The gene encoding UDP-GlcNAc:dolichol phosphate N-acetylglycosamine-1-phosphate transferase (GPT), the enzyme that initiates the pathway for the biosynthesis of asparagine-linked glycoproteins, is ubiquitously expressed in eukaryotic cells. However, its expression in the mammary gland is developmentally and hormonally regulated; transcription of the mouse mammary GPT gene is stimulated by the lactogenes hormones, insulin, glucocorticoid, and prolactin. The involvement of cis-acting elements in regulating the expression of the mouse GPT (mGPT) gene was investigated by transient transfections of various GPT promoter/luciferase (Luc) constructs into primary mouse mammary epithelial cells. A series of 5′-deletions of the GPT promoter identified a distal negative regulatory region (base pairs −1057 to −968) and deletion of this region results in enhanced hormonal induction (~7-fold) with no effect on basal promoter activity. Electrophoretic mobility shift assays (EMSA) performed with nuclear extracts from different developmental stages of mouse mammary gland demonstrated that the binding activity of the nuclear proteins to the distal negative regulatory region was predominant in virgin stage as compared with pregnant and lactating stages. EMSA performed with nuclear extracts from virgin explants showed that the binding activity was markedly decreased after cultivation with the combination of the three lactogenes hormones. DNase I footprinting analysis identified two pentamer direct repeat motifs, AGGAA and GAAAC, within the negative regulatory region. EMSA competition experiments showed that mutations within the direct repeats failed to compete for binding of the nuclear proteins to labeled wild type oligonucleotide. Transcription from the promoter containing the mutated direct repeats was increased greatly, consistent with the conclusion that these motifs functions in vivo to repress GPT gene expression. These data suggest the importance of the negative regulatory region in hormonal control of mGPT gene expression in mammary gland.

Asparagine-linked glycosylation in eukaryotes is initiated by a stepwise, dolichol-linked assembly of GlcC1Man9GlcNAc2-P-P-dolichol and its transfer en bloc to the nascent polypeptide in the endoplasmic reticulum, followed by co- and post-translational modifications of the tetradeacascaride in the secretory pathway (1). The enzyme UDP-GlcNAc:Dol-P GlcNAc-1-P transferase (GPT)1 catalyzes the first and the committed step, i.e. the transfer of GlcNAc-1-P from UDP-GlcNAc to Dol-P to form GlcNAc-P-P-Dol, in the assembly of the oligosaccharide precursor. It is, therefore, a potential target for the overall regulation of oligosaccharide assembly and protein N-glycosylation.

GPT has been purified to apparent homogeneity from bovine mammary gland (2); the cDNAs encoding GPT enzyme have been isolated from cDNA libraries prepared from mRNA of tunicamycin-resistant Chinese hamster ovary cell lines (3, 4) and mouse mammary gland (5). The GPT gene has been cloned from yeast (6), Leishmania (7), and mouse (8). These studies show that GPT is a multispans hydrophobic protein in the endoplasmic reticulum, and its catalytic site has a cytoplasmic orientation (9, 10). We previously reported that the mouse GPT gene maps to the chromosome 17 (5), is encoded in nine exons spanning 7.5 kilobases (kb), and its promoter is of the housekeeping type (8). The GPT gene promoter lacks TATA box, is GC-rich, and has multiple transcription initiation sites spread over a large region. These features are consistent with the ubiquitous occurrence of N-linked glycosylation process, and the presence of GPT mRNA in many tissues examined. However, an earlier report showed that the activities of three key enzymes of the dolichol cycle, namely GPT, GDP:Man-Dol-P mannosyltransferase, and UDP-GlcDol-P glucosyltransferase, are developmentally regulated in mouse mammary gland (11).

The mammary gland offers a unique model for studying the regulation of protein N-glycosylation. It synthesizes and secretes only a very limited number of well characterized N-linked glycoproteins, e.g. b-lactalbumin and transferrin. The gland is intensely modulated by mammogenic and lactogenic hormones as it repeatedly cycles between dormancy, growth, and differentiation upon pregnancy, lactation, and post-lactational regression throughout the reproductive life of the female (13). A recent study from our laboratory showed that GPT enzyme activity, immunoreactive GPT protein, and GPT mRNA are developmentally regulated in mouse mammary gland (12). All three parameters are low in tissues from virgin and pregnant animals, increase steadily during lactation, reaching a peak around mid to late lactation, and decline thereafter in glands of post-lactating animals. Furthermore, GPT synthesis is stimulated at the level of RNA in mouse mammary explants and primary mammary epithelial cell cultures under the synergistic influence of insulin, prolactin, and...
hydrocortisone (12). These studies suggest that the development- 
mental and hormonal regulation of GPT gene expression is 
mediated at the transcriptional and/or post-transcriptional 
levels. 

