Hormonally Regulated Double- and Single-stranded DNA-binding Complexes Involved in Mouse β-Casein Gene Transcription*

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Transcription of the 252-base pair-long mouse β-casein gene promoter is induced by the synergistic action of insulin, prolactin, and glucocorticoid in a primary mammary epithelial cell culture. The promoter contains a region termed block C having a highly conserved sequence and position among many casein genes. Mutation of block C reduced the response of the promoter to lactogenic hormones 84%. Nuclear extracts from lactating mouse mammary glands contained both a double-stranded and a single-stranded DNA binding protein complex (DS1 and SS), which specifically bind to the sequences AAATTAGCATGT and CCACAA of block C, respectively. The DS1 and the SS protein complexes were approximately 400 and 280 kDa, respectively. Each complex contained a DNA-binding component(s) having a molecular mass of approximately 120 kDa for DS1 and 80 and 65 kDa for SS. Deoxycholate, which interferes with the protein-protein interactions, inhibited the binding activities of DS1 and SS. The maximal increase in the binding activity of DS1 and SS in the mammary gland occurred during pregnancy and during lactation, respectively. In organ culture, the DS1 activity is increased by epidermal growth factor or prolactin in combination with insulin, whereas the SS activity is enhanced by insulin, prolactin, and glucocorticoid. These results suggest that multiprotein complexes binding to the double- and single-stranded DNA of block C mediate hormonal induction of β-casein gene transcription.

Caseins are the major milk proteins produced by the lactating mammary gland. The transcription of casein genes is regulated by the interplay of various hormones such as insulin, prolactin, and glucocorticoid. This system serves as a good model to study the molecular mechanisms whereby hormones regulate mammalian gene transcription. Prolactin and glucocorticoid are required for enhancement of both transcription of casein genes and stability of the transcripts (1), whereas cocorticoid are required for transcription but not for stabilization of casein mRNAs (2). Transfection of reporter plasmids containing 5'-flanking region of the β-casein gene to either primary mouse mammary epithelial cells (3) or a rat mammary epithelial cell line, HC11 (4), also demonstrated that all three hormones are required for full induction of the casein gene transcription and that the proximal region of the promoter, −258/+7 of mouse gene (5) or −355/+1 of rat gene (4), is sufficient for hormonal induction.

At least seven promoter regions of mouse, rat, bovine, and rabbit casein genes have been cloned (6–11). Alignment of their DNA sequences has revealed three highly conserved sequences, designated as blocks A, B, and C, in each promoter region (Ref. 6 and Fig. 1A). Blocks A and B share a similar sequence, whereas the sequence of block C is quite different from those of block A and B. The presence of nuclear proteins binding to these three regions was detected in the lactating mouse mammary glands (5) as well as a rat mammary epithelial cell line, HC11 (12). These proteins are likely candidates for transcription factors involved in casein gene expression.

Recently a protein that binds to block B was cloned and sequenced (13). This protein has been named mammary gland-specific factor (MGF3 or STAT5) and was shown to be a member of the cytokine-regulated transcription factor gene family (13). Several lines of evidence indicate that prolactin stimulates the phosphorylation of MGF, which then participates in the transcriptional activation of the β-casein gene by binding to block B and probably block A (4, 14). These findings relating to MGF suggest that the highly conserved regions of the casein gene promoter serve as a major site for transcriptional regulation wherein hormonally induced transcription factors act.

Another well conserved sequence, block C, is present approximately 20 base pairs upstream of a TATA box and approximately 40 base pairs downstream of block B. Its position also is well conserved among casein gene promoters (Fig. 1A). At present, however, the role of block C in regulating casein gene transcription has not been elucidated. Moreover, the nuclear protein(s) that bind to this region have not been characterized. Recently several groups reported that single-stranded DNA binding proteins play a role in transcriptional regulation (15–21). For example, a sequence-specific, single-stranded DNA binding factor (STR; Refs. 16 and 22) that binds to the distal region of rat β-casein gene promoter has been shown to have a suppressive role. These studies led us to investigate both double- and single-stranded DNA binding proteins that bind to block C.

