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Lactogenic Hormonal Induction of Long Distance Interactions between β-Casein Gene Regulatory Elements*[S]

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Lactogenic hormone regulation of β-casein gene expression in mammary epithelial cells provides an excellent model in which to study the mechanisms by which steroid and peptide hormone signaling control gene expression. Prolactin- and glucocorticoid-mediated induction of β-casein gene expression involves two principal regulatory regions, a proximal promoter and a distal enhancer located in the mouse approximately −6 kb upstream of the transcription start site. Using a chromosome conformation capture assay and quantitative real time PCR, we demonstrate that a chromatin loop is created in conjunction with the recruitment of specific transcription factors and p300 in HC11 mammary epithelial cells. Stimulation with both prolactin and hydrocortisone is required for the induction of these long range interactions between the promoter and enhancer, and no DNA looping was observed in nontreated cells or cells treated with each of the hormones separately. The lactogenic hormone-induced interaction between the proximal promoter and distal enhancer was confirmed in hormone-treated primary three-dimensional mammary acini cultures. In addition, the developmental regulation of DNA looping between the β-casein regulatory regions was observed in lactating but not in virgin mouse mammary glands. Furthermore, β-casein mRNA induction and long range interactions between these regulatory regions were inhibited in a progestin-dependent manner following stimulation with prolactin and hydrocortisone in HC11 cells expressing human PR-B. Collectively, these data suggest that the communication between these regulatory regions with intervening DNA looping is a crucial step required to both create and maintain active chromatin domains and regulate transcription.

The transcription of the β-casein milk protein gene is induced synergistically by the lactogenic hormones prolactin (Prl)[2] and glucocorticoids together with local growth factors, cell-cell and cell-substratum interactions that activate specific transcription factors and change chromatin structure. These alterations in chromatin structure include nucleosome remodeling (1) and post-translational modification of histones, both at the nucleosome level and at the level of larger chromatin domains.

In a previous study (2) using chromatin immunoprecipitation (ChIP) analysis, we examined the dynamics of recruitment of different transcription factors at the hormonally activated β-casein promoter proximal, as well as the more distal mouse β-casein enhancer, the latter located >6 kb upstream of the transcription start site (3). For simplicity, these regulatory elements are referred to as the "proximal promoter" and "distal enhancer." Hormonal stimulation of cells with Prl alone resulted in a rapid recruitment of Stat5 to the β-casein promoter and enhancer, and reciprocally the dissociation of YY-1 from the proximal promoter, but this was not sufficient to promote β-casein gene transcription. β-Casein gene transcription required treatment with both prolactin and glucocorticoids and the synergistic interaction of the glucocorticoid receptor and the LAP (liver-enriched activating protein) isoform of C/EBPβ followed by stable association of p300 and phosphorylated RNA polymerase (pol) II at the promoter and enhancer.

Because C/EBPβ (LIP isoform, liver-enriched transcriptional inhibitory protein) interacts directly with YY-1 (4) and YY-1 interacts with several histone deacetylases (5, 6), it is possible that interactions between YY-1 and C/EBPβ isoform LIP are responsible for the repression of β-casein promoter in the absence of lactogenic hormones through the recruitment of histone deacetylases. However, ChIP assays using antibodies to HDAC1 did not reveal a correlation of HDAC1 association with the dynamics of YY-1 disassociation at the proximal promoter (2). These data are consistent with results demonstrating that the YY-1-HDAC1 complex was not supershifted by HDAC1 antibodies (7) and suggest that a histone deacetylase other than HDAC1 is most likely associated with YY-1 resulting in transcriptional repression. One possible candidate could be HDAC3 that possesses histone deacetylase activity, represses transcription when tethered to a promoter, and binds the YY-1 transcription factor (8).

Transcriptional enhancers function at a distance from their target genes to help facilitate the formation of stable transcrip-

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[2] The abbreviations used are: Prl, prolactin; PR, progesterone receptor; 3C, chromosome conformation capture; ChIP, chromatin immunoprecipitation; pol, polymerase; p-pol, phosphorylated pol; MEC, mammary epithelial cell; EGF, epidermal growth factor; hPR-B, human PR-B; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GR, glucocorticoid receptor; HC, hydrocortisone.

tion preinitiation complexes. However, the question of how these distant activators interact with their target genes still remains open. The similar dynamics of assembly of transcription factors, the co-activator p300 and RNA pol II, as well as histone acetylation at the proximal promoter and the distal enhancer (2) suggested that these two regulatory regions might communicate with each other through protein-protein interactions, forming a chromatin loop. It is possible that a looping structure between promoter and enhancer provides binding surfaces that increase preinitiation complex recruitment and enhance the transition from transcription initiation to elongation (9).

The chromosome conformation capture (3C) assay is a quantitative method that allows identification of physical interactions between chromatin segments of up to several hundred kilobases apart (10–14). In this assay, chromatin fragments are fixed with formaldehyde, followed by digestion with restriction enzymes and ligation under dilute DNA concentrations, which promotes intramolecular ligation of cross-linked fragments over intermolecular ligation of random fragments. The cross-links are then reversed, and DNA is purified. Quantitative real time PCR is used to amplify DNA fragments containing novel ligation products using primers specific for the region under investigation (24). The development of 3C technology has contributed greatly to our understanding of long range chromatin architecture, and specifically, it has revealed that transcriptional regulatory DNA elements often form looped structures with proximal promoters to regulate gene expression. This method has been used to analyze the higher order chromatin structure at the β-globin (11, 14), T_H2 cytokine loci (15), the prostate-specific antigen (16), and snail genes (17) to list a few examples. Thus, the 3C assay has now become one of the essential tools for studying the relationship between nuclear organization and transcription.

Progesterone is known to repress lactogenic hormone induction of the β-casein gene expression in the mammary gland during pregnancy (18–20). The mechanism of the inhibitory action of progesterone has been investigated in mouse mammary epithelial cells, and it has been demonstrated that progesterone inhibits both PRL induction and glucocorticoid potentiation of β-casein transcription through a direct cross-talk of the progesterone receptor (PR) with the Prl/Stat5 signaling pathway in a manner dependent on PR interaction with the β-casein promoter (21). Progesterone treatment was also shown to induce recruitment of PR to the enhancer and disrupted the interactions of the enhancer with Stat5, GR, C/EBPβ, p300, pol II, and acetylation of H3 causing sustained repressive modifications of chromatin.[3]

In this study using ChIP assays, we first determined the dynamics of HDAC3 association with the β-casein gene proximal promoter and demonstrated a correlation in YY-1 and HDAC3 dissociation from the promoter after treating HC11 cells with Prl and HC. By using the 3C assay, we next demonstrated that hormonal treatment with Prl and HC induced a physical interaction between the β-casein gene proximal promoter and distal enhancer forming a chromatin loop structure. No DNA looping was observed in nontreated cells or cells treated with HC or Prl alone. Thus, for the first time we have demonstrated that the synergistic interactions between steroid receptors and peptide hormone-induced signal transduction pathways are required for the formation of these long range chromatin interactions. To test this hypothesis further, we used the three-dimensional mammary acini culture model, in which primary cultures of mammary epithelial cells (MECs, derived from mice 14 to 18 days pregnant) were grown on a layer of extracellular matrix (Matrigel). We demonstrate that lactogenic hormone treatment (insulin-hydrocortisone-prolactin) of these primary three-dimensional cultures induces the DNA loop between the β-casein promoter and enhancer, thus confirming the results obtained with the HC11 cell line. Additionally, we applied the 3C analysis to primary MECs isolated from the virgin and lactating mouse mammary glands to confirm that these long range chromatin interactions are regulated during normal mammary gland development. Finally, the long range interactions between the promoter and enhancer were inhibited by progestin/PR. Based on our previous results (2) and the results from this study, we suggest a model for the assembly of a multiprotein complex at the β-casein gene, and we propose that hormone-dependent formation of the active chromatin loop between the proximal promoter and distal enhancer is a key step required to achieve stable transcription.

