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Oct-1 functions as a transactivator in the hormonal induction of β-casein gene expression

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Abstract The ubiquitous transcription factor Oct-1 is involved in the hormonal regulation of the transcription of the major milk protein β-casein through an interaction with the prolactin receptor, the STAT-5, and the glucocorticoid receptor (GR). In this study, this interaction was further demonstrated using Oct-1-deficient cells. In addition, Oct-1 mRNA expression is shown to increase during pregnancy and reach the highest levels during early lactation in mouse mammary gland. In reconstituted COS-7 cells, the endogenous Oct-1 binding activity rapidly increased within 5 min upon the lactogenic hormone treatment, indicating potential post-transcriptional/translational modification of Oct-1 by prolactin and glucocorticoids. Furthermore, STAT-5B was as effective as STAT-5A in the interaction with Oct-1 during hormonal induction, and a GR mutant, which carries mutations at multiple potential phosphorylation sites, functioned similarly to the wild-type GR, indicating that these phosphorylation sites may not be involved in the interaction of GR with Oct-1 on the β-casein gene promoter.

Keywords Oct-1 · Transcription factor · β-Casein · Transactivator

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Introduction

Transcription of the major milk protein β-casein is synergistically activated by the lactogenic hormones prolactin and glucocorticoid through interactions of hormonal signaling molecules STAT-5 and glucocorticoid receptor (GR) [1, 2]. The proximal promoter region of β-casein gene contains two STAT-5 binding sites and multiple half-palindromic glucocorticoid response elements (GREs). STAT-5, when phosphorylated upon prolactin stimulation, can dimerize and translocate into the nucleus, then bind to β-casein promoter, and activate its transcription [2]. The GR, after releasing from the heat shock protein complexes upon binding to the glucocorticoid, also translocates into the nucleus and only binds to the half GREs on β-casein promoter efficiently in the presence of activated STAT-5 [1]. The direct interaction between GR and STAT-5 may enhance the DNA binding ability of STAT-5 through prolonging its dephosphorylation process [1].

There are two isoforms of STAT-5: STAT-5A and STAT-5B, both of which are expressed in the mammary gland. The isoforms share more than 90% identity in their amino acid sequences, though they have slightly different C-termini [3]. STAT-5A is the dominant isoform in the mammary gland. STAT-5A and STAT-5B can form both homo- and hetero-dimers after phosphorylation, which then show similar binding activity to the β-casein promoter [4]. However, there is evidence indicating that the STAT-5A and STAT-5B might be activated by distinct signal pathways and may be involved in different functions. For example, prolactin and src/abl kinases have different effects on the nuclear translocation of STAT-5A and STAT-5B [5]. The mammary alveolar development is dramatically reduced in STAT-5A-deficient mice, but not in STAT-5B-deficient mice [6].

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It has been speculated that the activation/deactivation of GR may also be mediated by a phosphorylation/dephosphorylation system. Mouse GR contains multiple phosphorylation residues (serines #122, 150, 212, 234, 315, and 412 and threonine #159), which are potentially involved in ligand-binding-dependent phosphorylation. Mutations of these serine/threonine residues remarkably decreased the effect of glucocorticoid on a minimal reporter (2× GRE-TATA promoter) [7], but not on a 2× GRE-truncated thymidine kinase promoter [8], indicating that the effect of phosphorylation of GR on its transactivation activity may be promoter specific.

The ubiquitous transcription factor Oct-1 may also play an important role in the hormonal induction of β-casein expression. Our previous studies has identified that Oct-1 may be the double-strand DNA binding protein which specifically binds to the Block C region of the β-casein promoter [9, 10]. The binding activity can be induced by the lactogenic hormones in vivo [10]. Mutation of the Oct-1 binding site and reversal of its orientation can dramatically reduce the hormonal induction of the promoter activity [9]. In addition, our recent study indicated that Oct-1 transactivates hormonal induction of the β-casein expression by functional interactions with STAT-5, GR, and the prolactin receptor (Prl-R) [9]. In transient transfection experiments in reconstituted lactogenic hormone-responsive COS-7 cells, the hormonal induction of the β-casein promoter activity was much stronger when Oct-1 is cotransfected with Prl-R and STAT-5 or GR or both [9].