The aim of our studies is to identify cis-acting elements 
involved in the developmental and hormonal regulation of GPT 
gene in the mouse mammary gland. In this report we show that 
the pentamer direct repeat motifs, AGGAA and GAAAC, within 
the distal negative regulatory region of the mGPT promoter 
play an important role in developmental and hormonal regu-
lation of GPT gene expression in mouse mammary gland. 

EXPERIMENTAL PROCEDURES 

Materials—Restriction enzymes, T<sub>a</sub> DNA ligase, bacterial alkaline phosphatase, and tissue culture media were from Life Technologies, Inc.; S1 nuclease and the Klenow fragment of DNA polymerase I were from Pharmacia Biotech Inc.; 5'-labeled radiolabeled probes were from Du-
Pont NEN; luciferase assay system, β-galactosidase assay system and 
and fmol™ sequencing system were from Promega Biotech; GeneAmp DNA 
amplification reagent kit was from Perkin-Elmer; TA Cloning™ system was from Invitrogen Corp; oligonucleotide primers were synthesized by Oligos Etc. Inc.; female mice (C3H/HeN) were from the animal center of 
the National Institutes of Health. 

Production of the mGPT Promoter—All promoter deletion plasmids were ob-
tained by subcloning Smal- and Xho-cut products of the polymerase 
chain reaction (PCR) in the corresponding sites of pGL2 Basic Lucifer- 
ase vector (Promega). In all PCR amplification reactions, the template was the genomic subclone Eco4 DNA (8). The upstream primers were 
complementary to the 15 base pairs (bp) of the 5'-end of the desired 
deletion flanked by a Smal site and six additional nucleotides in the 
5'-end. The downstream primer was complementary to the 5' noncoding 
sequence (bp –15 to –5) of the mouse GPT gene flanked by a Xho site 
and six additional nucleotides in the 5'-end. Mutated constructs were 
obtained by PCR-based site-directed mutagenesis method (14). Briefly, 
two primary PCR reactions were performed to produce two overlapping 
DNA fragments containing the same mutation. The two outermost primers 
were wild type and the inner two primers contained indicated mutations (Fig. 5B). The upstream and down-
stream outermost primers were complementary to the 5' noncoding 
sequences bp –1161 to –1147 and bp –15 to –5, respectively, of the 
mouse GPT gene flanked by either a Smal site (upstream) or a Xho site 
(downstream) and six additional nucleotides in the 5'-end. The up-
stream outermost primer and the downstream inner primer were used 
as one pair and the upstream inner primer and downstream outermost 
primer as another pair in the primary PCR amplifications. Excess 
primers were removed by gel electrophoresis. Small aliquots of purified, 
primary PCR products were then mixed, denatured, reannealed, and 
subjected to five cycles of PCR amplification without primers to allow the 
two fragments to recombine and extend. The resulting products were 
themselves served as templates in the secondary PCR amplifications, using only 
the outermost two primers. The sequences of all constructs were veri-
fied by DNA sequencing using the fmol™ sequencing system. 

Preparation of Nuclear Extracts—Nuclear extracts were prepared 
from mammary glands of virgin (4 months old), pregnant (days 15–19), 
and lactating (days 5–10), post-lactating (1 month) mice according to 
the procedure of Dignam et al. (15). Briefly, tissues were homogenized using 
a Dounce glass homogenizer with B type pestle in two volumes of 10 mM 
HEPES (pH 7.9), 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, and 0.5 mM EDTA. The 
homogenate was centrifuged at 12000 × g for 10 min to isolate crude 
nuclei. Non-histone proteins were extracted from the nuclei in two 
volutions of 10 mM HEPES (pH 7.9), 0.25 M NaCl, 1.5 mM 

MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonfyl fluoride, and 
and 0.5 mM DTT with a Dounce glass homogenizer. The suspension was 
rotated slowly for 30 min and then centrifuged at 25000 × g for 30 min. 
The resulting clear supernatant was dialyzed against 50 volumes of 20 mM 
HEPES (pH 7.9), 20% (v/v) glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM 
phenylmethylsulfonfyl fluoride, and 0.5 mM DTT. After dialysis, 
nuclear extracts were aliquoted and stored at –80°C until use. Protein 
concentrations were determined by the BCA assay, using bovine serum 
albumin as standard, according to the manufacturer’s instructions (Pierce). 

