The 5' Enhancer of the Mouse Mammary Tumor Virus Long Terminal Repeat Contains a Functional AP-2 Element*

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The mouse mammary tumor virus (MMTV) retrovirus causes mammary adenocarcinomas in mice by proviral insertion near members of the wnt family of proto-oncogenes, leading to their deregulation and cellular transformation. The 5' end of the MMTV long terminal repeat (LTR) has been implicated in tissue-specific activation of these genes. In this study, we characterize an enhancer element (Ban2; -1075 to -978) at the 5' end of the MMTV LTR. We show that this enhancer is 5-fold more active in a murine mammary carcinoma cell line (341) than in a fibroblast cell line (NIH3T3), and is inactive in the liver carcinoma cell line HepG2. Mutagenesis of the enhancer reveals four cis-acting elements that are required for maximal activity. DNA-binding proteins that interact with each of the four elements have been identified. One of these factors, designated mp6, is either identical to, or closely related to, the transcription factor AP-2. The mp5/AP-2 DNA binding activity co-migrates with recombinant AP-2 and is supershifted by anti-AP-2 antibodies. We also show that the lack of enhancer activity in HepG2 cells results from the absence of AP-2 protein in these cells. Co-transfection of an AP-2 expression vector restores the activity of this enhancer in HepG2 cells, and requires an intact mp5-binding site.

MMTV predominantly causes mammary adenocarcinomas but can also less frequently cause T-cell leukemias, thymomas, and kidney adenocarcinomas in mice (2–5). It is of great interest to understand why MMTV primarily causes mammary adenocarcinomas even though the virus infects several tissue types. In mammary tumors MMTV has been found to be integrated near several endogenous proto-oncogenes called wnt genes (6). The wnt genes are developmental genes, normally expressed only during embryogenesis, that become deregulated by proviral insertion. It is the aberrant expression of these proto-oncogenes that contributes to neoplastic transformation. wnt-2 gene regulation has been shown to be refractory to hormone stimulation; the locus is constitutively expressed in MMTV-induced mammary tumors, indicating that activation of wnt-2 is due to long terminal repeat (LTR) elements independent of the hormone response elements (7). The fact that MMTV predominantly causes mammary adenocarcinomas suggests that retroviral activation of the wnt genes is driven by mammary-specific transcriptional elements of the provirus. In addition, viral integration upstream of the wnt genes almost always occurs in the opposite transcriptional orientation relative to the activated gene, suggesting that the MMTV promoter must be positioned correctly for deregulated expression of the wnt genes (7, 8).

The contribution of the 5' end of the LTR to the tissue specificity of MMTV expression has been studied in transgenic mouse models and in cultured cells. Stewart and colleagues reported that a growth hormone reporter gene driven by an LTR with a deletion between -863 and -110 had a restricted tissue distribution, similar to a construct driven by the full-length 1320 bp LTR (9). This study suggested that sequences from -1185 to -863 along with the MMTV minimal promoter were sufficient for tissue specific expression of the LTR. In a later study, Ross and colleagues (10) showed that a slightly smaller fragment of the LTR (-1160 to -987) can direct expression of a heterologous promoter to the mammary gland in transgenic mice. Our previous work also identified a sequence at the 5' end of the MMTV LTR (-1180 to -968) that has enhancer activity specific to cultured mammary cells (11). A factor binding to this region, designated mp4, was detected by electrophoretic mobility shift assays (EMSAs), and was shown to generate a DNase I footprint between -1078 and -1052. Cato and colleagues (12) reported that a larger DNA fragment (-1094 to -739) directed mammary-specific transcriptional activity in cultured cells, and showed that several proteins bound to this region by in vitro DNAse I footprinting. Ishimoto and colleagues (13) have also localized a mammary specific activity to a 5' fragment of the LTR flanked by two BamHI restriction enzyme sites (-1075 to -978), designated Ban2.

In this study, we show that when assayed in DNA transfection experiments, the Ban2 enhancer cloned upstream of a heterologous promoter stimulates basal transcription to a level 5-fold greater in mammary cells (34i) than in fibroblasts.
(NIH3T3) and is inactive in liver carcinoma cells (HepG2). We have characterized this enhancer in mammary cells and non-mammary cells by using site-directed mutagenesis to determine which sequences are required for transcriptional activity. Four elements have been identified as contributing to the activity of this enhancer. One element, termed mp5, contains a consensus AP-2 DNA-binding site (5'GCCNNNGGC-3') (14). AP-2 was originally identified as a sequence-specific DNA-binding protein required for the function of several viral and cellular enhancer elements. There are functional AP-2-binding sites in the enhancers of the SV40, HTLV-1 viruses, and the metallothionein IIa, proenkephalin, keratin K14, and murine histocompatibility complex H-2k genes (14, 15).