In this study, we examined the interactions of Oct-1 with two different isoforms of STAT-5 as well as a GR mutant, which carried the mutations at potential phosphorylation sites, in hormonal induction of β-casein transcription. We also examined the induction of Oct-1 binding activity by the lactogenic hormones in vitro and Oct-1 expression in the mammary gland of different developmental stages.

Materials and methods

Plasmid constructs, cell culture, transient transfection, and luciferase assay

Mouse STAT-5A and STAT-5B expression plasmids, mSTAT-5a/pECE and mSTAT-5b/pECE, were provided by Dr. Wolfgang Doppler [11]. Mouse GR expression plasmid mGR/pSV2Wrec and its mutant A4/pSV2Wrec were provided by Dr. John Cidlowski [7]. All other plasmids and constructs have been previously described [9].

The COS-7 cell culture and transfection procedures have been previously described [9]. An Oct-1-deficient (Oct-$1^{-/-}$) mouse embryo fibroblast (MEF) cell line was obtained from Dr. Dean Tantin and was cultured as previously described [12]. Cells were transiently transfected with plasmid DNA using Fugene 6 (Roche) following the manufacturer's instructions. After 48 h, prolactin (Prl) and dexamethasone (Dex) were directly added to the medium to the final concentrations of 5 μg/ml and 0.1 μM, respectively. For luciferase assay, cells were harvested after 24 h of hormonal treatment. In all experiments, TK-Renilla luciferase control plasmid (pRL-TK, Promega) was used as an internal control to normalize transfection efficiency. For Oct-1 binding assay, the cells were harvested at 0 time, 5, 10, 15, 30 min, 1.5, 3, 6, 12, 18, 24, and 48 h after the hormonal treatment.

Preparation of nuclear extracts and gel electrophoresis mobility shift assays (EMSA)

Nuclear extracts were prepared from COS-7 cells and from the MEF cells as described previously [13]. Nuclear extracts of HeLa cells were purchased from Promega and used as a positive control. Protein concentration of the nuclear extracts was measured by using the BIO-RAD Protein Assay kit (BIO-RAD). Nuclear extracts (5 μg for COS-7 cells; 20 μg for Oct-1-deficient MEF cells) were incubated for 30 min with a Block C oligonucleotide probe, end labeled with [γ-^{32}P] ATP, and loaded on a 4% (w/v) nondenaturating polyacrylamide gel as previously described [10, 14]. For the binding reactions with antibodies, antiserum or normal rabbit IgG (negative control) was added into the reactions and incubated on ice for 30–60 min before adding the labeled probe.

Animals and isolation of mammary gland epithelial cells

Mammary glands were collected from C57BL/6 mice in different developmental stages. The mammary epithelial cells were isolated following an established procedure [15]. Briefly, intact mammary glands were freshly dissected and minced with a razor blade and incubated for 1 h with gentle shaking in Hank's balanced salt solution (HBSS) containing 0.004% DNase and 0.1% collagenase at 37°C. Three batches of isolated epithelial cells from each stage were prepared. The cell suspension was then fractionated on a 38% continuous Percoll (Sigma) gradient. The pure epithelial cells were washed in HBSS, frozen in liquid nitrogen, and stored at −80°C until RNA preparation.

RNA extraction, reverse-transcription, and quantitative real-time PCR

Total RNA from mouse mammary epithelial cells was isolated by Trizol (Invitrogen) following the manufacturer's instructions. RNA integrity was verified by electrophoresis of an aliquot on a 1% agarose gel. Of total RNA, 2 μg was

reverse-transcribed in a 25-μl reaction mixture using random primer Oligo-dT_{18} (Sangon, Shanghai, China) and Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega). The transcribed cDNA was subjected to the quantitative RNA analysis using DNA Engine Opticon-2 (MJ Research) and DyNAmo SYBR Green qPCR commercial kits (Finnzymes, Finland). The primers for β-actin, the reference gene, were: 5′-TAGACTTCGAGCAGGAGATG-3′ (forward) and 5′-CCACCAGACAGCACTGTGTT-3′ (reverse). The primers for Oct-1 were: 5′-AAATGACTTCAGCCAAACC-3′ (forward) and 5′-CAAACACGAATCACCTCCT-3′ (reverse). The annealing temperatures were 62 and 66°C, respectively. The PCR reaction consisted of 5.0 μl of SYBR Green qPCR mix, 1.0 μl of cDNA, 3.6 μl of double distilled water, and 0.4 μl of primer pairs (25 μmol/l each) in a total volume of 10 μl. Cycling conditions were 50°C for 2 min followed by 95°C for 5 min, 35 cycles of 95°C for 30 s, 62 or 66°C for 60 s, and 72°C for 30 s. The melting curve program was 65–95°C, with a heating rate of 0.1°C/s and continuous fluorescence measurement. All samples were measured in triplicate. The relative mRNA levels of Oct-1 were determined using the relative standard curve method as described previously [16].

