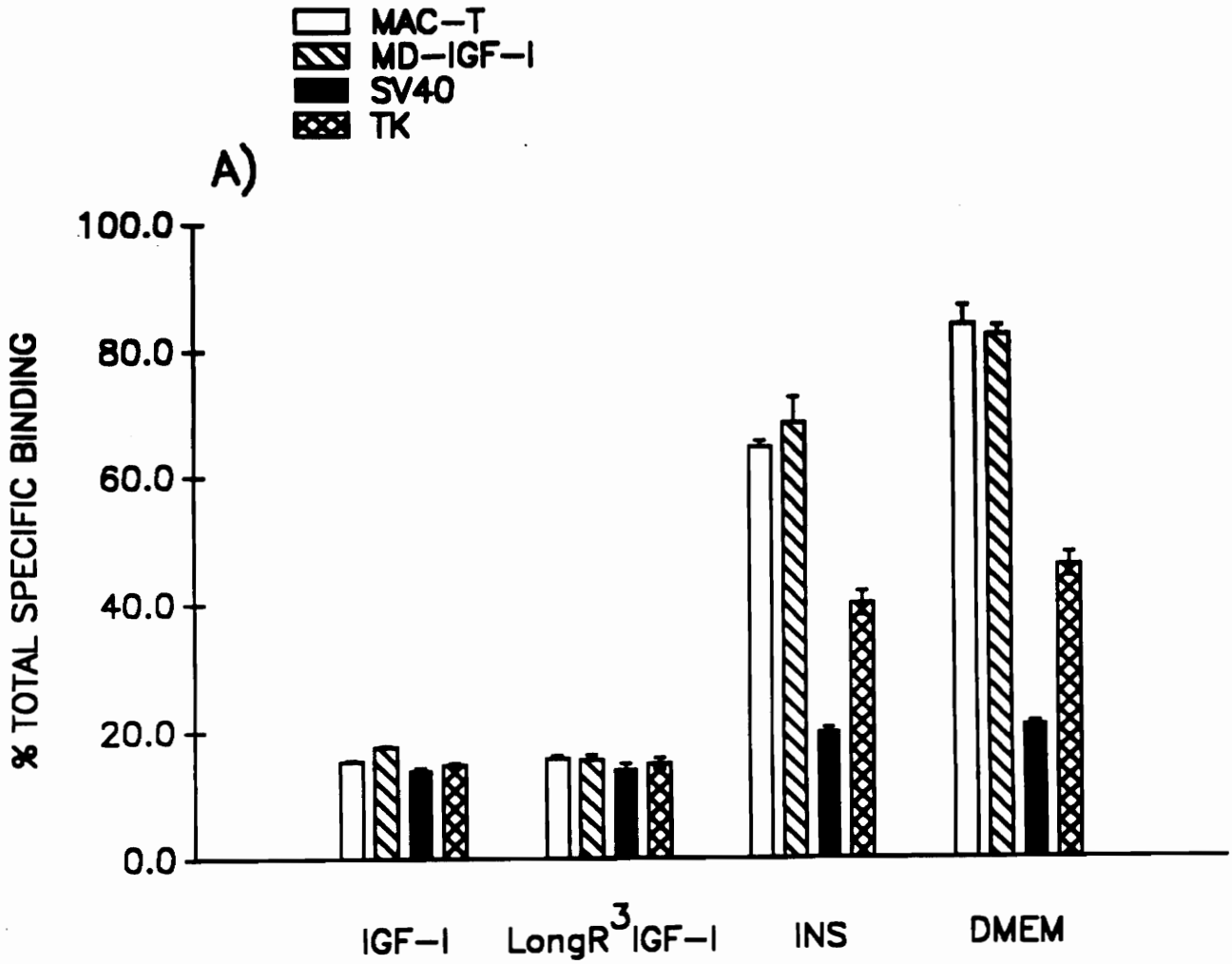

Figure 9A.[^{125}I]-LongR^{3}IGF-I displacement from parental MAC-T and IGF-I transfected cells. Cells were plated at a density of 1.5 x 10^5 in 24-well tissue culture plates. After 24 h, media were removed and cells cultured at 4^o C for 72 h in receptor buffer containing 100 ng [^{125}I]-LongR^{3}IGF-I and increasing amounts of unlabeled LongR^{3}IGF-I. Data are plotted as specific binding activity expressed as a function of total radioactivity.

