A Composite Enhancer Element Directing Tissue-specific Expression of Mouse Mammary Tumor Virus Requires both Ubiquitous and Tissue-restricted Factors*

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Mouse mammary tumor virus (MMTV) expression is restricted primarily to mammary epithelial cells. Sequences responsible for both the mammary-specific expression of MMTV and the activation of cellular oncogenes are located within two enhancer elements at the 5′-end of the long terminal repeat. Whereas the Ban2 enhancer (−1075 to −978) has been well characterized, the mammary-specific enhancer of MMTV from −958 to −862 has only recently been recognized as a key determinant of mammary-specific oncogene activation by MMTV. The present study identifies and characterizes three binding sites located within this element. Transient transfection of deletion and mutation constructs shows that all three sites contribute to the basal expression of MMTV in mammary cells. One of the binding activities (footprint I) is restricted to mammary cells, whereas the other two sites bind factors found in both mammary and nonmammary cells. The multimerized mammary-specific enhancer of MMTV on its own can enhance a minimal promoter in a mammary-specific fashion. However, the FpI binding site alone cannot mediate this effect. Thus, it is the binding of multiple factors in a combinatorial fashion that mediates the mammary-restricted expression of MMTV.

Infection by MMTV1 leads to the development of mammary adenocarcinomas in laboratory mice (1, 2). The retrovirus is passed horizontally by ingestion of milk-borne infectious viral particles or vertically, in certain strains, via the inheritance of an active proviral copy (3). Although MMTV is latently transforming, no oncogene is encoded in the viral genome (4). Integration of the provirus near the int genes results in the inappropriate expression of these cellular oncogenes and initiates events that lead to the formation of mammary tumors (5).

Entry into cells is achieved by the virion binding to a membrane receptor, which is expressed in many tissues (6). Low levels of MMTV RNA are detected in the epithelial cells from several tissues, such as salivary gland, lungs, kidney, and testes, as well as lymphoid tissue, but expression is approximately 500-fold higher in the lactating mammary gland (7). The MMTV LTR is widely used to direct the expression of transgenes to the mammary gland, but the basis for this specificity is not completely understood. The tissue specificity of MMTV expression is not due to its inducibility by steroid hormones. There are active and functional glucocorticoid receptors in most mouse tissues (8), yet MMTV expression is primarily detected in the mammary gland (7).

The 5′-end of the MMTV LTR contains enhancer elements that direct MMTV expression in a mammary-specific fashion (reviewed in Ref. 9). A complex array of protein binding sites in this region has been identified by gel shift and footprinting assays (10–15). One region, termed the Ban2 enhancer, contains binding sites for four different proteins, including AP-2, an Ets-related factor, a member of the CTF/NF-I family, and an uncharacterized factor mp4 (11, 14, 15). Recently, we identified a second region in the 5′-end of the MMTV LTR that we termed the mammary-specific enhancer of MMTV (MEM element) (16). This element is important for both the mammary-specific expression of MMTV and the activation of cellular proto-oncogenes. Although the MEM element acts synergistically with the Ban2 enhancer in the MMTV LTR, multimerization of the MEM element itself can confer mammary cell-specific transcriptional activation (16).

In this study, we show that the MEM element is a composite element displaying at least three distinct footprinted domains that function synergistically. Deletion or mutation of individual binding sites decreases transcription from the MMTV promoter by up to 90% in mammary cells. Two of the domains are bound by ubiquitous factors, one of which is probably a member of the NF-1 family. At least one of the binding activities, corresponding to the protected region footprint I (FpI), appears to be restricted to mammary cells. However, multimerization of the FpI binding domain alone cannot activate transcription in mammary cells. Thus, it is the combination of multiple binding sites, one of which may be mammary-specific, that contributes to the mammary tropism of MMTV.

EXPERIMENTAL PROCEDURES

DNase I Footprinting—The DNase I footprinting assay was performed as described by Dynan (17). A 215-bp fragment of the MMTV promoter encompassing −969 to −754 of the MMTV LTR was uniquely labeled at the 5′-end of the noncoding strand. Approximately 80,000 cpm of probe were incubated with increasing amounts of crude nuclear extracts and 2 μg of poly(dIC) in a final reaction volume of 50 μl for 15 min on ice. Fifty μl of a solution containing 5 mM CaCl2 and 10 mM MgCl2 were added to each tube. A variable amount of DNase I was then added to each reaction and incubated for 1 min at room temperature. The reactions were terminated by the addition of 90 μl of stop buffer (200 mM NaCl, 30 mM EDTA, 1% SDS, and 100 μg/ml yeast tRNA), extracted with phenol/chloroform, and ethanol-precipitated. DNA pellets were resuspended in 5 μl of 0.1 N NaOH/formamide (1:2, v/v)
containing xylene cyanol and bromphenol blue. The samples were boiled for 2 min, loaded onto a thin 5% polyacrylamide gel, and run in 1× TBE for ~2 h at 1600 V. Following electrophoresis, the gel was dried and subjected to autoradiography.