Statistical analysis

The statistical analyses of the normalized luciferase activities and Oct-1 mRNA levels in the mammary glands of different stages were carried out using Minitab 14 software. Comparisons between two groups were carried out using a *t*-test. A one-way ANOVA with Turkey's post hoc analysis was performed when comparisons were among more than two groups. Significant differences ($P < 0.05$) are noted with symbols in figure.

Results

Developmental regulation of Oct-1 expression in mouse mammary epithelial cells

We first examined the Oct-1 expression in the mammary gland epithelial cells at different developmental stages. As shown in Fig. 1, the expression of Oct-1 mRNA was upregulated in the mammary epithelia from the stage of virgin to early, middle, and late pregnancy stages and reached the highest levels in the lactation stage.

Lactogenic hormones induce endogenous Oct-1 binding activity to the β-casein promoter

In our previous study, we reconstituted COS-7 cells to be lactogenic hormone-responsive by transfection with STAT-5A, Prl-R, and GR expression plasmids and demonstrated that Oct-1 activates hormonally induced β-casein promoter activity in a dose-dependent manner [9]. We then investigated whether the hormones could induce endogenous Oct-1 binding to the β-casein promoter in this cell system. The nuclear extracts were prepared from the reconstituted COS-7 cells transfected with or without an Oct-1B expression plasmid, treated with Prl and Dex for different times, and subjected to EMSA assay with a radiolabeled double-stranded oligonucleotide probe that encompasses the Block C region of the β-casein promoter [10]. As shown in Fig. 2a and b, Oct-1 binding to block C is rapidly doubled in 5 min after the lactogenic hormone treatment, reached the highest level after 3 h, temporarily dropped back to the level seen before hormonal treatment, and then increased and remained at a level 45% higher than the basal level. The top binding complex showed a more significant response to hormones. Oct-1 binding specificity was confirmed by a supershift assay with anti-Oct-1 antibody and by adding unlabeled Block C oligonucleotide competitor to the reaction (Fig. 2c). A similar pattern of Oct-1 binding in response to lactogenic hormones was also observed in the cells transfected with exogenous Oct-1 (data not shown).

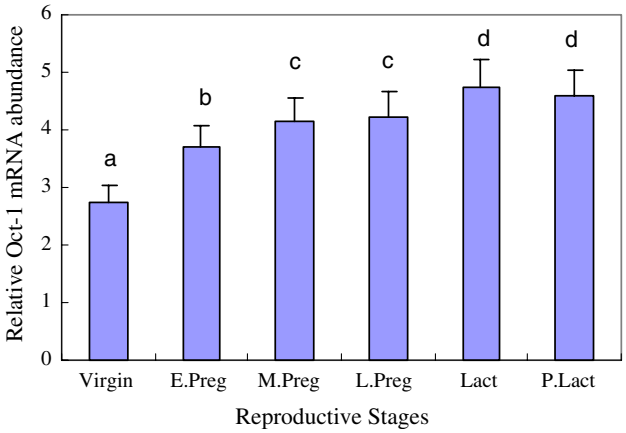


Fig. 1 Oct-1 mRNA levels in mouse mammary gland epithelial cells at the different reproductive stages. Total RNA were isolated from the mammary epithelial cells collected from mouse mammary glands at the stages of virgin, early pregnancy (5–7 days, E.Preg), mid-pregnancy (11–13 days, M.Preg), late pregnancy (17–19 days, L.Preg), lactation (7–9 days, Lact), and post-lactation (22–24 days, P.Lact). The Oct-1 mRNA levels were measured by quantitative real-time PCR and normalized by β-actin levels. *Bars* with *different letters* are significantly different ($P < 0.05$)

Oct-1 can efficiently interact with both isoforms of STAT-5 as well as GRs that are mutated at various potential phosphorylation sites

To investigate whether Oct-1 interacts with STAT-5A and STAT-5B differently during hormonal induction of β-casein promoter activity, an Oct-1 expression plasmid

