Characterization of an NF-1/CTF Family Member as a Functional Activator of the Mouse Mammary Tumor Virus Long Terminal Repeat 5′ Enhancer*

(Received for publication, May 28, 1996, and in revised form, August 20, 1996)

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The long terminal repeat of the mouse mammary tumor virus restricts virus expression primarily to the mammary epithelium. The extreme 5′ end of the long terminal repeat contains an enhancer that has been associated with tissue-specific expression of the virus. A total of six functional cis-acting elements have been identified in the enhancer. Although proteins binding to these elements have been reported, only one has been identified; this factor, mp5, is identical or closely related to the transcription factor AP-2 (Mellentin-Micholotti, J., John, S., Pennie, W. D., Williams, T., and Hager, G. L. (1994) J. Biol. Chem. 269, 31983–31990). The other factors are hitherto unidentified and poorly described. We report here the characterization of another of the six elements, previously referred to as the F3 site (Mink, S., Hartig, E., Jennewein, P., Doppler, W., and Cato, A. C. (1992) Mol. Cell. Biol. 12, 4906–4918). We show that the F3 binding activity and AP-2 act synergistically to enhance mouse mammary tumor virus-directed transcription, but only in the presence of glucocorticoid hormone. The F3 element has an NF-1-like half-site, but the activity recognizing this element has binding characteristics distinct from the NF-1/CTF family as well as the rest of the CCAAT-binding proteins. We conclude that the F3 activity represents a new member of the NF-1/CTF family.

The MMTV1 retrovirus induces cancer in susceptible mice by proviral inserational mutagenesis. Why carcinogenesis is restricted primarily to breast tissue is a major unsolved question. Mammary adenocarcinomas arise when the provirus integrates in the vicinity of one of a series of protooncogenes, including members of the Wnt gene family, FGF3, FGF4, and int-3 (Refs. 3 and 4; see Refs. 5 and 6 for review). The provirus, which has been detected up to 10 kilobases away from Wnt genes, is most frequently oriented so that transcription is directed away from the Wnt gene promoter, consistent with the action of an enhancer (7–10). Thus, inappropriate activation of a protooncogene promoter in a tissue-specific fashion has been the leading model for inserational mutagenesis.

The MMTV LTR contains a series of four steroid hormone-responsive elements, which confer strong transcriptional induction by steroid hormone receptors, including glucocorticoid, progesterone, androgen, and mineralocorticoid (11–14) (see Ref. 15 for review). It is noteworthy, however, that the deregulation of int-2 as a result of LTR enhancer action is independent of steroid hormones (16). The ability of the LTR to direct mammary tissue-specific transcription was first reported by Stewart et al. (17). Sequences responsible for tissue-specific or cell line-specific transcription were later reported by several groups to reside at the very 5′ end of the LTR (2, 18–20). Enhancer activity for this region has been reported to lie between positions −1166 and −987 in transgenic mice experiments (20) and between positions −1075 and −978 (Ban2 fragment) in transient transfection experiments (1, 19). MMTV LTR transcriptional activity has also been detected in other tissues including salivary glands, kidney, lung, prostate gland, testes, and lymphoid tissue (20–23), albeit at lower levels than in mammary tissue (22).

A total of six functional cis-acting elements have been reported in this region (1, 2, 18). Several sequence-specific DNA-binding proteins have been detected that bind to these elements, but none have been shown to be uniquely present in breast tissue or in cells derived from mammary tissue. These activities have been designated mp4 and mp5 (1, 18) and F2, F3, F11, and F12 (2). Using a DNA fragment from −1094 to −739 of the MMTV LTR (E1 fragment), Mink et al. (2) demonstrated that mutations in F3, F12, or F2 dramatically reduced the ability of the enhancer to promote mammary cell line-specific transcription from a heterologous thymidine kinase promoter, indicative of synergistic action of these elements. The E1 fragment was found to be more active in an orientation opposite to the transcriptional direction of the thymidine kinase promoter. The F2 and F12 elements were shown to have homology to elements in other milk protein genes, including the promoters of whey acidic protein, α-lactalbumin, and β-lactoglobulin. The F3 element was found to have sequence similarity to an NF-1 half-site, while the nature of the F11 binding activity remains unknown (2). We previously reported that the mp4 element, situated between F11 and F3, adds little to the activity of the enhancer cloned upstream of a heterologous thymidine kinase promoter (1). In contrast, the mp5 element, positioned between F3 and F12, constitutes an important factor for the integrity of the enhancer. In addition, we demonstrated that the activity binding to mp5 is either identical to or closely related to transcription factor AP-2 (1). Finally, in agreement with the results reported by Mink et al. (2), we found F3 and F12 to be significant in the function of the MMTV enhancer (1).

