Transactivation through Ets and Ap1 Transcription Sites Determines the Expression of the Tumor-suppressing Gene Maspin

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Abstract
Tumor invasion and metastasis are processes poorly understood at the molecular level. Maspin is a serine protease inhibitor (serpin) with tumor-suppressing function in the mammary gland. Maspin gene expression is decreased with malignancy and is lost in metastatic cells. We show in this report that differential expression of maspin in normal and carcinoma-derived mammary epithelial cells is regulated at the transcriptional level. We have identified the Ets and Ap1 sites in the maspin promoter that are active in regulating maspin expression in normal mammary epithelial cells but inactive in tumor cells. The Ets site alone is sufficient to activate transcription in a heterologous promoter, whereas the Ap1 site cooperates with Ets in activation. The enhancing function by Ets and Ap1 elements is decreased in primary tumor cells (21NT) and is abolished in invasive tumor cells (MDA-231). Thus, loss of maspin expression during tumor progression results at least in part from the absence of transactivation through the Ets and Ap1 sites.

Introduction
Proteases and protease inhibitors are known to play important roles in tumor invasion and metastasis (1). Proteinase degradation of the extracellular matrix is a prerequisite to invasion and metastasis; proteinase inhibitors function to prevent this process. Recently, a novel serpin called maspin, expressed in normal human mammary epithelial cells, was identified in this laboratory (2). Maspin is down-regulated in primary breast tumor cells, and its expression is lost in metastases, as shown by Northern analysis and by immunostaining of normal and tumor specimens from surgery. Maspin expression decreased with increasing malignancy of primary tumors and was absent from lymph node and distant metastases (2).

Functional studies demonstrated that the maspin protein, whether expressed in tumor transfectants or added as recombinant maspin to tumor cells, inhibited invasion in a Boyden chamber assay (3). Similarly, tumor cells were inhibited in motility by maspin, both in a Boyden chamber assay and as seen by direct video time lapse microscopy (4). Furthermore, in the nude mouse assay, maspin tumor transfectants were inhibited in tumor growth and metastasis (2).

These results identify maspin as a tumor suppressor gene that acts at the cell surface by inhibiting invasion and motility. This study addresses the question of how maspin expression is lost in tumor cells. By functional analysis of the promoter, we conclude that this loss results at least in part from the absence of transactivation through Ets and Ap1 elements in the promoter.

AP1 is a transcription factor consisting of the heterodimers of FOS and JUN family proteins (5). ETS binding proteins belong to a large transcription factor family (6, 7). The ETS proteins are found to be involved in tumorigenesis and metastasis (8–10). For example, ETS was found to be overexpressed in metastatic mammary adenocarcinomas (9). E1AF, another ETS protein, promoted tumor cell motility and invasiveness (10). Reports have been focused on the involvement of ETS proteins in transcriptional regulation of proteases, including collagenase (11), stromelysin (12), gelatinase B (10), factor IX (13), urokinase-type plasminogen activator (14, 15), and granzyme B (16). In all of these cases, the expression of proteases was mediated through the Ets element or the combination of Ets and Ap1 sites. Because the activities of proteases and protease inhibitors have to be well balanced in vivo (1), their expressions might be regulated by the same mechanism. The signal transduction pathway that controls the activity of ETS factors for the expression of proteases may be shared for the target protease inhibitors to assure a quick response to the unbalance of either side. The homeostasis is well maintained in normal cells until other genetic events occur. One example occurs during tumorigenesis, when the balance is shifted toward overproduction of proteases in tumors (1, 17–19) and down-regulation of a protease inhibitor such as maspin, which we will describe in this report. Our finding provides the first example of a tumor-suppressing protease inhibitor, maspin, which is transcriptionally regulated through Ets and Ap1 sites.
### Results

**Expression of Maspin in Tissues and Mammary Epithelial Cells and Carcinomas.** The *maspin* gene was originally isolated from normal mammary epithelial cells. To evaluate the tissue expression pattern and the cell specificity of *maspin*, we performed Northern blot analysis with RNAs from several human cell lines as well as with tissue blots containing RNAs from human tissues (Clontech). *Maspin* is highly expressed in 70N and 76N normal mammary epithelial cells, down-regulated in 21NT and 21PT primary tumors, and silent in a series of metastatic tumor cells (Fig. 1A). The gene is not expressed in cells of non-epithelial origin, such as 56NF1 (mammary fibroblast cells), FS2 (foreskin fibroblast cells), and U937 (human monocytic cells). Interestingly, it is expressed at low levels in HeLa cells, which are cervical carcinoma-derived cells of epithelial origin.