In this study we examined the functional role of block C in the hormonal induction of mouse β-casein gene transcription. Transfection experiments using a primary mammary epithelial cell (PMME) culture system indicated that block C is crucial for the β-casein gene transcription induced by lactogenic hormones. Our studies also revealed the presence of nuclear protein complexes in the lactating mammary gland that bind to both double-stranded and single-stranded DNA within block C. The appearance of these binding complexes in the mammary gland is hormonally regulated and undergoes developmental changes during the periods of pregnancy and lactation.

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The abbreviations used are: MGF, mammary gland-specific factor; EGF, epidermal growth factor; CAT, chloramphenicol acetyltransferase; DTT, dithiothreitol; PMME, primary mammary epithelial cell; DEPC, diethylpyrocarbonate.
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**A**

| Block A (MBG sites) | Block B (MBG min) | Block C (MBG sites) |
|--------------------|------------------|-------------------|
| -150              | -130             | -110              |
| -97               | -76              | -55               |
| -42               | -21              | -1               |
| -1               | -1               | -1               |

*Fig. 1. Structure of the proximal promoter region of mouse β-casein gene (A) and alignment of sequence between block C and TATA box of various casein promoters (B). The positions of blocks A, B (MGF binding site C and TATA box are shown by box pair distance from the transcriptional initiation site, denoted as + 1. Sequences of block C and TATA box are shown by boldface letters.*

**EXPERIMENTAL PROCEDURES**

Reagents—Bovine prolactin (number B-1) was obtained from the Hormone Distribution Program (NIDDK, National Institutes of Health). Hydrocortisone and mouse epidermal growth factor (EGF) were purchased from Calbiochem and Collaborative Research (Bedford, MA), respectively. Crystalline porcine zinc insulin was a gift from Eli Lilly (Indianapolis, IN).

Animals—Age-matched virgin (3-month-old) and pregnant (10–12 days gestation) C3H/HeN female mice were obtained from the Animal Center of the National Institutes of Health. Animal care and study protocols were in full compliance with the National Institutes of Health guidelines.

Organ Culture—Mammary explants from pregnant mice (in the second half of their first gestation) were cultured in the presence of various hormones as described previously (23). The concentrations of hormones used were as follows: insulin, 5 μM; hydrocortisone, 1 μM; prolactin, 5 μM; EGF, 50 ng/ml. After culture, explants were processed for preparation of nuclear proteins as described below.

Plasmids, Transfections, and Chloramphenicol Acetyltransferase (CAT) Assays—The plasmid, pD9 (5) was digested with HindIII and SphI to remove wild-type β-casein promoter. DNA fragments were subjected to gel purification to obtain a reporter plasmid bearing the bacterial CAT gene. Mutated DNA fragments corresponding to the 5' Flanking region (−245/+7) of mouse β-casein gene were prepared by a polymerase chain reaction-based mutagenesis method (24). These fragments were ligated into the CAT reporter plasmid. All insert sequences were verified by the dye-deoxy sequencing method (25). The preparation and culture of PMMSs, transfection, and CAT assays were performed as described previously (3) with minor modifications. Antibiotics added to culture media were penicillin G at 100 units/ml, streptomycin at 100 μg/ml, and amphotericin B at 250 ng/ml. Twenty micrograms of CAT plasmid was cotransfected with 5 μg of β-galactosidase expression plasmid (pSVβ-gal, Promega) in each culture dish, and transfection efficiency was normalized to β-galactosidase activity (26).

