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Progesterone Receptor Repression of Prolactin/Signal Transducer and Activator of Transcription 5-Mediated Transcription of the β-Casein Gene in Mammary Epithelial Cells

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Prolactin (PRL) and glucocorticoids act synergistically to stimulate transcription of the β-casein milk protein gene. Signal transducer and activator of transcription 5 (Stat5) mediates PRL-dependent *trans*-activation, and glucocorticoid potentiation occurs through cross talk between glucocorticoid receptor (GR) and Stat5 at the β-casein promoter. In the mouse, progesterone withdrawal leads to terminal differentiation and secretory activation of the mammary gland at parturition, indicating progesterone's role in repressing milk protein gene expression during pregnancy. To investigate the mechanism of the inhibitory action of progesterone, experiments were performed with cell culture systems reconstituted to express progesterone receptor (PR), the PRL receptor/Stat5 signaling pathway, and GR, enabling evaluation of PR, GR, and Stat5 interactions at the β-casein promoter. With COS-1, normal murine mammary gland, HC-11, and primary mammary epithelial cells, progestin-PR directly repressed the PRL receptor/Stat5a signaling pathway's mediation of PRL-induced β-casein transcription. Progestin-PR also inhibited glucocorticoid-GR enhancement of PRL induced *trans*-activation of β-casein. Inhibition depended on a functional PR DNA binding domain and specific PR-DNA interactions at the β-casein promoter. Chromatin immunoprecipitation assays in HC-11 cells revealed recruitment of PR and Stat5a to the β-casein promoter by progestin or PRL, respectively. Recruitment was disrupted by cotreatment with progestin and PRL, suggesting a mutual interference between activated PR and Stat5a. Without PRL, progestin-PR also recruited Stat5a to the β-casein promoter, suggesting that recruitment of an unactivated form of Stat5a may contribute to inhibition of β-casein by progesterone. These results define a negative cross talk between PR and Stat5a/GR that may contribute to the physiological role of progesterone to repress lactogenic hormone induction of the β-casein gene in the mammary gland during pregnancy. (*Molecular Endocrinology* 21: 106–125, 2007)

DEVELOPMENT OF THE mammary gland during pregnancy is under the control of progesterone and prolactin (PRL). Progesterone directly stimulates epithelial cell proliferation leading to extensive ductal side branching and alveolar morphogenesis. PRL is also required for alveolar development; however, it has both systemic effects, maintaining the corpus luteum required for progesterone production, and direct effects on development of alveoli (1–5). During pregnancy, progesterone has the additional function of inhibiting secretory activation and terminal differentiation until after parturition by inhibiting milk protein synthesis and tight junction closure; the latter is important for preventing reflux of accumulated milk into the interstitial space (5–10). In the mouse, a precipitous decline in circulating progesterone at parturition leads to increased milk protein production and tight junction closure (11–13). Progesterone receptor (PR) expression in the mouse mammary gland declines

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Abbreviations: C/EBPβ, CCAAT/enhancer-binding protein-β; ChIP, chromatin immunoprecipitation; CMV, cytomegalovirus; DBD, DNA-binding domain; DCC, dextran-coated charcoal; Dex, dexamethasone; DTT, dithiothreitol; EGF, epidermal growth factor; ER, estrogen receptor; FBS, fetal bovine serum; GR, glucocorticoid receptor; GRE, glucocorticoid response element; HMGB, chromatin high-mobility group protein; JAK2, Janus kinase 2; mAB, monoclonal antibody; MEC, mammary epithelial cell; MMTV, mouse mammary tumor virus; MOI, multiplicity of infection; NMuMG, normal murine mammary gland; pfu, plaque-forming unit; PR, progesterone receptor; PRE, progesterone response element; PRL, prolactin; PRLR, PRL receptor; PRLRL, long form of PRLR; S5RE, STAT5 response element; SDS, sodium dodecyl sulfate; STAT5, signal transducer and activator of transcription 5.

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progressively during pregnancy, suggesting that lowered levels of PR at parturition also contribute to secretory activation (14). However, a fall in circulating progesterone does not occur in all species. In humans, progesterone levels remain high during the onset of labor, and studies have suggested that PR function is inhibited at parturition through multiple molecular mechanisms (15–17). Thus release from inhibition by progesterone appears to be a common mechanism required for terminal differentiation of the mammary gland and, depending on the species, is due either to inhibition of PR function or to withdrawal of progesterone.

The primary hormone responsible for induction of milk protein gene expression is PRL with a strong potentiation effect by glucocorticoids (18–20). The most extensively studied milk protein gene is β-casein, and cell culture systems have defined the mechanisms of hormonal regulation. Induction of β-casein transcription by PRL is mediated by Stat5 (signal transducer and activator of transcription), a member of a transcription factor family that transmits signals from cytokine and growth factor receptors to nuclear target genes (21–23). PRL binding activates PRL receptor (PRLR) through dimerization and tyrosine phosphorylation by Janus kinase 2 (JAK2) (24, 25). Stat5 is recruited by activated PRLR and phosphorylated by JAK2 on a conserved tyrosine (Y694 or Y699, depending on species), resulting in its dimerization, translocation to the nucleus, and binding to a specific 9-bp palindromic consensus sequence (TTCnnnGAA). The β-casein promoter contains Stat5 binding sites, and PRL activation has been shown to be dependent upon Stat5 binding. Stat5 contains a carboxyl-terminal transcriptional activation domain that binds coactivators such as p300/cAMP reponse element-binding protein-binding protein) (26) and is phosphorylated on serine residues (27). Additionally, the corepressor, silencing mediator of retinoid and thyroid receptor, interacts with Stat5, and its dissociation has been suggested to be required for cytokine-induced activation of Stat5 (28). Of the two closely related Stat5 proteins, Stat5a has been demonstrated to be more important for PRL-dependent mammary gland development and lactation than Stat5b, which appears to be required for GH signaling (29, 30).

Mammary gland-specific expression of milk protein genes does not occur by the action of tissue-specific transcription factors, but rather through combinatorial interactions with composite response elements present in the promoter and upstream enhancer regions. Stat5 and other transcription factors that interact with the β-casein gene are not mammary gland specific; rather, they are expressed in a broad range of tissues (18, 19). The β-casein promoter contains closely spaced binding sites for Stat5, CCAAT/enhancer-binding protein-β (C/EBPβ), and YY-1 and has multiple half-glucocorticoid response elements (GREs) that are potential binding sites for glucocorticoid receptor (GR) (Fig. 1A). Cooperative interactions between these elements and their functional importance for tissue-specific gene activation by lactogenic hormones have been shown by site-directed mutagenesis studies and gene deletions in mice (31, 32). Although PRL alone can induce β-casein expression through Stat5, GR and C/EBPβ are not capable of inducing transcription in the absence of a Stat5. However, these two transcription factors strongly synergize with Stat5 to generate a much more robust induction than exhibited by PRL alone (31). Glucocorticoid potentiation has been reported to occur through multiple mechanisms. At high concentrations of GR, potentiation was shown to require interaction of GR with DNA-bound Stat5 and the presence of the N-terminal activation function domain 1, suggesting that GR functions as a Stat5 coactivator (33, 34). At low receptor concentrations, potentiation required integrity of the glucocorticoid response element (GRE) half-sites, suggesting that GR-DNA binding is also required (35, 36). Potentiation has also been reported to involve GR, promoting sustained tyrosine phosphorylation of Stat5a and stabilizing Stat5-DNA binding (37). In addition to positive *trans*-acting factors, the transcription factor YY1 (yin and yang), which has the capacity to act as a repressor or an activator, has been shown to repress β-casein expression in the absence of lactogenic hormone and to be dissociated from its binding site in response to PRL, indicating that induction of β-casein transcription also involves a relief from inhibition (31, 38, 39).

Progesterone receptor (PR), a member of the nuclear hormone receptor superfamily, contains a C-terminal ligand-binding domain, a DNA binding domain (DBD), and multiple transcriptional activation domains. In response to binding progesterone, PR is activated by dissociation from protein chaperones, dimerization, binding to specific palindromic progesterone response elements (PREs) in target genes, and recruitment of coactivators that enhance receptor-mediated transcription by promoting chromatin remodeling and assembly of a productive transcription complex (see review in Ref. 40). In addition to binding PREs, PR and other steroid receptors can regulate transcription through protein-protein interaction with other transcription factors either by tethering to another DNA-bound transcription factor, or by cooperative interactions between adjacent DNA sites (41–43). In most vertebrate species, PR is produced from a single gene as two receptor isoforms, N-terminally truncated PR-A and full-length PR-B (see review in Ref. 44). PR-B is generally a stronger activator of gene transcription than PR-A, whereas PR-A can act as a ligand-dependent repressor of PR-B and other steroid receptors including estrogen receptor (ER). Genetic targeting experiments in mice have also shown distinct biological roles for PR-A and PR-B *in vivo*. PR-A is important for mediating the actions of progesterone in the uterus and ovary, whereas PR-B is more important in the mammary gland (45, 46).

The suppressive effect of progesterone on milk protein gene expression was initially shown by experi-

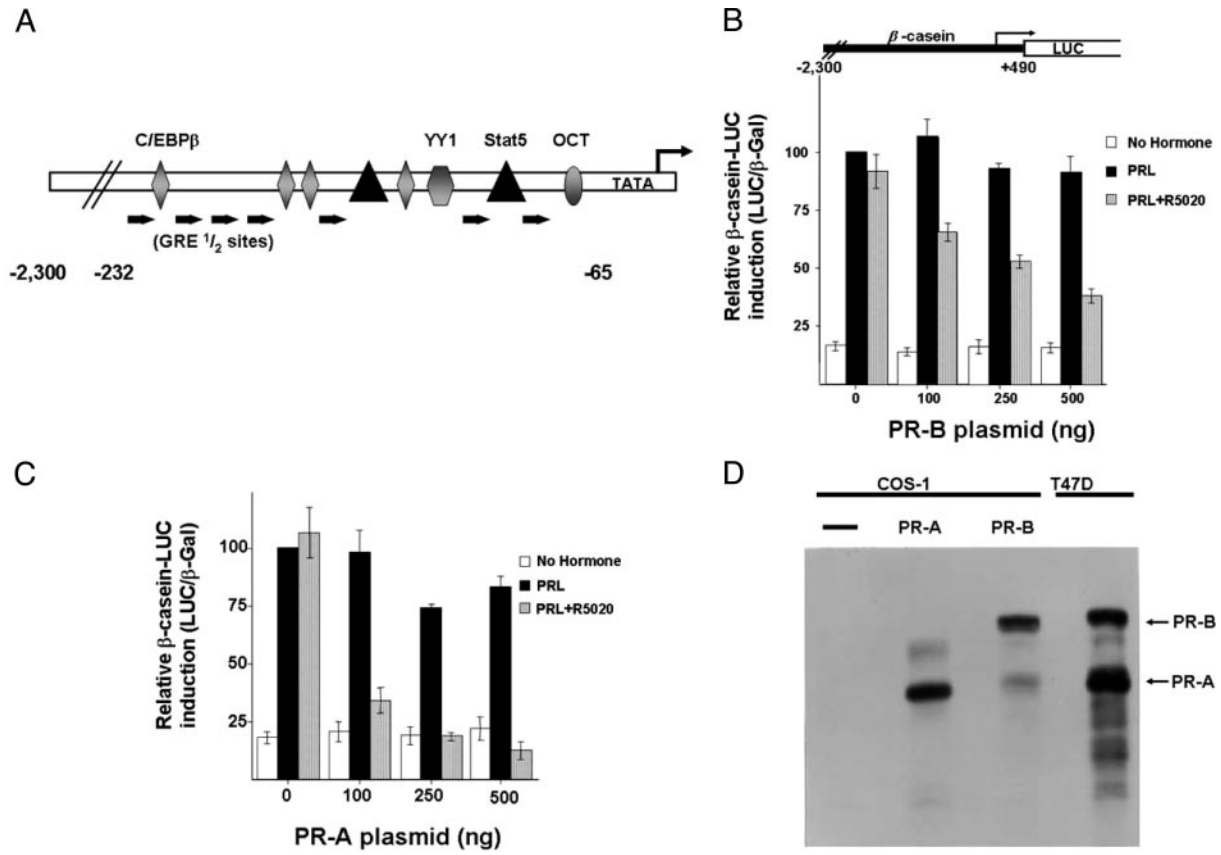


Fig. 1. Progestin-Dependent PR-Mediated Inhibition of PRL Induction of β-Casein Gene Activation in a Reconstituted Cell System

