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Prolactin-mediated gene activation in mammary epithelial cells

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Mammary epithelial cells grow and develop with the onset of sexual maturity. In addition, lobular alveolar structures are formed during pregnancy, and quiescent differentiated cells secrete high levels of milk proteins after parturition. These events are governed by multiple hormones and growth factors and involve the sequential and synergistic action of functionally distinct signal transduction pathways. Milk protein genes have been analyzed and composite response elements have been identified in the promoter sequences. Transcription factors, which relay the hormonal signals, bind to these sequences. The factor that confers prolactin stimulation to milk protein gene transcription has recently been identified. MGF/Stat5 is a latent transcription factor that becomes activated by a tyrosine-specific protein kinase, Jak2, associated with the prolactin receptor. Tyrosine phosphorylation converts the latent factor into one with DNA-binding and transcriptional activation potential. The regulation of MGF/Stat5 *in vitro* and *in vivo* indicates that it is a central component of the lactogenic hormone signaling pathway. Involvement of MGF/Stat5 in the signaling by other cytokines indicates that the same factor might be involved in regulation of growth-promoting genes, primarily in hematopoietic cells.

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Introduction

The post-natal development of the mammary gland and mammary epithelial cells has been extensively studied. The morphogenesis of the gland has been described and the signals governing differentiation and organ-specific gene expression have been identified. Cell contacts in the mammary gland [1] and basement membrane interactions [2–5] are important for the establishment of the differentiated phenotype. Lastly, cell hormone interactions are responsible for the induction of milk protein gene transcription [6–8]. The multitude of regulatory components and signals has complicated the molecular biological analysis of gene regulation in mammary epithelial cells. Some signals have been found to act in a sequential fashion, others act synergistically or antagonistically. Only the development of *in vitro* cell culture systems has allowed the defined manipulation of single variables and their correlation with particular phenotypic effects. This has led to a molecular biological approach to the study of lactogenic hormone action and the characterization and cloning of central components. Insights gained in the *in vitro* cultured cells have been verified *in vivo*.

In this review, we describe the steps leading to the discovery of MGF/Stat5. This factor confers the

transcriptional regulation by prolactin to the milk protein genes in mammary epithelial cells. MGF/Stat5 has been identified as a member of a transcription factor family, signal transducers and activators of transcription (Stat), that mediates the action of many different cytokines.

Hormonally responsive mammary epithelial cell lines

In the cell clone HC11, isolated from mouse mammary epithelial cells, it has been shown that the endogenous β -casein milk protein gene is rapidly induced by the lactogenic hormones without any requirements for extracellular matrix components or co-cultivation with other cell types [9,10]. This cell line has greatly facilitated the investigation into the molecular mechanisms of lactogenic hormone action and allowed the identification of regulatory DNA sequences in the promoter regions of the milk protein genes and specific nuclear interacting factors.

Transfections of HC11 cells with rat β -casein promoter-reporter gene constructs led to the delimitation of promoter sequences responsive towards lactogenic hormones [11]. It was found that prolactin and gluco-

Abbreviations

DIF—differentiation-induced factor; EGF—epidermal growth factor; IL—interleukin; MGF—mammary gland factor; WAP—whey acidic protein.

corticoid hormones synergistically induce expression of a transfected β -casein promoter construct when the cells are kept in the presence of insulin. All three lactogenic hormones (insulin, glucocorticoids, and prolactin) are required for transcriptional induction of the β -casein gene promoter, but a short segment of the promoter is sufficient to confer the response. Kinetic experiments indicated that prolactin and glucocorticoids control transcription by distinct mechanisms [12]. Dexamethasone increases the sensitivity of the cells to respond to prolactin. This increase in sensitivity is slow and can be rapidly reversed by withdrawal of dexamethasone.

In subsequent studies, it was shown that an interdependence exists between the signaling pathways. Lactogenic hormone action was investigated during the simultaneous activation of growth factor receptors. It was found that activation of epidermal growth factor receptor, but not of the ErbB-2 receptor, prevents lactogenic hormone induction in HC11 cells [13]. Stimulation of HC11 cells with Neu differentiation factor/herregulin induces phosphorylation of ErbB-2 and ErbB-3 and stimulates HC11 cell proliferation. Like epidermal growth factor (EGF), heregulin inhibits the lactogenic hormone induced expression of β -casein at the level of transcription [14]. Growth-promoting effects of EGF and the differentiation-specific signaling of the lactogenic hormones appeared to be mutually exclusive. However, the lactogenic hormone response was not counteracted by all proliferative signals. Activation of *c-myc* gene expression in mammary epithelial cells did not interfere with β -casein gene transcription [15,16].