DNase I Footprinting—The probe used was double-stranded DNA 
fragment from bp –1161 to –984 (F1). The F1 was end-labeled 
coding strand with [α-<sup>32</sup>P] ATP using the Klenow fragment of DNA 
polymerase I. Different amounts of nuclear extracts were incubated in a 
20 μl reaction mixture containing 10 mM Tris (pH 7.5), 100 mM NaCl, 
2.5 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 1 μg of poly(dG-dC)-poly(dG-dC), 6.5% 
glycerol, and 1 mM DTT and 50,000 cpm end-labeled probe for 20 min at 
temperature. After the incubation, 0.99 units of DNase I (Phar-
macia) were added, and digestion was carried out at room temperature 
for 60 s. The reaction was stopped by the addition of 60 μl of 4 × DNase 
stop solution containing 768 mM NaAc, 128 mM EDTA, and 0.65% 
SDS. The products were extracted by phenol/chloroform (1:1) and then 
separated by electrophoresis on 8% polyacrylamide, 8 x urea gels. The 
gel was then dried and subjected to autoradiography. G + A reaction 
was performed by the Maxam and Gilbert method (16). 

Electrophoretic Mobility Shift Assays—The double-stranded F1 
fragment and WT oligonucleotide (–1050 to –1004 bp) were used as probes. WT was end-labeled with [α-<sup>32</sup>P]ATP using T<sub>4</sub> polynucleotide 
kinase. The binding conditions for EMSA were same as described 
for the footprinting experiments except that 2 μg of nuclear extract was 
added in each reaction. After 20 min of incubation at room temperature, 
the resulting complexes were subjected to electrophoresis in 5% poly-
acrylamide gels in 0.5 × TBE buffer. Competition experiments were 
performed by adding the unlabeled oligonucleotides before the addition 
of the labeled probe. The DNA-protein complexes were visualized by 
autoradiography. 

Cell Cultures and Transfection—For primary cell culture, mammary 
epithelial cells were isolated from mammary glands of pregnant mice by 
enzymic dissociation followed by differential centrifugation as described 
previously (17). 1.5 × 10<sup>5</sup> cells were plated on a 35-mm plastic 
culture dish coated with Matrigel (0.3 ml/dish, Collaborative Research, Inc.). 
Cells were cultured at 37°C in 5% CO<sub>2</sub> in 3 ml of Dulbecco’s modified 
Eagle’s medium containing 10% fetal bovine serum, antibiot-
ics, and the indicated hormones. Cells were transfected with 20 μg of an 
appropriate plasmid DNA and 5 μg of pSV-β-galactosidase plasmid/dish, 
using the calcium phosphate-precipitation method (18). Unless 
otherwise stated, the final concentration of added hormones was as 
follows: porcine insulin (I), 5 μg/ml; hydrocortisone (H), 1 μg/ml; and 
bovine prolactin (P), 5 μg/ml. Medium was changed every other day. 
Cells were cultured for 5 days and harvested by treatment with 0.3% 
collagenase, 1% dispase. Cell extract was prepared by three cycles of 
freeze-thawing in 0.25 M Tris/HCl (pH 7.8) followed by centrifugation at 
16,000 × g. For primary cell culture, luciferase reporter constructs were 
prepared from mammary glands of virgin mice and cultured as described 
(19). 

Luciferase and β-Galactosidase Assays—Luciferase and β-galactosi-
dase enzyme assays were performed according to the manufacturer’s 
instructions (Promega). Luciferase activity was normalized for transcrip-
tion efficiency relative to that obtained with the reference β-galac-
tosidase plasmid pSV-β-galactosidase control vector. 

RESULTS 

The sequence of the mGPT promoter revealed the presence of 
several potential binding sites for transcription factor such as 
Sp1 and AP2, sequences similar to the half-palindromic CTF/ 
NF1 element, and a 13-bp direct repeat motif (8). Using a 
commercial luciferase reporter cell culture system we targeted 
sequently systematically to eliminate these potential binding sites 
to determine whether the sites and/or any other cis-acting 
elements were involved in developmental and hormonal regu-
lation of GPT gene expression. The Luc constructs containing 
the deleted promoter fragments were transiently transfected in 
the PMME cells and the basal and induced activities were 
determined (Fig. 1). Deletion from bp –1489 to –264 had little 
effect on the basal level of Luc activity. When the 5' deletion 
reached to bp –175, the basal level of Luc activity dropped to 
half, indicating that the sequence between bp –264 and –175 is 
important for the basal promoter activity. Since the major 
transcription initiation sites were located in this region (8), the 
possibility that removal of these sites caused the reduction of 
promoter activity could not be ruled out. 