Very little is known about the identity of the proteins binding to these elements, with the exception of the AP-2 activity. Several of the enhancer factors appear to function synergisti-
cally (2), and the enhancer seems to interact directly or indirectly with the hormone response elements in homologous MMTV enhancer/promoter constructs (18, 19). We report here that a small 65-bp fragment of the MMTV Ban2 enhancer, encompassing only the F3 and mp5 elements, has an activity comparable with that of the larger Ban2 fragment (~1075 to ~978) when examined in context with the natural MMTV promoter. Moreover, these elements only enhance transcription when the promoter has been induced by steroid hormone. We show that the activity binding to the F3 element in HeLa cells is antigenically related to the NF-1/CTF family of nuclear transcription factors. However, the F3 activity is clearly distinct from this family of proteins with respect to DNA-binding characteristics, sensitivity to proteolytic cleavage, and the size of proteins cross-linked to the binding site. We therefore argue that the F3 activity represents a new branch of proteins in the NF-1/CTF family.

MATERIALS AND METHODS

Plasmid Constructs and in Vitro Translation—The p200/110uc plasmid was constructed by cloning a polymerase chain reaction product (C3H LTR sequence from ~200 to ~110) containing XhoI and BglII restriction enzyme sites into a XhoI/BglII-restricted pT81 vector (24). The pT81 contains thymidine kinase promoter sequences from ~81 to ~52 relative to the start of transcription on the minus strand. This procedure swapped the thymidine kinase promoter sequence from ~81 to ~52 with the MMTV LTR sequence from ~200 to ~110. The pBn2~200/110uc was constructed by cloning a polymerase chain reaction product (C3H LTR sequence from ~1076 to ~978) containing HindIII and KpnI restriction enzyme sites into the HindIII/KpnI-restricted p200/110uc. All other plasmids were constructed by cloning 60-bp-long double-stranded oligonucleotides represented the sequences from ~1060 to ~1000 of C3H LTR with HindIII and KpnI overhangs (wild type sequence, coding strand: 5′-AGCTTCTCATTCTCTGCTGCGAACTTGGCATAGCTCTGCTTTGC-3′; mutations are described under “Results”) into p200/110uc digested with HindIII and KpnI. All constructs were verified by DNA sequencing.

The CTF1 protein used in electrophoretic mobility shift assay was in vitro translated from the circular plasmid pTCTF1 (1 μg translation reaction) using a TnT reticulocyte lysate IVT kit (Promega). The pTCTF1 plasmid contains the CTF1 sequence (25) excised from the pT7 vector (1). The pTCTF1 plasmid (p560) and cloned into pSG5 (Stratagene) just downstream of the T7 promoter. The pCTF1 plasmid (p560) was a kind gift of Robert Tjian.

Cell Culture and DNA Transfections—All cell lines were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 50 μg/ml gentamicin at 37 °C and 5% CO₂. The 34i cell line is derived from a mammary tumor induced in a C3H mouse by the C3H-S strain of MMTV (26). The 34i cell line was derived from mouse mammary cell line C127 by transformation with the episomal bovine papillomavirus vector pm18 (27). In the 31270 cell line was constructed by cloning a polymerase chain reaction product (C3H LTR sequence from ~861 of the MMTV LTR was radioactively labeled) in vitro in the presence of [35S]methionine and immediately loaded on a 5% nondenaturing polyacrylamide gel and run at 4°C at 250 V. Supershift assays were identical to the mobility shift assay, except that 1 μl of antibody was included in the incubation mixture.

For protease clamping EMSA, a series of identical assays were set up, and the protein-DNA complex was allowed to form, as described above, by incubation for 20 min on ice. Trypsin was then added to the EMSA mixture in a range from 5 to 60 μg/assay, and incubation was continued for 10 min on ice. The protein-DNA complexes were purified. The amount of 200 probe ranged from 10 to 50 fmol/assay. After incubation, the reaction was loaded directly on a 5% nondenaturing polyacrylamide gel and run at 4°C at 250 V.