Complementary to the study of milk protein genes in cultured cells, transgenic mice have been used to elucidate regulatory sequences in these gene promoters and the function of specific transcription factors. The rat β -casein gene promoter [17] and the whey acidic protein (WAP) gene promoter have been extensively investigated [18–21].

Transcription factors involved in lactogenic hormone action

The multitude of signals regulating the individual milk protein genes and the composite response elements found in their promoter sequences make it likely that a number of different transcription factors are involved in their transcriptional regulation. The delimitation of promoter sequences relevant for the lactogenic hormone induction in transfected cultured cells and in transgenic mice has provided the information necessary for factor identification. Insulin, glucocorticoids, and prolactin are required for the induction of two of the major milk protein genes, the β -casein and WAP genes. Glucocorticoids exert their transcriptional effect via the glucocorticoid receptor, a ligand-dependent transcription factor. Glucocorticoid receptor binding sites have been found in the promoter regions conferring the lactogenic hormone response [22]. Sequence motifs that represent half-sites of the classic glucocorticoid receptor

binding sites have been identified and interpreted as delayed secondary glucocorticoid-response elements. Cooperation between glucocorticoid receptor and other transcription factors is likely. Glucocorticoid regulation of the WAP gene promoter seems to involve indirect effects. Alterations in the chromatin structure of the distal promoter region are effected by glucocorticoids [23].

A region in the β -casein gene promoter has been identified that confers negative regulation. This repression of transcription in the uninduced state is mediated by a promoter element located at position -110 to -150 and comprises two interacting protein-binding sites. One of the nuclear factors interacting with the proximal site within the repressor region has been identified as the nuclear factor YY-1 [24]. Mutations in this regulatory region result in an increase in constitutive transcription. The YY-1-binding site is located in close proximity to that for the prolactin-regulated factor MGF/Stat5. Both proteins seem to compete for binding to overlapping sequences [24,25].

Regions have been identified in the WAP promoter that contribute to tissue-specific and stage-specific expression. A region between -120 and -100 is important for hormone-independent promoter function. Analysis of specific binding proteins has revealed that members of the Ets family of DNA-binding proteins recognize this site [26]. Analysis of this factor-binding site in transgenic mice revealed that the Ets-binding site plays a critical role in the activation of WAP promoter transgenes during pregnancy, but is not required for high expression during the lactation period. Ets proteins might function as stage-specific transcriptional activators in the developing mammary gland [27].

A pregnancy-specific mammary nuclear factor has been found to bind to two regions in the mouse β -casein gene promoter. This factor is regulated by progesterone and is involved in the repression of gene transcription during pregnancy. Progesterone suppresses hormone induction by the lactogenic hormones prolactin, hydrocortisone, and insulin [28].

Additional DNA sequence elements in the promoter of the β -casein gene have been identified. A negative regulatory element in the region between -221 and -183 and a positive regulatory element between -183 and -170 have been found. The promoter element between -194 and -163 binds to sequence-specific single-stranded DNA-binding proteins that exclusively recognize the upper DNA strand. They play a role in the repression of transcription. The DNA-binding activity of these nuclear proteins is hormonally controlled. Their repressive function is active in late pregnancy and post-lactation, but not during lactation. They might be sequestered by a single-stranded nucleic acid protein binding site in the β -casein mRNA during the lactation period [29,30].

NF-1 has been identified as a major factor regulating the transcription of the WAP gene. It binds to a

DNase I hypersensitive site between positions -800 and -700 in the promoter region. This site is critical for the transcriptional activation of the mouse WAP and the sheep β -lactoglobulin genes [31*]. Additional specific nucleic binding sites in the mouse β -casein gene promoter have been found by DNase I footprinting analysis and band-shift assays [32]. A summary of the nuclear factor binding sites identified in the proximal essential region of the β -casein gene promoter is shown in Fig. 1.