A, Schematic representation of the β-casein promoter with binding sites for different transcription factors. *Arrows* depict putative GRE half-sites, *diamonds* are C/EBPβ sites, *hexagons* are YY1 sites, *triangles* are Stat5 sites, and *ovals* are OCT-1 sites. B and C, COS-1 cells were cotransfected with a β-casein (−2300/+490)-LUC reporter gene and expression plasmids for rat PRLRL (100 ng), Stat5a (100 ng), and varying amounts (100–500 ng) of human PR-B (B) and human PR-A (C). At 24 h after transfection, cells were treated with ovine PRL (1 μg/ml) alone or in combination with R5020 (100 nM) for 24 h, and lysates were prepared and assayed for reporter gene activity. Luciferase activity corrected for variation in transfection efficiency with an internal CMV-β-galactosidase reporter was set to 100% for PRL treatment alone in the absence of PR, and all other data were calculated as luciferase activity relative to the 100% control. Values are averages ± SEM from four independent determinations. D, Levels of ectopically expressed human PR-A and PR-B proteins in COS-1 cells relative to endogenous PR in T47D breast cancer cells as detected by immunoblot with the anti-PR mAb, 1294. OCT, Octamer-binding transcription factor.

ments in which ovariectomy of pregnant animals resulted in transient lactogenesis and accumulation of β-casein mRNA and protein. This phenomenon was prevented by the administration of progesterone at the time of ovariectomy, but not by other hormones, including estrogen, PRL, or glucocorticoids (6–9). Experiments with organ cultures from mammary gland explants of pregnant mice similarly demonstrated that progesterone inhibited β-casein mRNA accumulation stimulated by PRL (47–49). The mechanism by which progesterone inhibits lactogenic hormone induction of β-casein gene expression is not well defined. Because PR is expressed in only a fraction of mammary epithelial cells (MECs) in late pregnancy, this suggests that some of the inhibitory effect may occur indirectly through progesterone regulation of paracrine factors that are capable of acting on PR-negative cells. For example, progesterone decreases the sensitivity of mammary epithelium to PRL during pregnancy by suppressing PRL up-regulation of the PRLR (50, 51). Progesterone has also been reported to induce a DNA binding activity, termed pregnancy-specific nuclear mammary factor, that has been proposed to act as a transrepressor of the β-casein gene promoter (52, 53).

To explore whether this suppressive function of progesterone involves direct cross talk of PR with GR and PRLR/Stat5 signaling pathways, we have examined interactions of PR, GR, and Stat5a at the β-casein promoter in different cell culture systems including transiently transfected COS-1, normal murine mammary gland (NmuMG), and HC-11 cell lines, and primary mouse MECs. Under all experimental conditions, PR in a progestin-dependent manner directly interfered with the PRLR/Stat5 signaling pathway and its ability to induce transcriptional activation of the β-casein gene. PR also inhibited the potentiation effect of

glucocorticoids-GR. Inhibitory activity was partially dependent on a functional PR DBD and binding of PR to GRE-half-sites in the β-casein promoter. Using a chromatin immunoprecipitation (ChIP) assay with HC-11 cells revealed that PR and Stat5 are recruited independently to the β-casein promoter in response to their cognate activating hormone, whereas assembly of PR and Stat5 was disrupted by cotreatment of cells with PRL and progestin. These results suggest that a mutual interference between PR and Stat5a at the β-casein promoter contributes to the repressive effect of progesterone on lactogenic hormone induction of β-casein. An additional sequestering mechanism was suggested by the finding that progestin in the absence of PRL, recruited unactivated Stat5a to the β-casein promoter. These data contribute to defining the combinatorial transcription factor interactions that mediate the repressive effect of progesterone on transcriptional activation of the β-casein gene.

RESULTS

Progesterone Inhibition of PRL Induction of β-Casein Gene Transcription Involves Progesterone Receptor (PR) Interference with PRLR/Stat5 Signaling

To examine whether progesterone and PR have a direct influence on PRL induction of β-casein gene transcription, a reconstitution cell cotransfection system was used. PRLR, Stat5a, and human PR were ectopically expressed in COS-1 cells that are deficient in these components of progesterone and PRL signaling, along with a β-casein-LUC reporter gene. The LUC reporter gene was linked to the −2300 to +490 promoter region of the rat β-casein gene that contains proximal Stat5 response elements (S5REs), multiple half-glucocorticoid response elements (GREs), and sites for various other transcription factors (Fig. 1A). PRL treatment resulted in a 6- to 7-fold induction of β-casein-LUC expression, and this was inhibited by the synthetic progestin R5020 in a manner dependent on expression of either PR-B or PR-A (Fig. 1, B and C). Furthermore, inhibition was dependent on the amount of PR transfected, with PR-A mediating a complete inhibition and PR-B mediating a reduction to approximately 35% that induced by PRL at the highest amount of transfected PR. This difference in PR isoforms was not due to a different level of protein expression, because PR-A and PR-B were expressed in comparable amounts as determined by immunoblotting (Fig. 1D). PRL induction was dependent on expression of Stat5a, indicating that progesterone inhibits the PRLR/JAK2/Stat5 signaling pathway (Fig. 2). β-Casein-LUC expression was not stimulated by progestin in the absence of PRL, whereas R5020, under the same cell conditions, stimulated induction of a PRE_2-tk-luciferase reporter containing a classical palindromic PRE (Fig. 2). These results, taken together, indicate that progesterone is capable of inhibiting PRL induction of β-casein gene transcription due to PR interfering with the PRL/Stat5 signaling pathway in a cell-autonomous fashion.

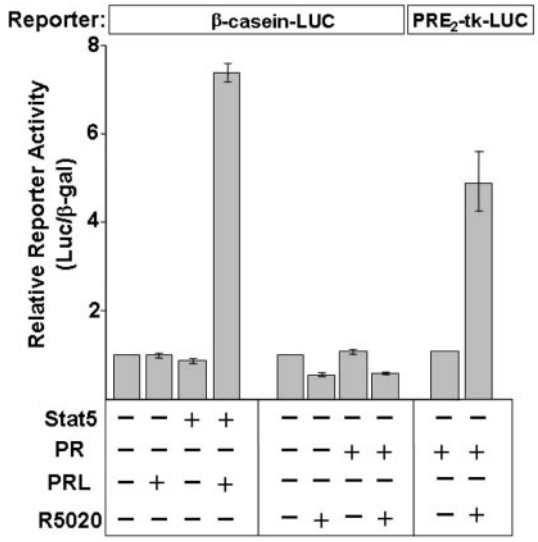


Fig. 2. PR Inhibits Stat5a-Dependent PRL Induction of β-Casein Gene Activation

COS-1 cells were cotransfected as in Fig. 1, with the β-casein (−2300/+490)-LUC reporter and expression plasmids for rat PRLRL, Stat5a, and human PR-B. Cells were treated with vehicle (ethanol), PRL (1 μg/ml), or R5020 (100 nM), and luciferase activity was determined and expressed as relative activity as in Fig. 1, B and C. Under the same transfection conditions, cells were also cotransfected with PRE2-tk-LUC and human PR-B. Values represent averages ± SEM from three independent experiments.

Progesterone Inhibits Glucocorticoid Potentiation of PRL Induction of β-Casein Gene Activation

To determine the influence of progesterone on glucocorticoid potentiation of PRL induction of β-casein gene transcription, COS-1 cells were transfected with the same plasmids as in Fig. 1 plus a vector for expression of GR. In confirmation of earlier reports (20, 33, 35), treatment with the synthetic glucocorticoid dexamethasone (Dex) alone did not stimulate β-casein-LUC expression, whereas Dex, in a GR-dependent manner, substantially enhanced PRL stimulation from an approximately 8-fold to about a 30-fold induction (Fig. 3). In cells transfected with PR-A, treatment with R5020 inhibited the synergistic induction stimulated by Dex and PRL to that of the basal promoter activity in the absence of hormone (Fig. 3). Thus, in addition to inhibiting activation of the PRL/Stat5 signaling pathway, PR interferes with glucocorticoid GR-dependent potentiation of PRL induction of β-casein gene activation.

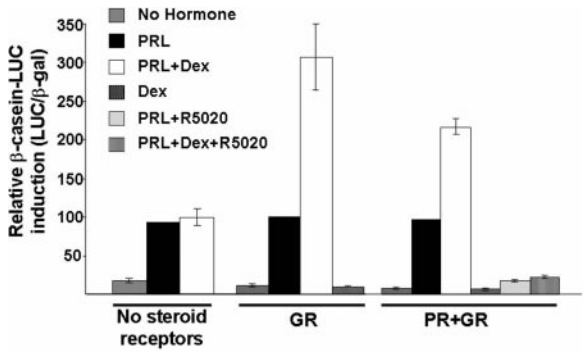


Fig. 3. PR Inhibits Glucocorticoid Potentiation of PRL-Induced Activation of β-Casein Gene Activation

COS-1 cells were cotransfected as in Fig. 1, B and C, with β-casein (−2300/+490)-Luc reporter, rat PRLRL, Stat5a, and human PR-A (500 ng) or GR (100 ng). At 24 h after transfection cells were treated another 24 h with ovine PRL (1 μg/ml), PRL (1 μg/ml) + Dex (100 nM), Dex (100 nM), PRL (1 μg/ml) + R5020 (100 nM) or PRL (1 μg/ml) + Dex (100 nM) + R5020 (100 nM). Luciferase was assayed and expressed as relative activity as in Fig. 1, B and C. Values are averages ± SEM from triplicate independent determinations.

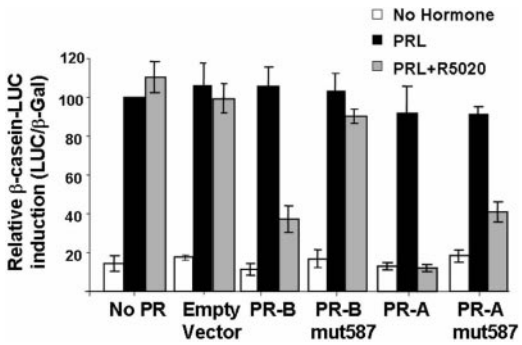


Fig. 4. Inhibition of PRL-Induced β-Casein-LUC Gene Activation Involves the DBD of PR

COS-1 cells were cotransfected as in Fig. 1 with β-casein (−2300/+490)-LUC reporter and expression plasmids for PRLRL, Stat5a, PR-A(500 ng), PR-B (500 ng), PR-Bmut587 (cys to ala) (500 ng), PR-Amut587 (cys to ala) (500 ng), or an empty vector. Cells were treated with vehicle, PRL (1 μg/ml), or PRL (1 μg/ml) + R5020 (100 nM), and luciferase was measured and expressed as relative activity to the 100% PRL-induced level in the absence of PR. The values are averages ± SEM from three independent determinations.

Inhibition of PRL Induction by Progesterone Involves PR Binding to GRE Half-Sites in the β-Casein Promoter

A cysteine residue (587) in the first zinc finger of the DBD that is critical for structural integrity of the DBD (40, 44) was mutated to an alanine in the context of PR-A and PR-B. This mutation was confirmed by EMSA to abolish specific high-affinity binding to a PRE DNA probe (data not shown). When transfected into mammalian cells, PR-A mut587 and PR-B mut587 were expressed as intact proteins at the same level as wild-type PR, indicating that these mutant PRs are not intrinsically unstable in cells (data not shown). In COS-1 reconstitution experiments, R5020 failed to inhibit PRL-induced stimulation of β-casein-LUC expression in cells expressing PR-B mut587, whereas progestin inhibition was significantly reduced but not eliminated in cells expressing PR-A mut587 (Fig. 4). These results suggest that a functional DBD is required for PR-B to mediate this inhibitory activity of progesterone, whereas DNA-dependent and DNA-independent pathways appear to be involved in inhibition mediated by PR-A.