Oligonucleotides for Electrophoretic Mobility Shift Assays—All oligonucleotides were annealed with a complimentary oligonucleotide and used in double-stranded form. Sequences represent the coding strand; mutated bases are underlined: F3 (~5′-1050 to ~1020), 5′-CTCTGCTGCAACTTGGCATAGCTCTGCTTTGCG-3′, F3/′-1076 to ~1075, 5′-AGCTTCTCATTCTCTGCTGCGAACTTGG-3′; NF-1/CTF family.

Galacto-Light Plus kit according to the manufacturer’s directions (Tropix). The system used a chemiluminescence substrate, which luminesces when cleaved by β-galactosidase. The β-galactosidase activity was measured using a Berthold MicroLumat LB 96 P luminometer. All transfections were done in duplicate. Luciferase activity is reported as normalized to β-galactosidase activity.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assays (EMSA)—HeLa nuclear extract was prepared from cells grown in suspension culture previously (29). The protein concentration of the extract ranged from 10 to 15 mg/ml.

EMSA analysis was performed as follows. 10–15 μg of nuclear extract was incubated for 30 min on ice in gel shift buffer (10 mM Tris-HCl (pH 7.9), 150 mM NaCl, 4 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol), and 3 μl of poly(dI-dC) with 100,000 cpm of double-stranded, end-labeled probe (~15–20 ng/μl volume) were phosphorylated as single-stranded oligonucleotides with T4 polynucleotide kinase in the presence of [γ-32P]ATP (>5000 Ci/mmol). Complementary strands were annealed, and the double-stranded probe was gel-purified. The amount of 200 probe ranged from 10 to 50 fmol/assay. After incubation, the reaction was loaded directly on a 5% nondenaturing polycrylamide gel and run at 4°C at 250 V.

Antibodies—Anisotserum 2902 is a rabbit anti-peptide antiserum raised against a peptide derived from the C terminus of CTF1 (sequence in Ref. 25) with the protein sequence NH₂-His-Leu-Asn-Pro-Gln-Asp-Pro-Leu-Lys-Asp-Leu-Val-Ser-Leu-Ala-Cys-Asp-COOH. Serum pre2902 is the preimmune serum of 2902. Both antisera were kind gifts of Naoko Tanese.

UV Cross-linking of F3-binding Proteins to F3 Probe—The binding reaction mix of HeLa nuclear extract or in vitro translated CTF1 and F3/NF-1 probes was identical to the EMSA described above. After incubation for 30 min on ice, each binding reaction mix was transferred to a paraffin film mounted on a layer of ice. The binding reactions were UV-irradiated with 600 mJ of UV light in a Strataphlin (Stratagen) and immediately loaded on a 5% nondenaturing polyacrylamide gel and run at 4°C at 250 V. After the run, the gel was exposed for 15 min on a PhosphorImager screen (Molecular Dynamics). Mobility shifts identified on the gel were excised. Ten μl of SDS-loading buffer (50 mM Tris-HCl, pH 6.8, 10 mM dithiothreitol, 2% SDS, 0.1% bromphenol blue, and 10% glycerol) was added to each gel piece in a microcentrifuge tube, and the tubes were incubated for 10 min at 95°C. The gel pieces were then loaded on a 7.5% polyacrylamide gel which luminesces when cleaved by β-galactosidase. The β-galactosidase activity was measured using a Berthold MicroLumat LB 96 P luminometer. All transfections were done in duplicate. Luciferase activity is reported as normalized to β-galactosidase activity.

DNase I Footprinting—A standard EMSA binding reaction was set up as described above, except that approximately 55 μg of HeLa nuclear extract was used, and 25,000–50,000 cpm of end-labeled DNA fragment was used per reaction. This was necessary to get quantitative binding of the probes. For coding strand footprints, a DNA fragment representing the sequence from ~1195 to ~861 of the MMTV LTR was radiactively

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labeled at the 5' end, and for noncoding strand footprints, a DNA fragment containing the sequence from +1345 to −893 was labeled at the 3' end. After a 30-min incubation on ice, increasing amounts of DNase I (RQI DNase I, 1 unit/μl, Promega) were added, and digestion was allowed to proceed for 1 min on ice. The reaction was terminated by the addition of 3 μl of 0.5 M EDTA. Twenty μg of yeast RNA was added, and the reaction was phenol/chloroform-extracted, ethanol-precipitated, and loaded on an 8% urea denaturing polyacrylamide gel.