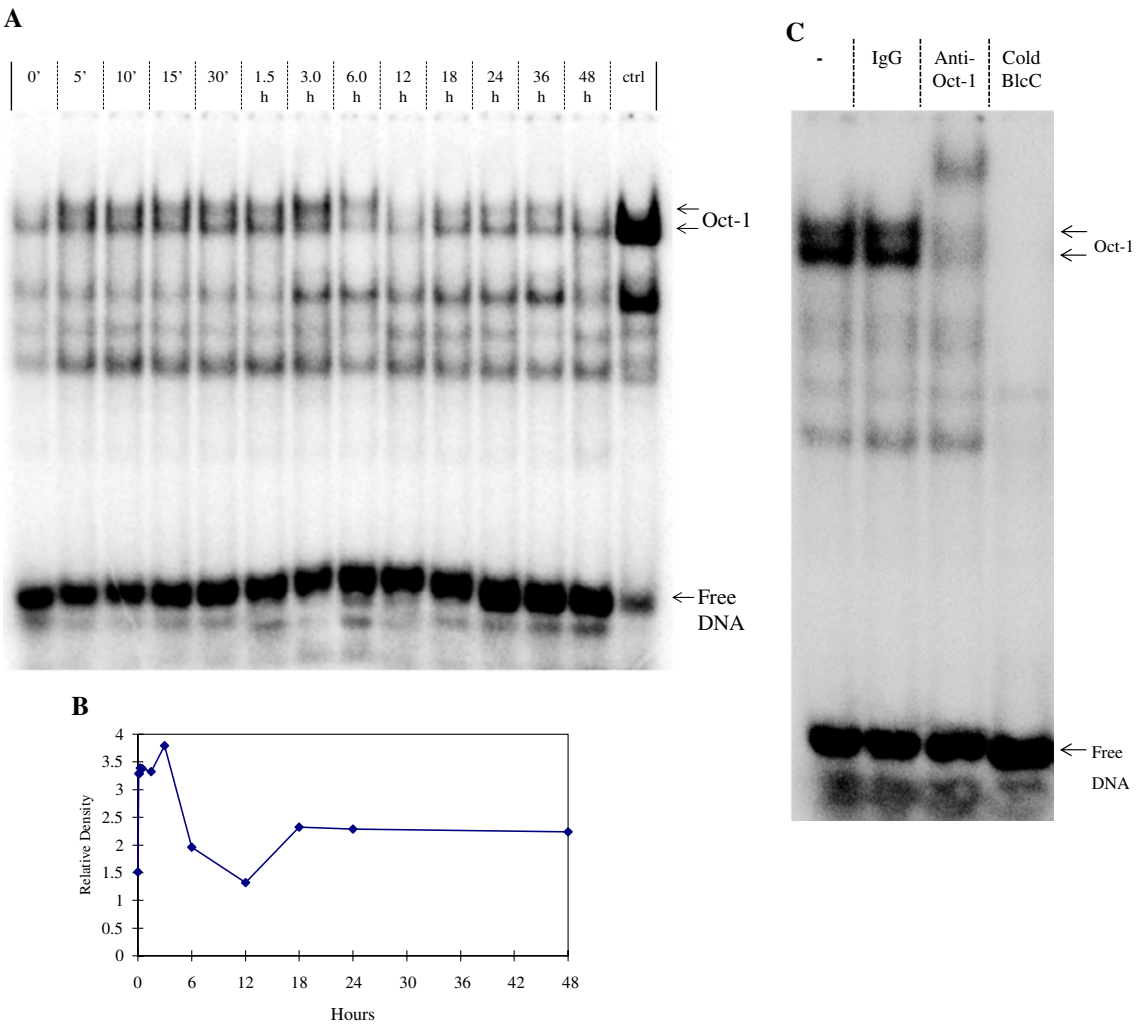


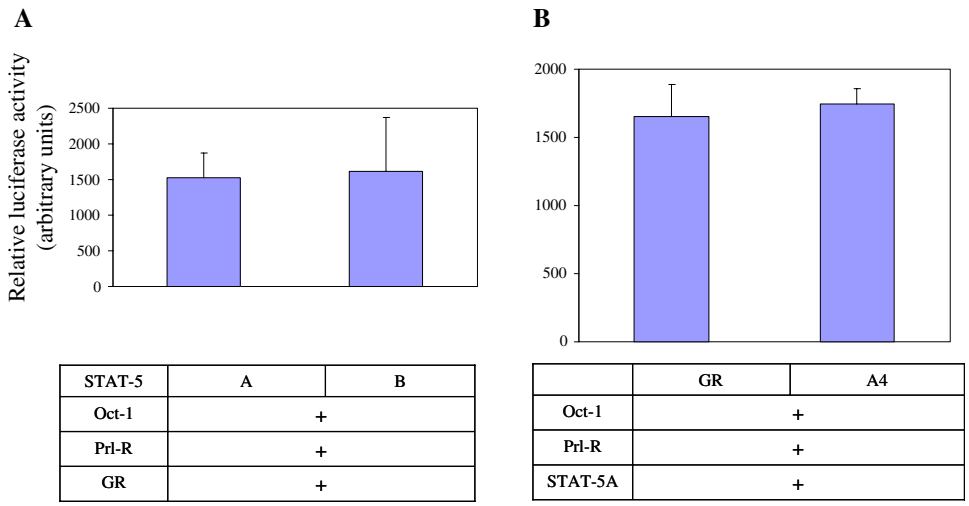
Fig. 2 Kinetics of Oct-1 binding to the β-casein promoter by lactogenic hormone treatment. **a** COS-7 cells were transfected with the expression plasmids of Prl-R, STAT-5, and GR and then treated with Prl and Dex. Nuclear extracts were prepared at different times after hormonal treatment and examined by EMSA with a radiolabeled Block C probe in a 4.0% polyacrylamide gel. Oct-1 complexes are indicated with *arrow*. **b** The intensity of the Oct-1 complexes in (**a**) was quantified, and the data are represented graphically. **c** Verification of Oct-1 complex specificity was carried out by addition of an unlabeled Block C oligonucleotide competitor to the reaction (cold BlcC) and by supershift assay of the nuclear extract at 3 h after hormonal treatment in (**a**) with either IgG or anti-Oct-1 antibody

was co-transfected into COS-7 cells with STAT-5A or STAT-5B in the presence of Prl-R and GR before cells were treated with Prl and Dex. No significant difference was observed between STAT-5A and STAT-5B in their functional interaction with Oct-1 during hormonal induction (Fig. 3a). Without transfection with STAT-5A and STAT-5B, the hormonal induction was marginal [9].

In addition, the effect of potential phosphorylation sites of GR on the interaction of GR with Oct-1 in hormonal induction was investigated by comparison of GR with its mutant A4, which carries multiple mutations at serines 150, 212, 220, and 234 (change to alanine). Mutant A4 exhibited similar activity to wild-type GR on the mouse mammary tumor virus (MMTV) promoter, but significantly lower activity in a minimal reporter