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# Transcriptional analysis of the *mts1* gene with specific reference to 5′ flanking sequences

(promoter region/chloramphenicol acetyltransferase assays/methylation)

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**ABSTRACT** **The *mts1* gene is specifically expressed in certain metastatic tumors but not in their nonmetastatic counterparts. It is also expressed in several normal cell and tissue types that exhibit the ability to be motile. The gene was cloned from both mouse and human sources and the 5′ flanking regions were sequenced. The sequencing data revealed a 135-base-pair region of high homology between the mouse and human *mts1* gene. This homology was observed in the vicinity of the TATA box. The 5′ region of the *mts1* gene was also observed to have a high degree of homology to some known promoter and enhancer sequences. To determine the role this region plays in regulating the transcription of *mts1*, promoter analysis was performed. Sixteen constructs were prepared in which the chloramphenicol acetyltransferase gene was fused to different regions of the mouse *mts1* promoter. These constructs were analyzed in transient transfection assays in two related cell lines derived from mouse mammary adenosarcomas: CSML-0, a nonmetastatic cell line with low levels of *mts1* expression, and CSML-100, a metastatic cell line with high levels of *mts1* expression. Results of our transient transfection assays in conjunction with results obtained from *in vitro* and *in vivo* footprinting of the promoter region show no evidence of cis-acting control elements important for the transcriptional regulation of *mts1* in these cell lines. A few nucleotides upstream of the TATA box are sufficient for maximal levels of *mts1* transcription. Because no cis-acting control elements were found, restriction of *mts1* transcription in CSML-0 cells must exist on some other level. *mts1* was found to be hypermethylated in CSML-0 cells but not in CSML-100 cells. The possible role of methylation in progression of the nonmetastatic CSML-0 adenosarcoma cell line toward the metastatic CSML-100 adenosarcoma cell line is discussed.**

Many cancer-related deaths result from the ability of the malignant neoplasm to colonize other organs at a distance from the primary tumor mass (1). Although metastasis is a clinically relevant process, little is known about the molecular mechanisms that govern it. In an effort to better understand the mechanisms of tumor progression, differential screening was performed with cDNA libraries generated from related nonmetastatic and metastatic mouse mammary adenosarcoma cell lines, CSML-0 and CSML-100, respectively. In this way the cDNA for the mouse *mts1* gene, expressed at high levels in metastatic cells and low levels in nonmetastatic cells, was cloned (2). A region of the *mts1* cDNA was then used as a probe to screen a mouse liver genomic library; a 17-kilobase genomic clone of *mts1*, containing two introns and three exons, was obtained (3). The 5′ flanking region of the *mts1* gene was sequenced by chemical degradation and compared to the nucleotide sequences in the GenBank data base (March 1989). This region of the *mts1* gene was found to be highly homologous to the promoter regions of rat fibrinogen and human prothrombin as well as to the enhancer sequences of simian virus 40 (SV40) (3). The human *mts1* gene has also been cloned and sequenced (see Fig. 1, and E.R. and E.L., unpublished data). The 5′ flanking regions of the mouse *mts1* and the human *mts1*¶ were compared, and a region of high homology was observed. In addition, the rat homolog of *mts1* was cloned by differential screening of a cDNA library generated from a highly metastatic radiation-induced rat thyroid tumor (IR6-mts; S.Z. and M. Salim, unpublished data).

The *mts1* gene or its cDNA was independently cloned by several other groups. These groups used differential screening to clone the gene from the following sources: quiescent mouse fibroblasts after serum stimulation (4), PC12 cells after nerve growth factor stimulation (5), myoepithelial-like cells (6), and the BALB/c3T3 established cell line (7). *mts1* encodes a 101-amino acid protein belonging to the S100 subfamily of small $Ca^{2+}$ binding proteins (see ref. 8 for a review on S100 proteins). The S100 subfamily consists of nine members, which have primary structures that are well conserved among the vertebrate species studied. They are acidic, of low molecular mass (10–12 kDa), and bind $Ca^{2+}$ through an EF hand structure. The genes encoding these proteins are structurally related and share a high degree of homology in coding regions, particularly those regions that code for $Ca^{2+}$ binding domains. In addition, at least three of the genes belonging to this family (*mts1*, calcyclin, and 42C) are clustered at the same locus of mouse chromosome 3 (9). It is possible that related and clustered genes may share some common mechanisms of transcriptional regulation. An example of this can be seen in X chromosome inactivation (10, 11). Although genes that code for S100 proteins may have some mechanisms of transcriptional regulation in common, additional levels of gene regulation are necessary to confer the temporal and tissue-specific expression of S100 gene products such as 42C (12), MRP8 and MRP14 (13), and calcyclin (14).