Prolactin action and inducible genes

The simultaneous activation of multiple receptors during lactogenic hormone action made it difficult to correlate individual transcription factors with individual hormonal signals. The development of a cell culture system in which individual signals could be manipulated and others held constant allowed researchers to focus on prolactin signal transduction. The WAP gene promoter [33,34], the *anx* Icp35 gene promoter [35], the α s1-casein gene promoter [36], and the β -lactoglobulin gene promoter [37] allowed comparisons with the β -casein gene promoter and facilitated the search for DNA elements conferring prolactin regulation. As prolactin action is not restricted to mammary epithelial cells, promoter elements in other cell types have been found to be valuable. The prolactin-dependent rat T-lymphoma Nb2 cells express genes (clone 15 and interferon regulatory factor 1 [38,39]) that allow conclusions to be drawn about promoter sequences conferring the prolactin response.

Another reason to focus on the prolactin aspect of lactogenic hormone regulation was the rapid progress made in the study of the prolactin receptor. Cloning of the gene and the structural analysis of the prolactin receptor contributed important insights [40]. The prolactin receptor belongs to the cytokine growth hormone/prolactin receptor superfamily. These receptors associate with cytoplasmic tyrosine kinases of the Jak family. Prolactin receptor activation results in the tyrosine phosphorylation of the kinase, the receptor, and cellular substrates that confer the transcriptional response [41*].

Discovery, purification and molecular cloning of MGF/Stat5

A strongly conserved sequence element is present in the region between -80 and -100 of casein genes from different species. Using DNase I footprinting analysis, methylation-interference assays, and band-shift analysis, a specific nuclear factor interacting with this sequence was found. This factor recognizes the DNA sequence 5'-ACTTCTTGGAATT-3'. It is present in the mammary gland tissue of lactating animals [42] and is termed mammary gland factor (MGF). Three binding sites for MGF have also been found in the β -lactoglobulin gene promoter [43] and the sequence is conserved in the α -casein and β -casein gene promoters of cows and rodents at position -87 to -99. Bovine and rodent MGF have identical DNA-binding properties and form protein-DNA complexes with the same electrophoretic migration properties. MGF has been