Nuclear Extracts—Crude nuclear extracts were prepared from the cell lines listed in Table I according to the method of Dignam et al. (18). Ten to 20 plates of cells (T-175 flasks or 15-cm diameter dishes) were grown to confluency and harvested to make each extract. Protein concentrations were determined according to Bradford (19). Typical yields ranged from 5–15 mg/ml. Extracts were aliquoted, flash frozen in liquid nitrogen, and stored at −70 °C.

Plasmids—The MMTV LTR in the MMTV-ehloaromphenicol acetyltransferase (CAT) vectors is a chimera derived from the C3H and GR strains of MMTV (GenBank™ accession numbers J02274 and V01175, respectively). The chimeric LTR has GR sequences from −291 to +83 and C3H sequences from −1194 to −292 and from +84 to +99.

Constructs with deletions in the MMTV LTR were made by cutting the vector with the indicated restriction enzymes, filling in the overhangs, and religating. A double-stranded 15-mer oligonucleotide containing only the FpI site was multimerized and cloned upstream of the basal MMTV promoter. Constructs that contained four or six wild-type copies of the FpI site and a construct with six mutant copies were chosen and confirmed by sequencing.

Cell Culture, Transient Transfections, and Assays for Reporter Gene Activity—Transient transfections were performed in T47D(A1–2) human breast cancer (20) and Ltk– mouse fibroblast cell lines using the DEAE-PO4 salt/mg/Me3SO shock method, as described previously (18). In T47D(A1–2) cells, CAT reporter gene activity was normalized using pCH110, a plasmid expressing T47D(A1–2) cells, using pSVL, a luciferase reporter gene driven by the Rous sarcoma virus LTR (22). Cells were harvested 70 h after transfection for determination of protein concentration, CAT, β-galactosidase, and luciferase activity in the extracts (16, 19, 23, 24).

Electrophoretic Mobility Shift Assay (EMSA)—EMSA probes were labeled by filling in the 5′-overhangs with Klenow, [α-32P]dCTP, and cold nucleotides. A 20-μl reaction containing 15–25 μg of crude nuclear extract and 1–2 μg of poly[d(C·C)] was incubated for 15 min at 4 °C in binding buffer (50 mM KCl, 10 mM Tris, pH 7.5, 5% glycerol, 1 mM EDTA, 2 mM MgCl2, 0.8 μg of gelatin, 1.5 mM dithiothreitol). In some cases, cold competitor was then added and incubated for 10 min at 4 °C. Finally, labeled probe (30,000 cpm) was added to each reaction and incubated 15 min at 4 °C. The samples were loaded on a precooled 4% polyacrylamide gel and run in 0.25× TBE at 250 volts for 2 h at 4 °C. The gel was dried and subjected to autoradiography.

RESULTS

Multiple Binding Sites within a 107-bp Region of the MMTV LTR—In our previous study (16), we identified a region in the 5′-end of the MMTV LTR that plays a role in the activation of cellular oncogenes, termed the MEM element. To begin to understand the mechanistic basis of the tissue specificity of MMTV, DNase I footprinting analysis was performed to identify protein binding sites in the MEM element (Fig. 1). Nuclear extracts from a human mammary carcinoma cell line (T47D) and a mouse fibroblast cell line (Ltk–) were compared. FpII was the binding site and reduced activity by 60% (Fig 3B). A series of mutations along the FpI site (FpI mut 1–4) resulted in 50–80% reductions of promoter activity. The spatial arrangement of the three binding sites of the MEM element is also critical for their functional synergy. A 15-bp oligonucleotide was inserted at the Bsu36I site separating FpI from FpII/III by 1.5 turns of the DNA helix while leaving the binding sites intact. This alteration in spacing resulted in a 70% drop in promoter activity. It appears that all three binding sites may be necessary for transcriptional activity. This speculation was confirmed by making clustered point mutations in the three binding sites individually to assess the contribution of each subdomain. A 5-bp change in the FpIII binding site reduced activity of the MMTV promoter in mammary cells by 70%, and a 3-bp change in FpIII/mammary cell-activating factor site reduced activity by 60% (Fig 3B). A series of mutations along the FpI site (FpI mut 1–4) resulted in 50–80% reductions of promoter activity. The spatial arrangement of the three binding sites of the MEM element is also critical for their functional synergy. A 15-bp oligonucleotide was inserted at the Bsu36I site separating FpI from FpII/III by 1.5 turns of the DNA helix while leaving the binding sites intact. This alteration in spacing resulted in a 70% drop in promoter activity. It appears that all three binding sites are...
needed, intact and in the correct context, in order to achieve mammary-specific transcription.

