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YY1 Represses β-Casein Gene Expression by Preventing the Formation of a Lactation-Associated Complex

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Site-specific mutagenesis of the highly conserved milk box (−140 to −110) region suggested that β-casein expression is regulated by a hormone-mediated relief of repression (M. Schmitt-Ney, W. Doppler, R. K. Ball, and B. Groner, Mol. Cell. Biol. 11:3745–3755, 1991). However, when this sequence was placed upstream of a heterologous thymidine kinase promoter, it activated reporter gene expression. This apparent paradox was resolved when the *trans*-acting factor YY1, capable of acting as both a positive and negative regulator, was shown to interact with the milk box region, using bacterially expressed YY1 and specific oligonucleotide and antibody competition experiments. Second, it was demonstrated that extracts prepared from several cell types contained a protein(s) interacting with the mammary gland-specific factor (MGF) binding site, previously shown to be required for β-casein promoter activity (Schmitt-Ney et al., Mol. Cell. Biol. 11:3745–3755, 1991). Sequence analysis of this site revealed similarity to the gamma interferon-activated sequence, suggesting that MGF may be related to the stat91 signaling protein. Finally, using an oligonucleotide encompassing both the YY1 and MGF sites, we detected a slow-mobility complex only in extracts from mammary glands at late pregnancy and lactation (lactation-associated complex [LAC]). Site-specific mutation of the YY1 binding site led to an enhancement in LAC DNA binding activity, while mutation of the MGF site decreased detectable LAC. These results support a model in which lactogenic stimuli lead to a decrease in YY1 binding, and subsequent increased formation of LAC at a nearby binding site, to stimulate β-casein transcription.

The caseins are encoded by a family of milk protein genes present as a large cluster on mouse chromosome 5 (9). The casein gene family is regulated in synchrony (48) in response to various developmental cues, including changes in the concentrations of several lactogenic hormones (48), the levels of certain growth factors (47), and components of the extracellular matrix (37, 46). Studies in transgenic mice and in transfected cells have revealed that both proximal and distal genetic regulatory elements are required for the appropriate regulation of casein gene expression (30). The proximal region of the β-casein gene promoter is sufficient, however, to elicit appropriate hormonal regulation in transfected mouse mammary epithelial cells (38) and developmental regulation in transgenic mice (21). More distal genetic elements appear to be required for efficient expression of the individual genes within the casein gene locus (30).

Several highly conserved sequence motifs have been identified in the proximal promoters of the calcium-sensitive casein genes (51); each of these regions has been distinguished as a binding site for putative *trans*-acting factors (10, 19, 38, 50). Further deletion and site-specific mutational analyses of the β-casein proximal promoter have defined a minimal ~100-bp region necessary for proper hormonal regulation of a heterologous reporter gene (6a, 49). Within this region, several putative *trans*-acting factor binding sites exist; two are of particular interest. A positively acting sequence, centered around −90 relative to the transcriptional start site, was reported to be a binding site for a hormone-regulated, 89-kDa mammary gland-specific factor, designated MGF (38). Site-specific mutagenesis of the MGF binding site led to complete inactivation of a −300 to +1 β-casein promoter-reporter construct. The second of these interesting regions, originally termed the milk box consensus (17), at −140 to −110, was shown to repress reporter gene expression. Deletion or site-specific mutagenesis of the milk box region led to increased basal activity of a β-casein–chloramphenicol acetyltransferase (CAT) construct. To date, however, no single binding region which is able to confer proper hormonal regulation of a reporter gene in cells has been characterized, suggesting that cooperative factor interactions may be required for appropriate hormonal regulation of β-casein transcription.

To begin to decipher this complex factor interplay, the *trans*-acting factor–DNA interactions at these two important sites in the β-casein promoter were further characterized. Initially, the ubiquitous *trans*-acting factor YY1 (yin and yang 1) (41) was identified in mammary epithelial cell extracts and shown to bind in the β-casein repressor milk box region. Second, an MGF DNA binding activity was observed in whole cell extracts (WCE) and nuclear extracts of several different cell types, suggesting that a protein(s) interacting with this site is ubiquitous and not mammary gland specific. Sequence analysis of the MGF binding site revealed a striking similarity to the recently described gamma interferon (IFN-γ)-activated sequence (GAS), present in a number of genes (27). Thus, one of the factors interacting with the MGF site may be the stat91 signaling protein (42) or a close relative thereof. Finally, electrophoretic mobility shift analyses (EMSAs), using an oligonucleotide encompassing both the MGF and YY1 binding sites, revealed a slow-mobility complex present only in extracts from mammary glands at late pregnancy and lactation. This binding activity was designated LAC, for lactation-associated complex. YY1 binding to the β-casein promoter appeared to block the formation of LAC, and cooperative interactions between factors binding near the MGF and YY1 sites appeared to be required for LAC formation. A model is proposed in which a repressor protein, YY1, blocks the formation of an activator DNA binding complex, LAC. Hormonal stimuli

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lead to a decrease in YY1 binding and the formation of LAC, activating β-casein transcription.

(Preliminary results of a portion of the work described herein were published previously [31].)

MATERIALS AND METHODS

Cell culture. HC11 cells were grown on plastic culture dishes. Growth medium consisted of RPMI 1640 with 10% bovine calf serum, 5 μg of insulin per ml, 2 mM glutamine (JRH Biosciences), 10 ng of epidermal growth factor per ml, and 50 μg of gentamicin (Sigma) per ml. For hormonal induction of β-casein expression (prolactin induced), cells were maintained at confluence for 2 days in growth medium and then switched at day 3 postconfluence to a priming medium for 24 h. Priming medium contained 10% dextran-charcoal stripped horse serum (JRH Biosciences) and included no epidermal growth factor; all other ingredients were the same as for growth medium. At days 4, 5, and 6 postconfluence hydrocortisone (1 μg/ml; Sigma) and ovine prolactin (1 μg/ml; prepared fresh daily; National Institutes of Health) were added to fresh priming medium. At day 7 of confluence, cells were harvested by scraping. Control (uninduced) HC11 cells were not exposed to prolactin but otherwise treated identically. Cell pellets were quick frozen in liquid nitrogen and stored at −70°C. Induction of β-casein expression was determined by Northern (RNA) analysis, as described previously (4), with a random oligonucleotide-primed, ^{32}P-labeled exon 7 cDNA from the rat β-casein gene. Transfection of HC11 and NIH 3T3 cells with β-casein–CAT constructs was performed as described previously (7).

Extract preparation. WCE were prepared by a modification of the procedure of Zimarino and Wu (52). Frozen cell pellets were resuspended in 1 volume of Wu buffer [10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES; pH 7.9), 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM dithiothreitol, 5% glycerol] with various protease inhibitors (1 mM soybean trypsin inhibitor, 1 mM benzamidine, and 1 mM phenylmethanesulfonyl fluoride) containing 400 mM KCl and centrifuged at 14,000 × *g* for 30 min at 4°C. One milliliter of the supernatant was added to 250 μl of saturated (at 0°C) ammonium sulfate, incubated at 0°C for 20 min, and centrifuged at 14,000 × *g* for 10 min. The supernatant was removed, and 400-μl aliquots were added to 600 μl of saturated ammonium sulfate. After another 20-min incubation at 0°C and 10-min centrifugation at 14,000 × *g*, the supernatant was discarded and the pellet was resuspended in Wu buffer containing 100 mM KCl. The resuspended extracts were dialyzed for 1 h in a microdialysis unit (Bethesda Research Laboratories) versus 1 liter of Wu buffer containing 100 mM KCl. The exclusion limit of the dialysis membrane was 6 to 8 kDa. For tissue WCE, 0.2 g of frozen tissue was minced, placed in 1 ml of modified buffer A (10 mM HEPES [pH 7.9], 0.3 M KCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM dithiothreitol, 10% glycerol, 0.25% Nonidet P-40, 0.15 mM spermine, 0.5 mM spermidine, protease inhibitors as in Wu buffer), and dissociated with a Brinkmann Polytron for 1 min at a setting of 6. Subsequent steps were the same as for cell extracts except that tissue extracts were dialyzed against modified buffer A. Nuclear extracts were prepared by the method of Dignam et al. (6). Protein concentrations were determined by a Bio-Rad assay.