**In Vivo Exonuclease Footprinting**—Nuclei were prepared as described previously. Ten μg of nuclei per condition were digested with Hha I at 5–10 units/μg of DNA to open up chromatin. The nuclei were then treated with either λ exonuclease (Life Technologies, Inc.) at a final concentration of 200 units/ml or T7 exonuclease (U.S. Biochemical Corp.) at a final concentration of 2000 units/ml at 30 °C for 15 min in Workman & Langmore buffer (50 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.5 mM MgCl₂, 1 mM β-mercaptoethanol (300) in a 200-μl reaction volume. The reactions were terminated with an equal volume of 2% SDS, 0.2 mM NaCl, 10 mM EDTA, 10 mM EGTA, and 50 mM Tris-HCl (pH 8.0) and further incubated with 10 μg/ml protease K for 3 h to overnight at 37 °C. The genomic DNA preparations were phenol/chloroform-extracted and ethanol-precipitated, resuspended in 30 μl, and restricted with a secondary enzyme to aid solubility. Fifteen μl was used in a thermally cycled primer extension reaction. The sequence of the primer used for the primer extension was 5'-AAGAGTCAGGGTGAGGC-3'. The final volume of the polymerase chain reaction was 30 μl, and dNTPs were at a final concentration of 0.3 mM. Reactions were subjected to electrophoresis on 8% denaturing gels.

### RESULTS

The Activity of the MMTV LTR 5' Enhancer Is Primarily Due to the Action of the F3 Binding Activity and AP-2—We wanted to determine which elements of the Ban2 enhancer (−1075 to −978) are important for its function. Mutation of the F3 and mp5 elements eliminated more than 80% of the activity (Ref. 1 and data not shown). We therefore focused initially on these elements. The activities of the Ban2 enhancer and a 65-bp enhancer encompassing the F3 and mp5 elements were tested in transient transfection assays in 34i (mouse mammary) and HeLa (human cervical carcinoma) cells. Mutations were cloned upstream of the MMTV LTR sequences from −200 to +110 driving the luciferase reporter gene. The Ban2 enhancer and 65-bp minimal enhancer both function efficiently in the transcriptionally induced state (i.e. after cells have been treated with dexamethasone) but are essentially inert in the basal transcription state (Fig. 1, compare Basal Activity with Induced Activity in panels B and C). The region encompassing the F3 and mp5 elements is as active as the whole Ban2 fragment (Fig. 1, compare lanes 4 and 7 in panels B and C). However, when either the F3 or mp5 elements are mutated, enhancer-driven transcriptional activation drops to the activity observed in the construct containing only the proximal promoter sequences (Fig. 1, compare lanes 1, 2, and 5 with lane 6 in panels B and C). In contrast, a mutation upstream of the F3 element has no effect on the minimal enhancer's function (Fig. 1, lane 3 in panels B and C). Hence, it seems that the F3 and AP-2 elements act synergistically to enhance transcription but only when the homologous promoter has been transcriptionally induced by hormone.

As opposed to previous reports (2, 19), we have found that MMTV LTR constructs are transcriptionally active in HeLa cells (Fig. 1). However, this is only the case when they are cotransfected with an expression plasmid encoding the glucocorticoid receptor. This shows nevertheless that the inactivity of MMTV LTR constructs in HeLa cells is not due to the lack of factors binding to the 5' enhancer, but rather to an intrinsic low level of GR in the HeLa cells.

**Characteristics of the F3 Element Binding Activity**—The 65-bp minimal enhancer (encompassing the F3 and mp5 elements) confers most of the activity of the Ban2 enhancer. We previously characterized the mp5 element as a member of the AP-2 family (1), and we now focus on the F3 element. A probe representing the sequence from −1054 to −1020 of the MMTV LTR, which only can bind the F3 activity, was prepared. A series of mutations spanning this region was created for use as competitor proteins in EMSA analysis to determine the bases critical for binding of the F3 activity. The EMSA study shows, first, that F3 binding is specific since it is competed by wild type F3 but not the unrelated ATF sequence (Fig. 2, lanes 5 and 13). The EMSA competition also shows that the N5 box-like half-site of F3 is important for binding (Fig. 2, lane 6). Moreover, mutations in the sequence downstream of this half-site, denoted the N5 box in Fig. 2, are critical for binding of the F3 activity (Fig. 2, lane 8). The F3 element does not have an obvious 3' NF-1-like half-site, and mutations in sequences downstream of the N5 box do not interfere with the binding of the F3 activity (Fig. 2, lanes 9–11). Mutations in sequences upstream of the