Our results indicate that the mp5 DNA-binding protein is AP-2 or an AP-2 family member. We show that in vitro translated, recombinant human AP-2 binds specifically to the mp5 recognition site, and that the AP-2-specific DNA-protein complex co-migrates with the mp5 activity found in mammary cell extracts. We also show that two antibodies to different regions of human AP-2 can supershift the mp5 protein in both human and murine extracts. In addition, we co-transfect a Ban2 reporter plasmid and a eukaryotic expression vector encoding AP-2 into a cell line (HepG2) that lacks endogenous AP-2 and show that co-transfection of AP-2 bicistronic plasmid, the other Ban2 enhancer: mp5/AP-2 is not the only protein responsible for activation of this enhancer. Functional mutagenesis studies show that three other factors, mp4, F3/NF1, and F12, are necessary for optimal expression of this enhancer. Taken together, our results suggest that the Ban2 enhancer is regulated by a multifactor complex containing at least AP-2, mp4, F3/NF1, and F12.

**MATERIALS AND METHODS**

**Plasmid Constructs and in Vitro Translation**—The wild type pBan2TK81 plasmid was constructed by cloning a polymerase chain reaction product (C3H LTR sequence from -1078 to -978) containing HindIII and KpnI restriction enzyme sites into a pTK81 vector digested from the osteocalcin promoter and was kindly provided by Dr. Catharine L. Smith. (14). The AP-2 protein was translated from the circular pT7PSalAP-2 in vitro translation (IVT) expression vector, pT7PSalAP-2, which has been described previously (14). The pT7PSalAP-2 vector contains an N-terminal peptide and has been described previously (14). Ab53 was made to an N-terminal peptide and has been described previously (14).

**Transient Transfection Assays**—Transient transfection assays were performed as described previously using the calcium phosphate technique (11). All cells were plated at 1-2.5 x 10^6 in six-well dishes 1 day prior to transfection. In 34I and 3T3 cells, 1 μg of each reporter plasmid was co-transfected with 50 ng of a plasmid encoding SV40-β-galactosidase (pCH110, Pharmacia Biotech Inc.). The pCH110 plasmid served as an internal control for transfection efficiency. In HepG2 cells, 1 μg of each reporter was co-transfected or without 50 ng of pSV2CAT. Luciferase activity was measured using a Berthold LB980LuminoMeter as described previously (11). β-Galactosidase activity was measured using a Hoesch TTO 100 fluorometer using manufacturer recommended protocols. In brief, 5-10 μl of extract were incubated in a final volume of 200 μl containing 2vol. of HCl (pH 7.4). 150 μM NaCl, 3 mM MgCl2, 3 mM EDTA, 1 mM dithiothreitol, 4% glycerol and 1 μg of poly(dI-dC) with 40,000 cpm of 32P end-labeled double-stranded probe in a 10-20 μl volume. In the assays which contained IVT-AP-2 protein, 1 μl of IVT extract from a typical 50-μl TNT reaction was used. Mobility shift probes 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 AP-2 protein ranged from 0.1 to 40 fmoi per binding assay. All competitors were double-stranded and used at 100-fold excess molar concentration (100 x) to that of the labeled probe.

For AP-2 supershift assays, 10 μg of cell extract or 1 μl of IVT-AP-2 was preincubated with 1.5 μl of antibody in TBST (10 mM Tris (pH 8.0), 150 mM NaCl, 0.05% Tween 20) in an 8-μl volume for 60 min on ice. The protein-Ab solution was then incubated on ice for 10 min with 10 μl of 2 x gel shift buffer and 1 μg of poly(dI-dC) in a final volume of 19 μl. Finally, 40,000 cpm of labeled probe (1 μl) was added and the mixture was incubated on ice for an additional 30 min. DNA-protein complexes were resolved on a 4% nondenaturing polyacrylamide gel run at 25 V in 0.5×TBE. The bands were then visualized using a PhosphorImager screen which was analyzed with a Molecular Dynamics phosphorImager. Transient transfection assays were performed as described previously using the calcium phosphate technique (11). All cells were plated at 1.5-2.5 x 10^6 in six-well dishes 1 day prior to transfection. In 34I and 3T3 cells, 1 μg of each reporter plasmid was co-transfected with 50 ng of a plasmid encoding SV40-β-galactosidase (pCH110, Pharmacia Bio-}