In this study we have examined the transcriptional regulation of the *mts1* gene. This may provide us with information on the specific mechanism of *mts1* gene regulation as it relates to metastasis and tumor progression. In addition, we may gain some insight into whether the genes coding for S100 proteins share some common transcriptional regulatory

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Abbreviations: SV40, simian virus 40; CAT, chloramphenicol acetyltransferase.

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¶The sequences reported in this paper have been deposited in the GenBank data base [accession nos. M88460 (mouse) and M88459 (human)].

mechanisms. Due to the homology of the 5′ flanking region of *mts1* with known promoter and enhancer sequences, particular attention was given to this region to determine if it was involved in the transcriptional regulation of the *mts1* gene. Our studies indicate that the 5′ flanking region of the *mts1* gene is not important for regulating gene expression. Instead, transcriptional regulation of the *mts1* gene is seen to correlate with the methylation pattern of the gene.

## MATERIALS AND METHODS

**Plasmid Constructions.** The published plasmid p-266CAT (previously referred to as p155CAT) (3) contains 266 base pairs of the 5′ flanking region of *mts1*, the first exon, the first intron, and the nontranslated part of the second exon fused to the chloramphenicol acetyltransferase (CAT) gene. A *Bgl* II–*Hin*dIII restriction fragment containing the upstream region of *mts1* from −1897 to −267 was subcloned into p-266CAT to yield p-1897CAT. All other constructions were obtained by treating the p-1897CAT plasmid with BAL-31 nuclease. The 5′ borders were determined by sequence analysis. The control plasmids p+65CAT, p+261CAT, and p+651CAT were made by deleting, respectively, the *Sph* I (in the polylinker of the p-41CAT construct)–*Nhe* I, *Sph* I–*Sac* I, and *Sph* I–*Pst* I restriction fragments from the p-41CAT construct. *Nhe* I, *Sac* I, and *Pst* I sites are all located in the first intron of the *mts1* gene (3). The y series of plasmids were constructed by subcloning the *Hin*dIII–*Nhe* I restriction fragments from p-1897CAT, p-268CAT, p-121CAT, p-75CAT, and p-41CAT into the polylinker of the Promega enhancer vector, which contains the SV40 enhancer but does not contain its own promoter.

**Cell Lines.** Cells from the mouse adenosarcoma cell lines CSML-0 and CSML-100 (15, 16) were grown in 5% $CO_2$/95% air at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal bovine serum (GIBCO). Cells were transfected by using the calcium phosphate method from the Promega mammalian transfection kit. As a control, the reporter plasmid pβGal was used, in which a *Pst* I–*Hin*dIII restriction fragment containing the bacterial β-galactosidase gene and an SV40 RNA polyadenylylation signal were subcloned under the control of the Rous sarcoma virus promoter. β-Galactosidase and CAT assays were performed as described (17).

***In Vitro* Footprinting Analysis.** Nuclear extracts of CSML-0 and CSML-100 were prepared as described by Dignam *et al.* (18). After lysis of cells, the protease inhibitors pepstatin A, aprotinin, and bestatin were added to give final concentrations of 1 μg/ml, and benzanidine and phenylmethylsulfonyl fluoride were added to give final concentrations of 0.5 mM. The DNA probes were made by cutting the plasmid p-159CAT with *Hin*dIII and labeling either the 5′ ends with [$\gamma$-$^{32}$P]ATP or the 3′ ends with [$\alpha$-$^{32}$P]dATP (19). The DNA was then treated with *Hae* III and run on a polyacrylamide gel to isolate the labeled DNA fragment containing the upstream region (−41 to −130) of the mouse *mts1* gene.