Preparation of Nuclear Extracts and Gel Shift Assays—Mammary glands were cut into pieces, placed in buffer A (20 mM Hepes, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM EDTA, and 0.3 mM sucrose) for 15 min and homogenized with a Dounce homogenizer (20 strokes with a type B pestle). The nuclei were sedimented by brief centrifugation, washed two times with buffer A without 0.3 mM sucrose, and then resuspended in 2 volumes of buffer B (20 mM Hepes, pH 7.9, 0.3 mM NaCl, 1.5 mM MgCl2, 1 mM EDTA, 12% glycerol, 2.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 μM pepstatin A, and 18 μM aprotinin). After being stirred for 30 min, the mixture was centrifuged at 18,000 × g for 20 min. The supernatant was diluted with buffer C (20 mM Hepes, pH 7.9, 1 mM EDTA, and 2.5 mM DTT) to increase NaCl concentration to 0.1 M, and concentrated with a Centricon-10 filter (Amicon). All procedures were performed at 4°C. Aliquots were stored at −70°C for later use.

Gel shift assays were performed using either double-stranded or single-stranded oligonucleotides corresponding to the −69/-38 region of the mouse β-casein gene promoter. The coding strand was end-labeled with [γ-32P]ATP (Amersham Corp., 6000 Ci/mmol) using T4 polynucleotide kinase (Stratagene). To prepare the double-stranded probe, the labeled oligonucleotide was hybridized to the noncoding strand. Both oligonucleotides were purified by electrophoresis in a 10% polyacrylamide gel to remove unincorporated nucleotides and nonhybridized oligonucleotides. Nuclear extracts were incubated with a 32P-labeled probe (3 × 106 cpm) in 25 μl of binding buffer (12 mM Hepes, pH 7.9, 12% glycerol, 60 mM NaCl, 1.5 mM MgCl2, and 2.5 mM DTT) with 3 μg of bovine serum albumin (Promega), 1.5 μg of poly(dI-dC) (Sigma), and with or without competitor oligonucleotides bearing wild-type or mutated sequences (Table 1). Following a 30-min incubation at 25°C, the reaction mixtures were subjected to electrophoresis in a 6% polyacrylamide gel in TBE buffer (pH 8.3, 90 mM Tris base, 90 mM boric acid, and 0.5 mM EDTA). The gels were dried, and the bands were detected by autoradiography. For quantitation of band activities, gels corresponding to retarded bands were cut out, and their radioactivities were measured by a liquid scintillation counter (Beckman LS3801).

**RESULTS**

**Block C Region within Mouse β-Casein Promoter Is Important for Transcriptional Induction by Lactogenic Hormones in PMME—We prepared a series of β-casein promoter-CAT gene constructs bearing various mutations within or outside of block C (Table I) and examined their response to the lactogenic hormones, insulin, prolactin, and hydrocortisone using the PMME transfection system (3). A construct bearing the −245/+7 region of mouse β-casein promoter was used as a wild-type construct (pD30wt). The CAT activity of the wild-type construct increased approximately 18-fold in the presence of the combination of three hormones, insulin, hydrocortisone, and prolactin compared with the level in the insulin-treated control (Fig. 2, A and B). Omission of hydrocortisone or prolactin resulted in very low CAT activities, which were approximately 5% of the level induced by the three hormones. Constructs pD30.3, pD30.4, and pD30.5 bearing mutations within block C showed reduced induction, 23, 29, and 45%, respectively, compared with the wild-type construct (Fig. 2B). In addition, a double mutation (pD30.35) resulted in further de-
Table I

| CAT construct | Gel shift competitor | Sequence
|-------------|---------------------|--------|
| pD30 wt     | WT                  | A T C T T A C A A A C C A A A A T T A G C A T G T C A T T A |
| pD30.1      | M1                  | G A A T |
| NA          | M2                  | G T T T |
| pD30.3      | M3                  | G T G T |
| pD30.4      | M4                  | T T T T |
| pD30.5      | M5                  | T A A T C |
| NA          | M6                  | G T A C |
| NA          | M7                  | T T |
| NA          | M8                  | A A |
| NA          | M9                  | |
| NA          | M10                 | T C |
| pD30.35     | M3+5                | G G T G T - T A A T C |

*Each β-casein promoter-CAT construct bears the same sequence as indicated.

