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The Nuclear Factor YY1 Participates In Repression of the β-Casein Gene Promoter in Mammary Epithelial Cells and Is Counteracted by Mammary Gland Factor during Lactogenic Hormone Induction

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Expression of the β-casein milk protein gene in the mammary epithelial cell line HC11 is primarily regulated at the transcriptional level. A 338-bp segment of promoter sequence 5′ of the transcription start site is sufficient to confer inducibility by the lactogenic hormones insulin, glucocorticoid hormone, and prolactin. Positively and negatively acting promoter elements and specific DNA binding proteins have been identified. The binding of the mammary gland factor MGF to a site between −80 and −100 is indispensable for hormonal induction of transcription. Binding of MGF activity to DNA is greatly enhanced by the action of the lactogenic hormones. Repression of transcription in the uninduced state is mediated by a promoter element located adjacent to the MGF binding site at positions −110 to −150. This repressor element consists of two interacting protein binding sites. A nuclear factor that binds specifically to the proximal site between positions −110 and −120 has been characterized and found to be identical with the nuclear factor YY1 (δ, NF-E1). YY1 does not bind to the distal site. The simultaneous mutation in the proximal and the distal sites results in high, hormone-independent transcription. This finding suggests that YY1 plays a functional role in the repression and acts in conjunction with a second DNA binding protein. Comparison of YY1 DNA binding activity in uninduced and hormone-induced cells showed that relief of repression is not mediated by changes in the concentration or binding affinity of YY1. Infection of HC11 cells with a YY1-expressing recombinant retrovirus resulted in overexpression of YY1 but did not suppress hormonal induction. The addition of purified MGF decreased YY1 binding to its DNA recognition site in vitro. This finding indicates that MGF regulates the DNA binding activity of YY1 and thereby may cause the relief of transcriptional repression.

β-Casein is an abundant milk protein expressed in mammary epithelial cells during lactation. Its expression is dependent on the synergistic action of the lactogenic hormones insulin, glucocorticoid hormone, and prolactin (6). Transcriptional regulation of the β-casein promoter has been identified as a major control mechanism influenced by the action of the steroid and peptide hormones (4). Several crucial aspects of lactogenic hormone regulation can be studied in the established mammary epithelial cell line HC11 (1). These cells have been very valuable for the elucidation of the sequential hormonal requirements for β-casein gene induction and have been used to define the role of the lactogenic hormones as well as the role of epidermal growth factor (EGF) in the induction process (6, 16, 29). Both positive and negative effects on β-casein gene transcription have been observed (16, 35).

Promoter-reporter gene constructs have been used to delimit the sequence elements in the β-casein gene promoter required for lactogenic hormone induction (7, 27). A promoter fragment of 338 bp, located 5′ of the transcription start site, has been shown to be sufficient to confer hormone inducibility to a linked reporter gene. Several nuclear DNA binding proteins which bind specifically in this region were identified (29). Best characterized among these proteins is the mammary gland-specific nuclear factor MGF. This factor binds to the region between −80 and −100 which is highly conserved in milk protein gene promoters of different species. Gene transfer experiments with mutated variants of the β-casein gene promoter into HC11 cells have shown that an intact MGF binding site is indispensable for the hormonal induction of transcription (29, 30). MGF binding activity is regulated in vivo and in vitro. High levels were found in nuclear extracts derived from mammary cells of lactating mice and in HC11 cells induced with lactogenic hormones. The regulation of the DNA binding activity of MGF is at least in part due to its state of phosphorylation (30, 31).

The introduction of mutations into the promoter sequence and gene transfer into HC11 cells revealed the presence of additional regulatory elements in the β-casein gene promoter. A negatively acting element was found in the region adjacent to the MGF binding site. This element located between −110 and −150 seems to be constituted by two interacting half sites. Mutations in the distal half site (−135 to −145) or in the proximal half site (−110 to −120) by themselves had little effect on the rate of transcription. The simultaneous mutations of both sites resulted in a high level of hormone-independent transcription. Two specific DNA-protein complexes were formed when DNA from this region was used as a probe in band shift experiments. These complexes were down-regulated in extracts of induced cells. These observations led us to propose that one mechanism by which hormones regulate β-casein transcription is the relief of repression (29).

We have further investigated the DNA-protein complexes formed by this promoter region and found that a novel nuclear factor binds to the proximal half of the negative

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regulatory element. After determination of the DNA binding specificity, proteolytic cleavage pattern, and molecular weight, we conclude that this factor is identical with the recently cloned nuclear factor YY1 (34). Several names have been used, depending on the origin of the factor (δ, CF-1, and NF-E1), and transactivating and repressing functions have been ascribed to it (14, 23, 25). This factor binds to a negative regulatory element in the c-*myc* promoter and interacts with a tissue-specific repressor (18, 25). YY1 also negatively regulates the serum response element of the c-*fos* promoter (9) and CArG boxes of muscle-specific genes, i.e., of the skeletal α-actin promoter (9, 19, 25), and the cardiac-specific expression of the creatine kinase-M gene (37). δ was shown to activate transcription of mouse ribosomal protein genes that lack a TATA box from binding sites upstream of the transcription start site (14). YY1 is involved in negative and positive regulation of transcription of many viral genes (2, 5, 8, 41). Two YY1 binding sites at −60 and at +1 of the adeno-associated virus P5 promoter mediate repression of transcription by YY1, an effect which is relieved by the adenovirus transforming protein E1A (34). In addition, YY1 can stimulate initiation of transcription from the +1 site in the absence of a TATA box (33). NF-E1 is also involved in the negative regulation of the κ 3′ enhancer (23) and binds adjacent to a negative regulatory element in the immunoglobulin heavy-chain (IgH) μE1 site (23, 25). Stage-specific silencing of γ- and ε-globin genes is mediated by YY1 (10, 24).

YY1 plays a functional role in repression of the β-casein gene. Mutations of the proximal element which cooperate with mutations in the distal element to relieve transcriptional repression abolish binding of YY1. YY1 does not bind to the distal element. This strongly indicates that YY1 represses transcription of the β-casein gene, possibly in conjunction with a nuclear factor bound to the distal element. We investigated whether the modulation of YY1 activity is involved in the lactogenic hormone induction of the β-casein promoter and found that the concentration and binding affinity of YY1 were unchanged when the uninduced and induced states of the cells were compared. Also, ectopic expression did not interfere with the induction process. Using purified MGF, however, we observed that MGF can suppress YY1 binding to an adjacent binding site in the β-casein promoter and might thereby trigger the relief of repression.