EXPERIMENTAL PROCEDURES

Reagents—Prolactin was kindly provided by the National Hormone and Pituitary Program (NIDDK, National Institutes of Health, Bethesda) and was used at a concentration of 1 μg/ml. Hydrocortisone (H-4001) was purchased from Sigma and used at a concentration of 1 μg/ml. R5020 (promegestrone; 17α,21-dimethyl-19-norpregna-4,9-diene-3,20-one) was obtained from PerkinElmer Life Sciences. The protease inhibitor mixture was purchased from Roche Applied Science. HindIII was obtained from Invitrogen. T4 DNA ligase was purchased from New England Biolabs (Ipswich, MA) and was used at a concentration of 4000 Weiss units/reaction.

Adenovirus—Human PR-B was expressed from an adenovirus vector and prepared as described previously (21). For adenovirus infection, cells were transduced with hPR-B at a concentration of 50 plaque-forming units/cell.

Cell Culture—HC11 mammary epithelial cells were grown in 150-mm dishes at 37 °C and 5% CO_2 in RPMI media supplemented with 10% bovine calf serum (SAFC Biosciences), 2 mM glutamine (SAFC Biosciences), 50 μg/ml gentamycin (Sigma), 5 μg/ml bovine insulin (Sigma), 10 ng/ml murine EGF (SAFC Biosciences). After cells reached confluency, they were grown for an additional 3 days (at this point cells were infected with adenovirus for experiments involving progesterone receptor recruitment) and then incubated in priming media (0.5 M glutamine, 5 μg/ml insulin, 10% stripped donor horse serum, RPMI 1640 media) for 48 h. Cells were treated with hormones HC (1 μg/ml), Prl (1 μg/ml), and R5020 (10 nM) for various periods of time.

Primary MECs were isolated from 12 mice, which were between 14 and 18 days pregnant, and single cell suspensions

[3] A. C. Buser and D. P. Edwards, unpublished observations.

were generated as described by Welm *et al.* (22). To generate mammary acini, the final cell pellet was resuspended in plating medium (F-12 Nutrient Mix medium containing 10% fetal bovine serum, 10 ng/ml EGF, 5 μg/ml insulin, 1 μg/ml hydrocortisone, and 50 μg/ml gentamycin) and was added to 60-mm tissue culture dishes, which were pre-coated with 360 μl of growth factor-reduced Matrigel, at a density 10^6 cells per dish. Isolated mammary MECs were maintained in plating medium for 6 days followed by 2 days of incubation in priming medium (F-12 Nutrient Mix/gentomycin medium containing 5 μg/ml insulin) and then 2 days of incubation in differentiation medium (F-12 Nutrient Mix/gentamycin with hydrocortisone (1 μg/ml), insulin (5 μg/ml), and prolactin (3 μg/ml)). For 3C assays performed from mammary gland tissue, primary mammary epithelial organoids were isolated as described by Fata *et al.* (23).

RNA Isolation and Analysis—Total RNA was isolated from untreated- or hormone-treated HC11 cells, primary cultures, and mammary gland samples using TRIzol reagent (Invitrogen). For HC11 cells, 500 μl of TRIzol was added, and cells were harvested and homogenized by passing the solution through a pipette tip several times. For primary cultures, 500 μl of TRIzol was added directly to acini cultures grown on Matrigel after removal of media and rinsing with phosphate-buffered saline. For mammary gland samples, 30 mg of tissue was homogenized in 500 μl of TRIzol using a Polytron. The amount of total RNA extracted from either mammary gland tissue, primary cultures, or HC11 cells was measured by absorbance at 260 nm using a NanoDrop spectrophotometer. The cDNA was generated from 5 μg of total RNA by using the Superscript First-Strand Synthesis System (Invitrogen). Quantification of β-casein expression was performed by real time PCR as described previously (2).

ChIP Assay—ChIP was performed as described previously (2). Anti-YY1 (c-20) (sc-281) and anti-HDAC3 (07-522) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Upstate (Charlottesville, VA), respectively. Results were quantified by using real time PCR with SYBR Green on an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA).

3C Assay—The 3C assay in HC11 cells was performed as described previously (14) with minor modifications. Cells (3×10^7) were dissolved in 45 ml of priming media and cross-linked with formaldehyde (1% final) for 10 min at room temperature. The cross-linking reaction was stopped by adding glycine to a final concentration of 0.125 M following incubation at room temperature for 5 min and then stored on ice for 15 min. Cells were centrifuged at 2000 rpm for 10 min, resuspended in 1 ml of ice-cold lysis buffer (10 mM Tris, pH 8.0, 10 mM NaCl, 0.2% Nonidet P-40) supplemented with 0.1 ml of protease inhibitor mixture, and incubated on ice for 15 min. Then nuclei were released by Dounce homogenization with 20 strokes (pestle B) on ice. After centrifugation at 5000 rpm, nuclei were washed with 0.5 ml of 1× restriction enzyme buffer and then resuspended in 125 μl of the same buffer. The nuclei suspension was then divided into five individual tubes (each tube contained 25 μl, which is $\sim 6 \times 10^6$ cells), centrifuged, and resuspended in 362 μl of 1× restriction enzyme buffer each. SDS was added to a final concentration of 0.1%, and the nuclei were incubated at 65 °C for 10 min. Triton X-100 was then added to the final concentration of 1% to sequester the SDS. Digestion was performed with 400 units of HindIII at 37 °C overnight. The restriction enzyme was inactivated by raising SDS concentration to 2% and incubating at 65 °C for 30 min. The reactions were diluted into 8 ml of ligation reaction buffer containing 1% Triton X-100, 50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 1 mM ATP, and 4000 units of T4 DNA ligase, and incubated at 16 °C for 2 h. Proteinase K (100 μg) was added, and samples were incubated overnight at 65 °C to reverse the cross-links. Samples from five individual reactions were extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1, v/v), combined together, and ethanol-precipitated. After centrifugation at 10,000 rpm, DNA was purified again with phenol/chloroform/isoamyl alcohol (25:24:1, v/v) and precipitated with ethanol. To remove extra salt, DNA was washed five times with 70% ethanol. Samples were dissolved in TE buffer, pH 8.0, and treated with 0.5 μg of RNase A for 15 min at 37 °C.