*Maspin* RNA was not expressed in heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, ovary, colon, and leukocyte (Fig. 1, B and C) but was expressed in prostate, thymus, testis, and small intestine.

**Cloning and Sequencing of the Upstream Promoter.** A genomic DNA library was screened with 32P-labeled *maspin* cDNA probe but failed to identify the first exon, suggesting the presence of a large intron between a noncoding exon 1 and exon 2. Using a YAC6 clone containing a cluster of serpins including *maspin*, we screened genomic DNA with a 50-mer oligonucleotide (OL1) from the 5'-untranslated region. A positive clone was identified containing a 1.2-kb 5'-flanking region, a 9-kb intron 1 sequence, and a partial exon 2 sequence. A subclone was isolated; partial sequencing data showed that it contained the first exon and upstream

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4 The abbreviations used are: YAC, yeast artificial chromosome; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; MAF, mammary cell activating factor.
region of maspin (Fig. 2). The major transcription start site was numbered +1 (by primer extension analysis, data not shown). Exon 1 consists of nucleotides from +1 to +184. The genomic DNA sequence from +115 to +184 nucleotides completely matches with our cDNA sequence in that region. Exon 2 starts at +185, which is 23 bp 5' of an ATG site. Computer analysis of the upstream region of the maspin gene revealed the presence of a number of potential transcription regulatory sites.

A 1-kb Upstream Region Is Sufficient for Activating Transcription of Maspin in Normal Breast Cells. Several well-known transcription factor binding sites, Ets, Ap1, Ap2, and GRE, are located within the 1-kb promoter region. Is this region sufficient for activating transcription of maspin? To find out, a fragment of 1043 bp (-956 to +87) was fused with the CAT gene to generate pCAT(956). The construct was transfected into normal mammary epithelial cells (70N), primary tumor cells (21NT), and metastatic tumor cells (MDA-MB231). CAT activity was assayed and normalized to pKTCAT (Fig. 3). A 15.6-fold elevated CAT activity was found in 70N cells. The activity decreased to 2-fold in 21NT cells, and no detectable CAT activity was found in MDA-MB231 cells compared to the negative control pKTCAT. When a CMV-CAT positive control vector was transfected into 70N, the activity was about 50-fold stronger than the maspin promoter. The same maspin promoter is about 8-fold stronger in 70N than in the 21NT cells. Quantitation of Northern blots indicated that the expression level of maspin was down-regulated 10-fold in the primary breast tumor cell line 21NT compared with 70N normal cells and abolished in metastatic tumor cell lines. These data indicate that maspin exogenous promoter strength mimics the endogenous RNA expression level, and that the 1-kb region is sufficient for turning on maspin transcription.

Functional Analysis of the Maspin Promoter. To identify the functional maspin promoter elements, progressive deletion mutants were constructed. Deletion constructs were transfected into 70N normal mammary epithelial cells and MDA231 tumor cells, and their relative CAT activities were assayed (Fig. 4). CAT activity is expressed relative to that of pKTCAT in the same cells. Deletion from -956 to -386, which removed the distal Ets site did not change the activity. Further deletion up to -112 bp did not alter the activity significantly. Well-known elements are present in this region, including the distal Ets, Ap2, and GRE sites. The deletion from -112 to -90 bp, however, which removed the proximal Ets element, completely abolished the CAT activity of 70N extracts. The level of pKT(90) was comparable to that of the negative control vector, which does not contain a promoter. These data demonstrate that the proximal Ets is the major
positive cis element within 1 kb responsible for up-regulation of maspin in normal mammary epithelial cells.

The constructs were tested in breast carcinoma MDA-MB231 cell extracts (Fig. 4). None of the deletions gave any CAT activity significantly higher than that of the negative control vector, showing that these tumors were unable to activate transcription. These results also suggest that down-regulation of the maspin gene in MDA-MB231 cells is not likely due to transcription repression through the negative cis elements but rather due to the loss of transactivation.