Hormonally induced Luc activity was increased dramatically by 
the removal of the sequence between bp –1057 and –968 
and construct B7 (7-fold) was one of the most active con-
structs analyzed. The hormone inducibility was greatly re-
duced when the sequence between bp –264 and –92 was de-
leted (Fig. 1). Again, the decrease in induction activity could 
result from the removal of either positive regulatory element(s) 
or the major transcription initiation sites in this region. These
results demonstrate that a distal fragment from \(-1057\) to \(-968\) behaved as a negative regulatory region and is crucial for induction of promoter activity by the lactogenic hormones. The region involved in the negative regulation contained a 13-bp direct repeat sequence, TGACTGGGAGGAA, at bp \(-1052\) to \(-1039\) and bp \(-1033\) to \(-1020\). Since some transcription factors act through binding to direct repeat sequences (such as vitamin D, thyroid hormone, and retinoic acid response elements, AGGTCA) (20), it was speculated that this direct repeat sequence was involved in binding a trans-acting factor that down-regulates GPT expression. To examine the possible presence of trans-acting factors that interact with the negative regulatory region, EMSA were performed using a 178-bp DNA fragment FP1 that encompasses the negative regulatory region and nuclear extracts prepared from mouse mammary glands of different developmental stages. As shown in Fig. 2A, FP1 formed a major shifted band with the nuclear proteins from virgin mouse mammary gland. The complex was abolished by addition of 50-fold molar excess of either unla beled FP1 (data not shown) or a much shorter oligonucleotide WT (lane 3). In contrast, when an unrelated oligonucleotide FP2 was used as a nonspecific competitor, it failed to abolish the binding (lane 2). In addition, the amount of the complex increased with increasing protein concentration (lanes 4–6), suggesting that it was a result of specific interactions. The binding activity to the FP1 was highest in virgin stage (Fig. 2B, lane 1) and was lowest in lactating stage (lane 3). The binding activities were intermediate at pregnant stage (lane 2) and returned to a higher level in post-lactating stage (lane 4). Results from EMSA experiment involving mixing of nuclear extracts from the virgin and lactating tissues showed the same binding pattern as virgin nuclear extract alone (data not shown), suggesting that lactating extracts did not contain any factor(s) that inhibit the DNA-protein complex formation. Since the adipocytes comprise the most mass in the virgin tissue, whereas the epithelial cells predominate in the lactating tissue (13), we examined the possibility that alterations in cell population were responsible for the results relating to the developmental changes. Fat cells were removed from mammary tissues of different developmental stages, and the resulting epithelial enriched cell fractions were used to prepare nuclear extracts for EMSA experiments. The results were similar to those obtained from the intact glands (Fig. 2C). Possible variations in the efficiency of nuclear isolation and extraction were examined by EMSA using consensus oligonucleotide sequence for known transcription factor, mammary gland factor (MGF). MGF activity in mice is strongly increased at the end of pregnancy and is maintained at high levels during lactation (38). As shown in
Regulation of GlcNAc-1-P Transferase

Fig. 2. Developmental profiles of nuclear factors that specifically bind to sequence –1161 to –983 of mGPT promoter. Double-stranded FP1 fragment was end-labeled by the Klenow reaction. A, increasing amounts (2, 4, and 6 μg) of protein from virgin nuclear extracts were used (lanes 4–6); lane 1, no extract (NE); lanes 2 and 3, 50-fold excess of unlabeled oligonucleotide FP2 (nonspecific, bp –776 to –623) and WT (specific, detailed in the legend to Fig. 5), respectively, were used as competitors. B, 2 μg of protein from nuclear extracts of virgin (V), pregnant (P), lactating (L), and post-lactating (PL) mouse mammary glands were used (lanes 1–3) in electrophoretic mobility shift assays. D, end-labeled oligonucleotide for MGF (nucleotides –104 to –84, 5′ CGTAGACTTCTGGAAATTGAA3′) and 2 μg of protein from nuclear extracts of virgin (V), pregnant (P), lactating (L), and post-lactating (PL) mouse mammary glands were used (lanes 2–4). Lane 1, no extract (NE).