MATERIALS AND METHODS

Cell culture. HC11 cells were grown in RPMI 1640 medium with 10% fetal calf serum, 5 μg of bovine insulin per ml, 10 ng of murine EGF per ml, 50 μg of gentamicin per ml, and 2 mM glutamine. Hormone induction was performed by the addition of 0.1 μM dexamethasone (Sigma) and 5 μg of ovine prolactin (Sigma) per ml in the absence of EGF. Control cells were kept for the same time without dexamethasone and prolactin and in the absence of EGF (1, 6).

Nuclear and cytoplasmic extract preparation. Cells were washed twice in phosphate-buffered saline (PBS) and scraped into PBS. After centrifugation, cells were lysed in 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9)–10 mM KCl–0.2 mM EDTA–0.5 mM dithiothreitol (DTT)–0.6% Nonidet P-40 (NP-40)–0.5 mM phenylmethylsulfonyl fluoride for 10 min on ice. After centrifugation at 2,000 rpm for 5 min, the cytoplasmic fraction was separated from the nuclei, cleared of debris by centrifugation at 13,000 rpm for 10 min, and used for Western blot (immunoblot) analysis. Proteins were eluted from the nuclei in 25% glycerol–20 mM HEPES (pH 7.9)–0.5 mM EDTA–0.5 mM DTT–0.5 mM phenylmethylsulfonyl fluoride and, unless indicated otherwise, 400 mM NaCl. Elution of proteins was performed on ice for 30 min. For stepwise elution of proteins from the nuclei, centrifugation and elution were repeated as described above, using increasing concentrations of NaCl as indicated.

Expression of YY1 in bacteria and partial purification of the recombinant protein. The cDNA of the δ factor (14) was cloned into the *Eco*RI site of plasmid pFlag (International Biotechnologies, Inc.). The δ-Flag fusion protein was expressed in *Escherichia coli* CC118 by induction with isopropyl thiogalactopyranoside (IPTG). It was not possible to use the Flag epitope for the purification of the recombinant protein. δ-Flag did not bind to the M1 anti-Flag affinity column but was recognized by the M2 anti-Flag antibody in Western blots. The δ-Flag fusion protein was partially purified on an Ni^{2+}-nitrilotriacetate agarose column (Qiagen), taking advantage of the presence of 12 consecutive histidines in the δ protein. Preparation of bacterial protein extracts and purification were performed under denaturing conditions as instructed by the manufacturer. Following elution from the Ni^{2+}-nitrilotriacetate agarose column, the δ-Flag fusion protein was freed from urea on a Sephadex G-50 column (Pharmacia) and renatured in the buffer used for proteins recovered from sodium dodecyl sulfate (SDS)-polyacrylamide (PAA) gels as described below. Uninduced extracts, which did not contain detectable δ-Flag fusion protein, were treated in the same way and used as a control.

Probe preparation. Oligonucleotides were annealed and 5′ end labelled with [γ-^{32}P]ATP (Amersham) and T4 polynucleotide kinase (Boehringer) according to standard procedures.

Band shift analysis and proteolytic clipping band shift assays. Electrophoretic mobility shift assays were performed as described by Schmitt-Ney et al. (29), with minor modifications. Labeled probe (10,000 cpm) was incubated in a 20-μl final volume containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, 2 μg of poly(dI-dC), and 2 μg of nuclear extract proteins. Unlabeled oligonucleotides were added as a competitor in a 100-fold molar excess as indicated. Following 30 min of incubation at room temperature, electrophoresis was performed on a 4% PAA gel in 0.25× Tris-borate-EDTA. Proteolytic clipping band shift assays (32) were performed under the same reaction conditions. After 20 min of incubation, *Staphylococcus aureus* V8 protease (Boehringer) was added as indicated, and the reaction was allowed to proceed for another 10 min (amounts of V8 protease and time intervals as described by Riggs et al. [25]).

When purified sheep MGF was used, the band shift conditions were the same as specified above except that 20 μg of bovine serum albumin (BSA) was added to each reaction. Purified MGF was in buffer E (50 mM NaCl, 10 mM Tris [pH 7.5], 0.1 mM EDTA, 5% glycerol, 0.1% NP-40, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg of BSA per μl). Different volumes due to different amounts of MGF were adjusted by adding the respective volume of buffer E. Purified sheep MGF was kindly provided by F. Gouilleux and H. Wakao.

Southwestern (protein-DNA) blotting and renaturation of proteins from SDS-PAA gels. Southwestern blotting was performed as described by Hubscher (15), with the following modifications. Nuclear extracts were size fractionated on SDS–7.5% PAA gels. Proteins were electrotransferred onto nitrocellulose filters (Schleicher & Schuell). Proteins bound

to the filter were denatured in 6 M guanidinium-HCl–50 mM Tris-HCl (pH 8)–0.1 M KCl–0.1% NP-40 for 1 h at room temperature. Renaturation was performed in 10% glycerol–5% milk powder–20 mM Tris (pH 8)–50 mM KCl–0.2 mM EDTA–0.1% NP-40–1 mM DTT for 1 h at room temperature. Filters were hybridized for 1 h at room temperature with 2×10^6 to 4×10^6 cpm of labeled oligonucleotides per ml in renaturation buffer containing 0.25% instead of 5% milk powder and 5 μg of poly(dI-dC) per ml. Filters were washed three times in renaturation buffer without milk powder, air dried, and exposed to X-ray film.

Recovery and renaturation of protein from SDS-PAA gels was performed as described by Hager and Burgess (11) and Li et al. (20), with modifications. After electrophoresis, the protein-containing lanes were cut into 5-mm wide slices and crushed into a paste. Proteins were eluted for 12 h in a buffer containing 150 mM NaCl, 20 mM HEPES-NaOH (pH 7.5), 5 mM DTT, 0.1 mM EDTA, 0.1% SDS, and 0.1 mg of BSA per ml. Proteins were precipitated with 4 volumes of cold acetone by incubation for 45 min on dry ice and collected by centrifugation at 12,000 × *g* for 30 min. The dried pellet was incubated at room temperature for 20 min with 6 M guanidine-HCl in 20 mM HEPES-NaOH (pH 7.5)–100 mM NaCl–1 mM DTT–0.1 mM EDTA–0.1 mg of BSA per ml. The solution was diluted 50-fold with 20 mM HEPES-NaOH–50 mM NaCl–0.1 mM EDTA–2 mM $MgCl_2$, 0.1 mg of BSA per ml–17% glycerol and incubated overnight at room temperature. The protein fractions were then analyzed in band shift assays.

Western blot analysis. Cytoplasmic extracts were prepared as described above. Immunoblot analysis was performed as described by Happ et al. (12). The cytoplasmic proteins were separated by electrophoresis on SDS–12.5% PAA gels. Proteins were electroblotted onto polyvinylidene difluoride membranes (Bio-Rad). β-Casein was detected with a rabbit anti-mouse polyclonal antibody and a goat anti-rabbit IgG coupled to alkaline phosphatase (Southern Biotechnology). The blot was developed in a buffer (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 5 mM $MgCl_2$) containing *p*-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate as instructed by the manufacturer (Bio-Rad).