A hyphen represents the same residue as that of wild type.

The number refers to the position from the transcription initiation site.

Wild type.

NA, not available.

FIG. 2. Effect of various mutations within block C on transcriptional induction of mouse β-casein gene by lactogenic hormones. A, effect of various hormone combinations on induction of CAT activity of pD30 wild-type construct (bearing −245/+7 region of β-casein promoter). Mouse mammary epithelial cells were prepared from mice in midpregnant stage (12–14 days of gestation) and cultured for 5 days in the presence of the indicated hormones. Concentration of insulin (I), hydrocortisone (H), and prolactin (P) used were 5 μg/ml, 1 μg/ml, and 5 μg/ml, respectively. B, the basal (black bar) and induced (shaded bar) CAT activities of various constructs. The basal and induced activities were determined in culture with insulin and with insulin/hydrocortisone/prolactin, respectively. The activities were calculated relative to the induced activity of pD30wt construct (−1) in each transfection experiment. Means of relative values ± S.E. from three to six independent transfection experiments are presented.

crease in CAT induction to 16% of the induced level of wild-type promoter. In contrast, a construct bearing a mutation outside of block C (pD30.1) showed almost the same level of induction as wild-type. All CAT reporters used here produced similar levels of basal CAT activities when PMME cells were cultured with insulin alone.

Double-stranded and Single-stranded Binding Activities to Block C Are Detected in Lactating Mouse Mammary Glands—To detect nuclear proteins that bind to either double-stranded or single-stranded DNA within block C, nuclear extracts prepared from mammary glands of lactating mice were analyzed by a gel shift assay. The probe used was either double-stranded (DS) or coding strand (SS(+)) or noncoding strand (SS(−)) DNA corresponding to the −69/−38 region of mouse β-casein promoter.

The DS probe formed at least three bands when incubated with mammary nuclear extracts (Fig. 3A). The upper major band (DS1) showed clear sequence specificity because the band was competed completely by wild type or M7 and partially by M3, M8, or M10 but not by the others (Fig. 3A, Table I). The intensity of the middle minor band (DS2) was rather faint in this experiment. However, other experiments using a partially purified fraction of nuclear proteins, in which DS2 binding activity was enriched, indicated that DS2 had the same sequence specificity as DS1 (data not shown). The lowest band was considered to be nonspecific because it did not show clear sequence specificity in competition experiments.

The SS(+) probe formed two binding complexes (Fig. 3B). The lower band was judged to be nonspecific because wild type and all mutated competitors were equally effective. The upper band (SS) was specific because wild type, M1, M2, M9, and M10 all competed efficiently, M5, M6, M7, and M8 competed less efficiently, and M4 showed no competition. M3, which showed substantial competition in DS1 binding, was a poor competitor in SS binding. Fig. 3C showed that SS(+) did not compete in DS1 complex formation (lane 3), suggesting that DS1 did not bind to SS(−). On the other hand, the SS complex formation was inhibited by the wild type of DS competitor (lane 7), suggesting that SS did bind to DS. These results implied that DS1 required double-stranded DNA for binding, whereas SS could bind to both single- and double-stranded DNA in block C. These findings suggested that these two binding factors are different entities.

The SS(−) probe formed a broad band, which was considered to be nonspecific because of no competition by a 30-fold molar excess of SS(−) competitor (Fig. 3C, lanes 7 and 10). In the presence of SS(+) competitor, appearance of the DS complex was found (Fig. 3C, lane 9). This was likely due to formation of DS probe as a result of annealing of SS(+) competitor to SS(−) probe during the binding reaction.