The MEM Element Can Function as an Independent Enhancer Unit—In the context of the MMTV LTR, the MEM element acts in synergy with the Ban2 element (16). To test whether the MEM element on its own is sufficient to enhance transcription, three or four copies of the enhancer were placed upstream of a minimal MMTV promoter (Fig. 3A). The minimal promoter had no activity in T47D(A1–2) cells. A single MEM element had little effect on this promoter. However, adding back three or four copies of the MEM element rendered the minimal promoter 5–10 times more active than the full-length MMTV LTR in mammary cells (Fig. 3B). In fibroblast cells (Fig. 3C), there was no change in the transcriptional activity of any of the constructs. These data support the hypothesis that the MEM element is a key determinant in the mammary-specific expression of MMTV.

The MEM Element Is Not Involved in the Hormone Response—Studies on the hormonal regulation of MMTV have provided many of the insights that have shaped the current understanding of what is now termed “classical” hormone response elements. MMTV has a well characterized cluster of hormone response elements located between −80 and −200 in the MMTV LTR (26) that mediate the prodigious induction of promoter activity resulting from exposure to the appropriate steroid hormones. We wanted to test whether the MEM element was involved in the hormone responsiveness of the MMTV LTR. The constructs diagrammed in Fig. 2A were transiently transfected into T47D(A1–2) and Ltk− mouse fibroblast (C) cells were transiently transfected with 2 μg of the reporter construct and 0.2 μg of the internal control plasmid. Bar graphs represent the averages of three to six experiments in which each condition was performed in duplicate, with the error bars representing the S.E. Results are expressed as a percentage of the activity measured for the control MMTV construct, which was set at 100%. The average raw CAT value for the control MMTV construct was 1.49 pmol/min, with an assay background of 0.15 pmol/min in T47D(A1–2) cells and 1.54 ± 0.13 pmol/min in Ltk− cells.

Characteristics of the FpI Binding Activity—To confirm the mammary-specific nature of the FpI-binding protein, a panel of nuclear extracts from 20 different cell lines (Table I) were tested for FpI binding activity. The extracts were bound to a probe containing the FpI binding site, and the resultant EMSA is shown in Fig. 5. Three DNA-protein complexes were formed

![Figure 2](image-url)  
**Figure 2.** Effect of deletions or mutations in the MEM element on MMTV promoter activity. A depicts the MMTV-CAT expression vector and the deletions or mutations that were introduced into the MEM element. The three protected regions identified by DNase I footprinting are indicated at the top in the schematic of the wild-type MEM element. Substitution mutations are indicated by the black stars, with the base pair changes shown to the side. T47D(A1–2) mammary carcinoma (B) and Ltk− mouse fibroblast (C) cells were transiently transfected with 2 μg of the reporter construct and 0.2 μg of the internal control plasmid. Bar graphs represent the averages of three to six experiments in which each condition was performed in duplicate, with the error bars representing the S.E. Results are expressed as a percentage of the activity measured for the control MMTV construct, which was set at 100%. The average raw CAT value for the control MMTV construct was 1.49 pmol/min, with an assay background of 0.15 pmol/min in T47D(A1–2) cells and 1.54 ± 0.13 pmol/min in Ltk− cells.