Plasmid construction. The 1.9-kb mouse cDNA fragment encoding the δ transcription factor (kindly provided by R. P. Perry) was derived from the TC-δ plasmid described by Hariharan et al. (14). The δ fragment was cloned into the *Eco*RI site of the retroviral vector pLXSN (kindly provided by Miller [21]). In pLδSN, δ is expressed from the long terminal repeat of Moloney murine sarcoma virus.

Cell transfections and infections. Virus was generated from the plasmid forms of the retroviral vector pLδSN or pLXSN by transient transfection of omega E packaging cells (kindly provided by H. Land [22]), using standard calcium phosphate transfection methods. Ten micrograms of plasmid was used in each transfection. HC11 cells were infected with the virus-containing medium as described by Miller and Rosman (21). After selection in G418 (0.2 mg/ml), stably transfected cell colonies were pooled; >200 colonies were derived in each of three independent experiments. To produce stable vector-producing omega E cells, transfection was performed as described above. Cells were selected in G418 (1 mg/ml), and stably transfected cell colonies were pooled and grown to confluency. The virus-containing medium was then used to infect HC11 cells. The number of stably transfected HC11 cell colonies was >500 in each of three independent experiments.

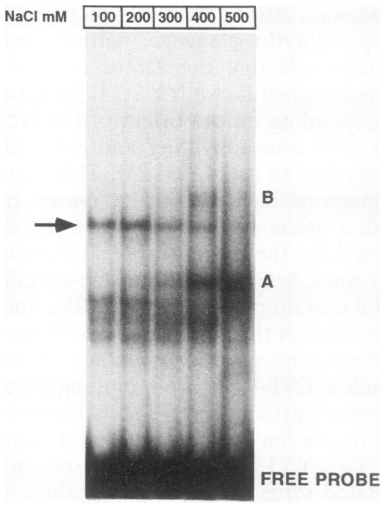

FIG. 1. The nuclear factor that binds to the repressor element of the β-casein promoter elutes at low salt concentrations from the nuclei of HC11 cells. Proteins were eluted from the nuclei of HC11 cells at the increasing salt concentrations indicated. The same nuclei were used in successive extractions. Band shift experiments were performed as described in Materials and Methods. The radioactively labeled DNA probe comprised the repressor element of the β-casein promoter, positions −98 to −154. The majority of the novel factor (arrow) elutes at NaCl concentrations of between 100 and 300 mM (lanes 1 to 3). The factors giving rise to complexes A and B, described before by Schmitt-Ney et al. (29), are eluted at 400 and 500 mM NaCl (lanes 4 and 5).

RESULTS

Differential salt extraction reveals a nuclear factor which binds to the repressor region of the β-casein promoter. A DNA fragment that comprises the β-casein promoter region from −98 to −154 was used as a probe in gel shift experiments to identify nuclear factors which bind to the negative regulatory element of the β-casein promoter. Proteins were eluted from nuclei of HC11 cells with increasing concentrations of NaCl. Two DNA-protein complexes (A and B) were observed in the high-salt eluates (400 and 500 mM) (Fig. 1, lanes 4 and 5) and have been described previously (29). At 100 to 300 mM NaCl, a new complex migrating between A and B was found (Fig. 1, arrow). This factor, which is unstable in 400 mM ammonium sulfate (not shown), was not detected in our earlier studies.

DNA binding specificity of the repressor element-binding factor. We have subdivided the repressor element into a distal and a proximal half site. These sites are schematically indicated in Fig. 2. Several fragments derived from the repressor region of the β-casein gene promoter were used to delimit the binding site of the nuclear factor observed in Fig. 1. The band shift experiment shown in Fig. 3, lane 2, indicates that the promoter sequence from −98 to −130 is sufficient for factor binding. This sequence has been called probe 6 and is shown in Fig. 4. The novel factor specifically binds to the 3′ half site of the negative regulatory element, as determined by competition assays shown in Fig. 3, lanes 5 to 9. The mutated variants of probe 6 (6.2 and 6.3; Fig. 4) fail to compete for factor binding. When mutated oligonucleotides were introduced as probes in the band shift experiments, we observed that the replacement of the inverted repeat region within the factor binding site (probe 6rm) still prevents factor binding (Fig. 3A, lane 1). The factor also does not bind to

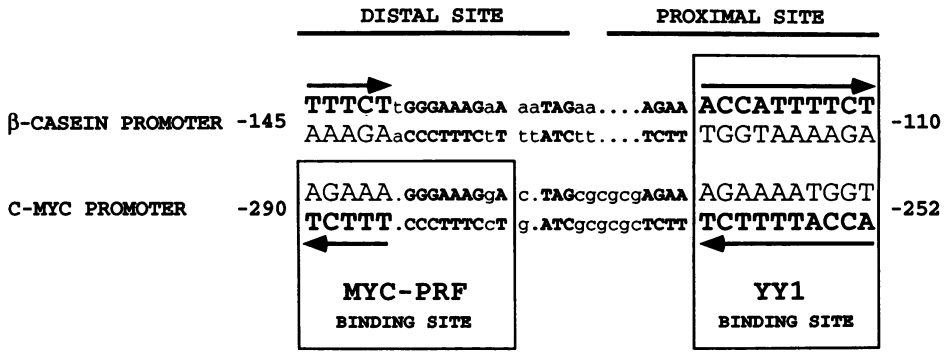

FIG. 2. Sequence comparison between the β-casein and c-*myc* promoters. The β-casein promoter sequences from −145 to −100 comprise the distal and proximal sites of the repressor element. The c-*myc* promoter sequences from −290 to −252 contain the PRF and the YY1 binding sites (18). Factor binding sites are indicated by boxes. Identical sequences are shown in boldface. Elements present on both promoters but in reversed orientation are indicated by arrows.

sequences from −154 to −122 (probe 1) comprising the 5′ half site of the repressor element (lane 4). This result indicates that factor binding is not mediated by the inverted repeats (TTTCTT. . .AAGAAA as discussed in reference 29), which are also present in the 5′ half site.

Factor binding to the labeled probe 6 is competed for by an excess of nonradioactive probe 6 (Fig. 3, lane 6) or mutated probe 6.4 (lane 9). We conclude that the sequence AGAAACCATTT is essential for factor binding. This sequence is conserved in the region between −110 and −130 of the rat and mouse β-casein gene promoters, and very similar sequences were found in the rabbit (AGAAATCATTT), cow, and goat (AGAATTCATTT) β-casein promoters. An oligonucleotide corresponding to the cow and goat sequence (probe 6tm; Fig. 4) binds the factor (lane 11), but with lower affinity than probe 6 does (lane 10).