A modified protocol (24) was adapted to perform 3C assays in primary three-dimensional acini cultures grown on Matrigel and in MECs isolated from mammary gland tissues. To release acini from Matrigel prior to fixation with formaldehyde, incubation at 37 °C for 1 h using Dispase reagent (BD Biosciences) was performed, and the reaction was stopped by adding 10 mM EDTA, followed by incubation for 30 min at room temperature.

We used BAC RP23-457P20, which contains the αs1-, β-, and γ-casein genes (3) to generate a PCR template that consists of all possible ligation products in the region under investigation in equimolar amounts. This template is used to normalize for differences in primer efficiency between the used primer pairs. BAC DNA (20 μg) was digested to completion with HindIII, and DNA was religated as described (25).

Primers for the 3C assay were chosen close to the HindIII sites (119 bp from the HindIII at promoter, 5′-CAA CTA CAT GTT CCT CCA GCC AAG TGA-3′, and 79 bp from HindIII site at BCE, 5′-TTT GAG GCC TTC TTC TGC TCC TTC AG-3′), and amplification of the ligated product resulted in a 255-bp fragment. In addition a distal reverse primer was chosen to serve as a control (39 bp from HindIII site ~112 kb from β-casein promoter, 5′-CAT GAG TGA TGC AAA AGC AAC TTG ATG-3′) with PCR amplification of the ligated product yielding a 211-bp fragment. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. The bands of interest were excised from the gel, and DNA was purified using the QIAquick spin columns (Qiagen) and analyzed by DNA sequencing. The partial sequences are listed in supplemental Fig. S1.

Quantitative Real Time PCR Analysis of the Ligation Products—Quantitative real time PCR was performed on ABI Prism 7700 Sequence Detection System using SYBR Green as a marker for DNA amplification. The linear range of amplification was determined for the experimental 3C templates and control 3C template by making serial dilutions (from 1:10 to 1:2000). Real time PCR was performed with 5 μl of 3C DNA template (at different dilutions) using 40 cycles of a three-step amplification (94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s). The relative amounts of experimental 3C templates were deter-

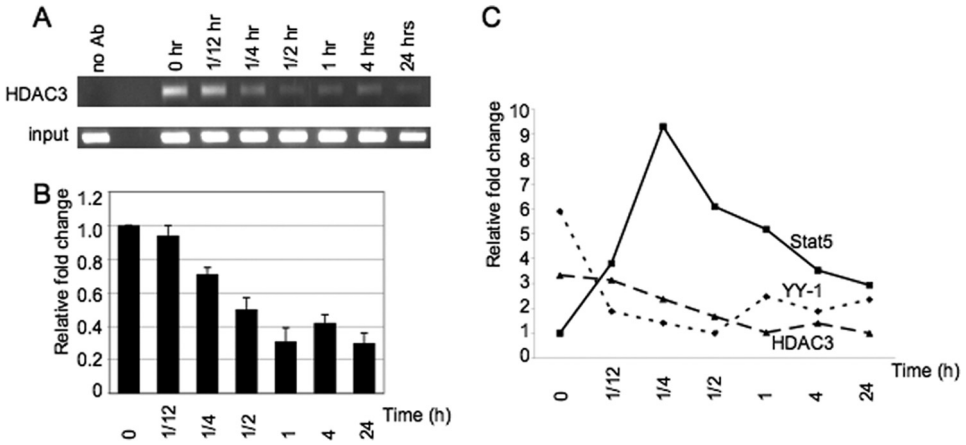

FIGURE 1. **Reciprocal regulation of histone deacetylase HDAC3 and YY-1 as compared with Stat5 binding at the β-casein proximal promoter in HC11 cells stimulated with lactogenic hormones.** *A,* ChIP assays were performed in untreated cells and cells treated with HC and Prl for different periods of time using antibodies (*Ab*) to HDAC3. PCR was performed using primers specific for β-casein promoter followed by gel electrophoresis. *B,* presence of HDAC3 at different time points was measured by quantitative real time PCR. Immunoprecipitation data were normalized to input DNA, and the amounts were expressed as the fold-change relative to untreated cells. *C,* reciprocal dynamics of Stat5 and HDAC3:YY-1 binding to the proximal promoter in cells stimulated with hormones for different periods of time.

mined by comparison to a standard curve generated by serial dilutions of the control BAC 3C DNA template. In addition, all PCRs were normalized to GAPDH to equalize for small differences in template amount or quality. All PCRs were run in triplicate. All differences reported were statistically significant ($p < 0.05$) using the Student's *t* test. Representative experimental data for all the dilutions of one experiment are shown in supplemental Fig. S2. The 3C assay was performed for at least two different cell culture experiments with essentially similar results.

RESULTS

Reciprocal Interactions of HDAC3 and YY-1 as Compared with Stat5 at the β-Casein Proximal Promoter—We demonstrated previously using quantitative ChIP assays that YY-1 is bound to the β-casein promoter and represses β-casein expression in the absence of lactogenic hormones (2). We hypothesized that this repression may occur because of interactions between YY-1 and C/EBPβ isoform LIP through the recruitment of histone deacetylases. However, when we performed ChIP assays using antibodies to HDAC1, we did not observe any correlation of HDAC1 association with the dynamics of YY-1 disassociation at the proximal promoter. Here we tested HDAC3, an ortholog of the yeast transcriptional regulator RPD3 that possesses intrinsic histone deacetylase activity (6, 8).

ChIP assays performed using antibody to HDAC3 in HC11 cells treated with Prl and HC, showed a transient decrease in chromatin association (about 2-fold) at the proximal promoter in the first 30 min (Fig. 1, *A* and *B*). These results show a good correlation with the rapid disassociation of YY-1 from the proximal promoter followed hormonal stimulation (Fig. 1*C*). The maximal dissociation of YY-1 in cells treated with both hormones was observed by 15–30 min, in a time frame when Stat5 binding was maximal. Thus, in HC11 cells treated with lactogenic hormones, Stat5 and YY-1 (together with HDAC3) display a reciprocal relationship in their association with the β-casein promoter; when Stat5 binding increases, YY-1 and HDAC3 decreases. These data suggested that YY-1 represses β-casein gene expression in the absence of prolactin through its association with HDAC3 and that lactogenic hormones act by relieving this repression.

3C Analysis Demonstrates Lactogenic Hormone-dependent Long Range Interactions between the β-Casein Proximal Promoter and Distal Enhancer in HC11 Cells—In our previous experiments, we were able to detect an increased level of phosphorylated RNA polymerase II (p-pol II) within the distal enhancer of the β-casein gene that is located between −6 and −6.4 kb upstream of the start site of transcription (2). The association of p-pol II at both regulatory regions suggested either the possibility of a long range interaction between the β-casein promoter and enhancer or, alternatively, independent recruitment of RNA pol II and perhaps even the transcription of short RNAs at the distal enhancer. Because detection of these small RNA transcripts has been problematic, we decided to first determine whether DNA looping might occur. Accordingly, we performed 3C assay in HC11 cells untreated and treated with hormones, singly and in combination for different periods of time.