Gel retardation assays were performed as described (19). Reaction mixtures were prepared by mixing the following reagents: 2 μl of poly(dI-dC) at 1 mg/ml, 2 μl of 5× buffer (150 mM KCl/30 mM Hepes·KOH, pH 7.9/20 mM Tris·HCl, pH 7.9/5 mM EDTA/5 mM dithiothreitol/25% glycerol/bovine serum albumin at 1.5 mg/ml), 3 μl of nuclear extract (13 μg of protein), 1 μl of end-labeled DNA fragment ($3 \times 10^4$ cpm), and 2 μl of doubly distilled $H_2O$. Polyacrylamide gel electrophoresis was carried out as described (19); however, a higher ratio (40:1) of acrylamide to *N*,*N*′-methylenebisacrylamide was used.

Methylation interference analysis was performed as described (19).

***In Vivo* Footprinting Analysis.** *In vivo* footprinting analysis was conducted by using the ligation-mediated PCR method (20). Primers used were homologous to the noncoding strand of the first exon of the mouse *mts1* gene: 1, ACCAAGAGAGAGGAAGTGCTGAATAG; 2, GAAGTGCTGAATAGAGAGGTTTGGA; 3, GCTGAATAGAGAGGTTTGGGGAGAGCC. The temperature used for annealing of the primers was $t_m$ + 2°C.

**Generation of a Calcyclin Probe and Hybridization to Mouse DNA.** A probe identical to the second exon of the human calcyclin gene was synthesized by using PCR. Genomic DNA isolated from human hepatoma cell line HepG2 was used as the template, and the following primers were used: CTCCAAGCCCAGCCCTCAGC and CGAGCCAATGGTGAGCTCT. The conditions of the PCR reaction were as follows: 94°C for 1 min, 63.5°C for 2 min, and 72°C for 3 min for 30 cycles. The product of the reaction was eluted from a polyacrylamide gel and was subjected to ≈20 PCR cycles under the above conditions with the addition of [$\alpha$-$^{32}$P]dCTP. Conditions of hybridization were 37°C for 18 hr in 50% formamide, 2× standard saline citrate, 10× Denhardt's solution, 0.3% SDS, and salmon sperm DNA at 100 μg/μl.

All other DNA manipulations (isolations, *Msp* I/*Hpa* II digests, Southern blots, etc.) were carried out as described by Maniatis *et al.* (17) or as described in catalogs from which reagents were purchased.

## RESULTS

**Comparison of the 5′ Flanking Regions of the Human and Mouse *mts1* Genes.** The 5′ flanking regions of mouse *mts1* [clone λ3 (3)] and its human homolog (clone λ44; E.R. and E.L., unpublished results) were sequenced and compared. Fig. 1 is a comparison of the two sequences. A region of high homology (≈80%) can be seen from the first transcribed nucleotide to 135 nucleotides upstream of the transcription start site. Two of the three known *mts1* sequences identified by computer analysis to be homologous to known eukaryotic enhancer or promoter sequences lie within the region of high homology between the mouse and human *mts1*. These regions are homologous to the SV40 enhancer (21) and the promoter of the human prothrombin (22) gene. The conserved nature of this region in the two species indicates that it may be necessary for the transcriptional regulation of *mts1*.

***In Vitro* Footprinting and Gel Retardation Analysis Identify Two Major and Two Minor DNA–Protein Complexes in the Upstream Region of *mts1*.** Gel retardation analysis was performed to identify protein binding sites in the upstream region of *mts1*. The −41 to −130 *mts1* fragment was combined with nuclear extracts from CSML-0 and CSML-100 to yield two major (C1 and C2) and two minor (C1a and C1b) complexes (Fig. 2*A*), which appeared in all experiments conducted. Other minor complexes were observed on occasion. The extracts from CSML-0 and CSML-100 produced qualitatively similar results; however, the C1 complex appeared to be more abundant when extracts from CSML-100 cells were used.