EMSA. All steps were carried out at room temperature unless otherwise noted. WCE (10 μg) were diluted to 10 μl with 100 mM KCl-containing Wu buffer and preincubated with 4 μg of poly(dI-dC) · poly(dI-dC) (Pharmacia) and unlabeled competitor oligonucleotides for 10 min. Ten microliters of binding mix [2.5 mg of bovine serum albumin per ml, 8% Ficoll, 0.1 mg of $pd(N)_5$ (Pharmacia) per ml] containing approximately 0.5 ng of ^{32}P-labeled probe (ca. 50,000 cpm) was then added. Binding was allowed to proceed for 10 min. For the anti-YY1 supershift assay, 0.5 or 1.0 μl of a polyclonal antibody was subsequently added, and the incubation continued for 20 min. Samples were analyzed on either 4 or 6% 0.5× Tris-borate-EDTA-containing polyacrylamide (38:2 acrylamide/bisacrylamide ratio) gels at a constant 10 V/cm. Gels were vacuum dried for 45 min at 75°C onto Whatman 3MM paper.

Bacterial expression vectors. The expression vectors encoding glutathione *S*-transferase (GST)-YY1 (GST:YY1/SB) and ΔYY1 (GST:YY1/SH) were prepared by subcloning the *Sty*I-*Bam*HI fragment containing the entire coding region of the YY1 cDNA (amino acid residues 1 to 414) and the *Sty*I-*Hin*dIII fragment encoding amino acid residues 1 to 331 from pGem:YY1 (41) into the *Eco*RI-linearized, blunted GST expression vector pGEX-2T(128/129) (15). GST fusion junctions were sequenced with a Sequenase kit (U.S. Biochemical Corp.) to confirm reading frame integrity.

Fusion proteins. GST fusion proteins were prepared essentially as described previously (45), with the following modifications. Overnight cultures of *Escherichia coli* BL21 transformed with pGEX-2T(128/129) or the YY1 recombinants described above were diluted 1:10 in Luria-Bertani medium containing ampicillin (50 μg/ml) and grown for 1 to 2 h. Isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma) was then added to a final concentration of 0.1 mM, and the cultures were incubated for another 3 to 4 h. Cells were pelleted and sonicated in 50 mM Tris (pH 8.0)–50 mM NaCl–2 mM EDTA–2 mM phenylmethanesulfonyl fluoride–0.1 mg of lysozyme per ml–120 U of aprotinin (Sigma) per ml–1 mM dithiothreitol, 1% Triton X-100, 10 μg of DNase I per ml–10 mM $MgCl_2$ at moderate settings until no longer viscous (20 to 30 s). Cell debris was then pelleted at 13,000 rpm for 10 min at 4°C. The supernatant was mixed with glutathione-agarose (Sigma) at 4°C for 30 min. The beads were then pelleted and washed several times with ice-cold NETN (20 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40). Fusion proteins were eluted by incubating the beads at 4°C for 10 min in 10 mM reduced glutathione. Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by Coomassie blue staining.

Antibodies. Rabbit polyclonal antibodies against recombinant human YY1, chicken α-actin, and the chicken serum response factor (SRF) were kindly provided by Robert Schwartz and Te Chung Lee and have been described previously (22).

Oligonucleotides. Oligonucleotides were labeled, using the Klenow enzyme (Boehringer Mannheim Biochemicals) to fill in 5′ overhangs, with α-^{32}P-dideoxynucleotides as described by Maniatis et al. (25). The sequences of the coding strands of double-stranded oligonucleotides are as follows: M, 5′-GAA TTT CTT GGG AAA GAA AAT AGA AAG AAA CCA TT TT CTA-3′; B, 5′-TAATCATGTGAACTTCTTGGAATTAAG GAACTTTTGAATAT-3′; M/B, 5′-AAATAGAAAGAAAC CAT TT TC TA ATC AT GTG AA CTT CT TGGAATTAAGG AA-3′; M/B (mutant MGF), same as M/B except in the MGF binding site (5′-AATTAAAAGAA-3′); M/B (mutant YY1), same as M/B except in the YY1 binding site (5′-AAATTT-3′); YY1 wild type, 5′-GATCCACCCAAATATGGCGACGAGA TC-3′; YY1 mutant, 5′-GATCCACCCAAATATGGATCCG AGATC-3′; and C, 5′-TGAATTGCTGCCTTGTTTAATGT CCCCCAGAATTTCTTGGAAAGAAAAT-3′. Note that the B and M/B oligonucleotides contain two G-to-A base pair transitions at positions −100 and −82 compared with the

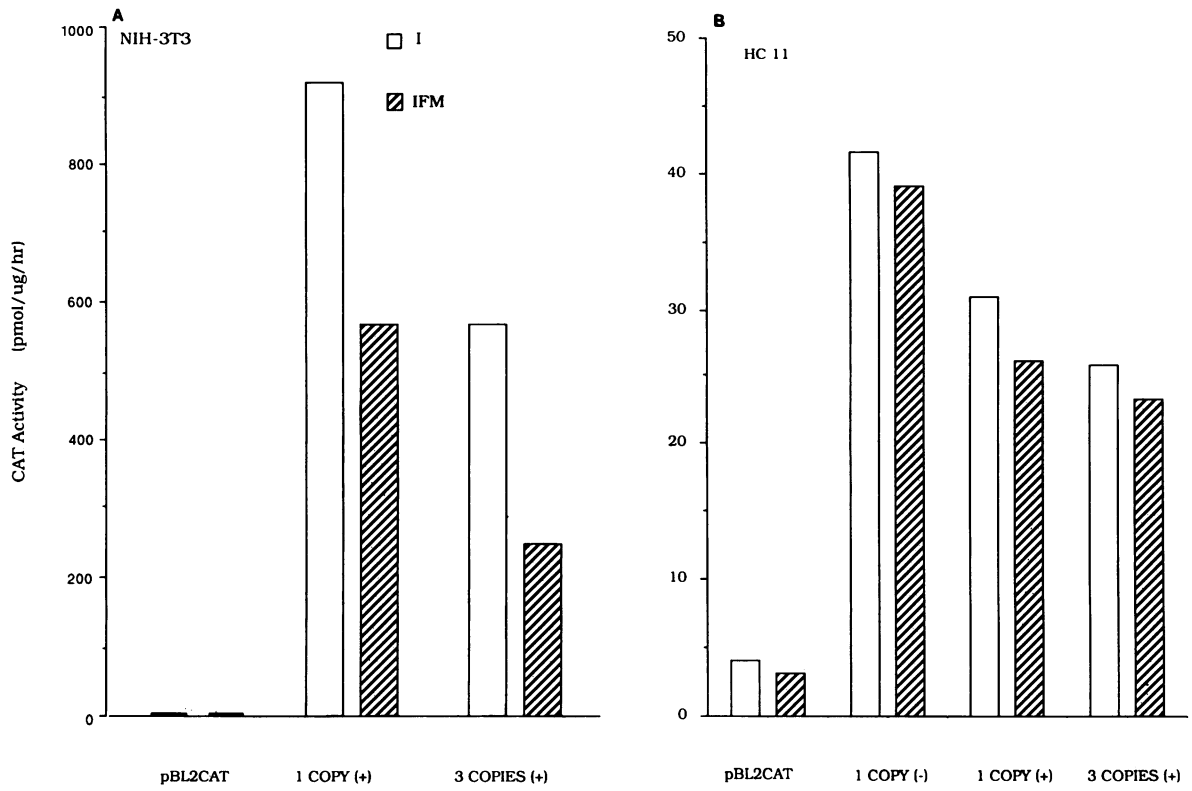

FIG. 1. Effect of the milk box consensus sequence on the activity of a heterologous promoter. One or three copies of the −140 to −108 oligonucleotide sequence containing *Xba* linkers were inserted at the *Xba*I site of the pBLCAT vector (22). The orientations of the inserts are shown as (+) to indicate their 5′-to-3′ orientation and (−) to indicate the opposite orientation, relative to the start site of transcription in the β-casein promoter. Hormonal treatment (insulin alone [I] or insulin, hydrocortisone, and prolactin [IFM]) and determination of CAT activity are described in Materials and Methods.

wild-type rat β-casein sequence (51). Neither of these guanosines interacts with MGF, however, as determined by dimethyl sulfate interference assay (8a, 38), and no differences have been observed in EMSA using the wild-type sequences.

