THE ROLES OF C/EBPβ AND C-JUN IN TRANSCRIPTION OF THE GENE ENCODING THE MURINE PROGESTERONE RECEPTOR

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ABSTRACT

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Progesterone (P) and its receptor, the progesterone receptor (PR), are important for mammary gland development. Moreover, P/PR signaling also contributes to mammary tumorigenesis. Thus, studying the mechanism of PR expression is important in breast cancer research. C/EBPβ-deficient mice and mice blocked for AP-1 activity show similar defects in mammary gland development as do PRB-deficient mice, especially during pregnancy, suggesting that these transcription factors might act in the same pathway or may regulate overlapping sets of downstream target genes. An overall decrease in PR observed in sexually mature wild type mice fails to occur in C/EBPβ-deficient mice, while no alterations in C/EBPβ expression are observed in PR-deficient mice. Moreover, AP-1 has been found to regulate PR expression. These observations suggest that C/EBPβ and AP-1 act upstream of PR. This leads us to study the possibility that C/EBPβ and AP-1 are required for PR expression.

We examined whether C/EBPβ participated in the transcriptional regulation of PR expression in the mammary gland. Transient co-transfection of a PR promoter-reporter construct with expression vectors that individually express C/EBPβ isoforms (LAP1, LAP2, or LIP) into a mouse mammary carcinoma cell line revealed that all C/EBPβ isoforms, surprisingly including LIP (the shortest isoform lacking transactivation domains), can transactivate the PR promoter. Importantly, we found that LIP, in particular, robustly synergizes with an AP-1 member, c-Jun, to drive PR transcription. Consistent with significant roles for C/EBPβ and c-Jun in PR expression, knockdown experiments showed that endogenous levels of C/EBPβ and c-Jun

expression were sufficient to drive the PR promoter-reporter. Additionally, overexpression of LIP elevated PR protein expression from the intact endogenous gene encoding PR. Furthermore, *in vivo* immunofluorescence studies showed that C/EBPβ and PRA expression are mutually exclusive in the mammary epithelium, while PRB is only expressed in cells that express C/EBPβ. This suggests an important role for C/EBPβ in PRB expression during pregnancy. Then, we studied the mechanism by which LIP and c-Jun synergistically activate the PR promoter. We demonstrated in the reporter assay that the integrity of C/EBP- and AP-1-binding sites was required for the respective C/EBPβ and c-Jun activities on the PR promoter. Moreover, we showed in ChIP assay that efficient promoter occupancy of both LIP and c-Jun and their synergistic transactivation of the PR promoter required at least one C/EBP- and one AP-1-binding site. In addition, as indicating in the sequential ChIP assay, C/EBPβ and c-Jun simultaneously occupied PR promoter. This leads us to propose a model where the synergy of C/EBPβ and c-Jun in transactivation of the PR promoter is dependent on the two factors mutually stabilizing their recruitment to the PR promoter. Collectively, our data suggest a critical role for C/EBPβ, particularly LIP, in PRB expression.

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CHAPTER 1

LITERATURE REVIEW

Progesterone Receptor (PR)

Progesterone (P4) plays critical roles in regulating normal female reproductive functions in ovulation, preparation of the uterus for implantation and pregnancy, development of the mammary gland for lactation, and the regulation of labor (Graham and Clarke, 1997). P4 effects are largely mediated through the progesterone receptor (PR), a member of the family of nuclear steroid receptors. PR also plays a major role in the diagnosis and treatment of breast cancers.

Progesterone Receptor Structure

PR belongs to the nuclear steroid receptor superfamily (Bain et al., 2007). All members of this family share the following structural domains: a C-terminal ligand-binding domain (LBD), a hinge region, a well-conserved DNA-binding domain (DBD), and a less conserved N-terminal regulatory domain (NTD) containing activation function domains (Figure 1).

In humans and rodents, PR exists as two main isoforms, PRA and PRB. These two isoforms are encoded from a single gene to give rise to two different transcripts as a result of transcription from two alternative promoters (Kastner et al., 1990; Kraus et al., 1993). PRA and PRB are identical in their LBD, DBD, and activation function 1 and 2 (AF1 and AF2) domains. The AFs are essential for transcriptional activity of PR. AF1 in the NTD is constitutively active in a ligand-independent manner, whereas AF2 in the LBD depends on ligand binding to induce a conformational change (Gao and Nawaz, 2002) that allows interaction with cofactors, such as steroid receptor coactivators SRCs, nuclear receptor interacting protein 1 NRIP1 (RIP 140), mediator complex DRIP/TRAPS (Hill et al., 2012), and CBP/p300 integrator complex (Kamei, 1996).

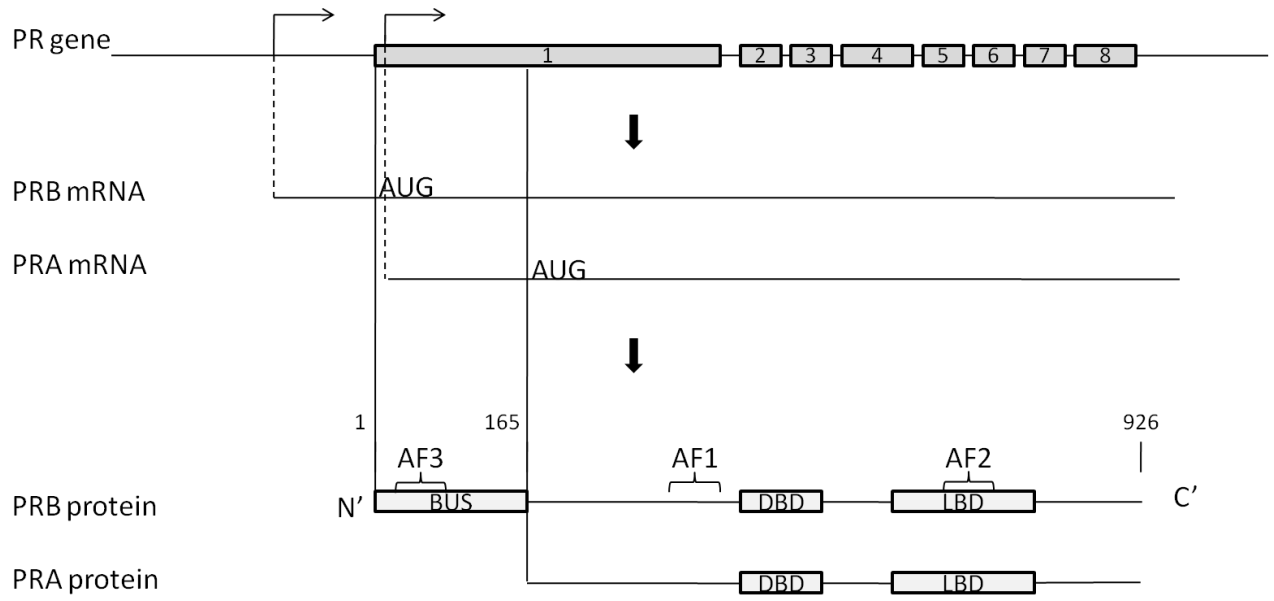

Figure 1.1. Structure of mouse progesterone receptor. Schematic representation of the mouse progesterone receptor gene and its two isoforms. Mouse PR gene with eight exons. Two differential promoters (PRB promoter and PRA promoter) give rise to two transcripts PRB mRNA and PRA mRNA, and consequent PRB isoform and PRA isoform, respectively. PRB differs from PRA by an extension of 164 amino acids at its N'-terminus, forming B-upstream segment (BUS). DNA binding domain (DBD), ligand binding domain (LBD), and three activation functions (AF1, AF2, and AF3) are indicated.

However, PRB differs from PRA by an extension of 164 amino acids at its N'-terminus, which is named as the B-upstream segment, BUS. BUS contains a third PRB-unique AF (AF3) which determines the differential functions of PRB in comparison to PRA. Since AF3 is suggested to interact with coactivators, PRA, lacking this AF3, inefficiently recruits coactivators GRIP1 and SRC-1 (Giangrande et al., 2000). AF3 is also shown to synergize with AF1 and AF2 (Takimoto et al., 2003; Tung et al., 2006) by cooperative interaction of a PR multimer complex bound to multiple progesterone responsive elements (PREs) (Tung et al., 2006). The AF3 is unaffected by an inhibitory function (IF) domain which suppresses the transcriptional activity of AF1 and AF2. The lack of AF3 in PRA contributes to its weaker transactivation function by permitting the inhibitory effect of IF on AF1 and AF2 functions (Hovland et al., 1998). Moreover, the BUS of PRB is also suggested to stabilize an active conformation of PRB (Bain et al., 2001). Gene expression profiling showed that form of PRB carrying site-specific mutation of AF3 ineffectively regulates PRB-dependent genes, but does not convert its transcriptional functions to those of PRA (Tung et al., 2006). In addition, the BUS may also affect the differential localization of PRA and PRB, and, thus consequentially, their distinct biological activities. PRA is mostly nuclear, whereas PRB is located both in the nucleus and cytoplasm in the absence of hormone (Lim et al., 1999).

A third truncated PR isoform, PRC, which is restricted primarily to the cytosolic fraction, lacks the N-terminal region and one zinc finger of DBD, and, thus, it is unable to bind DNA. However, PRC is still able to interact with hormone and heterodimerize with other PR isoforms, and consequent increase their activity in T47D breast cancer cell line (Wei et al., 1990, 1996, 1997). Moreover, alternative splicing is reported to generate PR variants lacking various whole, multiple or partial exons. These variants, which have been found in normal and cancer breast cell

lines and tissues, may compete for dimerization with other isoforms or for cofactors (Cork et al., 2008; Richer et al., 1998). Together, these variants may alter P4 responsiveness by leading to lower levels of PRA and PRB activity.

Progesterone Receptor Function

Newly synthesized PR is associated with a chaperone complex containing heat shock proteins (Catelli et al., 1985; Picard, 2006). In the absence of progestin (P), this complex maintains PR in an inactive form in the cytoplasm, generates an appropriate conformation for ligand binding, and prevents PR from proteolytic damage (Catelli et al., 1985; Picard, 2006; Pratt et al., 2004; Smith, 1993; Smith et al., 1990). P-bound PRs are dissociated from the heat shock proteins, translocate to the nucleus, and bind to PR binding sequences or progesterone response elements (PREs) in the promoters of target genes in form of either homo- or heterodimers (Gao and Nawaz, 2002). After binding, modulation of target gene expression may be altered by sequential interaction with either coactivators or corepressors (McKenna et al., 1999; Smith and O'Malley, 2004).

Since most of genes regulated by P do not contain consensus PREs (Richer et al., 2002), it is suggested that in addition to direct binding of PR to PREs, PRs can be recruited indirectly by other factors to promoters without PREs. These factors, such as AP-1 (Bamberger et al., 1996), Sp1 (Gao et al., 2001; Owen et al., 1998; Tang et al., 2002), NF-κB (Kalkhoven et al., 1996), Stat5 (Richer et al., 1998), Stat3 (Proietti et al., 2005), Cre/AP-1 (Tseng et al., 2003), CEBPβ (Christian et al., 2002), bind to DNA through their specific binding sites and tether PR to the target promoter through protein-protein interactions.

PR can also exert transcriptional activity in a ligand-independent manner. Unliganded PRA is reported to both up- and down-regulate a number of target genes. Most of these genes are

not regulated by P, suggesting that these genes have different functions compared to those involved in P4-driven processes (Jacobsen et al., 2005). For example, ECM adhesion genes DSCAM, ITGA3, CD44, PCDH1, and IGF-binding protein 5 (IGFBP5) and cell aggressiveness markers such as ITGA3 are up-regulated by unliganded PR (Jacobsen et al., 2005). In addition, unliganded PR is reported to bind to DNA and interact with a multi-repressor complex on 20% of hormone-inducible genes. These genes are maintained in an inactive state by this PR-corepressor complex. The presence of P may induce a conformational change to dissociate corepressors from the complex with the sequential association of coactivators to induce transcription of the target genes (Vicent et al., 2013). Thus, the structure of PRA and PRB determine their association with coregulators of transcriptional activity, such that PRA has higher affinity for corepressors and PRB has higher affinity for coactivators (Giangrande et al., 2000). A consequence of this is that both the availability of P and the PRA:PRB ratio affect the outcome of P stimulation.

In addition to PR genomic functions in direct modulation of target gene expression, PR can also act in a non-genomic manner through association with several signaling pathways and cytoplasmic signaling molecules. For example, P treatment rapidly and transiently activates protein kinases in breast cancer cells, such as MAPK, PI3K, and c-Src kinases (Boonyaratanakornkit et al., 2001; Migliaccio et al., 1998; Saitoh et al., 2005). The mechanism for this activity involves a specific polyproline motif in the N'-terminal domain of PR that is capable of interacting with the SH3 domains of Src family kinases in a progestin-dependent manner (Boonyaratanakornkit et al., 2001). This ultimately activates Ras and/or Raf1 and the ERK/MAPK pathways. P-induced c-Src/MAPK activation is disrupted by mutation of the polyproline motif, but not the DBD that is essential for the transactivation function of PRB. In addition, a homolog of human PR, Xenopus classical PR (XPR), is reported to directly associate

with active kinases PI3K and p42 MAPK in oocytes on induction by P4 (Bagowski et al., 2001). This mechanism is consistent with the rapid response to P that is observed in a non-genomic context.

PRE-containing promoters have been used to study PR as a transcriptional activator. Comparison of PRA to PRB by cotransfection isoform-specific PR expression plasmids with a PRE-containing reporter plasmid reveals differential activity between isoforms. PRB in all cell types is a potent transcriptional activator, while PRA exhibits a cell- and promoter-specific activity (Mote et al., 2007). Additionally, PRA is a weaker transcriptional activator and can act as a trans-repressor of PRB (Mote et al., 2007).

The study of PR isoform-specific gene signatures in a natural gene context with intact promoter regions and the expression of potentially PR-interacting transcription factors and cofactors may avoid the artifacts of artificial PRE-containing promoter-reporters. Expression profiling data from PR-negative T-47D breast cancer cells stably expressing either only PRA or PRB reveals distinct activities of the two isoforms (Jacobsen et al., 2005; Richer et al., 2002). The transcriptional activity of PR is shown to be isoform-, tissue- and promoter-specific. PRA and PRB regulate different subsets of genes. Under P treatment, the majority of genes are regulated uniquely by PRB, some genes by PRA, and a few genes by both (Richer et al., 2002). When the ligand-bound form of either isoform regulates the expression of a particular gene, PRB is transcriptionally more active than PRA (Richer et al., 2002). However, for those genes regulated by PR in a ligand-independent fashion, PRA is the stronger transactivator (Jacobsen et al., 2005). These studies demonstrate that homodimers have capacity to regulate target genes, in either P-dependent or -independent manners.

The aforementioned approaches explored the function of individual isoforms, however, without comprehensively examining the function of heterodimers. Thus, these results fail to reflect the co-expression of PRA and PRB, which often occurs physiologically (Mote et al., 2002). Co-expression yields three possible dimeric forms: PRA:PRA, PRB:PRB, and the PRA:PRB heterodimer. Cells with co-overexpression of both PRA and PRB at similar levels have a small number of P-responsive genes (Graham et al., 2005) which are shared with cells overexpressing single PR isoforms (Richer et al., 2002). This suggests that heterodimers and homodimers have differential impact on gene regulation.

In T-47D cell line, P-responsive gene profiles are largely unaffected by PRA:PRB ratio alteration from 1.8 to 5.1 (Graham et al., 2005) and repressive effects of PRA against PRB transcription activity are not observed unless PRA was more than 15-fold in excess over PRB (McGowan and Clarke, 1999). This supports the possibility that the presence of heterodimer is more important in determining the gene targets as long as the PRA isoforms not far exceed PRB isoforms.

Comparison between normal and breast cancer cells after P treatment shows the distinct expression profiles of genes between the two cell types with few overlapping genes (Graham et al., 2009), suggesting a dominant influence of tumorigenesis on P-driven transcription. Similarly, little overlap is reported between PRA-positive adult and pubertal mammary epithelial cells (Santos et al., 2009), suggesting dominant influence of the developmental stage of the cell.

Clearly, differences in structures, cis-element sequence, and interactions with other transcription factors and co-regulators determine each isoform's distinct activities in mammary gland regulation, differentiation, proliferation, and response to P.

Progesterone Receptor Role in Mammary Gland Development

The mammary gland is a hormone-responsive organ that develops primarily after birth. The gland dramatically develops during puberty, and more subtly changes with the hormonal fluctuations of the menstrual cycle. The tissue proliferates and differentiates during pregnancy to prepare for lactation, and then sequentially regresses at involution. Then, this cycle will repeat in the next pregnancy.

Progesterone, an ovarian steroid hormone, is produced at its highest levels in the luteal phase of menstrual cycle. During pregnancy, high levels of P are required to maintain pregnancy. P largely exerts its effects through its receptor – PR (Obr and Edwards, 2012).

In human mammary gland, both PR isoforms are often co-expressed in the same cells with similar levels (Mote et al., 2002) and PR levels are unchanged between the follicular and luteal phases of menstrual cycle (Graham and Clarke, 1997).

In mouse, expression of PRA and PRB are temporally and spatially separated during mammary gland development. PRA is the only expressed isoform during ductal development of the virgin mammary gland, and is at its highest levels during puberty. PRA-positive cells do not incorporate BrdU, thus they are not proliferating. The percentage of PRA-positive cells is reduced in the mature gland, further reduced in pregnancy. PRB is expressed robustly in majority of cells during alveologenesis in pregnancy, particularly at side branches and in developing alveolar lobules. At this stage, co-expression of PRA and PRB is detected in a small subset of cells. Moreover, PRB-positive cells incorporate the proliferation marker BrdU and stain for cyclin D1 during pregnancy, indicating that PRB-expressing cells are proliferative during pregnancy. No PR expression is observed during lactation (Aupperlee et al., 2005). Thus, PRA is dominant in the virgin gland and PRA-positive cells do not proliferate, while PRB is expressed

most highly in the pregnant gland and PRB-positive cells are highly proliferative. This implies that PRA and PRB are differentially regulated and that each isoform mediates a distinct response to P.

PR function in the mammary gland development has been studied in both knockout (KO) and transgenic mice. Mice lacking both PR isoforms (PRKO) exhibit normal ductal development in virgin glands, but impaired ductal branching and lobuloalveolar development during pregnancy (Lydon et al., 1995). This indicates that PRs are required for proliferation of the ductal epithelium and for differentiation of alveolar lobules during pregnancy, which are induced by prolonged treatment with E and P. Given the different transcriptional activities of the two isoforms, elucidation of the specific physiological function of each isoform requires mice that are selectively deficient in individual isoforms. PRAKO mice do not exhibit failure in the responses of the mammary gland to P (Mulac-Jericevic et al., 2000). The E and P-induced morphological changes of mammary glands in PRAKO mice are similar to those in wildtype, while these responses are inhibited in PRKO mice. Studies of PRAKO mice suggest that PRB is necessary and sufficient for the normal P-mediated proliferative and developmental responses of the mammary gland, and that functional expression of PRA is not required. In contrast to PRAKO mice, PRBKO mice exhibit a significant decrease in the proliferation of ductal and alveolar epithelial cells in response to P. As a result, fewer side branches and reduced lobuloalveolar development are observed in PRBKO mice (Mulac-Jericevic et al., 2003).

Transgenic mice overexpressing PRA or PRB both exhibit abnormal mammary development. PRA transgenic mice show increased ductal branching, epithelial cell hyperplasia, and a disorganized basement membrane (Shyamala et al., 1998), while PRB transgenic mice show reduced ductal development, precocious alveolar development, but no apparent tumor-

associated abnormalities (Shyamala et al., 2000). Together, this indicates that PRB plays a role in alveologenesis, while PRA plays a role in side branching.

P/PR signaling also contributes to mammary tumorigenesis. While the two isoforms are co-expressed at similar levels in the normal human breast, this ratio is altered in tumorigenesis, consistent with the aberrant response to hormones of breast cancer cells. A high PRA/PRB ratio is observed in some breast tumors, which are more aggressive and resistant to endocrine therapies (Hopp, 2004; Mote et al., 2002). Breast cancer patients with this high ratio have a higher probability of relapse than patients with lower ratios of PRA/PRB (Hopp, 2004). Consistent with this, transgenic mice with overexpression of PRA exhibit an abnormal developmental phenotype with hyperplasia (Shyamala et al., 1998). Predominance of PRA may lead to down-regulation of genes associated with differentiation and apoptosis, but up-regulation of genes related to tumor development and metastasis. Indeed, overexpression of PRA in cultured T47-D human breast cancer cells leads to changes in morphology and adhesive properties (McGowan and Clarke, 1999). Moreover, PRA transgenic mice show differential gene expression in comparison to wildtype mice, as decreased levels of p21, increased level of cyclin D1 (Chou et al., 2003), decreased TGFb1 activation (Simian et al., 2009), which correlate with high proliferation. Collectively, the data suggest that PRA may be more involved in the severity of breast cancer than PRB. PRA is often associated with high proliferation and a more aggressive tumor phenotype, while PRB is associated with differentiation of the mammary gland.

In addition, PRKO mice showed a reduction in mammary tumor incidence when treated with DMBA compared to wildtype mice (Lydon et al., 1999), indicating a critical role of PR in promoting tumorigenesis.

Collectively, the expression of the two PR isoforms is highly regulated and their

comparative expression levels ensure the appropriate cellular response to progesterone.

Regulation of Progesterone Receptor Expression

The status of chromatin has been reported to have effects on PR expression. Gene silencing mediated by methylation of DNA at cytosine-guanine dinucleotides (CpG) has been reported for the PR gene. The human PR gene has a CpG island in its 5' regulatory regions. Early reports using Southern analysis of DNA digested with methylation-sensitive restriction enzymes showed that this CpG island was hypermethylated in 40% of PR-negative breast tumors and cancer cell lines (MDA-MB-231, MDA-MB-468, and MDA-MB-435), but not in normal breast specimens and PR-positive breast tumors and cancer cell lines (MCF-7, T-47D, and ZR75) (Lapidus et al., 1996; Yang et al., 2001). Moreover, the study of isoform-specific promoters showed that the PRA promoter and the PRB promoter were methylated in 7.7% and 92.2% of breast cancers, respectively (Gaudet et al., 2009). Hypermethylation of PRB promoter has been also observed in numerous dysplasias of human reproductive tissues, such as uterine endometrial carcinoma (Sasaki et al., 2001), in endometriotic epithelial cells (Wu et al., 2006), adenomyosis (Jichan Nie et al., 2010), and sporadic breast cancer (Mc Cormack et al., 2008). Hypermethylation of PRA was shown in constitutive anti-progestin-resistant tumors, where PRA is silenced (Wargon et al., 2011). However, reduction of DNA methylation levels by DNA methyltransferase inhibitor was insufficient to reactivate PR gene expression in MDA-MB-231 cells, which are negative for ER and two PR isoforms. Thus other remodeling chromatin mechanisms such as histone acetylation and methylation may be a further requirement for PR gene transcription (Ferguson et al., 1998).

Histone modification, such as histone methylation and histone acetylation, of the PR gene has been reported. Using the MCF-7 breast cancer cell model, a ChIP assay showed that histone

H3 lysine 4 (H3K4) methylation at the region containing the +571bp ERE half-site near PRA transcription start site was associated with gene activation, and this controlled PR upregulation by estradiol treatment (Stratmann and Haendler, 2011). In the same study, JARID1A, a H3K4 demethylase that specifically removes H3K4 methylation, is found to bind to this region to counter E-upregulation of PR expression. Similarly, one physiological phenomenon observed in the human myometrium during active labor is the increase of H3K4me3 at the PRA promoter. Even though the occupancy of SMYD3, a co-activator of ER-mediated transcription that methylates H3K4 at EREs, was enriched at PRA promoter with no labor association in myometrium, JARID1A occupancy, which demethylates H3K4me3 and represses PR expression, was reduced at labor stage (Chai et al., 2014). They suggested that interaction of SMYD3 and JARID1A would determine H3K4 methylation status, and consequent PRA expression at this stage. Together, those epigenetic changes lead to differential expression levels of PR isoforms.

Two separate promoters for PRA and PRB have been reported in human and rat. Each promoter gives rise to the expression of different mRNAs. Human PR contains two distinct promoters, a PRB promoter from -711 to +31bp relative to transcription start site for PRB and a PRA promoter from +464 to +1105bp (Kastner et al., 1990) . Human studies have found that 17β-estradiol (E2) and ER are the major factors regulating PR. E responsiveness is mediated through non-canonical EREs, as well as other binding sites for additional transcription factors. In the human gene, an ERE half-site with two adjacent Sp1 sites in the +571/+595 region, two Sp1 sites in the -80/-34 region, and a +90AP-1 site have been identified for ER-mediated upregulation of PR, whereas +745AP-1 site with an adjacent imperfect ERE is repressive.

The ERE half-site with two adjacent Sp1 sites at +571/+595 is located in the PRA-specific region of the human PR promoter. In vitro binding assays confirm that Sp1 and ER

directly bind to their respective sites and form a Sp1-ER-DNA complex in this region (Petz and Nardulli, 2000). This study found that ER enhances Sp1 binding, and that ER fails to bind to a mutant ERE half site. Further studies examined the individual contributions of each site to E2-mediated transcription of PR (Petz et al., 2004a). The two Sp1 sites gave rise to an additive rather than a cooperative or synergistic effect. Studies in intact cells detect Sp1 occupancy on the endogenous ERE/Sp1 site with or without E2 treatment, suggesting its role in the basal PR expression in the absence of E. Basal expression of a TATA-CAT reporters inserted with an oligo containing mutated single or both Sp1 sites of +571 ERE/Sp1 sequence are decreased in comparison with the wildtype reporter. Together observations suggest that Sp1 by itself may activate basal expression of PRA, whose start site is about 150bp downstream. Moreover, mutation of both Sp1 sites decreases promoter activity more than does mutation of a single site, while mutation of the ERE site increases transcription. However, ERα occupancy is only observed after E2 addition and enhanced Sp1 binding in vitro, suggesting that this ERE/Sp1 site modestly modulates PR transcription.

In addition to this half ERE/Sp1 site, another ERE half-site is found between the two PR promoters to enhance PR expression. This site is adjacent to an AP-1 site at +90. In gel mobility shift assay, ER binding enhances AP-1 (c-Jun and c-Fos) protein binding. In the endogenous promoter of intact cells, this region shows substantial occupancy by ER, c-Fos, and c-Jun in ChIP assays (Petz et al., 2002). This AP-1 site that interacts with EREs is not well conserved across species (our observation), so the role AP-1 in E2-responsiveness of PR may not be a global phenomenon.