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The F3 Activity Has Binding Features Different from the NF-1 Family of Nuclear Factors—To examine how closely the binding patterns of the F3 activity and the NF-1 family of nuclear factors are related, we prepared an F3-element probe in addition to the F3 element probe used above. A set of mutations was prepared in the 5' half-site and the N5 box of both elements, and in the 3' half-site of the NF-1 binding element, for competition in gel mobility shifts. The EMSA competition (Fig. 3) shows that mutations of competitor DNAs in the 5' half-sites of the F3 and NF-1 elements, as well as the 3' half-site of the NF-1 element, eliminates the ability to compete both F3 and NF-1 mobility shifts (Fig. 3B, lanes 2, 4, 6, and 7 for F3 shifts, and lanes 9, 11, 13, and 14 for NF-1 shifts). However, when the N5 boxes of the two elements are mutated, the F3 mutant competitor loses the ability to compete, while the NF-1 mutant competitor is unaffected by the mutation (Fig. 3B, compare lanes 3 and 5 for F3 shifts and lanes 10 and 12 for NF-1 shifts). Thus, both the NF-1-like half-site and the N5 box are critical for the binding of the F3 activity, while only the 5’ and 3’ half-sites (and not the N5 box) are critical for NF-1 binding. This is further underscored by the finding that the F3 activity does not shift the F3 element with the N5 box mutation prepared as probe, while NF-1 readily shifts the corresponding mutated probe for the NF-1 element (Fig. 3B, compare lanes 15 and 16). The latter mobility shift is competed by a wild type NF-1 element competitor and by the NF-1 element with a

NF-1-like half-site have, as expected, no effect on binding of the F3 activity (Fig. 2, lanes 2–4). Although the F3 activity does not behave like an NF-1 family member, an NF-1 element, derived from the MMTV proximal promoter, fully competes for binding of the F3 activity (Fig. 2, lane 12). These findings show that the sequences important for binding of the F3 activity are an NF-1-like half-site and what would correspond to the box separating the two half-sites in the consensus NF-1 element, called the N5 box.

The anatomy of the sites is delineated with boxed sequences. The mutated sequences of the oligonucleotides used as competitors in the competition EMSA in B are also shown along with their designations. The upper sequence is the wild type, and the lower sequence shows the mutation. Panel B shows a competition EMSA using either HeLa nuclear extract or in vitro translated CTF1 with F3 and NF-1 sequences as probes and as competitors. Arrows identify specific mobility shifts. Panel D shows an autoradiogram of an SDS-polyacrylamide gel of proteins UV cross-linked to the F3 element and NF-1 binding site; lane 1, proteins from HeLa nuclear extracts cross-linked to the NF-1 binding site; lane 3, proteins from HeLa nuclear extracts (>6 months old) cross-linked to the F3 element; lane 4, proteins from HeLa fresh nuclear extract cross-linked to the F3 element.
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Knowing that the F3 mobility shift is competed very well by an NF-1 element (Fig. 2, lane 12), it was of interest to examine whether the F3 element would compete an NF-1 mobility shift. Fig. 3C shows that an NF-1 mobility shift using in vitro translated CTF1 as a protein source is only weakly competed by the F3 element while readily competed by an NF-1 element (Fig. 3C, compare lanes 5, 6, and 7). In accordance with this finding, the in vitro translated CTF1 does not shift the F3 element (Fig. 3C, lane 4). The F3 element does not compete an NF-1 mobility shift at all when using HeLa nuclear extract instead of in vitro translated CTF1 (data not shown), but it competes an F3 shift just as well as an NF-1 element competitor (Fig. 3C, lanes 1–3).

Since the binding characteristics of the F3 and NF-1 elements are significantly different, it was of interest to examine whether different protein factors, as judged by molecular weight, bind to these two elements. HeLa nuclear extract and in vitro translated CTF1 were incubated with radioactively labeled F3 and NF-1 elements, respectively. Proteins bound to the probes were UV cross-linked and submitted to EMISA. The mobility shifts were excised from the gel, and the cross-linked proteins in these shifts were separated by SDS-polyacrylamide gel electrophoresis. In vitro translated CTF1 cross-linked to the NF-1 element had a mobility similar to that of a 75-kDa protein (Fig. 3D, lane 1). The NF-1 and F3 elements were also cross-linked to a protein from HeLa extract 75 kDa in size as well as a 65-kDa protein (Fig. 3D, lanes 2 and 3, respectively). However, in addition, two proteins of 105 and 46 kDa were cross-linked only to the F3 element (Fig. 3D, lanes 3 and 4). The 105-kDa protein appeared with freshly prepared extract, while the 46-kDa protein was observed when extracts stored for a longer time (>6 months) were used.

