Transcription Factor Activities and Gene Expression during Mouse Mammary Gland Involution

Running Title: Gene Regulation during Mammary Gland Involution

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Maintenance of mammary epithelial differentiation and milk production during lactation is a consequence of milk removal and the presence of lactogenic hormones, particularly glucocorticoids, insulin and prolactin. After weaning the fall in lactogenic hormones and milk stasis lead to involution, a process that is mainly characterized by three events: *(i)* downregulation of milk protein gene expression, *(ii)* loss of epithelial cells by apoptosis and, *(iii)* tissue remodeling and preparation of the gland for a new pregnancy. Each of these processes is likely to depend on the activity of specific sets of transcription factors in the mammary epithelium and stroma that ensure the timely and spatially coordinated expression of critical gene products such as mediators of apoptosis (e.g. caspase-1) and regulators of tissue remodeling events (e.g. matrix metalloproteinases). Here we describe signal transduction events such as activation of protein kinase A and JNK and changes in the activity of several transcription factors including Stat5, Stat3, NF1, Oct-1, and AP-1 during the early and late phases of mammary gland involution. We discuss their possible role in regulating and coordinating involution with emphasis on the apoptotic process of involution.

KEY WORDS: Apoptosis, AP-1 (Fos/Jun), Gene Expression, JNK, NF1, Oct-1, STAT, Transcription Factor

ABBREVIATIONS: PRL, prolactin; JNK, c-Jun N-terminal kinase; STAT, signal transducer and activator of transcription; GR, glucocorticoid receptor

INTRODUCTION

A change in the pattern of gene expression allows a cell to switch from proliferation to differentiation or, alternatively, to die by apoptosis, depending on the biological context. Generally, gene expression is tightly controlled by extracellular factors such as hormones, growth factors, differentiation factors, survival factors, death factors, or cell-cell interactions and cell-matrix interactions. The information provided by these extracellular factors is sensed by specialized receptors and translated via signal transduction cascades to the nucleus where transcription factors are instructed to change the pattern of gene expression. In the mammary gland gene expression can be studied *in vivo* during the proliferative phase of epithelial cells at puberty and pregnancy, during the differentiation phases at late pregnancy and the transition to lactation, and during involution when epithelial cells die by apoptosis and the gland is remodeled (1-10). Many genes that may play a role during these distinct phases of development of the mammary gland have been identified. However, only a few signal transduction pathways and transcription factors have been clearly linked to the regulation of gene expression during postnatal mammary gland development: the best characterized is prolactin (PRL) signaling during lactation (5, 9, 10).

Below, we summarize and discuss the current view of some signal transduction events and changes in the gene expression patterns that occur during early involution of the mouse mammary gland and we present a hypothesis for the involvement of transcription factors in these processes.

CHANGES IN GENE EXPRESSION DURING EARLY AND LATE PHASES OF INVOLUTION

Mouse mammary gland involution occurs in two distinct phases when experimentally induced by forced weaning (11). The first phase is initiated within a few hours after removal of the pups and is morphologically characterized by an accumulation of milk in the alveolar lumen and limited apoptosis of epithelial cells (8, 12). The initial phase of involution can be reversed up to about 1.5 days after weaning when epithelial cell apoptosis starts to dominate the process (13, 14). Three to 5 days after weaning (depending on the mouse strain) the second phase of involution is initiated. It is morphologically characterized by degradation of the basement membrane, a collapse of alveoli, infiltration of macrophages and restructuring of the gland to a virgin-like state. During the second phase, apoptosis of epithelial cells continues until 50% to 80% of the epithelial cells have been cleared from the gland (12).

Gene expression in the early phase of involution

Both stages of involution are associated with characteristic changes in the patterns of gene expression (8, 11, 15, 16). A well-documented example is the downregulation of the mRNA coding for milk protein genes like WAP and β-casein during the first phase of involution (8). Based upon nuclear run on assays it was also observed that the major histocompatibility (MHC) class I gene, the histone H2B gene, and the small nuclear RNA genes U1a and U1b are transcriptionally downregulated within 4 days after weaning (15).

The down-regulation of lactation specific genes during the first phase of involution is paralleled by activation of new sets of genes, some of the products which may be responsible for the induction and/or execution of epithelial cell apoptosis. Some may also trigger events responsible for the initiation of the second phase of involution. Caspase-1 (ICE) and Bax are putative effector genes for apoptosis both of which were found to be transiently induced after weaning with maximum expression around day 3 of involution (17-19).