![Figure 3](image-url)  
**Figure 3.** A multimerized MEM element has enhancer activity. Schematics of the constructs used in transient transfections are shown in A. The shaded box spanning from HinfI to CiaI represents the 107-bp MEM element containing the three protected regions (FpI, FpII, and FpIII). Three or four copies of the MEM element were placed upstream of a minimal MMTV promoter, with the direction of the arrow denoting the orientation of the MEM element. The constructs were tested by transient transfection of either T47D(A1–2) cells (B) or Ltk− cells (C). Results are expressed as a percentage of the activity measured for the full-length MMTV construct included in each experiment.
with T47D extracts. Complex 1 was present in five of eight mammary extracts. In contrast, the complex was not found in any of 12 extracts from nonmammary cells both epithelial and nonepithelial in origin. Complex 2 was seen with all the extracts, and the mobility of a third, faster migrating complex (complex 3) varied among different extracts and was not always present. The presence of more than one EMSA complex using a FpI probe explains the partial footprinting seen using Ltk

\[ \text{FpI} \]

fibroblast extracts (Fig. 1). However, there is a mammary-restricted binding activity associated with the FpI protected region, corresponding to complex 1.

Next, probes containing the four different FpI mutations (mut1–4) were used to test for FpI binding (Fig. 6A). Compared with a wild-type FpI sequence, none of the four mutants were able to bind complex 1 well. Additionally, mut2 did not form complex 2, and mut3/mut4 could not form complex 3. Because all four mutations lead to a reduction in the transcriptional activation by the MEM element (Fig. 2B), complex 1 exhibits the best correlation between FpI binding activity and transcriptional activity.

In order to determine the specificity of binding to the FpI probe, a competition EMSA was performed with a variety of competitor DNAs (Fig. 6B). A 10-fold molar excess of cold wild-type FpI oligonucleotide efficiently competed for binding of all the complexes. The FpI mutants could only compete for binding of the complexes they formed in Fig. 6A. Thus, mut2 was unable to compete well for complex 2 binding, and neither

**FIG. 4.** The MEM element is not involved in the steroid hormone responsiveness of the MMTV LTR. T47D(A1–2) (A) or Ltk– (B) cells were transfected with MMTV-CAT or the indicated mutant MMTV-CAT vectors and then treated with 40 nM dexamethasone for 20 h before harvest. The data represent three to seven experiments ± 1 S.E. Results are expressed as a percentage of the activity measured for the full-length MMTV construct included in each experiment. The average raw CAT value for the full-length MMTV construct was 79.0 pmol/min, with an assay background of 0.16 pmol/min in T47D(A1–2) cells and 72.3 ± 0.13 pmol/min in Ltk– cells.

**FIG. 5.** The complex 1 FpI binding activity is found exclusively in mammary cell lines. EMSA experiments were performed using nuclear extracts made from the cell lines listed in Table I and a 33-bp probe containing the FpI binding site. Three specific complexes were identified and are indicated on the left. A fourth, faster migrating complex, is a nonspecific band that could be largely eliminated by the inclusion of poly[d(I-C)] during the incubation. Lanes 1–8 depict results with extracts from the indicated mammary cell lines, and lanes 9–20 represent nonmammary cell lines.

**TABLE I**

| Cell lines used to make nuclear extracts for EMSA | Species |
|-----------------------------------------------|---------|
| I. Mammary epithelium (carcinoma) |         |
| T47D(A1–2) | Human |
| MCF-7 | Human |
| ZR75.1 | Human |
| DU44775 | Human |
| MDA 231 | Human |
| II. Mammary epithelium (normal) |         |
| MCF-12A | Human |
| HBL-100 | Human |
| COMMA1D | Mouse |
| III. Nonmammary (epithelial) |         |
| Ishikawa | Endometrial adenocarcinoma |
| HTR-8 | Placental trophoblasts |
| HeLa | Cervical carcinoma |
| CHO | Ovary |
| LNCaP | Prostate adenocarcinoma |
| SW-13 | Adrenal cortical carcinoma |
| HepG2 | Hepatocellular carcinoma |
| NCI-H460 | Large cell lung carcinoma |
| IV. Nonmammary (other) |         |
| Ltk– | Fibroblast |
| COS-1 | Kidney/fibroblast |
| S49 | T cell lymphoma |
| U937 | Monocyte/lymphoma |
mut3 nor mut4 could compete for complex 3. The FpI mutants, with the exception of mut1, could not compete for complex 1 very efficiently. Two unrelated oligonucleotides could not compete for the binding of any of the complexes, demonstrating the specificity of the nuclear extracts for binding the FpI sequences.