These experiments demonstrate that the sequence of the N5 box in the NF-1 element is nonessential for NF-1 binding, while the corresponding sequence in the F3 element is essential for binding of the F3 activity. Also, in vitro translated CTF1 does not interact with the F3 element probe but binds strongly to the NF-1 element probe. Finally, the F3 element, but not the NF-1 element, binds a 105- and perhaps a 46-kDa protein.

The F3 and NF-1 Nuclear Factors Share both Structural Similarities and Dissimilarities—As shown above, proteins binding to the F3 and NF-1 elements have different DNA binding characteristics, although the mobility shifts seem to co-migrate. Hence, it was of interest to investigate whether these nuclear factors are structurally related. For this purpose we took two approaches: 1) analysis of antigenic relatedness employing EMISA supershifts, and 2) analysis of resistance to proteolytic degradation of the factors when bound to DNA using proteolytic clipping EMISA. The supershifts were performed using rabbit anti-NF-1 antiserum. An antiserum raised against a peptide representing a sequence from the C terminus of CTF1 (Ab 2902) supershifted both F3 and NF-1 mobility shifts (Fig. 4A, compare lanes 1 and 3 and lanes 2 and 4). The preimmune rabbit antiserum (Ab pre2902) had no effect on the mobility shifts (Fig. 4A, lanes 5 and 6). The proteolytic clipping EMISA (Fig. 4B) showed that both the F3 and NF-1 mobility shifts were cleaved to one end product. However, the proteolytic end product of the F3 shift had a slower mobility than that of the NF-1 shift (Fig. 4B, compare lanes 11 and 12). Both proteolytic end products were competed by an NF-1 element (Fig. 4B, lanes 13 and 14), indicating that they were not degradation products of the nonspecific bands present in the intact mobility shifts (Fig. 4B, lanes 1 and 2). These results show that the F3 activity and NF-1 present in HeLa nuclear extract share antigenic and, therefore, structural similarities, but at the same time differ in their sensitivity to trypsin (and Pronase; data not shown) when complexed with DNA, indicating structural differences.

Identification of the F3 Element in Vitro by DNase I Footprint Analysis—To identify the sequences interacting with the F3 activity in the context of a larger fragment of the enhancer containing the AP-2 element, we performed a DNase I footprint analysis on an MMTV LTR fragment from −1185 to −861 for the coding strand footprint and from −1185 to −893 for the noncoding strand footprint. The coding strand footprint (Fig. 5A) shows that the F3 activity protects a long region of DNA from −1044 to −1026, which covers the NF-1-like half-site and the N5 box. In the noncoding strand footprint, the F3 activity
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FIG. 5. DNase I footprinting of the MMTV LTR 5' enhancer. For footprinting of the coding strand (A) a HindIII/ClaI fragment (−1195 to −861) was labeled at the HindIII site, and for footprinting of the noncoding strand a HindIII/AvaII fragment (−1195 to −893) was labeled at the AvaII site. HeLa nuclear extract was used for footprinting on both strands. The coordinates and positions of the footprints are shown in both panels. The lighter shaded part of the boxes representing the F12 footprint on the noncoding strand refers to a weakly protected region. Lanes A/G in panels A and B are chemical-sequencing reactions of the corresponding probe run in parallel. Coordinates are relative to the transcription start site.

The coordinates of two other footprints observed, mp4 and F12, are in agreement with previously published results (mp4 (18) and F12 (2)). Interestingly, a new footprint, designated mp6, is detected further upstream on the LTR from −1083 to −1075 on the coding strand and from −1093 to −1062 on the noncoding strand. The involvement of the mp6 binding activity in enhancer function awaits further investigation.