The nuclear factor which binds to the proximal repressor half site is identical with YY1. The nucleotides shown to be important for factor binding in the proximal site of the β-casein promoter region were compared with the binding sequences of known transcription factors. We found that the nucleotides identified above also occur in the consensus binding site CCATnT of the nuclear factor YY1 (34). This factor binds to a repressor element in the c-*myc* promoter, to the IgH-μE1 site, and to the skeletal α-actin promoter (25). The sequence ACCATTTTCT, comprising the factor binding site in the β-casein promoter, is present, in reversed orientation, in the binding site of YY1 in the c-*myc* promoter. To show the identity of the DNA binding specificity of the nuclear factor which binds to the negative regulatory element of the β-casein promoter with YY1, we performed band shift and competition assays with oligonucleotides derived from the β-casein promoter, the c-*myc* promoter, and the IgH-μE1 site. A complex with identical electrophoretic mobility was detected with all three probes (Fig. 5, lanes 1, 2, and 6). Binding competition with the labeled probes was observed when nonradioactive probe 6 was included (lanes 4 and 8) but not when a mutated variant of probe 6 (probe 6rm; lanes 5 and 9) was used.

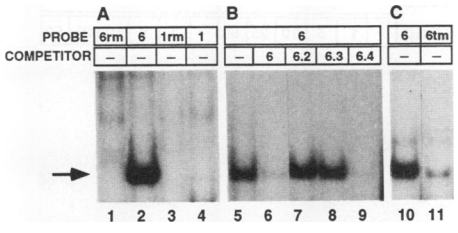

FIG. 3. DNA binding specificity of the repressor-binding factor. (A) Band shift assays were carried out with the radioactively labeled oligonucleotide 6 (lane 2), 6rm (lane 1), 1 (lane 4), or 1rm (lane 3), shown in Fig. 4. The probes were incubated with nuclear extracts from HC11 cells obtained with 200 mM NaCl. The nuclear factor binds to probe 6, which corresponds to the 3′ half site of the repressor element of the β-casein promoter, but not to a mutated form of the same oligonucleotide or to probe 1, which comprises the 5′ half site of the repressor element shown in Fig. 2. (B) Band shift assays were carried out in the presence of competing, unlabeled oligonucleotides. Binding of the nuclear factor to the radioactively labeled probe 6 is competed for by a 100-fold excess of unlabeled oligonucleotides 6 (lane 6) and 6.4 (lane 9) but not by a 100-fold excess of unlabeled oligonucleotide 6.2 (lane 7) or 6.3 (lane 8), shown in Fig. 4. (C) Band shift assay with oligonucleotides 6 (lane 10) and 6tm (lane 11) as radioactively labeled DNA probes. Probe 6tm represents the sequence of the cow and goat β-casein promoters corresponding to the proximal site of the repressor element. Oligonucleotide sequences are shown in Fig. 4.

To further ascertain the relatedness between the factors, we performed band shift assays after the proteolytic clipping of the DNA-protein complexes. Proteolytic clipping was carried out with increasing amounts of V8 protease. When the IgH-μE1 fragment was used as a probe, the resulting clipping pattern was identical to the pattern previously described for CF-1 (25) (Fig. 6, lanes 1 to 6). The same pattern of four indicative partial complexes is obtained with probe 6 (lanes 7 to 12).

The apparent molecular mass of the HC11 nuclear factor was estimated by Southwestern blotting analysis. A nuclear protein with an apparent electrophoretic mobility of approximately 60 kDa relative to a standard set of globular protein markers was specifically recognized by probe 6 but not by the mutated probe 6rm (Fig. 7A, lanes 1 and 2, respectively). This result was confirmed by a band shift analysis of the renatured proteins found in the 60-kDa range of the gel. Protein was eluted from the SDS-PAA gel fractions, renatured, and tested in a band shift assay. The proteins originating from the 60-kDa region of the gel (F60; Fig. 7A) were compared with the repressor-binding protein obtained from the crude nuclear extract. Complexes of identical electrophoretic migration were observed (Fig. 7B, lanes 2 and 3).

The identical DNA binding properties, the indicative proteolytic cleavage pattern, and the apparent molecular

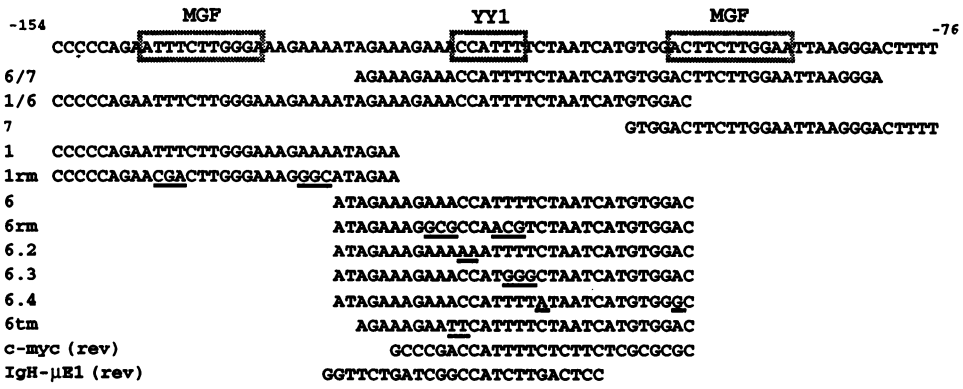

FIG. 4. Oligonucleotides used in band shift and competition assays. The rat β-casein promoter sequences from −154 to −76 include the MGF binding site and the repressor element. Factor binding sites are indicated. Mutations are underlined. Oligonucleotide 6/7 (−127 to −81) comprises the MGF binding site and the proximal half of the repressor element. Oligonucleotide 1/6 (−154 to −98) comprises the repressor element. Oligonucleotide 7 (−103 to −76) comprises the MGF binding site. Oligonucleotide 1 represents the distal half site of the repressor element from −154 to −124. In oligonucleotide 1rm, the two inverted repeats are mutated. Oligonucleotide 6 represents the proximal half site of the repressor element from to −129 to −98. The mutations introduced in oligonucleotide 6rm affect the inverted repeats. In oligonucleotide 6tm, the factor binding site is changed according to the sequences present on cow and goat β-casein promoters. Oligonucleotide c-myc comprises the sequences from −247 to −273 of the c-*myc* promoter. Oligonucleotide IgH-μE1 comprises the sequences of the murine IgH intronic enhancer from 368 to 344. rev, reverse orientation.

mass of 60 kDa indicate that the nuclear factor which binds to the negative regulatory element of the β-casein promoter is very similar to or identical with YY1.