The same group found E2-responsive elements in the PRB promoter region located from -80 to -34, containing two Sp1 sites, but without direct binding of ERα (Schultz et al., 2003).

However, ERα is able to enhance Sp1 binding to its site. Mutation of these Sp1 sites leads to inefficient Sp1-DNAcomplex formation in vitro and substantially decreased promoter activity. The Sp1 site proximal to transcriptional initiation is more potent than the distal Sp1 site in E2-responsive activation of PR, as shown in a promoter-reporter assay utilizing site-specific mutations plus coexpression of ERα.

An additional E2-responsive AP-1 site with an adjacent imperfect ERE has been reported for the PR promoter at +745bp. Cotransfection for ERα and c-Jun/c-Fos expression repressed wildtype 1.5kb promoter activity (containing sequence from -711 to +817), whereas E2 and ERα enhanced transcription in a promoter where this AP-1 site had been mutated, indicating that inhibitory effect of E2 at this site on the PR gene was mediated in an AP-1-dependent manner. The binding of ERα to this ERE site enhances the binding of c-Jun and c-Fos in vitro. In ChIP assay of the intact endogenous gene, the complex of c-Fos and ERα with DNA was observed in the presence of E2, while a c-Jun-containing complex was present with and without E2 treatment (Petz et al., 2004b). This +745 site is adjacent to the PRA transcription start site at +737bp, thus it is suggested to modulate the expression of PRA.

Because PR levels increase in cyclin D1 transgenic mice with E2 treatment and decrease in cyclin D1 KO mice (Yang et al., 2010), cyclin D1 was examined for its role in transactivation of the PR gene (Yang et al., 2010). Cyclin D1 stimulated human PR expression via an enhancer in the 3'-untranslated region (UTR) which is bound by ERα in a ChIP assay. This UTR when cloned to a heterologous promoter could enhance transcription in response to E2 and cyclin D1. This enhancer region conferred significantly activated reporter expression to the PR promoter region. In experiments examining overexpression or knockdown of cyclin D1, the UTR-containing promoter was responsive to changes in cyclin D1 levels (Yang et al., 2010). In

addition to cyclin D1 modulation of PR expression, there is an auto-inhibitory loop where liganded PR suppresses cyclin D1 expression (Groshong et al., 1997).

In the rat PR gene, two distinct promoters, a distal -131/+65bp PRB promoter and a proximal +461/675bp PRA promoter have also been identified (Kraus et al., 1993). Unlike the human PR gene where E can induce the activity of both isoform-specific promoters and can up-regulate the expression of both isoforms, only the rat proximal PRA promoter is induced by E2 in MCF7 cells. These studies found five widely distributed E-responsive regions containing four imperfect ERE sites and four ERE half-sites. The enhancers constructed by PR fragments of five EREs showed both promoter specificity and E2-responsiveness. The position and spacing of the ERE regions determined E2-mediated cooperative effects on PR expression among the respective elements (Kraus et al., 1994).

In the rabbit, PRB is the only isoform expressed and its transcript is produced from a single promoter (Savouret et al., 1991). An ERE at the translational initiation site confers E-dependent upregulation of PR.

In addition to the effects of EREs and transcription factor binding sites proximal to the PR gene in the 1-2kb sequence in the vicinity of the transcription start sites (TSS), a long range transcriptional mechanism of ER-dependent upregulation of PR is also reported (Bonéy-Montoya et al., 2010). In particular, eight EREs, which had been shown to bind ERα are located in a region from 311 kb upstream to 4 kb downstream of the PRB TSS. These distal regions cooperatively interact with TSS proximal regions and increase the sensitivity of the PR gene to E-mediated upregulation.

There is evidence supporting the notion that other E/ER-independent factors are also involved in regulating PR expression. For example, PR expression does not change during the

hormone fluctuations associated with the menstrual cycle (Mote et al., 2002), so some mechanism that attenuates hormonal influences must be at play. PR was still detected in ERαKO mice (Korach, 2000), so the basal level of PR expression must depend on other factors that are not hormone-dependent for their activity. In ER+/PR- breast tumors, the expression of ER is clearly insufficient for PR expression, even though ER is fully functional in these tumors (Cui et al., 2003a). Clearly, PR transcription utilizes some E-independent regulatory mechanisms.

Although the two Sp1 sites in the -80/-34bp sequence play a role in E2-mediated transcription, another study found in that region a -67bp binding site for the homeobox protein HOXA5, which was involved in hormone-independent PR expression. In vitro binding of HOXA5 to this site and upregulation of PR expression were observed with a wildtype but not a HOXA5 binding site mutant promoter sequence (Raman et al., 2000).

GATA5 has also been implicated in PR regulation (Huggins et al., 2006). The human PR gene has a +331G/A polymorphism that correlates with breast cancer risk. GATA5, which is strongly expressed in breast cancer cell lines, binds adjacent to the polymorphism at +311 site and activates the transcription of human PR promoter, especially the PRB promoter. The +331A allele is more strongly activated by GATA5 than the +331G allele, and is associated with increased breast cancer risk.

Another level of transcriptional regulation of PR expression occurs through positive and negative autoregulation that is cell type and tissue-specific.

For example, in rabbit PRB, an ERE for E-dependent upregulation of PR also controls P/PR-dependent downregulation of PR, which requires the DNA binding domain of PR and not as a result of decreased affinity of ERE; however, the mechanism is unclear (Savouret et al., 1991). P blocks the stimulatory effects of E2 on both PR mRNA and protein in the rat uterus

(Kraus and Katzenellenbogen, 1993). P represses both basal and E2-stimulated human PR transcription rate and mRNA concentration without affecting the activity of PRB (−711/+31) and PRA (+464/+1105) promoters in T-47D human breast cancer cell lines (Graham et al., 1995). The fact that multiple examples exist for negative P-dependent autoregulation of PR suggests that the expression levels of these receptors must be limited or that signaling must be transient rather than sustained.

In addition, PR exerts a positive feedback loop for its own expression by activating its own promoter (Tang et al., 2002). In human endometrial stromal cells treated with the PR ligand medrozyprogesterone acetate (MPA), PRA, and to lesser extent PRB, significantly induces the transcription of PR promoter reporters. Since PR ligand binding domain, but not DNA binding domain is required, this transactivation is not mediated through a PR binding site, but an Sp1 site at -49/-43bp, where site-specific mutation abrogates PR-mediated transactivation function. Moreover, the physical interaction between Sp1 and PR has been identified, suggesting a model in which Sp1 may recruit PR (Tang et al., 2002).

Provocatively, this positive loop extends to other transcription factors that influence PR expression. Liganded PR increases C/EBPβ levels in human breast cancer cells (Richer et al., 2002) and in mouse mammary glands (Aupperlee et al., 2009), and c-Jun, as well, is P-regulated (Alkhalaf and Murphy, 1992). Our own research (presented in this dissertation) shows the activity of C/EBPβ and c-Jun in transcription from the PR promoter. This draws a picture of a regulatory network where P enhances the expression of its own receptor through the activation of several transcription factors.

In addition to E2, other signaling pathways regulate PR expression in different cell types, such as follicle stimulating hormone (FSH), luteinizing hormone (LH) and forskolin (all of

which elevate cAMP) in rat preovulatory granulosa cells (Clemens et al., 1998; Sriraman et al., 2003). In rat, distal (-131/+65bp) promoter shows low basal and small induced level to forskolin, whereas proximal (+461/+675bp) promoter exhibits higher basal activity and response to forskolin. The putative ERE-like region (ERE3) in the rat proximal PR promoter requires for forskolin-induce activity of reporter construct containing ERE3 region ligated with PR promoter. The distal PR promoter contains a GC rich region with a CCAAT box at -68 bp and a GC box for putative Spl binding at -47 bp. This GC rich region binds Sp1, but does not bind either C/EBPα or C/EBPβ in vitro. They suspected that Sp1/Sp3 synergized with factors binding to ERE3 to transactivate forskolin-induced distal promoter (Clemens et al., 1998). In another report (Sriraman et al., 2003), forskolin/phorbolmyristate (PMA) significantly transactivates the mouse PR promoter reporter (-348/+680bp). Similarly to the rat promoter, the distal promoter (-348/+64) and proximal promoter (+420/+680bp) show similar activity and response to forskolin. Mutation of either the CCAAT box or the Sp1 site in GC region of distal promoter did not alter transcriptional function, even though they identified in vitro that NFY-B, Sp1/Sp3, and GATA-4 bound to a CCAAT box, GC box, and a GATA site, respectively. The proximal promoter region confers PMA-responsiveness, this effect is mediated by two Sp1/Sp3 binding sites +440/+461 and +473/+490. Among of two, mutation +473/+490 Sp1 site results in total loss of proximal promoter activity, 70-80% reduction of forskolin/PMA-induced intact longer (-145/+680bp) promoter, and more severe reduction of longer promoter when combination with mutated distal GC box (Sriraman et al., 2003).

In breast cancer cells, downregulation of PR mRNA transcription by IGF-I (Insulin like growth factor I), which is mediated through the PI3K/AKT/mTOR signaling pathway, depends upon PR promoter context (Cui et al., 2003a). Interestingly, IRS-2, which is utilized by IGFs for

signal transduction in cells, is transcriptional induced by liganded PRB (Cui et al., 2003b). This suggests that PR has a negative feedback loop by indirectly suppressing its expression through IRS-2, an element for IGF-I effectiveness. Together with the positive feedback loops discussed above, this negative feedback loop may sensitively fine-tune the expression of PR.

In sum, PR gene contains multiple regulatory elements operating at both the transcriptional and post-transcriptional levels in a species, tissue (e.g., breast vs. reproductive organs), cell (e.g., normal vs. neoplastic), PR isoform (i.e., PRA vs. PRB), ligand, and coactivator-specific manner. In addition to the mechanisms discussed here, PR is also regulated by post-translational modifications, including phosphorylation, ubiquitination, sumoylation, and acetylation. These modifications affect PR functions at multiple levels to alter PR stability, localization in the cell, interaction with co-factors/co-regulators, and transcriptional activity. These mechanisms also show species, tissue, cell, isoform, ligand, and co-regulator specificity to provide a very complex regulatory system.

CCAAT/Enhancer Binding Protein (C/EBP) family

CCAAT/enhancer-binding proteins (C/EBPs) are widely expressed transcription factors involved in many biological processes, including energy metabolism, immunity, inflammation, hematopoiesis, adipogenesis, osteoclastogenesis, cell cycle, and cellular proliferation and differentiation (Tsukada et al., 2011). The C/EBPs comprise a family of basic leucine zipper transcription factors with six identified members: C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε, and C/EBPζ (Ramji, 2002; Zahnow, 2009). The C/EBPα, -β, -γ, -δ and genes are intronless, whereas C/EBPε and -ζ contain two and four exons, respectively. The C/EBPγ, C/EBPδ, and C/EBPζ genes produce single proteins. However, the C/EBPα and C/EBPβ genes generate multiple isoforms by the alternative use of translation initiation sites from the same mRNA. The

C/EBPε. gene also produces multiple isoforms; however this is accomplished by alternative promoter usage and differential splicing.

C/EBP family members consist of three structural regions: an N'-terminal activation domain (AD), a basic amino acid-rich DNA binding domain, and a leucine zipper domain that allows dimerization. The two latter domains are referred to as a bZIP region. Their N'-terminal region, which is least conserved across the different family members (20% shared sequence identity), contains activation domains that interact with the basal transcription apparatus and several transcriptional coactivators. C/EBPζ lacks defined activation domains and possesses a less conserved DNA binding domain that fails to bind the C/EBP consensus binding site. It generally acts as a dominant negative inhibitor of C/EBP-dependent transcription by forming heterodimers with other C/EBP family members that fail to bind the C/EBP consensus binding site. C/EBPζ:C/EBPβ heterodimers are known to activate endoplasmic reticulum stress response genes through a non-consensus binding site. C/EBPγ also lacks defined activation domains and has generally been seen an inhibitor of C/EBP-depend transcription, although it has been shown to have a stimulatory effect in the activation of IL-6 and IL-8 transcription (Gao et al, 2002).

In addition to N-terminal activation motifs, negative regulatory regions have also been identified in some C/EBPs. For example, C/EBPβ has two N'-terminal negative regulatory domains, RD1 and RD2, of which deletion mutations or phosphorylation result in increased protein activity. RD1 suppresses transcriptional activity by inhibiting AD through direct interaction, while RD2 inhibits the DNA binding activity (Williams et al., 1995)

The highly conserved bZIP domain (90% shared sequence identity) is required for DNA binding and for forming homodimers or heterodimers within the C/EBP family. The multiple isoforms among C/EBPα, -β, and -ε provide even greater diversity in dimer formation than that

derived from six simple family members. There is also limited dimerization with members of other leucine zipper protein families, such as the AP-1 family. C/EBPs bind as a dimer to a consensus DNA binding site: RTTGCGYAAY, where R is A or G, and Y is C or T. However, numerous variations of the consensus site containing a conserved half-site followed by a more divergent sequence with at least 2 bp of the consensus are functional (Tsukada et al., 2011)

The differential transactivation activity of various C/EBP dimmers for particular target genes is dependent on not only the DNA binding specificities and affinities, but also the family member and isoform composition of the dimer. In turn, the composition and formation of C/EBP dimers depends on tissue- and developmental stage-specific expression of various C/EBP family members and isoforms. Also, specific post-translational modifications and the availability of co-regulators and other transcription factors contribute to the transactivation activity of C/EBP transcription factors. On the other hand, the structural similarities among all the C/EBP family members and isoforms can lead to their functional redundancy, depending on their co-expression in a specific cell type.

C/EBPβ

The C/EBPβ gene has been cloned from the human, mouse, rat, cow, wild boar, chicken, frog, sea slug, salmon, and zebrafish. In all species, it is encoded by an intronless gene containing a single exon. In the mouse, the C/EBPβ gene is transcribed into a single 1.4-Kb mRNA. C/EBPβ expression is widely regulated by multiple mechanisms. At the transcriptional level, C/EBPβ expression is regulated by a number of transcription factors, including CREB/ATF, Sp1, c-Jun/ATF2, KLF4, Nrf2, tethering of Stat3, and autoregulation by C/EBPβ itself (Berrier et al., 1998; Birsoy et al., 2008; Hou et al., 2012; Lin et al., 2002; Niehof et al., 1997, 2001a, 2001b) . Posttranscriptional control of C/EBPβ gene expression also has been

reported. AU-binding protein HuR interacts with the 3'-UTR of C/EBPβ mRNA to enhance mRNA stability and translation in anaplastic large cell lymphomas (Bergalet et al., 2011). The expression of the multiple C/EBPβ isoforms is regulated translationally. In the mouse, the three isoforms are formed by either leaky ribosome scanning or differential initiation of translation using alternative in-frame AUG sites (Zahnow, 2009): full-length 38-kDa liver-enriched activator protein (LAP1), 35-kDa N-terminally truncated LAP2, and 21-kDa N-terminally truncated liver inhibitory protein (LIP). In the mouse, LAP1 comprises the two conserved regulatory domains found in other C/EBP proteins, as well as the bZIP region. Additionally, the first N-terminal 21 amino acids constitute a regulatory domain with chromatin remodeling activity. The smaller LAP2 lacks the first 21 amino acids of the N-terminus, but is otherwise identical to LAP1. In many cell types and tissues, LAP2 is the most active and abundant isoform compared to LAP1 and LIP. The shortest truncated isoform, LIP, lacks all of the N-terminal activation domains. Heterodimerization of LIP with LAP isoforms generally represses the transactivation function of LAPs. Moreover, LIP homodimers have higher affinity for DNA binding than LAP1 and LAP2, and thus can effectively compete with these activating isoforms for DNA binding. LIP is generally considered as a dominant negative isoform, although there are instances where it can participate in transactivation. For instance, LIP can participate in activation of the IL-6 promoter (Hu et al., 2000).

LAP1 is translated at the first in-frame start codon. The translation of LAP2 and LIP is controlled by the 5' UTR containing an upstream AUG (uAUG) and a small upstream open reading frame (uORF). This region, located between the first start codon for LAP1 and the second start codon for LAP2, forms a stable secondary hairpin structure, which interferes with scanning ribosomes. Translation starting at uAUG prevents initiation at the LAP2 initiation

codon adjacent to this uAUG, allowing ribosomes to re-initiate translation at the downstream LIP initiation codon. However, failure of initiation at uAUG allows translation to initiate at the LAP2 AUG codon to produce LAP2 protein (review in (Zahnow, 2009)).

Translation initiation factors, such as eIF-2 and eIF-4E, have been shown to contribute to the differential translation C/EBPβ isoforms. Increased activity of eukaryotic translation initiation factors eIF-2 and eIF-4E through inactivation of RNA-dependent protein kinase (PKR) and activation of mammalian target of rapamycin (mTOR) favor LIP expression, whereas decrease of eIF and inhibition of mTOR activity enhance expression of LAPs (Calkhoven et al., 2000; Jundt et al., 2005). Moreover, some specific RNA binding proteins, such as CUG repeat binding protein (CUGBP1) and calreticulin, interact with the uORF structure in the 5'- end of the C/EBPß mRNA and induce translation from downstream start codons (Timchenko et al., 2002, 1999).

In addition to the 5'-UTR/uORF mechanism, LIP can also be generated through proteolytic cleavage of full-length C/EBPβ by a C/EBPα -dependent mechanism (Welm et al., 1999), by in vitro proteolysis (Baer and Johnson, 2000), or by the activity of the proteolytic enzyme calpain (Wei et al., 2006).

The functions of C/EBPβ depend on not only differential translation products and the resulting isoform ratio, but also post-translational modifications that are linked to multiple signaling pathways. These modifications, which include phosphorylation, sumoylation, acetylation, and methylation, alter C/EBPβ function, transcriptional activity, efficiency of DNA binding, and interaction with other co-factors. C/EBPβ is normally maintained in repressed state which is controlled by two regulatory domains (Kowenz-Leutz et al., 1994; Williams et al., 1995). This inhibited conformation is relieved and transcriptional function activated by

phosphorylation of multiple C/EBPβ serine and threonine residues by kinases: Ras–MAPK, growth factors and glycogen synthase kinase 3b (GSK3b), calcium/calmodulin-dependent protein kinase, ribosomal S6 kinase, protein kinases A and C, the cyclin-dependent kinase pathway CDK1–CDK2–CCNA2 (cyclin A) (Zahnow, 2009).

There is also a crosstalk between phosphorylation and methylation. In the absence of activating phosphorylation, C/EBPβ is methylated at conserved K39 and R3 residues by histone-lysine N-methyltransferase - H3 lysine-9-specific 3 (G9a) and protein arginine methyltransferase 4 (PRMT4/CARM1), respectively, and maintained in an inactive or repressed state; however, phosphorylation of C/EBPβ at MAP kinase site (chicken Thr220 and human Thr235) in the RD disrupts the interaction between the C/EBPβ protein and those methyltransferases (Kowenz-Leutz et al., 2010; Pless et al., 2008).

Acetylation of C/EBPβ modification may either activate or repress its activity, depending on the acetyltransferase and sites. Increased acetylation of a cluster of lysine residues from amino acid 98 to 102 by GCN5 and PCAF during glucocorticoid treatment enhances C/EBPβ transcriptional potential (Wiper-Bergeron et al., 2007). Similarly, acetylation at Lys39 increases the transactivational activity of C/EBPβ on promoters for C/EBPα and c-Fos (Ceseña et al., 2007). However, acetylation at Lys215 or Lys216 decreases C/EBPβ binding activity, whereas deacetylation by HDAC1 is required for C/EBPβ transactivational function on *Id-1* gene (Xu et al., 2003).

Sumoylation, which correlates with gene silencing, is also reported with C/EBPβ. Human LAP1, but not LAP2, can be sumoylated on Lys173 (Lys132 in mouse) by SUMO-2 and SUMO-3. This sumoylation of LAP1 requires Lys173 and the first 23 N'-terminus amino acids unique to LAP1 of human C/EBPβ. The sumoylated form of LAP1 impairs the transactivational

function of LAP1 on cyclin D1 (CCND1) gene (Eaton and Sealy, 2003)

Constitutive expression of C/EBPβ is high in the liver, intestine, lung, adipose tissue, spleen, kidney and myelomonocytic cells (Ramji and Foka, 2002). In these instances, LAP1 is generally expressed at lower levels than LAP2 and LIP. In most of cases, LAP2 is regarded as the most transcriptionally active isoform, while LIP is an inhibitor of C/EBP-dependent transcription (Zahnow, 2009).

The ratio of LAP:LIP has been shown to be altered in ER stress response, in mammary gland development, in liver development, regeneration, and lipopolysaccharide (LPS)-mediated acute phase response, and in some cancer diseases (Li et al., 2008; Zahnow, 2002). These observations indicate that this ratio has a substantial function importance in these processes and similarly to dimerization between different C/EBP family members, dimerization among C/EBPβ isoforms can determine its functional activities, including DNA binding, transactivation, cross-talk with signaling pathways, and interaction with other transcription factors/co-regulators.

LAP1 and LAP2 differ by an additional 21 amino acids at the N-terminus of LAP1. This structural difference may determine the functional distinction between the two isoforms through their abilities to form specific folded structures and consequent distinct protein-protein interactions. LAP2 is generally the more active of the two isoforms, although the activity of these two isoforms is both dependent upon the specific transcriptional target and the activity of specific signaling pathways. Unlike LAP2, LAP1 is reported to be a repressor in certain contexts. The 21 amino acid N'-terminus contains a motif allowing interaction with the enabled/VASP homology 1 (EVH1) domain of Homer-3. This interaction suppresses C/EBPβ transcriptional activity (Ishiguro and Xavier, 2004). In addition, N'-terminus of LAP1 is required for binding and recruiting SWI/SNF to the promoter of Ric-8B gene encoding for a guanine exchange factor

(GEF), resulting in negative regulation of this gene expression. However, methylation of R3 prevents the interaction and consequently inhibits LAP1 repressive effect on Ric-8B gene (Aguilar et al., 2014; Grandy et al., 2011). Moreover, 21-amino acid region also plays a role in LAP1 susceptibility to sumoylation. Sumoylation is specific for LAP1, and results in LAP1 inactivation. Consequently, LAP1 fails to transactivate cyclin D1 gene. Whereas LAP2, lacking this modification, is able to activate this gene (Eaton and Sealy, 2003). Other genes repressed by sumoylated LAP1 include peroxisome proliferator-activated receptor β/δ (PPARβ/δ) (Di-Poï et al., 2005) and cyclooxygenase-2 (cox-2) through recruiting HDAC4 by LAP1 (Wang et al., 2008). This effect is target gene-specific, as sumoylation does not inhibit the activity of LAP1 in IL-4 transcription and relieves the repressive effects of LAP1 on c-myc transcription (Berberich-Siebelt et al., 2006). Additionally, there are instances where LAP2 preferentially binds co-activators, while LAP1 with its additional amino acids cannot. For example, Nopp140 synergizes with LAP2, but not LAP1, to transactivate the AGP promoter-reporter. LAP2 also has higher basal activity than LAP1 in AGP transactivation (Lee et al., 1996).

However, LAP1 is also has unique activation functions. LAP1 activates manganese-containing superoxide dismutase (MnSOD), while LAP2 and LIP inhibit IL-1β-dependent induction of this gene (Qiu et al., 2008). Moreover, LAP1, but neither LAP2 nor LIP, is regulated in a redox-responsive manner, in which the formation of a Cys11-Cys33 (i.e., positions 11 and 33) disulfide bond in LAP1 represses its activity and allows its activation by a redox switch to up-regulate IL-6 expression in the presence of LPS (Su et al., 2003). There are other examples for LAP1-specific activation functions. Unlike repressive effect in Ric-8B gene, the interaction of chromatin remodeling complex SWI/SNF and the LAP1-specific N-terminal region results in activation of a number of myeloid target genes (Kowenz-Leutz and Leutz, 1999). Unmethylation

of R3 residue in LAP1-specific N'-terminus enhances SWI/SNF recruitment, eventually leads to target gene activation (Kowenz-Leutz et al., 2010).

Surprisingly, LIP, although lacking any defined activation domains, has also been reported to play a role in activating some genes via its interactions with other transcription factors. LIP and PRB cooperatively activate both the mouse mammary tumor virus (MMTV) promoter, which has several consensus PREs, and a synthetic construct consisting of two palindromic PREs upstream of the minimal dPRL promoter region (Christian et al., 2002). This synergy requires the binding of ligand-bound PRB to its binding site with LIP acting as a coactivator. LIP also interacts with Runx2 to promote osteoblast differentiation (Hata et al., 2005) and cooperates with NF-κB for LPS-induced transcription of IL-6 promoter in B lymphoblast (Hu et al., 2000). LIP is also the most active C/EBPβ isoform in activation of the CDH3 promoter in breast cancer cells (Albergaria et al., 2013), and up-regulates the transcription of the chemokine receptor CXCR4 through direct binding to the CXCR4 promoter (Park et al., 2013). It has been suggested that the bZIP domain of C/EBPβ, which comprises the majority of LIP, serves as a scaffold for mediating the association of various transcriptional activators with their target genes (Hata et al., 2005; Tominaga et al., 2008). Moreover, the mutated C/EBPβΔSpl (covalent to LIP protein) activates cyclin D1-responsive promoters similarly to cyclin D1 by itself, while the LAP1 wildtype protein reduces the cyclin D1-induced promoter activity, this indicates that LIP may act as a competitive inhibitor to the repressive form of LAP1(Lamb et al., 2003). These models are not mutually exclusive of each other.

The synergistic effects of C/EBPβ with other factors which bind adjacent to C/EBP site on DNA have been widely reported. However, the cooperation of C/EBPβ and other factor from separated sites on a promoter has also been reported. C/EBPβ directly interacts with cMyb in a

distant manner, 80 bp away (Tahirov et al., 2002).

Role of C/EBPβ in Mammary Gland Development

Low levels of C/EBPα expression during mammary gland development and the absence of any effects on gland morphology in C/EBPα KO mice suggests that C/EBPα plays a relatively minor role in mammary epithelial cell biology (Robinson et al., 1998; Seagroves et al., 1998). However, C/EBPβ and C/EBPδ show dramatic changes in expression levels and have functions during mammary gland development.

C/EBPδ mRNA levels are low throughout pregnancy and lactation, but are elevated transiently at the onset of involution (Gigliotti and DeWille, 1998). C/EBPδ is involved in the initiation growth arrest and apoptosis during involution (O'Rourke et al., 1999; Thangaraju et al., 2005). Further, the mammary glands of nulliparous C/EBPδ KO mice show ductal hyperplasia in gland, as well as delayed involution (Gigliotti et al., 2003). These latter results indicate that C/EBPδ functions not only in involution, but in growth regulation during development in nulliparous mice.