GRE2-reporter system [7]. As shown in Fig. 3b, no significant difference was observed between GR and A4 in Oct-1's activation of the β-casein promoter by lactogenic hormones. Without transfection with GR and A4, the hormonal induction was marginal [9]. Thus, the potential phosphorylation sites of GR at serines 150, 212, 220, and 234 may not be involved in the interaction of GR with Oct-1 in hormonal induction.

Activation of the hormonal induction of β-casein promoter activity by Oct-1 in Oct-1-deficient cells

Recently, an Oct-1-deficient (Oct-$1^{-/-}$) MEF cell line was developed and obtained from Dr. D. Tantin [12]. This cell line was used to investigate the degree of endogenous Oct-1's role in hormonal induction of the β-casein promoter.



STAT-5	A	B
Oct-1	+	
Prl-R	+	
GR	+	

	GR	A4
Oct-1	+	
Prl-R	+	
STAT-5A	+	

Fig. 3 Comparisons of STAT-5A and STAT-5B (**a**) as well as GR and GR mutant on serine phosphorylation sites (A4) (**b**) in their functional interactions with Oct-1 in hormonal induction of β-casein promoter activity. (**a**) In 12-well plates, LHRRWT/pGL$_3$ (0.2 μg) and expression plasmids of Oct-1B, GR, and Prl-R (0.2 μg each) were co-transfected into COS-7 cells with a plasmid expressing either STAT-5A or STAT-5B (0.2 μg). **b** LHRRWT/pGL$_3$ (0.2 μg) and expression plasmids of Oct-1B, STAT-5A, and Prl-R (0.2 μg each) were co-transfected into COS-7 cells with a plasmid expressing either wild type or GR mutant A4. Cells were then treated with Prl and Dex. Reporter luciferase activities were expressed as the mean ± SE of one experiment in triplicate. Three independent experiments were carried out. Data from one representative experiment are shown