To confirm the formation of binding complexes and to localize the direct binding sites in the block C region, DEPC interference assays were performed. DEPC modifies the N-7 position of free purines in single- and double-stranded DNA, which results in interfering protein-DNA interaction (27). DNA fragments bound to proteins were isolated, cleaved at the position of modification by the treatment of piperidine, and subjected to gel electrophoresis. Nuclear extracts from lactating mammary glands were incubated with either DS or SS(+) oligonucleotides chemically modified at A and G residues by DEPC. When a DS probe labeled at either the coding or noncoding strand was used, interference of A and G residues occurred at positions corresponding to those mutated in M4, M5, and M6 competitors (Fig. 4, A and B, closed circle). When a labeled SS(+) probe was used, residues at positions corresponding to those mutated in...
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Fig. 3. Competition gel shift assay of the binding complex formation on the block C region. Three micrograms of nuclear extract from mammary glands obtained from mice in the 3rd through the 7th days of lactation was incubated with an end-labeled double-stranded (A) or a single upper strand (B) DNA corresponding to the -69/-38 region of mouse β-casein promoter in the presence of 50-fold molar excess of double-stranded (A) or 20-fold molar excess of single-stranded (B) unlabeled competitors. The sequence of competitors (M1 through M10) is shown in Table I. Three, five, or two micrograms of nuclear extract from lactating mice was allowed to bind to DS, SS(+), or SS(−) probe, respectively (C). Thirty-fold molar excess of unlabeled DS (lanes 2 and 6), SS(+)(lanes 3 and 5) or SS(−)(lane 10) wild-type competitor was added to the binding mixture before the addition of labeled probes. WT, wild-type; NO, no competitors. Specific complexes, DS1, DS2, and SS, are shown by arrows.

M4 were interfered with (Fig. 4C, closed circle). We found hypersensitive sites at adenine residues positioned at −55 and −57 on the double-stranded probe (Fig. 4B), and also at −49 on the single-stranded probe (Fig. 4C). These hypersensitive sites could result from binding of proteins to the adjacent nucleotides. The results of interference assays indicated that the binding sequences of DS1 and SS involved 5′-AAATTAGCATGT-3′ and 5′-CCACAA-3′, respectively.

Changes in the Binding Activities of DS1 and SS in the Mammary Gland at Various Reproductive Stages—To determine developmental changes in DS1 and SS binding activities in the mammary gland, nuclear extracts prepared from mammary glands of virgin, pregnant, lactating and postlactating mice were subjected to gel shift assays (Fig. 5, A and B). The change in DS1 and SS binding activities during pregnancy and lactation was estimated by determining the radioactivities of corresponding bands. The levels of DS1 and SS binding activities relative to their peak values, i.e., on the 17th to 19th day of pregnancy for DS1 and the 7th to 10th day of lactation for SS, are shown in Fig. 5C. The DS1 binding activity was low in the extracts of virgin and postlactating animals but showed an apparent increase on the 17th to 19th day of pregnancy. Thereafter it increased further to reach nearly the plateau level on the 17th to 19th day of pregnancy. In contrast to the DS1 binding activity, the elevated level was maintained during lactation and then decreased in the postlactating stage.

Effects of Various Hormones on the Binding Activities of DS1 and SS in Cultured Mammary Gland—The growth and differentiation of the mammary gland can be induced by the appropriate combination of hormones and growth factors in an organ culture system (29). Mammary cell proliferation is stimulated by the combination of insulin and EGF, whereas casein gene expression is induced by insulin, hydrocortisone, and prolactin. Both insulin and EGF are also important for mammary epithelial cells to express the casein gene because cultivation of mammary cells in the presence of insulin and estrogen decrease cell growth and differentiation of the mammary gland (3, 23). This in vitro system was used to examine the influence of hormones on the binding activities of DS1 and SS. Mammary gland explants from pregnant mice were cultured in the presence of hormones as indicated. During culture with the combination of insulin and EGF, the level of DS1 binding activity increased on day 1, reached a maximum on day 2, and remained elevated up to day 3. The initial level of DS1 binding activity decreased in the presence of insulin or EGF alone. The combination of insulin and prolactin or insulin, prolactin, and hydrocortisone increased the level of DS1 binding activity, but the combination of insulin and hydrocortisone did not (Fig. 6A).