Fig. 3. Combination of lactogenic hormones IHP reduces the binding between the nuclear factors in virgin mammary explants and sequence –1161 to –983 of mGPT promoter. End-labeled FP1 was incubated with 2 μg of protein from nuclear extracts of virgin mouse mammary gland explants cultured in the presence of indicated combinations of hormones for 72 h. T0, uncultured explants. The designations for hormones are as follows: I, insulin; E, epidermal growth factor; H, hydrocortisone; P, prolactin.

To evaluate the effect of spacing between the direct repeats on tissue and FP1 probe. The nuclear extracts protected two regions (Fig. 4). The sequence of region I is 5′-AGGAAGAAAACTTGAC-3′, between bp –1029 and –1044, and the sequence of region II is 5′-AGGAAGCCAAAGC-3′, between bp –1010 and –1025 (Fig. 5A). Their positions overlap with the 13-bp direct repeat sequence. Comparison of these two sequences revealed the presence of two direct repeats AGGA and GAAAC. The AGGA direct repeat is separated by 14 bp, and the GAAAC direct repeat is separated by 11 bp.

As mentioned above, direct repeats of the hexamer AGGTCA can serve as response elements for vitamin D, thyroid hormone, or retinoic acid (20–22). The specificity of the response appears to reside in the spacing between the hexamers. To test whether the direct repeats found in mouse GPT gene are responsible for the observed DNA-protein interaction, a 46-bp double-stranded oligonucleotide probe WT (bp –1050 to –1004) containing these sites along with oligonucleotides containing the mutated sites in the direct repeats were used in EMSA competition experiments (Fig. 5B). As shown in Fig. 5C, the WT probe formed a complex with the nuclear extracts from the virgin tissue and the formation of this complex was efficiently competed by excess unlabeled WT oligonucleotide (lanes 2 and 3). Oligonucleotides MM1 and MM2, which had the mutations at the both sites of the direct repeats AGGA and GAAAC, respectively, failed to compete, suggesting that these sites were critical to binding (Fig. 5C, lanes 4 and 5). Since the right half-sites of these two pentamers were overlapped, it was difficult to differentiate their effect on the binding. Therefore, mutations were introduced only at the half-sites. Both oligonucleotides M1 and M1–2, which were mutated at the left and right half-site of the direct repeat AGGA, respectively, showed some competition (Fig. 5C, lanes 6 and 7), whereas MML mutated at both left half-sites of AGGA and GAAAC had weaker effect on the binding (lane 8). In contrast, oligonucleotide M2 with mutations at the left half-site of the direct repeat GAAAC competed the binding substantially (lane 9). These results suggested that both direct repeats are involved in DNA-protein binding and AGGA plays a more important role. To evaluate the effect of spacing between the direct repeats on the binding, oligonucleotides with deletions and insertions between the direct repeats were used in EMSA experiment (Fig. 5B). SM2 and SM4 with two and four base deletions, and SM13 with three base insertions between the two footprints, com-
peted the binding completely (Lanes 11–13), indicating that spacing is not important. However, MS4, deleted four bases within the footprint I, failed to compete efficiently (lane 10), whereas MS2–5 deletion with two bases within the footprint I and three bases between the two footprints competed the binding completely (lane 14). These data suggest that sequences within the footprints are important for DNA-protein contact.

The results from the EMSA experiments indicated the ability of the GPT promoter sequences between bp −1050 and −1004 to form a specific complex with binding proteins in the nuclear extracts of virgin tissue. In order to assess the functional importance of these DNA-protein interaction, 12 GPT/Luc expression constructs, containing the mutations used in the competition EMSA experiments, were prepared. These constructs were transfected into PMME cells in parallel with the unmutated GPT/Luc construct WT (Fig. 6). The WT construct produced the same pattern of transcriptional activity as observed in previous experiments (compare Fig. 6 to Fig. 1, B4). The MM1 and MM2 constructs had dramatically increased induction activity. M1, M2, and MM1 constructs also showed much higher induction activity than the WT, indicating that mutations at half-site of AGGAA pentamer alone could partially relieve the repressive effect of the negative regulatory region. M2 construct showed only half of the induction activity compared to the M1, consistent with the competition EMSA results that AGGAA is more important than GAAAC. All spacing mutant constructs had no effect except for MS4, and MS4 caused an increase in induction activity. These results suggest that the DNA-protein complex between the factor in the virgin extracts and the sequence containing the direct repeats suppressed the expression of the GPT gene.