Loss of Transactivation through the Ets and Ap1 in Tumor Cells. To further confirm that the Ets site is involved in transcriptional activation of maspin, we investigated the ability of Ets to enhance transcription by cloning the Ets site (−112 to −90 bp) into the pBLCAT2 vector, which contains no enhancer but a minimal strength thymidine kinase promoter.

Ets has been reported to cooperate with Ap1 in transcriptional activation (11, 14, 20). To test the role of the Ap1 site in the maspin promoter, we subcloned the fragment containing the proximal Ets and Ap1 sites (−112 to −47 bp) and the
one containing the Ets site and mutated Ap1 site into pBLCAT2 to generate pEts/Ap1CAT and pEts/mAp1CAT. To test the effect of Ets mutation on transcriptional activation, we mutated the Ets site in the pEts/Ap1CAT construct to generate pmEts/Ap1CAT. These constructs were transfected into 70N, 21NT, and MDA-MB231 cells. As shown in Fig. 5, the presence of the proximal Ets site greatly increased the CAT activity of pBLCAT2 in 70N. The mutation at the Ets site abolished the activity. The pEts/Ap1CAT construct had a dramatic increase in transcription activation over pEtsCAT alone, whereas pEts/mAp1CAT has the same range of activity as pEtsCAT. These data demonstrate that Ets alone is sufficient to activate transcription, Ap1 is involved in transcriptional activation of maspin in 70N cells, and that Ap1 cooperates with Ets in this process.

The enhancing ability of Ets was decreased for pEtsCAT in 21NT cells, indicating the impaired transcriptional activation through the Ets site in the primary mammary tumor cells. Moreover, the cooperative transactivation between Ets and Ap1 was lost in 21NT cells. Both transactivation through Ets
and cooperation between Ets and Ap1 were lost in metastatic MDA-MB231 cells.

Discussion

We have demonstrated that expression of maspin, a tumor-suppressing serpin, is regulated at the transcriptional level. Functional analysis has demonstrated that Ets and Ap1 elements in the promoter activate transcription of maspin in normal mammary epithelial cells. The Ets element cooperates with a downstream Ap1 element to activate maspin transcription. Deletion or mutation of these elements abolished or attenuated promoter activity. Loss of expression of maspin results from the absence of transcriptional activation through the Ets and Ap1 sites.

Recently, a mouse homologue of maspin has been isolated in this laboratory. Tissue distribution of mouse maspin has been studied with the mouse maspin cDNA probe. The expression pattern in mouse resembles that of maspin in human tissues, confirming the highest level of expression in mouse mammary gland. The mammary gland undergoes structural and biochemical changes continuously from embryo to aging females. Several well-known milk proteins, such as whey acidic protein, lactalbumin, and lactoglobulin, are considered as markers for the differentiated function of the mammary epithelium. A mammary cell specific enhancer (the binding site for MAF) has been identified in the promoters of genes encoding several milk proteins. This MAF factor belongs to the ETS family of transcriptional binding proteins, as was demonstrated by an electrophoresis mobility shift assay experiment in which MAF binding complexes were competed by several high affinity Ets sites recognized by the majority of the known members of the ETS family. The maspin promoter contains two ETS binding sites. CAT assays indicated that the proximal Ets element mediates the cell type-specific expression in human mammary epithelial cells. These results have demonstrated that maspin, as a gene well expressed in the mammary gland, is regulated by an ETS factor, as are genes encoding milk proteins, such as whey acidic protein, lactalbumin, and lactoglobulin.

The ETS proteins share a common DNA-binding domain that recognizes the consensus sequence GGA(A/T) (5, 6). Binding of ETS protein is often associated with the binding of other proteins, which could serve to stabilize the interaction between the ETS DNA-binding domain and DNA, as proposed in a model by Petersen et al. (26). On the other hand, the binding protein may serve as coactivator. For example, JUN and PNT, a Drosophila ETS protein, act synergistically to activate the promoter containing Ap1/Ets elements in the R7 photoreceptor induction (27). ETS binding protein cooperates with AP1 in the collagenase promoter to achieve a maximum level of transcription activation (11). These synergistic activations are present in the promoters of many protease genes (11, 12, 15).