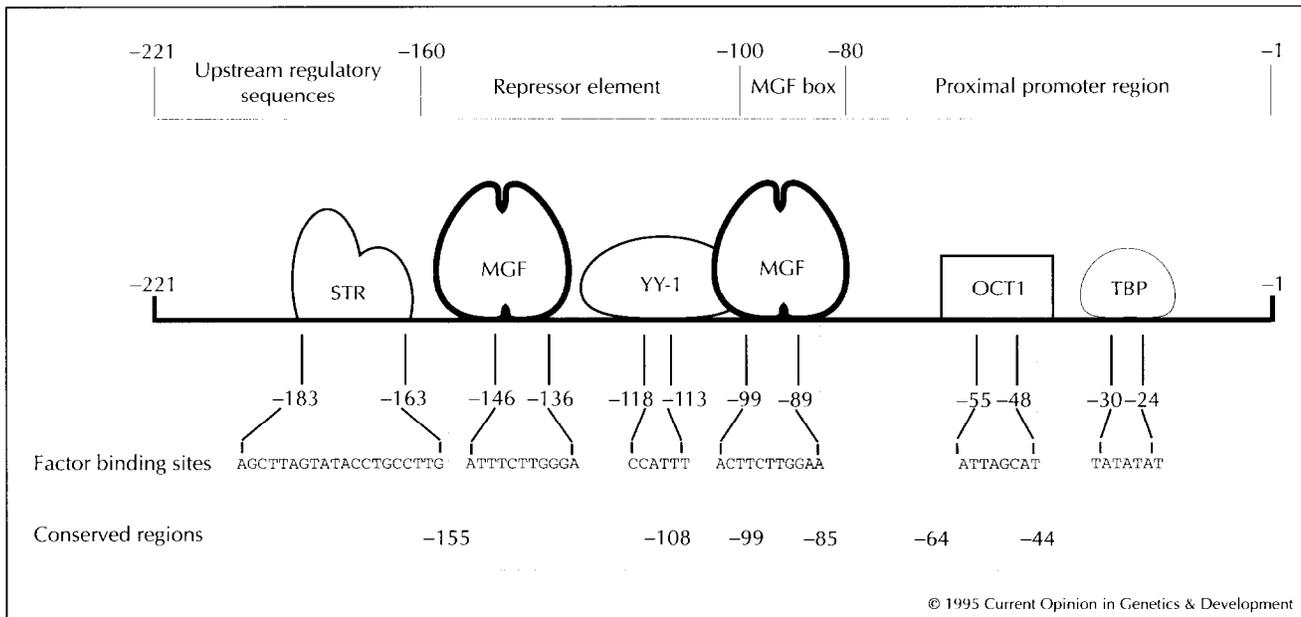


Fig. 1. Nuclear factor binding sites in the β -casein gene promoter. The binding sites for nuclear factors are indicated in the lower part of the picture. The sequence-specific single-stranded DNA-binding factor (STR) recognizes the upper strand of the region between -163 and -183 and has a repressing role on transcription in the absence of hormones. Two binding sites for MGF/Stat5 have been identified. A low-affinity site is present between positions -136 and -146 and a high-affinity site between positions -89 and -99. The recognition sequences for the YY-1 factor are present between positions -113 and -118. The region between -100 and -160 also exerts repressing functions. Downstream of the high-affinity MGF-binding site, an OCT1-binding site and a proximal promoter region have been identified. TBP, TATA-binding protein.

purified from nuclear extracts of mammary epithelial cells from lactating rats [44] and lactating sheep [45**] by sequence-specific DNA-affinity chromatography. Proteins of 84 kDa and 92 kDa were obtained, partial amino acids sequences were determined, and a cDNA clone of MGF was isolated. MGF comprises 794 amino acids and has sequence homology with members of the Stat family of transcription factors. It is encoded by mRNAs of 6.5 kb and 4.5 kb. mRNA expression was found in mammary tissue, ovaries, thymus, spleen, kidney, lung, muscle, and adrenal gland. Homologs of sheep MGF/Stat5 have been cloned from human cells [46] and two isoforms of mouse Stat5 with 96% sequence identity have been characterized [47,48*].

Regulation of MGF/Stat5 activity in cultured cells

The mechanism of prolactin activation of MGF/Stat5 has been studied in COS cells co-transfected with cDNA encoding Stat5 and the prolactin receptor. Prolactin treatment of the transfected cells caused activation of Stat5 within minutes. This activation is independent of ongoing protein synthesis. Tyrosine kinase inhibitors prevented Stat5 activation and treatment of activated Stat5 with tyrosine-specific protein phosphatases abolished its DNA-binding activity. The prolactin response of the β -casein milk protein gene promoter was observed in cells co-transfected with a β -casein promoter reporter construct. Substitution of the tyrosine at position 694 in MGF/Stat5 by a phenylalanine residue prevented phosphorylation upon prolactin induction and transcriptional transactivation [49**]. MGF/Stat5 was also able to enhance the *in vitro* transcription from a β -casein gene promoter [50]. These experiments formally established MGF/Stat5 as the mediator of prolactin-induced gene transcription. Prolactin was also able to induce MGF/Stat5 in HC11 cells [51]. In these cells, however, the synergistic action of glucocorticoids, insulin, and prolactin is necessary for the induction of the β -casein gene promoter. The fact that prolactin efficiently activates the promoter in transfected COS cells might be a result of the high expression of MGF/Stat5 in the transiently transfected cells. A very strong MGF/Stat5 signal may make the cooperating signals superfluous. Induction of MGF/Stat5 by prolactin and experimental evidence for its phosphorylation in confluent cultures of mammary epithelial cells was also obtained by Welte *et al.* [52].

Cross-talk between signal transduction pathways is observed when lactogenic hormone induced signal transduction is related to the action of protein kinase C. Downregulation of protein kinase C inhibits β -casein protein expression induced by the lactogenic hormones in HC11 cells. This inhibition occurs at the level of transcription and coincides with an inhibition of MGF/Stat5 activation [53]. A similar observation was made when the influence of EGF and signals downstream of the EGF receptor on the lactogenic

hormone response were studied. EGF, activated Ha-Ras, or activated v-Raf block the lactogenic induction of MGF/Stat5 and β -casein gene transcription [54].

Regulation of MGF/Stat5 activity in the mammary gland during pregnancy and lactation

The important role that MGF/Stat5 plays in the conferral of the prolactin response has been corroborated through observations in animal tissues. Using band-shift analysis, it was found that nuclear extracts from mammary glands of mice taken towards the end of pregnancy and during the lactation period exhibit high MGF/Stat5 activity. Withdrawal of suckling pups from their mothers during the lactation period causes a strong and rapid decrease of MGF/Stat5 DNA-binding activity. Restoration of the pups to their mothers increases MGF/Stat5 DNA binding to maximum levels within four hours [55]. In the lactating animals, MGF/Stat5 activity is regulated by suckling, milk stasis, and systemic hormone signals. In addition to the systemic signals, signals originating in the mammary gland cooperate in the *in vivo* regulation of MGF/Stat5 activity [56].