**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 was isolated from a mammary tumor induced in a C3H mouse by the C3H-3 strain of MMTV (17). NIH3T3-L1 cells are mouse embryo fibroblasts. T47D and MCF7 cells are derived from a human breast ductal carcinoma and an adenocarcinoma, respectively. HeLa and HepG2 cells are derived from a human cervical carcinoma and a liver carcinoma, respectively. All cell lines except 34I were obtained from the American Type Culture Collection (ATCC).
An MMTV Enhancer Contains a Functional AP-2 Element

Fig. 1. DNA transfection analysis of the Ban2 enhancer. A, diagram of the MMTV LTR showing the U3 and U5 regions and the location of fragments that have been shown to be important for tissue specificity. The line labeled Transgene represents a fragment that directs the expression of a heterologous promoter to mammary gland in transgenic mice (10). The fragments Ban2 and Pst-Hinfl were shown to be mammary-specific enhancers when driving the MMTV minimal promoter and in the case of Ban2 a heterologous promoter (11, 13). B, activity of the Ban2 enhancer in 34i, 3T3, and HepG2 cells. pTK81 (Vector) is standardized to 1, therefore the y axis represents the ratio of pBan2TK81/pTK81 (Ban2) for each cell line. The bar graph shows the mean and S.E. for five DNA transfection experiments for 34i and 3T3 and four experiments for HepG2 done in duplicate on separate days. The a axis represents luciferase/β-galactosidase activity.

The affinity purified anti-glutathione S-transferase antibody was kindly provided by Dr. Emil F. Michelotti.

RESULTS

A 5' MMTV Enhancer Is Preferentially Expressed in Mammary Cells—We evaluated the activities of various fragments in the 5' LTR to determine their ability to direct tissue-specific expression of a heterologous promoter. We determined that the Ban2 fragment (−1075 to −978) was sufficient to direct preferential expression in mammary cells. For this experiment, the Ban2 fragment was cloned directly upstream of the herpes simplex I thymidine kinase (TK) promoter in the pTK81 plasmid (16). Either pBan2TK81 (Ban2) or pTK81 (Vector) were transfected into murine mammary carcinoma cells (34i), murine fibroblasts (NIH3T3), or human liver carcinoma cells (HepG2). The data in Fig. 1 shows that this enhancer stimulates the TK81 promoter 41-fold in 34i cells and 8-fold in 3T3 cells; the Ban2 enhancer is inactive in HepG2 cells. As a positive control to show that the TK81 promoter is functional in HepG2 cells, we transfected a plasmid construct (pAP1TK81) which contains 3 AP-1 sites directly upstream of the TK81 promoter, pAP1TK81 was stimulated 294-fold above the pTK81 vector alone thereby confirming that the TK promoter is capable of being activated in HepG2 cells (data not shown). These results suggest that the Ban2 enhancer is preferentially active in mammary cells.

Identification of an Activity Binding to the Region—In addition to the previously published DNA-binding

Fig. 2. EMSA using the mp5 sequence as a probe to delineate the mp5 DNA-binding site. A, the coding strand of double-stranded oligonucleotides used for this experiment. The oligonucleotide designated mp5 contains sequences from the C3H MMTV LTR. Coordinates are labeled relative to the start site of transcription. The competitors are identical in sequence to the mp5 probe except for the indicated mutations. The dashes indicate the inferred binding site based on the mobility shift data in B. The consensus AP-2 DNA binding sequence is shown as previously determined by Williams et al. (16). B, EMSA to determine which sequences are required for mp5 DNA binding activity in 34i cells. mp5 is the 32P-labeled probe and the competitors (shown in A) are used at 100 x probe concentration. Lane 1 has no extract and lane 13 has an unrelated ATF-binding site (see "Materials and Methods"). The mutation in the wapl (wapm) oligonucleotide targets a sequence shown to be important for wap gene promoter activity (22). The open arrow denotes the specific mp5 band and the shaded arrow denotes a second binding activity that is discussed in the legend to Fig. 6.