To identify the precise location of the complexes bound to the −41 to −130 *mts1* fragment, methylation interference assays were performed on both the coding and the noncoding strands. As shown in Fig. 2*B*, guanosine residues involved in the formation of complex C1 are located between nucleotides −106 and −118, and guanosine residues involved in the formation of complex C2 are located between nucleotides −66 and −76 (Fig. 2 *B* and *C*). The methylation interference pattern of the total preparation of the minor complexes C1a and C1b appears to be similar to that of the major complex C1 (Fig. 2*B*).

***In Vivo* Footprinting Analysis Does Not Identify Any DNA–Protein Complexes Upstream of *mts1*.** *In vitro* methylation

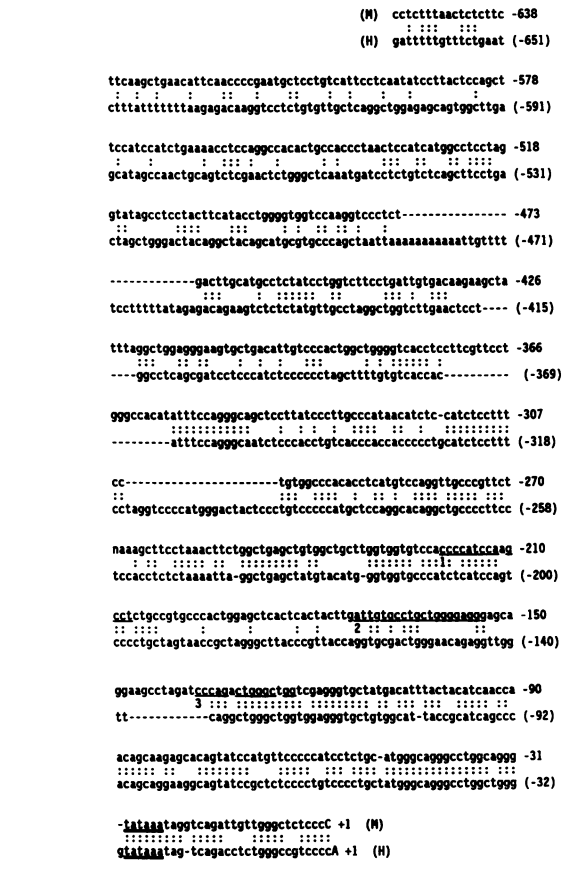

FIG. 1. Comparison of the 5′ flanking regions of the mouse (M) (3) and human (H) *mts1* genes. 1, region of homology with the SV40 enhancer; 2, region of homology with the 5′ flanking sequence of the human prothrombin gene; 3, region of homology with the 5′ flanking sequence of the rat fibrinogen gene.

interference assays demonstrated the existence of two major protein binding regions in the upstream region of the mouse *mts1* gene, independent of the source of extract used. However, *in vivo* methylation interference experiments revealed no protein binding sites upstream of the TATA box, regardless of the source of extract used (Fig. 2*D*).