The FpI-binding Protein Alone Cannot Activate Transcription—

To test whether the FpI binding region was itself sufficient to activate transcription in mammary cells, reporter gene constructs containing either a wild-type or mutant 35-bp oligonucleotide spanning the FpI binding site were multimerized and cloned upstream of a minimal MMTV promoter (Fig. 7A). As before, the minimal MMTV promoter (ΔSt) showed almost background levels of transcriptional activity in T47D(A1–2) cells (Fig. 7B). Adding back four or six copies of the wild-type FpI probe failed to enhance the activity of the minimal promoter further. Therefore, the FpI domain requires the other domains of the MEM element to enhance transcription in mammary cells, even though the FpI domain alone appears to have mammary cell-specific binding activity.

Homology to a C/EBP Binding Site—
The sequence of the FpI binding site shows similarity to the consensus binding sequence for the C/EBP family of transcription factors, including two CAAT-box motifs (Fig. 8A). A set of reciprocal competition gel shift assays revealed some similarities between FpI binding and C/EBP binding. EMSA experiments using T47D nuclear extracts and either a 33-bp FpI probe or a 20-bp C/EBP probe were performed and a 50-fold molar excess of competitor DNA was added. In Fig. 8B, both the 33-bp FpI oligonucleotide and a shorter 21-bp FpI oligonucleotide competed for binding to the FpI probe (lanes 2 and 3). Interestingly, a C/EBP binding site was also able to efficiently compete for the binding of complex 1 and partially for complex 2 (Fig. 8B, lane 4). The binding was specific, as a mutant C/EBP oligonucleotide could...
not compete for the complexes (lane 5). When a C/EBP binding site was used as a probe, both FpI oligonucleotides competed for binding almost as well as the C/EBP oligonucleotide (lanes 7–9). Again, binding to the C/EBP probe was specific because a mutant C/EBP binding site did not compete for the complexes (lane 10). These findings raise the possibility that a C/EBP family member contributes to the mammary-specific expression of MMTV and are discussed more fully below.

**DISCUSSION**

The 5′-end of the MMTV LTR is responsible for both the mammary-specific expression of the virus and the activation of cellular oncogenes (16). The fact that MMTV induces tumors primarily in the mammary gland appears to be linked with mammary-specific expression of the virus. A number of binding sites for nuclear proteins have been reported in this region of the LTR. These fall within two regions defined as having mammary-specific transcriptional activity: the Ban2 enhancer and the MEM element. When the MEM element is multimerized upstream of a minimal MMTV promoter, it can enhance transcription in a mammary-specific fashion. Similar experiments with the Ban2 enhancer show that it, too, can enhance transcription on its own (10, 15). However, we have shown that inactivation of either the Ban2 enhancer or the MEM element in the context of the LTR abrogates both the MMTV promoter and its ability to activate a nearby proto-oncogene promoter (16). Thus, the Ban2 and the MEM elements function synergistically when contained in a full-length LTR.

The Ban2 enhancer contains binding sites for four different proteins, including AP-2, an Ets-related factor, a member of the CTF/NF-1 family, and an uncharacterized factor mp4 (11, 14, 15). The Ban2 fragment (−1075 to −978) upstream of the thymidine kinase promoter is active only in mammary cells and not in HepG2 liver cells (10, 14). A 150-bp fragment (−1166 to −987) containing this enhancer can also target a transgene to the mammary gland of mice when linked to the SV40 promoter (27). Even when present as a single copy and independent of the MEM element, the Ban2 enhancer was able to direct mammary-specific transcription. This may be because the Ban2 enhancer is separated from the negative regulatory elements contained in the full-length MMTV LTR, or due to the use of a stronger heterologous promoter, rather than the very weak MMTV basal promoter. However, there were higher levels of transgene expression when the Ban2 and MEM elements were both present (10).

The MEM element has only recently been recognized as a distinct functional element (16). We have now characterized the functional interplay of the binding sites within the MEM element that constitute the active, tissue-specific enhancer. DNase I footprinting analysis revealed three protected regions within this element in extracts from T47D mammary carcinoma cells (Fig. 1). All three sites contribute to the mammary-specific expression of the virus. Deletions that removed one, two, or all three of the binding sites reduced transcriptional activity by 85–96%, and base substitutions in individual sites reduced transcriptional activity by 50–80%. These results with the MEM element contrast with the Ban2 enhancer, in which clustered point mutations in all four binding sites were required to reduce the activity by 80% (14). The spatial context of the MEM element binding sites with respect to one another is also important. When the three binding sites are left intact but a 15-bp insertion is introduced, the LTR can no longer support transcription in mammary cells. Thus, not only are all three binding activities of the MEM element important for the mammary-specific expression of MMTV, it may be that physical interaction between the factors or a common coactivator is required for MEM element activity.