A number of experimental controls are required to validate the results of the 3C assay. First, we generated a BAC control template that represented all possible ligation products in equimolar amounts. This BAC control template was used for normalization of signal intensities obtained by quantitative real time PCR of cross-linked 3C templates (correction for the PCR amplification efficiency). Second, we determined the range of template concentration required for linear PCR product formation. Third, to compare the cross-linking frequencies between the 3C template of interest and a 3C template that represents interactions between unrelated regions, two sets of DNA primers were designed as follows: Bp290_Be5401, flanking restriction sites in the promoter and enhancer region (255-bp PCR product), and Bp290_Be474, in the promoter and a distal region, which is 112 kb from the promoter (211-bp PCR product) (Fig. 2*A*).

We performed 3C analysis in untreated HC11 cells and cells treated with HC and Prl for 15 min, 1 h, and 24 h. The purified ligation products were amplified using both Bp290_Be5401 and Bp290_Be474 primer sets (Fig. 2*B*). Both PCR products were confirmed by DNA sequencing (supplemental Fig. S1). The 3C assay with both primers sets for PCR amplification was repeated in three independent experiments and revealed similar results.

For accurate quantification of different ligation products, we performed real time PCR in a linear range for each DNA amplification reaction. The linear range of amplification was determined by serial dilutions (supplemental Fig. S2). Using this approach a 4–5-fold increase in the frequency of interactions

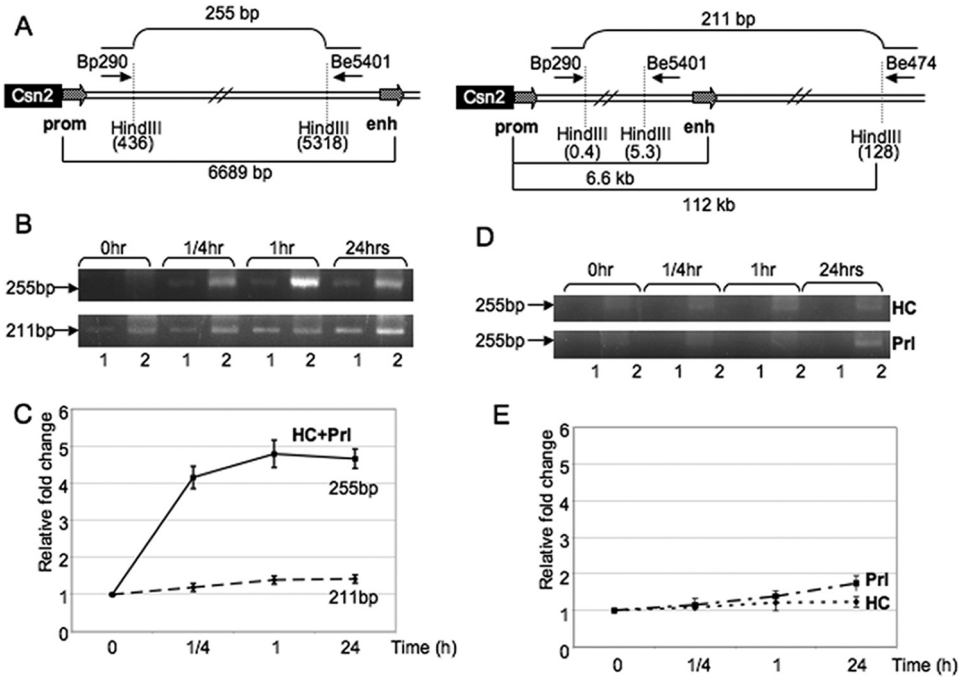

FIGURE 2. **3C analysis indicates that long range interactions between the β-casein gene proximal promoter and distal enhancer are observed only following hormonal stimulation of HC11 cells with both prolactin and glucocorticoids but not in cells treated separately with these hormones.** *A,* schematic representation of β-casein regulatory regions, primers, and amplicons used in PCR analysis. Cross-linked chromatin from nontreated and hormone-stimulated HC11 cells was digested with HindIII. The ligated DNA was PCR-amplified with primers as indicated. Two different primer sets, Bp290_Be5401 and Bp290_Be474, were designed to compare cross-linking frequency between restriction fragments in the promoter (*prom*) and enhancer (*enh*) region (255-bp PCR product) and between the promoter and a distal regions (112 kb from promoter; 211-bp PCR product). The sequences of the primers are given under "Experimental Procedures." *B,* 3C assay was performed using HindIII-digested chromatin from untreated HC11 cells and cells treated with HC and Prl for 15 min, 1 h, and 24 h. Agarose gel electrophoresis of the PCR products obtained with primer set Bp290_Be5401 indicates interactions between the promoter and enhancer (*upper panel*), whereas the primers Bp290_Be474 represent the random ligation efficiency (*lower panel*). *Lanes 1* and *2* show PCR products obtained from the diluted 1:10 and undiluted 3C templates, respectively. *C,* interaction frequency at different time points was measured by quantitative real time PCR. PCR products obtained with the 3C templates for both primer sets were normalized to control BAC template, and the amounts were expressed as the fold-change relative to untreated cells. The results shown are averages of at least three different amplifications with five different dilutions in each linear range with the standard deviations. *D,* 3C assay was performed using HindIII-digested chromatin from untreated HC11 cells and cells treated with HC (*upper panel*) or Prl (*lower panel*) for 15 min, 1 h, and 24 h. The PCR products (255 bp) using the Bp290_Be5401 primer set were run on agarose gels. *Lanes 1* and *2* showed PCR products obtained from the diluted 1:10 and undiluted 3C templates, respectively. *E,* interaction frequency at different time points was determined as described above.

between promoter and enhancer (255-kb PCR product) in cells treated with HC and Prl *versus* nontreated cells was observed (Fig. 2*C*). In contrast, no increase (less than 1.5-fold) in the frequency of random nonfunctional interactions (211-kb PCR product) was detected (Fig. 2*C*). These data suggest that the physical interactions between the promoter and enhancer of the β-casein gene are specific and are induced only after stimulations of HC11 cells with HC and Prl.

No Association between the β-Casein Proximal Promoter and Distal Enhancer Is Observed in HC11 Cells Treated with Prl or HC Separately—In HC11 cells treated with HC alone, we observed a statistically significant increase in GR binding to both promoter and enhancer within 15 min (2). Histone acetylation directly initiated by GR recruitment was also found highly increased at both regulatory regions of β-casein gene, although no direct DNA-binding sites for GR had been identified at the distal enhancer. To test the possibility that the presence of both hormones is not absolutely required for promoter-enhancer communication through DNA looping, we performed 3C assays in HC11 cells treated with HC or Prl separately (Fig. 2, *D* and *E*). Treatment with HC alone for 15 min, 1 h, or 24 h produced no detectable increase in interactions between the promoter and enhancer (255-kb PCR product) as compared with nonstimulated cells. Similar results were obtained for HC11 cells treated with Prl alone for 15 min and 1 h, and a small but insignificant (less then 2-fold) increase in 255-kb PCR product was detected at 24 h. For both treatments, no increase in the frequency of random nonfunctional interactions (211-kb PCR product) was found (data not shown). These results correlate with the increase in β-casein mRNA accumulation in HC11 cells stimulated with HC and Prl either singly or in combination (2), and suggested that stimulation with both the steroid and peptide hormones is absolutely required for these interactions between the promoter and enhancer.