Although the Ets element in the maspin promoter is a well-conserved site for the binding of PEA3 protein, a subfamily of ETS factors, our data indicate that PEA3 does not bind to the Ets site of maspin promoter in normal mammary epithelial cells. A gel-shifting experiment with antibody against PEA3 does not block or supershift the ETS-DNA complex (data not shown). Others have also shown that PEA3 protein is overexpressed in invasive tumors (9, 10), suggesting that PEA3 is not likely to positively regulate maspin expression. The actual ETS involved in the transcription regulation of maspin remains to be identified.

We have found that the Ets element cooperates with the Ap1 site in the maspin promoter to activate transcription in normal mammary epithelial cells. A single Ets site works as an enhancer in both 70N and 21NT cells but not in MDA-MB231 cells. The enhancing ability is greatly reduced in 21NT cells compared to that in 70N cells. When both Ets and Ap1 elements are present, we observed cooperative activation. This cooperation was not present in 21NT primary tumor cells, as was shown in CAT assays with the pEts/Ap1CAT construct.

A few other features about the maspin gene are noteworthy: (a) cloning and sequencing of the maspin promoter revealed the presence of multiple regulatory cis elements: Ets, Ap1, Ap2, and GRE. The upstream Ets and Ap1 sites are inactive in our assay system but may be involved in other types of regulation during mammary gland development, requiring additional factors for activity; (b) there is no TATA box in the maspin promoter. Primer extension analysis indicates the presence of multiple transcription start sites (data not shown), which are characteristic of TATA-less promoters; (c) maspin belongs to the serine proteinase inhibitor superfamily located at chromosome 18q21.3 (28). Others have shown that a cluster of serpins, including maspin, SCCA1, SCCA2, and PAI2 are located in the same chromosomal region (29). Based on this knowledge, we cloned the maspin promoter from a YAC clone containing DNA corresponding to this family of serpin genes. Because the genes are closely linked, they may have evolved by gene duplication. Thus, the regulation of other genes in this cluster may be similar to that of maspin.

In conclusion, differential expression of maspin mRNA in normal mammary epithelial cells and carcinomas is regulated at the transcriptional level. Expression of maspin in normal mammary epithelial cells is mediated mainly by the Ets and Ap1 elements. Loss of expression of maspin in tumor cells is due to the loss of transactivation through the Ets site and loss of cooperative interaction through the Ets and Ap1 sites. Study of the maspin promoter not only helps to understand the mechanism of gene regulation but also offers an opportunity for reexpression of maspin in tumors by therapeutic intervention. Further studies are planned to screen for strong inducers of maspin expression in mammary tumor cells by the mechanism of transcriptional up-regulation.

Materials and Methods

Cell Lines and Media. Normal human mammary epithelial cells (HMECs; 70N, 76N, and 81N) were from reduction mammoplasties as described.

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5 M. Zhang, S. Sheng, N. Maass, and R. Sager. mMaspin, the mouse homologue of a human tumor suppressor gene, inhibits mouse mammary tumor invasion and motility, submitted for publication.
Tumor cell lines were from the American Type Culture Collection. The 21T cell lines were from a progression series derived from the mammary carcinoma cells of a single patient (31). Both normal and tumor cells were cultured in DFCI-1 medium (30).

**Northern Blot Analysis.** Total cellular RNA was prepared as described previously (32). Twenty µg of total RNA were fractionated on 1% agarose, 1.7M formaldehyde gels, transferred to Zetaprobe (Bio-Rad) membrane in 20× SSC, and baked for 1 h at 80°C. Blots were probed with a 2.5-kb EcoRI/XhoI fragment of the maspin cDNA plasmid. 36B4 was used as an internal loading and transfer control (33).

**Promoter Cloning and Sequencing.** YAC genomic DNA subclones were screened with a [70] end-labeled antisense OL1 primer (see oligonucleotide sequences below) as a probe. A positive clone was identified and subcloned into pBluescript SK+ vector to generate pSKmas1 plasmid. The pSKmas1 was partially sequenced to confirm the presence of promoter, exon 1, and the intron 1 boundary. DNA sequencing was performed using an ABI 373A automated DNA sequencer in the Dana-Farber Cancer Institute core facility.