Although the β-casein promoter does not contain a palindromic GRE, it does have GRE half-sites flanking either side of the S5RE (see Fig. 1A). Because PR and GR can recognize the same or similar DNA sequences (40, 44), we next determined whether PR can bind to the GRE half-sites in the β-casein promoter. EMSAs were performed with purified PR using an oligonucleotide probe corresponding to −110 to −70 of the rat β-casein promoter that contains the S5RE and 5′- and 3′-flanking GRE half-sites. Human PR was expressed in the baculovirus insect system, activated by binding to R5020 and purified to more than 90%, as described in *Materials and Methods*. Stat5a was coexpressed as a recombinant full-length protein in the baculovirus system, along with JAK2 kinase to phosphorylate and activate Stat5a and was partially purified by heparin sepharose as previously described (54). Previous studies in our laboratory have shown that the chromatin high-mobility group proteins-1 and -2 (HMGB-1/-2), facilitate binding of PR to specific target DNAs, particularly weak divergent elements such as half-hormone response elements. HMGB-1/-2 are not stable components of the enhanced PR-DNA complex and dissociate under EMSA conditions (55, 56). As shown by the EMSA in Fig. 5, PR in the presence of HMGB-1 bound specifically to the β-casein DNA probe in a dose-dependent manner. The presence of PR in the DNA complex was shown by supershift with a PR-specific antibody (Fig. 5A), and specificity of binding was determined by competition with excess unlabeled β-casein promoter DNA probe whereas an unrelated DNA probe failed to compete (data not shown). To localize the site(s) of PR binding, wild-type and mutant forms of the β-casein probe were analyzed by EMSAs (Fig. 5, B and C). PR binding was severely diminished by substitution mutations in both the 5′- and 3′-GRE half-sites flanking the Stat5 response element. Of the two half-sites, the 5′-site was more important. Mutations in the 3′-half-site alone had little effect on PR binding, whereas 5′-half-site mutations reduced PR binding the same as mutations in both 5′- and 3′-half-sites (Fig. 5, B and C). Nucleotide substitutions in the S5RE did not alter PR binding, whereas Stat5a binding, as expected, was dependent on the integrity of the S5RE (Fig. 5, B and C). These results show that PR binds to GRE half-sites with a preference for the 5′-site.

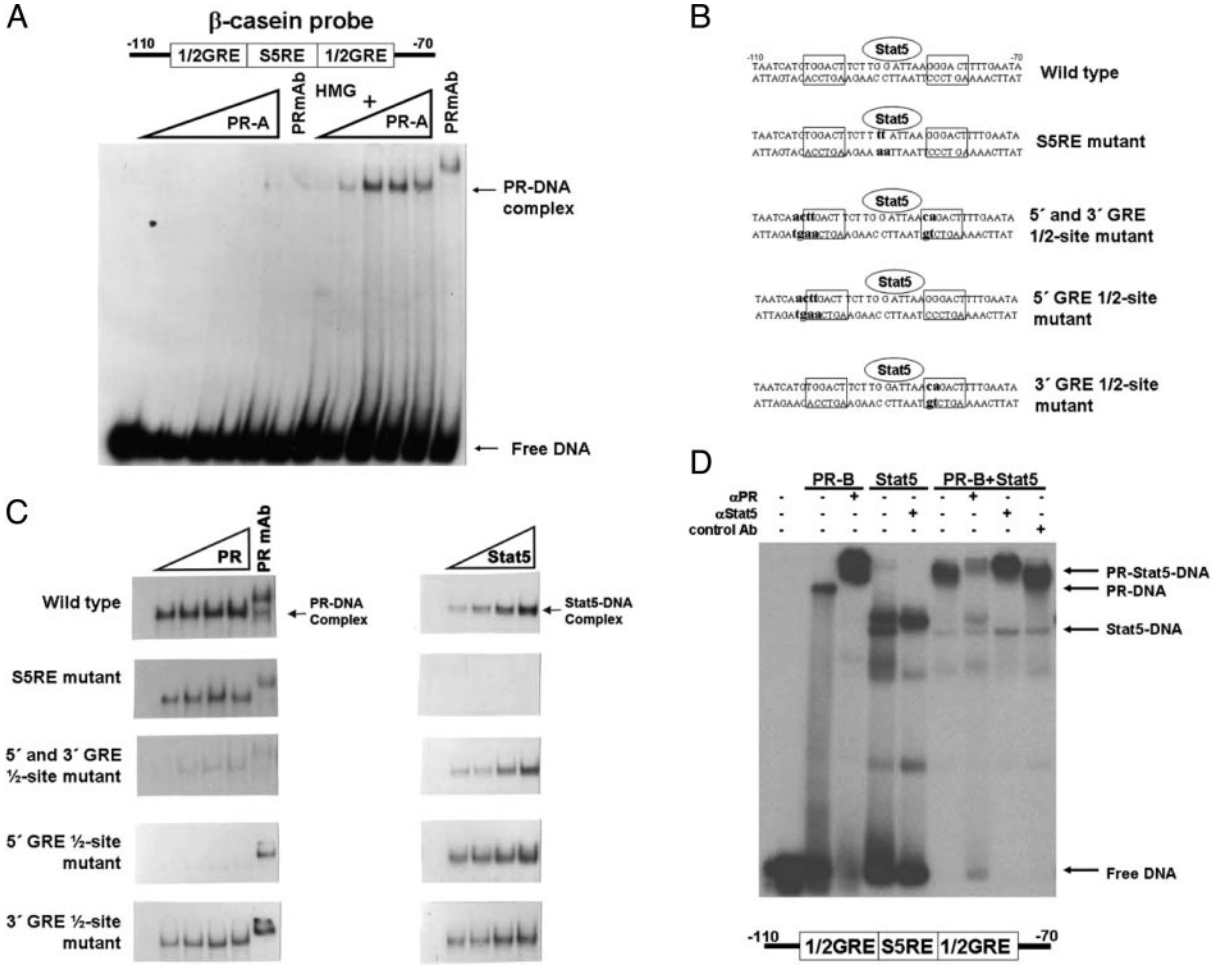


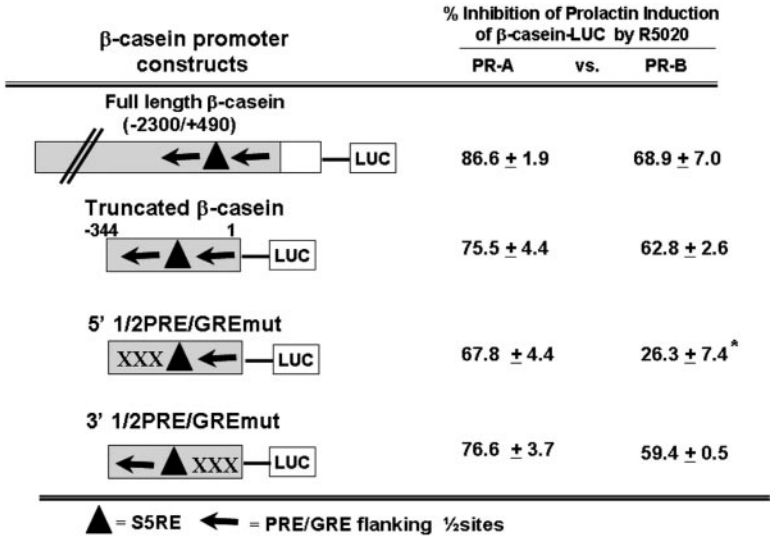
Fig. 5. PR Binds to GRE Half-Sites in the Proximal β-Casein Gene Promoter

A, EMSA of PR binding to a double-stranded oligonucleotide probe corresponding to −110 to −70 bp of the rat β-casein promoter containing the S5RE and 3′- and 5′-flanking GRE half-sites. Varying concentrations (10–200 nM) of purified baculovirus-expressed PR-A (bound to R5020) were reacted with a limiting concentration of ^{32}P-labeled DNA probe (0.3 ng) in the absence and presence of HMGB-2 (500 ng). Free and bound DNA complexes were separated by electrophoresis on native 5% polyacrylamide gels and detected by autoradiography. Supershift of the complex with PR-specific antibody (1294) is shown in the *far right lane*. B, Schematic of double-stranded oligonucleotide ^{32}P-labeled β-casein promoter probes containing point mutations in either the Stat5 response element or 3′- and 5′-GRE half-sites. Mutations are indicated by *bold letters*. C, EMSAs of purified Stat5 and PR binding to the ^{32}P-labeled DNA probes shown in panel B above. Only regions of gels with upshifted DNA complexes are shown; the free DNA has been cut off. *Left panels*, Varying concentrations (25–100 nM) of PR were reacted with a limiting concentration (0.3 ng) of ^{32}P-labeled DNA probes. Supershift of PR-DNA complexes with PR-specific antibody (1294) is in the far right lane. *Right panels*, Varying concentrations (500 ng to 1.25μg) of Stat5a were reacted with a limiting concentration of ^{32}P-labeled DNA probe. D, EMSA of PR-B and Stat5a binding individually and together as a ternary complex with a DNA probe containing the β-casein promoter sequence (−110 to −70). The ^{32}P-labeled DNA probe (0.3 ng) was reacted with PR-B (75 nM) alone without and with a PR-specific antibody (1294), with Stat5a (200 ng) alone without and with a Stat5a-specific antibody (mouse monoclonal) or a mixture of PR-B (75 nM) and Stat5a (200 ng), without and with antibodies to PR (1294), Stat5a, or a control unrelated antibody. Ab, Antibody.

The close positioning of the S5RE and the 5′-GRE half-site raises the possibility that PR could act by interference with Stat5a binding to its site in the β-casein promoter. To test this, EMSAs were performed with a mixture of PR and Stat5a in addition to PR or Stat5a alone. The mobility of the binary Stat5/DNA complex is distinct from that of the slower mobility PR-B/β-casein DNA complex (Fig. 5D). PR-B and Stat5, together in the same reaction, generated a higher mobility complex than either protein-DNA complex alone, and this new complex was supershifted by a PR-specific (1294) or a Stat5-specific (L-20) antibody, but not by an unrelated antibody control (Fig. 5D). These results indicate that PR and Stat5 are able to directly cobind as purified proteins *in vitro* with a β-casein promoter DNA probe.

To map the region of the promoter required for the inhibitory effect of progesterone, a LUC reporter was

Table 1. Progesterone Repression of β-Casein is Partially Dependent on the 5′ GRE Half-Site within the Proximal Promoter



COS-1 cells were transfected with plasmids for PRLR, Stat5a, and PR along with the β -casein promoter reporters: wild type (−2300/+490), truncated (−344 to +1), truncated (−344 to +1) containing mutant 5′ GRE half-sites, or mutant 3′ GRE half-site. Cells were treated for 24 h with PRL (1 μg/ml) or PRL + R5020 (100 nM). Luciferase was determined and normalized as described, and results are presented as activity relative to prolactin induction set at 100%. The values are averages ± SEM from three determinations. The percent inhibition of the 5′ PRE/GREmut mediated by PR-B(*) was statistically significant as determined by a Student's *t* test, $P < 0.5$.

used that contains a truncated β-casein promoter from −344 to +1 bp. In the COS-1 cell reconstitution assays, the proximal promoter between −344 to +1 was sufficient for full PRL induction and for mediating inhibition by progesterone (Table 1). Mutations in the 3′-GRE half-site had minimal influence on R5020 inhibition of PRL induction, whereas mutations in the 5′-GRE half-site significantly diminished the inhibitory effect of R5020 mediated by PR-B, but had minimal effect on PR-A-mediated inhibition (Table 1). These functional results correlate with *in vitro* DNA results, supporting the conclusion that PR binding to the 5′-GRE half-site is important for the inhibitory effect of progesterone on PRL induction of β-casein gene transcription, whereas the PR-A isoform requires an additional DNA-independent mechanism.

Inhibition of PRL/Stat5 Signaling at the β-Casein Promoter Is a General Property That Extends to Mouse PR in Mammary Epithelial-Derived Cells

The experiments described above were performed with human PR. To determine whether this inhibitory action is a more general characteristic of PR and occurs in MECs, we have performed similar reconstitution experiments with mouse PR in both COS-1 and NMuMG cells. NMuMG cells are immortalized epithelial cells derived from normal murine mammary gland (57). Because of a general lack of constructs for mouse PR, these experiments and others described below required construction of vectors for expression of mouse (ms)PR-A and msPR-B in mammalian cells, recombinant adenovirus vectors, and baculovirus vectors for production of mouse PR in insect cells. Using purified baculovirus-produced mouse PR as an antigen, rabbit polyclonal antibodies were generated for use in immunoblot detection of mouse PR. When mouse PR was expressed in NMuMG cell reconstitution experiments, a significant progestin-dependent inhibition of PRL induction of β-casein-LUC was detected. This effect was dependent on the amount of msPR transfected and gave a maximal inhibition of 60% with msPR-B, and 55% inhibition with mouse PR-A (Fig. 6). Inhibition was comparable to that mediated by human PR under the same conditions in NMuMG cells, except that the larger inhibition obtained with human PR-A (70%) *vs.* human PR-B (55%) was not observed with the mouse PR isoforms (Fig. 6). Mouse PR isoforms expressed in COS-1 cells mediated similar results to that in NMuMG cells (data not shown). The 100% inhibition mediated by human PR-A in COS-1 cells was not observed with mouse PR-A in either COS-1 or NMuMG cells. Nonetheless, the progesterone-dependent inhibition mediated by either isoform of mouse PR was substantial and experimentally significant. These results show that progestin inhibition of PRL induction of β-casein gene transcription is a general property of progesterone receptors that extends to mouse PR and cells of mammary epithelial origin.