Hormonal Regulation of Long Range Interactions between the β-Casein Proximal Promoter and Distal Enhancer in Three-dimensional Acini Cultures—We next asked whether the interaction between the promoter and enhancer of the β-casein gene observed following lactogenic hormone stimulation in HC11 cells also occurred in primary three-dimensional mammary acini cultures, and whether it also was regulated during normal mammary gland development. To answer the first question, we adapted the 3C analysis for use in the three-dimensional mammary acini cultures, in which primary MECs are grown on a layer of Engelbreth-Holm-Swarm-based extracellular matrix, Matrigel. For these experiments, we isolated primary MECs from 14- to 18-day pregnant mice. As reported previously (26), after culturing for 2 days on Engelbreth-Holm-Swarm, primary MECs formed distinct, rounded structures (Fig. 3*A*). By 6 days, the cells had formed hollow acini-like spheres (Fig. 3*A*) that look similar to secretory epithelial cells *in vivo*. During mid- to late pregnancy, the mammary gland is exposed to high levels of placental lactogen and glucocorticoids and already expresses high levels of β-casein mRNA (27). Therefore, to suppress and then re-induce β-casein gene expression, primary cultures were grown on Matrigel in serum-free, EGF-free, insulin-supplemented medium for 2 days, followed by treatment with Prl and HC for 2 days. To confirm an increase at the level of β-casein gene expression after stimulation of primary cultures with hormones, quantitative PCR was

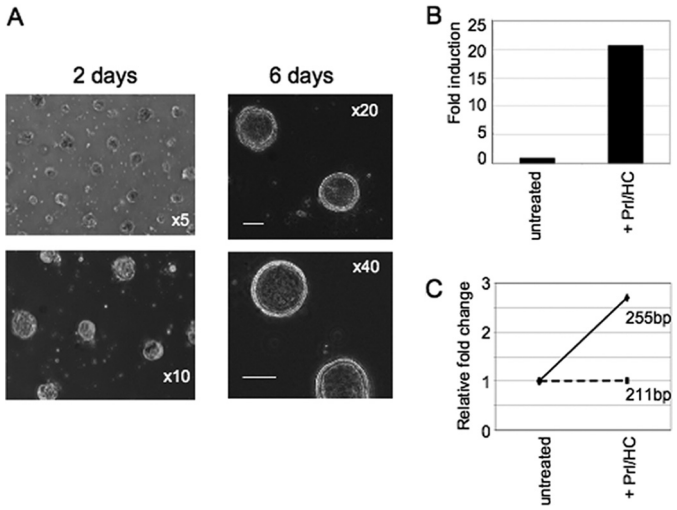

FIGURE 3. **3C analysis detects hormonally regulated long range interactions between the β-casein proximal promoter and distal enhancer in three-dimensional acini cultures.** *A,* light microscope images of three-dimensional structures grown on Matrigel (*bar,* 20 μM). MECs were isolated from mid-late pregnant mice (pooled from 12 to 14 mice), plated, and grown on Matrigel for 6 days. Cultures were incubated for 48 h in medium without serum and EGF followed by hormonal stimulation for an additional 48 h. *B,* total RNA prepared from three-dimensional acini in the absence (*untreated*) and presence of hormones (+*Prl/HC*) was reverse-transcribed and amplified using exon VII primers specific to the β-casein gene. The accumulation of transcripts was measured by quantitative real time PCR. Each value was corrected by GAPDH and expressed as a relative fold induction. *C,* 3C assay was performed using HindIII-digested chromatin in both untreated three-dimensional acini cultures and in cultures treated with lactogenic hormones. The interaction frequency was measured by quantitative real time PCR as described above. PCR products obtained with the 3C templates for both primer sets were normalized to the control BAC template, and the amounts are expressed as the fold-change relative to untreated cells.

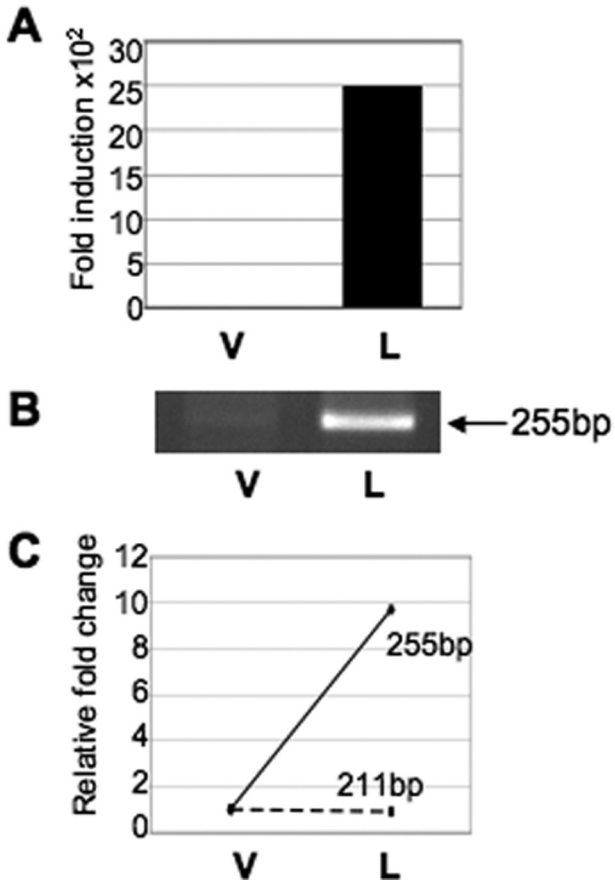

FIGURE 4. **Developmentally regulated chromatin interactions between the proximal promoter and distal enhancer at the β-casein gene.** *A,* total RNA prepared from mammary epithelial cells isolated from the mammary glands of virgin (*V*) or lactating (*L*) mice was reverse-transcribed and amplified using exon VII primers specific to β-casein. The accumulation of transcripts was measured by quantitative real time PCR. GAPDH primers were used to normalize for mRNA integrity. *B,* 3C assay was performed using HindIII-digested chromatin from primary MECs isolated from mammary glands of virgin (*V*) and 8-day lactating mice (*L*). The PCR product (255 bp) using the Bp290_Be5401 primer set was separated by agarose gel electrophoresis. *C,* interaction frequency was measured by quantitative real time PCR. The amount of PCR product obtained with the 3C template of primary MECs isolated from 8 days of lactation was expressed as a fold-change relative to the amount of PCR products obtained with a 3C template of primary MECs isolated from the virgin mice. Both PCR products were normalized to a control BAC template. The 3C assay was performed in two different experiments with essentially similar results. Representative results of one of the experiments are shown.