A comparison of the isoforms of Stat5 (Stat5a and Stat5b) has been carried out. Similar levels of Stat5a and Stat5b mRNA were found in most tissues of virgin and lactating mice. Both Stat5a and Stat5b mediate prolactin-induced transcription upon transfection into COS cells. An increase in Stat5 expression is observed during pregnancy and coincides with the activation of milk protein gene transcription [48*]. The two genes encoding Stat5a and Stat5b have also been studied in rat mammary gland [57]. The levels of Stat5a mRNA and protein were measured during pregnancy and were found to be highest in late pregnancy. They slightly decreased during lactation and did not correlate quantitatively with milk protein expression.

MGF/Stat5 is activated by numerous cytokines and growth factors

It was initially assumed that the regulation of milk protein genes in mammary epithelial cells would be affected by a tissue-specific transcription factor. Prolactin is active in many organs, though, so it was not surprising to find MGF/Stat5-like activity in different prolactin-responsive cells, such as Nb2 T-lymphoma cells. However, it was surprising to find that MGF/Stat5 activation is not restricted to the prolactin receptor signaling. Co-transfection of MGF/Stat5 cDNA and growth hormone receptor cDNA into COS cells showed that growth hormone treatment of the transfected cells results in MGF/Stat5 activation [58]. In addition to prolactin and growth hormone, erythropoietin and granulocyte/macrophage colony stimulating factor were also shown to be able to induce MGF/Stat5 [59]. The intracellular domain of the erythropoietin receptor crucial for MGF/Stat5 activation has recently been identified [60].

These results suggest that MGF/Stat5 not only regulates physiological processes in mammary epithelial cells, but also plays an important role in hematopoietic cells. This hypothesis gained credence when the connections between MGF/Stat5 and additional cytokines were discovered. Stat5 can also be activated by interleukin (IL) 2. The cytoplasmic region of the IL-2 receptor β c-chain is required for this activation [61]. These experiments also showed that Stat5 activation is not restricted to receptors activating Jak2, but can also be affected by Jak3. IL-2-mediated activation of Stat5 has also been observed in the transformed human lymphocyte cell line [46], a cytotoxic T-cell line CTLL-2 [62], and Nb-2 lymphoma cells [63].

IL-3, like IL-2, is an important regulator of hematopoietic cells and exerts its effects partially on the activation of specific transcription factors. Characterization of the IL-3-induced factor shows that a mouse homolog of MGF/Stat5 can be activated in response to IL-3 [47,64,65]. Further inducers of MGF/Stat5 were found when a differentiation-induced factor (DIF) in myeloid cells was investigated [66]. The treatment of myeloid cells with colony-stimulating factor 1, granulocyte/macrophage colony stimulating factor or interferon- γ results in the induction of DIF. The DIF complexes are partially made up of MGF/Stat5 or closely related proteins.

Thrombopoietin is a cytokine that regulates the levels of circulating platelets and controls proliferation and differentiation of megakaryocytes. It acts via the thrombopoietin receptor, c-Mpl. Activation of c-Mpl by thrombopoietin or constitutive activation of a transforming viral variant, v-Mpl, in COS cells or UT7 cells resulted in MGF/Stat5 activation [67]. Finally, the signal transduction pathway through Stat5 seems to

extend to growth factors that bind to tyrosine kinase receptors. Intraperitoneal treatment of mice with EGF [68] or heregulin (M Jeschke, B Groner, unpublished data) causes the activation of MGF/Stat5 in liver cells.

The prolactin response factor MGF/Stat5 plays a role in differentiation-specific gene expression and growth control

The Jak/Stat pathway has now been well established as one arm of cytokine-mediated signal transduction [69,70]. Cytokine receptor stimulation results in the recruitment and activation of non-receptor protein tyrosine kinases that trigger their signaling cascade. Tyrosine phosphorylation of the kinase itself, the intracellular domain of the receptors, and cellular substrates are observed. The intracytoplasmic region of the cytokine receptors seems to be able to recruit different cellular substrates and ultimately determine the specificity of the signaling events. A scheme for the prolactin-induced activation of MGF/Stat5 is shown in Fig. 2.