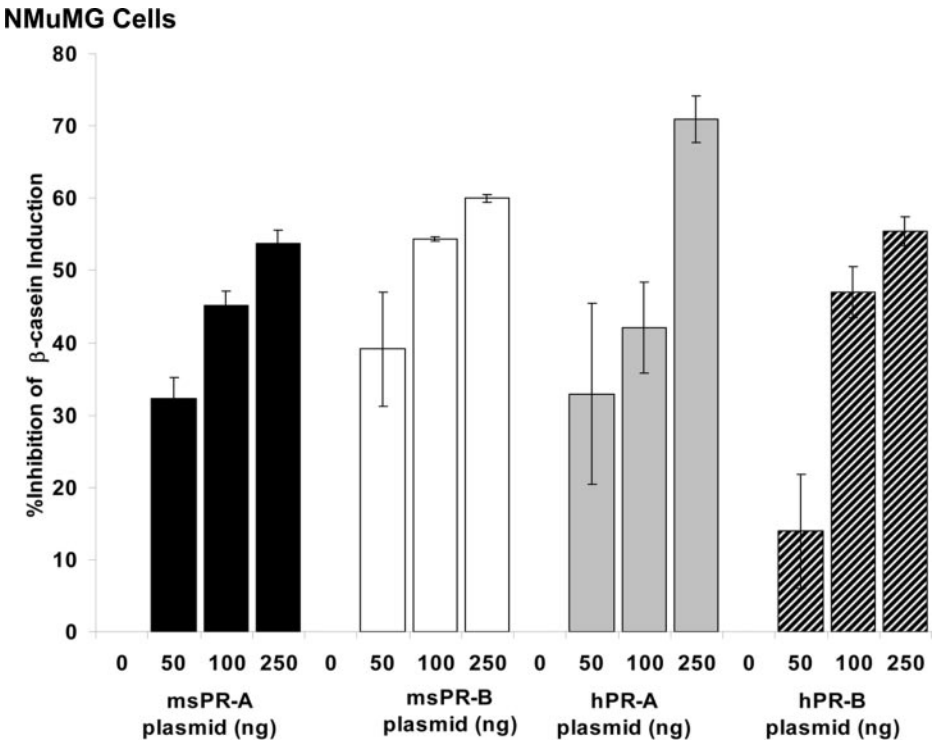


Fig. 6. Both Mouse and Human PR Mediate Inhibition of PRL Induction of β-Casein Gene Transcription in Mammary Epithelial-Derived Cells

NMuMG cells were cotransfected with rat PRLR (100 ng), Stat5a (5 ng), β-casein luciferase (200 ng), and varying amounts (as indicated in figure) of mouse or human PR-A and PR-B expression vectors. At 24 h post transfection, cells were treated for another 24 h with ovine PRL (1 μg/ml), or PRL (1 μg/ml) + R5020 (10 nM), and luciferase activity was measured. The data were calculated as percent inhibition of PRL-induced luciferase activity by R5020, and the values represent averages ± SEM from three independent determinations. ms, Mouse; h, human.

PR-Dependent Inhibition of PRL/Glucocorticoid Induction of the Endogenous β-Casein Gene in Primary MEC Cultures

A primary MEC culture system was developed to determine whether PR can mediate progesterone-dependent inhibition of PRL/glucocorticoid induction of the endogenous β-casein gene. Primary MECs typically lose expression of steroid receptor; thus PR was introduced by transduction with recombinant adenovirus vectors using a protocol adopted from Watkin *et al.* (58). Mammary glands were excised from midpregnant C57/BL6 mice, and epithelial cells were enriched by treatment with 0.3% collagenase followed by plating and growth for 48 h as a monolayer on a collagen I matrix. Cells were then harvested and incubated in suspension culture for 1 h at 37 C with recombinant adenovirus vectors encoding mouse PR-A, mouse PR-B, or a Lac Z control at a multiplicity of infection (MOI) of 50 plaque-forming units (pfu) per cell. Cells were then replated and grown on Matrigel for 2 d. Differentiated cells that grew out on Matrigel were then treated for 2 d with hormones, and cell lysates were analyzed by immunoblot for mouse PR and β-casein protein. As shown in Fig. 7A, there is little evidence of endogenous mouse PR in cells transduced with control adenovirus encoding Lac Z, or in uninfected cells (data not shown). Mouse PR-A and PR-B are present in approximately equal amounts in adenovirus transduced cells, and as evidence that expressed PR is functional, treatment with R5020 resulted in an upshift in electrophoretic mobility of PR-A and PR-B (Fig. 7A). The upshift is due to increased phosphorylation of PR in response to binding hormone and correlates with hormone-induced activation of receptors (59). No β-casein was detected in the absence of hormone, whereas treatment with PRL and glucocorticoid induced a robust expression of β-casein protein (Fig. 7B). In the absence of PR (Lac Z vector control), R5020 had no effect on PRL/glucocorticoid induction, whereas as in the presence of mouse PR, a substantial R5020-dependent inhibition of induction of β-casein expression was observed (Fig. 7, B and C). Similar results were obtained in MECs expressing mouse PR-A (Fig. 7B) or PR-B (Fig. 7C). Quantitation of immunoblot data from replicate experiments is represented in Fig. 7C and shows an approximately 65% inhibition of β-casein protein induction mediated by either isoform of mouse PR. Thus, results of transfection experiments with β-casein reporter genes were recapitulated in primary MECs with the endogenous

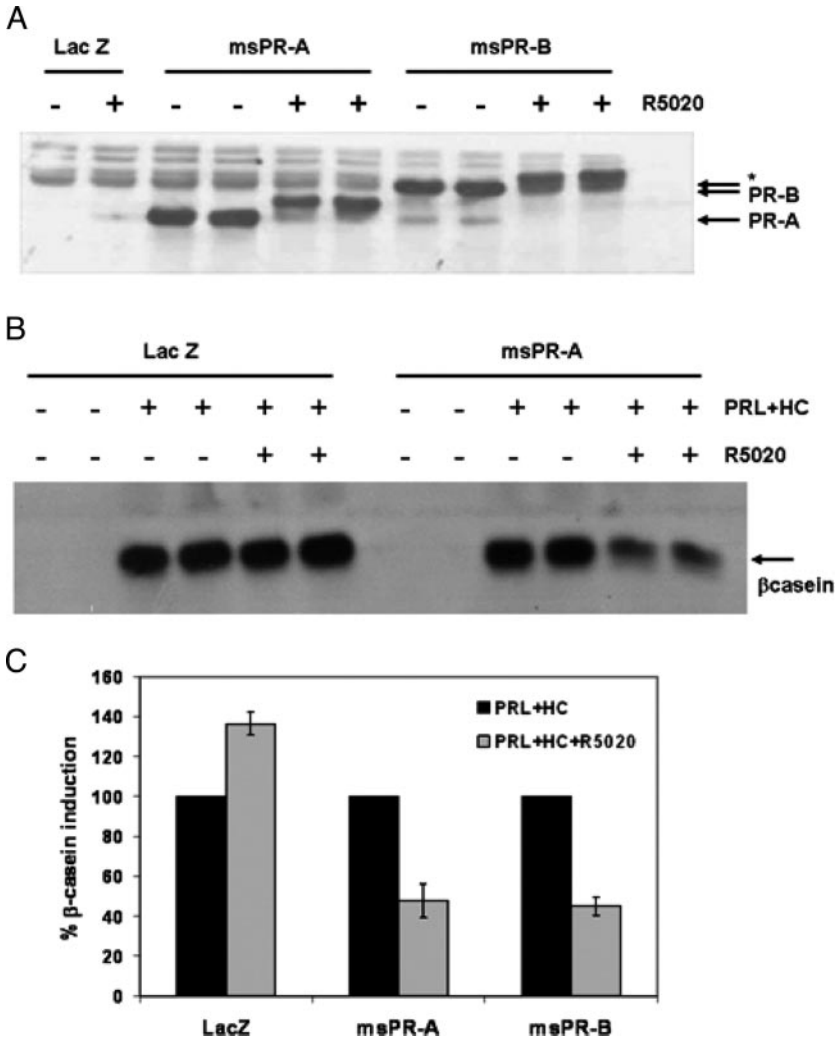


Fig. 7. Progestin Inhibits PRL Induction of the Endogenous β-Casein Gene in Primary Mouse MECs

A, Western blot of msPR expressed in primary MECs. MECs were grown on collagen for 2 d before resuspension and were transduced for 1 h at 37 C with recombinant adenoviruses encoding LacZ, mouse PR-A, or mouse PR-B. Cells were then replated on a thin layer of matrigel and treated with 100 nM R5020 or EtOH (− control) for 48 h. MECs were harvested and PR protein was assayed by immunoblotting using a polyclonal antibody to mouse PR. *, Nonspecific band. B, Primary mouse MECs were transduced as in panel A above and treated for 48 h with either EtOH (− control), ovine PRL (3 μg/ml), or ovine PRL (3 μg/ml) + R5020 (100 nM). Cells were harvested and β-casein protein expression was assayed by immunoblotting using a polyclonal antibody to mouse β-casein. C, Quantitation of β-casein protein expression. Immunoblots of β-casein protein expression were scanned with a Storm phosphorimaging system and data were analyzed using ImageQuant 5.0 software. Data were calculated as the percent intensity of β-casein protein bands in the presence of PRL and R5020 to that in the presence of PRL alone,which was set to 100%. Ratios were calculated separately for each treatment group: LacZ, mouse PR-A, and mouse PR-B. The values are averages ± SEM from three independent determinations. ms, Mouse; HC, hydrocortisone.

β-casein gene and the PRLR/Stat5a signaling pathway.

ChIP Detection of PR and Stat5a Recruitment to the β-Casein Promoter and Disruption of Assembly by Treatment with Both PRL and Progestin

HC-11 cells derived from mouse mammary epithelium are an immortalized cell line that retains many of the characteristics of normal differentiated mammary epithelium, including PRL induction of β-casein gene expression when grown in the presence of epidermal growth factor (EGF), insulin, and glucocorticoids (60). To create a convenient cell line to measure hormone effects on β-casein transcription, we stably transfected HC-11 cells with a β-casein-luciferase reporter gene that contains the same −2300-bp promoter used in transient transfection assays (Fig. 1). Subclones of HC-11 with stably integrated β casein-luc were selected that exhibited robust PRL/glucocorticoid induction, and one of these lines (HC-11.7E) was used for

ChIP assay. Because HC-11 cells do not express PR, receptors were introduced as above by transduction with recombinant adenoviral vectors for 48 h, but using human PR-B instead of msPR, because of the more efficient human PR-specific antibody (1294) for ChIP assay. PRL in the presence of glucocorticords induced β-casein-LUC expression in HC-11.7E cells (Fig. 8A). In cells transduced to express PR (PR-B), R5020 treatment inhibited lactogenic hormone induction in a PR dose-dependent manner, resulting in an approximately 75% reduction at the highest amount of transduced PR (Fig. 8A). Thus, similar to COS-1, NmuMG, and primary MECs, a progestin-PR-dependent inhibition of β-casein induction was observed in HC-11 cells.