performed on mRNA isolated from treated (+Prl/HC) and control (untreated) three-dimensional acini cultures (Fig. 3*B*). Under these conditions, approximately a 20-fold increase of β-casein mRNA expression was observed in hormone-treated as compared with untreated cultures. Next, to determine whether lactogenic hormone induction of β-casein mRNA in primary three-dimensional acini cultures was accompanied by changes in long range chromatin interactions, we performed 3C analysis on these primary cultures, followed by quantitative real time PCR (Fig. 3*C*). In three-dimensional acini treated with HC and Prl, we observed a 2.7-fold increase in the frequency of interactions between promoter and enhancer (255-kb PCR product) as compared with untreated cultures. At the same time, no increase in the frequency of random ligation nonfunctional interactions (211-kb PCR product) was detected. These data correlate with our observations in HC11 cells and suggest that lactogenic hormones also regulate long range chromatin interactions between the promoter and enhancer of the β-casein gene in primary mouse MEC three-dimensional cultures.

Developmental Regulation of β-Casein Gene Long Range Chromatin Interactions—To determine whether the physical interaction(s) between the β-casein promoter and enhancer were regulated during normal mammary gland development, we next applied 3C analysis to primary MECs isolated from the mammary glands of virgin (4–8 pooled) and lactating mice (8 days of lactation) (Fig. 4). As expected, a dramatic increase (2500-fold) of β-casein mRNA expression was observed in the mammary gland of lactating as compared with virgin mice (Fig. 4*A*). Next, we performed 3C analysis in primary MECs isolated from these stages in development. Using quantitative real time PCR, we detected an ~10-fold increase in the frequency of interactions between promoter and enhancer (255-kb PCR product) in MECS isolated from mice during lactation as compared with virgin mice, yet no increase in the frequency of random ligation, nonfunctional interactions (211-kb PCR product) was detected (Fig. 4*C*). These results were validated in two independent experiments. An even greater increase in the frequency of interactions between promoter and enhancer (up to 20-fold) and the expression of β-casein mRNA (3200-fold) was observed when comparing lactating to diestrus-staged virgin mice (data not shown).

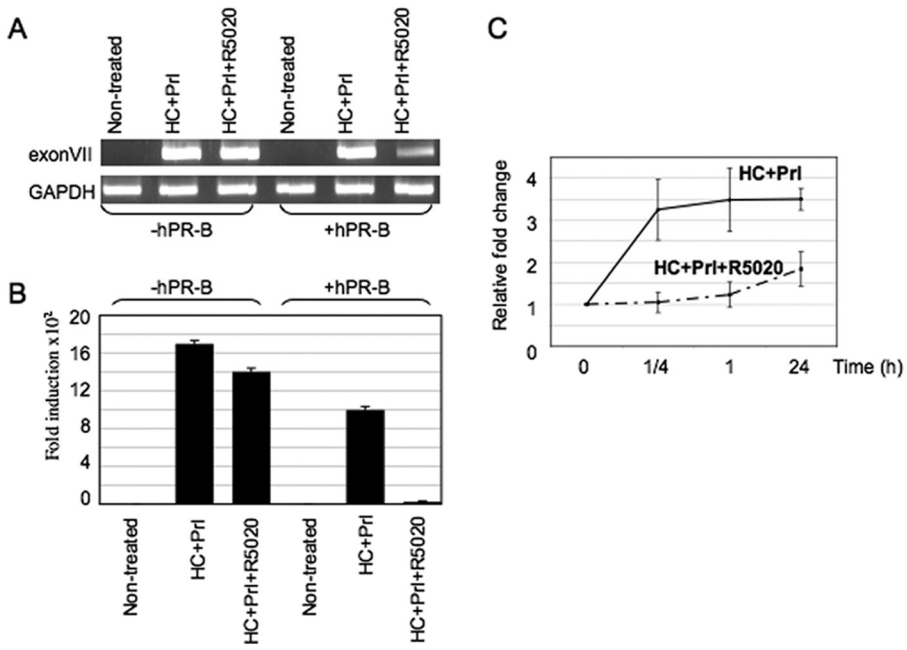

FIGURE 5. **PR in a progestin-dependent manner represses lactogenic hormone induction of β-casein mRNA expression and inhibits long range interactions between the β-casein gene promoter and enhancer induced by prolactin and glucocorticoids.** *A,* HC11 cells were infected with a recombinant adenovirus encoding hPR-B and treated for 24 h with HC + Prl or HC + Prl + R5020. Noninfected cells treated for 24 h with hormones served as a control for β-casein expression. Total RNA was isolated from untreated and treated cells. RNA then was reverse-transcribed and amplified using exon VII primers specific to β-casein and GAPDH primers to control for mRNA integrity followed by gel electrophoresis of PCR reactions. *B,* accumulation of transcripts after stimulation of cells with hormones was measured by quantitative real time PCR. GAPDH was used as an internal control. *C,* HC11 cells were infected with a recombinant adenovirus encoding hPR-B. The 3C assay was performed using HindIII-digested chromatin from untreated cells and cells treated with HC + Prl or HC + Prl + R5020 for 15 min, 1 h, and 24 h. The interaction frequency was measured by quantitative real time PCR as described above. PCR product (255 bp) obtained with the 3C templates for Bp290_Be5401 primer set was normalized to the control BAC template, and the amounts are expressed as the fold-change relative to untreated cells and presented as mean ± S.D. ($n = 3$); $p < 0.05$.

PR/Progestin Inhibits the Long Range Interaction between the β-Casein Promoter and Enhancer in Cells Treated with Lactogenic Hormones—To determine whether progesterone can inhibit β-casein induction in a progesterone receptor-dependent manner, we introduced human PR-B into HC11 cells, which normally lack this receptor, by adenovirus infection, and we treated these cells with or without HC and Prl, with HC, Prl, or R5020 for 24 h. We used noninfected HC11 cells treated with hormones in the same way as a positive control for β-casein induction. RNA was isolated, reverse-transcribed, and amplified using exon-VII primers specific to β-casein and cDNA (Fig. 5*A*). β-Casein mRNA levels were quantified by quantitative PCR (Fig. 5*B*). In noninfected cells treated with HC and Prl for 24 h, we observed a robust, ~1700-fold induction of β-casein mRNA compared with nontreated cells. Interestingly, for noninfected cells treated with hormones in the presence of R5020, we observed a slight decrease in the level of β-casein mRNA (by 17%). An even greater decrease in β-casein mRNA accumulation (about 40%) was detected in cells infected with hPR-B and treated with HC and Prl (without R5020). However, we observed a much more dramatic decrease (more than 30-fold) in β-casein mRNA induction in cells both infected with hPR-B and treated with hormones in the presence of R5020. These data suggested that the progesterone receptor in a progestin-dependent manner inhibits Prl/glucocorticoid induction of the endogenous β-casein gene expression in HC11 cells.