C/EBPβ mRNA levels are detectable in the virgin gland, elevated during pregnancy, decline slightly in mid-lactation, and activated again during involution (Gigliotti and DeWille, 1998; Robinson et al., 1998). At protein levels, LAP1 and LAP2 are detectable and vary in expression to some degree at all stages, with increased expression of LAP2 at the onset of pregnancy and very low LIP levels in the virgin gland that are dramatically increased during pregnancy and decreased to virgin gland levels during lactation (Raught et al., 1995; Seagroves et al., 1998). The variation in LIP expression during pregnancy suggests it, among other C/EBPβ isoforms, specifically plays a key role at that stage. The expression of PRB is also elevated at this time (Kariagina et al., 2008).

Mice lacking all three C/EBPβ isoforms exhibit reduced ductal development, decreased branching, and low proliferative rate. This is especially evident in abnormal alveologenesis with only limited lobuloalveolar development and ductal side branching during conditions of pregnancy mimicked by estrogen/progesterone treatment (Robinson et al., 1998; Seagroves et al., 1998). This phenotype is similar to that observed in PRKO mice, specifically PRBKO mice (Lydon et al., 1995; Mulac-Jericevic et al., 2003) . While C/EBPβ levels are unchanged in PRKO mice (Seagroves et al., 2000), an increased number of PR-positive cells and increased mRNA levels are in C/EBPβ KO mice (Seagroves et al., 2000). Further indication of a relationship between C/EBPβ and PR expression is that an overall decrease in PR that is observed in sexually mature wild type mice fails to occur in C/EBPβ KO mice, while no alterations in C/EBPβ expression are observed in PR-deficient mice (Seagroves et al., 2000). This is consistent with C/EBPβ acting upstream of PR. Moreover, uniform distribution of PR in all stages rather than switching to non-uniform pattern between 8-12 weeks of age and dramatically reduced proliferation of epithelial cells in E+P treatment are observed in C/EBPβ KO mice. The non-uniform distribution of PR-positive cells was proposed to initiate alveolar development through the juxtacrine signaling to affect the proliferation of PR-negative cells (Seagroves et al., 2000). C/EBPβ KO mice actually showed increased numbers of ERα/PR positive cells, yet these cells fail to proliferate in response to E and P (Grimm et al., 2005). The lack of hormone-responsive proliferation was attributed to increased active TGF-β1, reduced cyclin E expression, increased p27 stability, decreased levels of phosphatase cdc25a, and absence of cdk2 kinase activity (Grimm et al., 2005). Collectively, the data from C/EBPβ KO mice suggest a role in the control of cell fate determination and proliferation in the mammary epithelium.

Since LAP2 is the most active isoform in most systems and organs, mice lacking only

LAP2 were generated by a knock-in mutation that removes the LAP2 translational initiation codon (Uematsu et al., 2007). In these mice, the defects in mammary gland development and differentiation which are observed C/EBPβ KO mice are not apparent. Moreover, homozygous C/EBPβ$^{\Delta uORF}$ female mice which fail to express LIP, while still expressing LAP1 and LAP2, show normal mammary gland development and function (Wethmar et al., 2010). Together, in contrast to C/EBPβ KO mice, the deficiency of either LAP2 or LIP alone does not produce a mammary gland phenotype, implying that both LAP2 and LIP are redundant in regard to mammary gland development, or that LAP1 is the critical isoform for mammary gland development. On the other hand, transgenic overexpression of LIP increases mammary epithelial proliferation leading to mammary hyperplasia and infrequent carcinoma development (Zahnow et al., 2001). Since LAP1 expression levels in the mammary gland are quite low (Zahnow, 2002), it is unlikely to be the critical isoform for mammary gland development. We hypothesize that since LIP is highly regulated during the course of pregnancy, it is likely a key player in the regulation of mammary gland development in pregnancy. LAP2 clearly has some degree of redundancy to LIP, but is essential in regulating some genes expressed in the terminally differentiated gland, such as β-casein (Doppler et al., 1995; Raught et al., 1995) and whey acidic protein (Seagroves et al., 1998) . These two genes were significantly inhibited in C/EBPβ KO mice (Robinson et al., 1998; Seagroves et al., 1998).

Mice deficient for these following genes fail to undergo normal lobuloalveolar morphogenesis during pregnancy: cyclin D1 (Fantl et al., 1995; Sicinski et al., 1995), PR (Lydon et al., 1995), Id2 (Mori et al., 2000), C/EBPβ (Robinson et al., 1998; Seagroves et al., 1998), Stat5 (Liu et al., 1997), RANKL/RANK (Fata et al., 2000), PRL/PrlR (Brisken et al., 1999; Ormandy et al., 1997). Among those genes, C/EBPβ directly binds to promoters and regulates

the expression of cyclin D1 (Bundy and Sealy, 2003; Eaton et al., 2001), Id2 (Karaya et al., 2005), PrlR (Goldhar et al., 2011; Hu et al., 1998), and up-regulates PR (Chapter 2).

Collectively, the literature describe C/EBPβ as a key regulator in the functional differentiation of mammary epithelial cells and as essential for proper proliferation and differentiation in response to hormone stimulation during mammary gland development, particularly pregnancy. Moreover, each isoform has both overlapping and distinct roles in growth and differentiation of the mammary gland.

Activating Protein-1 (AP-1) family

AP-1, a basic region-leucine zipper family of transcription factors, includes 4 sub-families Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra-1, Fra-2), Atf (ATF2, LRF1/ATF3, B-ATF, JDP1, JDP2) and Maf (c-Maf, MafB, MafA, MafG/F/K and Nrl) (Shaulian and Karin, 2002). All members share a bZIP region: a leucine zipper domain that mediates dimerization and a basic amino acid-rich DNA binding domain. Fra-1, Fra-2, and several alternatively spliced variants of FosB, FosB1 and FosB2, lack transcriptional activation domains and can act as inhibitors to the transactivation activity of other family members. Similarly to other bZIP families of transcription of factors, members of the AP-1 family need to dimerize in order to bind DNA. Its consensus DNA-binding site is TGA(C/G)TCA. However, variation in recognition sequences has been observed in the promoter and enhancer regions of many genes. These variant DNA binding elements are suggested to contribute to the differential binding of various AP-1 dimer complexes to target genes (Chinenov Y 2001).

While the Jun and ATF proteins can form homodimers, the Fos proteins cannot homodimerize but must heterodimerize with Jun members to form active and stable complexes. c-Maf and Nrl dimerize with both c-Jun and c-Fos, whereas MafB, MafF, MafG and MafK

dimerize with only Fos, but not Jun (Meng and Xia, 2011). The composition of AP-1 dimers formation is dependent upon the abundance and expression levels of family members, which are cell type- and tissue-specific in their expression, resulting, along with differential binding to variants in the consensus DNA binding sequence, in AP-1 specificity for individual target genes. Each AP-1 dimer differs in its transcriptional function and DNA binding affinity. Moreover, time-dependent changes in dimer composition are considered to dramatically impact the expression of early and late genes in response to various signaling stimuli (Chinenov Y 2001). AP-1 can act as a transcriptional activator or repressor, depending on dimer composition, gene context, and interaction with distinct coactivators or corepressors (Shaulian E 2002).

Individual AP-1 family members, as revealed in various knockout mouse models, have both redundant functions which can be compensated by other family members, and specific functions unique to the family member which cannot be substituted by other family members (Chinenov Y 2001). c-Jun, c-Fos and FosB usually exhibit potent transactivational functions, while JunB, JunD, Fra1, and Fra2 are less active and even repress the activity of other AP-1 family members (Hess J 2004).

c-Jun

c-Jun is an intronless gene that is regulated by multiple mechanisms. The presence of multiple transcription factor binding sites and regulatory regions have been identified within the c-Jun promoter, and their distinct usage in controlling c-Jun expression is dependent on the specific cell type and stimulus studied (Agarwal et al., 2008; Clarke et al., 1998; Rozek and Pfeifer, 1993, 1995; Sharma et al., 2003; Wong et al., 1999). This complexity within the c-Jun promoter implies that the existence of these elements ensures the effective response to a variety of physiological conditions. For example, in Mink lung epithelial cells (Mv1Lu) and

immortalized human keratinocyte cells (HaCaT), Smad and AP-1 sites are critical for TGFβ-mediated c-Jun promoter activity (Wong et al., 1999), whereas in HeLa cells, ATF1 and MEF2D and their binding sites are required for EFG and Rac/Cdc42-induced c-Jun expression (Clarke et al., 1998). In addition to its regulation by other transcription factors, c-Jun is subject to positive autoregulation by direct binding of c-Jun to an AP-1 site in its own promoter (Angel et al., 1988). Thus upon rapid activation by stimuli, activated c-Jun, in turn, is responsible for sustaining its long-term effects by inducing overexpression of itself.

c-Jun function is also changed by some post-translational modifications, and the interplay of different medication will precisely regulate c-Jun activity function. Phosphorylation of c-Jun has both stimulatory and inhibitory effect on c-Jun function. N-terminal phosphorylation at Ser63/Ser73 and Thr91/Thr93 has positive effect on c-Jun transactivational potential (Minden et al., 1994; Papavassiliou et al., 1995; Pulverer et al., 1991). Phosphorylation of these N'-terminal sites either (1) transactivates c-Jun by facilitating the its interaction with coactivator CREB binding protein (Bannister et al., 1995; Tsai et al., 2008), or (2) activates c-Jun in a de-repression manner through dissociating c-Jun from histone deacetylase 3 (HDAC3) repressor complex (Weiss et al., 2003), or methyl-CpG-binding domain protein 2/nucleosome remodeling deacetylase (Mbd3/NuRD) repressor complex (Aguilera et al., 2011), or nuclear receptor corepressor (NCoR) complex (Ogawa et al., 2004). In contrast to the N-terminal phosphorylation, the C-terminal phosphorylation (residues 227-252), mediated by GSK3 in vitro, decreases c-Jun DNA-binding activity (Boyle et al., 1991). Under treatment with stimuli, such as tumor promoter phorbol-ester (TPA), c-Jun is rapidly activated by phosphorylation of N'-terminal residues, which consequently stimulates the dephosphorylation of the C'-terminal sites (Boyle et al., 1991; Papavassiliou et al., 1995; Pulverer et al., 1991)

Acetylation of Lys271 at DNA-binding domain, mediated by p300, is required for the repression of collagenase promoter by adenovirus early region 1A (E1A) (Vries et al., 2001). Moreover, acetylation also leads to rapid degradation of Drosophila homolog of c-Jun, Jra, by facilitating poly-ubiquitination. However, acetylation level is significant reduced in the presence of phosphorylation signal or phosphorylation-mimic mutant Jra forms, suggesting the collaboration between dramatic activation by phosphorylation and proper degradation by acetylation of Jra (Zhang et al., 2013).

Sumoylation of c-Jun at Lys257 and Lys229 down-regulates its dimer activity, and subsequent transcriptional activity (Bossis et al., 2005; Muller et al., 2000). JNK-mediated phosphorylation decreases, whereas loss of functional phosphorylation sites, Ser63 and Ser73, increases SUMO-1 modification of Lys229 (Muller et al., 2000).

c-Jun can be subjected for multi-ubiquitination at the δ domain (amino acids 31-57), that targets c-Jun for proteolytic degradation through the proteasome 26S pathway (Musti et al., 1996; Treier et al., 1994). In non-stimulated cells, human de-etiolated-1 (hDET1) promotes c-Jun ubiquitination by assembling a multisubunit ubiquitin ligase containing DNA damage binding protein-1 (DDB1), cullin 4A (CUL4A), regulator of cullins-1 (ROC1), and constitutively photomorphogenic-1 (Wertz et al., 2004) (Wertz 2004). In T cells, Itch, a homology to the E6-associated protein C terminus (HECT) domain-containing E3 ligase, promotes c-Jun ubiquitination under JNK activation. The process depends on Itch conformational change and its subsequent ligase activity, rather than phosphorylation of c-Jun Ser63/Ser73 (Gao 2004). In response to prolong osmotic stress, PHD/RING finger domain of MEKK1, an E3 ligase, mediates c-Jun ubiquitination (Xia 2007). Phosphorylation at Thr239/Ser243 mediated by GSK3, but not Ser63/Ser73, leads to its interaction with FBW7 ubiquitin ligase complex and

subsequent destruction of c-Jun (Wei et al., 2005). Phosphorylation at Ser63/Ser73 or Thr91/Thr93 prevents c-Jun from ubiquitination-mediated degradation (Fuchs et al., 1996; Musti et al., 1997). However, contradict study in neuronal cells reported the requirement of JNK-mediate Ser63/Ser73 phosphorylation for polyubiquitination by the E3 ligase FBW7-containing Skp1/cullin/F-box protein complex (Nateri et al., 2004). It is possible that depending on the cell/tissue types and their specific regulatory induction and existed E3 ligases, c-Jun ubiquitination will be differentially regulated to ensure the precise c-Jun activity.

Even though all Jun family members share high homology in their amino acid sequences, especially in their transactivation and DNA binding domains (Volt 1990), c-Jun has the highest binding affinity of any family member to DNA containing an AP-1 site, and is thus regarded as the most active transcription factor in the Jun family (Ryseck and Bravo, 1991). Indeed, c-Jun is able to transactivate promoters carrying a single AP-1 site, while JunB only has activating function on promoters with multiple binding sites (Deng and Karin, 1993). The heterodimer of c-Jun with Fos family members exhibits more stability (Halazonetis et al., 1988; Kouzarides and Ziff, 1988) and higher activity (Ryseck and Bravo, 1991) than does the c-Jun homodimer.

Role of c-Jun in Normal Mammary Gland Development

AP-1 expression has been studied only in the context of involution in mammary gland development. c-Fos, JunB, JunD show dramatically increased expression, and c-Jun slightly increased expression at one day post-lactation (Marti et al., 1994). The composition of AP-1 dimers detected in the mouse mammary gland at this stage is largely c-Fos/JunD, and AP-1 DNA binding activity is transiently increased. These changes are associated with programmed cell death of epithelial cells. AP-1 DNA-binding activity dramatically increased in late pregnancy through lactation, and then decreased after involution (Malewski et al., 2005).

Although not extensively studied at stages of mammary gland development other than involution, AP-1 is critical for mammary epithelial proliferation and normal postnatal mammary gland growth, as shown in mice carrying an inducible AP-1 inhibitor (Tam67), a dominant negative truncated form of c-Jun lacking a transactivation domain (Shen et al., 2006). Blockade of AP-1 activity results in reduced gland size, as well as reduced branching and endbud formation in prepubertal and pubertal glands, and decreased branching and fewer terminal ends in E+P-stimulated mammary glands. However, c-Jun KO mice showed similar mammary gland ductal branching and terminal endbuds as did wildtype mice at 8 weeks and 2 days post involution (Katiyar et al., 2010). Together, these findings suggest that either AP-1 family members have redundant roles in mammary gland development, or that an AP-1 family member(s) other than c-Jun is essential for normal development.

The expression of each AP-1 member has been studied in breast tumor and adjacent normal tissues (Kharman-Biz et al., 2013). In tumors, Fra1, Fra2, JunB and JunD expression levels are elevated in comparison to adjacent normal tissue, while c-Fos and c-Jun levels are reduced. This is consistent with earlier findings that c-Fos and c-Jun expression levels and their transcriptional function are higher in normal cells and immortal human mammary epithelial cells than in breast cancer cells (Smith et al., 1997). FosB is also higher in normal lobules and ducts compared to carcinomas. The reduced FosB expression in carcinomas correlates with a lack of ER and PR expression (Milde-Langosch et al., 2003), while its increased expression in normal mammary structures is associated with ER and PR expression. Fra1 expression is particularly elevated in ER-, PR-, and triple-negative tumors (Kharman-Biz et al., 2013).

Consistent with an important role in mammary gland development, AP-1 also directly regulates several essential genes for mammary gland development. AP-1 binds and regulates the

expression of cyclin D1 (Albanese et al., 1995, 1999; Shen et al., 2006; Watanabe et al., 1996) and PRL (review in (Shen et al., 2006)). AP-1 putative binding sites have been identified in the intergenic region near to Stat5a promoter (Crispi et al., 2004). AP-1 regulates PR expression through the +90 AP-1 site (Petz et al., 2002) and +745 AP-1 site (Petz et al., 2004b) in an ERα-dependent manner. Jun down-regulates PR transcription by binding to the +745 site in the absence or presence of E2, indicating that Jun homodimer occupancy at this site would maintain the low basal level of PR expression in untreated cells. In contrast, Jun up-regulates PR by binding to the +90 site only in the presence of E2. It is possible that in the presence of E2, the expression of PR will be determined by the presence of co-activators or co-repressors for flanking sequences around stimulatory +90 site or inhibitory +475 site, respectively. From these observations, they suggested a model of dynamic and flexible regulation of PR expression in the response to environmental and cellular influences.

Molecular mechanisms of synergistic transactivation

Transcriptional regulation is the major mechanism controlling gene expression, which is driven by multiple regulatory proteins, including RNA polymerase, many transcription factors, and co-activators. However, there are differential requirements for individual transcription factors and co-activators in specific cell types, tissues, and developmental stages. The requirement for specific transcription factors ensures precise spatial, temporal, and expression levels of gene under physiological conditions. Transcription factors can contribute to specific gene expression in either an additive or synergistic manner. An additive effect is one in which promoter activity in the presence of two factors is similar to the sum of their individual activities, while a synergistic effect is one in which promoter activity in the presence of two factors is greater than the sum their individual activities.

A requirement for synergism in transcriptional activation ensures that a gene will only be activated to a high level of expression if the correct positive combination of factors are available, and this in turn often requires two signaling pathways. Synergy over long distances between two transcription factors is carried out through DNA looping to bring the factors in close proximity, which requires their physical interaction. Effective DNA looping is exhibited with DNA separation of at least 500 bp length or longer between two factors (Mossing and Record, 1986). The synergy in short distances may be explained by the factors' cooperative binding. Whether occurring over short or long distances on the DNA, the ultimate proximity of the two factors may provide a unique structure for recruitment of multiple co-activators (Carey and Smale, 2001). It is clear that synergy between transcription factors requires that their binding sites be co-localized on the genome at specific and non-random distances from each other. Cooperativity in binding results in an increased DNA affinity of the second factor after the first factor binding. One mechanism for this is DNA binding of the first factor stabilizing a DNA conformation that confers accessibility to the second factor (Courey, 2009). For example, as reported for the positive regulatory domain II (PRDII) in the context of the Interferon β (IFNβ) enhancer, binding of a high mobility group protein (HMG), HMG I(Y), to the AT-rich sequence leads to DNA unbending. This appropriate conformation enhances p50/p65 binding to NF-κB site on PRDII (Falvo et al., 1995). The more common mechanism is the mutual dependence of bound transcription factors for binding, in which one factor stabilizes the binding of the other and vice versa (Courey, 2009). In this mechanism, the co-expression of two factors results in higher levels of DNA occupancy than could be achieved by either factor alone and, consequently, synergistic transactivation of target gene. In the absence of either transcription factor or mutation of either's DNA binding site, the promoter activity will be decreased significantly as a result of

destabilization of the activator complex. For example, on IL-6 promoter, the synergy of C/EBPβ and NF-κB in stimulation of IL-6 promoter requires both C/EBP and NF-κB protein expression and their intact sites. In the presence of expression vectors for both C/EBPβ and p65/p50, the IL-6 promoter activity increases dramatically comparing to that when single factor is overexpressed. In addition, mutations of either C/EBP or NF-κB sites effectively impair their synergistic effect (Matsusaka et al., 1993). Synergy may also be determined by the more efficient recruitment of a co-activator when both factors occupy the activated gene; the transcription factor complex may provide a high affinity surface for co-activator binding (Courey, 2009). For example, recruitment of CBP/p300 coactivator by efficient interaction with the novel activating surface assembled from activation domains of p65, IRF1, and ATF2/c-Jun results in the synergistic transactivation of IFNβ gene (Merika et al., 1998). Ultimately, this cooperative binding enhances the recruitment of general transcription machinery.

In Chapter 3, we are explore the following possible mechanisms for synergy between C/EBPβ and c-Jun: 1) Two factors bind independently to two separate sites on the DNA; 2) Two factors bind to two separate sites on the DNA in a mutually dependent manner; 3) One factor binds to DNA, while the other factor does not bind directly to the DNA, but rather physically interacts with the first factor; 4) Two factors bind to multiple redundant sites on the DNA. In the last instance, the redundancy may ensure promoter activity when transcription factor concentrations are limited, and levels of gene expression may be dependent on the concentrations of the respective factors. This latter scenario is not mutually exclusive to the other mechanisms.

Cooperative activity of C/EBP and AP-1

The phenotypes of C/EBPβ KO mice and Tam67-expressing (i.e., AP-1 inhibited) mice are similar, suggesting that C/EBPβ and AP-1 may co-regulate the same genes. Indeed, C/EBP

and AP-1 have been found to functionally cooperate in the regulation of a number of genes, dependent upon tissue and cell type. Specifically, interaction and cooperation between AP-1 and C/EBP play role in regulation of many genes, including: melanoma differentiation associated (mda7) (Madireddi et al., 2000), class A scavenger receptor (SR-A) (Mietus-Snyder et al., 1998), c4.4a (Fries et al., 2007), TNF-stimulated gene 6 (TSG-6) (Klampfer et al., 1994), tumor necrosis factor α (TNFα) (Zagariya et al., 1998), endothelin B receptor (ETB receptor) (He et al., 2013), and cyclooxygenase-2 (cox-2) (Wang et al., 2006). Additionally, C/EBP and AP-1 can heterodimerize to activate the PU.1 and FosB promoters through this hybrid protein binds to a hybrid site which contains a half site of C/EBP and a half site of AP-1 (Cai et al., 2008; Hong et al., 2011).

Moreover, in genome analysis of ER binding sites, AP-1 and C/EBP putative binding sites have been identified among enriched motifs (including AP-1, Oct, and C/EBP motifs) within the regions bound by ERα in MCF7 breast cancer cells (Carroll et al., 2006), suggesting that the collaboration of AP-1 and C/EBP may play key roles in regulating of a number of genes which are inducible by E/ERα, including PR gene.

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CHAPTER 2

C/EBPβ LIP AND C-JUN SYNERGIZE TO REGULATE EXPRESSION OF THE PROGESTERONE RECEPTOR

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Abstract

C/EBPβ is a critical transcription factor in the regulation of mammary gland proliferation and development. Experiments with C/EBPβ knockout mice demonstrate a requirement of C/EBPβ for ductal morphogenesis and alveologenesis. Progesterone is critical for proliferation and alveologenesis in the adult mammary gland, and experiments with progesterone receptor (PR) knockout mice show a similar requirement of progesterone receptor B (PRB) for alveologenesis. We examined whether C/EBPβ participates in the transcriptional regulation of PR expression in the mammary gland. Transient co-transfection of a PR promoter-reporter with expression vectors that individually express C/EBPβ isoforms into a mammary tumor cell line revealed that all C/EBPβ isoforms, surprisingly including LIP, can transactivate the PR promoter. Importantly, we found that LIP, in particular, robustly synergizes with c-Jun to drive PR transcription. Consistent with significant roles for C/EBPβ and c-Jun in PR expression, knockdown experiments showed that endogenous levels of C/EBPβ and c-Jun expression are sufficient to stimulate the PR promoter-reporter. Additionally, overexpression of LIP elevates PR protein expression from the intact endogenous PR gene. In the course of pregnancy, both PRB and the relative abundance of LIP among C/EBPβ isoforms increase. Consistent with a role in the expression of PRB, *in vivo* immunofluorescence studies showed that the localization of C/EBPβ and PRA expression are mutually exclusive in the mammary epithelium, while PRB is expressed in cells that express C/EBPβ. Collectively, our data suggest a critical role for C/EBPβ, particularly LIP, in PRB expression.

Introduction

C/EBPβ is a critical transcription factor in the regulation of mammary gland proliferation and development. Experiments with C/EBPβ-deficient mice demonstrate a requirement for C/EBPβ in ductal morphogenesis and alveologenesis (Robinson et al., 1998; Seagroves et al., 1998). C/EBPβ occurs in three isoforms in mammary and other tissues: C/EBPβ p38 (LAP1) and C/EBPβ p35 (LAP2), both potent transcriptional activators, and C/EBPβ p20 (LIP), a truncated form generally reported to inhibit C/EBP-dependent transcription (reviewed in Zahnow, 2002). During the course of pregnancy in the mouse, C/EBPβ protein expression increases, with LIP expression being particularly elevated, not being detectable in the virgin mammary gland (Seagroves et al., 1998).

Progesterone (P) signaling through the progesterone receptor (PR) is also a critical factor for proliferation, ductal morphogenesis and alveologenesis in the adult mammary gland (reviewed in Fendrick et al., 1998; Shyamala et al. 1998; Aupperlee et al., 2005; Aupperlee and Haslam, 2007). Furthermore, experiments with mice deficient in PR isoform B (PRB) show a requirement of PRB for alveologenesis (Lydon et al., 1995; reviewed in Conneely et al., 2002).

The block to alveologenesis in both C/EBPβ- and PRB-deficient mice suggests that these transcription factors might act in the same pathway or may regulate overlapping sets of downstream target genes. An overall decrease in PR observed in sexually mature wildtype mice fails to occur in C/EBPβ-deficient mice, while no alterations in C/EBPβ expression are observed in PR-deficient mice (Seagroves et al., 2000). This is consistent with C/EBPβ acting upstream of PR. PR isoform A (PRA) is the predominant isoform of PR expressed in the mammary glands of virgin adults, with its expression dramatically decreasing at pregnancy and PRB being expressed

with alveolar development during pregnancy (Aupperlee et al., 2005). This raises the possibility that C/EBPβ is required for the differential upregulation and localization of PRB expression that is observed during pregnancy.

In this report, we examined whether C/EBPβ participates in the transcriptional regulation of PR expression in the mammary gland. Transient co-transfection of a PR promoter-reporter with expression vectors that individually express C/EBPβ isoforms into a mammary tumor cell line revealed that all C/EBPβ isoforms, surprisingly including LIP, can transactivate the PR promoter. Importantly, we found that LIP, in particular, robustly synergizes with c-Jun to drive PR transcription. Consistent with significant roles for C/EBPβ and c-Jun in PR expression, knockdown experiments showed that endogenous levels of C/EBPβ and c-Jun expression are sufficient to stimulate the PR promoter-reporter. Additionally, overexpression of LIP elevates PR protein expression from the intact endogenous gene encoding PR. Furthermore, *in vivo* immunofluorescence studies showed that the localization of C/EBPβ and PRA expression are mutually exclusive in the mammary epithelium, while PRB is expressed in cells that express C/EBPβ. Collectively, our data suggest a critical role for C/EBPβ, particularly LIP, in PRB expression.