For ChIP assays, HC-11.7E cells transduced to express PR-B were treated for 1 h with either R5020, PRL, or both hormones together. Immunoprecipitation

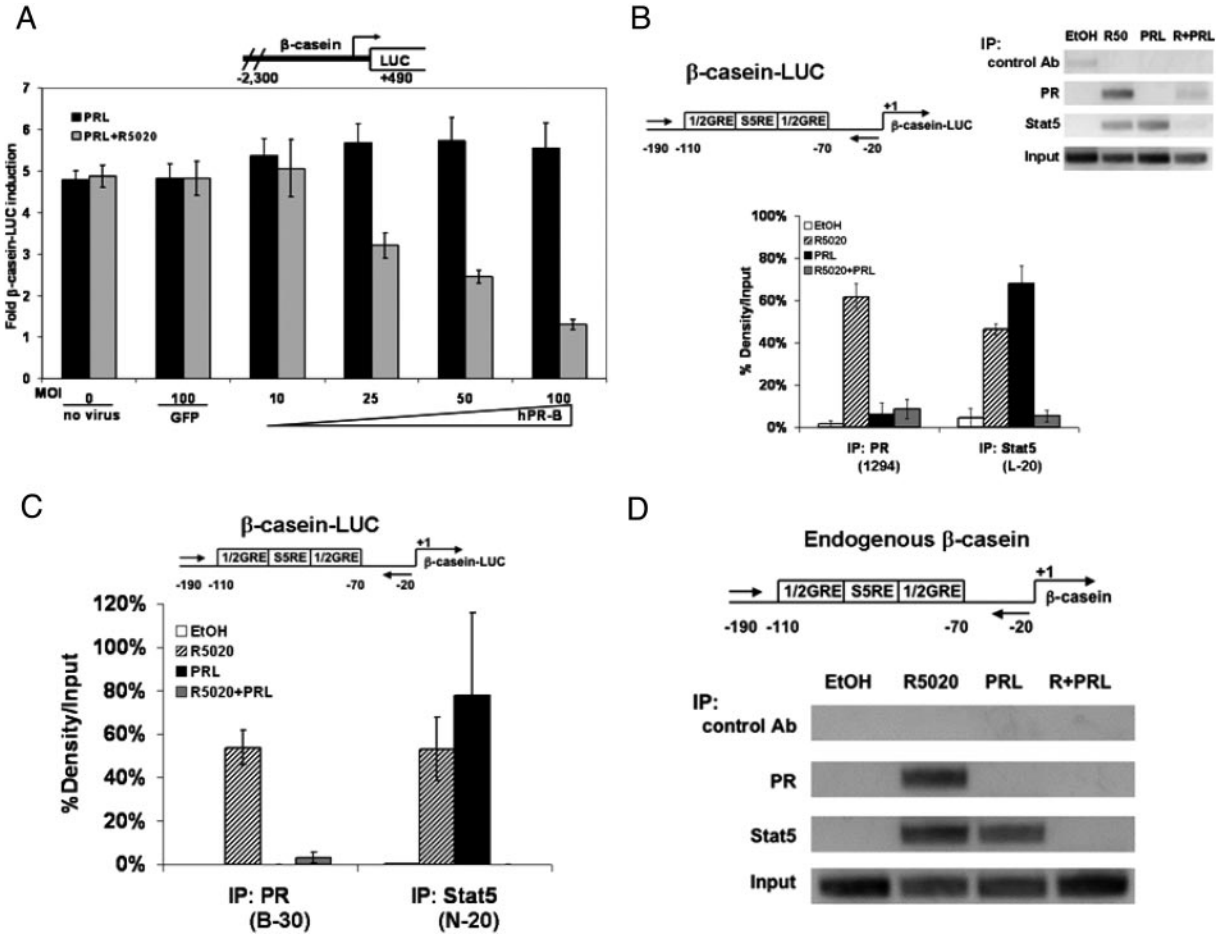


Fig. 8. Recruitment of PR and Stat5a to the β-Casein Promoter in HC-11 Cells Detected by ChIP Assay Was Disrupted by the Presence of Both Hormones, Progestin and PRL

A, HC-11 cells stably transfected with the β-casein (−2300/+490)-LUC reporter construct (HC-11.7E) were transduced for 24 h with an adenovirus expression vector for human PR-B (MOI of 50 pfu/cell). Cells were then incubated for another 24 h with EtOH (− control), ovine PRL (3 μg/ml), or ovine PRL (3 μg/ml) + R5020 (100 nM), and luciferase was measured and data expressed as fold β-casein induction. Values are average determinations ± SEM from triplicate indepdendent determinations. B, HC-11.7E cells were transduced with 50 pfu/cell of human PR-B encoding adenovirus and then treated for 1 h with either EtOH (− control), ovine PRL (3 μg/ml), R5020 (100 nM), or ovine PRL (3 μg/ml) + R5020 (100 nM). Cells were then harvested for ChIP assay using PCR primers flanking the S5RE and the two GRE half-sites within the proximal promoter of β-casein. *Upper left*, Schematic of the primers for ChIP assay (*upper right*), representative agarose gel of PCR products of input DNA, and from immunoprecipitation by control antibody, PR mAB (1294), or Stat5 polyclonal antibody (L-20). *Lower panel*, Quantification of ChIP assays from multiple experiments using Syngene Genetools software. Data represent average density of immunoprecipitations as a percentage of total input DNA adjusted after subtracting signals obtained with control antibody. The values are averages ± SEM from three determinations. C, ChIP assay described in panel B was repeated with a second set of PR (B-30) and Stat5 (N-20) antibodies. The values are averages ± SEM from three determinations. D, ChIP assay, as described in panel B above with 1294 PR antibody and L-20 Stat5a antibody, was performed with endogenous β-casein gene in parental nontransfected HC-11 cells. The agarose gel is representative of three independent experiments. Ab, Antibody; IP, immunoprecipitation; GFP, green fluorescent protein; R, R5020.

of cross-linked DNA fragments was performed with a PR-specific monoclonal antibody (mAb) that interacts with the N-terminal domain common to PR-A and PR-B (1294), a Stat5a-specific antibody (L-20) that interacts with the C-terminal domain, and a control unrelated antibody. After reversal of cross-links, immunoprecipitated DNA fragments were amplified by PCR with primers that span the proximal β-casein promoter between −190 to −20 containing the Stat5 response element and flanking half-GREs (Fig. 8B). As anticipated, little to no Stat5a or PR was assembled at the β-casein proximal promoter in the absence of hormone whereas treatment with PRL stimulated recruitment of Stat5a, and treatment with R5020 induced recruitment of PR (Fig. 8B). Unexpectedly, R5020 treatment also recruited a significant amount of Stat5a in the absence of PRL, whereas the converse was not true. PRL had no effect on PR interaction with the β-casein promoter (Fig. 8B). Cotreatment of cells with PRL and R5020 resulted in a complete loss of recruitment of PR and Stat5a that occurred in the presence of either R5020 or PRL alone (Fig. 8B). To determine whether lack of detection of PR and Stat5 interaction with the β-casein promoter in the presence of both hormones (PRL and progestin) could be due to a change in conformation of the proteins and occlusion of antibody-binding sites, the ChIP experiment was repeated with a different set of antibodies including B-30 mAb to the unique N-terminal extended region of PR-B and N-20, which interacts with the N-terminal domain of Stat5a. Similar results were obtained with this set of antibodies; PR and Stat5a were recruited to the β-casein promoter in response to treatment with their cognate activating hormone, whereas no interaction of PR or Stat5a was detected in the presence of both PRL and R5020 (Fig. 8C). Recruitment of Stat5a by R5020 in the absence of PRL was also detected with a different Stat5 antibody (Fig. 8C). The same ChIP results were obtained with the endogenous β-casein gene in nontransfected HC-11 cells (Fig. 8D), indicating that stably transfected HC-11 is a relevant experimental system. These ChIP results indicate that PR and Stat5a can assemble independently at the β-casein promoter in response to their cognate activating hormones and that lack of detection of PR and Stat5a interaction in the presence of both PRL and progestin is most likely due to disassembly of the PR/Stat5a complex. Thus in intact cell nuclei with chromatin-integrated β-casein, it appears that progesterone inhibition of PRL induction of gene transcription involves disassembly of the Stat5a complex at the promoter through a mutual interference with PR.

Progestin-PR Dependent Recruitment of Stat5a to the β-Casein Promoter in the Absence of PRL

To further investigate the mechanism by which progestin promotes recruitment of Stat5a to the β-casein promoter in the absence of PRL, we asked whether Stat5a was recruited by PR to a PRE-controlled MMTV target gene that lacks Stat5 response elements. As expected, ChIP assay with T47D breast cancer cells containing a stably integrated MMTV gene (61) detected recruitment of PR to the promoter in response to treatment of cells for 1 h with R5020. No recruitment of Stat5a was detected in response to either PRL or R5020, suggesting that progestin-PR recruitment of Stat5 is promoter context dependent, requiring a composite PR/Stat5 binding site (Fig. 9).

Treatment of cells with R5020 failed to stimulate tyrosine phosphorylation of Stat5a as detected by immunoblotting with a Stat5a tyrosine Y694/699-specific antibody (Fig. 10). In contrast, PRL, under the same conditions, stimulated tyrosine phosphorylation of Stat5a without a change in total Stat5a. Furthermore, R5020 cotreatment did not influence PRL-induced tyrosine phosphorylation of Stat5a, indicating that inhibition of PRL induction of β-casein gene transcription by progesterone does not involve blocking this Stat5 activation step. Similar results on tyrosine phosphorylation of Stat5a were observed with endogenous PR and Stat5a in T47D breast cancer cells, with cotransfected PR and Stat5a in COS-1 cells, and with endogenous Stat5a and ectopically expressed PR in HC-11 cells (Fig. 10, A–C). These results suggest that PR in a progesterone-dependent manner is capable of stimulating recruitment of an unactivated non-tyrosine-phosphorylated form of Stat5a to the β-casein promoter.

DISCUSSION

Studies described herein provide evidence that progesterone receptor interacts in a combinatorial manner with other transcription factors at the β-casein promoter and acts to suppress both PRL induction of transcription mediated by Stat5a, and the synergistic induction achieved by glucocorticoid and PRL. The suppressive effect of progestin and PR was not restricted to a single cell type or experimental condition. Progestin-PR-dependent suppression was observed in COS-1 and NMuMG MEC reconstitution systems in which PR, GR (only in COS-1), PRLR, Stat5, and a β-casein reporter gene were introduced by transient transfection (Figs. 1D, 4, and 6). Similar results were obtained in HC-11 MECs with endogenous PRLR/Stat5 and GR signaling pathways, and in primary mouse MECs with all endogenous factors (Fig. 7). Furthermore, we observed similar results with human and mouse PR indicating that repression of β-casein transcriptional activation is a general property of PR.

Because PR and GR can recognize the same or similar DNA sequence response elements, we explored whether PR can bind to GRE half-sites in the β-casein promoter and whether this interaction was important for mediating the inhibitory effects of progestins. By EMSA, PR bound to a β-casein proximal promoter DNA probe in a manner dependent on the

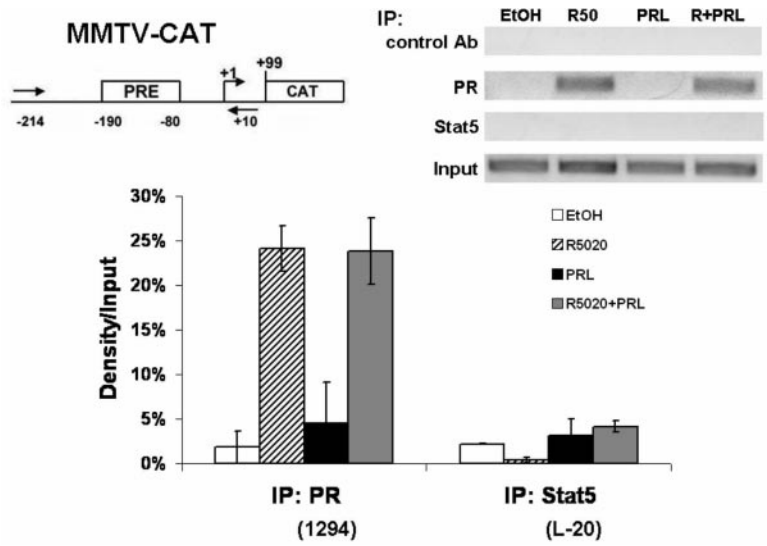


Fig. 9. PR Does Not Recruit Stat5a to Classical PRE of the MMTV Gene

T47D breast cancer cells containing a stably integrated MMTV gene fused to chloramphenicol acetyltransferase (CAT) were treated for 1 h with either EtOH (− control), ovine PRL (3 μg/ml), R5020 (100 nM), or ovine PRL (3 μg/ml) + R5020 (100 nM). Cells were then harvested for ChIP assay using PCR primers to the GRE/PRE sites located between −190 and −80 in the LTR of the MMTV promoter. *Upper left*, Schematic of the primers used for ChIP assay of the hormone response element (HRE) in the MMTV promoter. *Upper right*, Representative agarose gel of PCR products from input DNA and from immunoprecipitation by control antibody, PR mAB (1294), and Stat5 polyclonal antibody (L-20). *Lower panel*, Quantification of ChIP assays using Syngene Genetools software. Data represent average density of immunoprecipitations as a percentage of total input DNA after subtraction of signal obtained with a control antibody. The values are averages ± SEM from three determinations. Ab, Antibody; IP, immunoprecipitation; R, R5020.

integrity of the 5′-GRE half-site, indicating that half-GREs are indeed potential binding sites for PR (Fig. 5). By ChIP assay, PR in a progestin-dependent manner was also recruited to the proximal β-casein promoter in HC-11 cells, confirming PR interaction in nuclei in the context of chromatin (Fig. 8, B–D). The importance of PR interaction with GRE half-sites in mediating functional response to progestin was shown by the fact that a DNA binding-deficient PR-B with a point mutation in the DBD exhibited a complete loss of repression activity and a partial loss in the context of PR-A (Fig. 4). Point mutations in GRE half-sites in the β-casein reporter gene also resulted in a substantial loss of the repression activity of PR-B, whereas the activity of PR-A was minimally affected, indicating the involvement of additional DNA-independent mechanisms for the A isoform of PR (Fig. 4 and Table 1). It should be noted that the more efficient and complete inhibition of PRL transactivation obtained with human PR-A in COS-1 cells was not observed with mouse PR-A or with human PR-A in MECs, suggesting this may be a cell type-specific or conditional property of human PR-A.