We then asked the following question: does this inhibition by progesterone affect the HC- and Prl-induced long range interactions between the β-casein gene promoter and enhancer? To answer this question, we employed the 3C assay in HC11 cells infected with recombinant adenovirus encoding hPR-B and treated with hormones for 15 min, 1 h, and 24 h in the absence and presence of R5020 (Fig. 5*C*). In the absence of R5020, the PCR amplification product (255 kb), representing promoter/enhancer interactions, showed a marked increase in hPR-B containing cells within 15 min after treatment with hormones as compared with nontreated hPR-B-containing cells. By using quantitative real time PCR, we determined that this increase was about 3.5-fold (Fig. 5*C*), which is slightly lower than that observed in non-hPR-B-containing cells (4-5-fold) (Fig. 2*C*). However, in the presence of R5020, the amount of the 255-kb PCR product was significantly lower compared with nontreated hPR-B-containing cells; almost no increase was found at 15 min or 1 h following hormonal treatment and only 2-fold at 24 h (Fig. 5*C*). Once again, no increase in the frequency of random nonfunctional interactions (211-kb PCR product) was found for both treatments (−/+R5020) in infected cells (data not shown). Similar results were obtained with the 3C assay in two independent experiments.

DISCUSSION

Specific transcriptional activation of a gene is believed to be a multistep process that involves recruitment of transcription factors and co-modulatory proteins, remodeling of chromatin structure, including nucleosome remodeling with post-translational modifications of histones, and assembly of a preinitiation complex. Distal enhancers that are remote from the genes they activate may help recruit complexes that accomplish these chromatin alterations and sometimes recruit RNA polymerase II. However, the mechanism by which distal enhancers participate in the recruitment of chromatin remodeling complexes and pol II, which eventually implement their activity at the promoters, is not yet completely understood. The currently favored model involves the establishment of an open chromatin domain accomplished by direct interactions between elements of the distal enhancer and gene promoter by DNA looping (28–32). The existence and nature of long range interactions were rather speculative and illusive until the development of molecular methods such as "RNA TRAP" (tagging and recovery of

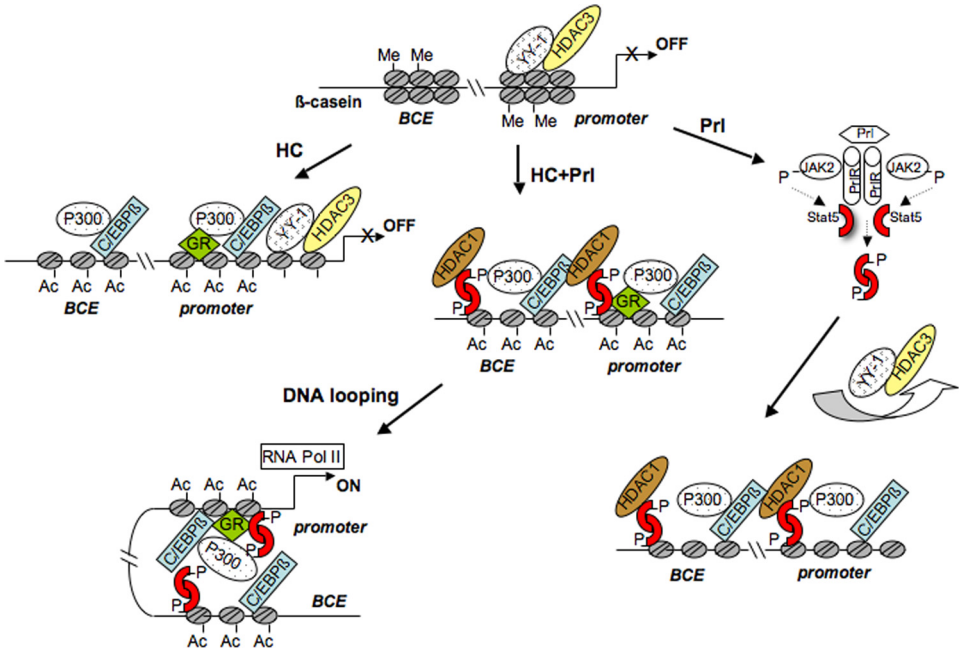

FIGURE 6. **A model to explain the sequential formation of complexes leading to the activation of β-casein gene expression following hormonal stimulation.** In the absence of lactogenic hormones, YY-1 binds to the β-casein promoter presumably interacting with the LIP isoform of C/EBPβ and HDAC3, which in turn results in the formation of dimethyl K9 H3 associated with a repressive chromatin structure. PrlR activation of Stat5 dimerization via Jak2 phosphorylation followed by nuclear translocation and DNA binding promotes the rapid displacement of YY-1 and HDAC3 from the β-casein promoter. Activated Stat5 then binds to sites adjacent to the C/EBPβ-binding sites at both the promoter and enhancer regulatory regions and recruits HDAC1 (2). Consequently, HDAC1 may deacetylate C/EBPβ LAP, and once deacetylated, LAP-LAP homodimers and/or LAP/LIP heterodimers can bind with a high affinity to their cognate DNA-binding sites as well as interact with other transcription factors, such as GR. Following hormonal stimulation with Prl and HC, the transcription factors Stat5, GR, and C/EBPβ rapidly bind to their respective response elements within β-casein regulatory regions and recruit p300 through protein-protein interactions. The recruitment of the co-activator p300 facilitates histone acetylation that in turn modifies chromatin organization. Interactions between open chromatin structures at the promoter and enhancer mediated through these transcription factors and co-activators enable direct protein-protein contacts facilitated by DNA looping. The formation of the active chromatin loop between distant regulatory elements facilitates binding of the basal transcriptional machinery to the DNA template and initiates transcription. Prolactin alone recruits Stat5 to a complex that is competent to recruit pol II and stimulates a low level of transcription after 24 h. However, GR recruitment is essential to increase histone acetylation resulting in an open chromatin structure at both regulatory regions. Thus, the combined treatment with both hormones is required for the formation of an active chromatin loop between the proximal promoter and distal enhancer to achieve maximal β-casein gene transcription. For simplicity, potential GR-Stat5 and GR-C/EBPβ interactions with the distal enhancer are not shown in this figure but may help facilitate long range looping. *BCE*, β-casein enhancer; *Ac*, histone acetylation.

associated proteins) (33) and 3C (10–14) made the analysis of this level of chromatin organization possible.

In our study we focused on the mechanism of hormonal regulation of β-casein gene expression in normal mammary epithelial cells. In our previous experiments (2) using ChIP assays, we have determined the dynamics of recruitment/occupancy of transcription factors Stat5, GR, C/EBPβ, and YY-1 at the hormonally activated β-casein proximal promoter and distal enhancer. In addition, we investigated the recruitment of p300, which is involved in β-casein expression and possesses post-translational modification activity. We also studied the dynamics of histone acetylation and established the time course of RNA pol II and phosphorylated RNA pol II accumulation at both regulatory regions of the hormonally activated β-casein gene. These data provided us with valuable information on the assembly of the multiprotein complexes and chromatin modifications at both β-casein regulatory regions that are about 6 kb apart. Here, by using the 3C assay, we have demonstrated that the hormonally activated β-casein proximal promoter and distal enhancer communicate with each other through the direct physical interactions forming a chromatin loop. These studies have now been incorporated into a working model (Fig. 6) that helps to define how the signaling pathways controlled by lactogenic hormones are integrated to regulate β-casein transcription.