proteins that have been shown to interact with the Ban2 enhancer region (11-13), we had detected an activity by in vitro DNase I footprinting that bound between −1032 and −1005 in the human mammary carcinoma cell line T47D.2 We designed an EMSA probe, designated mp5 (−1044 to −1004), which encompassed the DNase I footprint. Several mutations of this DNA sequence were used in gel mobility shift competitions to determine critical sequences for mp5 binding. Fig. 2A shows a diagram of the oligonucleotides that were used for this competition experiment. The EMSA competition (Fig. 2B) shows that the mp5 activity denoted by the open arrow is specific; it is competed by unlabeled wild type mp5 but not the unrelated sequence ATF (lanes 3 and 13). Oligonucleotides containing

2 P. Lefebvre, unpublished data.
mutations in the wild type sequence, mp5m, 633, 635 (lanes 4, 6, and 7), do not compete for binding of this activity to the mp5 oligonucleotide, suggesting that the sequences mutated in these oligonucleotides are important for mp5 binding. Oligonucleotides wapm, 661, 733, 735, 737, and 739 competed the mp5 shift as efficiently as wild type mp5, indicating that the sequences mutated in the oligonucleotides are outside of the mp5-binding site. This shows that the mp5 DNA binding activity minimally requires sequences within a 12-bp fragment from -1024 to -1012. A Findpatterns search comparing this sequence with sequences in the Transcription Factor data base showed that this element contains a consensus AP-2-binding site (Fig. 2A) (20, 25). AP-2 is expressed in a tissue-specific manner and was previously shown by Northern blot analysis to be present in the mammary carcinoma cell line MCF7 (21). We next determined if this element or the previously characterized elements were functional in DNA transfection experiments in both mammary and non-mammary cells.

Mutational Analysis of 4 Elements in the Ban2 Enhancer— We synthesized specific DNA mutations in the Ban2 enhancer to analyze which DNA sequences contribute to its activity in 34i and 3T3 cells. The mutations are diagrammed in Fig. 3A and include 3 elements which had been previously defined by in vitro DNase I footprinting and mobility shift assay (mp4, F3/NF1, and F12) and mp8, a novel DNA binding activity (11, 12). Each of the Ban2 fragments containing a mutation was cloned into pTK81 and the plasmid constructs were transfected into both 34i and 3T3 cells. The data shown in Fig. 3B indicates that the Ban2 element is regulated in mammary cells by the combinatorial activity of at least 3 DNA binding activities. The mutation of a single binding site is not sufficient to eliminate the activity of this enhancer. However, in 34i cells 82% of the activity is lost when all four sites are mutated. The most dramatic effects of single mutations are seen when the mp5 (56% reduction) or F3/NF1 (52% reduction) binding sites are mutated. There is also a contribution of the F12 site to this activity since this mutation causes a 43% drop in activity. In 3T3 cells (Fig. 3C), the most dramatic effects are seen with the mp4, F3/NF1, and F12 mutations that have reductions of 41, 39, and 33%, respectively. The mp5 mutation has the least effect in 3T3 cells (16% reduction) which is in contrast to its marked effect in 34i cells (56% reduction). In addition, mp4 has a greater effect in 3T3 cells (41% reduction) than in 34i cells (21% reduction). In summary, the Ban2 enhancer requires mp5, F3/NF1, and F12 in 34i mammary cells and mp4, F3/NF1, and F12 in 3T3 fibroblasts for optimal activity. The results of this mutation series prompted us to analyze the DNA-binding factors which interact with these regions in both mammary (34i) and non-mammary (3T3) cells.

Four DNA-binding Proteins Interact with Sequences in the Ban2 Enhancer—DNA transfection experiments showed that at least four elements are required for optimal activity of the Ban2 enhancer in two different cell types. We employed mobility shift assays to determine if there are sequence-specific DNA-binding proteins associated with each of these elements. We first tested whether the proteins that have been reported to bind to the F3/NF1 and F12 elements are present in 34i and 3T3 nuclear cell extracts. In the following EMSA experiments, the sequences of the oligonucleotides as shown under "Materials and Methods" were used. Fig. 4A shows a mobility shift assay using a labeled F3/NF1 probe (1051 to 1019). We find that the F3/NF1 activity is present in both extracts and that the specific band competes with both unlabeled F3/NF1, and consensus NF1 (MMTV, -80 to -60) oligonucleotides (lanes 3, 5, 8, and 10) but not with an unrelated ATF-binding site or an oligonucleotide containing a mutation in the consensus NF1