The level of SS binding activity was increased by the combination of insulin, prolactin, and hydrocortisone, whereas the
Multiple Proteins—cultured cells.

The original sample applied (Fig. 3) as a control. C, the level of binding activity in each band was evaluated by determining its radioactivity. These results were presented relative to the mean of the peak activity, 17th to 19th day of pregnancy for DS1 and 7th to 10th day of lactation for SS. The relative values of mean ± S.E. of each time point were obtained from three independent experiments.

In addition, we attempted to examine the effect of hormones on the binding activities of DS1 and SS in mouse mammary epithelial cells in primary culture. However, it was difficult to obtain nuclear extracts from cultured cells in adequate quantity and quality because those cells cultured on the Matrigel needed to be extensively treated with trypsin and EDTA to detach them from the substratum. This treatment produced a number of problems in isolating nuclear binding proteins from cultured cells.

Both DS and SS Binding Protein Complexes Are Formed by Multiple Proteins—Molecular weights of the DS and the SS binding protein(s) in nuclear extracts from lactating mammary gland were determined by gel filtration column chromatography using various molecular weight marker proteins (Fig. 7A). The original sample applied (AP) had a major binding activity, DS1, and a minor binding activity, DS2. Upon chromatography, DS1 and DS2 binding activities were eluted at different fractions. The peak activity of the DS1 and the DS2 binding was at fractions 17 and 24, respectively. The molecular mass of DS1 and DS2 binding proteins was estimated to be 400 and 230 kDa, respectively (Fig. 7B). The peak activity of the SS binding was found at fraction 21, which corresponded to approximately 280 kDa (Fig. 7C). Such large molecular weights of these proteins suggested that they were composed of multiple protein components.

In order to detect protein molecules binding directly to DNA probes, cross-linking experiments were performed. Using nuclear extracts, we found a single protein binding to the DS probe in the DS1 band (Fig. 8A) and two proteins binding to the SS (+) probe (Fig. 8C). We also performed similar experiments using partially purified extracts in which DS2 binding activity was enriched. Again, a single protein binding to the DS probe was found (Fig. 8B). The molecular mass of the DNA binding protein was approximately 120 kDa for DS1 and 80 kDa for DS2. The molecular masses of two DNA binding proteins for the SS complex were estimated to be 80 and 65 kDa. These values were much smaller than those of the corresponding complexes determined by gel filtration experiments.

Deoxycholate is a mild ionic detergent which, when used at low concentrations, is known to disrupt protein-protein interactions but not DNA-protein interactions (30, 31). As shown in Fig. 8D, the addition of deoxycholate at very low concentrations to the binding reaction inhibited the formation of DS or SS bands and produced no bands migrating faster (Fig. 8, D and E). This suggested that disruption of DNA-binding complexes by deoxycholate produced no DNA binding component of smaller size. The effect of Nonidet P-40, a nonionic detergent, was also analyzed. Nonidet P-40 (0.3%) had no effect on the DS complex formation by itself but prevented the disruptive effect of deoxycholate in the complex formation. This might be due to the effect of Nonidet P-40 to sequester deoxycholate from solution (31). As for the SS complex, Nonidet P-40 like deoxycholate, inhibited the formation of the complex. The results presented above suggested that all three DNA-binding complexes, DS1, DS2, and SS, are composed of multiple proteins.
here that the mouse dependent transcription factor, MGF (12). We demonstrate has not been elucidated, whereas block B and probably block A serves regions, designated as blocks A, B, and C, in the promoter. Thirty micrograms of partially purified extract (mixture of fractions 25–28 obtained from gel filtration column chromatography (see Fig. 7)) was allowed to bind to a double-stranded (B) 5-bromo-2-deoxyuridine-substituted DNA probe corresponding to the −69/−38 region of the mouse β-casein promoter. Thirty micrograms of partially purified extract (mixture of fractions 25–28 obtained from gel filtration column chromatography (see Fig. 7)) was allowed to bind to either double-stranded (A) or coding strand (C) 5-bromo-2-deoxyuridine-substituted DNA probe corresponding to the −69/−38 region of the mouse β-casein promoter.