RESULTS

The β-casein repressor milk box region increases reporter activity. On the basis of site-specific mutational analysis of the −110 to −148 region of the β-casein promoter, the mechanism of regulation of β-casein gene expression was suggested to be a hormone-mediated relief of repression (38). To test this possibility, one or three copies of the milk box repressor region (−140 to −106 relative to the transcriptional start site; see also Fig. 2) were linked to a heterologous constitutive thymidine kinase (*tk*) basal promoter (from position −105) in front of a CAT reporter gene and stably transfected into both NIH 3T3 and HC11 mammary epithelial cells. HC11 is a clonal mammary epithelial line capable of producing β-casein in response to prolactin while grown on plastic (2). CAT activity in pooled transfected HC11 and 3T3 cells was quantitated in the presence of insulin alone or in the presence of insulin, hydrocortisone, and prolactin (Fig. 1). No significant differences in CAT activity were observed in any of the transfectants in response to the addition of lactogenic hormones. However, NIH 3T3 (Fig. 1A) and HC11 (Fig. 1B) cells containing one or three copies of the milk box consensus displayed an unexpected hormone-independent increase in CAT activity over the parental vector alone. This increase also appeared to be independent of the orientation of the milk box consensus.

This result suggested that the milk box consensus region could act either as a hormone-responsive repressor or as a hormone-independent activator of gene transcription, depending on promoter context. A careful sequence analysis revealed that the milk box contained a consensus binding site for the ubiquitous *trans*-acting factor YY1, a member of the GL1-Krüppel factor family. Members of this family are able to both positively and negatively regulate transcription, depending on factor concentration (13) and binding site context (1). In fact, when multimers of a c-*myc* YY1 (CF1) binding site were cloned upstream of the identical *tk* promoter-CAT construct, results quite similar to those summarized in Fig. 1 for the milk box consensus were reported: transfection of the c-*myc* construct into L or plasmacytoma cells also led to an increase in CAT activity in comparison with cells containing only the parental vector (29). Thus, the presence of a YY1 binding site may explain why the milk box sequence acts as a repressor region in the β-casein promoter but as an activator in the context of the *tk* promoter. The putative YY1 binding site in the β-casein promoter also displayed identical guanosine contacts, as determined by dimethyl sulfate interference assay, as YY1 binding sites in other promoters (8a, 11, 40). While these results were suggestive that YY1 plays a role in the regulation of casein gene expression, it remained to be established whether YY1 actually interacted with this region of the β-casein promoter.

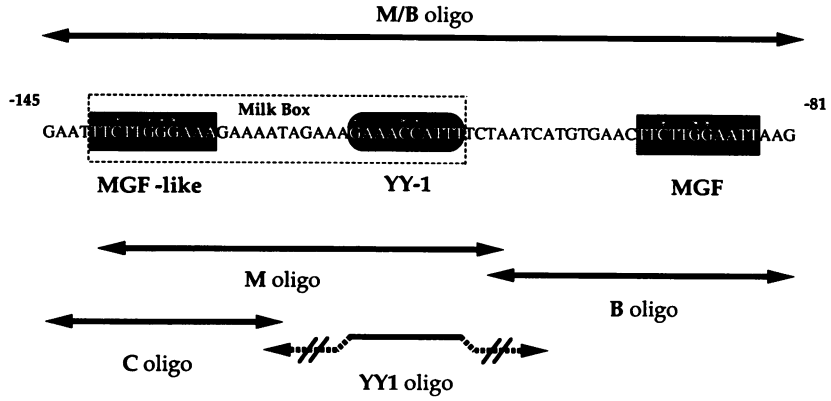

FIG. 2. Diagrammatic representation of oligonucleotides utilized in EMSA. Sequences are given in Materials and Methods. The YY1 oligonucleotide (oligo) shares a 7-of-13-bp similarity with the β-casein promoter at the consensus YY1 site. It is otherwise nonidentical. *, protected residues as determined by dimethyl sulfate interference assay (8a, 39). The mutant MGF M/B oligonucleotide contains two G-A transitions at the protected guanosine residues in the MGF sequence (−89 and −88). The mutant YY1 M/B oligonucleotide contains two C-T transitions at the protected residues in the YY1 binding consensus region (−114 and −113).

YY1 is present in mammary epithelial cells and binds in the milk box repressor region of the β-casein promoter. (i) An unrelated YY1 oligonucleotide abolishes M oligonucleotide EMSA complexes. To confirm the presence of a bona fide YY1 binding site in the β-casein milk box sequence, EMSAs, utilizing a 40-bp ^{32}P-labeled oligonucleotide (M; Fig. 2), were conducted to identify *trans*-acting factors interacting with this region. Two specific complexes, one of slow mobility and a second with faster mobility (most likely resulting from partial proteolysis [23]), were detected in WCE prepared from HC11 cells (Fig. 3). An oligonucleotide competitor containing only the consensus YY1 binding site was used to demonstrate that these complexes contained YY1. The YY1 competitor oligonucleotide (Fig. 2) was derived from the serum response element of the chicken α-actin proximal promoter (22) and shares no similarity with the β-casein promoter except for a 7-of-13-bp match in the consensus YY1 binding site of the milk box region. A fivefold excess of the competitor wild-type YY1 oligonucleotide was sufficient to abolish EMSA complexes observed with WCE isolated from both prolactin-induced (Fig. 3, lanes A to D) and uninduced (lanes H to K) HC11 cells. A mutant YY1 oligonucleotide, differing in three nucleotides and with a 100-fold-lower binding affinity (22), only slightly decreased the intensity of EMSA complexes even at a 100-fold excess of competitor (lanes E to G and L to N).

No qualitative or quantitative changes were detected in DNA binding activity in WCE prepared from prolactin-induced and uninduced HC11 cells (Fig. 3, lanes A and H). As expected, YY1 binding activity was also detected in several other cell types (lanes O to T) (16). WCE prepared from rat

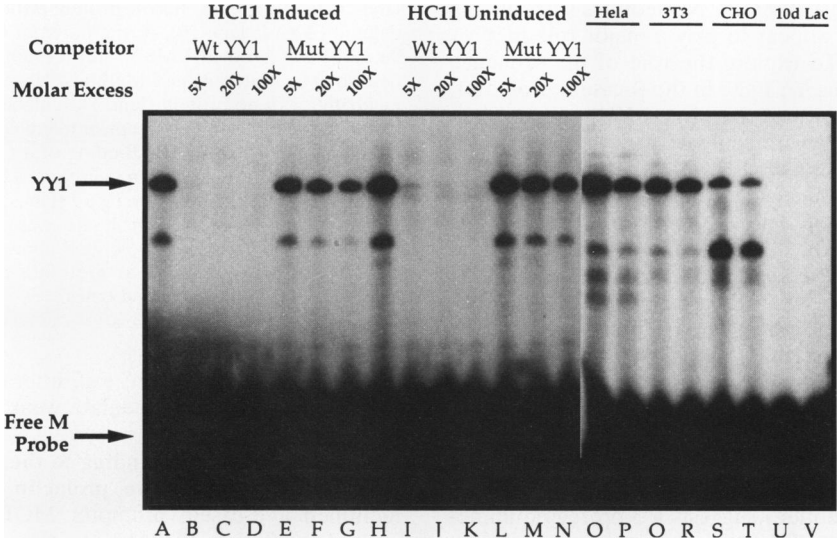

FIG. 3. Identification of factors binding in the milk box region (−140 to −107) of the rat β-casein promoter. Ten micrograms of WCE protein from prolactin-induced (lanes A to G) and uninduced (lanes H to N) HC11 cells and 10 μg (lanes O, Q, S, and U) or 5 μg (lanes P, R, T, and V) of WCE from other cell lines (HeLa, NIH 3T3, or CHO) or rat mammary tissue at 10 days of lactation (10d Lac) were subjected to EMSA with ^{32}P-labeled M oligonucleotide as described in Materials and Methods. Unlabeled wild-type YY1 oligonucleotide (Wt YY1) was used in competition analysis and added at the indicated molar excess to EMSA binding reactions prior to addition of labeled M oligonucleotide. A mutant form of the YY1 oligonucleotide (Mut YY1), differing in 3 bp and with a 100-fold-lower binding affinity for YY1, was also used.

mammary glands throughout all stages of development displayed no binding activity to the M oligonucleotide (lanes U and V and data not shown). Thus, a putative YY1 complex detectable in HC11 cells and other cell lines, but not in mammary tissue, was abolished with an unrelated oligonucleotide containing only a consensus YY1 binding site and was not abolished with a mutant form of this oligonucleotide.