![Diagram](image-url)
An MMTV Enhancer Contains a Functional AP-2 Element

FIG. 4. EMSA using F3/NF1, F12, and mp4 as probes in 34i and 3T3 cell extracts. The open arrows indicate the specific band for each gel shift. The sequences of both probes and competing oligonucleotides are shown under “Materials and Methods” and competitors are used at 100 x probe concentration. A, EMSA using an F3 32P-labeled probe in 34i and 3T3 nuclear cell extracts. B, EMSA using an F12 32P-labeled probe in 34i and 3T3 nuclear cell extracts. C, EMSA using a mp4 32P-labeled probe in 34i and 3T3 nuclear cell extracts.

not competed by oligonucleotides containing ATF, NF1, or mp5-binding sites (lanes 5, 6, 7, 11, 12, and 13). The F2 element (−905 to −870) was also reported by Cat0 and colleagues (12) to contain an F12 DNA-binding site. In addition to F3/NF1 and F12, we have identified an activity binding to the mp4 DNA sequence (−1075 to −1055) in both 34i and 3T3 cells (Fig. 4C). The mp4 DNA binding activity is specific because it is competed by an unlabeled mp4 oligonucleotide (lanes 3 and 6). The mp4m mutation targets a cis-acting sequence (ACAAAG) which is present in the murine wap promoter and other mammary-specific promoters and has been shown to be important for mammary-specific expression of the wap gene (22). Since the mp4m mutation partially competes the specific band (lanes 4 and 7), we hypothesize that either the mutation still allows for a weak mp4 interaction or that the mp4 protein is not the same protein that binds to the ACAAAG consensus sequence found in the wap promoter.

The DNA binding activity that we have designated mp5 is present in all cell types analyzed except for the liver carcinoma cell line HepG2. Fig. 5 shows that there is a specific shifted complex present in the mammary cell lines T47D, MCF7, 34i, and the non-mammary lines HeLa and 3T3 using mp5 as a probe. This band is competed by unlabeled wild type mp5 (lanes 3, 6, 9, 16, and 19) but not an oligonucleotide containing a mutation in the mp5 consensus AP-2 site (lanes 4, 7, 10, 17, and 20). The HepG2 extract is competent for EMSA studies because the mp4, F3/NF1, and F12 DNA binding activities are all present in HepG2 cells (data not shown).

The mp5 probe (−1044 to −1004) partially overlaps the F3/NF1 probe (−1051 to −1019) and contains an NF1 half-site (TGGCA) at its 5′ end. Therefore, we designed an experiment to test whether the upper band (shaded arrow in Figs. 2B and 5) represents an NF1 DNA binding activity in 34i cells. In Fig. 6, we used a 32P-labeled mp5 probe and competed with unlabeled NF1, NF1m, ATF, or Met, an oligonucleotide containing a consensus AP-2 site from the human metallothionein IIA promoter (15). The upper band (shaded arrow) is competed by mp5, mp5m, or NF1 (lanes 2, 3, and 4) but not by NF1m or ATF (lanes 5 and 6). This shows that the upper band contains an NF1-like DNA binding activity. The Met oligonucleotide competes the upper and lower shift indicating that AP-2 may be required for the NF1-like binding activity and that there may be cooperative binding between mp5/AP-2 and NF1. Alternatively, the upper band may represent an NF1 homo or heterodimer binding exclusively to the probe, although the MMTV (−81 to −59) NF1 mobility shift has a faster mobility than the mp5 shift (data not shown). The faint broad band in the HepG2 mp5 shift with no competitor competes with both wild type mp5.
and mp5m which both contain the NF1 half-site (Fig. 5; compare lanes 11, 12, and 13). Additional studies show that this activity is competed with NF1 but not NF1m indicating that this band most likely represents an NF1 homodimer DNA binding activity (data not shown).

HepG2 is the only cell line tested that does not contain the mp5 binding activity and it has been previously shown to be lacking in AP-2 (15). This data in combination with the fact that there is a consensus binding site for AP-2 in the mp5 element prompted us to investigate whether mp5 is in fact AP-2.