DNA binding activity of YY1 is not directly affected by lactogenic hormone induction in HC11 cells. The binding of YY1 to the repressor region of the β-casein gene promoter and the relief of transcriptional repression through the lactogenic hormones made it reasonable to suspect that the hormones might directly influence the interaction of YY1 with its DNA binding site. We investigated whether the DNA binding activity or the concentration of YY1 is altered in hormonally induced HC11 cells in comparison with non-induced cells. For this purpose, nuclear extracts were prepared from cells induced with glucocorticoids and prolactin or control cells cultured with insulin only. Nuclear proteins were eluted from nuclei at different salt concentrations and analyzed in a band shift assay (Fig. 8). YY1 was found in similar concentrations in the uninduced (lanes 1 to 6) and the induced (lanes 7 to 12) states of the cells. β-Casein induction was measured to confirm the induced state of the cells (data not shown). Thus, the relief of repression by hormones is not due to a change in concentration or DNA binding activity of YY1.

Overexpression of YY1 in HC11 cells is not sufficient to prevent hormonal induction of β-casein synthesis. To investigate the effects of deregulated expression of YY1 on β-casein induction, we infected HC11 cells with a recombinant retrovirus (LδSN) (21) which directs the expression of the mouse homolog of YY1, called δ, and the selectable marker gene *neo*. Nuclear proteins were extracted from infected cells, and YY1 levels were quantitated in a band shift experiment. Figure 9A, lane 3, shows that the cells infected

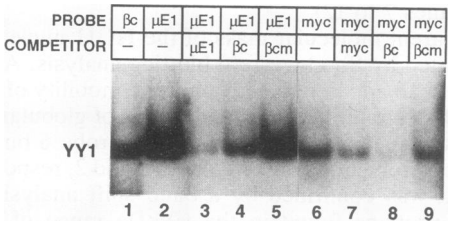

FIG. 5. YY1 and the nuclear factor bound to the repressor element of the β-casein promoter show similar electrophoretic mobilities and DNA binding properties. Band shift and competition assays were performed as described in the text, using nuclear extracts from HC11 cells. DNA probes and competitors: βc, β-casein oligonucleotide 6; βcm, β-casein oligonucleotide 6rm; μE1, YY1 binding site on the IgH-μE1 element; myc, YY1 (CF-1) binding site on the c-*myc* promoter. These sequences are detailed in Fig. 4. Competitors were used in a 100-fold molar excess.

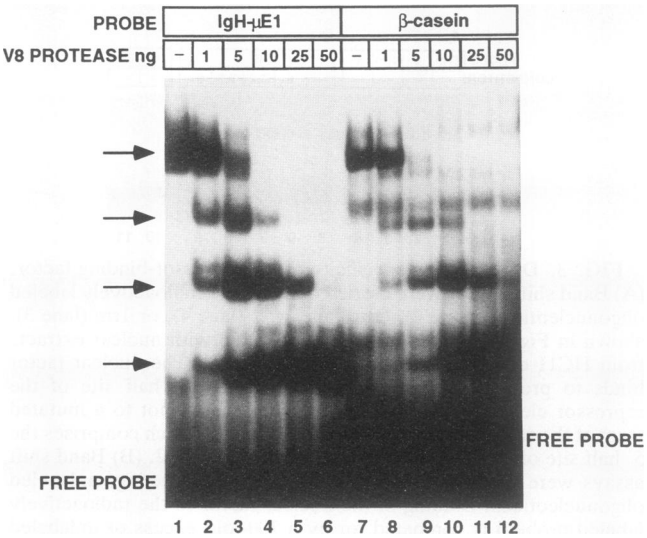

FIG. 6. Proteolytic clipping patterns. Nuclear extracts from HC11 cells were incubated with probe 6 of the β-casein promoter or with a probe comprising the IgH-μE1 site. The indicated amounts of V8 protease were added to the reaction mixtures, and proteolytic clipping was allowed to proceed for a fixed time interval. The resulting protein fragments (arrows) were visualized in band shift experiments.

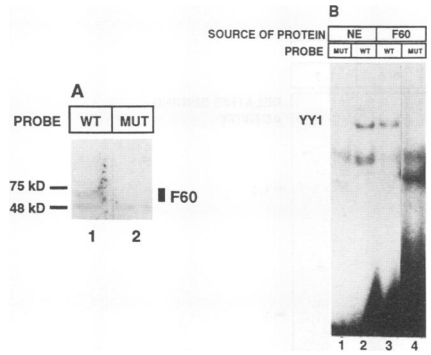

FIG. 7. The apparent molecular mass of the repressor-binding protein is approximately 60 kDa. (A) Nuclear extracts from HC11 cells were probed with the radioactively labeled wild-type oligonucleotide 6 (WT) or the mutated oligonucleotide 6rm (MUT) in a Southwestern analysis. A nuclear protein with an apparent molecular mass of about 60 kDa (F60) is specifically recognized by the oligonucleotide comprising the wild-type sequence but not by the mutated version. Nonspecific binding of nuclear factors to both probes is also observed. (B) Band shift assays were performed with the probes indicated and either nuclear extracts from HC11 cells (NE) or the fraction of proteins with an electrophoretic mobility of about 60 kDa (F60), which had been eluted from an SDS-PAA gel and renatured.

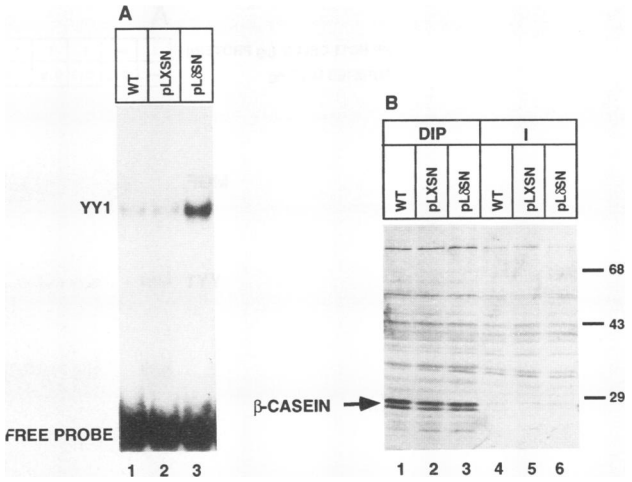

FIG. 9. Five- to tenfold overexpression of YY1 does not suppress β-casein induction by lactogenic hormones. The cDNA of the δ (YY1, NF-E1) factor was cloned into the viral vector pLXSN. HC11 cells were infected with this vector (pLδSN) or with a vector without an insert (pLXSN). After G418 selection, stably transfected clones were pooled and hormonal induction was performed as described for Fig. 9. The nuclear protein fractions were tested in band shift assays (A). The cytoplasmic protein fractions were analyzed in Western blots (B). (A) Band shift assays were performed with 2 μg of total nuclear proteins from induced cells in each sample. The radioactive DNA probe was oligonucleotide 6. (B) Western blots were performed as described in Materials and Methods, using an antiserum which detects the β-casein protein (arrow). Cells induced with the lactogenic hormones dexamethasone, insulin, and prolactin (DIP) or uninduced cells (I) were tested. WT refers to uninfected HC11 cells.