Using *in vitro* DNA binding assays with a DNA probe, we found that PR activated by R5020 and Stat5a activated by phosphorylation with JAK-2 can bind simultaneously to the proximal β-casein promoter to form a ternary PR/Stat5/DNA complex (Fig. 5D). By ChIP assay PR or Stat5a was recruited to the β-casein promoter in response to treatment of cells with either progestin or PRL, respectively; however, neither PR nor Stat5 was recruited after 1 h treatment of cells with both hormones (Fig. 8, B–D). Because the same results were obtained by ChIP assay with antibodies to different regions of PR and Stat5a, this suggests that adding PRL and progestin together prevents recruitment of both PR and Stat5a to the promoter, rather than inducing a change in the conformation of these proteins that obstructs antibody recognition. There are several possible explanations for the apparent difference between *in vitro* DNA binding and ChIP results. First, the orientation of the adjacent Stat5a and PR binding sites (GRE half-sites) may simply be different in the DNA probe *in vitro vs.* DNA in the context of nucleosomes in nuclei. Second, treatment of cells with PRL and progesterone may stimulate posttranslational modifications (presumably phosphorylation) that affect protein-protein interactions in a manner that disrupts assembly of PR and/or Stat5a with the promoter. Third, PR and/or Stat5a *in vivo* may interact with other protein factors that disrupt their interaction with the β-casein promoter. Because dissociation of YY1 is required for PRL induction of β-casein, this raises the possibility that PR could stabilize YY1 interaction in a manner that reduces Stat5 binding, and Stat5a binding, in turn, may be required to stabilize PR interaction (32, 38, 39). Lastly, ChIP experiments were performed only at a single time point of hormone addition (1 h), raising the possibility that a sequential mechanism may operate to expel Stat5a from the β-casein pro-

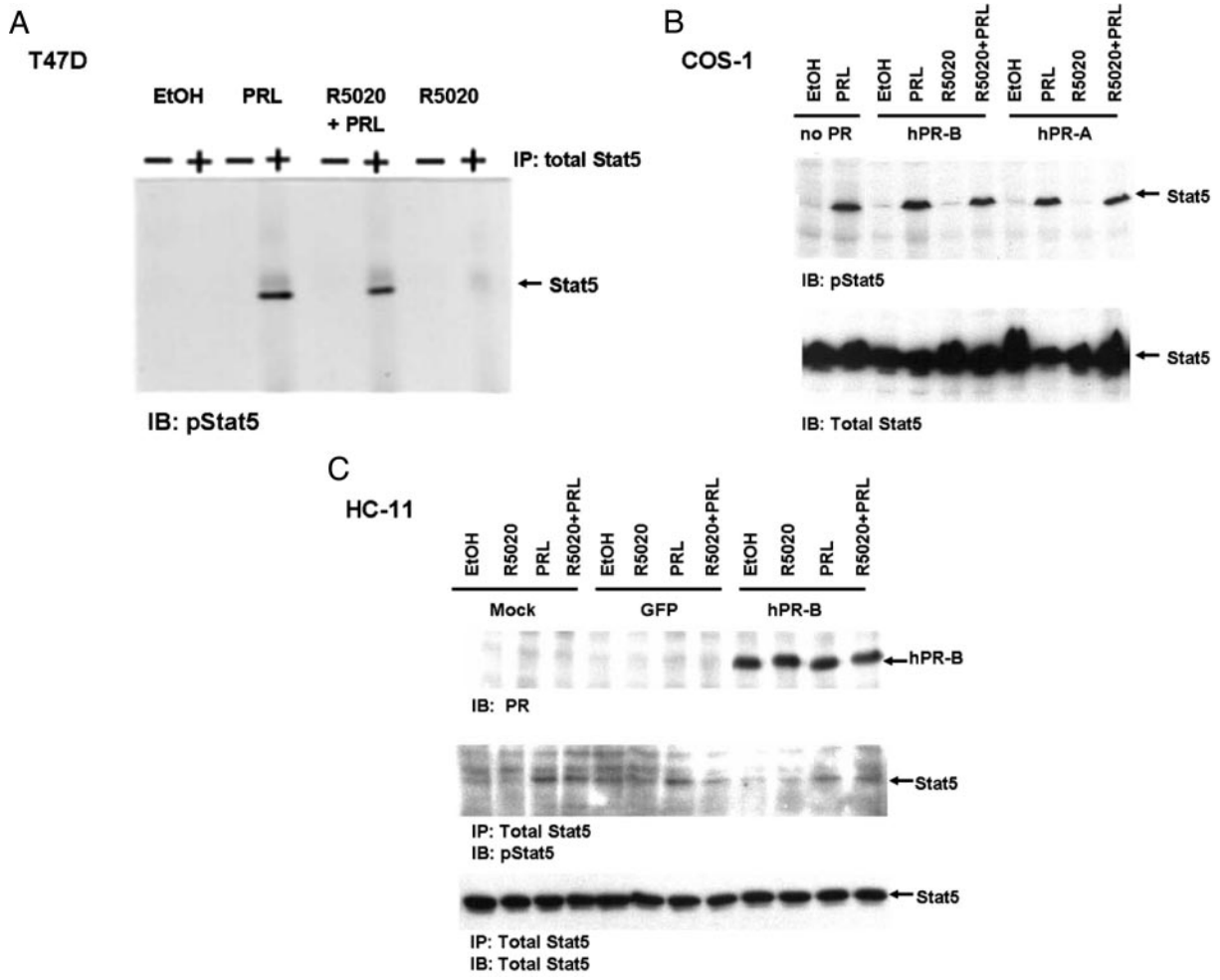


Fig. 10. Progesterone Does Not Affect PRL-Induced Tyrosine Phosphorylation of Stat5a

A, Human breast carcinoma T47D cells were treated with vehicle (ETOH), PRL (1 μg/ml), PRL (1 μg/ml) + R5020 (100 nM), or R5020 (100 nM) for 30 min. The cells were then lysed, and equal amounts of protein were immunoprecipitated with anti-Stat5, L-20 antibody (+), or a control unrelated antibody (−). The immunoprecipitates were then probed with antiphosphotyrosine Stat5 antibody (Tyr694/699). B, Monkey kidney COS-1 cells were transiently transfected with Stat5a alone or in combination with either human PR-B or human PR-A and then treated with vehicle, PRL (3 μg/ml), R5020 (100 nM), or PRL (3 μg/ml) + R5020 (100 nM) for 1 h. The cells were then lysed and equal amounts of protein were probed by Western blot. *Upper panel*, Tyrosine-phosphorylated Stat5a (Tyr694/699); *lower panel*, total Stat5a (L-20). C, Mouse mammary epithelial HC-11.7E cells were transduced with either adenovirus encoding GFP as a control vector, or human PR-B at an MOI of 50 pfu/cell, or no adenovirus (Mock), and were then treated with vehicle, R5020 (100 nM), PRL (3 μg/ml), or PRL (3 μg/ml) + R5020 (100 nM) for 1 h. The cells were then lysed, and equal amounts of protein were analyzed by immunoblot for PR (1294) (*upper panel*) or were immunoprecipitated with antibody to total Stat5a and the immunoprecipitate was analyzed by immunoblot for tyrosine-phosphorylated Stat5a (Tyr694/699) (*midpanel*) or total Stat5a (L-20) (*lower panel*). GFP, Green fluorescent protein; IB, immunoblotting; IP, immunoprecipitation.

moter. PR may first contact the β-casein promoter and cycle on and off with time of hormone treatment resulting in expulsion of Stat5a. A sequential mechanism would be consistent with the requirement of the PR DBD and PR-DNA for the repressive effect of progesterone on PRL induction of β-casein gene transcription. These results, taken together, are consistent with the conclusion that progestin inhibition of PRL induction of β-casein gene transcription involves a mutual interference of PR and Stat5 binding that is dependent on the presence of both PRL and progesterone.

Progesterone could potentially repress PRL/R/Stat5a signaling by inhibiting more proximal steps of Stat5a activation including tyrosine phosphorylation by Jak2, or subsequent dimerization and nuclear translocation of Stat5a. However, as determined by immunoblotting with an affinity-purified antibody to tyrosine 694/699 of Stat5a, progestin did not inhibit PRL-induced phosphorylation of Stat5a under any conditions examined including that of endogenous Stat5a in T47D breast cancer cells and HC-11 cells, and transfected Stat5a in COS-1 cells (Fig. 10). Para-

doxically, we observed a progestin-PR-dependent recruitment of Stat5a to the β-casein promoter in the absence of PRL as detected by ChIP assay (Fig. 8, B–D), indicating the formation of a ternary PR/Stat5a/DNA complex under these conditions. This result is consistent with a previous report of Richer *et al.* (62), showing progestin-induced nuclear translocation of Stat5 in T47D breast cancer cells. In cotransfected COS-1 cell experiments we similarly observed by immunofluorescence a progestin-PR-dependent nuclear translocation of Stat5a in the absence of PRL (Buser, A. C., and D. P. Edwards, unpublished data). Stat5a recruited to the β-casein promoter by progestin appears to be unactivated because progestin did not stimulate tyrosine phosphorylation of total cellular Stat5 (Fig. 10). However, it is possible that progestin stimulates tyrosine phosphorylation of a small fraction of total cellular Stat5a that is recruited to the β-casein promoter, but is below the level of detection by phosphoimmunoblotting. ChIP assay with antibodies to total and phosphorylated Stat5a will be required to distinguish between these possibilities.

How progestin promotes nuclear translocation and recruitment of Stat5a to the β-casein promoter is not known. A physical interaction between PR and Stat5a has been reported previously by coimmunoprecipitation assay of Hela cell nuclear extracts cotransfected with Stat5a and Flag-tagged PR-B (62). However, the interaction did not appear to be hormone dependent (62). We did not observe an efficient interaction of PR and Stat5a by coimmunoprecipitation assay of T47D cells either in the absence of hormone or after short-term (30 min) treatment of cells with R5020, PRL or both hormones together. Yet, under the same conditions, an efficient coimmunoprecipitation of GR and Stat5a was detected in cells treated with glucocorticoid and PRL (Gass-Handel, E. K., and D. P. Edwards, unpublished data) consistent with previous reports of physical interaction of GR and Stat5 (33). Thus, whether PR and Stat5 make protein-protein interactions in the absence of DNA is not clear. Progestin-PR-dependent recruitment of Stat5a by ChIP assay was not observed with the PRE-containing MMTV promoter (Fig. 9), suggesting that PR recruitment of Stat5a does not rely simply on protein-protein interaction but is promoter context dependent. Thus a combination of protein-protein and protein-DNA interactions by PR and Stat5a at the β-casein promoter may be required. The functional consequence of progestin-PR-dependent recruitment of Stat5a to the β-casein promoter is not known. Stat5a does not appear to function as a PR coactivator, because progestin did not induce transcription of the β-casein gene under conditions where PR recruits Stat5a. If, in fact, Stat5a recruited by progestin-PR in the absence of PRL has bypassed the PRLR/Jak2 signaling pathway and is not tyrosine phosphorylated, it could contribute to the inhibitory effect of progesterone by acting as a dominant negative for any active Stat5a. Further studies will be required to determine the role of progestin-PR-dependent recruitment of Stat5a to the β-casein gene in the absence of PRL.

Several studies have reported interactions between Stats and different classes of steroid receptors indicating that cross talk between these two distinct families of transcription factors activated by different signaling pathways may be of general functional relevance. GR interaction with Stat5a results in a strong enhancement of Stat5a-mediated gene transcription and plays a role in potentiating PRL induction of milk protein gene expression in the mammary gland. The potentiation effect of glucocorticoids requires both GR-Stat5 protein interaction and the integrity of GRE half-sites in the β-casein promoter, suggesting an involvement of GR-DNA binding (20, 33–36). GR and Stat5a can both recruit the histone acetyl transferase (HAT) p300 coactivator to the β-casein promoter in a manner that correlates with functional synergy between PRL and glucocorticoids and acetylation of core histones H3 and H4 (26, 32), suggesting that GR and Stat5a, as a unit, may be required for efficient recruitment of coactivators and subsequent chromatin remodeling and transcription. Because PR and GR are closely related and can bind to the same DNA sequences, this raises the interesting question of how these two receptors can have opposite effects on PRL induction of β-casein, and how PR can inhibit the potentiation effect of glucocorticoids. It is possible that PR binds with higher affinity to the GRE half-sites and effectively competes with GR. Doppler *et al.* (36), by use of PR/GR chimeras, showed that the strong enhancement activity toward PRL-induced *trans*-activation of β-casein that is lacking in PR resides in the N-terminal domain of GR. Also, the DBD of GR was shown to be dispensable for glucocorticoid potentiation activity (34), whereas the DBD of PR is important for the inhibitory activity of progesterone (Fig. 4). Clearly, further studies are required to determine the mechanism responsible for the opposing actions of GR and PR.