The mp5 DNA Binding Activity Is Related to AP-2—If the mp5 protein is antigenically related to AP-2, then the EMSA mp5 band should be supershifted by antibodies to AP-2. Ab53 and Ab57 are two affinity purified anti-AP-2 polyclonal antiserum made against peptides from different regions of the human AP-2 protein. We tested each anti-AP-2 antibody in two human (T47D and HeLa) and two murine (34i and 3T3) cell extracts and the results are shown in Fig. 7. As a positive control, we show that in vitro translated AP-2 (IVT-AP-2) is quantitatively supershifted by Ab53 (lanes 13 and 14). There are no nonspecific bands present when either Ab53 or Ab57 are added to probes in the absence of extract or when an unrelated anti-glutathione S-transferase affinity purified antibody is added to the extract (data not shown). In each cell extract, both Ab53 (lanes 2, 5, 8, and 11) and Ab57 (lanes 3, 6, 9, and 12) are capable of supershifting the mp5 DNA binding activity.

This experiment also shows, importantly, that IVT-AP-2 binds to the mp5/AP-2 element present in our mp5 probe and that IVT-AP-2 co-migrates with the mp5 DNA binding activity in our cell extracts (compare lane 13 to lanes 1, 4, 7, and 10). In addition, we have performed EMSA protease clipping assays with the mp5 probe which show that the limit digest of T47D, HeLa, 34i, and 3T3 cell extracts co-migrate with IVT-AP-2 (data not shown). The amino acid sequences of the human and murine AP-2 proteins are 99% identical so it is predicted that the protein in murine extracts would have a similar protease digestion profile (23).

In summary, the above experiments show that the mp5 activity (now designated mp5/AP-2) is either AP-2 or an antigenetically related family member. To test for functional involvement of the AP-2 protein in the activity of the Ban2 enhancer, we conducted AP-2 expression experiments in a cell line that lacks endogenous AP-2.

AP-2 Is Required for the Activity of the Ban2 Enhancer in HepG2 Cells—It has been previously shown that the cell line HepG2 lacks endogenous AP-2 activity (15). We demonstrate here that the Ban2 enhancer has no stimulatory activity in HepG2 cells (Fig. 1), and that the mp5/AP-2 DNA binding activity is absent in nuclear extracts from these cells (Fig. 5, lanes 11, 12, and 13). The mp4, F3/NF1, and F12 DNA binding activities are all present in HepG2 cells (data not shown), indicating that mp5/AP-2 may be critical for the activity of this enhancer. We co-transfected a eukaryotic expression vector for AP-2 (pRSVAP-2) with the pBan2TK81 construct into HepG2 cells. In this assay, we tested for the activity of the Ban2 enhancer in the presence or absence of exogenous AP-2. As a positive control for the efficiency of expression of the pRSVAP-2 vector, we utilized a control vector, pAP-2BCAT, containing 3 AP-2 DNA-binding sites cloned directly upstream of the E1b TATA box of pBCAT (15). In this case, the pAP-2BCAT vector was stimulated 5-fold in the presence of co-transfected pRSVAP-2, indicating that the expressed AP-2 protein is functional in HepG2 cells. The RSV promoter vector alone has no effect on the activity of pBan2TK81 or pTK81 (data not shown).

Fig. 8 shows the results of DNA transfection experiments in HepG2 cells. The data is presented as the ratio of activity for cells co-transfected with Ban2 reporters and pRSVAP-2, relative to the activity observed with cells that had only Ban2 reporters (AP-2+/AP-2−). The pBan2TK81 vector was stimu-
An MMTV Enhancer Contains a Functional AP-2 Element

The Ban2 enhancer activity in HepG2 cells is required for the activity of this enhancer in HepG2 cells. In addition to the wild type enhancer, we tested the mutation series that was used in the initial characterization of the enhancer (Fig. 3A). The mp4 and F12 mutations have no significant effect on the ability of AP-2 to stimulate the enhancer. In contrast, the enhancers containing the mp5 mutation alone (mp5m), or all four sites mutated ((4x)m), are not capable of responding to AP-2. This shows that the mp5/AP-2 sequence element that we have mutated in both of these constructs is the target for exogenous AP-2 activity. We note that the F3 mutation is compromised by 30% in its ability to respond to AP-2. This is further evidence that there may be a functional interaction between F3/NF1 and AP-2.