**DISCUSSION**

The present work is part of a study focused on the identification of cis-acting elements involved in the developmental and hormonal regulation of GPT gene in the mouse mammary gland. GPT has a ubiquitous function in eukaryotic cells, and GPT gene has several characteristics of a housekeeping promoter. Such promoters often lack both TATA and CCAAT boxes, are GC-rich, and contain one or several Sp1 binding sites. The housekeeping genes are expressed in all cells, and their expression is not subject to control by environmental factors such as hormones (23). The mGPT gene is unusual in that its promoter contains characteristics of housekeeping genes but its activity is modulated during the developmental state of the mammary gland and by hormones.

Removal of consensus sequences for several potential transcription factors within the promoter region of mGPT gene indicated that these sequences, e.g. a CCAAT box (−1487), two Sp1 binding sites (−754 and −25), and an AP-2 binding site (−577), were not important for expression of the GPT gene in transient transfections of PMME cell assays. On the other hand, a region from bp −1057 to −968 was identified to be critical for developmental and hormonal regulation of GPT.
**Fig. 6. Transient expression of GPT/Luc mutated constructs in PMME cells.** Mutated oligonucleotides used in competition EMSA were used as primers to generate mutated GPT/Luc constructs, which contain sequence −1161 to −5 of the GPT gene. The transfection experiments were performed as described in Fig. 1. The Luc activity was determined and normalized to β-galactosidase activity measured in the same sample. The values are presented as the percent Luc activity relative to IHP-treated MM1 construct (100%). Thin lines represent S.E.; n = 3.

| WT | MM1 | MM2 | M1 | M1-2 | MML | M2 | MS4 | SM2 | SM4 | SMI3 | MS2-5 |
|----|-----|-----|----|------|-----|----|-----|-----|-----|------|-------|
| ![Graph](image) | ![Graph](image) | ![Graph](image) | ![Graph](image) | ![Graph](image) | ![Graph](image) | ![Graph](image) | ![Graph](image) | ![Graph](image) | ![Graph](image) | ![Graph](image) | ![Graph](image) |

Relative Luciferase Activity

---

gene in mouse mammary gland. This region contained a 13-bp direct repeat sequence, which was a potential site for binding a trans-acting factor. Deletion of this region resulted in the enhancement of hormonally inducible promoter activity. EMSA experiments using a DNA fragment targeted to this region revealed that a nuclear protein(s), a trans-acting repressor, could specifically bind to this region. The binding activity changed during the development of the mammary gland such that it was high in virgin and post-lactating glands, intermediate in pregnant gland, and low in lactating gland. The binding activity was also hormonally regulated since mammary explants cultured with lactogenic hormones, IHP, showed greatly reduced DNA-protein complex formation. Our data are consistent with the results from previous studies that GPT enzyme activity is developmentally and hormonally regulated in the mammary gland. It is high in the lactating gland (11, 12), as well as in the mammary explants cultured in the presence of IHP.

DNase I footprinting analysis identified that the presence of two pentamer direct repeats, AGGAA and GAAC, within the protected sequences. EMSA competition experiments, using a 46-bp oligonucleotide containing this direct repeat motifs and oligonucleotides with mutations at the direct repeats, confirmed that the direct repeat AGGAA is largely responsible for the observed DNA-protein interaction and the direct repeat GAAC is also involved. A dramatic increase in hormonally inducible transcription was observed after introducing the direct repeat mutations into Luc reporter constructs. This is consistent with the view that failure of the repressor, identified by in vitro EMSA, to bind in vivo resulted in the enhancement of transcription activity. The finding that two protected regions separated by 4-bp were identified by footprint analysis and that only one major band was detected by EMSA suggested that the repressor probably binds to this negative regulatory element as a dimer. The nature of this DNA repressor remains to be studied.