(ii) A recombinant GST-YY1 protein binds in the β-casein milk box region. Hundreds of genes encode GL1-Krüppel zinc fingers (3). To verify that the β-casein repressor region bound authentic YY1, in vitro binding analysis was conducted with a bacterially synthesized recombinant YY1 protein. A human YY1 cDNA (kindly provided by T. Shenk) was placed downstream of a GST sequence to produce a recombinant fusion protein. Lysates from *E. coli* expressing the GST-YY1 fusion formed specific DNA-protein complexes with both the M (Fig. 4A, lanes 6 to 9) and YY1 (Fig. 4B, lanes 6 and 8) oligonucleotides. Identical lysates from *E. coli* transformed with a YY1 cDNA lacking the Zn^{2+} finger-DNA binding region (Fig. 4A, lane 10; Fig. 4B, lanes 10 and 11) and from bacteria transformed with the parent plasmid (Fig. 4A, lane 11; Fig. 4B, lanes 12 and 13) did not exhibit DNA binding activity. Utilizing the M oligonucleotide as a ^{32}P-labeled probe, addition of excess M oligonucleotide (5- to 100-fold) decreased the intensity of endogenous (Fig. 4A, lanes 2 to 5) YY1-containing complexes and of the slower-mobility GST-YY1-containing complexes (Fig. 4A, lanes 6 to 9). A 50-fold molar excess of the YY1 oligonucleotide also abolished the GST-YY1 (Fig. 4B, lanes 7 and 9) and endogenous YY1 (Fig. 4B, lanes 3 and 5) EMSA complexes observed with the ^{32}P-labeled YY1 oligonucleotide.

A recombinant GST-YY1 fusion protein was therefore able to bind specifically to both the β-casein promoter and an oligonucleotide containing a consensus YY1 binding site, while a GST-YY1 protein lacking the DNA binding region was unable to bind to either oligonucleotide. These results indicate that authentic YY1 is able to bind specifically to the YY1 site in the β-casein milk box region.

(iii) The β-casein milk box binds YY1 with relatively low affinity. Previously described YY1 binding regions share only a 3-bp consensus sequence, CAT (12). Nucleotides surrounding this consensus, however, appear to play a major role in YY1 binding affinity (1, 22). To explore the role of the sequence surrounding the consensus YY1 site in the β-casein promoter, the relative binding affinities of the M and YY1 oligonucleotides for the YY1 protein were compared. Both oligonucleotides were used as competitors in EMSA with the heterologous oligonucleotide. Greater than a 100-fold molar excess of the M oligonucleotide competitor was required to abolish YY1-containing complexes on the ^{32}P-labeled YY1 oligonucleotide (data not shown). Conversely, a fivefold molar excess of the YY1 oligonucleotide abolished YY1-containing complexes observed with the M oligonucleotide as a probe (Fig. 3, lanes A to D). These data suggest that YY1 binds with at least a 20-fold-lower affinity to the milk box region of the β-casein promoter compared with a previously described YY1 site in the chicken α-actin gene promoter. A sequence comparison between these two sites and the mutant YY1 oligonucleotide (with at least a 100-fold-lower binding affinity for YY1 than the wild-type YY1 oligonucleotide) suggests a 3-bp region immediately 5′ to the conserved CCATNT sequence may be responsible for these differences in affinity. The β-casein (gAAAc cattt) and mutant YY1 (gTCGccatat) oligonucleotides both differ from the YY1 consensus oligonucleotide (gGATccatat) at these positions. Relatively low YY1 binding affinity in the β-casein promoter may play an important role in the regulation

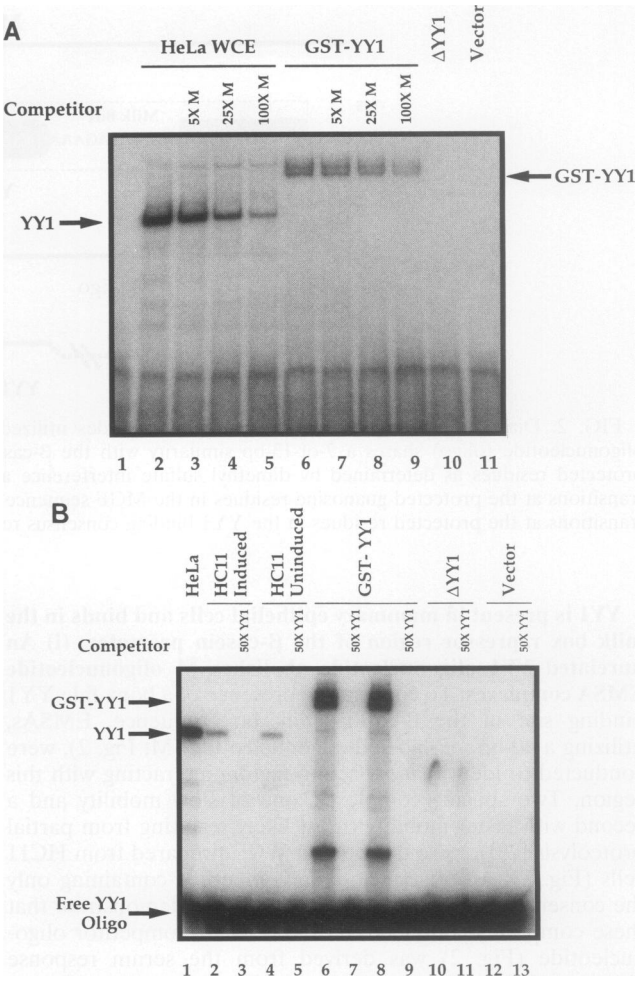

FIG. 4. (A) Binding of a GST-YY1 fusion protein to the M oligonucleotide. Ten micrograms of HeLa WCE (lanes 2 to 5) or 1 μg of lysates of *E. coli* expressing a full-length fusion protein (GST-YY1; lanes 6 to 9), a GST fusion protein with the DNA binding region deleted (ΔYY1; lane 10), or the parental GST vector alone (Vector; lane 11) was used in EMSA with ^{32}P-labeled M oligonucleotide as described in Materials and Methods. Negative control, labeled probe incubated with no protein (lane 1). Unlabeled M oligonucleotide was added to the binding reaction prior to labeled M oligonucleotide at the indicated molar excess. (B) Binding of a GST-YY1 fusion protein to the YY1 oligonucleotide. Ten micrograms of HeLa WCE (lane 1) and HC11 WCE (lanes 2 to 5) or 10 μg (lanes 6, 7, and 10 to 13) or 5 μg (lanes 8 and 9) of *E. coli* lysate was subjected to EMSA with ^{32}P-labeled YY1 probe as described above. Unlabeled YY1 oligonucleotide (oligo) was added at a 50-fold molar excess prior to ^{32}P-labeled probe in the indicated lanes.

of casein gene expression; e.g., interactions with other DNA binding proteins may modulate weak YY1 binding (see Discussion).

At least one protein binding to the MGF site is ubiquitous and is not responsive to prolactin. Previous studies have identified a β-casein promoter MGF binding site centered around −90 and 15 bp 3′ to the YY1 binding site (38). Site-specific mutational analysis showed the integrity of the MGF binding region to be essential for β-casein promoter activity; i.e. a 2-bp change at this site abolished both MGF binding and the activity of a −300 to +1 β-casein–CAT reporter construct (38). To further characterize factors inter-