It seems as though MGF/Stat5-mediated transcriptional induction couples to differentiation-specific gene transcription events in mammary epithelial cells and proliferation signals in hematopoietic cells. Suggestive evidence indicates that the inappropriate activation of Jak kinases and Stat proteins could also be involved in malignant transformation. A dominant mutation of the *Drosophila* homolog of the Jak kinase causes developmental defects [71] and hematopoietic neoplasias [72]. Elevated levels of Stat DNA-binding activity have been found in breast carcinomas [73], oncogene-induced mouse mammary carcinomas [54], and v-Mpl-transformed hematopoietic cells [67]. It is

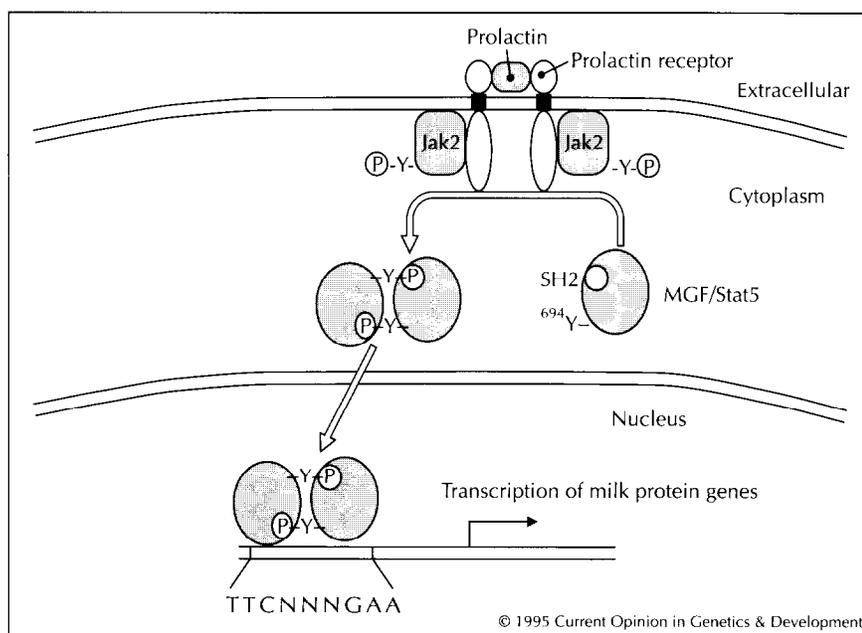


Fig. 2. MGF/Stat5 is a signal transducer and transcriptional activator. Upon binding of prolactin to the extracellular domain of the prolactin receptor, dimerization of the receptor takes place. Jak2 is associated with the intracellular domain of the prolactin receptor. This tyrosine-specific protein kinase becomes activated upon prolactin receptor dimerization. MGF/Stat5 is present as a latent factor in the cytoplasm and is a substrate of the Jak2 kinase. Upon phosphorylation at tyrosine residue (Y) 694, MGF/Stat5 dimerizes and assumes DNA-binding properties. The dimer of MGF/Stat5 binds to sequence elements present in target genes that have the consensus sequence 5'-TTCNNGAA-3' (where N can be any nucleotide).

conceivable that the activation of MGF/Stat5 is a normal proliferative signal in hematopoietic cells and a signal for differentiation-specific gene expression in mammary epithelial cells. Inappropriate activation of MGF/Stat5 in mammary cells at a differentiation stage before they have reached quiescence might confer a proliferative stimulus and thus contribute to their transformation.

Conclusions

The study of hormonally regulated gene expression in highly differentiated mammary epithelial cells led to the discovery and cloning of the transcription factor MGF/Stat5. This transcription factor is activated by prolactin, one of the lactogenic hormones. The regulation of its activity through tyrosine phosphorylation is an unusual feature for a transcription factor and defines it as a member of the Stat family. Its prominent induction during the lactation period initially led to the suggestion that it was a mammary-specific factor. The investigation of its mRNA expression pattern then showed that the gene is transcribed in most tissues. It was also found that the activation of MGF/Stat5 is not restricted to prolactin, but that a large number of cytokines (e.g. IL-2, IL-3, IL-5, granulocyte/macrophage colony stimulating factor, erythropoietin, thrombopoietin, and growth hormone) use MGF/Stat5 as a signaling intermediate. It is likely, therefore, that MGF/Stat5 plays a role in the transcription of differentiation-specific genes as well as growth-promoting genes. The cell type specific expression of individual cytokine receptors, the positively or negatively regulating properties of Stat5 isoforms and splice variants, and the composite nature of the promoter regions of the responsive genes will have to be considered as factors that determine the function of Stat5 in different cell types.

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