Negative regulatory mechanisms appear to be quite common in eukaryotic cells and negative elements are often associated with cell-specific gene expression (32). These promoters contain basal positive elements that bind ubiquitous transcription factors, which function in different cell types. Therefore, negative control mechanisms are required for their repression in these different cell types (33). On the other hand, ubiquitous expression of a promoter can be achieved by activation of different elements by nuclear proteins in different cell types (31). Although GPT gene is expressed in all cells, its expression in the mammary gland is regulated in a tissue-specific manner by lactogenic hormones during the development. Our data suggest that high levels of the repressor in the virgin tissue is responsible for the repression of promoter activity. Low levels of the repressor and the presence of possible mammary gland-specific positive factors in the lactating tissue confer the promoter full responsiveness to the hormonal induction. Therefore, developmental and hormonal regulation of GPT gene in mammary gland most likely involves a combination of both negative and positive elements that are modulated by changing in hormone milieu. Such promoter structure is correlated with the regulation of the GPT gene and the functions of the GPT enzyme, which participate not only in the constitutive pathway of N-linked glycosylation but also in the tissue-specific, hormonally regulated pathway to synthesize large amounts of N-linked glycoproteins such as α-lactalbumin in the lactating mammary gland. The β1,4-galactosyltransferase (β4-GT) gene is another example of a widely expressed gene whose expression involves negative regulatory element (29, 30). Both GPT and β4-GT have housekeeping function to participate in biosynthesis of N-linked glycoproteins. It was proposed that differential initiation from the two promoters of the β4-GT gene, a distal housekeeping promoter for the 4.1-kb transcript and a proximal mammary cell-specific promoter for the 3.9-kb transcript, is responsible for regulation of β4-GT enzyme levels. Binding of repressor(s) to the negative regulatory element could account for the repression of transcription from the 3.9-kb start site in all somatic tissues except the lactating mammary gland. Tissue-specific transcriptional regulation of genes that encode other enzymes involved in N-linked glycoprotein biosynthesis, e.g. α1,3-galactosyltransferase (34), α2,6-sialyltransferase (35, 36), and N-acetylglucosaminyltransferase I (37), also have been reported.

In GC-rich, Sp1 binding site-containing promoters, the ubiquitous promoter activity is due to the ubiquitous expression of the Sp1 protein (24). Binding of Sp1 has been shown to be critical for transcription initiation (25, 26). In the case of mGPT promoter, removal of the two perfect GC boxes did not affect the basal and inducible promoter activities, suggesting that these elements are inactive. However, the involvement of Sp1 could not be ruled out, because there are three imperfect copies of GC boxes with a 1-bp mismatch between bp −264 and −92. In addition, the transfection experiments indicated that this region may contain positive regulatory elements for both basal
Regulation of GlcNAc-1-P Transferase

11203

and inducible promoter activity. Furthermore, two copies of CCAAT box core sequence, CAAT, and two sequences similar to the half-palindromic CTF/NF1 element are also present in this region. A unique half-site CTF/NF1-binding protein has been identified in lactating mammary glands (27, 28) and implicated in the regulation of murine β4-GT gene (29, 30) and several milk protein genes (27, 28). CCAAT box-binding proteins have been shown to be the major factors involved in basal promoter activity of the cytosolic aspartate aminotransferase gene. This gene, like mGPT gene, is also ubiquitously expressed but regulated by hormones in a tissue-specific manner (31). The roles of these elements in regulation of mGPT gene are currently under investigation.

The nuclear protein(s) that binds to the negative regulatory element is present in all tissues examined, although the position of the shifted band differed a little among different tissues (data not shown). Relative higher levels were found in thymus, spleen, ovary, and virgin mammary gland. Among these tissues, thymus, ovary, and virgin mammary gland are targets of prolactin action. Although the mechanism of prolactin signal transduction is not completely understood, MGF has been shown to be a Stat (signal transducer and activator of transcription) protein and confer the prolactin response in β-casein gene (38–40). MGF binding site was found in the promoters of the genes of the most abundantly expressed milk proteins, namely caseins (41). However, MGF binding site is not present in the promoter of mGPT gene. It is possible that lactogenic hormones exert their influence partially through reduction of the binding of the repressor to the negative regulatory element in the mGPT promoter. Further purification and characterization of the repressor will be necessary to understand its mode of action and to determine whether it plays a more general role in transcriptional regulation of TATA-less housekeeping genes.

Acknowledgments—We thank Dr. Roland A. Owens for the critical reading of this manuscript and Margaret Kempf for the excellent secretarial support.