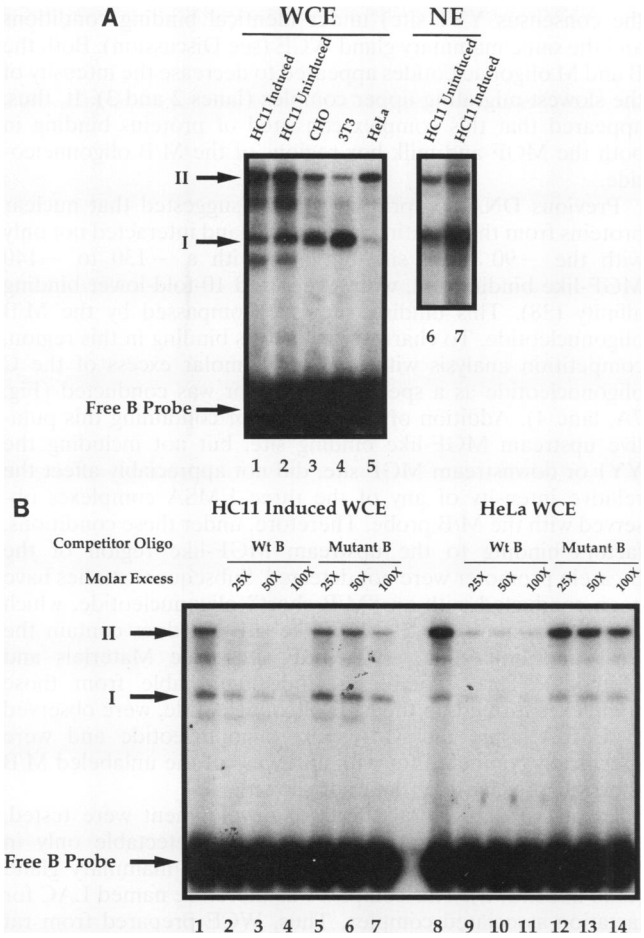

FIG. 5. (A) Binding of WCE prepared from several cell types to the B oligonucleotide, which contains the MGF binding site. Ten micrograms of WCE from prolactin-induced HC11 (lane 1) and uninduced HC11 (lane 2), CHO (lane 3), NIH 3T3 (lane 4), and HeLa (lane 5) cells and nuclear extracts (NE) prepared from prolactin-induced (lane 7) or uninduced (lane 6) HC11 cells were used in EMSA with ^{32}P-labeled B oligonucleotide (oligo) as described in Materials and Methods. (B) B oligonucleotide competition analysis; comparison of HC11 and HeLa WCE. Ten micrograms of WCE from HeLa (lanes 8 to 14) and prolactin-induced HC11 (lanes 1 to 7) cells was used in EMSA with ^{32}P-labeled B oligonucleotide. Unlabeled wild-type B oligonucleotide (Wt B; lanes 2 to 4 and 9 to 11) or mutant B oligonucleotide (Mutant B; lanes 5 to 7 and 12 to 14), differing in 2 bp previously shown to be contact sites for the MGF protein (8a, 39), was added prior to labeled B oligonucleotide at the indicated molar excess.

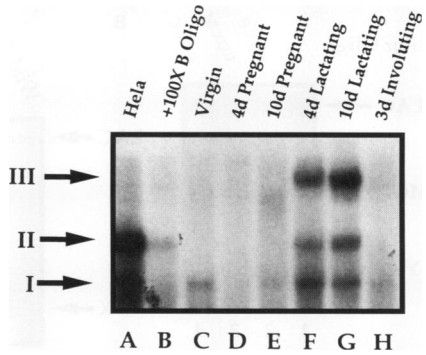

FIG. 6. Changes in B oligonucleotide binding activities throughout mammary gland development. Ten micrograms of HeLa WCE (lanes A and B) or rat mammary gland WCE from virgin rats (Virgin; lane C), rats at 4 days of pregnancy (4d Pregnant; lane D), 10 days of pregnancy (10d Pregnant; lane E), 4 days of lactation (4d Lactating; lane F), 10 days of lactation (10d Lactating; lane G), or 72 h after pup removal (3d Involuting; lane H) was used in EMSA with ^{32}P-labeled B oligonucleotide (Oligo). Specific DNA binding was shown by addition of a 100-fold molar excess of unlabeled B oligonucleotide (lane B).

acting with this region and to characterize changes in binding activity of these factors in response to prolactin and throughout mammary gland development, EMSAs were conducted with the B oligonucleotide (Fig. 2; −107 to −67), encompassing the previously described MGF binding region.

WCE and nuclear extracts prepared from confluent prolactin-induced and uninduced HC11 cells formed similar complexes with the B oligonucleotide (Fig. 5A, lanes 1, 2, 7, and 6); no quantitative or qualitative differences in DNA binding activities were observed after treatment with lactogenic hormones under these conditions. WCE prepared from other cell lines derived from different species and tissues also formed similarly migrating complexes under these conditions (lanes 3 to 5). All of these complexes could be specifically competed for with an excess of unlabeled B oligonucleotide at similar levels of competitor (Fig. 5B, lanes 1 to 4 and 8 to 11 and data not shown), suggesting they represent a specific interaction between DNA probe and binding factor(s). A mutant form of the B oligonucleotide, differing in two nucleotide pairs previously determined to be required for MGF binding and β-casein promoter function (Fig. 1) (8a, 38), did not appreciably decrease the observed binding of the MGF complex until present at a 100-fold molar excess of competitor (lanes 5 to 7 and 12 to 14).

Therefore, complexes with indistinguishable mobilities bound to the MGF site of the β-casein B oligonucleotide in EMSAs utilizing WCE from several cell lines; all of these complexes were shown to be specific. WCE and nuclear extracts of HC11 cells contained B oligonucleotide-binding activity, whether or not the cells had been exposed to prolactin. Thus, similar or identical factors capable of binding to the MGF site are present in HC11 and other cell lines, and prolactin treatment is not required for binding activity.

MGF DNA binding activity changes during mammary gland development. To explore changes in binding to the MGF site of the β-casein promoter throughout mammary gland development, WCE prepared from rat mammary glands at various stages of development (identical to those used with the M oligonucleotide) were used in EMSA with the B oligonucleotide (Fig. 6). Relatively low levels of DNA binding activity were observed in WCE isolated from mammary glands of virgin and pregnant animals (lanes C to E). An increase in binding activity was observed in WCE prepared from lactating mammary glands (lanes F and G). Pups were removed from one animal, and involuting gland extracts were prepared 72 h later. These extracts showed little DNA binding activity (lane H). Two complexes (complexes I and II, as seen with other cell lines; see also Fig. 5A) which were specifically competed for with a 100-fold molar excess of unlabeled B oligonucleotide were observed in HeLa WCE (Fig. 6, lane B). These complexes migrated at the same position as the two faster-migrating complexes observed in WCE isolated from lactating mammary glands. WCE prepared from mammary glands at 4 and 10 days of lactation, however, contained a third, more slowly migrating complex (complex III) not seen in any of the cell lines tested. To test for extract integrity, the identical mammary gland

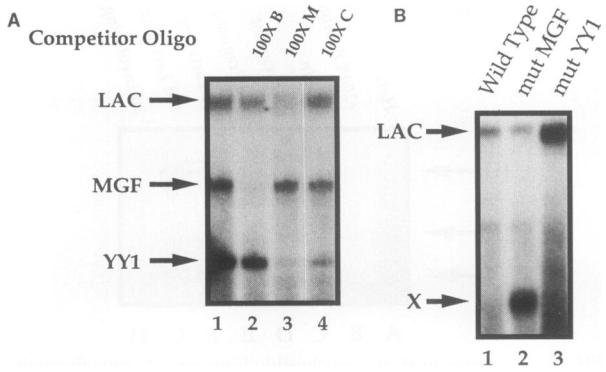

FIG. 7. (A) Competition analysis of WCE prepared from a rat mammary gland at 10 days of lactation. EMSA were performed with the M/B oligonucleotide as described in Materials and Methods. Unlabeled competitor oligonucleotides (Oligo) were added prior to labeled oligonucleotide at a 100-fold molar excess. (B) EMSA using wild-type and mutant M/B oligonucleotides. Ten micrograms of WCE from mammary glands at 4 days of lactation was incubated with ^{32}P-labeled wild-type (lane 1), mutant MGF (mut MGF; lane 2), and mutant YY1 (mut YY1) M/B oligonucleotides.

WCE were demonstrated to be capable of binding to the M/B oligonucleotide (data not shown).

These results were consistent with those reported previously (38) and indicated that MGF binding activity is developmentally regulated in the mammary gland. Minimal B oligonucleotide binding activity was observed in extracts prepared from nonlactating mammary tissue. Two complexes similar in mobility to those seen in extracts from various cell lines were detected, however, in lactating extracts. A unique slow-mobility complex was also detected in extracts from lactating mammary glands which was absent from nonlactating glands and several cell lines. A comparison of these results with those observed for HC11 WCE suggests that changes in MGF binding activity during development may not be due solely to the presence or absence of prolactin. Instead, MGF regulation appears to be more complex. It is also possible that hormonal regulation in HC11 cells is perturbed or that small changes in factor concentration cannot be detected with EMSA (see Discussion).