Identification of the F3 Element in Vivo by Exonuclease Footprint Analysis—The results presented above indicate that the F3 and AP-2 binding activities are present in HeLa cells and 34i cells. To examine whether F3 and AP-2 also load on the MMTV LTR template in vivo, we performed a λ and T7 exonuclease 5' boundary in vivo footprint analysis using nuclei isolated from a murine mammary cell line (3134 cells) and a nonmammary cell line (1361.5 cells). In both cell lines, the F3 5' boundary was detected at bp −1051 (Fig. 6, panels A and B). Also, the boundary was not affected by treatment with hormone (compare lanes 2 and 3 with lanes 5 and 6, and compare lanes 8 and 9 with lanes 11 and 12 in both panels). Two 5' boundaries were detected, corresponding to the AP-2 element at −1036 and −1033 (Fig. 6, panels A and B). Again, neither of these boundaries were affected by hormone treatment (Fig. 6, compare lanes 2 and 3 with lanes 5 and 6, and compare lanes 8 and 9 with lanes 11 and 12 in both panels).

Slight differences were observed between the boundaries determined using λ and T7 exonucleases. These variations are due to differences to the extent each enzyme will degrade DNA after encountering a protein factor.

These results are in good agreement with the in vitro DNase
FIG. 7. Summary of results. A shows the maximum protection footprints and their coordinates of the LTR enhancer reported in this paper as well as the functional footprints and their coordinates reported by Mink et al. (2). B shows the F3 element. The shaded boxes represent the DNase I footprints on the coding and noncoding strands. The sequence critical for binding of the F3 activity as identified by EMSA analysis is highlighted in boldface letters. The positions of the NF-1 like half-site and the N5 box are indicated above the sequence. C, a comparison of binding sequence preferences for CCAAT-binding proteins. The TGG sequence motif shared by all groups is boxed. Triangles denote points of contact between the nuclear factor and DNA as identified using the methylation protection assay. Solid triangles denote a stronger interference with DNA methylation, and open triangles denote a weaker interference with DNA methylation. The methylation interference pattern for F3 is taken from Mink et al. (2); the methylation interference patterns for NF-1/CTF binding to the NF-1 consensus site (NF-1) and for CP1 binding to the adenovirus major (AdML) are taken from Chodosh et al. (33); the methylation interference pattern for NF-Y binding to the murine major histocompatibility complex class II Ea gene (Ea) is taken from Dorn et al. (36); the methylation interference pattern for the coding strand only for C/EBP binding to the murine sarcoma virus LTR enhancer (MSV) is taken from Johnson et al. (32); the methylation interference pattern for α-CBF binding to the human α  subunit gene (hα) is taken from Kennedy et al. (34). For easier comparison, all sequences have been aligned so that the TGG motif appears on the upper strand, which means that the upper strand of the CP1 sequence represents the noncoding strand.

I footprints in Fig. 5. The exonuclease 5’ boundaries are all slightly offset (5–10 bp) relative to the DNase I footprints.

DISCUSSION

Several lines of evidence indicate that the enhancer located at the extreme 5’ end of the MMTV LTR is a major determinant of the tissue- and cell line-specific activation potential for MMTV, which is preferentially expressed in the epithelium of the murine mammary gland and in cell lines of mammary origin (1, 2, 18–20). To understand the mechanisms by which this enhancer functions, it is important first to identify and characterize the nuclear factors binding to this enhancer. We and others have now identified six functional cis-acting elements in the enhancer: mp4 and mp5 (1, 18) and F2, F3, F11, and F12 (2) (Fig. 7A). However, none of these factors have been shown to be restricted to mammary tissue or mammary cell lines.

The factor binding to the mp5 element was the first of the six binding factors to be identified. This factor is either the transcription factor AP-2 or a closely related family member (1). The F2 and F12 elements were shown to be represented in other milk protein genes from several species, including β-lactoglobulin, α-lactalbumin, and whey acidic protein, indicating a potential role of these elements in controlling tissue-specific gene expression (2). However, the identity of the factor(s) binding to F2 and F12, designated MAF, as well as the distribution profile of these elements in nonmammary genes, has not been established.

In this report, we have characterized the F3 element. We have provided evidence that the F3 element binds a protein or protein complex, which has some structural features in common with the NF-1/CTF family of nuclear factors, because it shifts with EMSA with an antibody directed against the C terminus of CTF1 (25). However, the sequence critical for binding of the F3 activity has two unusual features not shared by any reported NF-1/CTF related protein. 1) It contains only one NF-1-like half-site, and 2) five base pairs, designated the N5 box, immediately downstream of this half-site are equally important as the half-site for binding of the F3 activity (Fig. 7B). Other CCAAT-binding proteins, such as C/EBP (31, 32), CP1 (33), α-CBF (34), and NF-Y (35, 36) also only need one CCAAT-like sequence for binding, in addition to sequences either upstream or downstream of this site. However, none of these additional sequences share any homology with the N5 box of the F3 element (Fig. 7C). Thus, based on the binding sequence comparison, the F3 binding activity seems to be unrelated to the other single-site CCAAT-binding proteins (Fig. 7C).