**Oligonucleotides.** Oligonucleotides were synthesized by Aminco, Inc. (Boston). For annealing, pairs of sense and antisense oligonucleotides were mixed in equimolar amounts and annealed in 10 µl Tris (pH 8.0), 200 µM NaCl, and 1 µM EDTA by heating to 95°C for 5 min and cooling to room temperature over a period of 3 h. These were for genomic DNA screening (OL1), 5′-TCAGGCTGTGGACCAACGCTCGGTTCCTCTCGGTCTCCTCTGAG-3′, and antisense (OL3), 5′-TTTGC-CCAGGGGCTGAGCT; (OL2), 5′-GGGCTCGGGGCTGC-3′, for pEtsCAT plasmid construction: sense (OL4), 5′-GGGCTCGGGGCTGAGCT; and antisense (OL5), 5′-TCTCTGAGGAGAGCTGAG-3′ for pEtsAP1CAT and pEts/Al1CAT plasmid construction: sense (OL6); 5′-GCCCTGGTACCAAGAGGAGATCCAGG-3′, and antisense (OL8); 5′-GCTCTGAGGAGAGGAGGCTG; CT.

**Construcrs.** pSkmas1 was digested with HindIII (at +87 bp) and blunt ended by T4 DNA polymerase, and followed by ligation to a HindIII linker. Subsequent digestion with HindIII (at +87 bp) and XbaI (at +956 bp) generated a XbaI-HindIII fragment containing the promoter, which was directionally subcloned into pHCAT promoterless vector (34) to generate pHT(856). Progressive deletions of pHT(856) were made either by restriction enzyme digestion or by exonial transfection. pHT(586) was constructed by digestion of pHT(856) and the removal of the fragments of Pst-Pst, Xba-Pst, Xba-SnaB, and Xba-Stu, respectively. The linearized DNA were blunt ended by T4 DNA polymerase and ligated. pHT(265), pHT(172), pHT(112), and pHT(17) were generated by digestion of pHT(856) with Smal and XbaI, followed by exonial transfection. The linearized DNA fragments were filled in by a large Klenow fragment and ligated. The exonial deletion constructs were sequenced to confirm the sequence of deletion.

The construction of pEtsCAT, pairs of OL2 and OL3 oligonucleotides were annealed as described above. The annealed product was phosphorylated by T4 polynucleotide kinase and ligated to the SacI, Smal sites of pBlact2 (33) to generate pEtsCAT. For the construction of pEts/Al1CAT and pEts/Ma1, POR fragments (~120 to ~47 bp, using OL4/OL5 or OL4/OL6 primers) were digested with XbaI and cloned into the XbaI and BamHI sites of pBLECAT. The pEtsAP1CAT was generated by site-directed mutagenesis (QuickChange site-directed mutagenesis kit; Stratagene) using pEts/Al1CAT plasmid as a template and OL7/OL8 oligonucleotides as primers. All four constructs were sequenced to confirm the sequence of fragments in a single copy.

**Transformation and CAT Assay.** CAT constructs were made as illustrated in Fig. 4A. Cells were plated at 1 × 10⁶/p100 and grown to about 75% confluence. DNA was transfected by the method of modified DEAE-Dextran (Promega Corp., Madison, WI). The amounts of DNA used were: 10 µg reporter plasmid, except for pCMVCAT in which only 2 µg of DNA was used. One µg of pCMVgαl was used as an internal control for transfection efficiency. Forty-eight h after transfection, cells were harvested in 0.25 µl Tris (pH 8.5)-15% glycerol. The extracts were made by three cycles of freeze-thaw. The β-galactosidase activity in the extracts was calculated as described (32). Twenty units of extracts (calculated by β-galactosidase activity) were used for each CAT assay, except for transfection with pCMVCAT positive control, in which only 10 units of extracts were used because of high activity. CAT assay was performed as described by Gorman et al. (38). Quantification of acetylated CD4 and acetylated chloramphenicol was performed by cutting out the appropriate regions of the silica gel TLC plate and counting in BioFluor (DuPont, Wilmington, DE).

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**References**