Materials and methods

Mice: Female BALB/c mice were purchased from Harlan (Indianapolis, IN). All animal experimentation was conducted according to standards approved by the All University Committee on Animal Use and Care at Michigan State University.

Cells and cell culture: MC7-L1, MC4-L2, and MC4-L3 are mammary epithelial cell lines derived from murine mammary ductal carcinomas (Lanari et al., 2001). These cell lines express

both ER and PR. MC7-L1 and MC4-L2 are hormone responsive *in vitro* and MC4-L3 is hormone responsive *in vivo*. Cells were maintained in DMEM-F12 (1:1) medium supplemented to 5% FCS, 100 units/ml penicillin and 100mg/ml streptomycin. Experiments were carried out with charcoal-stripped FCS in the absence of antibiotics. Cells were cultured at 37°C with 5% CO_2.

Expression vectors and promoter-reporters: For transient transfections, murine C/EBPβ isoforms were individually expressed from pcDNA3.1 (Invitrogen, Carlsbad, CA). pcDNA-LIP has been described (Dearth et al., 2001). pcDNA-LAP2 (plasmid 12557; Addgene, Cambridge, MA) has been described (Basu et al., 2011). pcDNA-LAP1 was derived from a plasmid containing the complete coding sequence of murine C/EBPβ inserted between the EcoRI and HindIII sites of pcDNA3.1 (a gift from Dr. Peter Johnson, NCI-Frederick, Frederick, MD). The AUG translational start sites for LAP2 and LIP were mutated to GCG and a consensus Kozak sequence introduced upstream of the LAP1 translational start, mutating GCGTTCATG to GCCACCATG (mutated bases underlined) by site-directed mutagenesis. pCMV-c-Jun has been described (McCabe at al., 1996).

The tandem PR promoter-reporter (Figure 2.1) consists of the region -2502 to +753 base pairs (bp) in relation to the predicted PRB transcriptional start inserted between the Asp718 and NcoI sites of pGL3-Basic (pGL3B) (Promega, Madison, WI). The PR promoter region was isolated by PCR amplification of C57/Bl6 genomic mouse DNA using the following primers: -2502 to -2471 bp, 5'-ACATGGTACCAGCGTGTCACCTGGCACAGA-3' (containing an underlined Asp718 site); +771 to +753 bp, 5'-CTGTCCATGGACACGTCCGAGTGCTGGCT-3' (containing an underlined NcoI site). TA cloning placed the PCR fragment into vector pCR2.1 (TA Cloning Kit; Invitrogen, Life Technologies, Grand Island, NY). The promoter fragment was

then excised with Asp718 and NcoI, and inserted into pGL3B. 2xPRE-TK-luc is plasmid 11350 (Addgene; Giangrande et al., 2000), and contains two copies of a consensus P response element (PRE) upstream of the human thymidine kinase promoter. pRL-SV40 expresses Renilla luciferase from the SV40 early enhancer-promoter (Promega).

Transient transfections: Transient transfections were conducted in either 12-well cell culture plates or 6 cm culture plates containing 1 ml/well or 4 ml/plate, respectively, of DMEM-F12 supplemented to 5% charcoal-stripped FCS. 12-well cell culture plate was seeded with $5x10^4$ cells/well and 6 cm culture plates with $5x10^5$ cells 24 h prior to transfection. FuGene 6 Transfection Reagent (Promega) and plasmid DNAs were mixed at a 2:1 ratio (volume:weight) in 100 μl or 1 ml of serum-free medium for 12-well and 6 cm culture plates, respectively. This mixture was incubated at room temperature for 30 min before addition to cell cultures. The DNA, totaling 500 ng or 5 μg per individual 12-well and 6 cm culture plate transfection, respectively, comprised 100 ng or 1 μg of tandem PR promoter-reporter, 1 ng or 10 ng of pRL-SV40, and varying amounts of C/EBPβ expression vector and/or c-Jun expression vectors, as indicated in figure legends. All quantities of expression vector are expressed as ng per $5x10^4$ cells. Cells were assayed 24 h after transfection. For experiments utilizing 2xPRE-TK-luc, a slightly modified transfection protocol was used. 200 ng of the 2xPRE-TK-luc promoter-reporter was transiently co-transfected with DNA totaling 400 ng, comprising 100 ng pcDNA-LIP and/or 100 ng pCMV-c-jun, or 200 ng pcDNA. These transfections were performed in a 12-well plate format and assayed 36 h after transfection. Transfected cells were harvested, lysed, and analyzed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase values were normalized to the Renilla luciferase values as a control for transfection

efficiency between individual samples. All transfections were carried out in duplicate and repeated at least three times.

In transient transfections assayed by the isolation of RNA or nuclear protein extracts, $5x10^5$ cells were transfected in 6 cm culture plates under conditions similar to those described above. The quantities of expression plasmids are indicated in figure legends. Cells were harvested for RNA isolation or nuclear extracts as described below.

RNA isolation and quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. For RNA isolation in the experiments examining PR promoter-reporter, the Agilent Total RNA Isolation Mini Kit (Agilent Technologies, Santa Clara, CA) was used to eliminate residual transfected plasmid DNA. RNA was reverse-transcribed into cDNA using the RT^2 First Strand Kit (Qiagen, Germantown, MD) according to the manufacturer's protocol. cDNA reactions lacking reverse transcriptase were performed to confirm elimination of DNA. cDNAs (20 μL) were diluted to150 μl with deionized H_2O. For qRT-PCR, each reaction (15 μl) included 7.5 μl of RT^2 SYBR Green qPCR Mastermix (Qiagen), 1 μl of diluted first-stand cDNA synthesis reaction, 1 μl of primer, and 5.5 μl of deionized H_2O. qRT-PCR was performed with the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Inc., Foster City, CA) using the following program: step 1: 95°C, 10 min; step 2: 40 cycles of 95°C for 15 sec, 56°C for 30 sec, and 72°C for 45sec; step 3: dissociation curve at 95°C for 1 min, 65°C for 2 min (optics off), and 65–95°C at 2°C per minute (optics on). Primers 5'-AAAGGATCCGCAGGTTCTC-3' and 5'-GTTCCATCTTCCAGCGGATA-3' were used for amplification of total PR transcripts. Primers

5’-CCCAGTTCTCAGACCAGACC-3’ and 5’-GTGGGATCTCCACCTCCTG-3’ were used for amplification of PRB transcripts. The positions of these primers are indicated in Figure 2.1.

Small interfering RNA (siRNA)

C/EBPβ siRNA (sc-29862), c-Jun siRNA (sc-29224), and control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology (Dallas, Texas). Transient transfections of siRNAs were performed in 12-well cell culture plates containing 1 ml/well of DMEM-F12 supplemented to 5% charcoal-stripped FCS. Each well was seeded with $5x10^4$ MC7-L1 cells 24 h prior to transfection. 200 ng plasmid DNA of the tandem PR promoter-reporter, 1 ng of pRL-SV40, and either 10 pmol C/EBPβ siRNA, 25 pmol c-Jun siRNA, both siRNAs, or 25 pmol control siRNA were diluted in 60 μl serum-free medium (solution A). 2 or 3 μl of Lipofectamine 2000 (Life Technologies), respectively for C/EBPβ or c-Jun and control siRNAs, was diluted in 60 μl serum-free medium, and incubated for 5 minutes (solution B). Then, solutions A and B were combined and incubated for another 20 minutes before being added into culture wells. Transfections were performed in duplicate, and repeated at least three times. Transfected cells were analyzed for luciferase activity 36 h after transfection as described for transient transfections.

Western analysis: Nuclear extracts were prepared as follows. Cells were washed in phosphate-buffered saline (PBS) and lysed in 15 mM KCl, 10 mM HEPES (pH 7.6), 2 mM MgCl2, 0.1 mM EDTA, 1mM DTT, 0.1% (volume/volume) Nonidet P-40, 0.5mM PMSF, 2.5 μg/ml leupeptin, 5 μg/ml antipain, and 5 μg/ml aprotinin for 10 min on ice. Nuclei were pelleted by centrifugation at 14,000×g for 1 min at 4^oC. Proteins were extracted from nuclei by 20 min incubation at 4^0C with vigorous vortexing in buffer C (420mM NaCl, 20mM HEPES (pH 7.9),

0.2mM EDTA, 25% [volume/volume] glycerol, 1 mM DTT, 0.5 mM PMSF, 2.5 μg/ml leupeptin, 5 μg/ml antipain, and 5 μg/ml aprotinin). Nuclear debris was pelleted by centrifugation at 13,000 rpm for 30 min at 4°C, and the supernatant extract was collected and stored at −80°C. The extracts (10 μg) were adjusted to 1x Laemmli sample buffer (Laemmli, 1970) and processed by sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis. The gel was transferred to a Protran membrane (Schleicher and Schuell Bio-Science, Inc., Keene, NH), and antigen-antibody complexes were visualized with the Enhanced Chemiluminescence Kit (Amersham Biosciences, Piscataway, NJ). The primary antibodies utilized were rabbit anti-C/EBPβ specific to the carboxyl terminus (C-19; 1:1000; Santa Cruz Biotechnology) and rabbit anti-β-tubulin (H-235; 1:1000; Santa Cruz Biotechnology). Anti-rabbit IgG horseradish peroxidase (HRP)-conjugate was used as a secondary antibody (1:3000; Promega).

Immunofluorescence: Dual immunofluorescence detection of PRA and C/EBPβ or PRB and C/EBPβ was performed using rabbit anti-PRA (1:100; A0098, Dako, Carpinteria, CA) or rabbit anti-PRB (1:800; B15; Kariagina et al., 2007) with mouse anti-C/EBPβ (1:50; sc7962, Santa Cruz Biotechnology) primary antibodies followed by appropriate secondary antibodies conjugated to Alexa 488 (Molecular Probes, Eugene, OR) or Alexa 546 (Molecular Probes). Nuclei were counterstained with 4', 6-diamidino-2-phenylindole, dilactate (DAPI) (1:10,000 w/v in H_2O).

For tissue sections, mice were killed and their mammary glands were fixed and paraffin-embedded for immunohistochemistry (Aupperlee et al., 2005). MC7-L1 cells were grown on a round microscope cover glass (Fisher Scientific, Pittsburgh, PA) with a starting density of $2x10^5$ cells in each well of 6-well cell culture plates. After 20 h, transfections were performed as

described above, using 0.5 μg of pcDNA or pcDNA-LIP. Transfected cells were incubated for 24 h prior to staining. Cell-coated cover glasses were fixed in 3.7% formalin for 30 min, rinsed twice in PBS for 5min, permeabilized in 0.5% (weight:volume) Triton X-100 for 15 min, rinsed again in PBS, and then blocked with PBS containing 2% BSA for 15 min. After blocking, cells were treated with primary antibodies overnight. The next day, slides were washed with PBS, incubated with secondary antibody for 30 min. After another rinse in PBS, slides were stained with DAPI for 5 min. Slides were mounted with fluorescence mounting medium and dried overnight. Stained tissue sections and cells were visualized and images captured using a Nikon inverted epifluorescence microscope (Mager Scientific, Dexter, MI) with MetaMorph software (Molecular Devices Corporation, Downington, PA).

Results

Examination of the DNA sequence in the region from -2494 to +769 of the PRB gene with MatInspector (Quandt et al., 1995; Cartharius et al., 2005) revealed a number of predicted C/EBPβ binding sites (Figure 2.1). In order to investigate the ability of C/EBPβ isoforms to stimulate transcription of the PR gene, we performed transient co-transfections of vectors that singly expressed LAP1, LAP2, and LIP into murine mammary carcinoma cell line MC7-L1 (Lanari et al., 2001) with the tandem PR promoter-reporter. The tandem PR promoter-reporter retains sequences allowing transcription from both the PRB and PRA transcriptional start sites.

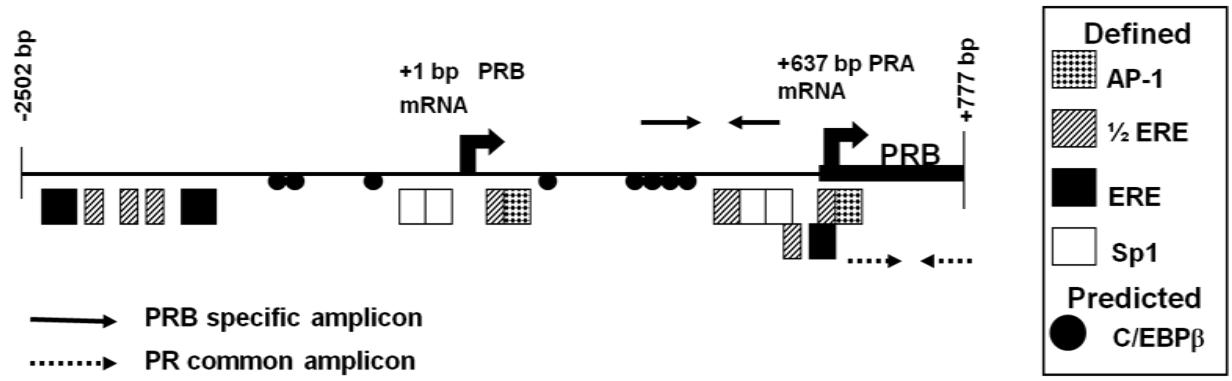


Figure 2.1. Map of the murine PR promoter region used in these studies.

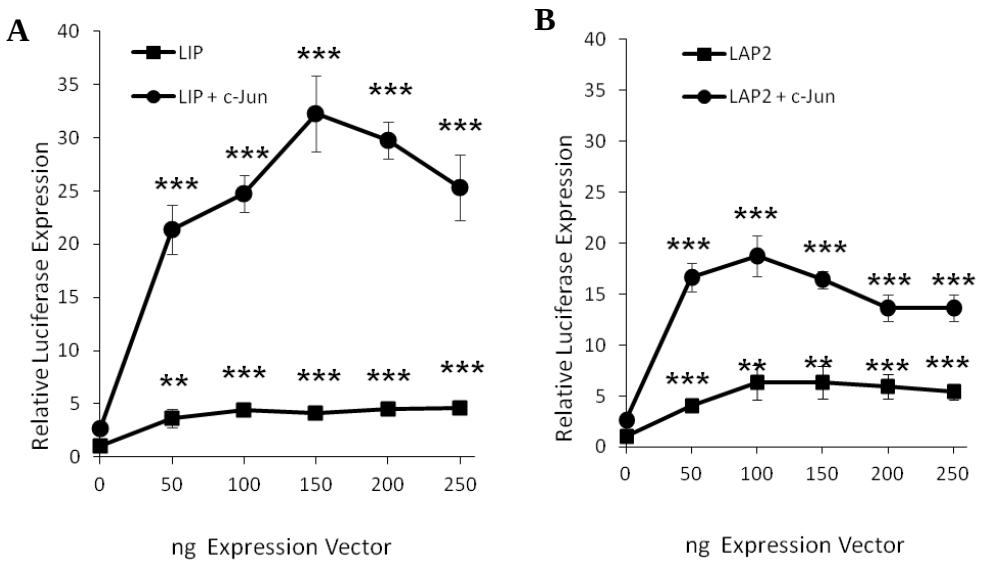


Figure 2.2. Transient co-expression of LIP and c-Jun synergizes to induce expression from the PR promoter-reporter. Transient co-transfection of C/EBPβ and c-Jun expression vectors were carried out in duplicate and repeated at least three times in murine mammary epithelial cell lines with the tandem PR promoter-reporter. (A, B, C, D) MC7-L1 cells. The varied nanogram quantities of the expression vector per $5x10^4$ cells are indicated below the x-axis. For panels A, B, and C, C/EBPβ expression vectors were co-transfected with 100 ng of c-Jun expression vector in some transfections. Luminometer values were normalized for expression from a co-transfected SV40 early enhancer/promoter-*Renilla* luciferase reporter. These values were then normalized to a relative value of 1.0 for cells receiving neither C/EBPβ nor c-Jun expression vector. The data presented are the means of at least three experiments with their standard error. *p* was calculated by Student's T-Test in comparison to the control transfection for transfections involving a single transfected expression vector, and in comparison to transfections involving a C/EBPβ vector alone for co-transfections involving both C/EBPβ and c-Jun expression vectors. *, $p<0.1$; **, $p<0.05$; ***, $p<0.01$. (E) MC4-L2 cells. 100 ng LIP and 100 ng c-Jun expression vectors per $5x10^4$ cells. Values were normalized and statistics performed as described above. (F) MC4-L3 cells. 50 ng LIP and 50 ng c-Jun expression vectors per $5x10^4$ cells. Values were normalized and statistics performed as described above. (G) A western blot confirms similar levels of C/EBPβ expression among the various isoforms in transfection of MC7-L1 cells. c-Jun is overexpressed in cells transfected for c-Jun expression. β-tubulin was detected as a loading control.

Figure 2.2 (Cont'd)

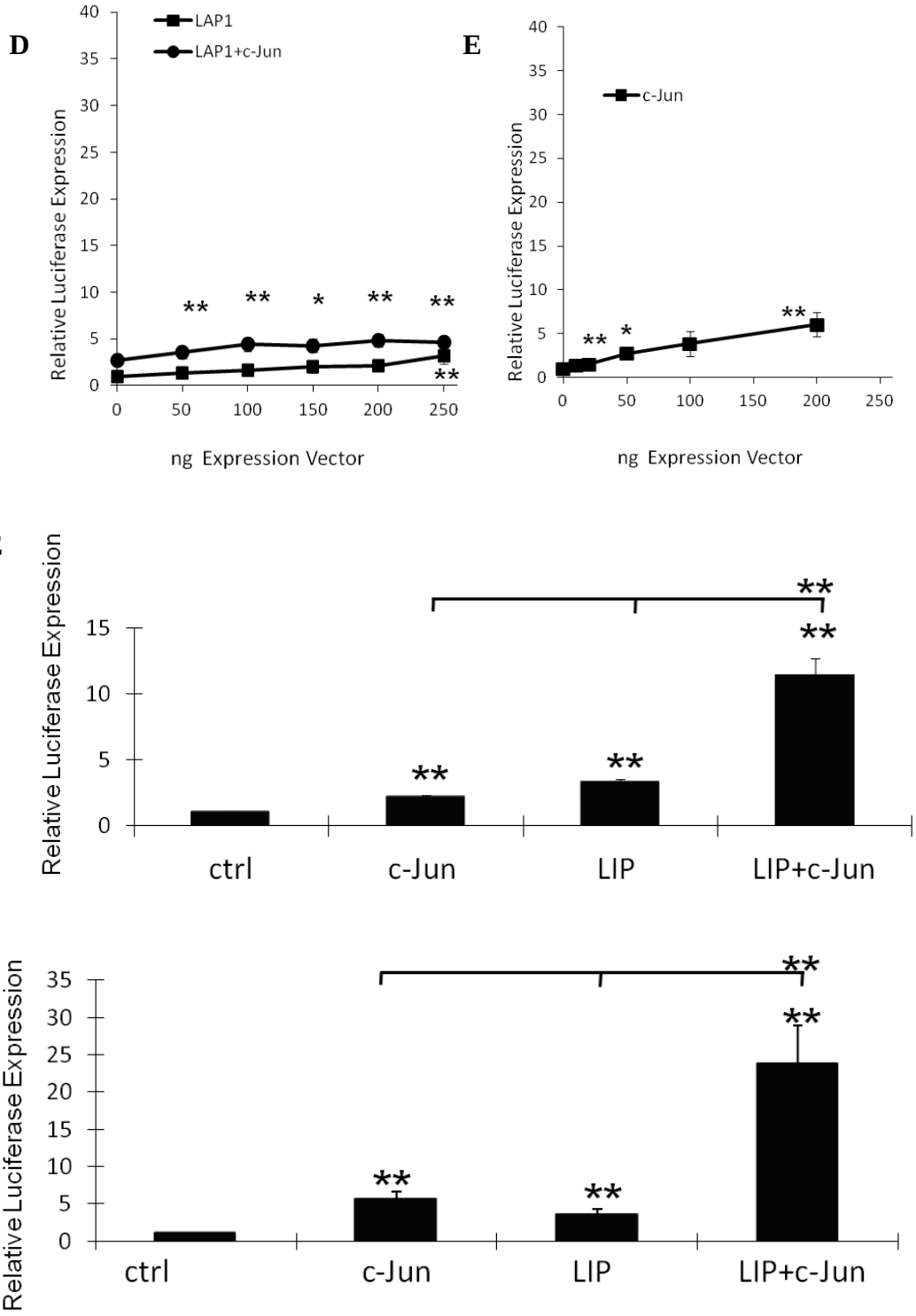
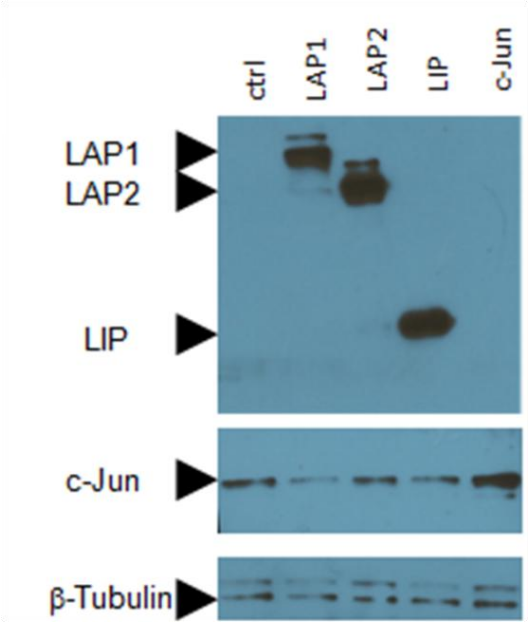


Figure 2.2 (Cont'd)

G



We chose MC7-L1 because its endogenous expression of PR assured its ability to support a response from the tandem PR promoter-reporter. All three C/EBPβ isoforms displayed modest activity (maximally 3 to 6-fold) in stimulating the PR promoter (Figure 2.2A).

Previous investigators demonstrated a role for AP-1 (i.e., c-Jun/c-Fos) in stimulating human PR promoter activity in conjunction with estrogen receptor and Sp1 binding (Petz et al., 2002; Shultz et al., 2005). Additionally, transgenic mice expressing inducible TAM67, a dominant negative truncated form of c-Jun that lacks a transactivation domain, showed reduced mammary ductal development at puberty, as well as in hormonally stimulated adults (Shen et al., 2006). We decided to examine how AP-1 overexpression might interact with C/EBPβ activation of the tandem PR promoter-reporter. To that end, LAP1, LAP2, and LIP were transiently co-expressed with c-Jun.

Co-expression of LIP with c-Jun showed a striking synergy in activation of the PR promoter (Figure 2.2A). Peak stimulation of the PR promoter averaged 32-fold under conditions where LIP and c-Jun singly provided 4-fold and 2.7-fold stimulation, respectively. This synergy was more modestly evident with LAP2 (Figure 2.2B), while LAP1 (Figure 2.2C) only showed additive levels of stimulation with co-expression of the two transcription factors. Expression of c-Jun by itself (Figure 2.2D) showed only modest stimulation of the PR promoter over a range similar to that of the individual C/EBPβ isoforms. Differences in activity were not due to differential levels of expression, as all of the C/EBPβ isoforms showed similar levels of expression in parallel transfections, and c-Jun did not induce C/EBPβ expression (Figure 2.2G). A similar synergy between LIP and c-Jun on PR promoter was also observed in MC4-L2 and MC4-L3 (Figure 2.2E and 2.2F), two other mammary carcinoma cell lines that show endogenous PR expression (Lanari et al., 2001).

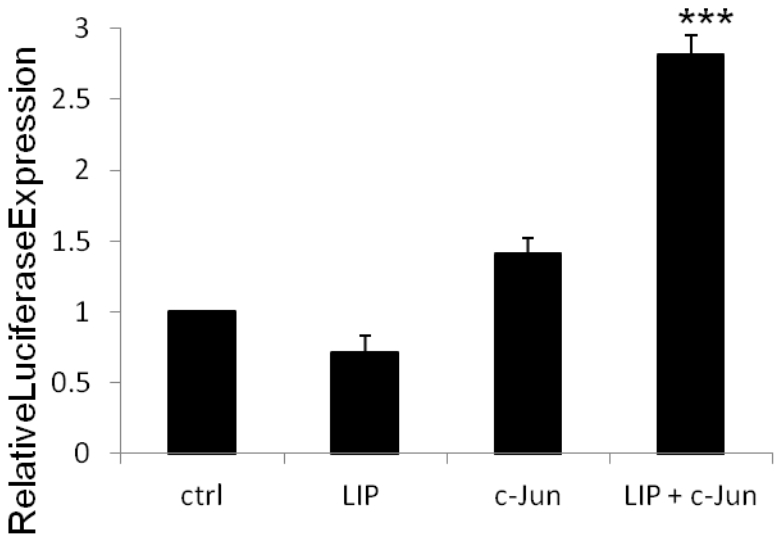


Figure 2.3. Co-expression of LIP and c-Jun increases 2xPRE promoter activity.
Transient co-transfection of C/EBPβ and c-Jun expression vectors were carried out in duplicate and repeated at least three times in MC7-L1 cells with the 2xPRE promoter-reporter. Values were normalized and statistics performed as described in Figure 2.1. ***, $p<0.01$.

Having observed the robust synergy between LIP and c-Jun in activating the PR promoter in a transient transfection system, we tested whether the intact endogenous PR gene would respond to these transcription factors. To that end, LIP and c-Jun overexpression was introduced transiently into MC7-L1 cells in co-transfections with the 2xPRE-TK-luc promoter-reporter. The activity of the 2xPRE promoter is dependent upon PR expression. While LIP or c-Jun overexpression by itself did not significantly increase 2xPRE activity, co-expression of LIP and c-Jun increased 2xPRE promoter activity 2.8-fold (Figure 2.3).

As a more direct assessment of the effects of LIP and c-Jun overexpression on endogenous PR gene expression, LIP and c-Jun expression was introduced transiently into MC7-L1 cells, and then PRA and PRB expression were examined by immunofluorescence. PRA and PRB expression were increased about 4-fold in LIP overexpressing cells (Figures 2.4A and B). C-Jun neither increased PR expression nor enhanced LIP stimulation of PR expression (data not shown) The lack of an effect from c-Jun overexpression in this assay may reflect a surfeit of c-Jun expression in the context of the diploid endogenous gene as opposed to more limited availability for the multiple copies of the transfected tandem PR promoter-reporter. Consistent with the notion that LIP transcriptionally activates the PR gene, PR RNA levels were increased 23-fold by LIP overexpression (Figure 2.4C).