Cross talk between ER and Stat5 has also been reported and, depending on the cellular context or experimental conditions, estrogen and ER have been observed to either repress or potentiate Stat5-mediated gene transcription. In HC-11 cells, ERα or ERβ potentiated PRL-induced Stat5a-mediated transcription of a β-casein reporter gene in a manner dependent on ER ligand, on an intact ER DBD, and on interaction of ER with Stat5 bound to DNA (63). ER potentiation of PRLR signaling mediated by Stat5a was also observed in MCF-7 breast cancer cells (64), but most other studies observed an estrogen-ER-dependent repression of Stat5-mediated transcriptional activity. In transfected human embryonic kidney 293 cells, ERα and ERβ repressed PRL-induced activation of a β-casein reporter gene mediated by Stat5a/b (64, 65). Repression required protein interaction between ER DBD and Stat5 and appeared to be mediated by a decrease in PRL-induced tyrosine phosphorylation of Stat5a and reduced nuclear translocation and binding

of Stat5a to DNA (64, 65). In HepG2 liver cells, an estrogen-ER-dependent repression of PRL activation of the Na+/taurocholate cotransporter polypeptide (ntcp) gene was demonstrated by a mechanism that also involved interference with the PRLR/Stat5a signaling pathway (66). In addition to cytokine receptors, growth factor receptors can mediate effects on gene expression by signaling through Stat5. In breast cancer cells, overexpressing EGF receptor, an estrogen-ER-dependent repression of EGF-induced activation of Stat5-mediated gene transcription was reported that appeared to account for the ability of estrogen to inhibit EGF-stimulated DNA synthesis (67). The physiological role of ER-Stat5 cross talk is not known other than to speculate that both ER and Stat5 are important in the mammary gland and that negative or positive functional cooperativity may have a role in specific developmental stages or functions of the mammary gland.

Much less is known about interactions of other steroid receptors with Stat5. Two survey experiments analyzed functional interactions of different classes of steroid receptors with Stat5 in COS-7 or CV-1 cell cotransfection assays (36, 68). Androgen receptor had no effect on Stat5a-mediated transcriptional activity, a minimal positive effect of mineralocorticoid receptor on Stat5a activity was observed, and conflicting results have been reported with PR. PR enhanced or had a minimal effect on PRL induction of Stat5-mediated transcription of β-casein, as compared with the more substantial enhancement observed by GR. Another study of the influence of progesterone on PRL-Stat5 signaling in T47DY breast cancer cells stably transfected with PR-B reported that pretreatment of cells with R5020 for 48 h was required for PRL-induced tyrosine phosphorylation of Stat5 and PRL induction of a β-casein-LUC reporter gene (62). Why progestin-PR-dependent repression of PRL/Stat5 signaling at the β-casein promoter was not observed in these other studies is not known. As with studies of ER-Stat5 cross talk, the nature of the effect on Stat5 activity may be dependent on cell type-specific factors or experimental conditions. Much higher concentrations of expression plasmids were used in COS-1 and CV-1 cotransfection experiments as compared with the present study, suggesting differences may be the result of overexpression of steroid receptors and Stat5. In T47DY experiments, cells were pretreated for 48 h with R5020 before PRL addition, indicating that progesterone sensitized cells to PRL by persistent changes in gene expression, as opposed to mediating a direct cross talk between PR and Stat5 signaling pathways (62). A requirement of progesterone pretreatment for PRL stimulation of tyrosine phosphorylation of Stat5 and induction of β-casein is not consistent with other results in the literature, including studies herein with parental T47D breast cancer cells, suggesting this may be a unique property of T47DY cells stably transfected with PR-B.

Immortalized cell lines derived from normal MECs, as well as primary MEC cultures, lose expression of PR and ER; thus, it was not possible in this study to evaluate endogenous PR in mediating effects of progesterone on the PRLR/Stat5a signaling pathway. However, an advantage of ectopically expressed PR is a versatility to show that inhibitory effects of progestin were dependent on expression of PR, to compare different forms of PR, and to analyze the dependence or influence of other transcription factors. A potential limitation of the experimental system is that overexpression may force PR interactions with other factors that may not occur with lower endogenous concentrations of PR. However, under all experimental conditions, a similar progestin-PR-dependent repression of PRL induction of β-casein gene expression was observed including lipid-mediated transient transfection of COS-1 and NMuMG cells that can result in high protein expression in a fraction of the cell population, and expression in HC-11 and primary MECs from adenoviral vectors under conditions that result in a more homogenous lower level of expression. Similar results were also obtained with reporter genes and endogenous β-casein, arguing against forced interactions. Moreover, the inhibitory activity of PR observed in these experimental systems is consistent with the role of progesterone in the mammary gland during pregnancy to suppress secretory activation and differentiation until after parturition.

To our knowledge, these are the first studies to extensively analyze cross talk between PR and the PRLR/Stat5 signaling pathway at the β-casein promoter. These results define a direct interaction between PR and the PRLR/Stat5 signaling pathway in the nucleus that may contribute to the role of progesterone to repress lactogenic hormone induction of milk protein gene expression in the mammary gland during pregnancy. In the mammary gland PR is expressed heterogeneously in epithelial cells (3), and it is likely that a paracrine mechanism contributes to the inhibitory action of progesterone on PR-negative cells. Because of the functional importance of this action of progesterone, it is reasonable to speculate that inhibition by progesterone is multifactorial, *i.e*. mediated by indirect paracrine and direct nuclear actions of PR. Further studies will be necessary to determine the contribution of direct PR cross talk at the β-casein promoter relative to paracrine effects of progesterone on PR-negative cells and to better define the paracrine factors and mechanisms.

MATERIALS AND METHODS

Materials

R5020 (promegestone; 17α,21dimethyl-19-norpregna-4,9-diene-3,20-one) was obtained from DuPont-New England Nuclear Research Products (Boston, MA), Dex was from Sigma (St. Louis, MO), and ovine PRL was provided by Na-

tional Institute of Diabetes and Digestive and Kidney Diseases (Bethesda, MD). 1294/H9 is a mouse IgG1 mAb produced against purified human PR that recognizes both the A and B isoforms of PR (69), and B-30 is a mAb that recognizes the N-terminal segment unique to human PR-B (70). N-terminal Stat5 (N-20) (sc836) and C-terminal Stat5 (L-20) (sc1081) polyclonal rabbit anti-Stat5 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal antiphosphotyrosine Stat5 antibody (Tyr694/699) (07–586) was purchased from Upstate Biotechnology (Charlottesville, VA). Polyclonal rabbit anti-β-casein antibody used in primary MEC experiments was obtained from Margaret Neville [University of Colorado at Denver and Health Sciences Center, Aurora, CO].

Plasmids

The mammalian cell expression plasmids, pCMV-human PR-A and human PR-B, containing the cDNA for human PR-B under the control of the cytomegalovirus (CMV) enhancer/promoter, have been described previously (55, 56, 61). The rat Stat5a cDNA was subcloned into pRcCMV expression vector (Invitrogen, Carlsbad, CA). The rat PRLR-long form expression vector pECE-PRLRL and β-casein (−2400/+490)-LUC reporter gene were described previously (32). Truncated β-casein (−344/+1), and mutant β-casein promoters driving chloramphenicol acetyltransferase gene were described previously (35). Baculovirus vectors for expression of murine Stat5a and murine Jak2 were obtained from James Ihle (St. Jude's, Memphis, TN) and Andrew Kraft (University of Colorado Health Science Center, Aurora, CO) respectively. Mouse PR-B cDNA was provided by Shyamala Gopalan (Berkeley, CA) and was cloned into the CMV-based mammalian cell expression plasmid pcDNAI (Invitrogen, Carlsbad, CA) by insertion into the *Bsp*HI/*Eco*RI site located in the multiple cloning site. The cDNA for mouse PR-A was prepared by partial digest of mouse PR-B cDNA, ligated into the pBlueBacHis2B transfer plasmid and then inserted into the *Bam*HI/*Eco*RV site in the multiple cloning cassettes of pcDNAI. pSV2neo was obtained from CLONTECH (Mountain View, CA).

Transient Transfections

Monkey kidney COS-1 cells were plated into six-well dishes (Falcon, Oxnard, CA) at a density of 1.65×10^5 cells per well in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT). Cells were grown at 37 C for approximately 24 h and allowed to reach 50–60% confluency. Cells were then transfected using Lipofectamine according to the manufacturer's instructions (Life Technologies, Inc., Gaithersburg, MD). DNA (1–2 μg total) and 2.25 μl of Lipofectamine were used per 35-mm well. After transfection, cells were maintained in serum-free DMEM containing 5 μg/ml insulin and 10 μg/ml apo-transferrin (Sigma, St. Louis, MO) throughout the experiment. After 24 h, cells were treated with the synthetic progestin R5020 and/or ovine PRL (1 μg/ml) in serum-free DMEM supplemented with insulin and apo-transferrin, as indicated in figure legends, and incubated for 24 h at 37 C.

Normal murine mammary gland (NMuMG) cells obtained from Gary Firestone (University of California, Berkeley, CA) were plated into six-well dishes (Falcon, Oxnard, CA) at a density of 1.5×10^5 cells per well in DMEM supplemented with 10% FBS. Cells were grown at 37 C for approximately 24 h and reached 60–70% confluency. Cells were then transfected using the Lipofectamine protocol above. After transfection, cells were maintained in DMEM containing 5% FBS treated with dextran-coated charcoal (DCC) to remove steroids. After 24 h, cells were treated with vehicle, R5020 and/or PRL (1 μg/ml) in DMEM supplemented with 5% FBS-DCC, 5 μg/ml insulin, and 1 μg/ml hydrocortisone.

Reporter Gene Assays

Cell monolayers in six-well dishes were rinsed twice with wash buffer [40 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1 mM EDTA] and cells were lysed directly in the well with 300 μl of lysis buffer consisting of wash buffer plus 0.5% Triton X-100. The lysates were removed and centrifuged at 12,000 rpm for 10 min at 4 C to pellet cell debris. Luciferase activity in lysates was measured using a Monolight 2001 luminometer (Analytical Luminescence Laboratories, San Diego, CA). Lysates (25 μl) were added to 0.35 ml luciferase assay buffer [100 mM K2HPO4 (pH 7.8), 15 mM $MgSO_4$, 5 mM ATP, and 1 mM dithiothreitol (DTT)]. Luciferase-mediated light output was assessed for 1 mM luciferin in the reaction chamber. To compensate for variation in transfection efficiency, cells were cotransfected with a CMV-β-gal reporter plasmid. Results were calculated as the ratio of LUC to β-gal activity. Normalization of LUC activity to 1.0 in the absence of hormone or to 100% in the presence of PRL, or PRL plus glucocorticoids, enabled calculation of average values between multiple experiments. Values for relative luciferase activity were calculated as averages from at least three independent determinations ± SEM.

Protein Purification of PR, HMGB-2, and Stat5a

Polyhistidine-tagged PR and HMGB-2 were expressed from baculovirus vectors in Sf9 insect cells and prepared as previously described (55, 56). Briefly, cells were lysed in the following buffer: 20 mM Tris-HCl, pH 8.0; 350 mM NaCl; 10 mM imidazole; 5% glycerol; and a cocktail of protease inhibitors. Cell lysates were centrifuged at 100,000 × *g* for 30 min, and the supernatant was used as soluble whole-cell extract. Whole-cell extracts were passed over nickel affinity resins (NTA; QIAGEN, Valencia, CA) in a column at a flow rate of 1–2 ml/min. The resins were washed with lysis buffer until the OD_{280} returned to the buffer baseline. Bound polyhistidine-tagged proteins were eluted under nondenaturing conditions by competition with 100 mM imidazole. For PR, DTT (1 mM), zinc chloride (1 μM), and $MgCl_2$ (1 mM) were added immediately to stabilize receptor DNA and steroid binding activity. For HMGB-2, 1 mm DTT was added to the eluate. Samples were dialyzed in elution buffer minus imidazole and were stored at −80 C in aliquots.