In the absence of lactogenic hormones, both β-casein regulatory regions exhibit a constitutive dimethylation of lysine 9 of histone H3,[3] a modification usually associated with repressive chromatin structure (34). YY-1 has been demonstrated to play a functional role in β-casein repression (35). The YY-1 site in the β-casein promoter is a low affinity site, which is presumably influenced by protein-protein interactions, including interactions with the LIP isoform of C/EBPβ (4). LIP can interact directly with YY-1 to recruit HDAC3 to promote a repressive chromatin state (5, 6). By using ChIP, we observed the disassociation of HDAC3 from the β-casein proximal promoter followed by stimulation with HC and Prl (Fig. 1, *A* and *B*), which correlated with the dynamics of YY-1 dissociation and demonstrated a reciprocal relationship with Stat5 binding (Fig. 1*C*). Therefore, Stat5 binding induced by prolactin appears to be responsible for the disassociation of both YY-1 and HDAC3 from the β-casein promoter.

Following stimulation with Prl, Stat5 rapidly becomes phosphorylated by JAK2, dimerizes, and translocates into the nucleus where it interacts with clustered Stat5-binding sites in both the β-casein promoter and enhancer. However, optimal β-casein gene expression is only achieved following stimulation by both prolactin and glucocorticoids, indicating the requirement for synergy between Stat5 and GR (36–40). Interestingly, in cells stimulated with Prl alone, we did not detect any changes in histone H3 acetylation compared with nontreated cells; however, in cells treated with HC only or in combination with Prl, we observed a rapid increase in acetylation at both the β-casein promoter and enhancer (2). This strict dependence of histone acetylation upon HC treatment strongly suggests that acetylation was directly initiated by GR recruitment to the β-casein gene. Seven half-palindromic DNA-binding sites for GR (1/2GREs) are present at the β-casein proximal promoter, but none have been identified at the enhancer region (3). The observed hyperacetylation at both regions of the β-casein gene

suggests that GR might play a bridging role in β-casein activation through interactions with both Stat5 and C/EBPβ as well as binding to different co-activators and co-modulators.

All three transcription factors Stat5, GR, and C/EBPβ interact with nuclear transcriptional co-activator p300 (41–42), which possesses histone acetyltransferase activity involved in chromatin remodeling and facilitates the binding of RNA polymerase II complex to the core promoter (43). We observed similar kinetics of Stat5 and C/EBPβ binding and the recruitment of p300 in cells stimulated with Prl at both β-casein gene regulatory regions (2). Moreover, treatment of cells with both HC and Prl resulted in a cumulative and sustained increase in p300 accumulation as compared with either hormone alone. These results suggested that co-activator p300 is recruited at the gene regulatory regions through the protein-protein interactions with the primary transcription factors following their DNA binding. Finally, the presence of phosphorylated RNA polymerase II at the distal enhancer region (2) supported the hypothesis that the proximal promoter and distal enhancer communicate with each other through protein-protein interactions facilitated by DNA looping. To test this hypothesis, we performed 3C assays in HC11 cells stimulated with HC and Prl singly or in combination (Fig. 2). These data revealed that direct physical interactions between the proximal promoter and distal enhancer of the β-casein gene are specific, and that they are induced exclusively in the presence of both hormones. We demonstrated further that these specific long range interactions detected in HC11 cells are also observed following lactogenic hormone induction in three-dimensional acini cultures (Fig. 3) and *in vivo* during lactation (Fig. 4). The communication between regulatory regions of the β-casein gene with intervening DNA looping appears to be a key step required to both create and maintain active chromatin domains and regulate transcription. The recent observation that sustained activation of Stat5 is necessary and sufficient for chromatin reorganization and β-casein transcription (44) supports this hypothesis. The precise protein-protein interactions, which facilitate this looping and the structure of this multiprotein complex, remain to be determined. Presumably this may involve either transcription factor interactions with large co-modulatory proteins, such as p300 at both regulatory regions, as well as the ability of several of these transcription factors to form both dimers and tetramers and to interact directly with each other.

The distance of interaction between the β-casein promoter and enhancer falls within the wide variety of interactions between distal and proximal regulatory regions observed in these types of studies. In general, 3C assays have revealed interactions from 1.9 kb to 1 Mb and even inter-chromosomal interactions (45–47). Estrogen-dependent loop formation of distal enhancer and promoter was reported for the *CA12* gene promoter and distal estrogen receptor-α binding enhancer located about 6 kb from the promoter (48), although estrogen-induced long range chromatin conformational changes can also involve enhancers that may be as far away as 1 Mb.[4]

Progesterone is known to repress lactogenic hormone induction of the β-casein gene expression in the mammary gland during pregnancy (18–20). To determine whether progesterone inhibits β-casein induction in HC11 cells in a progesterone receptor-dependent manner, we induced human PR-B in cells by adenovirus infection and characterized the kinetics of lactogenic hormone induction of β-casein mRNA accumulation in the presence and absence of the PR ligand R5020 (Fig. 5). A dramatic decrease (about 30-fold) of β-casein induction was observed in cells expressing hPR-B and treated with hormones in the presence *versus* absence of R5020 (Fig. 5*B*), suggesting that the expression of human PR-B by adenovirus in HC11 cells provides an appropriate model in which to examine interactions of PR with the endogenous β-casein gene. It was determined using ChIP assays that PR is recruited in a progestin-dependent manner to both β-casein gene regulatory regions and inhibits the assembly of Stat5, GR, C/EBPβ, and p300 as well as histone acetylation and the recruitment of RNA polymerase II required for gene activation.[3] A much smaller ligand-independent decrease in β-casein induction occasionally was observed following the expression of human PR-B by adenovirus in HC11 cells. This slight decrease in β-casein induction without R5020 may possibly be due to either an effect of unliganded PR on gene expression, because unliganded PR has been reported to induce a number of genes in T47D cells (49), or possibly to an indirect effect of unliganded PR interacting with Stat5 in the cytoplasm.[5] The level of PR expressed was less than that observed in breast cancer cell lines, like T47D, and PR was not recruited to the β-casein proximal promoter in the absence of ligand.

To study the effects of ligand-activated PR on chromatin looping, we performed 3C assays in HC11 cells expressing hPR-B following stimulation with HC and Prl in the presence and absence of R5020 (Fig. 5*C*). In the absence of R5020, we observed a 3.5-fold increase in the frequency of interactions between the promoter and enhancer in expressing cells; however, no significant changes were detected in the presence of R5020. These results suggested that PR/progestin inhibits long range interactions between the β-casein promoter and enhancer induced by lactogenic hormones resulting in a repressive chromatin domain that impedes transcription. The precise mechanism, by which this occurs, remains to be elucidated.

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