In order to examine whether the levels of C/EBPβ and c-Jun normally present in MC7-L1 cells can support PR expression, "gene knockdown" experiments were performed with siRNAs directed against the mRNAs of these transcription factors. SiRNAs directed against C/EBPβ and

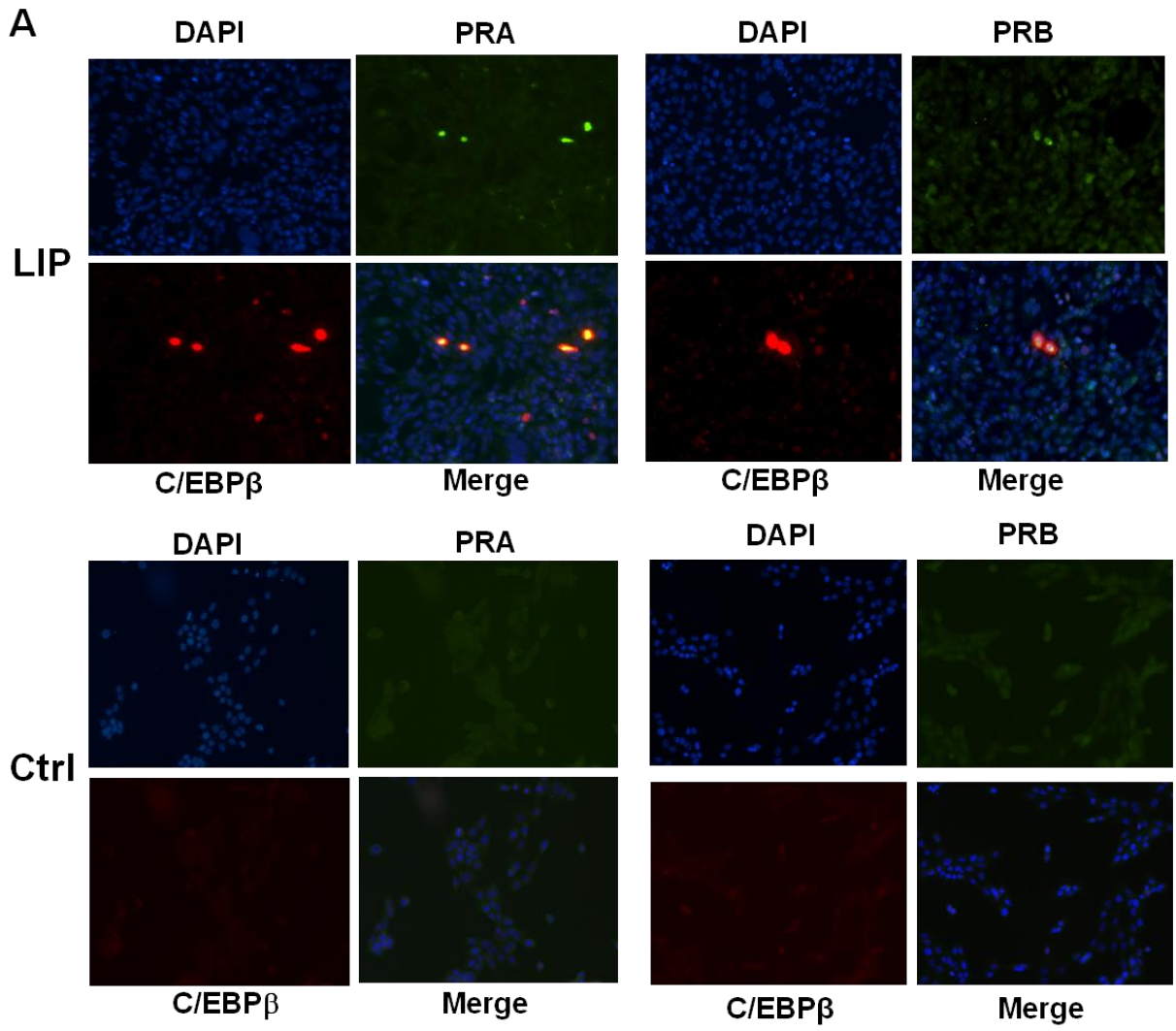
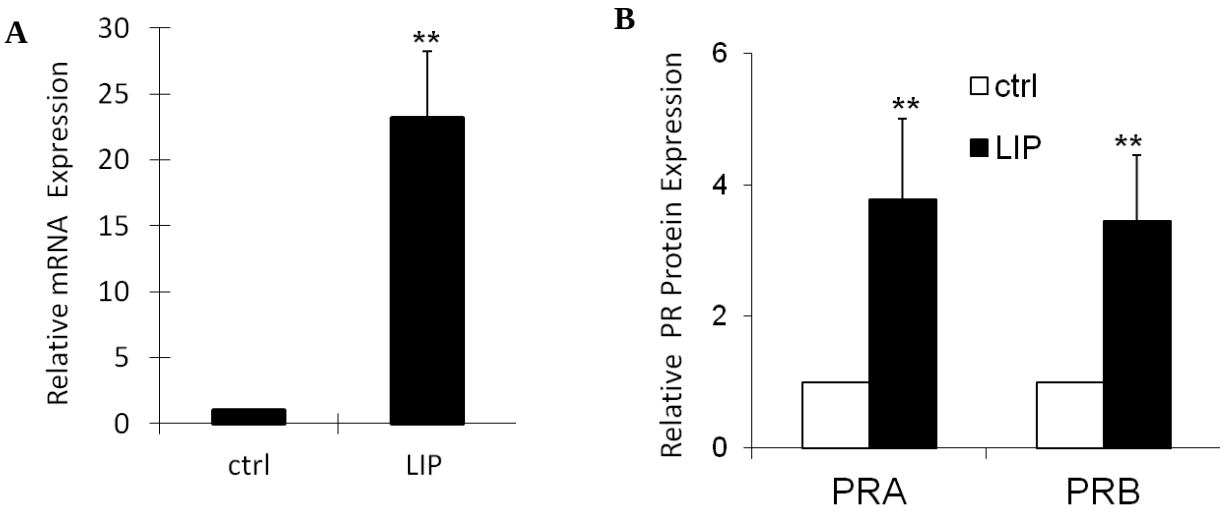


Figure 2.4. LIP expression induces endogenous PRA and PRB expression. Transient transfections of the LIP expression vector were carried out in MC7-L1 cells. (A) Immunofluorescent detection of PRA (green) and C/EBPβ (red) in cells transfected for LIP expression (LIP) and in cells transfected with empty vector (Ctrl). Nuclei were counterstained with DAPI (blue). (B) The immunofluorescence of PRA and PRB were measured in cells showing LIP overexpression after transfection with LIP expression vector (LIP) in comparison to the level of immunofluorescence in cells transfected with empty vector (ctrl). Values from LIP overexpressing cells were normalized to a relative value of 1.0 for cells transfected with empty vector. The data presented are the means of three experiments with their standard error. At least 80 LIP overexpressing cells were scored in each experiment. *p* was calculated by Student's T-Test in comparison to the control transfection. **, $p<0.05$. (C) The quantities of PR mRNA were measured by qRT-PCR in cells transfected for LIP expression (LIP) in comparison to cells transfected with empty vector (ctrl). Values were normalized to a relative value of 1.0 for cells receiving the empty vector control. The data presented are the means of three experiments with their standard error. *p* was calculated by Student's T-Test in comparison to the control transfection. **, $p<0.05$.

Figure 2.4 (Cont'd)



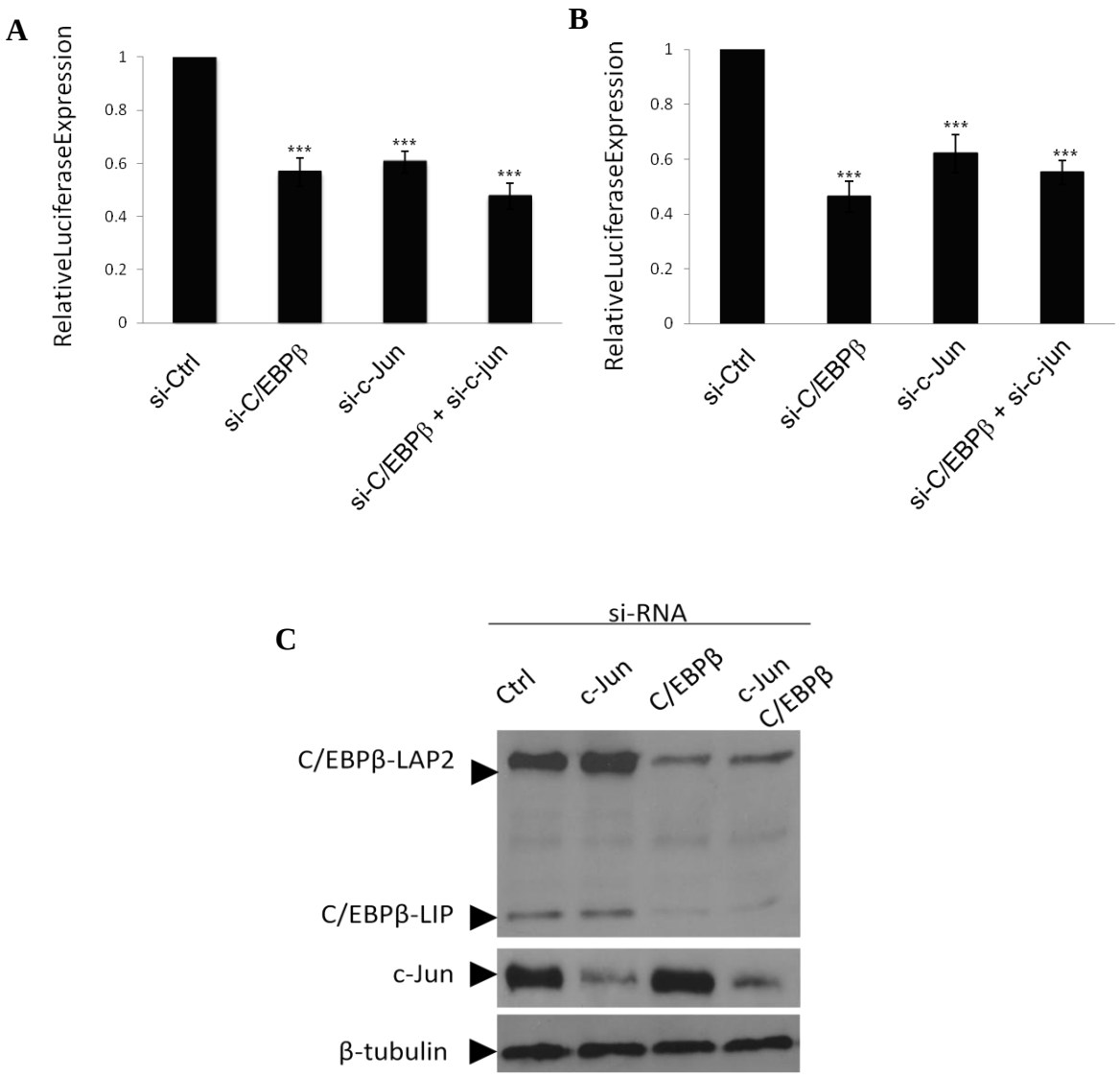


Figure 2.5. si-RNAs directed against endogenous C/EBPβ or c-Jun expression similarly suppress expression from the tandem PR and 2xPRE promoter-reporters. (A) Transient transfections of siRNAs directed against C/EBPβ (si-C/EBPβ) and c-Jun (si-c-Jun), as well as control siRNA (si-Ctrl), were carried out in MC7-L1 cells with the tandem PR promoter-reporter. Luminometer values were normalized for expression from a co-transfected SV40 early enhancer/promoter-*Renilla* luciferase reporter. These values were then normalized to a relative value of 1.0 for cells receiving control siRNA. The data presented are the means of at least three experiments with their standard error. *p* was calculated by Student's T-Test in comparison to the control transfection. ***, $p<0.01$. (B) Transient transfections of siRNAs directed against C/EBPβ (si-C/EBPβ) and c-Jun (si-c-Jun), as well as control siRNA (si-Ctrl), were carried out in MC7-L1 cells with the 2xPRE promoter-reporter. Data were processed as in (A). (C) A western blot confirms decreased C/EBPβ and c-Jun expression with transfection of their cognate siRNAs. β-tubulin was detected as a loading control.

c-Jun mRNAs were transiently transfected, either singly or together, with the tandem PR promoter-reporter. The activity of this promoter was reduced by about 40% by either siRNA alone, while co-transfection with both siRNAs reduced promoter activity by about 50% (Figure 2.5A). Similar reductions in basal activity were found for control promoter-reporters specifically dependent on C/EBP or c-Jun family transcription factors (data not shown). At the same time, protein levels of C/EBPβ isoforms and c-Jun were dramatically reduced by their cognate siRNAs (Figure 2.5C). This suggests that the endogenous levels of C/EBPβ and c-Jun expressed in MC7-L1 cells are sufficient to support PR transcription. Additionally, the fact that co-transfection with siRNAs for both C/EBPβ and c-Jun suppresses expression from the PR promoter little more than each siRNA alone is consistent with the notion that the activity of these transcription factors on the PR promoter is largely derived from their synergy.

In order to extend our examination of the effects of gene knockdown to the intact endogenous PR gene, we repeated the transfection of siRNAs directed against C/EBPβ and c-Jun mRNAs with the 2xPRE-TK-luc promoter-reporter. Similarly to the case with the tandem PR promoter, activity of the 2xPRE promoter was reduced by about 40 to 50% whether transfected singly or with both siRNAs (Figure 2.5B). This suggests a similar dependence of the intact endogenous PR promoter for endogenous levels of C/EBPβ and c-Jun expression as for the tandem PR promoter-reporter.

C/EBPβ expression, particularly LIP, increases during the course of pregnancy in the mouse (Seagroves et al., 1998). The block to alveologenesis in both C/EBPβ- (Robinson et al., 1998; Seagroves et al., 1998) and PRB-deficient mice (Lydon et al., 1995; Conneely et al., 2002), coupled with PRB being expressed with alveolar development during pregnancy (Aupperlee et al., 2005), led us to hypothesize that LIP may be associated PRB expression during

pregnancy. Immunofluorescent staining of PRA, PRB and C/EBPβ was performed on the mammary glands of nulliparous compared to 14-day pregnant mice. Staining revealed that the localization of PRA, the predominant PR isoform in the nulliparous mammary gland, was mutually exclusive of C/EBPβ (Figure 2.6A). In contrast, PRB, the predominant PR isoform in the pregnant mammary gland, largely co-localized with C/EBPβ (Figure 2.6B), consistent with a role for C/EBPβ in PRB expression.

Overexpression of LIP increased both PRA and PRB expression in MC7-L1 cells (Figure 2.4A and B), but the relative expression of PRA and PRB proteins is uncertain as these protein were detected with different antibodies. In order to assess differential expression of PRA and PRB, we performed a PCR assay utilizing primers specific to either a region common to both PRA and PRB transcripts or to an upstream region specific to PRB transcripts. Transcripts from the endogenous gene were not abundant enough to reliably assay (data not shown), so assays were performed on RNAs isolated from cells transfected with the tandem PR promoter-reporter. Consistent with a role for LIP in PRB expression, LIP overexpression increased the ratio of PRB transcripts to total PR transcripts by 1.8-fold (Figure 2.6C).

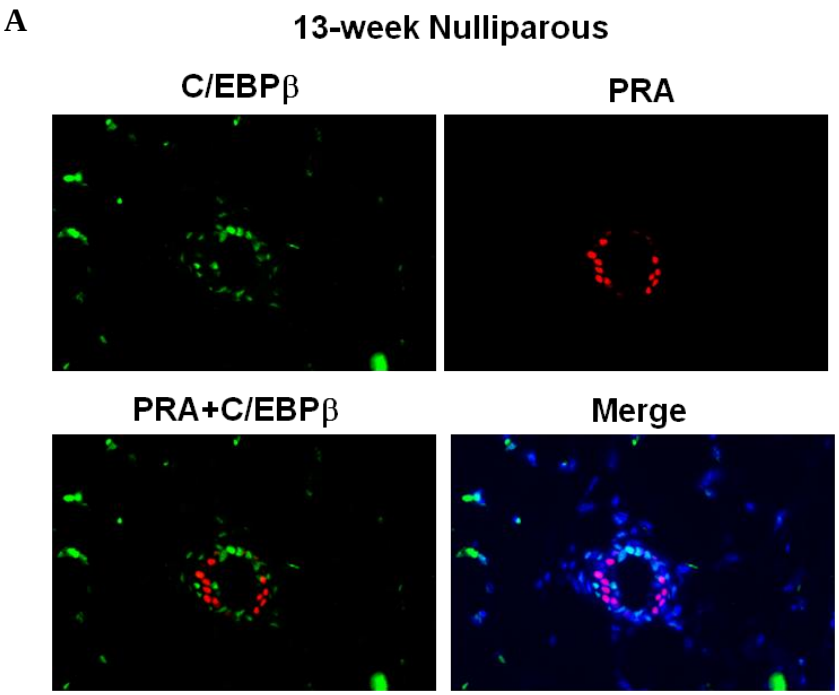
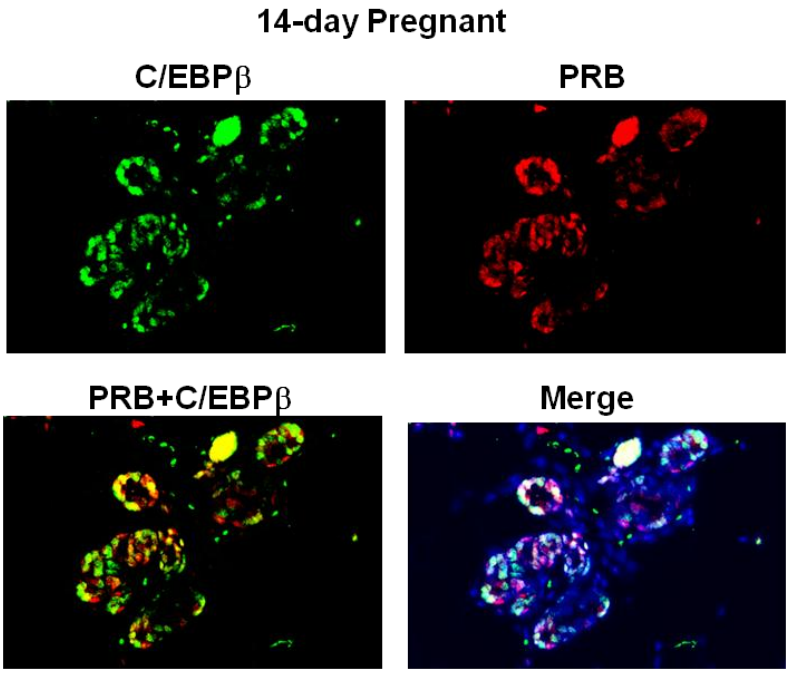


Figure 2.6. PRA expression is mutually exclusive of C/EBPβ, while PRB and C/EBPβ largely co-localize. (A, B) PRA (red) or PRB (red) and C/EBPβ (green) were detected by immunofluorescence in the mammary glands of 13-week old nulliparous mice and 14-day pregnant mice. Nuclei were counterstained with DAPI (blue). Representative tissue sections are shown with the percentages of PR and C/EBPβ co-localization. (C) The quantities of PR RNA transcribed from the tandem PR promoter-reporter were measured by qRT-PCR in cells transfected for LIP expression (LIP) in comparison to cells transfected with empty vector (ctrl). A ratio of PRB to total PR RNA was calculated and these values were normalized to a relative value of 1.0 for cells receiving the empty vector control. The data presented are the means of three experiments with their standard error. *p* was calculated by Student's T-Test in comparison to the control transfection. **, $p<0.05$.

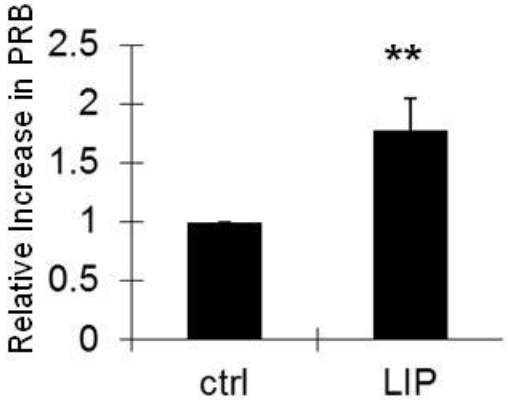
Figure 2.6 (Cont'd)

B



C/EBPβ+ also staining for PRB: 73%
PRB+ also staining for C/EBPβ: 65%

C



Discussion

The data presented in this paper support an important role for C/EBPβ in the transcriptional regulation of PR. The LIP isoform of C/EBPβ was particularly active in synergy with c-Jun on a PR promoter-reporter, and was capable of increasing PR protein expression in a murine mammary carcinoma cell line. Importantly, PRB expression in the mammary glands of pregnant mice co-localized with C/EBPβ expression, while PRA and C/EBPβ expression in nulliparous mice were mutually exclusive in their localization. LIP expression is particularly increased in the mammary glands of pregnant mice (Seagroves at al., 1998) at the same time when PRB becomes the predominant PR isoform in the murine mammary gland (Aupperlee et al., 2005). Both LIP (Seagroves at al., 1998) and PRB (Aupperlee et al., 2005) are also both down-regulated at lactation and involution. Viewed in this context, our results suggest a specific role for LIP in the expression of PRB. Observations in knockout mice are consistent with a role for C/EBPβ in regulating PRB expression. PRB-deficient mice in are defective in alveologenesis (Lydon et al., 1995; Conneely et al., 2002), as are C/EBPβ-deficient mice ((Robinson et al., 1998; Seagroves et al., 1998). Further, in contrast to alterations in PR expression that are observed in C/EBPβ-deficient mice, no alterations in C/EBPβ expression are observed in PR-deficient mice (Seagroves et al., 2000). This is consistent with our conclusion that C/EBPβ acts upstream of PR.

At the same time that knockout studies support the notion of an involvement of C/EBPβ in PRB regulation, there are, at least superficially, some inconsistencies. C/EBPβ-deficient mice show elevated numbers of PR-positive cells rather than a decrease in PR expression that night be expected (Seagroves et al. 2000). However, that study did not distinguish between PRA and

PRB, and, as those observations were made in nulliparous mice, they most likely represent PRA expression. The number of PR-positive cells did not decrease in sexually mature C/EBPβ-deficient mice and is not decreased by E plus P treatment that mimics pregnancy, as is the case in wildtype mice (Seagroves et al. 2000). Again, the interpretation of this lies with specific isoform expression that was not assessed. In wildtype animals, PRA decreases with maturity and is further diminished with pregnancy, and PRB is not evident until day 14 of pregnancy (Aupperlee et al., 2005). The failure to decrease PR expression in C/EBPβ-deficient mice may reflect a block in the transition to PRB expression that occurs with pregnancy. It would be informative to analyze the developmental expression of PRA and PRB in C/EBPβ-deficient mice.

Another question arises from studies showing that, in contrast to complete C/EBPβ deficiency, mice that fail to express LIP do not show defects in mammary gland development (Wethmar et al., 2010). Mice lacking LAP2 expression also have normal mammary gland development (Uematsu et al., 2007). These findings are entirely consistent with our findings that although LIP is the most robust isoform for transactivation of the PR promoter-reporter, LAP2 also has significant activity. As our results shows little activity for LAP1 on the PR promoter, a LAP1 knock-in on a C/EBPβ-deficient background would not be expected to rescue mammary gland development.

It is interesting that LIP, generally described as an inhibitor of LAP-mediated transcription (Descombes, and Schibler, 1991), is the C/EBPβ isoform that most robustly activates the PR promoter. There is precedent for activation of other genes by LIP, and, as in this report, LIP activity is associated with synergy with other transcription factors. LIP can transactivate the IL-6 promoter reporter in manner dependent upon an intact NF-κB site (Hu et al., 2000; Spooner et al., 2007). IL-8 is similarly activated through a synergy between C/EBPβ

and NF-κB, and this is, at least in part, mediated through cooperative DNA binding (Stein and Baldwin, 1993). LIP and PRB cooperate to transactivate the decidual prolactin and MMTV promoters (Christian et al., 2002). LIP and Runx2 cooperate to transactivate the promoters for alkaline phosphatase and osteoclacin (Hata et al., 2005). This latter activity is based upon the enhanced ability of ATF4-C/EBPβ (i.e., LIP) heterodimers to bind DNA and interact with Runx2 (Tominaga et al., 2008). Knockdown of C/EBPβ, c-Jun, or both all inhibited the PR promoter-reporter to a similar extent, implying a mutual dependence of the LIP and c-Jun for their synergistic activity. The basis for this synergy remains to be elucidated.

Progestin acting through PRB is reported to up-regulate C/EBPβ expression in a human breast cancer cell line (Richer et al., 2002) and P upregulates C/EBPβ expression in mouse mammary glands (Aupperlee et al., 2009). Progestin is also reported to up-regulate c-Jun in a human breast cancer cell line (Alkhalaf and Murphy, 1992). Thus, our finding of C/EBPβ and c-Jun synergy in regulating PR presents a robust mechanism for a positive feedback loop in PRB expression in the pregnant mammary gland. P decreases PRA and increases PRB levels in the normal adult murine mammary gland (Aupperlee and Haslam, 2007). Such a positive feedback loop may be critical in the action of P to promote PRB expression in pregnancy.

Acknowledgements

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CHAPTER 3

C/EBPβ LIP AND C-JUN SYNERGY ON THE PROGESTERONE PROMOTER IS BASED ON THEIR MUTUAL DEPENDENCE FOR EFFICIENT OCCUPANCY

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Abstract

Transcriptional regulation of PR by E and estrogen receptor (ER) is well described; however, expression of PR in mammary gland may be regulated by cross-talk of multiple hormones and growth factors leading to activation of multiple transcription factors and co-activators. We recently found that the LIP isoform of C/EBPβ synergizes with the AP-1 family member c-Jun to up-regulate expression from the PR promoter (Wang et. al., unpublished results). In this report, we have elucidated the synergistic mechanism of LIP and c-Jun to activate the PR promoter. We demonstrate that the integrity of C/EBP- and AP-1-binding sites is required for the respective C/EBPβ and c-Jun activities on the PR promoter, and that efficient promoter occupancy of both LIP and c-Jun, as well as their synergistic transactivation of the PR promoter, requires at least one C/EBP- and one AP-1-binding site. From these observations, we propose a model where the synergy of LIP and c-Jun in transactivation of the PR promoter is dependent on the two factors mutually stabilizing their recruitment to the PR promoter.

Introduction

Mammary gland development is dependent on the actions of three major hormones, estrogen (E), progesterone (P), and prolactin (Prl), and occurs largely after birth (Brisken and O'Malley, 2010). The mammary gland undergoes two hormonally regulated developmental programs, one at puberty and the other at pregnancy. Progesterone (P) and its receptor, the progesterone receptor (PR), are particularly important for mammary gland development and maintenance during pregnancy. P signaling through PR is required for normal mammary development, as ductal side branching is reduced and lobuloalveolar development at duct ends is absent in PR knockout mice (Lydon et al., 1995).