Less evident is the function of a number of other gene products which were found to be activated during the early phase of involution. These genes include the surface glycoprotein 2 (SGP-2 also called testosterone repressed prostate message-2, TRPM-2, or clusterin), p53, the immediate early genes c-*fos*, c-*jun*, *jun*B, *jun*D, the delayed early gene c-*myc* and the cell cycle control genes cyclin D1, D2 and D3 among others (8, 15, 16).

Gene expression in the late phase of involution

The second phase of involution is characterized molecularly by a transition in the ratio of extracellular proteinase inhibitors to proteinases (20). The expression of tissue inhibitors of metalloproteinase genes (TIMPs) is downregulated around day 3 of involution with the concomitant upregulation of a number of metalloproteinases and serine proteinases such as stromelysin-1, stromelysin-3, gelatinase A and urokinase-type plasminogen activator (uPA) (6-8, 11, 20). The products of these genes most likely contribute primarily to the destruction of extracellular matrix components (ECM) and, therefore, to the remodeling process.

SIGNAL TRANSDUCTION CASCADES DURING INVOLUTION

Changes in gene expression patterns are the result of changes in signal transduction cascades. In the mouse mammary gland some of the signals and signal transduction cascades that regulate the involution process have been described and the work of several laboratories shows that both systemic and local signals generated by the accumulation of milk regulate the involution process (5, 18, 21-23). In a simple scenario one might speculate that after weaning the systemic downregulation of the PRL or glucocorticoid levels results in shutting off intracellular signaling cascades that provide survival functions for epithelial cells (5, 7, 10, 21, 24). This is most likely reflected by the downregulation of several genes during early involution. Local signals resulting from engorgement may initiate new and independent signaling cascades that activate the apoptotic program during the first phase of involution. Indeed, sealing of a single gland during lactation was shown to provoke an accumulation of milk resulting in characteristic changes in gene expression and massive apoptosis of epithelial cells in sealed glands but not in the remaining glands of the same animals (18, 23).

PKA

In many cases, critical signals are mediated by protein kinases. Indeed, the activity of several kinases and the phosphorylation state of their substrates have been shown to change rapidly and dramatically during involution. For example protein kinase A (PKA) or a PKA-like enzyme activity was found to be 10 fold increased within hours after weaning (15). This activation is very likely regulated by local effects as sealing of a single gland during lactation was sufficient to cause kinase activation whereas no kinase activation was observed in contralateral, matched glands (23). Therefore, a local signal may be the initial event triggering this PKA-like activation. Although the finding of the activation of a PKA-like enzyme is very intriguing, it is not possible from the available evidence to conclude that this activity contributes directly to apoptosis of epithelial cells. But it is very likely that PKA stimulates the expression of several genes among them probably members of the *fos* and *jun* families of genes that were shown to be activated in this first phase of involution (15).

JNK activity

In addition to PKA, the c-Jun N-terminal kinase (JNK) may play a role during the first phase of mouse mammary gland involution. Figure 1 shows that JNK activity is induced *in vivo* from a low level at lactation (lane 2) to an elevated level at day 3 of involution (lane 3). Densitometric analysis revealed that the activity was induced more than 5 fold. An involvement of JNK in the activation of apoptosis was previously documented for various cell culture systems (25-28). c-Jun represents a major target for JNK and phosphorylation of c-Jun by JNK enhances its activity (29). Figure 2 demonstrates immunohistochemically an induction in the mammary epithelium and nuclear localization of c-Fos and c-Jun after weaning. No nuclear signals can be detected with antibodies specific for c-Fos (panel A) and c-Jun (panel C) in sections prepared from mammary gland at lactation. Three days after weaning nuclear staining was observed for c-Fos (panel B) and for c-Jun (panel D) in epithelial cells. The phosphorylation state of c-Jun can be monitored with an antibody that specifically recognizes phosphorylated serine 73 of c-Jun. Figure 2 shows that phosphorylated c-Jun was

absent or below the level of detection during lactation (panel E). Many epithelial cell nuclei stained positive for phosphorylated c-Jun at 2 days (data not shown) and 3 days of involution (panel F). These data indicate that JNK activation takes place in the epithelial compartment of the gland and that c-Jun is a target for JNK after weaning.

TRANSCRIPTION FACTOR ACTIVITIES AND APOPTOSIS

Weaning results in the downregulation of signal transduction events as exemplified by the PRL signaling and upregulation of signal transduction events such as the activation of JNK and PKA. Some of these changes most likely contribute directly to the characteristic changes in the patterns of gene expression, e.g. the inactivation of PRL-dependent WAP gene expression or the activation of *fos* and *jun* genes by PKA and/or JNK.