In addition to the unusual binding sequence preference, we have provided several lines of evidence to demonstrate that the F3 activity is distinct from NF-1/CTF (25, 37–40) and, hence, is likely to be a new family member. 1) In vitro translated CTF1 does not mobility-shift an F3 probe while strongly shifting an NF-1/CTF probe. This finding clearly argues against the F3 activity being identical to, or closely related to, an NF-1/CTF homodimer. Also, the F3 element only weakly competes the in vitro translated CTF1 off the NF-1/CTF probe and does not compete the NF-1 mobility shift, even at a 100-fold molar excess when using HeLa nuclear extract. 4 On the other hand, the NF-1/CTF binding sequence is a strong competitor for both an NF-1/CTF and an F3 mobility shift. This again indicates some similarity between the NF-1/CTF and F3 activities despite the several differences. 2) Trypsin treatment of the F3 and NF-1/CTF protein-DNA complexes shows that limited protease treatment degrades the DNA-bound F3 and NF-1/CTF protein complexes such that the proteolytic end products have clearly different mobilities. 3) UV cross-linking of F3 and NF-1/CTF protein-DNA complexes using HeLa nuclear extract have shown that two proteins of molecular mass 75 and 65 kDa are cross-linked to the NF-1 element, while two proteins of 105 and 46 kDa are cross-linked uniquely to the F3 element. Since the 105-kDa protein is cross-linked when using extract stored for less than 2–3 months and the 46-kDa protein appears only when extracts stored for longer periods of time (>6 months) are used, it seems likely that the 46-kDa protein is a degradation product of the 105-kDa protein, which has retained its F3 binding ability. The 75-kDa protein is identified as NF-1/CTF since in vitro translated CTF1 cross-linked to the NF-1 element migrated at 75 kDa. This somewhat larger size than the reported 60 kDa for CTF1 (25) is likely due to the cross-linked DNA fragment. The nature of the 65-kDa protein is uncertain, but it may be a degradation product. Thus, it is plausible that the F3 binding activity is a heterodimer composed of a protein either identical to CTF1 (25) or closely related to the NF-1/CTF family recognizing the NF-1-like half-site, and another protein with a molecular weight of approximately 105 kDa (less the

4 P. Kusk, unpublished observation.
contribution from the cross-linked DNA), which may recognize the N5 box. Originally, NF-1/CTF was found to bind to DNA as a homodimer (38). However, the existence of heterodimers of NF-1 and other factors has been suggested (41), and demonstrated to exist even in the absence of DNA (42). Thus, we propose that the F3 binding activity is a heterodimer composed of NF-1/CTF, or a closely related family member, and a new factor, which seems to recognize the sequence 5'-TAGCT-3'.

Here we have also demonstrated that the activity of a 65-bp minimal enhancer, containing only the F3 and mp5 elements, is comparable with the activity of the Ban2 enhancer, which includes the mp4 and F12 elements. Moreover, F3 and mp5 seem to work synergistically since mutations in either element eliminate enhancer function. Also, the DNase I footprints demonstrate that the F3 binding activity and AP-2 come into very close contact when bound to the enhancer. In fact the regions of interaction overlap by 4 bp, suggesting that the factors make direct protein-protein contacts, a notion that would fit well with the apparent functional synergy between these elements. Our finding that F3 is important for enhancer function is in agreement with the apparent functional synergy between these elements.

The observation that the Ban2 enhancer is hormone-independent for heterologous promoters, but synergistic with the MMTV 5' proximal promoter, is consistent with the model that this enhancer functions in proviral insertional mutagenesis. In conclusion, we have shown that the F3 element is an important component of the MMTV 5' Ban2 enhancer. This element binds an activity structurally related to transcription factor NF-1/CTF, but with a composite recognition sequence unrelated to that for any of the currently described CCAAT-binding proteins. Thus, the F3 binding activity is likely to be a new member of the NF-1/CTF family.