MEF cells were reconstituted to lactogenic hormonal responsiveness using the same method as those used in COS-7 cells by co-transfection of Prl-R, STAT-5, and GR expression plasmids. Oct-1 plasmid was co-transfected into the reconstituted MEF cells, followed by Prl and Dex treatment. As shown in Fig. 4a, the reconstitution of MEF cells was successful, as Prl and Dex induced the promoter activity by more than 50-fold in these cells. However, exogenous Oct-1 could only increase the hormonal induction further by 120%, similar to the induction in the COS-7 cells. However, residual Oct-1's binding activity could still be detected in Oct-1-deficient MEF cells as anti-Oct-1 antibody, but not anti-Oct-2, could still shift a weak complex on Block C oligonucleotide in the EMSA assay of the nuclear extract of Oct-1-deficient MEF cells (Fig. 4b). This residual Oct-1-binding activity was also shown by Wang et al. [12].

Discussion

In this study, we provided new lines of evidence that support the involvement of the ubiquitous Oct-1 in tissue-specific β-casein gene expression. First, the expression pattern of Oct-1 mRNA at different developmental stages is consistent with the β-casein gene expression in the mammary gland; and second, in reconstituted COS-7 cells, the lactogenic hormones can rapidly induce the endogenous and exogenous Oct-1 binding activity to the β-casein gene promoter. These new data are consistent with the previous evidence: (1) the Oct-1 binding activity is correlated well with the β-casein gene expression in the mammary gland at different developmental stages [17] and can be induced by the lactogenic hormonal treatment in the virgin mouse mammary gland in vivo [10, 17] as well as in the mammary epithelial cells in vitro [17]; (2) Oct-1 activates hormonal induction of β-casein gene expression in reconstituted COS-7 cells in a dose-dependent manner [9]; and (3) mutation and orientation changes of Oct-1 binding site in the β-casein gene promoter dramatically reduce the hormonal induction of β-casein gene transcription [9, 17].

The rapid increase of Oct-1 binding activity after lactogenic hormonal treatment indicates that the lactogenic hormones can modulate Oct-1 activity by post-transcriptional/translational mechanisms, potentially by phosphorylation. One example is that prolactin rapidly induces phosphorylation of STAT-5, which then forms dimers, translocates into the nucleus and binds to the β-casein gene promoter [1, 11]. Prolactin may also induce phosphorylation of Oct-1, as our previous study showed that the functional interactions of Oct-1 with STAT-5 and GR are prolactin signaling-dependent [9, 17]. Oct-1 has been shown to be phosphorylated by several kinases, including PKA [18, 19] and cyclic GMP-dependent protein kinase [20]. The importance of Oct-1 phosphorylation to its function has recently been demonstrated in a study in which mutations on multiple potential phosphorylation sites of Oct-1 diminished its rescue function in Oct-1$^{-/-}$ murine embryonic fibroblasts [21]. An alternative