with the LδSN virus express about 5- to 10-fold-higher levels of YY1 than do noninfected cells (lane 1) or cells infected with the retroviral vector (lane 2). The cells infected with LδSN were induced with lactogenic hormones, cytoplasmic proteins were extracted, and β-casein synthesis was quantitated. A Western blot experiment using an antiserum specific for mouse milk proteins shows the induction of β-casein (Fig. 9B). Induction was observed in all three cell lines analyzed (lanes 1 to 3), independent of the level of YY1 expression. Thus, a 5- to 10-fold overexpression of YY1 is not sufficient to suppress β-casein induction.

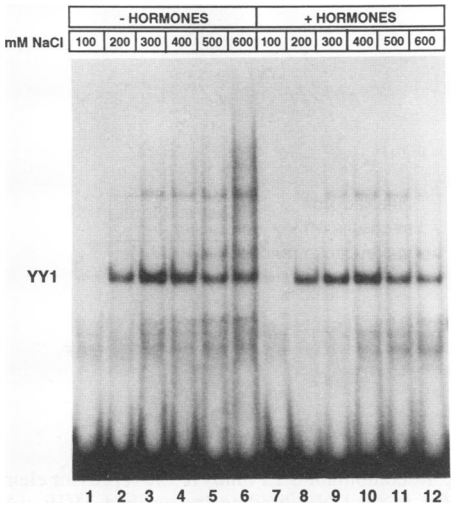

FIG. 8. DNA binding of YY1 is not regulated by hormones. HC11 cells were grown to confluency as described in Materials and Methods and kept in this state for 4 days. Then they were cultivated for another 4 days either without (lanes 1 to 6) or with (lanes 7 to 12) addition of the lactogenic hormones prolactin and dexamethasone. Nuclear extracts were prepared at the salt concentrations indicated, using a different dish of cells for each extraction. The amount of YY1 present in 2 μg of the nuclear protein fraction was monitored in band shift assays using oligonucleotide 1/6 as a radioactive probe. No significant difference in the amount of YY1 eluted at a given salt concentration was observed between extracts from induced and uninduced cells.

This result was corroborated by cotransfection experiments in which the YY1 expression vector pLδSN was introduced into HC11 cells together with a wild-type β-casein promoter-chloramphenicol acetyltransferase (CAT) construct or with the mutant constructs. These constructs have mutations in the distal binding site, in the proximal binding site, and in both binding sites of the repressor complex (29). Stable transfectants were selected, and YY1 overexpression was visualized by band shift analysis. No consistent effects of YY1 overexpression, however, were observed on the induced levels of CAT activity. The overexpression of YY1 is not sufficient to prevent the induction of the transfected β-casein promoter constructs.

MGF counteracts YY1 binding to its DNA recognition site. The experiments shown above suggest that neither the modulation of DNA binding activity nor the level of YY1 expression plays a role in the lactogenic hormone induction. We have observed earlier that the DNA binding activity of MGF is drastically regulated by lactogenic hormones both in vitro and in vivo. Since the binding sites of MGF and YY1 are very close to each other, separated by only one helical turn, it is possible that the two factors interact and influence each other's DNA binding activity. To investigate this possibility, we performed band shift assays with a β-casein promoter fragment which harbors binding sites for both factors (probe 6/7; Fig. 4). Nuclear extract from exponen-

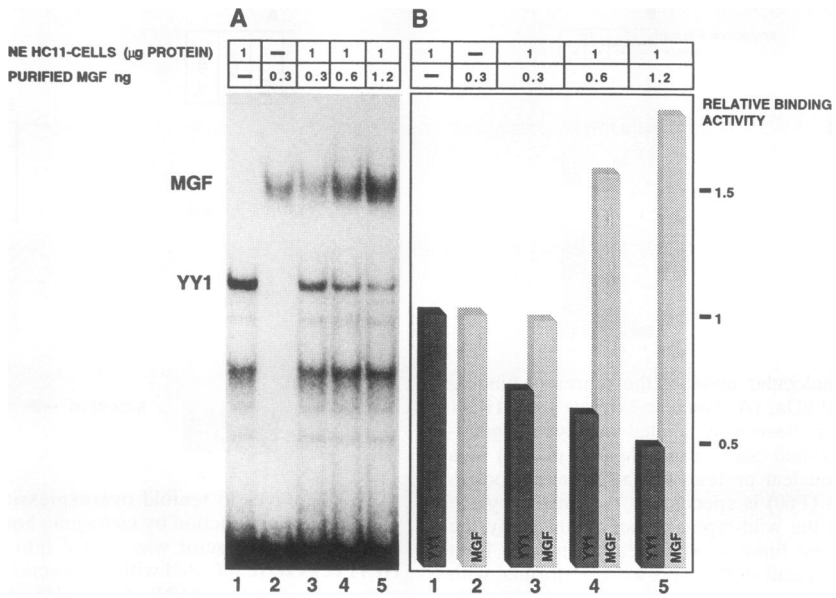

FIG. 10. YY1 and MGF compete for DNA binding. Band shift assays were performed with an oligonucleotide spanning the β-casein promoter sequences from −81 to −127 as a radioactive probe. The binding site of YY1 as well as the binding site of MGF are present in these sequences. Equal amounts of nuclear extracts from exponentially growing HC11 cells were incubated with the probe, and then different amounts (0.3 to 1.2 ng) of MGF purified from sheep mammary gland cells were added. Different volumes or protein concentrations were compensated for by adding the corresponding buffer or an equal amount of BSA, respectively. The bands representing YY1 and MGF were quantified with a PhosphorImager. The relative binding activities were calculated with respect to the binding activity of each factor in the absence of the other (lanes 1 and 2).

tially growing HC11 cells was used as a source of YY1. There is no detectable MGF activity in these extracts (Fig. 10, lane 1). Equal amounts of this extract were incubated with the radioactively labeled probe and increasing amounts of purified MGF (38). The resulting complexes were visualized after gel electrophoresis (Fig. 10A). The increase in the amount of MGF in the reaction mixture resulted in a stronger DNA-MGF band (Fig. 10A, lanes 3 to 5). The increase in MGF binding was paralleled by a decrease in YY1 binding. A complex of slower migration, predicted from the simultaneous binding of both factors to the probe, was not observed. This result indicates that YY1 and MGF compete for binding to the common promoter element. The binding activities of YY1 and MGF were quantitated (Fig. 10B). The binding activities of YY1 and MGF in reaction mixtures containing both factors were calculated relative to the binding activity obtained by each factor in the absence of the other. YY1 binding is markedly decreased upon addition of MGF (lanes 1 and 3), but binding of MGF is only slightly affected by the presence of YY1. This might reflect the higher affinity of MGF to the overlapping element and suggest a role for MGF in the relief of YY1-mediated repression.