**DISCUSSION**

Previously we reported the presence of three highly conserved regions, designated as blocks A, B, and C, in the promoters of many casein genes (6). The functional role of block C has not been elucidated, whereas block B and probably block A have been suggested to serve as an acting site of a prolactin-dependent transcription factor, MGF (12). We demonstrate here that the mouse β-casein gene promoter (−245/+7) containing all three blocks responded fully to lactogenic hormones, insulin, prolactin, and hydrocortisone when transfected into mouse PMME. Mutational disruption of block C impaired the response of the promoter to the lactogenic hormones as much as 84%, although the mutated promoter still retained its hormonal response (2.7-fold). These observations indicated that block C, like the other two conserved regions, is important for enhancement of the transcriptional activation by lactogenic hormones.

The results of gel shift assays using DNA probes corresponding to the block C sequence revealed the presence of two double-stranded DNA binding activities (DS1 and DS2) and one single-stranded (coding strand) DNA binding activity (SS) in the lactating mammary gland. The results obtained by competition experiments in gel shift assay and by DEPC interference assay indicated that the binding sequences of these complexes involved 5′-AAATAGCATGT-3′ for DS1 and 5′-CCACAA-3′ for SS. Nine out of 12 nucleotides of the DS1 binding sequence and seven out of eight nucleotides of the SS binding sequence are conserved among seven casein gene promoters (see Table I). As for DS2 complex, DEPC interference assay gave inconclusive results because of low levels of the complex formation. However, the results obtained by competition experiments suggested that DS2 complex involves the same binding sequence as DS1. At present, the relationship between DS1 and DS2 remains to be elucidated.

The formation of the double-stranded binding complex DS1 and the single-stranded binding complex SS involved a contiguous, partially overlapping element, located at −54/−43 and −59/−53, respectively, on the mouse β-casein gene promoter. Competition gel shift assays revealed that the sequence mutated in M3 was important for the binding of SS but less so for DS1 binding and that the sequence mutated in M5 was important for the binding of DS1 but not for SS binding. The data in Fig. 3C suggested that SS could bind to both single- and double-stranded DNA in block C, whereas DS1 could bind only to the double-stranded DNA. Transfection experiments showed that disruption of SS binding site (pD30.5) resulted in a greater decrease in hormonal induction of CAT activity than that of DS1 binding site (pD30.3) and pD30.5, suggesting that the binding of SS was important for the induction. Moreover, disruption of both
sites (pD30.35) caused an even greater decrease in the CAT activity, suggesting the possible interplay between DS1 and SS binding factors. On the other hand, these mutations did not affect the basal CAT activities. These results suggested that the DS1 and the SS complexes are formed on a contiguous element within block C and that they work cooperatively as positive regulators of hormonal induction of mouse β-casein gene transcription. It is possible that the initial binding of DS1 to block C results in conformational changes such as partial dissociation of the double-stranded DNA and subsequently allow SS to bind to block C. The difference in strand requirements between the two binding factors may provide the mean whereby the two factors interact with block C in turn to induce casein gene transcription. Replacement of DS1 binding by SS may occur at or near their binding sites, which have the overlapping recognition sequence.