Murine Stat5a and murine Jak2 were coexpressed from baculovirus vectors in Sf9 insect cells to yield expression of tyrosine-phosphorylated Stat5a. Sf9 cells grown in spinner flask suspension culture were coinfected with baculoviruses encoding Stat5a and Jak2 with a multiplicity of infection of 2 and 10 PFU/cell, respectively. At 48 h post infection, cells were harvested and lysed for 15 min on ice in the following buffer: 20 mM HEPES (pH 7.9); 50 mM sodium fluoride; 1 mM EDTA; 1 mM sodium vanadate; 2 mM DTT; 0.4 M NaCl; and a cocktail of protease inhibitors. Lysates were centrifuged at 40,000 rpm for 30 min and dialyzed in lysis buffer containing 50 mM NaCl, and supernatant was loaded on a heparin Sepharose (Pharmacia, Uppsala, Sweden) column and eluted with a NaCl (50 mM to 1 M) gradient in lysis buffer. Fractions were analyzed by silver stain and immunoblotting using anti-Stat5 antibody L-20 (Santa Cruz Biotechnology). Fractions containing Stat5a protein were assayed for functional Stat5 protein using a phosphotyrosine-specific antibody to Stat5a/b (Tyr694/699; Upstate Biotechnology).

EMSA

A 28-bp oligonucleotide containing a PRE/glucocorticoid response element derived form the MMTV-long-term re-

peat and a 35-bp oligonucleotide containing the proximal region of the rat β-casein gene promoter (−110 to −70) were used for EMSAs. The sequences of the coding strand: wild type, 5′-TAATCATGTGGACTTCTTGGAATTAAGGGACTTTT-3′; GASmut, 5′-TAATCATGTGGACTTCTTTTAATTAAGGGACTTTT-3′; GREmut, 5′-TAATCAAGCTTACTTCTTGGAATTAACAGACTTTT-3′; GRd, 5′-TAATCAAGCTTACTTCTTGGAATTAAGGGACTTTT-3′; GRe, 5′-TAATCATGTGGACTTCTTGGAATTAACAGACTTTT-3′. For PR/Stat5 cobinding experiments the sequence of the coding strand for β-casein probe was 5′-GATCCATGTGGACTTCTTGGAATTAAGGGACTTTT-3′. Oligonucleotides were designed with 4-bp overhangs and were labeled by Klenow polymerase filling of the overhangs with ^{32}P-labeled deoxynucleoside triphosphates (NEN Life Science Products, Boston, MA). Recombinant purified PR and Stat5a were incubated for 1 h at 4 C with ^{32}P-labeled DNA (0.3 ng) in a total reaction volume of 25 μl. Also included was 100 ng of poly (dA-dT) nonspecific competitor DNA. The DNA binding buffer contained 10 mM Tris-base, pH 7.4, 50 mM NaCl, 5 mM dithiothreitol, 2 mM $MgCl_2$, 10% glycerol, and 50 ng/ml of carrier ovalbumin. Samples (25μl) were electrophoresed on 5% polyacrylamide gels prepared at 40:1 (wt/wt) acrylamide-bis-acrylamide ratio using 20 mM Tris-acetate, 0.5 mM EDTA as the electrode buffer. To maintain constant temperature during electrophoresis, 4 C water was recirculated through the gel apparatus. Gels were dried and subjected to autoradiography. Quantification of protein-DNA complexes was carried out by direct scanning of dried gels for radioactivity using a series 400 PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and quantitated by Molecular Dynamics ImageQuant Program.

Immunoblot Analysis

Cell lysates containing equal amounts of total protein were solubolized in 1% sodium dodecyl sulfate (SDS) sample buffer and electrophoresed on 8–12% polyacrylamide SDS gels. Separated proteins were transferred to Immobilon-P PDVF membranes (Millipore Corp., Bedford, MA) and incubated with appropriate antibodies in TBS-T (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; and 0.1% Tween 20). Antibody interaction was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Arlington Heights, IL).

Adenovirus

Mouse PR-B and mouse PR-A cDNA were cloned into the pShuttle-CMV adenovirus shuttle vector at the *Hin*dIII/*Eco*RV sites of the multiple cloning cassette as previously described (71). Competent *E. coli* cells carrying pAdEasy-1 were cotransfected with either pShuttle-CMV-msPR-B or pShuttle-CMV-mouse PR-A that had been linearized by digestion with *Pme*I. Plasmids from individual colonies were screened for the presence of recombinant plasmids. The adenovirus vector chromosomes were released from the recombinant plasmids by digestion with *Pac*I and used to transfect human embryonic kidney 293 cells. Virus vectors were plaque purified, grown in small quantities, and tested by immunoblot for expression of the introduced genes. Positive stocks were grown in large scale, purified by CsCl step and isopycnic gradient centrifugation, and dialyzed *vs.* buffer containing 50% vol/vol glycerol as previously described (72). A recombinant adenovirus vector encoding human PR-A was constructed in a similar manner. Adenovirus transfer vectors encoding Lac Z, or green fluorescent protein under the control of the CMV promoter, were described previously (73).

Mouse PR Antibody

Mouse PR-B cDNA cloned into pBlueBacHis-2B baculovirus vector (Invitrogen) was expressed from baculovirus vectors in Sf9 insect cells and purified by nickel affinity resins as previously described (55, 56, 61). Rabbit polyclonal antibody to mouse PR-B was prepared commercially (Global Peptide, Inc., Ft. Collins, CO), and the total IgG fraction was purified by protein A-Sepharose affinity chromatography.

Primary MEC Cultures

Primary MECs were cultured according to Watkin *et al.* (58). Midpregnant, wild-type C57/BL6 mice were killed, and all 10 mammary glands were harvested from each mouse. Glands were manually homogenized, and the tissue was digested for 1 h at 37 C in 0.3% collagenase in Sigma's F10 media supplemented with 5% fetal calf serum, 0.15% trypsin, 10 mM HEPES, Na salt, and 15 mM $NaHCO_3$. Cells were spun down at 800 rpm for 3 min and washed with serum-free Ham's F12 medium (Sigma). The resulting cell pellet, enriched for epithelial cells, was plated on Collagen Type I (BD Biosciences, San Jose, CA)-coated plates at 10 μg/cm^2, at a density of 2.5×10^5 cells/cm^2, supplemented in Ham's F12 medium with 10% fetal calf serum and 1 mg/ml fetuin (Sigma). After cells had grown for 48 h at 37 C, they were harvested, suspended in Hams F12 medium supplemented with 10% FBS, and transduced in suspension at an MOI of 50 pfu per cell for 1 h at 37 C with recombinant adenovirus encoding Lac Z, mouse PR-A, or mouse PR-B. Cells were then spun down and replated onto 35-mm dishes coated with Matrigel at 20 μl/cm^2 (BD Biosciences, San Jose, CA) and grown in Ham's F12 medium plus 10% FBS for 48 h. Cells were then treated with either R5020 (100 nM), PRL (3 μg/ml), R5020 + PRL, or EtOH (no hormone). Cells were also maintained with insulin (5 μg/ml) and hydrocortisone (1 μg/ml) throughout hormone treatment. After 3 d, cells were washed and lysed in lysis buffer containing 100 mM Tris, pH 7.4; 2% Igepal; 60 mM KCl; 8 mM EDTA and 4 mM EGTA, and lysates were analyzed by immunoblotting.

HC-11 Stably Expressing β-Casein-LUC Reporter Gene

Differentiated mouse mammary HC-11 epithelial cells were cultured in RPMI-1640 medium (Life Technologies) supplemented with 5% FBS (Hyclone Laboratories), 5 μg/ml insulin, 10 ng/ml EGF, 100 U/ml penicillin, and streptomycin. Cells were plated on 100-mm dishes (Falcon, Oxnard, CA) at a density of 1.0×10^6 cells per well and grown at 37 C for approximately 24 h to reach 60–70% confluence. Cells were then transfected with Effectene according to the manufacturer's protocol (QIAGEN). DNA [1.0 μg β-casein(−2300/+490)-LUC + 0.1 μg pSV2-Neo] and 60 μl of Effectene reagent were added to each 100-mm culture dish. At 48 h after transfection, cells were treated with 200 μg/ml G418, the minimal effective dose leading to 100% cell death of nontransfected HC-11 cells within 48 h. Within 3–5 d single colonies were isolated and expanded. To assay for PRL induction of β-casein-LUC, cells were plated in six-well dishes at 1.5×10^5 cells per well in growth medium. At 48 h after plating, cells were washed free of serum and replaced with priming medium (PM) (RPMI-1640 supplemented with 5% DCC-FBS, 5 μg/ml insulin, and 1 μg/ml hydrocortisone), and grown for another 48 h and then treated for 48 h plus and minus 3 μg/ml ovine PRL. Cells were then harvested and assayed for luciferase activity as described above. HC-11 clones exhibiting robust PRL induction of β-casein-LUC in the presence of PRL were subcloned by serial dilution and reassayed for PRL induction. Clones and subclones were also assayed for PR-mediated inhibition of β-casein-LUC by infection of cells in suspension with PR adenovirus and treated with R5020, as in primary MECs above. One subclone, HC-11.7E, was selected for use in ChIP assays.

ChIP Assays

ChIP was performed as described previously with some modifications (61). Cells were cross-linked with 1% formaldehyde added directly to cell culture medium for 10 min at room temperature. Cell monolayers were washed three times with ice-cold PBS containing 1 mM phenylmethylsulfonylfluoride (PMSF) and protease inhibitors (1 mg/liter aprotinin and 1 mg/liter pepstatin) and harvested. Cells in suspension were incubated on ice for 10 min in DTT solution (1 mM DTT; 1.5 mM Mg_2CL_2; 10 mM KCl; 0.1% Nonidet P-40; 25 mM HEPES, pH 7.8) containing protease inhibitors and 1 mM PMSF and were lysed using glass-Teflon homogenization and centrifuged for 5 min at 2000 rpm. Cell pellets were resuspended in ChIP buffer (140 mM NaCl; 1 mM EDTA; 1% Triton X-100; 0.1% Na-deoxycholate; 0.1% SDS; 50 mM HEPES, pH 7.8) containing protease inhibitors and 1 mM PMSF. Sonication was performed using a Branson-450 Sonifier with microtip in 10-sec bursts followed by 1 min of cooling on ice for a total sonication time of 100 sec per sample. This resulted in DNA fragment sizes of 0.3–3.0 kb. Sonicated samples were centrifuged at 14,000 rpm for 15 min at 4 C, and the supernatants were diluted 10-fold in ChIP buffer containing protease inhibitors. Three percent of the supernatant was taken for input controls and processed with the immunoprecipitated samples at the point of the cross-linking reversal step. Remaining supernatants were incubated at 4 C overnight with appropriate antibodies: 15 μg of 1294, 15 μg B-30, 25 μg L-20, 25 μg N-20, or 12 μg of a control unrelated antibody (rabbit anti-mouse IgG). Immunocomplexes were collected with 25 μl of Protein G Sepharose 4 Fast Flow (GE Healthcare Biosciences AB, Uppsala, Sweden) (17-0618-01) for 1 h at 4 C with rotation. Beads were washed six times (5 min each) in ChIP buffer and twice in Tris-EDTA buffer. Complexes were eluted twice in 225μl of elution buffer (1% SDS; 1 mM EDTA; 50 mM Tris, pH 8.0). Formaldehyde cross-links were reversed by incubation at 65 C overnight followed by 1 μl Proteinase K (20 mg/ml) and 4 μl 0.5 M EDTA for 1 h at 42 C. DNA was recovered using phenol-chloroform extraction and ethanol precipitation. PCR-amplified DNA was analyzed by electrophoresis on ethidium bromide-stained 2% agarose gels, and relative band intensities were quantitated by an image scanner (Syngene), and band densities were quantitated using Syngene Genetools software. DNA immunoprecipitated with specific antibody was normalized by first subtracting signals obtained with control antibody and then expressing the normalized value as a ratio to input DNA.

PCR

PCR reactions were carried out in a GeneMate 20-well thermocycler. The following primers were used. β-casein promoter forward, 5′-CCAGCTTCTGAATTGCTGCC-3′; β-casein promoter reverse, 5′-GGTCTATCAGACTCTGTGAC-3′; MMTV promoter forward, 5′-GCGGTTCCCAGGGCTTAAGT-3′; MMTV promoter reverse, 5′-GGACTGTTCGAAGTTTACTC-3′. PCR was performed using 5 μl of DNA (input DNA was diluted 1:2) using 32 cycles of 30 sec at 95 C, 30 sec at 55 C, and 1 min at 72 C.

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