**DISCUSSION**

Transcriptional regulation of MMTV is complex; the LTR has been shown to contain multiple regulatory elements, both positive acting and repressive (24). We previously reported that the AP-2 activity has been shown in several systems to interact with other transcription factors which bind to overlapping or juxtaposed cis-acting sequences. First, we show that AP-2 binds to overlapping sequences in the human growth hormone gene in a mutually exclusive manner with NF1 (26). The DNA-binding domains of NF1 and AP-2 share no homology, but it may be significant that they both contain a proline-rich activation domain. Second, AP-2 binds to overlapping sequences with a factor called AP-3 on the "core" sequence of the SV40 enhancer and when AP-2 is bound to the element AP-3 is excluded (27). Third, AP-2 has been shown to bind to an enhancer element in the H-2k gene promoter which is activated by tumor necrosis factor-α and in tumor necrosis factor-α-treated cells AP-2 is replaced by an NFκB-like binding activity (28).

AP-2 activity is also regulated by several factors that inhibit its binding to DNA. It was recently shown that a second splice form of the AP-2 protein exists in several cell types. This protein, called AP-2B, results from differential exon usage and is identical to AP-2 at the N terminus but is lacking the DNA-binding domain (29). Buetting and colleagues (29) show by EMSA that AP-2B inhibits the ability of AP-2 to bind to DNA and by transfection analysis that AP-2B inhibits the functional activity of AP-2. They also show that AP-2 and AP-2B do not bind to each other directly but require an undefined factor that is present in rabbit reticulocyte lysate or mammalian cell extracts but not in bacterial extracts. This raises the possibility that differences in the levels of AP-2, AP-2B, and a potential bridging factor will affect gene regulation in different cell types.

AP-2 activity has also been shown to be inhibited by SV40 T antigen and HTLV-1 Tax which prevent the binding of AP-2 to DNA in DNase I footprinting and mobility shift assays, respectively (30, 31).

What is the mechanism by which the Ban2 enhancer is preferentially active in mammary cells? The lack of activity in HepG2 cells is straightforward; AP-2 is simply not present in these cells. The data of Ross and collaborators (10) suggests that a fragment encompassing the Ban2 region of the LTR manifests very pronounced tissue targeting activity in transgenic animals, even when attached to a heterologous promoter. The enhancer is not quiescent in all non-mammary cells in culture, however, as shown here for 3T3 cells. The enhancer is also modestly active in HeLa cells. Incomplete restriction of cell type specificity could have several explanations. Cells grown on plastic substrata in culture lose many of the features of primary cells. Assembly of an active multifactor complex on

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3 S. John, unpublished data.
4 J. Mellentin-Michelotti, unpublished data.
the enhancer in inappropriate cell types could result from aberrant modification of one or more of the factors, or loss of secondary factors blocking the binding of one of the activators. As noted above, an AP-2 variant that blocks normal AP-2 activity has already been described.

Alternatively, the activation potential of the Ban2 enhancer may be modulated in some cells by other repressive elements in the LTR. It is becoming increasingly clear that both positive and negative elements are responsible for selective expression of MMTV in the mammary gland. Several negative regulatory elements have been described. One DNA element (–428 to –364) has been characterized both in transfection experiments (33, 34) and by analysis of proviral sequences that are deleted (35). A second negative element (–156 to –162) has been defined between the two distal hormone responsive elements and has been shown to bind a nuclear factor (32).

Thus, the ability of the Ban2 enhancer to activate transcription may be conditioned by repressive elements between the enhancer and the promoter. In this context, it is important to note that the orientation of proviral elements activating the \textit{wnt}-2 proto-oncogene is almost always antiparallel with respect to the activated promoter, even with proviruses inserted at large distances from the oncogene (7, 8). Thus, the Ban2 enhancer is placed at the end of the LTR in the direction of the target promoter. The significance of such directional positioning of elements over large distances is unclear. The Ban2 element, however, is clearly a candidate for the major activator of cellular \textit{wnt} genes during proviral insertion and tumorigenesis.

In summary, we have now defined an activating element at the 5’ end of the MMTV LTR that provides one component of the cell specificity observed for expression from this promoter. The involvement of AP-2, or a closely related protein, has been established as one of the primary factors acting at this locus. Further efforts will now be directed at a complete delineation of the AP-2 forms present in mammary cells, post-translational modifications which may be specific to these cells, and the mechanisms involved in assembly of the complex multifactorial protein complex binding to this locus.

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