Sequence-specific, single-stranded DNA binding proteins have been reported to play an important role in the expression of various genes (15–21). Most of them bind solely to DNA. Recently novel single-stranded binding proteins were shown to bind to the contiguous sequence of a TTF-1 binding site in thyrotropin receptor gene promoter. TTF-1 is a double-stranded binding transcriptional factor. The authors speculated that single-stranded binding proteins positively regulate gene transcription in cooperation with TTF-1 (32). Another sequence-specific, single-stranded DNA binding factor (STR; Refs. 16 and 22) was reported to play a role in β-casein gene transcription. This factor was found to bind to the distal region of the β-casein gene promoter and to exert negative regulation. On the other hand, we found that the SS complex binds to the proximal region of the promoter and exerts positive regulation. Although the molecular mechanisms of action of these regulators have not yet been clarified, these findings suggest the importance of single-stranded DNA binding proteins for β-casein gene regulation.

UV cross-linking experiments indicated that a 120-kDa protein bound directly to the double-stranded DNA of block C. On the other hand, the molecular mass of the DS1 protein(s) complex was estimated to be 400 kDa by gel filtration chromatography. As for the SS complex, 80- and 65-kDa proteins were found to bind directly to single-stranded DNA, whereas the molecular mass of the entire binding complex was approximately 280 kDa. Thus, the values of molecular mass of the entire binding complex and DNA-binding component(s) are quite different. Although the reason for this difference is not known at the present time, one possibility is that the DS1 and SS complex formation involves multiple proteins, which include DNA binding and non-DNA binding proteins. The inhibitory effect of deoxycholate on the binding activities of both complexes suggested that these complexes were composed of multiple components. Moreover, deoxycholate treatment produced no DNA-binding complexes having smaller molecular weights, suggesting that full assembly is necessary for their DNA binding activities. Many transcription factors including Fos/Jun, ATF/CREB, MyoD, and E12/E47-like proteins are known to be multimeric complexes formed by protein-protein interactions (33–35).

Our studies showed that the appearance of the DS1 and SS binding complexes in the mammary gland undergoes developmental changes under the influence of hormones. The DS1 binding activity increased to a maximal level during pregnancy and was maintained at somewhat reduced levels during lactation. In contrast, the SS binding activity increased maximally during lactation. It is noteworthy that the levels of the two binding activities in the gland increase sequentially during the periods of pregnancy and lactation when casein gene expression increases progressively. On the other hand, their levels are low in virgin and postlactating periods when casein gene is not expressed. Thus, the appearance of the two binding activities correlate well with the change in casein gene expression.

It has been well established that the combination of EGF and insulin stimulates the formation of daughter cells in the mammary gland that express casein gene in the presence of insulin, prolactin, and hydrocortisone in vitro (29). Organ culture experiments using mouse mammary gland explants showed that the DS1 binding activity was increased by the combination of insulin and EGF, and to a somewhat lesser extent by the combination of insulin and prolactin. The SS binding activity was enhanced by the synergistic actions of insulin, prolactin, and hydrocortisone. These results indicated that the binding activities of the two complexes are differentially regulated by the different combinations of hormones during mammary gland development.

Prolactin, hydrocortisone, and EGF are also implicated in the mammary gland development during the periods of pregnancy and lactation. For example, both the plasma concentration of EGF and the number of EGF receptors in the gland increase during pregnancy (36, 37). During the lactating period, the number of EGF receptors in mammary tissue decreases, but both the plasma concentration of prolactin and the number of prolactin receptors in the gland increase (38). These changes in the levels of EGF and prolactin and their receptors could, at least in part, account for changes in the DS1 and the SS binding activity in the mammary gland during the periods of pregnancy and lactation. The findings presented in this study suggest that the hormonal induction of β-casein gene expression involves stimulation of binding activities of DS1 and SS, which, in turn, enhance the gene transcription via block C. These binding proteins are likely candidates for transcription factors that participate in the tissue- and stage-specific casein gene expression. Purification of SS and DS1 complexes would allow us to examine their predicted transcriptional activities using the in vitro transcription system. Moreover, cloning of these factors would make it possible to determine the relative roles of each by expressing them in hormone-responsive cell lines that are deficient in the SS/DS1 binding activities.

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