Cooperative interactions are required to form LAC. To study interactions between factors binding in this region of the β-casein promoter, a longer M/B oligonucleotide (Fig. 2), encompassing both the YY1 and MGF binding sites, was used in EMSA. To first determine the identities of the EMSA complexes formed on the M/B oligonucleotide, competition analysis was performed with different oligonucleotide competitors and WCE prepared from a rat mammary gland at 10 days of lactation (Fig. 7A). Three major EMSA complexes were observed (lane 1). Addition of a 100-fold molar excess of the B oligonucleotide (lane 2) completely abolished the middle complex without appreciably affecting the intensity of the fastest-migrating complex. The middle complex, therefore, appeared to contain MGF. Addition of a 100-fold molar excess of the M oligonucleotide (lane 3) completely abolished the fastest-migrating complex. Addition of the YY1 oligonucleotide also abolished the lower, fastest-migrating complex (data not shown). This lower complex, thus, appeared to contain YY1 or a similar protein. A cooperative interaction, however, with proteins binding to adjacent sites must be required to stabilize this YY1 complex, because no DNA-protein complexes were detected with the M oligonucleotide (containing the consensus YY1 site) under identical binding conditions and the same mammary gland WCE (see Discussion). Both the B and M oligonucleotides appeared to decrease the intensity of the slowest-migrating upper complex (lanes 2 and 3). It, thus, appeared that this complex consisted of proteins binding in both the MGF and milk box regions of the M/B oligonucleotide.

Previous DNA footprinting studies suggested that nuclear proteins from the lactating mammary gland interacted not only with the −90 MGF site but also with a −130 to −140 MGF-like binding site, with a reported 10-fold-lower binding affinity (38). This binding site is encompassed by the M/B oligonucleotide. To characterize factors binding in this region, competition analysis with a 100-fold molar excess of the C oligonucleotide as a specific competitor was conducted (Fig. 7A, lane 4). Addition of the competitor containing this putative upstream MGF-like binding site, but not including the YY1 or downstream MGF site, did not appreciably affect the relative intensity of any of the three EMSA complexes observed with the M/B probe. Therefore, under these conditions, factors binding to the upstream MGF-like region of the β-casein promoter were not detected. Subsequent studies have been conducted with an "M/B short" oligonucleotide, which does not contain the 5′ MGF-like site but does contain the YY1 and bona fide −90 MGF sites (see Materials and Methods). Three complexes, indistinguishable from those complexes formed on the M/B oligonucleotide, were observed in EMSA using the M/B short oligonucleotide and were specifically competed for with an excess of the unlabeled M/B short oligonucleotide (data not shown).

Extracts from several stages of development were tested, and the slowest-migrating complex was detectable only in WCE from the late pregnant and lactating mammary gland (data not shown). This complex was therefore named LAC for lactation-associated complex. Thus, WCE prepared from rat mammary glands at 10 days of lactation contained three complexes which bound to the M/B oligonucleotide. One complex appeared to contain MGF, which interacted with the −90 binding site. A second, faster-mobility complex contained YY1 or a closely related protein, and the third, slowest-mobility complex, termed LAC, was detected only in WCE isolated from late pregnant and lactating mammary glands. LAC was not completely abolished with a 100-fold molar excess of either the M or B oligonucleotide, suggesting that a cooperative interaction between factors binding at or near the MGF and YY1 sites is required for formation of this complex.

YY1 binding prevents the formation of LAC. (i) Mutations of the YY1 and MGF binding sites affect LAC binding activity. To further explore interactions between proteins binding to the M/B oligonucleotide, mutations were specifically introduced into either the MGF or YY1 binding site to create two mutant M/B oligonucleotides previously demonstrated to significantly decrease MGF or YY1 binding (see Fig. 2 and Materials and Methods). The mutant MGF oligonucleotide contains the same two base changes as the mutant B oligonucleotide used in Fig. 5B, while the mutant YY1 oligonucleotide contains two base changes previously determined to be contact sites for this milk box-binding protein (8a, 40). EMSAs were conducted with WCE isolated from mammary glands at 4 days of lactation and the wild-type (M/B short) or mutant M/B oligonucleotides (Fig. 7B). When the mutant M/B oligonucleotide containing an altered MGF binding site (Fig. 7B, lane 2) was used in EMSA, LAC formation, compared with the wild-type oligonucleotide (lane 1), was significantly decreased. A previously undetected complex with a faster mobility (X) was also observed. Antibodies against YY1 did not prevent the

formation of this complex, nor did a 100-fold molar excess of the YY1 oligonucleotide (data not shown). More importantly, LAC formation was increased markedly in EMSA containing the M/B oligonucleotide with specific mutations in the YY1 binding site (lane 3).

Therefore, LAC formation appears to require protein(s) binding to the previously described MGF binding site, as mutation of this site significantly inhibited LAC formation. Removal of MGF binding, however, did not appear to increase YY1 complex formation, using WCE prepared from lactating mammary tissue, but instead resulted in the detection of another complex with a slower mobility than the YY1-containing complex. In contrast, elimination of YY1 binding, through mutation of the YY1 binding site, led to enhanced LAC formation. Thus, the binding of YY1 to the M/B oligonucleotide appears to inhibit LAC formation, whereas binding of protein(s) at the MGF site is required for LAC formation.

(ii) An anti-YY1 antibody unmasks a complex with a mobility similar to that of LAC in HC11 cells. To further test the hypothesis that a YY1-like protein blocks the formation of LAC, HC11 WCE were depleted of endogenous YY1 protein by prior incubation with a polyclonal antibody directed against recombinant human YY1 (anti-YY1; Fig. 8A). LAC is normally undetectable in HC11 WCE under the conditions of this assay (Fig. 8A, lane 1). Addition of anti-YY1 (lanes 2 and 3), however, abolished the faster-migrating YY1-containing complex and led to the appearance of a slower-mobility complex which migrated with a mobility similar to that of LAC (compare with 4-day lactating WCE in lane 6). This complex observed in HC11 WCE, however, appears to be less diffuse and is detected at a much reduced level compared with the LAC complex observed in WCE prepared from lactating tissue (see Discussion). Addition of anti-YY1 to extracts from lactating tissue (lanes 7 and 8) abolished the YY1-containing complex but did not further enhance LAC. The appearance of the LAC-like complex with HC11 WCE was not the result of an antibody supershift, because removal of immunoglobulins from the HC11 WCE with protein A-Sepharose prior to gel electrophoresis did not diminish it (data not shown). Two irrelevant antibodies (antiactin [lanes 4 and 9] and anti-SRF [lanes 5 and 10]) did not affect the EMSA.

No difference in the LAC-like activity (as detected following the addition of anti-YY1) was observed when WCE prepared from prolactin-induced and uninduced HC11 cells were compared (Fig. 8B, lanes 5 and 8). Thus, this activity did not appear to be responsive to prolactin, as it was present even in confluent HC11 cells not exposed to prolactin. Because these analyses were conducted with WCE, however, differences in subcellular localization (i.e., cytoplasm versus nucleus) are not detected. It is also possible that prolactin responsiveness is altered in HC11 cells such that LAC no longer requires a prior prolactin stimulus. Further study is required to clarify these points. Incubation of HeLa WCE with anti-YY1 also revealed a small but detectable amount of a complex with a mobility similar to that of LAC (lane 2), suggesting that LAC (as with MGF) may not be restricted to the mammary gland. The precise identities of the proteins in this slowest-mobility complex, however, remain to be established. Again, an irrelevant control antibody (antiactin; lanes 3, 6, and 9) did not affect the EMSA. An antibody supershift was also detected in extracts isolated from both prolactin-induced and uninduced HC11 cells and HeLa cells (lanes 2, 5, and 8). The HeLa supershift appeared more intense and diffuse than the comparable HC11 complex and migrated at a slightly faster rate. There is, at present, no explanation for these differences between the HeLa and HC11 supershifts.