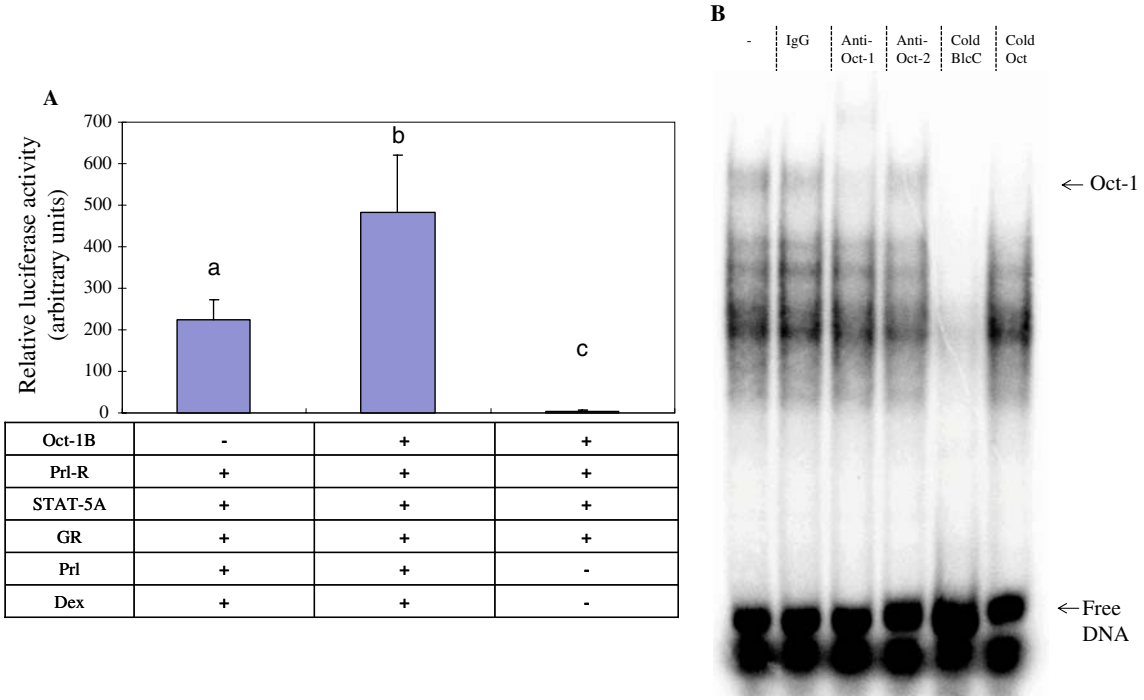


Fig. 4 Hormonal induction of β-casein promoter activity in Oct-1-deficient (Oct-$1^{-/-}$) cells. **a** Oct-1-deficient (Oct-$1^{-/-}$) mouse embryo fibroblast (MEF) cells were plated in six-well plates and transfected with or without 0.3 μg of mOct-1B/pcDNA3.1 plus 0.1 μg of each plasmid of Prl-R, STAT-5A, GR, and $LHRR_{WT}$/pGL3. The cells were then treated with or without Prl and Dex as indicated. Reporter luciferase activities were expressed as the mean ± SE of one experiment in triplicate. *Bars* with *different letters* are significantly different ($P < 0.05$). Three independent experiments were carried out. Data from one representative experiment are shown. **b** Nuclear extracts were prepared from untransfected and untreated MEF cells, and 20 μg of the extracts were subjected to EMSA using radiolabeled Block C oligonucleotide probe. Rabbit IgG, anti-Oct-1, anti-Oct-2, unlabeled block C oligonucleotide (cold BlcC), or unlabeled Oct-1 motif oligonucleotide (Cold Oct) was added to the binding reactions

explanation is that the phosphorylation of STAT-5 or other transcription factors in hormonal signaling may recruit Oct-1 to the β-casein gene promoter by direct interactions.

This study showed that both isoforms of STAT-5, STAT-5A and STAT-5B, can functionally interact with Oct-1 in hormonal induction of β-casein gene transcription. The structural difference between STAT-5A and STAT-5B is in their C-termini. Thus, our data suggests that the C-terminus of STAT-5 is not involved in the functional interactions with Oct-1 and GR. This is in contrast to the C-terminus of Oct-1, which has been shown to play a role in this process [9]. In addition, our data also showed that the phosphorylation sites of GR at serines 150, 212, 220, and 234 are not involved in the interactions of GR with Oct-1 and STAT-5 in hormonal induction. Although the phosphorylation of GR has been shown to play a role in regulating the expression of several glucocorticoid-induced genes [22], its role in hormonal regulation of β-casein gene transcription has not been observed.

The 50-fold induction of β-casein promoter activity by lactogenic hormones in reconstituted Oct-1-deficient (Oct-$1^{-/-}$) MEF cell line is consistent with our previous results. Oct-1 binding to the β-casein promoter activates the hormonal induction of the promoter activity, but this binding is not essential [9]. This also suggests that the presence of Oct-1 (binding-independent functions) may not be essential in the process. In addition, the exogenous Oct-1 activated the induction in Oct-1-deficient MEF cells to the same degree as in the COS-7 cells, which contain a low level of endogenous Oct-1 activity [14]. As we have demonstrated that the activation of the hormonal induction of the β-casein promoter by Oct-1 is dose-dependent [9], the low levels of Oct-1 activity may not be sufficient for the regulation process. However, since marginal Oct-1 binding was also observed in Oct-1-deficient MEF cells, whose nature is not known, this Oct-1 activity may have contributed to the results observed in the MEF cells.

In summary, in this study, we provide new lines of evidence which support the involvement of the Oct-1 in hormonal induction of β-casein gene expression.

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