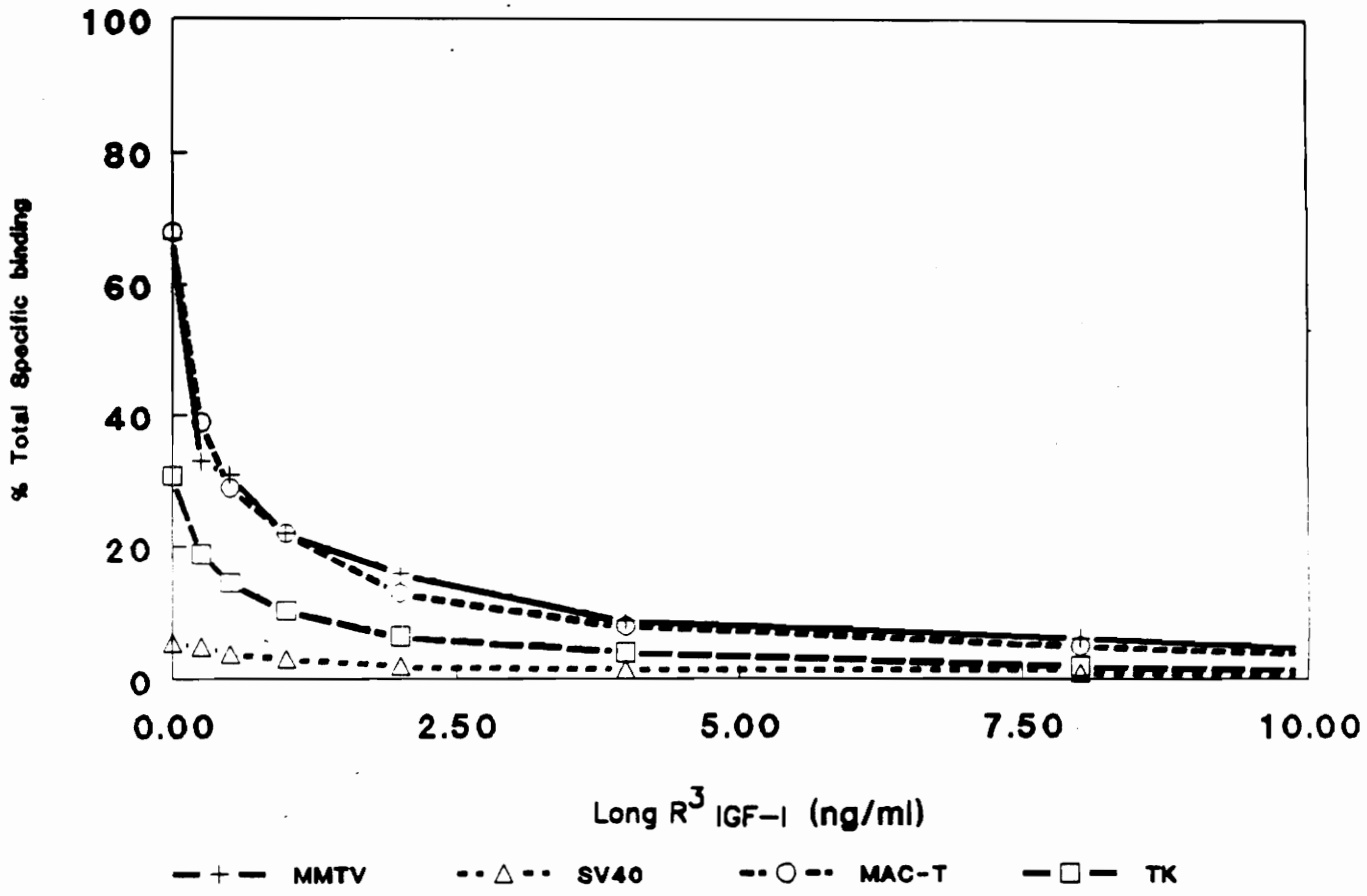

Figure 9B. [^{125}]I-LongR^3IGF-I specific binding in the presence of 100 ng/ml IGF-I, LongR^3IGF-I, and insulin.

TABLE 3. LIGAND BINDING ANALYSIS OF MAC-T AND IGF-I TRANSFECTED CELLS*

Cell Type	B_o $10^{-12}M/10^5$ cells	K_d $10^{-9}M/l$	r
MAC-T	21.5	5.5	.89
MD-IGF-I	19.3	7.6	.88
SV40-IGF-I	3.6	6.0	.88
TK-IGF-I	11.3	7.7	.93

*IGF-IR ligand binding studies were performed using Scatchard analysis in the presence of 0.5 ng/ml [^{125}I]-LongR^3IGF-I and increasing amounts of unlabelled LongR^3IGF-I. Affinity of binding was estimated by fitting a straight line to the data, for which correlation coefficients (r) were calculated. The slope was an estimate of the equilibrium dissociation constant (Kd) and the intercept an estimate of the binding capacity (B_o).

VITA

Donato Romagnolo was born on October 26th, 1957 in Solesino, Padova (Italy). He earned his Bachelor of Agricultural Science, in Animal Science at the University of Padova, Padova (Italy) in November 1983 and thereafter served as extension agent for the Agricultural Extension Program (Italy). The author began a Masters Program at the Virginia Polytechnic Institute and State University, Blacksburg, VA in January 1986 and received a Master of Science in Dairy Science, Dairy Nutrition in January 1988. Then, he joined the National Breeding Association, Italy as coordinator of the National Dairy Nutrition Program of the Ministry of Agriculture and Forestry, Italy, and later (1989-1990) the International Research Team of the Ralston Purina Int. as a ruminant nutritionist. In fall, 1990, he returned to the Virginia Polytechnic Institute and State University, Blacksburg, VA and began a doctoral program in Mammary Gland Biology, Lactation Physiology. The author received his Doctorate of Philosophy in Animal Science in July, 1993 and is a member of the American Association of Dairy Science, The American Association for the Advancement of Science, The Endocrine Society, Gamma Sigma Delta Society, and the Ordine dei Dottori Agronomi di Padova (Italy). Donato Romagnolo