PR exists as two isoforms, PRA and PRB. PRB comprises the identical amino acid sequence as PRA, but additionally contains an extension of 164 N-terminal amino acids (Jacobsen and Horwitz, 2012). In mouse, expression of PRA and PRB is temporally and spatially separated during mammary gland development (Aupperlee et al., 2005). PRA expression is dominant in the virgin and pubertal gland, is reduced in the mature gland, and is expressed at its lowest level in pregnancy. PRB is expressed robustly at pregnancy, particularly during alveologenesis at side branches and developing alveolar lobules. This indicates that PRB-expressing cells proliferate and differentiate during pregnancy. No PR expression is observed during lactation. Collectively, this implies that PRA and PRB are differentially regulated during the various stages of mammary gland development.

The individual roles of each isoform have been identified in mice specifically deficient in a single isoform. While ablation of PRA allows normal mammary gland development and response to hormones, ablation of PRB resulted in a defective phenotype, similar to that of mice

deficient in both isoforms, indicating that PRB is critical for mammary gland development during pregnancy (Mulac-Jericevic et al., 2003).

P/PR signaling-mediated cell proliferation, dysfunction in the P/PR pathway, and changes in the relative ratio of the two PR isoforms have all been shown to contribute to development of breast cancer. However, PR expression in breast cancer is a positive prognostic indicator for a better response to endocrine therapy and overall patient survival, and PR-positive tumors are more differentiated and less invasive than PR-negative tumors (Obr and Edwards, 2012). Moreover, similar levels PRA and PRB expression, which are detected in normal human breast tissues, are altered in tumors. The ratio of PRA:PRB is increased in more aggressive tumors (Hopp, 2004). Thus, elucidation of the mechanisms regulating PR expression, especially PRB, which correlates with better breast cancer prognosis, may be beneficial in developing therapeutic strategies for breast cancer that rely on modulating PR expression.

Transcriptional regulation of PR by E and estrogen receptor (ER) is well described (Jacobsen and Horwitz, 2012). However, PR is still expressed in ERα-deficient mice (Korach, 2000), and total PR levels do not fluctuate with the hormonal changes during the menstrual cycle (Mote et al., 2006). Further, loss of PR expression in ER-positive tumors does not result from non-functional ER (Cui et al., 2003). These observations indicate that other E-independent mechanisms contribute to regulation of PR expression. One other regulator of PR transcription is AP-1, which has been shown to both up- and down-regulate the human PR gene, respectively through a +90 AP-1 site (Petz et al., 2002) and a +745 AP-1 site (Petz et al., 2004). We recently found that the LIP isoform of C/EBPβ synergizes with the AP-1 family member c-Jun to up-regulate expression from the PR promoter (Wang, unpublished results).

When viewed in the context of the robust increase in LIP expression observed in mammary glands of pregnant mice (Seagroves et al., 1998), it is clear that the mechanism by which LIP acts with c-Jun to up-regulate PR expression may be significant to understanding the regulation of PR expression during murine pregnancy. Moreover, C/EBPβ-deficient mice (Robinson et al., 1998; Seagroves et al., 1998) and mice blocked for AP-1 activity (Shen et al., 2006) show similar defects in mammary gland development, as do PRB-deficient mice (Mulac-Jericevic et al., 2003), especially during pregnancy.

In this report, we have elucidated the mechanism by which LIP and c-Jun synergistically activate the PR promoter. We demonstrate that the integrity of C/EBP- and AP-1-binding sites is required for the respective C/EBPβ and c-Jun activities on the PR promoter, and that efficient promoter occupancy of both LIP and c-Jun, as well as their synergistic transactivation of the PR promoter, requires at least one C/EBP- and one AP-1-binding site. This leads us to propose a model where the synergy of LIP and c-Jun in transactivation of the PR promoter is dependent on the two factors mutually stabilizing their recruitment to the PR promoter. Surprisingly, the DNA binding and dimerization domains of the two transcription factors, rather than their known transactivation domains, are required for this synergy.

Material and methods

Cell lines: MC7-L1 is a murine mammary epithelial cell line derived from a ductal carcinoma (Lanari et al., 2001). Cells were maintained in DMEM-F12 (1:1) (Gibco, Life Technologies, Grand Island, NY) medium supplemented to 5% charcoal-stripped FCS, 100 units/ml penicillin and 100mg/ml streptomycin. Experiments were carried out with charcoal-stripped FCS in the absence of antibiotics. Cells were maintained in a humidified incubator at 37°C at 5% CO_2.

Expression vectors and promoter-reporters: Murine C/EBPβ isoforms were individually expressed from pcDNA3.1 (Invitrogen, Carlsbad, CA). pcDNA-LIP has been described (Dearth et al., 2001). pcDNA-LAP2 (plasmid 12557; Addgene, Cambridge, MA) has been described (Basu et al., 2011). pcDNA-LAP1 was derived from a plasmid containing the complete coding sequence of murine C/EBPβ inserted between the EcoRI and HindIII sites of pcDNA3.1 (a gift from Dr. Peter Johnson, NCI-Frederick, Frederick, MD). The AUG translational start sites for LAP2 and LIP were mutated to GCG and a consensus Kozak sequence introduced upstream of the LAP1 translational start, mutating GCGTTCATG to GCCACCATG (mutated bases underlined) by site-directed mutagenesis. pCMV-c-Jun has been described (McCabe at al., 1996). pCMV-TAM-67, expressing a dominant negative form of c-Jun that lacks amino acids 3 through 122, has been described (Brown et al., 1996). The expression vectors for A-C/EBP and A-fos under the control of the CMV promoter has been described (Ahn et al., 1998) and obtained from Addgene. The 2x-C/EBP-Luc reporter and 2x-AP-1 Luc reporter contain two canonical C/EBP binding sites and two AP-1 sites, respectively. The tandem PR promoter-reporter consists of the region -2494 to +769 base pairs (bp) in relation to the predicted PRB transcriptional start inserted between the Asp718 and NcoI sites of pGL3-Basic (pGL3B) (Promega, Madison, WI). The PR promoter region was isolated by PCR amplification of C57/Bl6 genomic mouse DNA using the following primers: -2502 to -2471 bp, 5'-ACAT<u>GGTACC</u>AGCGTGTCACCTGGCAC AGA-3' (containing an underlined Asp718 site); +771 to +753 bp, 5'-CTGT<u>CCATGG</u>ACACGT CCGAGTGCTGGCT-3' (containing an underlined NcoI site). TA cloning placed the PCR fragment into vector pCR2.1 (TA Cloning Kit; Invitrogen, Life Technologies, Grand Island, NY). The promoter fragment was then excised with Asp718 and NcoI, and inserted into pGL3B. The PRA (+379bp/+769bp), long PRB (-444bp/+399bp), short PRB (-444bp/+63bp) were

similarly constructed using the following primers for PCR amplification: PRA, +379 to +399 bp, 5'-GCTACTTCTTCCTGTCCTCAC-3', +751 to +769 bp, 5'-GCACGTCCGAGTGCTGGCT-3'; long PRB, -444 to -423 bp, 5'-AGCACCTGCAACTTCACCTCTG-3', +399 to +379 bp, 5'-GTGAGGACAGGAAGAAGTAGC-3'; short PRB, -444 to -423 bp; 5'-AGCACC TGCAACTTCACCTCTG-3', +43 to +63 bp, 5'-TAGCAGAATGTCAGA ATCCTC-3'. The minimal PRB (-117bp/+63bp) was generated by digestion of short PRB (-444/ +63) with SpeI and re-ligation. Mutations were introduced into the minimal PRB using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) following the manufacturer's protocol. Primers for mutagenesis are shown in the Table 3.1.

Site	Sequence	Primer sequence 5'-3'
B1	TGATTGGCTA TGATGGTCTA	TAGATCTAGCCAGTGATGGTCTAGGGAGGGGCTTTG
B2	CTTGCTAGAAAG CTGGTTATACAG	CCTAGAGCGCCAACGCTGGTTATACAGCTATGGAGCC
B3	CTTTGTAGTATT CTTTTTATTATT	Antisense: GAATCCTCGCACCCGTAAATAATAAAAGACTGATAGTGACAGTC
B4	TCTGACATTCTGCT TCTAAGATTGTGCT	Antisense GAATTCGCCCTTTAGCACAATCTTAGAATCCTCGCACCCG
J1	ACTGTCA AATGTAA	GAAAGCTATGGAGCCAGTCTAGAATGTAACTATCAGTCTTTGTAG
J2	TGACATT TATCATT	CGGGTGCGAGGATTCTATCATTCTGCTAAGGGCG
half B2J1		GTTATACAGCTATGGAGCCAGTCTAGAATGTAACTATCAGTC

Table 3.1. List of Primers for Site Mutagenesis.

DNA transfections and promoter-reporter assays: $5x10^4$ cells were plated in each well of a 12-well plate 24 h prior to transfection. Transient transfections were performed on the next day with 100 ng of promoter-reporter, 1 ng of pRL-SV40 as a control, and varied amounts of expression and control plasmids as indicated in figure legends totaling 500 ng of total DNA per well. Plasmid DNAs were mixed with 1 µl of FuGENE6 Transfection Reagent (Promega, Madison, WI) in 50 µl of serum-free DMEM-F12 medium. This mixture was incubated at room

temperature for 30 min before addition to culture wells. 24 h later, cell lysates were assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). The firefly luciferase values were from the promoter-reporters were normalized to the Renilla luciferase values from pRL-SV40 (Promega) to control for transfection efficiency between individual samples. All transfections were carried out in duplicate and repeated at least three times. In experiments involving the minimal PRB, luciferase values derived from pGL3B lacking any inserted sequences were subtracted from those of minimal PRB promoter-reporter to eliminate background levels of luciferase expression driven by C/EBPβ and c-Jun responsive sequences in the parent vector.

Chromatin immunoprecipitation (ChIP) and sequential ChIP assays: The PRB promoter-reporter was precut with ScaI to separate promoter sequences from the pGL3B backbone. Transient transfections were performed similarly to promoter-reporter assays, except $5x10^5$ cells were plated per 6 cm culture dish and the DNA transfection mix comprised 2 μg of precut promoter-reporter, 1.5 μg of each expression plasmid, an amount of "empty" control expression vector to total 5 μg of total DNA, and10 μl FuGENE6 in 100μl of serum-free DMEM/F12 medium. ChIP was performed 24 h after transfection.

Trypsinized cells were resuspended in DMEM/F-12 medium supplemented to5% FCS. The cells were then crosslinkedat1% formaldehyde for10 minutes at room temperature. Crosslinking was stopped by the addition of glycine to a final concentration of 0.125 M. The cells were washed twice with ice cold PBS, resuspended in cell-lysis buffer (5 mM Pipes [pH 8.0], 85 mM KC1, 0.5% NP40 with protease inhibitors [1mM dithiothreitol, 0.5 mM PMSF, 2.5 ug/ml leupeptin, 5 ug/ml antipain, 5 ug/ml aprotinin and 1 uMpepstatin A]), and then incubated on ice for 10 min. Nuclei were pelleted by centrifugation at 5000 rpm for 5 min at 4°C. The

nuclei were resuspended in nuclear lysis buffer (50 mMTris-Cl [pH 8.1], 10 mM EDTA, 1% SDS with protease inhibitors). The cells were then sonicated 10 timesat30% amplitude, 0.7s on and 1.3s off, over30-s intervals on ice.

Each 100μl chromatin was pre-cleared at 4°C overnight in900μl ChIP dilution buffer (0.01% SDS, 1.1% Triton X100, 1.2 mM EDTA, 16.7 mMTris-Cl [pH8.1], 167 mMNaCl with protease inhibitors) and 80μl of a 50% (v/v) Protein G-Agarose slurry containing 20μg salmon sperm DNA and 1 mg/ml BSA, and stored in 4°C for later use. Equal amounts of pre-cleared chromatin in ChIP dilution buffer were incubated with 2μg of either rabbit anti-C/EBPβ (C-19; Santa Cruz Biotechnology, Dallas, TX), rabbit anti-c-Jun (H-79; Santa Cruz Biotechnology), or normal rabbit IgG (Santa Cruz Biotechnology) at 4°C overnight. Immunoprecipitated products were collected after 2 h incubation with 50μl of pre-coated Protein G-Agarose beads (The Thermo Scientific™Pierce™). Protein G-Agarose beads were pre-coated with 20μg salmon sperm DNA and l mg/ml BSA at 4°C overnight, and stored at4°C for later use.

Immunoprecipitated material was washed sequentially in TSE buffer (20 mM Tris [pH 8.1], 0.1% SDS, 2mM EDTA, 1% Triton X-100), TSE buffer plus 150 mMNaCl, and TSE buffer plus 500 mM NaCl, and finally in buffer III (10 mMTris [pH 8.0], 1 mM EDTA, 0.25 M LiCl, 1% NP-40,1% deoxycholate). Each wash step was performed twice for 15 min at room temperature with a rotating plate. Beads were then washed twice in TE buffer (20 mM Tris [pH 8.0], 2 mM EDTA), and protein-DNA complexes were eluted in 500 μl of 0.1 M NaHC03-l% SDS for 30 min at 65°C. Crosslinking of the protein-DNA complexes was reversed and RNA and protein removed by overnight incubation with 2μl of 10mg/ml RNAseA at 65°C, followed by incubation with 2μl of 20mg/ml Proteinase K at 42°C for 2 hours. DNA was then purified by

phenol-chloroform extraction and ethanol precipitation, and subsequently dissolved in 50 μl of TE buffer.

DNA from the input and immunoprecipitated samples were subjected to quantitative polymerase chain reaction (qPCR) with specific primers (Table 2) in triplicate. qPCR was performed with the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Inc., Foster City, CA) using the following program: step 1, 95°C for 10 min; step 2, 40 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 30 s; step 3,72°C for 5 minutes. In experiments with the minimal PRB, qPCR was also performed with additional primers for the pGL3B backbone region as a control (Table 3.2). The amplified qPCR products from immunoprecipitated DNA are presented as the fold enrichment compared to normal rabbit IgG immunoprecipitation products.

Region	Primer Sequence 5'-3'
Minimal PRB	Forward CTAGCAAAATAGGCTGTCCC Reverse CTTTATGTTTTTGGCGTCTTCCA
Control Vector	Forward GCGACACGGAAATGTTGAATAC Reverse CTACGTGAACCATCACCCTAATC

Table 3.2. List of Primers for ChIP.

Sequential ChIP was performed similarly to ChIP. Briefly, 100 μl of crosslinked chromatin from transfected cells was incubated first with anti-C/EBPβ, the immunoprecipitated collected on Protein G-agarose beads, and then protein-DNA complexes eluted from the beads with TE containing 1% SDS. Each half of the eluted material was then subjected to a second immunoprecipitation (i.e., sequential ChIP) with either normal IgG, or anti-C/EBPβ, or anti-c-Jun. The supernatant from the first ChIP was subjected to a second ChIP with anti-c-Jun to detect

c-Jun-DNA complexes not associated with C/EBPβ. This procedure was also performed with an initial c-Jun ChIP.

Results

We previously demonstrated that C/EBPβ and c-Jun synergistically transactivate the tandem PR promoter-reporter (Wang et al., unpublished results; see Chapter 2). The truncated LIP isoform of C/EBPβ was particularly active in this synergy. To define which sequences of the tandem promoter (TR, -2494bp/+769bp) are required for the synergistic activity of C/EBPβ and c-Jun, we used four truncated versions of the PR promoter-reporter: PRA (+399bp/+769bp), long PRB (-444bp/+399bp), short PRB (-444bp/+63bp), and minimal PRB (-117bp/+63bp). First, the basal activity of these promoters was evaluated by transiently transfecting each promoter into MC7-L1 cells (Figure 3.1A). We found that the activities of the TR, PRA, long PRB, short PRB, and minimal PRB (mPRB) were respectively 5.6-fold, 8.5-fold, 12.5-fold, 21.5-fold, and 10.8-fold that of the pGL3B vector that lacks any PR sequences. The increase in promoter activity as a consequence of deletion suggested the presence of negative regulatory elements within the promoter regions between -2494bp to -444bp and +63bp to +399bp.

The activities of LIP and c-Jun were then examined individually and together in transient co-transfections with each promoter-reporter construct in MC7-L1 cells (Figure 3.1B). Both LIP and c-Jun were individually able to similarly transactivate all four promoters, indicating that all the promoter constructs retained responsive elements for LIP and c-Jun. Co-transfection of LIP and c-Jun showed synergistic activation of all of the promoter constructs, including mPRB.

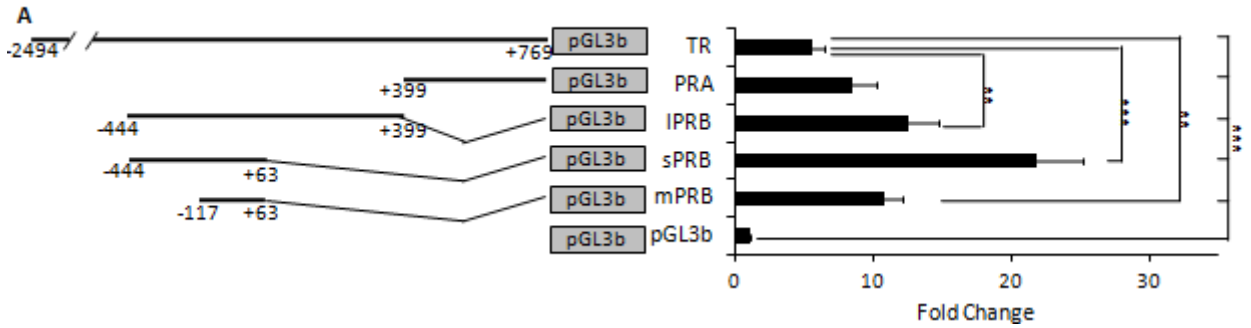
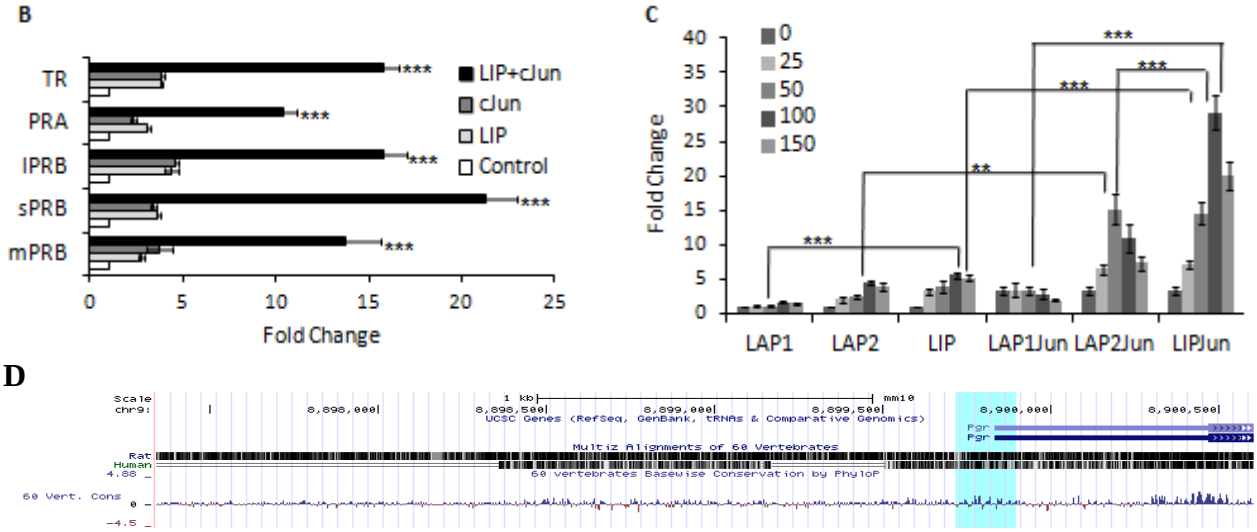


Figure 3.1. Characterization of Progesterone Receptor promoter.
(A) Basal promoter activity of truncated forms of PR promoter.MC7-L1 cells were transfected with 100ng of either full-length tandem promoter (TR) reporter (-2494/+769) or truncated forms (PRA (+399/+769), lPRB (-444/+399), sPRB (-444/+63), mPRB (-117/+63).The luciferase activity was normalized by Renilla reporter activity values. The fold change in activity of each promoter was presented as the ratio of its normalized luciferase activity to the control pGL3basic promoter, which was set as 1. Results are the mean±S.E. from three independent experiments; each experiment was performed in duplicate. *p* was calculated by Student's T-Test in comparison to the pGL3-basic plasmid transfection, and in comparison to tandem PR promoter transfections. *, $p<0.1$; **, $p<0.05$; ***, $p<0.01$. (B) Synergistic effect of C/EBPβ and c-Jun on truncated forms of PR promoter. MC7-L1 cells were transfected with a fixed amount of eitherTR reporter or truncated forms, plus individual or combined expression vectors for C/EBPβ-LIP and c-Jun. The luciferase activity value was normalized by Renilla reporter activity value. The fold change in activity of each promoter was presented as the ratio of its normalized luciferase activity to the activity in overexpression of control vector, which was set as 1. Results are the mean±S.E. from three independent experiments; each experiment was performed in duplicate. *p* was calculated by Student's T-Test in comparison to sum of folds in single factor transfection. *, $p<0.1$; **, $p<0.05$; ***, $p<0.01$. (C) Differential transactivation of three C/EBPβ isoforms.MC7-L1 cells were transfected with 100ng of minimal PRB reporter, an increasing amount (25, 50, 100, 150ng) of expression vector for each C/EBPβ isoform with or without 100ng of c-Jun expression vector. The fold change in activity of each promoter was presented as the ratio of its normalized luciferase activity to that activity in control expression vector transfection, which was set as 1. Results are the mean±S.E. from three independent experiments; each experiment was performed in duplicate. *p* was calculated by ANOVA in comparison between the co-transfection of LIP and c-Jun and co-transfections involving other C/EBPβ isoforms plus c-Jun, and in comparison between single transfection of each C/EBPβ vector alone and co-transfections of that certain C/EBPβ isoform and c-Jun expression vectors. *, $p<0.1$; **, $p<0.05$; ***, $p<0.01$. (D) Conservative regions. Conservative comparison of tandem promoter of mouse Progesterone Receptor with other species' sequences with mPRB region highlighted. (E) Putative C/EBP and AP-1 sites on mPRB. The putative C/EBP and AP-1 sites on mPRB.

Figure 3.1 (Cont'd)



E

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Mouse   CTAGTGAGT.AGCTGGAA.TTCCAACGCCAGAGATTTAGATCTAGCCAG
Rat     CTGATGTTCCAGGTGGAA.TGCCAACTCCAGT..TTTGGATCTCGCCAG
Human   ATAACG.....GGTGGAAATGCCAACTCCAGAGTTTCAGATCCTACCGG

          C/EBP(B1)
Mouse   TGATTGGC.TAGGGAGGGGCTTTGGGCGGG.CCTTCCTAGAGCGCCAAC
Rat     TGATTGGC.TAGGGAGGGGTTTGGGGCGGG.CCTTCCTAGAGCGCGGAG
Human   TAATTGGGGTAGGGAGGGGCTTTGGGCGGGGCCTCCCTAGAG.GAGGAG

            C/EBP(B2)                          AP-1(J1)
Mouse   GC.TTGCTAGAAAGCTATGGAGCCAGTCTAGA.CTGTCACTA.TCAGT.
Rat     GC.TTACTAGAAAGCTGTGGAGCCAGTCTAGA.CTGTCACTA.TCAGG.
Human   GCGTTGTTAGAAAGCTGTCTGGCCAGTCCACAGCTGTCACTAATCGGGG

              C/EBP(B3)                  C/EBP(B4) & AP-1(J2)
Mouse   ....CTTTGTAGTATTTACGGGTGCGAGGATTCTGACATTCTGCT
Rat     ....CACTGTAGTATTTACTGGTGTGAGGATTCTGCCTTTCTGCT
Human   TAAGCCTTGTTGTATTTGTGCGTGTGGG.....TGGCATTCT...
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Since the mPRB is capable of supporting the synergism of LIP and c-Jun, we first asked if the mPRB resembled the tandem PR promoter in terms of differential transactivation by the three C/EBPβ isoforms, LAP1, LAP2, and LIP. We previously found that, while all three isoforms could modestly activate the tandem PR promoter, LIP was the most active in synergy with c-Jun and LAP1 showed no synergy with c-Jun (Wang et al., unpublished results; see Chapter 2). Transient transfection with the mPRB promoter-reporter showed a similar pattern of activity (Figure 3.1C). We found synergy with c-Jun for LAP2 and LIP, but not for LAP1 at any concentration. Moreover, LIP was the most active isoform in the synergy with c-Jun. Since the activity of the mPRB in regard to C/EBPβ and c-Jun was virtually identical to that of the TR, , we decided to utilize the mPRB to study the mechanism of synergy between LIP and c-Jun in regulation of the PR promoter.

To identify the potential *cis*-acting elements for LIP and c-Jun that may mediate their synergistic transactivation of the PR gene, we used the UCSC Genome Browser (https://genome.ucsc.edu/) to align the 5′ region upstream of the PR coding sequence among available vertebrate sequences. Direct comparison of the genomic PR promoter sequences among multiple species showed that the mPRB sequence is among the limited regions of sequence homology shared across species (mouse mPRB has 86% and 73% identity with rat and human, respectively) (Figure 3.1D). The selection for conservation across species suggests that this regulatory sequence and its control elements may play a key role in regulating transcription of the PR gene *in vivo*. Further screening of the mPRB sequence for C/EBP and AP-1 consensus binding sites using MatInspector (Cartharius et al., 2005; Quandt et al., 1995) and TFSearch (Heinemeyer et al., 1998) software revealed four C/EBP sites (B1 to B4) and two AP-1 sites (J1 and J2) (Figure 3.1E). Among those sites, J2 and B4 overlap. However, these potential

transcription factor binding sites in this region are less conserved among species with a few mismatches (Figure 3.1E), indicating the possible species-specific mechanism of PR regulation by this region of promoter.