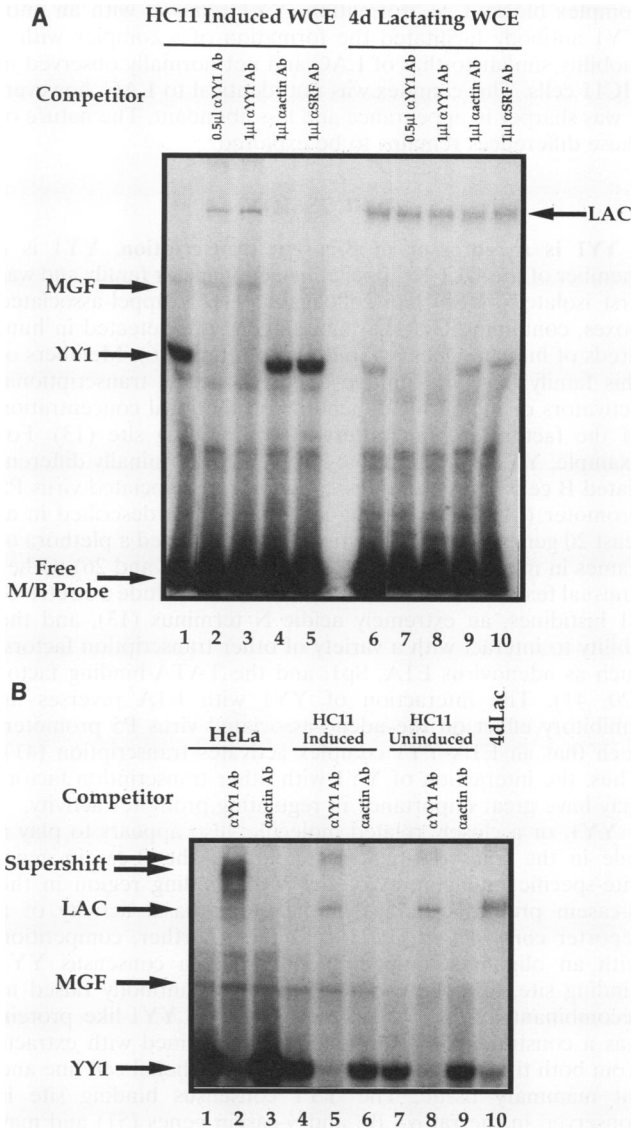

FIG. 8. (A) An anti-YY1 antibody unmasks a complex with a mobility similar to that of LAC in HC11 cells. Ten micrograms of WCE from a rat mammary gland at 4 days of lactation or prolactin-induced HC11 cells was used in EMSA with the ^{32}P-labeled M/B short oligonucleotide. WCE and M/B probe are in lanes 1 and 6. WCE were incubated with 0.5 μl (lanes 2 and 7) or 1.0 μl (lanes 3 and 8) of an anti-YY1 antibody (αYY1 Ab). As negative controls, WCE were incubated with 1 μl of an antiactin antibody (αactin Ab; lanes 4 and 9) or 1 μl of an anti-SRF antibody (αSRF Ab; lanes 5 and 10) as indicated in Materials and Methods. (B) The LAC-like complex and a YY1 supershift are present in both prolactin-induced and uninduced HC11 cells. Ten micrograms of HeLa (lane 1), prolactin-induced HC11 (lane 4), or uninduced HC11 (lane 7) WCE and WCE from rat mammary gland at 4 days of lactation (4dLac; lane 10) alone was incubated with ^{32}P-labeled M/B oligonucleotide. One microliter of anti-YY1 antibody (lanes 2, 5, and 8) or antiactin antibody (lanes 3, 6, and 9) was incubated with the binding reaction mixture as in Materials and Methods.

These experiments provide additional evidence for the presence of authentic YY1 in the mammary gland and HC11 WCE because an anti-YY1 antibody abolished the putative YY1-containing complex. In support of the hypothesis that the YY1

complex blocks LAC formation, YY1 removal with an anti-YY1 antibody facilitated the formation of a complex with a mobility similar to that of LAC and not normally observed in HC11 cells. This complex was not identical to LAC, however; it was sharper in appearance and less abundant. The nature of these differences remains to be explored.

DISCUSSION

YY1 is a repressor of β-casein transcription. YY1 is a member of the GL1-Krüppel *trans*-acting factor family and was first isolated by Shi and colleagues (41). Krüppel-associated boxes, containing Cys_2His_2 zinc fingers, are detected in hundreds of human genes by Southern blotting (16). Members of this family have the unique ability to act as transcriptional activators or inhibitors, depending on the local concentration of the factor (35) or context of the binding site (13). For example, YY1 activates the c-*myc* gene in terminally differentiated B cells (16) yet represses the adeno-associated virus P5 promoter (41). YY1 binding sites have been described in at least 20 genes, and the YY1 protein has assumed a plethora of names in many systems (e.g., references 8, 13, and 26). Other unusual features of this transcription factor include a stretch of 11 histidines, an extremely acidic N terminus (13), and the ability to interact with a variety of other transcription factors, such as adenovirus E1A, Sp1, and the TATA-binding factor (20, 41). The interaction of YY1 with E1A reverses an inhibitory effect on the adeno-associated virus P5 promoter, such that an E1A-YY1 complex activates transcription (41). Thus, the interaction of YY1 with other transcription factors may have great importance in regulating promoter activity.

YY1, or a closely related molecule, also appears to play a role in the transcriptional regulation of the β-casein gene. Site-specific mutagenesis of the YY1 binding region in the β-casein promoter led to an increased basal activity of a reporter construct in HC11 cells (38). Further, competition with an oligonucleotide containing only a consensus YY1 binding site, and the use of a polyclonal antibody raised to recombinant human YY1, revealed that a YY1-like protein was a constituent in EMSA complexes formed with extracts from both the murine HC11 mammary epithelial cell line and rat mammary tissue. The YY1 consensus binding site is conserved in the rat α-, β-, and γ-casein genes (51) and may therefore play a role in the synchronized regulation of casein gene expression.

Importantly, the YY1 complex observed in EMSA of rat mammary tissue most likely contains some other unidentified protein(s); while complexes were readily detectable in HC11 WCE, no complex formation was observed with the M oligonucleotide, containing the consensus YY1 binding region, with rat mammary extracts. A complex which was abolished with an anti-YY1 antibody was detected, however, with use of the longer M/B oligonucleotide, containing both the YY1 and MGF regions. An interaction with proteins binding at or near the MGF region, therefore, appears to be required for a stable YY1 interaction to be detected under these conditions. Consistent with this hypothesis, competition experiments suggested that the YY1 binding site in the β-casein milk box has a relatively low binding affinity for YY1 compared with a previously defined YY1 binding site in the chicken α-actin promoter (22).

We posit that the affinity of the YY1 binding site plays a major role in promoter regulation; e.g., a binding site to which YY1 has weak affinity may facilitate a mechanism whereby other hormone-responsive *trans*-acting factors interact with YY1 to modulate its activity.

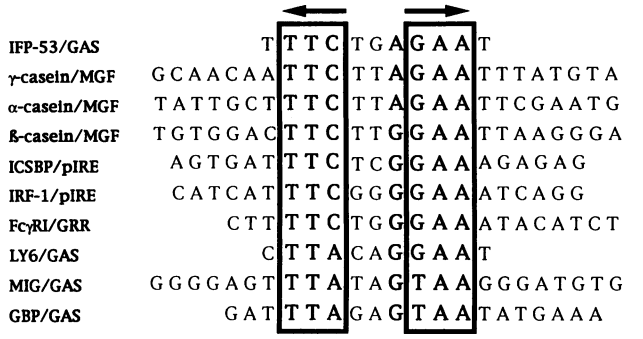

FIG. 9. The calcium-sensitive casein genes contain a consensus GAS. Shown is sequence comparison of casein proximal promoter MGF sites and GAS from several IFN-γ-responsive genes. (Adapted from reference 27.)

A protein binding to the MGF site is ubiquitous and may be related to the IFN-responsive stat91 *trans*-acting factor. The conserved motif centered around −90 contains a binding site for a factor designated MGF by Schmitt-Ney et al. (38). MGF purified from a bovine lactating mammary gland was reported to be an 89-kDa protein regulated positively by lactogenic hormones, with the highest observable DNA binding activity during lactation (49). Site-specific mutation of the putative MGF binding site resulted in the loss of β-casein promoter activity (40).