The competition for DNA binding between MGF and YY1 was confirmed by using purified recombinant YY1. YY1 was expressed in bacteria, purified by affinity chromatography, and tested for its binding to the repressor region probe 6 (Fig. 11A, lane 1). Binding was observed with the wild-type sequence of probe 6 but not with the mutated probe 6rm (lane 2). The simultaneous inclusion of YY1 and MGF into the DNA binding reaction (Fig. 11B, lane 3) showed a clear preference for MGF binding over YY1 binding.

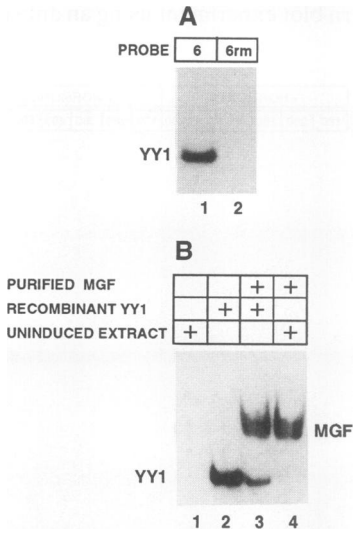

FIG. 11. Recombinant YY1 binds to the repressor element and is competed for in its DNA binding by purified MGF. (A) Binding specificity of recombinant YY1. Recombinant YY1 was partially purified from bacterial extracts and probed with the radioactively labeled wild-type oligonucleotide 6 (lane 1) or the mutated oligonucleotide 6rm (lane 2) in band shift assays. (B) MGF counteracts DNA binding of recombinant YY1. Band shift assays were performed with an oligonucleotide spanning the β-casein promoter sequences from −81 to −127 as a radioactive probe as described for Fig. 10. Approximately 1 ng of partially purified recombinant YY1 (lanes 2 and 3) or equal amounts of protein from uninduced bacterial extracts (lanes 1 and 4) were incubated with the probe; 1.3 ng of MGF purified from sheep mammary gland cells (lanes 3 and 4) or the same volume of the corresponding buffer (lanes 1 and 2) was added.

DISCUSSION

Several DNA sequence elements with different functional properties and specific nuclear protein binding interactions have been identified in the immediate 5′ region of the β-casein gene promoter. This study is concerned with the repressor element located in the region between −110 and −150 which is responsible for low transcription in the absence of lactogenic hormones. This element was initially discovered in a mutational analysis of the promoter. It was found that the repressor element is composed of a distal and a proximal half site. Mutations in the individual half sites had little influence on the transcriptional activity. The simultaneous mutation of both sites resulted in a high constitutive promoter activity (29). Here, we characterized the nuclear factor which interacts with the proximal half site of the repressor region. On the basis of its molecular weight, its DNA binding specificity, and its proteolytic clipping pattern, we conclude that the factor is identical with or very similar to the nuclear factor YY1.

The YY1 site with the sequence CCATTTTCT is present between −110 and −120 in the rat (17) and mouse (40) β-casein promoters. In the rabbit (36), goat (26), and bovine (3) β-casein promoters, the corresponding sequence TCATTTTCT also binds YY1, albeit with lower affinity. The consensus binding site of YY1 (also called CF-1, δ, or NF-E1) on different promoters consists of the core CCATnT (25). Exceptions are the ACATTTT sequence found in the adeno-associated virus P5 −60 promoter. Many additional sites with homology to this consensus sequence can be found in the upstream sequences of β-casein promoters. The sequence ACATTT at −230 of the rat β-casein promoter also binds YY1 (not shown). Additional putative YY1 binding sites are present in the transcribed regions of the mouse (40) and rat (17) β-casein genes, mostly in introns and in the 3′ flanking regions. For instance, at +530 in intron 1 of both genes, the sequence AGAAACCATGT, surrounded by clusters of the AGAA motif, is conserved. Another example is the sequence CCATCTTTCT at +2670 in intron 4 of the rat gene and the sequence CCATTTGTTTCTT at +2420 in intron 4 of the mouse gene. Both sites are very likely to bind YY1. The YY1 consensus binding site CCATnT is present eight times in the transcribed region of the mouse β-casein gene. The significance of these sites remains to be tested. YY1 was shown to activate transcription of ribosomal protein genes from binding sites upstream of the transcription start site (13, 14).

The specific binding of YY1 to the repressor region suggests that this factor is involved in the transcriptional repression of the β-casein gene promoter in the uninduced state. Since mutation of the YY1 binding site or of the distal site of the repressor element has only a small effect on transcription and YY1 itself does not bind to the distal site, we propose that repression is achieved by interaction of YY1 with a protein which binds the distal site of the repressor element. This protein has yet to be characterized. The interaction of YY1 with this nuclear factor can result in functional repression even if the complex of the proteins is anchored to the repressor element via only a single DNA binding site, the proximal site or the distal site. The situation is reminiscent of the interaction of YY1 (CF-1) with plasmacytoma repressor factor (PRF). This repressor factor has been found in differentiated B cells and acts on the c-*myc* promoter. The binding site of PRF is located at position −290 in the c-*myc* promoter just 5′ of the YY1 binding site. Binding of YY1 to the −290 element was greatly enhanced by complex formation with PRF (18). Deletion of the PRF binding site resulted in an increase in the rate of transcription. Mutation of the YY1 binding site had no effect (18, 25). In addition to the functional analogy, the repressor element of the β-casein promoter and the −290 element of the c-*myc* promoter share a high sequence homology (Fig. 2). The YY1 binding sites are identical in 10 bp but present in different orientations. The *myc*-PRF binding site with the sequence AGAAAGGGAAAGGA is located about 20 bp upstream of the *myc*-YY1 site (18). The distal element important for repression of the β-casein promoter has the sequence TT TCTTGGGAAAGAA and is located at the same distance from the YY1 binding site (29). Both elements share the sequence GGGAAAGPyA. The sequence TTTCT is present in the reversed orientation (AGAAA) on the c-*myc* promoter, like the YY1 site. On both promoters, the sequence AGAA is also present directly 5′ of the YY1 binding site and is contained in the YY1 binding site itself. The negative element of the β-casein promoter harbors six copies of the AGAA (or the inverted TTCT) motif. An AGAA is also contained in the MGF binding site.