REFERENCES
1. Kornfeld, R., and Kornfeld, S. (1985) Annu. Rev. Biochem. 54, 631–664
2. Shalitubai, K., Dong-Yu, B., Saxena, E. S., and Vijay, I. K. (1988) J. Biol. Chem. 263, 15964–15972
3. Zhu, X., and Lehman, M. A. (1990) J. Biol. Chem. 265, 14250–14255
4. Scocca, J. R., and Krag, S. S. (1990) J. Biol. Chem. 265, 20621–20626
5. Rajput, B., Ma, J., Muniappaa, N., Schantz, L., Naylor, S. L., Lalley, P. A., and Vijay, I. K. (1992) Biochem. J. 285, 985–992
6. Rine, J., Hansen, W., Hardeman, E., and Davis, R. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 6750–6754
7. Liu, X., and Chang, K. P. (1992) Mol. Cell. Biol. 12, 4112–4122
8. Rajput, B., Ma, J., and Vijay, I. K. (1994) J. Biol. Chem. 269, 9590–9597
9. Abejon, C., and Hirschberg, C. B. (1990) J. Biol. Chem. 265, 14691–14695
10. Kean, E. L. (1991) J. Biol. Chem. 266, 942–946
11. Vijay, I. K., and Oka, T. (1988) Eur. J. Biochem. 154, 57–62
12. Rajput, B., Muniappaa, N., and Vijay, I. K. (1994) J. Biol. Chem. 269, 16054–16061
13. Topper, Y. J., and Freeman, C. S. (1980) Physiol. Rev. 60, 1049–1106
14. Higuchi, R., Krummel, B., and Saki, K. R. (1988) Nucleic Acids Res. 16, 7351–7367
15. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) Nucleic Acids Res. 11, 1475–1489
16. Maxam, A., and Gilbert, W. (1980) Methods Enzymol. 65, 499–560
17. Yoshimura, M., and Oka, T. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 3670–3674
18. Chen, C., and Okayama, H. (1987) Mol. Cell. Biol. 7, 2745–2752
19. Yoshimura, M., and Oka, T. (1990) Endocrinology 126, 427–433
20. Urnesono, K., Murakami, K. K., Thompson, C. C., and Evans, R. M. (1991) Cell 65, 1255–1266
21. Naar, A. M., Boutin, J., Lipkin, S. M., Yu, V. C., Holloway, J. M., Glass, C. K., and Rosenfeld, M. G. (1991) Cell 65, 1267–1279
22. Yu, V. C., Delseret, C., Andersen, B., Holloway, J. M., Devary, O., Naar, A. M., Kim, S. Y., Boutin, J. M., Glass, C. K., Rosenfeld, M. G. (1991) Cell 67, 1251–1266
23. Dyrnan, W. S. (1996) Trends Genet. 2, 196–197
24. Briggs, M. R., Kadonaga, J. T., Belfi, S. P., and Tjian, R. (1986) Science 234, 47–52
25. Pugh, B. F., and Tjian, R. (1990) Cell 61, 1187–1197
26. Pugh, B. F., and Tjian, R. (1991) Gene & Dev. 5, 1935–1945
27. Watson, C. J., Gordon, K. E., Robertson, M., and Clark, J. (1991) Nucleic Acids Res. 19, 6603–6610
28. Mink, S., Hartig, E., Janneke, P., Popoff, W., and Cato, C. B. (1992) Mol. Cell. Biol. 12, 4960–4918
29. Harduin-Lepers, A., Shaper, J. H., and Shaper, N. L. (1993) J. Biol. Chem. 268, 14348–14355
30. Rajput, B., Shaper, N. L., and Shaper, J. H. (1995) Glycoconjugate J. 12, 476
31. Garlatti, M., Victor, T., Daheshia, M., Felleux-Duchê, S., Hanoune, J., Aggerbeck, M., and Barouki, R. (1993) J. Biol. Chem. 268, 6567–6574
32. Zhou, M.-D., Wu, Y., Kumar, A., and Siddiqui, M. A. Q. (1992) Gene Exp. 2, 127–138
33. Herschbach, B. M., and Johnson, D. (1993) Annu. Rev. Cell Biol. 9, 479–509
34. Jop andre, D. H., Shaper, N. L., Kim, D., Van den Eijnden, D. H., and Shaper, J. H. (1992) J. Biol. Chem. 267, 5534–5541
35. Paulson, J. C., Weinstein, J., and Schauer, A. (1989) J. Biol. Chem. 264, 10931–10934
36. Wang, X., Vertino, A., Eddy, R. L., Byers, M. G., J ani-Sait, S. N., Shows, T. B., and L au, J. T. Y. (1993) J. Biol. Chem. 268, 4355–4363
37. Yang, J., Buehrer, M., Liu, Y., and Stanley, P. (1994) Glycobiology 4, 703–712
38. Schmitt-Ney, M., Hopp, B., Ball, R. K., and Groner, B. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 3130–3134
39. Wakao, H., Gouilleux, F., and Groner, B. (1994) EMBO J. 13, 2182–2191
40. Gouilleux, F., Wakao, H., Mundt, M., and Groner, B. (1994) EMBO J. 13, 4361–4369
41. Schmitt-Ney, M., Doppler, W., Ball, R. K., and Groner, B. (1991) Mol. Cell. Biol. 11, 3745–3755