To learn which of these putative sites contribute to the observed synergy, we compared the activity of the wild-type mRRB promoter-reporter construct with that of mutant constructs in which the predicted binding site(s) were mutated. First, four mPRB promoter-reporters lacking individual C/EBP binding sites (B1, B2, B3, and B4) were generated by site-directed mutagenesis. Co-transfection of any of these mutant promoter-reporters with a constant amount of c-Jun and increasing amounts of LIP or vice versa resulted in a dose response similar to that of the wild-type promoter (Figure 3S.1A). Since multiple putative C/EBP binding are predicted, it is possible that the lack of effect in mutating a single site reflects their redundancy. To test this, a series of mutated promoter-reporters were constructed with double, triple, and quadruple binding site mutations, and used in transient transfections. Some of the double mutant promoters (B1B2, B1B3, B1B4, and B2B4) showed only slight impairment of the synergy between LIP and c-Jun (Figure 3S.1B). Three among the four triple mutant promoters (B1B2B3, B1B2B4, and B1B3B4) showed statistically significant reductions transactivation by LIP and c-Jun compared to that of wild-type (Figure 3.2A). These promoter-reporters not only showed reduced transactivation, but synergy between LIP and c-Jun was absent. A promoter carrying mutations in all four C/EBP binding sites (B1-4) completely abolished the synergy LIP and c-Jun (Figure 3.2A).

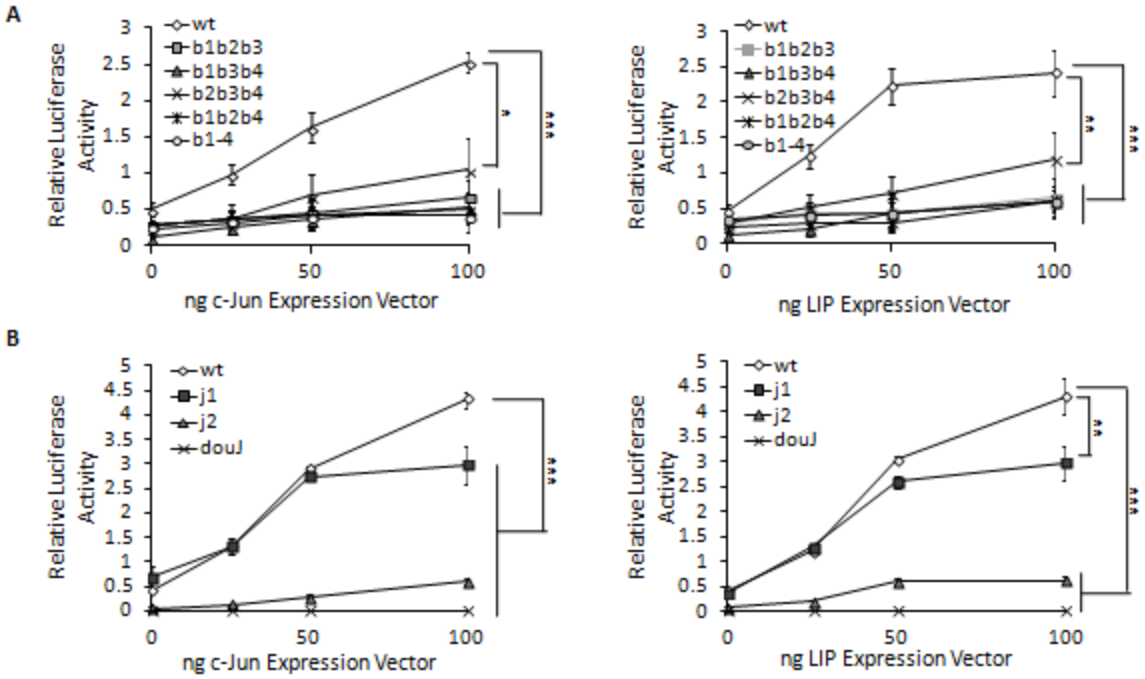
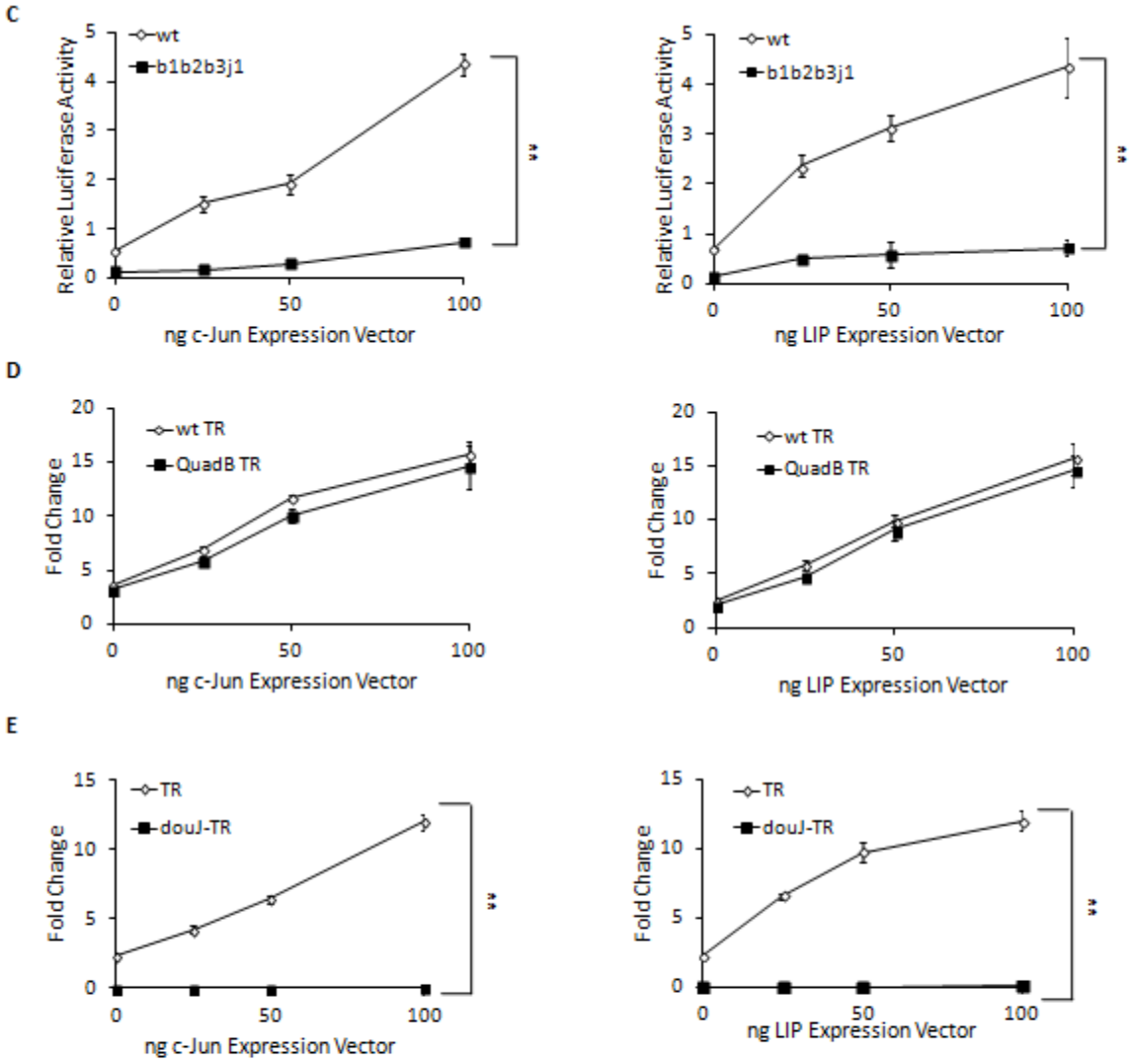


Figure 3.2: The effects of mutation of C/EBP and AP-1 sites on the promoter activity.
(A) mPRB with C/EBP mutated sites, (B) mPRB with AP-1 mutated sites, (C) mPRB with mutation of three C/EBP sites and one AP-1 site, (D) TR with four C/EBP mutated sites, and (E) TR with two AP-1 mutated sites. MC7-L1 cells were transfected with 100ng of wildtype mPRB or reporter promoters carrying mutations of indicated sites, plus either an increasing amount (25, 50, 100ng) of c-Jun expression vector (as indicated) and 100ng of LIP expression vector (Left Sides) or vice versa (Right Sides) The normalized luciferase activity of pGL3 was subtracted from the normalized luciferase activity of each promoter. The value of promoter activity of each promoter was presented as the relative activity after subtraction. Results are the mean±S.E. from six (A and B) or three (C, D, and E) independent experiments; each experiment was performed in duplicate. *p* was calculated by ANOVA in comparison between the wildtype promoter and mutant promoters. *, $p<0.1$; **, $p<0.05$; ***, $p<0.01$.

Figure 3.2 (Cont'd)



The same approach of mutating individual or both binding sites of the mPRB promoter-reporter was applied to the AP-1 sites (Figure 3.2B). c-Jun at 100ng induced J1 mutant and J2 mutant 2-3 folds, which were as similar as wildtype promoter. Both the J1 and J2 mutants still showed synergistic activation by LIP and c-Jun. However, these mutants had significantly reduced activity in response to LIP and c-Jun comparing to wildtype promoter. Among two mutants, J1 mutant retained higher levels of basal and induced activity. These results indicate that J1 is dispensable, while J2 is essential for full activity of the mPRB promoter and full synergism between LIP and c-Jun. Moreover, when both AP-1 binding sites were mutated, the promoter exhibited extremely low basal activity and failed to respond to either LIP or c-Jun alone, or LIP and c-Jun co-expression. This shows that a J2 binding site is essential for transactivation of mPRB by both C/EBPβ and c-Jun.

Since the B4 and J2 sites overlap, it suggested to us that it might constitute a regulatory unit that was sufficient for the synergy between LIP and c-Jun, where the two factors might bind in close juxtaposition. We examined whether a promoter that only retained the overlapping B4 and J2 sites could still respond synergistically to LIP and c-Jun, similarly to the wild-type mPRB. However, this mutant responded poorly to LIP and c-Jun co-transfection (Figure 3.2C). This suggests that the synergy between CEBPβ and c-Jun requires efficient DNA binding of each factor that might not be available from these overlapping sites.

To determine how the C/EBP and AP-1 binding sites that were examined in the mPRB influence transcription in the larger context of the 3.3kb tandem PR promoter, we introduced the same binding site mutations that promoter sequence. A version of the tandem PR promoter lacking all four C/EBP binding sites of the minimal PRB promoter showed neither decreased basal activity nor suppression of the synergy between LIP and c-Jun (Figure 3.2D). In contrast,

co-transfection of LIP and c-Jun failed to activate the tandem promoter containing lacking the two AP-1 binding sites identified in the minimal PRB promoter (Figure 3.2E).The retention of activity with C/EBP binding site mutations that severely cripple the minimal PRB promoter and the loss of activity with the AP-1 binding site mutations may reflect the redundancy of C/EBP binding sites in the longer promoter.

Since C/EBP and AP-1 sites were required for mRRB promoter-reporter activity, we next examined the physical binding of LIP and c-Jun to this minimal promoter region by a transient transfection ChIP assay. MC7-L1 cells were transfected with pre-cut mPRB promoter-reporter and expression vectors for LIP and c-Jun or control empty vector. C/EBPβ and c-Jun were detected on mPRB promoter with 143-fold and 64-fold enrichment relative to IgG, respectively (Figure 3.3A). These levels of occupancy were much higher than that observed with the control expression vector or in a region of the mPRB vector distal to the promoter, suggesting that occupancy of the promoter region containing the C/EBP and AP-1 binding sites is specific..

We hypothesized that the synergy between LIP and c-Jun is mediated through the two factors mutually stabilizing their occupancy of their respective binding sites. To address this question, ChIP was performed on transiently transfected reporter-promoters carrying the wild-type binding sites, or four mutated C/EBP binding sites and two wild-type AP-1 sites, or two mutated AP-1 binding sites and four wild-type C/EBP sites (Figure 3.3B). Mutation of all four C/EBP sites abolished virtually all binding of C/EBPβ in comparison to the wild-type promoter, consistent with the same mutant promoter having a very weak response to LIP in the promoter-reporter assay (Figure 3.2A). Similar to the case with the promoter lacking all four C/EBP

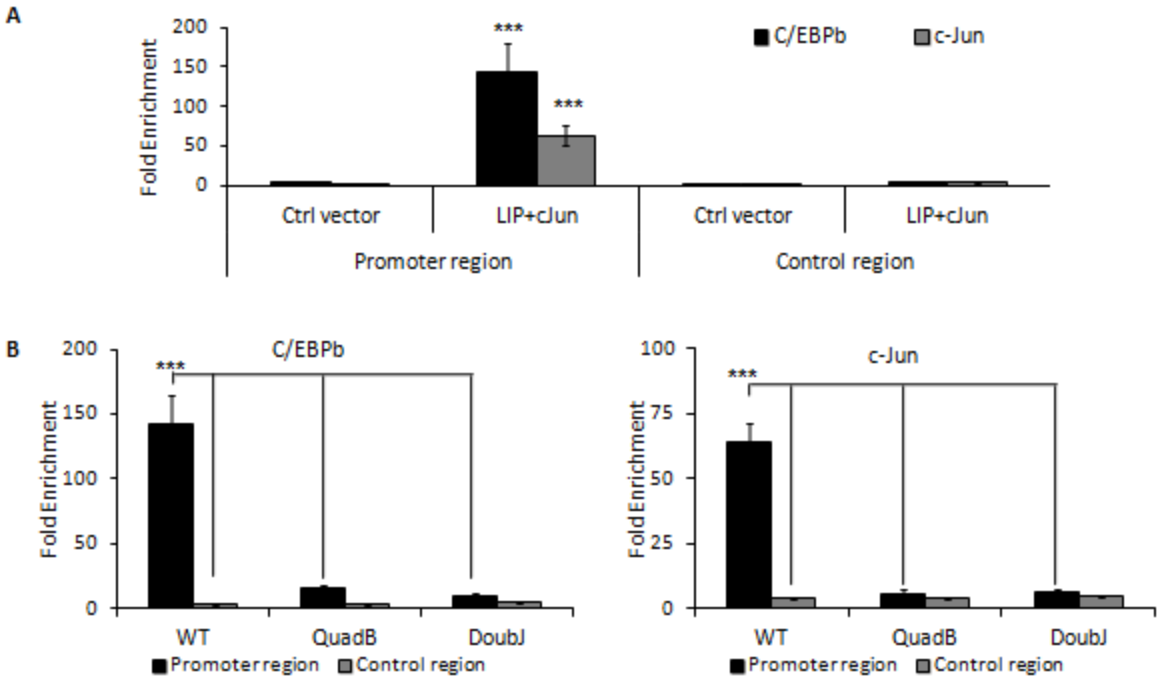


Figure 3.3. Differential occupancy of C/EBPβ and c-Jun on wildtype and mutant promoters.

(A) Occupancy of C/EBPβ-LIP and c-Jun on wildtypemPRB promoter in ChIP assays. MC7-L1 cells were transfected with pre-cut mPRB promoter and expression vectors for both C/EBPβ-LIP and c-Jun, or control expression vector (as indicated). Chromatin was precipitated with either C/EBPβ antibody (Black Colum) or c-Jun antibody (Grey Colum), or IgG. The DNA from precipitated chromatin was subjected to quantify by qPCR either with a primer pair for the promoter region (Left Side) or with another primer pair for the control region (Right Side). The value of qPCR-amplified product was calculated as the percentage of input, then the fold enrichment was presented here as the ratio of amplified product with specific antibody to that with nonspecific IgG antibody. Results are the mean±S.E. from three independent experiments. *p* was calculated for each IP with specific antibody by Student's T-Test in comparison to the control vector transfection, and in comparison to the control region. *, $p<0.1$; **, $p<0.05$; ***, $p<0.01$.

(B) Differential occupancy of C/EBPβ and c-Jun on wildtypeand mutant mPRBpromoter in ChIP assays. MC7-L1 cells were transfected with pre-cut mPRB promoter forms (WT: wildtypemPRB, QuadB: mPRB carrying four C/EBP mutated sites, DoubJ: mPRB carrying two AP-1 mutated sites) and expression vectors for both C/EBPβ-LIP and c-Jun. Chromatin was precipitated with either C/EBPβ antibody (Panel B Left) or c-Jun antibody (Panel B Right), or IgG. The DNA from precipitated chromatin was subjected to quantify triplicate by qPCR either with a primer pair for the promoter region (Black Colum) or with another primer pair for the control region (Grey Colum). The value of qPCR-amplified product was calculated as the percentage of input, then the fold enrichment was presented here as the ratio of amplified product with specific antibody to that with nonspecific IgG antibody. Results are the mean±S.E. from three independent experiments. *p* was calculated for each IP with specific antibody by Student's T-Test in comparison to the mutant promoter transfection for promoter regions, and in comparison to the control region. *, $p<0.1$; **, $p<0.05$; ***, $p<0.01$.

binding sites, mutation of both AP-1 binding sites abolished virtually all binding of c-Jun. Again this is consistent with this promoter's lack of activity in the promoter-reporter assay (Figure 3.2B). These results demonstrate that LIP and c-Jun bind specifically to these binding sites in the mRRB region of the PR promoter. It is particularly noteworthy that mutation of either the C/EBP or the AP-1 binding sites almost completely eliminated occupancy by both factors. Moreover, the ChIP products from the internal control region show no difference among the wild-type and mutant promoter-reporters, indicating that the differential occupancy of LIP and c-Jun between wild-type and mutant promoters was dependent upon the mutated binding sites rather than differences in the plasmid DNAs. Collectively, these findings are consistent with a cooperative model in which the stable recruitment and/or binding of these factors is mutually dependent.

Mutation of C/EBP and AP-1 sites on the minimal PRB promoter severely impairs the LIP and c-Jun transactivation and causes a dramatic decrease in occupancy of the two factors on DNA. This suggests that these two transcription factors need to bind DNA for their transactivation effects. To examine this further, we utilized A-C/EBP and A-Fos, dominant negative inhibitors that, respectively, prevent C/EBP and AP-1 from binding DNA (Ahn et al., 1998). Co-transfection of A-C/EBP with LIP blocked transactivation of tandem promoter reporter by LIP (Figure 3.4A). A similar result was seen in co-transfection of A-Fos with c-Jun. Further, the expression of either A-CEBP or A-Fos in co-transfection of LIP and c-Jun was sufficient to inhibit the synergy between LIP and c-Jun on the tandem PR promoter. Co-transfection of LIP, c-Jun, and both of their dominant-negative inhibitors suppressed promoter activation to basal levels. A-C/EBP inhibited C/EBPβ-mediated transactivation of a 2x-C/EBP promoter-reporter, and A-Fos inhibited c-Jun-mediated transactivation of a 2x-AP-1 promoter-reporter (Figure 3S.2C). Moreover, A-C/EBP and A-Fos were unable to inhibit basal activities of

2x-AP-1 and 2x-C/EBP, respectively (Figure 3S.2A). These observations confirmed their specificity and efficacy. Collectively, this is consistent with our hypothesis of mutual dependence of LIP and c-Jun for DNA binding.

We previously found that siRNA-mediated downregulation of C/EBPβ and c-Jun attenuated the activity of PR promoter, demonstrating that the endogenous levels of C/EBPβ and c-Jun play a significant role in activating the PR promoter (Wang et al., unpublished results; see Chapter 2). To test if A-C/EBP and A-Fos could prevent the endogenous C/EBPβ and c-Jun from activating the basal activity of the tandem PR promoter, an A-C/EBP and A-Fos were co-transfected with the tandem PR promoter-reporter (Figure 3.4B). Transfection of A-C/EBP had no significant effect on basal activity of the tandem PR promoter. A-C/EBP also failed to reduce the basal activity of the 2x-C/EBP promoter-reporter (Figure 3S.2B). However, A-Fos suppressed the basal activity of the tandem PR promoter in a dose-dependent manner, maximally inhibiting activity by more than 60% (Figure 3.4B). This latter result is consistent with the previous studies showing that intact AP-1 sites and c-Jun binding to be essential for transactivation of the PR gene.

To determine if both LIP and c-Jun co-occupy the minimal PRB promoter, we performed sequential ChIP (Figure 3.5). This procedure was either performed with an initial immunoprecipitation with anti-C/EBPβ or with anti-c-Jun. The recovered precipitate was then subjected to a second immunopreciptation with non-specific normal IgG, or the same antibody as in the initial immunoprecipitation, or the transcription factor antibody not used in the initial immunoprecipitation. Neither supernatant from the initial immunoprecipitation yielded product

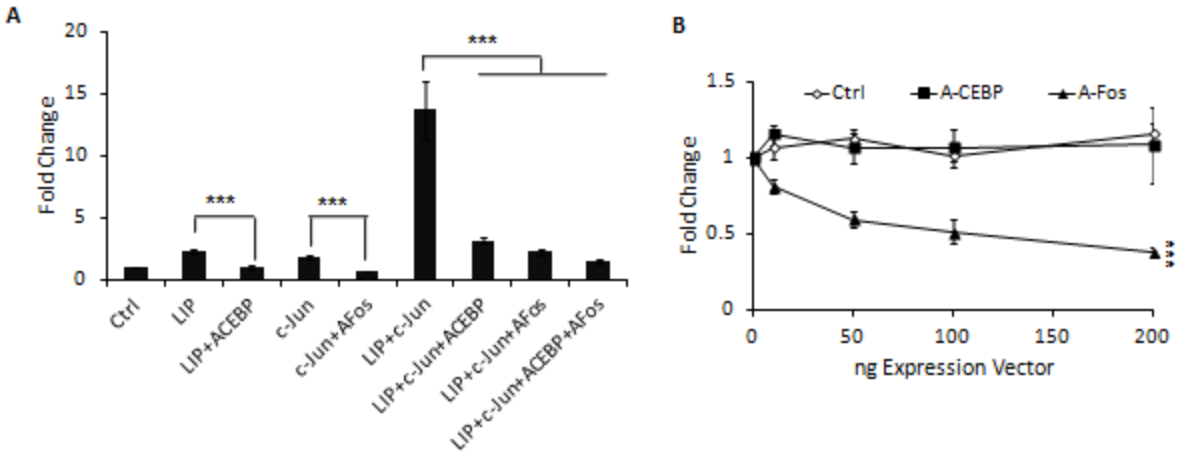


Figure 3.4. The effect of DNA binding inhibition of C/EBPβ and c-Jun on the promoter activity.

(A) Synergistic effect of C/EBPβ and c-Jun,(B) Basal activity.MC7-L1 cells were transfected with (A) 100ng of TR and 100ng of expression vectors for proteins (C/EBPβ-LIP, c-Jun, A-CEBP, and A-Fos) as indicated, or (B) 100ng of TR alone and an increasing amount (10, 50, 100, 200ng) of expression vectors for either A-CEBP or A-Fos. The fold change in activity of each promoter was presented as the ratio of its normalized luciferase activity to the activity in control expression vector transfection, which was set as 1. Results are the mean±S.E. from five independent experiments; each experiment was performed in duplicate. For (A) *p* was calculated by Student's T-Test in comparison to the single expression vector transfection and co-transfection with its dominant negative form, and in comparison between LIP and c-Jun co-transfection and LIP, c-Jun, and their dominant negative forms . *, $p<0.1$; **, $p<0.05$; ***, $p<0.01$. For (B) *p* was calculated by Student's T-Test in comparison to the control transfection.

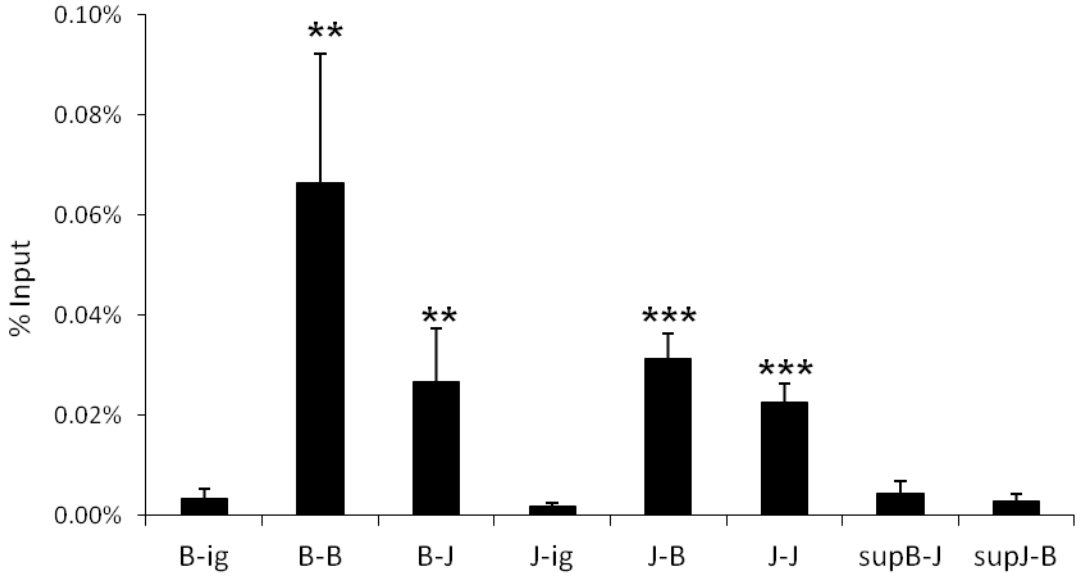


Figure 3.5: Co-occupancy of C/EBPβ and c-Jun in sequential ChIP assays.
MC7-L1 cells were transfected with pre-cut mPRB promoter and expression vectors for both C/EBPβ-LIP and c-Jun. Chromatin was first precipitated with C/EBPβ antibody. Then, precipitate product was equally subjected to either non-specific IgG antibody (B-ig), or C/EBPβ (B-B), or c-jun (B-J). The supernatant was subjected to c-Jun antibody (supB-J), as indicated. The order direction was similarly performed to collect chromatin for J-ig, J-B, and J-J. The DNA from precipitated chromatin was subjected to quantify triplicate by qPCR. The value of qPCR-amplified product was presented as the percentage of input. Results are the mean±S.E. from three independent experiments. *p* was calculated for each sequential IP by Student's T-Test in comparison to its IgG control in the second IP (B-ig, or J-ig) . *, $p<0.1$; **, $p<0.05$; ***, $p<0.01$.

in a second immunoprecipitation suggesting that no significant amount of c-Jun-bound complex remained in the supernatant after initial immunoprecipitation with anti-CEBPβ, and that no significant amount of LIP-bound complex remained in the supernatant after immunoprecipitation with anti-c-Jun. Importantly, after both initial immunoprecipitation with anti-C/EBPβ and anti-c-Jun, ChIP products were seen after the second immunoprecipitation with either anti-C/EBPβ or anti-c-Jun. This confirms that LIP and c-Jun are simultaneously bound to minimal PRB promoter region. These results support the model that LIP and c-Jun binding to the PR promoter are mutually dependent.