STAT factors

STAT (signal transducer and activator of transcription) proteins are transcription factors that play an important role in cytokine signaling (30). As described for other transcription factors STAT proteins are built of various domains which mediate both DNA binding and interactions with other proteins. STAT proteins contain Src-homology domains that recruit the proteins to tyrosine-phosphorylated docking sites located in cytoplasmic domains of activated cytokine receptors. After being tyrosine-phosphorylated by specific kinases of the Janus kinase (JAK) family of kinases (30), STATs leave the receptor and translocate into the nucleus where they bind to defined DNA sequences in the promoter/enhancer region of target genes. In mammary epithelial cells Stat5 is recruited to the PRL receptor and activated through tyrosine-phosphorylation by Jak2 (5, 30). Stat5 activity is induced in late pregnancy and stays high during lactation. It is essential for the development of functional alveoli and milk producing epithelial cells and is important for the expression of milk protein genes like WAP (5, 9, 10, 31, 32). After weaning Stat5 activity is downregulated within 24h by dephosphorylation (13) indicating that its activity is not further needed or is not compatible with early events of mammary gland involution. Stat5 impairment may be due to local signals since accumulation of milk by sealing a single gland was sufficient to cause a dephosphorylation of Stat5 (18). In epithelial cells, inactivation of Stat5 may be a major reason why WAP gene expression declines shortly after weaning.

Stat3, another member of the STAT family of proteins, was shown to be induced in the first phase of involution (10, 18). Similar to Stat5, Stat3 is regulated by local signals which are a consequence of the accumulation of milk (18). Again, tyrosine-phosphorylation is involved in the activation of Stat3. No function for Stat3 has been proposed so far in the mammary gland. It might be interesting to test whether Stat3 contributes to the activation of the caspase-1 gene as was shown for Stat1 in cells in culture (33).

NF1 proteins

CCAAAT-binding transcription factor (CTF)/nuclear factor 1 (NF1) is a family of transcription factors that have been shown to play important roles in tissue specific transcription of differentiation-associated genes in a number of tissues (34-37). NF1 proteins are composed of a conserved N-terminal DNA binding domain and a heterogeneous C-terminal proline rich activation domain. In the mammary gland NF1 proteins play a critical role in activating milk protein genes during lactation (31). The function

of NF1 proteins therefore resembles the role played by Stat5. Indeed, NF1 proteins were shown to cooperate with Stat5 in the expression of milk protein genes (31). Like Stat5, several NF1 proteins are rapidly downregulated after weaning (38). Interestingly, a new NF1 protein with a molecular mass of 74kDa is induced during the first phase of involution (38). This form of NF1 recognizes a defined DNA sequence in the SGP-2 (TRPM-2/clusterin) promoter providing support for the hypothesis that this NF1 (74kDa) protein contributes to the activation of SGP-2 expression during involution (38).

Oct-1

Nuclear run on assays showed that a number of housekeeping genes including histones and small nuclear RNA genes are turned off during involution (15). Oct-1 (octamer-binding transcription factor-1, OTF-1) is a ubiquitous transcription factor of the POU-homeo family of proteins which is important for the expression of these housekeeping genes (39-42). Oct-1 DNA binding activity is high at lactation and is lost within 4 days of weaning (15). It is possible that the inactivation of Oct-1 contributes to the observed inhibition of histone and small nuclear RNA gene expression. Oct-1 is inactivated at the protein level, either by a phosphorylation event or by proteolytic degradation, since Oct-1 mRNA is still expressed 4 days after weaning at levels similar to those observed during lactation (15). It was suggested that the loss of Oct-1 DNA binding is a direct consequence of PKA activation induced during involution since purified PKA inactivates Oct-1 DNA binding in extracts from mammary glands of lactating mice (15). Presently, it is not known whether the inactivation of Oct-1 is important in apoptosis of mammary epithelial cells. Oct-1 inactivation has been independently associated with cell death in various other models of apoptosis such as the castration-induced regression of the prostate (15) or the light-mediated death of photoreceptors in the retina (43). The observed inactivation of Oct-1 seems to be mediated by local signals: Oct-1 activity was lost in sealed glands and the impairment of Oct-1 DNA binding activity was reversible when lactation was resumed within 24h of weaning (14).