The significance of the AGAA motif is emphasized by its presence in the cell-type-specific repressor element of the IgH-μE1 enhancer (39). Here it is repeated twice and located adjacent to the YY1 binding site. We speculate that the AGAA motif might be important in complex formation of YY1 with other cellular factors. It could either allow DNA contacts of YY1 when bound in a complex or function as a recognition site for an additional factor which mediates complex formation of YY1 with other proteins. Interestingly, the AGAA motif is not present in promoter elements, where repression of transcription is mediated by the YY1 site alone. The YY1 site in the adeno-associated virus P5 promoter, for example, mediates repression without the requirement of additional sequences or complex formation of YY1 with other factors (34).

The proteins contained in complexes A and B (Fig. 1), previously shown to bind to the repressor element of the β-casein promoter, could be considered as interacting with YY1. There are several observations, however, which argue against this possibility. First, complexes A and B are formed by several other sequences in the β-casein promoter which have no apparent sequence homology with the repressor element (28, 28a). Second, an oligonucleotide comprising the negative element of the β-casein promoter with mutations in the YY1 site as well as in the distal site of the repressor element (oligonucleotide 1/6 with mutations shown in oligonucleotides 1rm and 6rm; Fig. 4) still forms complexes A and B indistinguishably from the wild-type oligonucleotide (not shown). Third, no higher-order complex could be detected when extracts containing YY1 were mixed with extracts containing complexes A and B (not shown).

Since YY1 is part of the repressor complex, it was reasonable to assume that its concentration or DNA binding affinity could also be subjected to the control of the lactogenic hormones. However, we found no decrease in the binding activity of YY1 after hormonal induction of HC11 cells. YY1 was also found to be present in similar amounts in extracts of cells from lactating mammary glands (data not shown). Thus, the relief of repression is not due to an influence of the lactogenic hormones on the concentration or binding affinity of YY1. These results are corroborated by our experiments in which the concentration of YY1 in mammary epithelial cells was artificially raised. Overexpression of YY1 was not sufficient to suppress β-casein expression or the expression of transfected β-casein–CAT con-

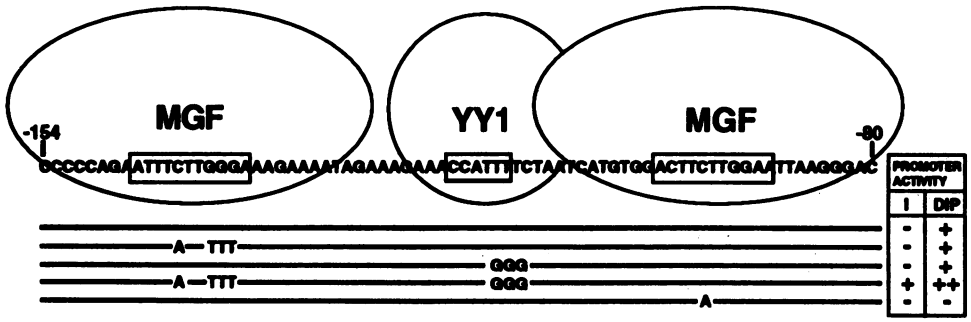

FIG. 12. Model for the dual function of MGF in the hormonal regulation of β-casein promoter activity and its interaction with YY1. The β-casein promoter sequences from −80 to −154 span the repressor element (−154 to −100) and the high-affinity MGF binding site (−80 to −100). An MGF binding site with lower affinity is present in the distal half of the repressor element. Promoter activities in the uninduced (insulin [I]) and induced (dexamethasone, insulin, prolactin [DIP]) states of the cells are indicated. Mutations that abolish YY1 binding are not sufficient to relieve repression. Equally, mutations in the distal half of the negative element have by themselves little effect on transcription. Simultaneous mutation of both sites results in high, hormone-independent transcription. This finding suggests that YY1 may play a role in repression by interacting with MGF, or another cellular factor, present at the distal half of the repressor element in the absence of hormone. Mutation analysis of the MGF binding site between −80 and −100 indicates that binding of MGF to this site positively regulates promoter activity. MGF assumes a high binding affinity for this site upon hormonal activation (30). Since the YY1 and the high-affinity MGF sites are overlapping, MGF binding could cause the replacement of YY1 and thereby counteract its repressor function. The promoter activity data are from Schmitt Ney et al. (29).

structs. These findings are consistent and indicate that repression and derepression are not regulated via changes in the concentration of YY1 and might be achieved by the interaction of YY1 with another nuclear factor.

We have previously described the nuclear factor MGF, which binds specifically to the region from −80 to −100 of the β-casein promoter. This site is located just 3′ of the YY1 binding site. A second binding site, which binds MGF with a somewhat lower affinity, is located 5′ of the YY1 binding site, in the distal half of the repressor region. The binding activity of MGF to its high-affinity binding site at −80 to −100 increases dramatically with the onset of lactation in vivo or the lactogenic hormone treatment of HC11 cells (29). The experiments shown here demonstrate that small amounts of purified MGF added to nuclear extracts containing YY1 or purified YY1 protein markedly decreased the binding of YY1 to a promoter fragment comprising the YY1 binding site and the adjacent MGF high-affinity binding site. The displacement of YY1 was a function of the amount of MGF added. No more slowly migrating complex which would indicate the simultaneous binding of both factors to the probe was detected, and the effect was observed at different probe concentrations (not shown).

Taking these observations into consideration, we propose a model in which MGF could play a dual role in the regulation of transcription (Fig. 12). In uninduced cells, MGF might be present on the distal site of the repressor element and form a functional complex with YY1. This complex might repress transcription. Upon hormonal induction, MGF might become phosphorylated. This modification could convert MGF to an activating form, which could be released from the complex with YY1 and bind with high affinity to the −80 to −100 site 3′ of the YY1 binding site. The binding of MGF to this site could result in the replacement of YY1 from its binding site as observed in vitro. This model predicts that MGF can assume a repressing and an activating function in collaboration with the ubiquitous factor YY1. Recent reports describe reminiscent scenarios in the c-*fos* and skeletal α-actin promoters. YY1 and the serum response factor (SRF) act as functional antagonists and compete for binding to overlapping binding sites (9). In contrast to our observations, the increase in concentration of the activator SRF is paralleled by a decrease in YY1 concentration in myoblasts (19). Overexpression of YY1 repressed basal and induced gene expression, which could be counteracted by overexpression of SRF (9). Modulation of YY1 function can also be observed by E1A. This oncoprotein can turn repression exerted from the YY1 binding site into activation, without altering YY1 DNA binding activity (34).

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