In promoter-reporter assays, LIP was the most active C/EBPβ isoform in synergy with c-Jun; LAP2 showed synergy with c-Jun but at a lower magnitude, while LAP1 did show any synergy with c-Jun (Wang et al., unpublished results; see Chapter 2). Having observed that the synergy of LIP with c-Jun correlated with their mutual dependence for occupancy on the minimal PR promoter (Figure 3.3B), we questioned whether differential occupancy among C/EBPβ isoforms with and without c-Jun would correlate with their relative activities in transactivating the PR promoter. To that end, we performed ChIP in cells transfected with the wild-type minimal PR promoter-reporter and expression vectors for LAP1, LAP2, or LIP in the absence or presence of an expression vector for c-Jun (Figure 3.6). The occupancy of singly expressed C/EBPβ isoforms was low and little different from that observed in negative controls for LAP1, LAP2, and LIP. The co-expression of c-Jun dramatically enhanced the occupancy of LIP and modestly enhanced the occupancy of LAP2, while having little effect LAP1 occupancy. Similarly, the occupancy of c-Jun was very low in single c-Jun overexpression, but slightly and dramatically increased in the presence of LAP2 and LIP, respectively. The significantly enhanced occupancy

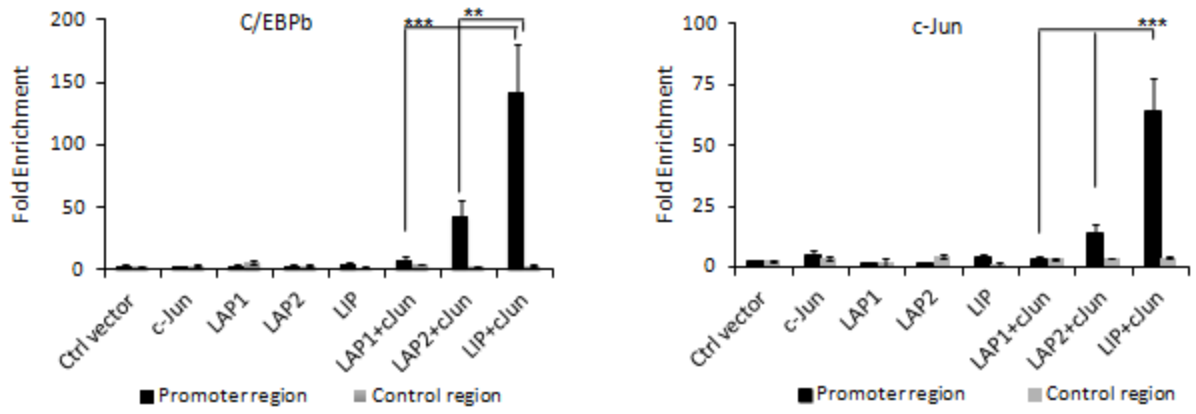


Figure 3.6: Differential occupancy of three C/EBPβ isoforms.
MC7-L1 cells were transfected with pre-cut mPRB promoter and either individual expression vector, or combined vectors for C/EBPβ isoforms and c-Jun, or control expression vector (as indicated). Chromatin was precipitated with C/EBPβ antibody or IgG. The DNA from precipitated chromatin was subjected to quantify triplicate by qPCR either with a primer pair for the promoter region (Black Colum) or with another primer pair for the control region (Grey Colum). The value of qPCR-amplified product was calculated as the percentage of input, then the fold enrichment was presented here as the ratio of amplified product with specific antibody to that with nonspecific IgG antibody. Results are the mean±S.E. from three independent experiments.MC7-L1 cells were transfected with pre-cut mPRB promoter and expression vectors for proteins as indicated. Chromatin was precipitated with either C/EBPβ antibody or c-Jun antibody. The fold enrichment was presented as the ratio of amplified product with specific antibody to that with nonspecific IgG antibody. Results are the mean±S.E. from three independent experiments. *p* was calculated for each IP with specific antibody by Student's T-Test in comparison to the co-transfection of LIP and c-Jun. *, $p<0.1$; **, $p<0.05$; ***, $p<0.01$.

observed with co-expression of LIP and c-Jun compared to that of either LAP1 or LAP2 with c-Jun correlated well with the synergistic transactivation effects seen in the promoter-reporter assay.

Discussion

The important role for PR in mammary gland development and the correlation of PR expression with the characteristics of various types of breast cancer require a comprehensive study of how PR expression is regulated. Differential PR expression levels and isoform expression between normal and breast cancer cells determine the action of the two PR isoforms in these two different conditions. Regulated expression of PR is required for appropriate mammary responsiveness to progesterone; thus, identification of the mechanisms that modulate the effects of PR may offer new strategies in breast cancer treatment. Our previous work showed that C/EBPβ and c-Jun synergistically activate the PR promoter (Wang et al., unpublished results; see Chapter 2). The LIP isoform of C/EBPβ, generally viewed as an inhibitor of C/EBPβ-mediated activation, was the most active isoform in this synergy, while LAP2 displayed more modest activity and LAP1 showed little activity. This current study provides mechanistic insight into how PR expression is regulated by C/EBPβ and c-Jun, and the mechanism of synergy between LIP and c-Jun on the PR promoter. These results expand our current knowledge on PR regulation.

C/EBP and AP-1 have been found to cooperate in the regulation of a number of genes, dependent upon tissue and cell type (Cai et al., 2008; Fries et al., 2007; He et al., 2013; Hong et al., 2011; Klampfer et al., 1994; Madireddi et al., 2000; Mietus-Snyder et al., 1998; Wang et al., 2006; Zagariya et al., 1998). The data that we present here support a model of mutual dependence of LIP, and presumably LAP2, and c-Jun for the transactivation of the PR promoter.

This mutual dependence appears to be based on each transcription factor requiring binding of the other for its own efficient DNA binding. This is particularly highlighted by our finding with the minimal PRB promoter that mutation of all C/EBP binding sites decreased both c-Jun's transactivation function and DNA occupancy, and vice versa for mutation of both AP-1 binding sites. Furthermore, the coincident binding of both LIP and c-Jun is supported by sequential ChIP studies.

Our data also demonstrate that the C/EBP binding sites are largely redundant. The mutation of one or two of the C/EBP binding sites in the minimal PRB promoter is not sufficient to impair the response to LIP and c-Jun. When the B1 C/EBP site by itself is retained, the response to LIP and c-Jun co-expression is dramatically reduced, but synergy between the two transcription factors is retained. It should be noted that when only one or two C/EBP sites are mutated, retention of the B1 site confers no more activity than retention of any of the other sites. Similarly, when the J2 site retained, there is a significant reduction in the response to LIP and c-Jun, but again synergy is retained. The B1 and J2 binding sites are the most effective C/EBP and AP-1 sites, respectively. Also consistent with the redundancy of C/EBP sites, mutation of the four C/EBP binding sites of the minimal PRB promoter in the context of the larger tandem PR promoter, which contains additional putative C/EBP binding sites, decreases neither basal nor LIP- and c-Jun-induced synergistic transactivation.

The physical interaction of C/EBPβ and c-Jun in vitro is mediated by bZIP regions of both proteins (Hsu et al., 1994). To explore if these two factors can interact without DNA binding in MC7-L1 cells, we performed co-immunoprecipitation assays. However, we did not observe C/EBPβ and c-Jun co-immunoprecipitation in the MC7-L1 extracts. It is possible that the interaction is not stable in vitro without binding to DNA, indicating the absolute requirement of

DNA binding for their function on the PR promoter. Similar result showed that C/EBPβ and c-Jun interact in vitro and in a DNA-dependent manner has been reported by using DNA binding assays with oligonucleotides derived from the TNFα promoter (Zagariya et al., 1998).

Using a deletion approach, we demonstrated that the synergy of LIP and c-Jun was observed in all of the truncated PRB promoter-reporters that we tested. This allowed identification of a minimal region amenable to a mechanistic analysis. This synergy had more significant impact on the promoter region associated with PRB isoform-specific expression than on the promoter region associated with PRA expression. This finding of an important role for C/EBPβ in PRB expression is consistent with the finding that C/EBPβ and PRB KO mice show a similar defective phenotype in mammary gland development at pregnancy (Mulac-Jericevic et al., 2003; Robinson et al., 1998; Seagroves et al., 1998) and that PRB and C/EBPβ co-localize in the *in vivo* staining experiments, while staining for PRA and C/EBPβ are mutually exclusive (Wang et al., unpublished data; see Chapter 2).

We found that the mouse -444bp/+63bp and -117bp/+63bp regions relative to the transcription start site for PRB are robustly responsive to LIP and c-Jun, and are sufficient to confer a synergistic response to LIP and c-Jun, similarly to tandem PR promoter. Moreover, in this well-conserved and short region of the minimal PRB promoter, we found four putative binding sites for C/EBPβ and two for c-Jun located near or overlapping to each other.

This region has variously been reported to be non-responsive or responsive to various factors depending upon the cell and tissue type, as well as specific stimuli. Previous studies of the mouse -368bp/+64bp region in rat primary granulosa cells (Sriraman et al., 2003) and the rat -131bp/+65bp region, which shares high conservation with the mouse sequence, in MCF-7 human breast cancer cells (Kraus et al., 1993) did not find these sequences to be responsive in

promoter-reporter constructs. However, these earlier studies examined either the roles of Sp1/Sp3, or the role of the estrogen receptor, respectively.

In this proximal promoter region, several other transcription factors and putative binding sites have been reported to regulate PR expression, dependent upon the specific cell type and/or inducing stimulus. In the mouse PR promoter, in the region surrounding the B1 site, NFY-B binds to a CCAAT sequence within B1, GATA-4 to a GATA site, and Sp1/Sp3 to a GC box when using granulosa cell extract (Clemens et al., 1998; Sriraman et al., 2003). Similarly, the rat and human sequences spanning the CCAAT sequence within B1 and the GC box were bound by Sp1 only when using rat granulosa cell extract and MCF7 cell extract (Park-Sarge and Sarge, 1995; Schultz et al., 2003; Tang et al., 2002). However, in the human PR gene, using human osteosarcoma SAOS2 cell extract, HOXA5 was found to bind a site that overlaps the CCAAT site in B1 (Raman et al., 2000). Mutation of the CCAAT site reduces cAMP-induced activity, but not the basal activity of this promoter (Park-Sarge and Sarge, 1995). However, we found that disruption of the CCAAT motif within the B1 site neither affected basal activity nor the response to LIP and c-Jun. Moreover, in the human PR gene in the MCF-7 cell line, mutation of the Sp1 binding site adjacent to the B1 CCAAT motif disrupts Sp1 complex formation and decreases transcription (Schultz et al., 2003). PR is clearly regulated in a tissue- and species-specific manner with different promoter elements and their cis-binding factors active in differing contexts. Presumably, this complexity allows regulation of PR expression under varying cellular and physiological conditions in different tissues, requiring the tissue- and cell type-specific interaction of multiple factors. Thus, the differential availability of specific factors determines the precise level and mechanism of PR regulation.

The introduction of the two mutated AP-1 binding sites (J1 and J2) into the 3.2kb tandem

PR promoter dramatically decreased the response of the larger promoter to LIP and c-Jun. This indicates that these two AP-1 sites are required for LIP and c-Jun induced transcription of both the PRB and PRA promoters. This is quite interesting because these two sites are proximal to the putative PRB promoter, yet can impact the activity of the downstream PRA promoter, suggesting that these AP-1 binding sites are critical elements of both the PRB and PRA promoters. There are no reports about AP-1 sites in minimal PRB promoter region (-117/+63) from any species. In human the PR promoter, an AP-1 site at +90bp is required for estrogen responsiveness of PR expression, but not for basal promoter activity (Petz et al., 2002). However, this site is not conserved in mouse and rat PR promoters.

We show a requirement for DNA binding in the synergistic transactivation function of LIP and c-Jun through A-C/EBP and A-Fos, dominant negative dimerization partners that prevent C/EBP and AP-1, respectively, from binding to DNA. Moreover, A-Fos alone can interfere with endogenous c-Jun activity to decrease basal promoter activity. However, A-C/EBP alone was unable to down-regulate activity of both tandem PR promoter and 2x-C/EBP promoter. This observation may be explained by inefficient formation of endogenous C/EBPβ and A-C/EBP heterodimers due to existing dimers are stable and C/EBPβ protein turnover is required to allow A-C/EBP entry into heterodimers. Indeed, formation of C/EBP dimmers through their leucine zipper domains leads to stabilize and prevent their degradation (Hattori et al., 2003).

Coupled with ChIP data on the mutant promoters that correlate decreased occupancy with decreased transactivation, these lines of evidence reveal that the synergistic effect of LIP and c-Jun requires DNA binding of both factors to the promoter; lack of either factor's binding blocks the transactivation of the promoter.

In a transient transfection assay with the minimal PRB promoter-reporter, we found that LIP and LAP2 synergize with c-Jun, while LAP1 does not. Moreover, LIP was the most active C/EBPβ isoform, inducing 30-fold activation with c-Jun, while LAP2 induced the promoter 15-fold with c-Jun. These results parallel the occupancy by these factors observed in a ChIP assay. Co-expression of LIP and c-Jun gave the highest level of promoter occupancy, LAP2 and c-Jun gave an intermediate level of occupancy, while LAP1 and c-Jun did not lead to a significant level of occupancy for either factor.

Although LIP lacks a transactivation domain and is generally regarded as a transdominant inhibitor of C/EBP activation, there are significant precedents for LIP having a positive transcriptional function. LIP can participate in LPS-induced transcription of the IL-6 promoter in a B lymphoblast cell line (Hu et al., 2000). It has also been ascribed unique properties in transcriptional activation and interactions apart from those of the other C/EBPβ isoforms.LIP specifically interacts with PRB to drive PRE-dependent promoters in endometrial stromal cells (Christian et al., 2002), and with Runx2 to promote osteoblast differentiation (Hata et al., 2005). LIP specifically up-regulates transcription of the CXCR4 gene through direct promoter binding (Park et al., 2013). It is also important for induction of CDH3 in breast cancer cells, where although all C/EBPβ isoforms activate the CDH3promoter, only LIP activation leads to increased protein levels (Albergaria et al., 2010, 2013). Moreover, since PRB and LIP are both up-regulated and co-localized in murine pregnancy, LIP is plausibly a major factor in driving PRB transcription during this stage.

APPENDIX

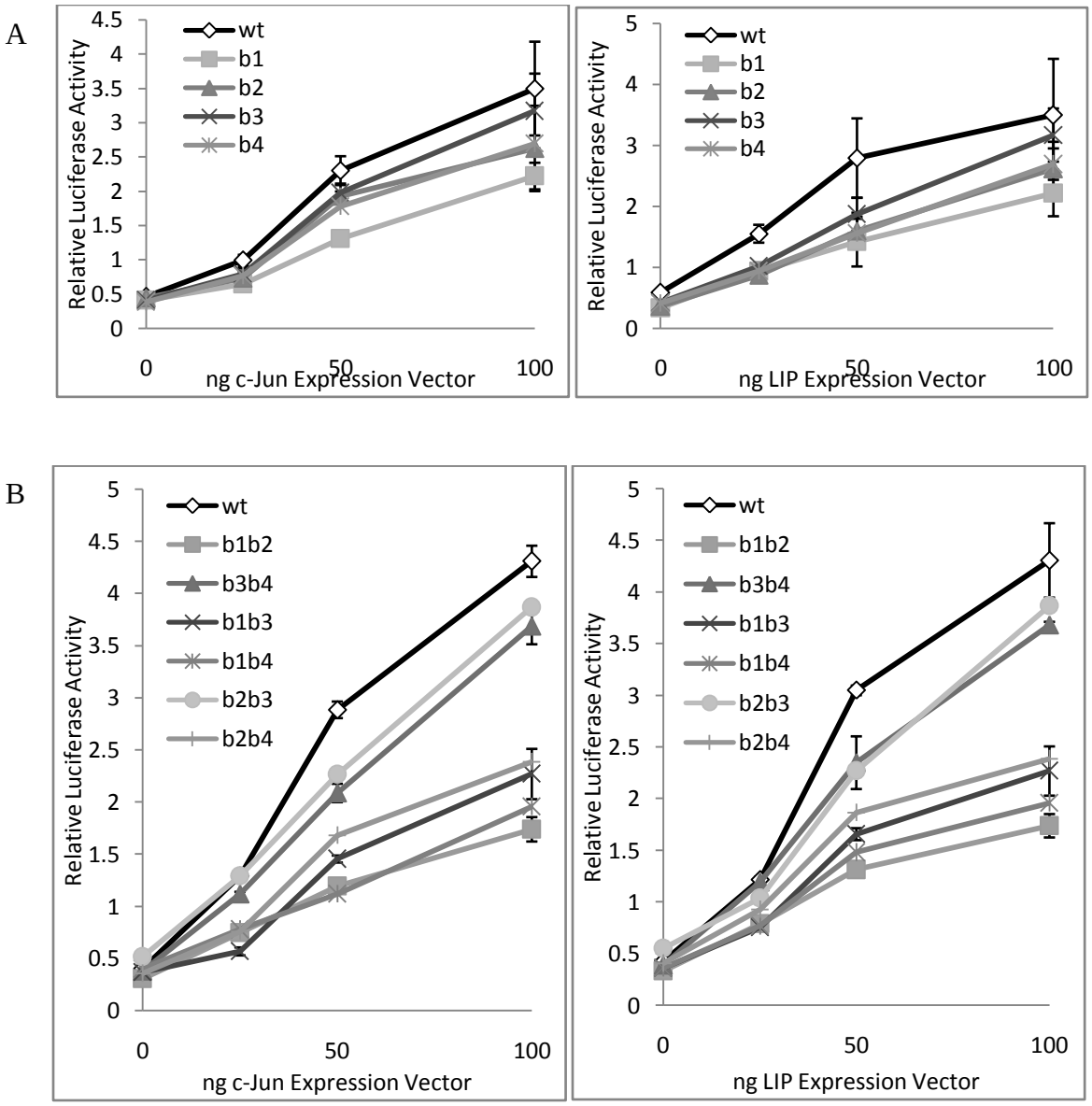


Figure 3S.1.The effects of mutation of C/EBP and AP-1 sites on the promoter activity.

mPRB with single (A) or double (B) C/EBP mutated site. MC7-L1 cells were transfected with 100ng of wildtype mPRB or reporter promoters carrying mutations of indicated sites, plus either an increasing amount (25, 50, 100ng) of c-Jun expression vector (as indicated) and 100ng of LIP expression vector (Left Sides) or vice versa (Right Sides) The normalized luciferase activity of pGL3 was subtracted from the normalized luciferase activity of each promoter. The value of promoter activity of each promoter was presented as the relative activity after subtraction. Results are the mean±S.E. from six (A and B) or three (C, D, and E) independent experiments; each experiment was performed in duplicate.

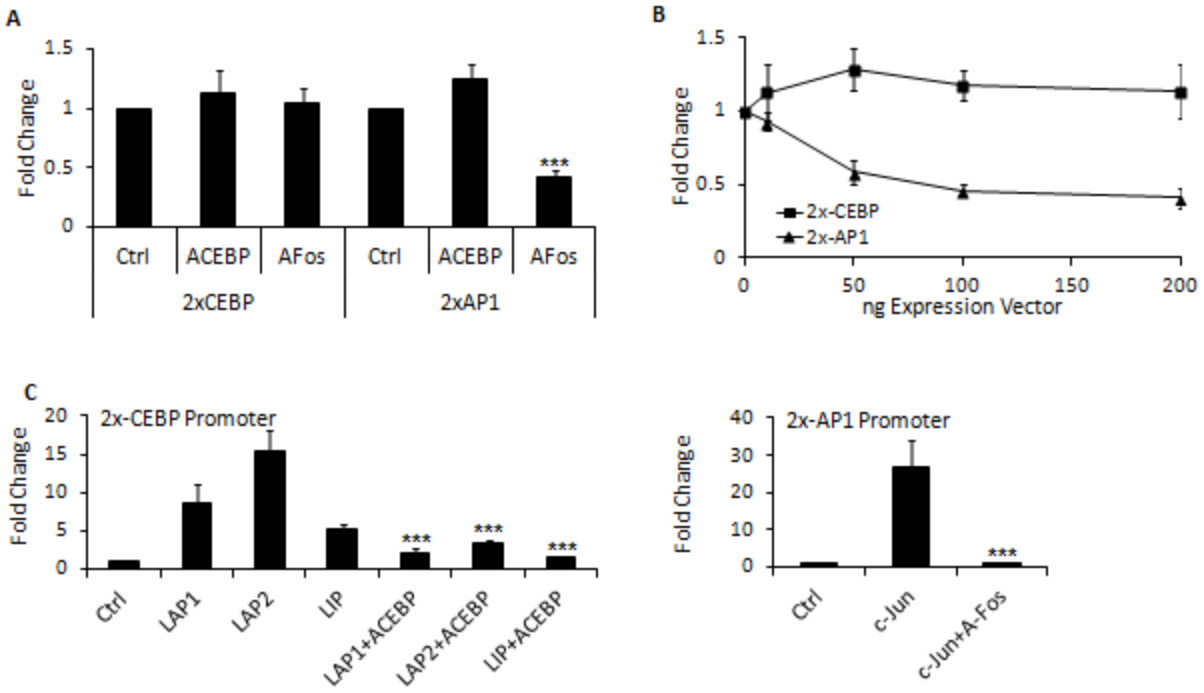


Figure 3S.2. The effects of A-CEBP and A-Fos on 2x-CEBP and 2x-AP-1 promoters.
(A) MC7-L1 cells were transfected with 100ng of promoter reporter (as indicated), and 100ng of either A-CEBP or A-Fos or control expression vector. The fold change in activity of promoter was presented as the ratio of its normalized luciferase activity to the control transfection, which was set as 1. (B) MC7-L1 cells were transfected with 100ng of either 2x-CEBP or 2x-AP-1 promoter reporters (as indicated), and an increasing amount of A-CEBP or A-Fos, respectively. The fold change in activity of promoter was presented as the ratio of its normalized luciferase activity to the control transfection, which was set as 1. (C) MC7-L1 cells were transfected with 100ng of either 2x-CEBP promoter and expression vectors for each C/EBPβ isoform in the absence or presence of A-CEBP (Left Side) or 2x-AP-1 promoter and expression vector for c-Jun in the presence or absence A-Fos (Right Side). The fold change in activity of each promoter was presented as the ratio of its normalized luciferase activity to the control transfection, which was set as 1.

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CHAPTER 4

CONCLUSION AND FUTURE RESEARCH

We demonstrated that C/EBPβ and c-Jun can transactivate PR promoter activity, both individually and in synergy. The synergy between C/EBPβ and c-Jun shows isoform specificity with LIP being particularly active with c-Jun, LAP2 less so, and LAP1 inactive. LIP has been found to be the most abundant isoform of C/EBPβ at pregnancy stage. However, c-Jun expression level and activity at this stage should be further investigated by using mammary gland extracts at pregnancy to perform western blots with antibody to c-Jun and EMSA with AP-1 probe.

Our current studies have used transient co-transfection of a PR promoter-reporter with expression vectors; a new experimental model in which C/EBPβ and c-Jun are overexpressed in a stable manner to better allow examination of regulation of the endogenous PR gene. C/EBPβ co-localizes with PRB in mammary epithelial cells at pregnancy, while it is mutually exclusive of PRA in the virgin gland. The PRB promoter was more responsive than the PRA promoter to transient co-expression of C/EBPβ and c-Jun. Moreover, differential function of each C/EBPβ isoform has been found in reporter assays with transfected PR promoter plasmids. LIP expression increases the ratio of PRB transcripts to total PR transcripts from the transfected tandem PR promoter-reporter. We will further confirm our current conclusions that LIP transactivates the PRB promoter by utilizing stable expression of each C/EBPβ isoform and c-Jun. This model will provide insight into the physiologic roles of C/EBPβ and c-Jun on activating specific endogenous PR isoform mRNAs and proteins.

Since we were unable to show binding of C/EBPβ and c-Jun on endogenous PR gene in transient transfection assay, where only a small portion of cells expressed the expression vectors, ChIP assays on the endogenous PR gene from stable transfected cells may be more successful for investigating the occupancy of each C/EBPβ isoform in the presence or absence of c-Jun, and vice versa, on promoter-specific PR gene regions. We expect to confirm the result observed with minmal PRB promoter model that LIP requires c-Jun for its binding, and vice versa.

Recent preliminary data showed that trichostatin (TSA), adeacetylase inhibitor, could enhance basal promoter activity (Figure 4.1.A). Moreover, overexpression of p300, an acetyltransferase, also increased LIP and c-Jun- mediated transactivation of PR promoter (Figure 4.1.B). We further study the involvement of p300 on PR gene regulation in the following

experiments. How p300 alone and in combination of LIP and/or can affect on PR promoter activity, and on endogenous PR expression. To evaluate if acetylation of C/EBPβ and/or c-Jun modulates their activity, acetylation level of C/EBPβ and/or c-Jun will be increased under overexpression of p300. However, if acetylation of these factors showed unaffected by p300, one explanation is that p300 would facilitate the recruitment of LIP and c-Jun to the promoter if enhanced occupancy of LIP and c-Jun on endogenous PR promoter will be confirmed in the presence of p300. The physical interaction of p300 will be determined by co-IP to further support for the hypothesis of transactivational complex formation among these three proteins. If we fail to show that the transactivational role of p300 is mediated through LIP and c-Jun, p300 may interact with other factors and induce an open chromatin state of PR promoter.

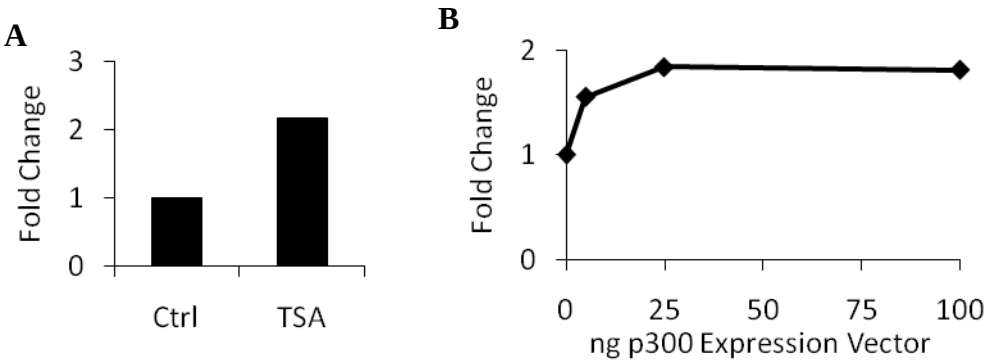

Figure 4.1. The effects of TSA and HAT on PR promoter activity. A) MC7-L1 cells were transfected with 100ng of tandem PR promoter in the presence or absence of TSA. The non-treated activity was normalized to a value of 1. B) MC7-L1 cells were co-transfected with 100ng of tandem PR promoter, 100ng of each expression vector for LIP and c-Jun, plus an increasing amount (5, 25, 100ng) of p300 expression vector. The promoter activity in the co-overexpression of LIP and c-Jun without p300 was set as 1.