AP-1

Among the most prominent genes activated during the early phase of involution are various members of the *fos* and *jun* gene families (15). Fos and Jun proteins together with ATF/CREB proteins collectively form the dimeric transcription factor AP-1 (44). Leuzine-zipper domains and adjacent basic regions in the Fos, Jun and ATF/CREB families of proteins mediate homo- and heterodimerization and DNA binding (45). AP-1 recognizes and binds to a set of closely related DNA sequences (called TRE for TPA response element or CRE for cAMP response element) that are located in the promoter/enhancer regions of many cellular and viral genes (46). AP-1 activity is regulated by the abundance of the Fos/Jun/ATF/CREB proteins, by post-translational modifications of these proteins and by interactions with other proteins (44, 47). The *fos* and *jun* genes are activated by many extracellular signals, *e.g.* growth factors, hormones, cytokines, and by various forms of cellular stress, *e.g.* UV light or irradiation (44, 46, 47). In the mammary gland *fos* and *jun* expression and the transient activation of AP-1 DNA binding activity are induced by local signals (23). Similar to PKA activation, sealing of a single gland during lactation was sufficient to cause AP-1 expression and transient elevation of AP-1 DNA

binding activity whereas in matched glands no comparable AP-1 activation was observed (23). After weaning the activation of AP-1 is reversible when lactation is resumed within 24h (14). PKA may contribute to the expression of AP-1 by phosphorylating and thereby activating specific transcription factors, including the transcription factor CREB (cAMP response element binding factor), with the ability to activate expression of members of the *fos* and *jun* families (48, 49). As explained above AP-1 activity is regulated not only by the abundance of Fos and Jun proteins but also by post-translational modifications of the proteins. For example phosphorylation of c-Jun at serine 63 and 73 by JNK significantly induces its transcriptional activity (29). Therefore JNK most likely directly contributes to AP-1 activation in the involuting gland by phosphorylating c-Jun (see also previous section).

Presently, it is difficult to asses the importance of AP-1 for the apoptotic process in mammary epithelial cells. The finding that c-Fos is essential for the initiation of apoptosis in nerve cells after NGF withdrawal (27), in retinal photoreceptors after light damage (50) and in prostate epithelial cells after castration (51) suggests that AP-1 may also be important for the initiation and/or execution of apoptosis in the mammary epithelium. After weaning several genes may be induced directly by AP-1. Besides members of the *fos* and *jun* family of genes, stromelysin, Bax and cyclin D genes are likely candidates. The best documented AP-1 regulated gene is stromelysin (52), a metalloproteinase whose expression is induced around day 3 of involution, with a peak of expression in the later phase of involution. It has been suggested that metalloproteinases contribute to apoptosis by degrading the extracellular matrix (19). Stromelysin expression and epithelial cell apoptosis can be blocked *in vivo* by the administration of steroid analogs that stimulate the glucocorticoid receptor (GR) (24). Interestingly, glucocorticoids inhibit neither the activation of the PKA-like activity nor the expression of AP-1 members suggesting that hormone-activated GR acts at the same level as or downstream of AP-1 (24). It is well documented that the GR has the ability to impair AP-1 function by directly binding to the AP-1 complex (53). It is therefore possible that the GR acts as survival factor, at least in part, by keeping in check the activity of transcription factors like AP-1 that have the capacity to regulate metalloproteinases.

CONCLUSIONS

In this review we present an overview of signal transduction cascades, transcription factor activities and changes in gene expression that have been shown to accompany the involution process in the mouse mammary gland. The inactivation of lactation-associated genes after weaning and the activation of involution-associated genes correlate with an inactivation and activation of a number of transcription factors. Figure 3 shows that the decline in expression of WAP and β-casein correlates with the inactivation of specific transcription factors such as Stat5, NF1 (114kDa, 68kDa, 48kDa) and Oct-1. The importance of Stat5 in regulating WAP expression has been clearly shown (5, 10, 31). A critical role for NF1 proteins in the regulation of milk protein genes has also been documented (31). To date, a functional link between the activation of involution associated genes like Bax, caspase-1, stromelysin and SGP-2 and the activation of the transcription factors Stat3, NF1 (74kDa) and AP-1 is much less substantiated (Figure 3). A possible connection between NF1 (74kDa) and

SGP-2 expression was recently found when increased DNA binding of this factor to the SGP-2 promoter was demonstrated after weaning (38). Stromelysin and Bax genes are likely factors regulated by AP-1. Furthermore, various *fos* and *jun* genes may also be regulated by AP-1. Interestingly, changes in the activity of transcription factors are largely dependent on local signals and they are reversible within 24 h after weaning. To further clarify the role of distinct transcription factors during involution of the mouse mammary gland, studies of transcription factor gene knockout mice and of animals overexpressing transcription factors during specific stages of the mammary gland development may be helpful.