In contrast to the results of these studies, EMSAs performed in our laboratory have shown that several cell lines contain a factor which binds to an oligonucleotide containing only the putative MGF site. WCE from several different cell types formed complexes with identical mobilities on the B oligonucleotide which were similarly competed for with a wild-type, but not a mutant, oligonucleotide. Sequence analysis of this region revealed that the MGF binding site shares a surprising similarity to the recently described GAS, contained in IFN-γ-responsive genes (Fig. 9). The GAS palindrome is the binding site for a 91-kDa *trans*-acting factor, now referred to as stat91 (42), which is tyrosine phosphorylated in the cytoplasm and then translocates to the nucleus (36). stat91 appears to be involved in the signaling pathways for several different hematopoietin/cytokine receptor family members as well as other receptor types, including IFN-α/β, IFN-γ, epidermal growth factor, platelet-derived growth factor, colony-stimulatory factor 1, and interleukin-10 (18, 33, 34, 43). The presence of IFN (44) or other cytokine receptors in most cell types, along with the associated signal transduction machinery, could explain why apparent MGF binding activity is detected in many different cell extracts.

Preliminary studies conducted in our laboratory have indicated that several specific antibodies directed against stat91 (kindly provided by K. Ozato, D. Finbloom, and J. Darnell) do not block LAC formation on the M/B oligonucleotide. These antibodies were, however, able to abolish complex formation on an IFN response element incubated with nuclear extracts from IFN-γ-stimulated B cells (kindly provided by K. Ozato). Interestingly, however, a polyclonal antibody directed against the stat91-related family member stat113 (the 113-kDa component of the IFN-α/β-stimulated ISGF3α complex; antibody kindly provided by J. Darnell) inhibited LAC formation on the M/B oligonucleotide (data not shown). It has been recently suggested that the 113-kDa protein of the stat family, like stat91, may be phosphorylated and bind specifically to DNA in

response to hormonal stimulation (14). Further studies will be required to determine the precise relationship between MGF, LAC, and a stat113-like protein in the mammary gland.

If MGF is a member of the stat91 family, what then might account for the observation of MGF binding activity in both whole cell and nuclear extracts prepared from untreated HC11 cells? One possibility is that the growth of HC11 cells at confluence, and in the presence of 10% stripped horse serum for several days, a condition known to be required for prolactin induction of β-casein gene expression (2), activates either an integrin-regulated or a cytokine-mediated signal transduction pathway (32); this could lead to activation of stat91 or related family members. It is of interest that cell substratum and prolactin response elements have been mapped to similar regions of the bovine β-casein gene (37). Alternatively, highly homologous members of the stat91 family may bind to the same DNA sequence, such that different protein complexes are indistinguishable in EMSA. In this regard, it appears stat91 may bind as either a homo- or heterodimer to GAS in the IFN-γ-responsive and c-*fos* promoters (27, 34).

A model for hormonal control of β-casein expression. The complex that we have named LAC shares some characteristics with MGF but also displays several unique properties. The ontogeny of LAC during mammary gland development is similar to that of MGF; DNA binding of both factors is first detected in mammary gland extracts during late pregnancy, increases during lactation, and disappears upon involution. Like MGF, LAC complex formation is sensitive to both alkaline and acid phosphatase treatment (data not shown). Unlike MGF, which was reported to be a single DNA-binding protein (49), LAC appears to be made up of a complex of proteins. Support for the importance of cooperative interactions between *trans*-acting factors in LAC complex formation was obtained through experiments in which specific oligonucleotide competitors, containing either the MGF or YY1 binding region alone, only partially decreased the intensity of the LAC complex. Addition of lactating extracts to the mutant M/B oligonucleotide containing an altered MGF binding site resulted in significantly decreased LAC formation, compared with a wild-type M/B probe, and the formation of a faster-migrating complex (complex X) which does not appear to contain YY1. Thus, two lines of evidence suggest MGF may be a constituent of LAC, but LAC appears to also contain one or more proteins interacting with MGF and binding to a DNA site near the YY1 region.

Mutation of the YY1 binding site in the β-casein proximal promoter has been shown previously to increase the basal activity of a reporter construct (38). The present study demonstrates that a similar mutation also led to an increase in the formation of LAC. Taken together, these data suggest that a YY1-containing complex interferes with LAC formation. Additionally, the LAC complex, once formed, appears to be quite stable: recombinant YY1 when added to WCE from lactating mammary glands either before, at the same time as, or after the ^{32}P-labeled probe was unable to displace LAC (data not shown). This result could be due to differences between recombinant YY1 protein and the YY1-containing complex in the rat mammary gland, such as phosphorylation of YY1 or an unknown YY1-binding partner which may be required for efficient competition with LAC. Conversely, LAC formation does not appear to inhibit YY1 binding; mutation of the MGF site has no apparent effect on YY1 complex formation. Identification of the proteins making up the YY1-containing, LAC, MGF, and X complexes will be required in order to understand fully the interactions among the members of these complexes.

We were unable to demonstrate a prolactin requirement for formation of the YY1, MGF, or LAC complexes. The role of prolactin per se in the regulation of β-casein transcription, thus, remains unclear. The binding activities of all three *trans*-acting factors were observed, however, to change throughout mammary gland development (unpublished observation). The activities of these factors must, therefore, respond to changes in the hormone-growth factor-extracellular matrix milieu, but prolactin alone may not be sufficient in HC11 cells to mimic this effect. The levels of YY1, MGF, and LAC in HC11 cells appeared different from those in the lactating mammary gland. YY1 binding activity is extremely high and LAC activity is very low in HC11 cells compared with the lactating gland. This is not completely unexpected, because the HC11 line was cloned from the COMMA-D cell strain (5), originally derived from a mid-pregnant mouse; WCE from virgin or early pregnant mammary glands contained significantly higher YY1 DNA binding activity than WCE from lactating glands (data not shown). Thus, increased DNA binding activity of LAC may be difficult to detect in HC11 cells. Further, because only about 10% of the cells in a given culture dish produce β-casein in response to prolactin (2), very subtle changes in LAC or YY1 activities in WCE may be difficult to detect with EMSA.

The simplest model to explain these results posits that YY1 acts as a repressor on the β-casein promoter by blocking the formation of a positive complex, LAC. LAC formation appears to require the cooperative interaction between two or more *trans*-acting factors binding at or near both the MGF and YY1 sites (but not requiring the same base pair contacts as YY1). Lactogenic signals probably activate several signaling cascades which impinge upon LAC. Phosphorylation may effect nuclear translocation and DNA binding of MGF, which we suggest may be a stat91-like protein, and one constituent of LAC. Lactogenic signals may also impinge upon proteins in the YY1-containing complex, decreasing its DNA binding affinity and relieving YY1-mediated repression, thus allowing LAC to assemble on the DNA. The formation of LAC then recruits transcriptional machinery necessary to activate β-casein expression.

Precedence exists for this model in other systems, in which YY1 is a transcriptional repressor replaced by a positively acting factor. For example, in the chicken skeletal α-actin gene promoter, a YY1 binding site overlaps an SRF binding region (22). During myogenesis, the DNA binding activity of SRF increases while that of YY1 decreases; Gualberto and colleagues (11) suggest that increasing SRF levels overcome an inhibitory effect of YY1 by displacing the YY1 protein from its cognate site. Similarly, the ε-globin gene silencer contains overlapping YY1 and GATA-1 binding sites (28). Peters et al. (28) propose that during fetal development, expression of the ε-globin gene occurs when GATA-1 levels are high and able to displace YY1 from its DNA binding site.

We have only begun to define the mechanisms involved in the regulation of casein gene expression. Characterizing changes in *trans*-acting factor occupancy on the β-casein proximal promoter has increased our understanding of casein control but cannot explain responses to the complex developmental cues involved in the control of lactation. Further experimentation will be required to fully understand the various regulatory networks controlling lactation.

In summary, analysis of *trans*-acting factors interacting with the β-casein promoter has revealed that (i) YY1 binds in the repressor milk box region, (ii) the MGF binding site shares similarity with the GAS, and apparent MGF binding activity is detected in many cell types, suggesting that MGF may be related to the stat91 signaling protein, (iii) a cooperative

interaction between factors binding near both the YY1 and MGF sites appears to be required for the formation of an EMSA complex (LAC) detectable only in extracts from late pregnant and lactating mammary glands, and (iv) mutation of the YY1 binding site increased LAC formation, suggesting that YY1 binding on the β-casein promoter inhibits LAC assembly. These results support a model for lactogenic activation of β-casein expression in which the activator DNA binding complex, LAC, in response to lactogenic stimulation, replaces a repressor protein, YY1, to activate β-casein transcription.

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ADDENDUM IN PROOF

After this article was submitted, another article (V. S. Meier and B. Groner, Mol. Cell. Biol. **14**:128–137, 1994) reported similar results concerning the role of YY1 in the regulation of β-casein gene expression.

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