In dissecting the MEM element, we have focused on the FpI protected region, because DNase I footprinting analyses revealed a difference in binding activity in extracts from T47D mammary carcinoma cells and fibroblasts. EMSA studies indicated three activities in T47D cell extracts that bound a FpI oligonucleotide. In order to be certain that there was a mammary-specific binding activity associated with the FpI factor, we assayed the binding activity of nuclear extracts from 20 cell lines representing mammary carcinomas, nontumor mammary epithelium, nonmammary epithelial cells, and nonepithelial cells (Table I). Complex 1 was found exclusively in mammary cells and was present in the majority of the mammary lines examined. A second complex, complex 2, was found in all cell types. Finally, a third, faster migrating complex was present in many cases, but its mobility varied depending on the cell line. Four different clustered point mutations within the FpI binding site decreased MEM element activity. Complex one correlated best with this functional data, as it was the only one of the three complexes of which the binding was decreased by all four mutations.

The FpI region cannot mediate mammary-specific transcription on its own, however, even in multiple copies. When the FpI binding site is multimerized upstream of a minimal MMTV promoter and separated from other two MEM element-binding proteins, there is no increase in transcriptional activity in a mammary cell line. Thus, the MEM element behaves as a composite enhancer, the function of which is dependent on functional synergy between tissue-specific (or at least tissue-restricted) and nonspecific factors.

The binding of multiple transcription factors to a composite enhancer element to allow cell-type specific transcription has been observed in a number of tissues, including the mammary gland, liver, lymphoid cells, heart, and muscle (28–32). This combinatorial effect circumvents the requirement for tissue-specific transcription factors and instead mediates tissue-restricted gene expression by combining both ubiquitous and tissue-enriched transcription factors in a novel way. No factor specific to the mammary gland has been identified, although a couple of binding proteins were named for that putative property. Mammary gland factor is identical to Stat5 and is expressed in many tissues (33). Mammary gland factor/Stat5 does, however, mediate the prolactin signal and is important for the expression of milk proteins, such as β-casein and whey acidic protein (28). Recently, a functional Stat5 binding site has
been identified in the middle portion of the MMTV LTR and is important for the mammary-specific expression of MMTV (34). The mammary cell-activating factor was found to be a member of the Ets family of transcription factors and is not restricted to the mammary gland (35). Its activity has not been well characterized, but because of such a binding site in the Ban2 enhancer, it does play some role in the mammary-specific regulation of MMTV. Fig. 9 outlines the binding sites located within the two enhancer elements in the 5′-end of the MMTV LTR. Of the eight binding sites, six binding activities are found in both mammary and nonmammary cells. The uncharacterized mp4 factor was reported to show binding activity in mammary cell lines only (11), and the FpI complex 1 binding activity was shown to be restricted to mammary cell lines in this study. We found that the FpI region binds ubiquitous factors as well. An earlier study (10) that termed this region P4 observed nonspecific binding activities but not a mammary-specific complex as we have seen. Because we did not see the mammary-specific complex (complex 1) in all mammary cell lines, this may explain the discrepancy.

The competition EMSA data (Fig. 8) raise the interesting possibility that the FpI-binding protein may be a member of the C/EBP family of transcription factors (36). The 3′-half of the binding site is well conserved, and it has been documented that C/EBP binding sites may show significant homology to only one half of the palindrome (37). Three of the six isoforms, C/EBP α, β, and δ, are expressed in the mouse mammary gland and are important for its development and function (38–40). C/EBP β is important for the mammary-specific expression of β-casein and whey acidic protein, in conjunction with other ubiquitous transcription factors, such as Stat5, YY1, NF-I, and the glucocorticoid receptor (28). Although the sequence of the FpI binding site, along with EMSA data, is consistent with a member of the C/EBP family, other data weigh against this conclusion. In contrast to the marked heat stability characteristic of C/EBP proteins, the FpI binding activity is heat labile at temperatures above 37 °C. When nuclear extracts were heated to 60 °C for 1, 2, 3, 4, or 5 min and used in EMSA analysis, the extracts retained the ability to form a complex with a C/EBP palindrome nor disrupted the complexes. Finally, none of the C/EBP family of transcription factors. It is apparent that a complex interaction of many transcription factors in a combinatorial fashion is required to allow the mammary-specific expression of MMTV. The mechanism by which combinatorial interactions are translated into tissue-specific, developmentally appropriate activity is a key question in the regulation of transcription.

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