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FIGURES

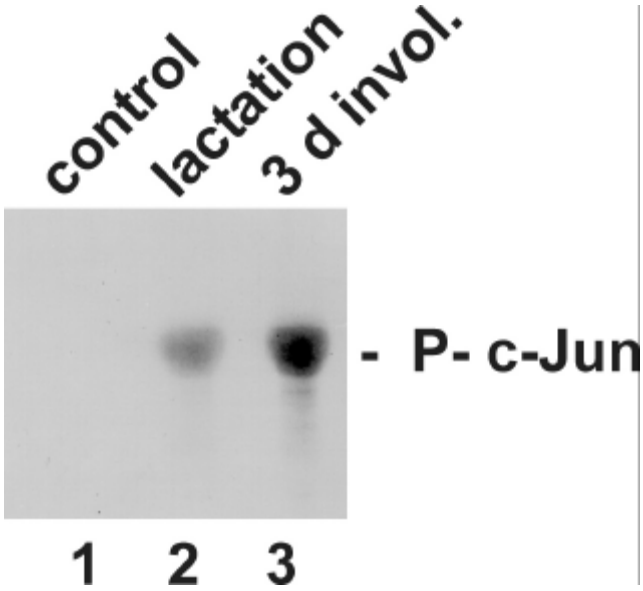

Figure 1 **Induction of JNK during involution.** c-Jun binding proteins were isolated from nuclear extracts prepared from mammary glands at lactation (lane 2) or at 3 days of involution (lane 3) using GSH-agarose beads containing recombinant GSTc-Jun: Beads were washed and incubated in the presence of 5 μCi γ-ATP for 30 min at 30°C. Proteins were washed, boiled in SDS loading buffer, separated on an SDS-PAGE and exposed for 15 min. Lane 1 represents beads incubated in the absence of extract.

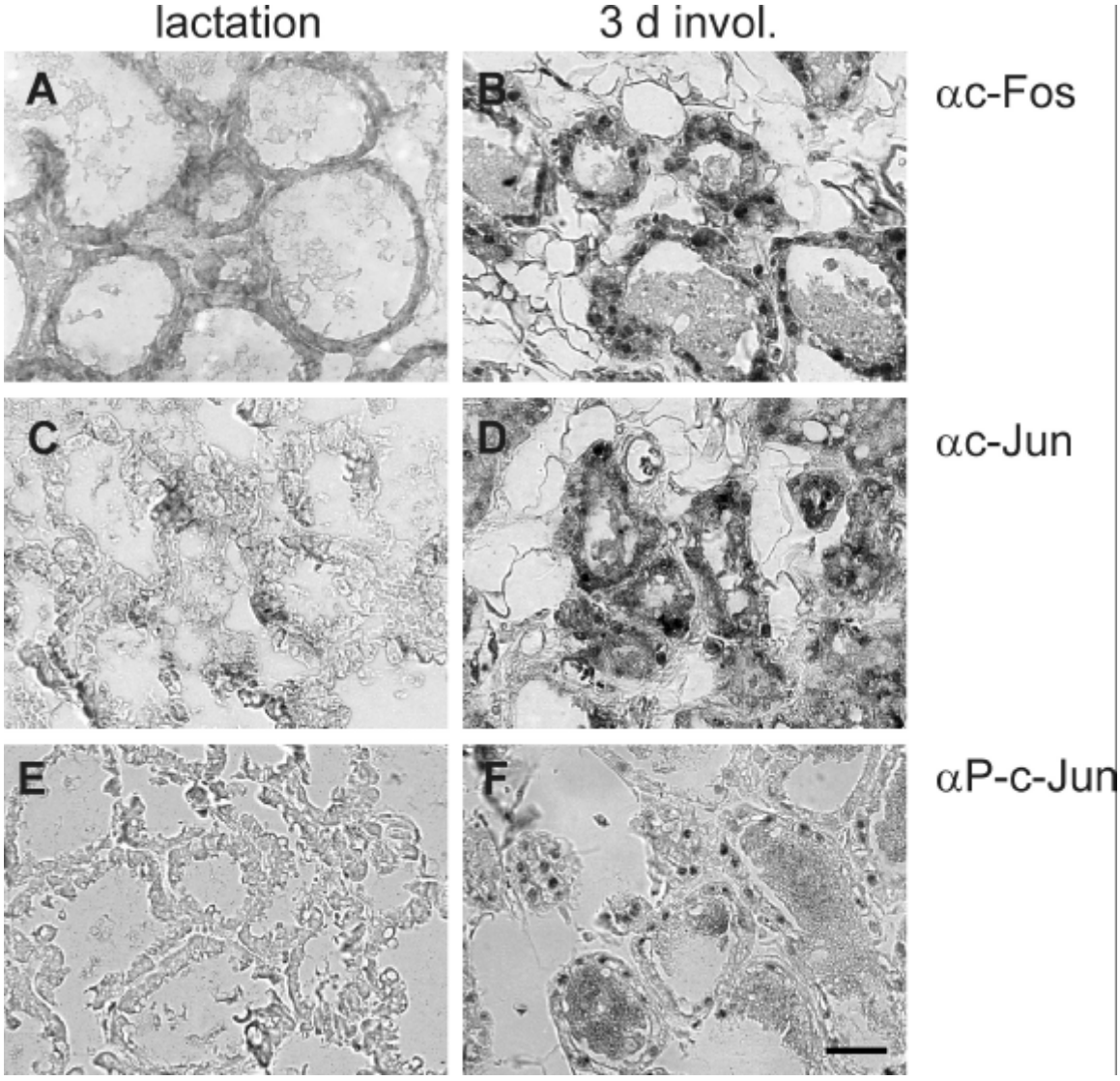

Figure 2 **Immunohistochemical analysis of c-Fos, c-Jun and phospho-c-Jun.** Paraffin sections were prepared from mammary glands at lactation (panels A, C, E) or at 3 days of involution (lanes B, D, F) and processed for immunohistochemistry using c-Fos specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA) (panel A and B), c-Jun specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA) (panels C and D) or a phospho-c-Jun (Ser73) specific antibody (New England BioLabs) (panels E and F). Antibodies were visualized using peroxidase labeled polymer and AEC chromogen as substrate (DAKO, Glostrup, Denmark). The same magnifications were used for all panels and the bar represents 25 μm.

Figure 3

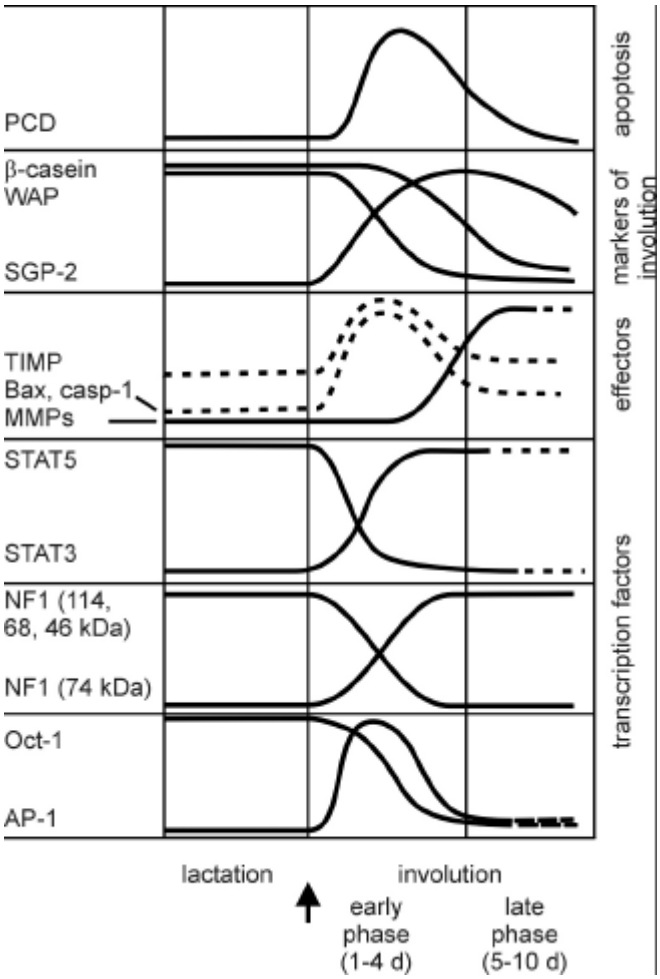

Figure 3 **Changes in the level of gene expression during involution.** Schematic representation of the relative frequency of programmed cell death, expression levels of milk protein genes and putative cell death-effector genes during lactation and early and late phases of involution. In the lower panels changes in the relative levels of several transcription factor activities are shown during the same periods of development (for further details see text).