**CAT Constructs with Different Lengths of *mts1* 5′ Flanking DNA Revealed No Cis-Acting Elements Important for Promoter Activity.** To fully determine the importance of various areas of the upstream region of *mts1*, constructs were made in which different regions of the 5′ flanking area of *mts1* were fused to the bacterial CAT gene. Sixteen constructs were made (see *Materials and Methods*), and the results of experiments performed with two of these constructs are shown (Fig. 3*C*). The 3′ border of all constructs is an *Nco* I site near the ATG codon from the second exon of the mouse *mts1* gene. All the constructs contained the untranslated portion of the second exon, the first intron, and the first exon. They could be distinguished from each other by the length of the 5′ flanking region. To show that no internal promoter activities exist, three control plasmids were constructed in which the sequence acting as the *in vivo* promoter was eliminated (p+65CAT, p+261CAT, and p+651CAT). The 3′ border of the *mts1* insert in these three constructs is the *Nco* I site of the second exon, and their 5′ borders vary: they begin at nucleotides 65, 261, and 651, respectively. The constructs were transfected into CSML-0 cells and CSML-100 cells, and CAT activities in appropriate extracts were examined. To interpret the data accurately, it was necessary to transfect the cells from all the experiments with a β-galactosidase vector (pβGal), which served as an internal control to monitor relative transfection efficiencies between different dishes of cells. Fig. 3 *A* and *B* represent transient expression assays in which p-41CAT (which represents the shortest 5′ flanking region used, containing only 41 nucleotides upstream of the transcription start site) and p-1897CAT (which represents the longest 5′ flanking region used, containing 1897 nucleotides upstream of the transcription start site) were transfected into CSML-100 (Fig. 3*A*) or CSML-0 cells (Fig. 3*B*). A diagramatic representation of the plasmids used is shown in Fig. 3*C*. Approximately equal levels of CAT activity were seen with both these constructs, as well as with the 14 others tested. Extracts prepared from cells transfected with the three control plasmids p+65CAT, p+261CAT, and p+651CAT demonstrated 13-fold, 7-fold, and 15-fold less CAT activity than extracts prepared from cells transfected with the promoter-containing constructs (data not shown). Although the level of CAT activity is lower in the control constructs than in the promoter-containing constructs, the detection of a small amount of CAT activity indicates the existence of a weak promoter activity in the first intron of the *mts1* gene. In determining the importance of *mts1* upstream sequences on promoter activity, an internal promoter activity may make the data difficult to interpret. To eliminate the influence of the internal promoter on CAT activity, a new set of constructs was prepared (y series). Different *mts1* upstream sequences were cloned into the Promega enhancer vector. This vector contains the SV40 enhancer, but it is missing the SV40 promoter. Each *mts1* fragment subcloned into this vector contained the same 3′ end (+65), thereby eliminating the internal promoter region. The 5′ ends of the *mts1* fragments subcloned into the Promega enhancer vector were as follows: −1897, −268, −121, −75, and −41. All constructs from this series had approximately the same level of promoter activity (data not shown). Therefore, 11 nucleotides upstream of the TATA box (p-41CAT and y-41CAT) are sufficient for maximal levels of transcription from the *mts1* promoter (Fig. 3). Small differences in CAT activities of different extracts were not reproducible. All constructs tested were able to express the CAT gene after transfection into CSML-0 cells (Fig. 3*B*), where transcription of the *mts1* gene is not detected.

**Importance of Methylation in Transcriptional Regulation of the *mts1* Gene.** The ability of all the constructs to express the CAT gene in CSML-0 cells at approximately the same level as in CSML-100 cells suggests that some transcriptional regulatory mechanism exists, which is lost during the cloning procedure. Because methylation has been implicated as a possible mechanism of gene regulation and because methylation may be lost during the cloning procedure, we decided to examine the methylation pattern of *mts1* in CSML-100 cells and CSML-0 cells. To determine whether methylation of *mts1* is important in regulating its transcription, DNA isolated from CSML-0 and CSML-100 cells was digested with the isoschizomers *Msp* I and *Hpa* II. The digested DNA was then run on an agarose gel, blotted, and hybridized to a labeled *mts1* cDNA probe (plasmid p271) (Fig. 4*A*). To ensure that the restriction digests were complete, the filter was stripped and rehybridized to a probe homologous to the second exon of the calcyclin gene (Fig. 4*B*). Unlike the calcyclin gene (which is expressed in both CSML-0 and CSML-100 cells), the *mts1* gene is hypermethylated in CSML-0 cells. This is in good agreement with the known lack of expression of the *mts1* gene in these cells.

## DISCUSSION

The 5′ flanking regions of mouse and human *mts1* were sequenced and compared, revealing an area of high homology from the transcription start site to 135 base pairs upstream of this site. However, no cis-acting elements important for modulation of *mts1* transcription were identified in this region by transient expression assays or by *in vivo* dimethyl sulfate footprinting analysis. The two constructs p-41CAT and

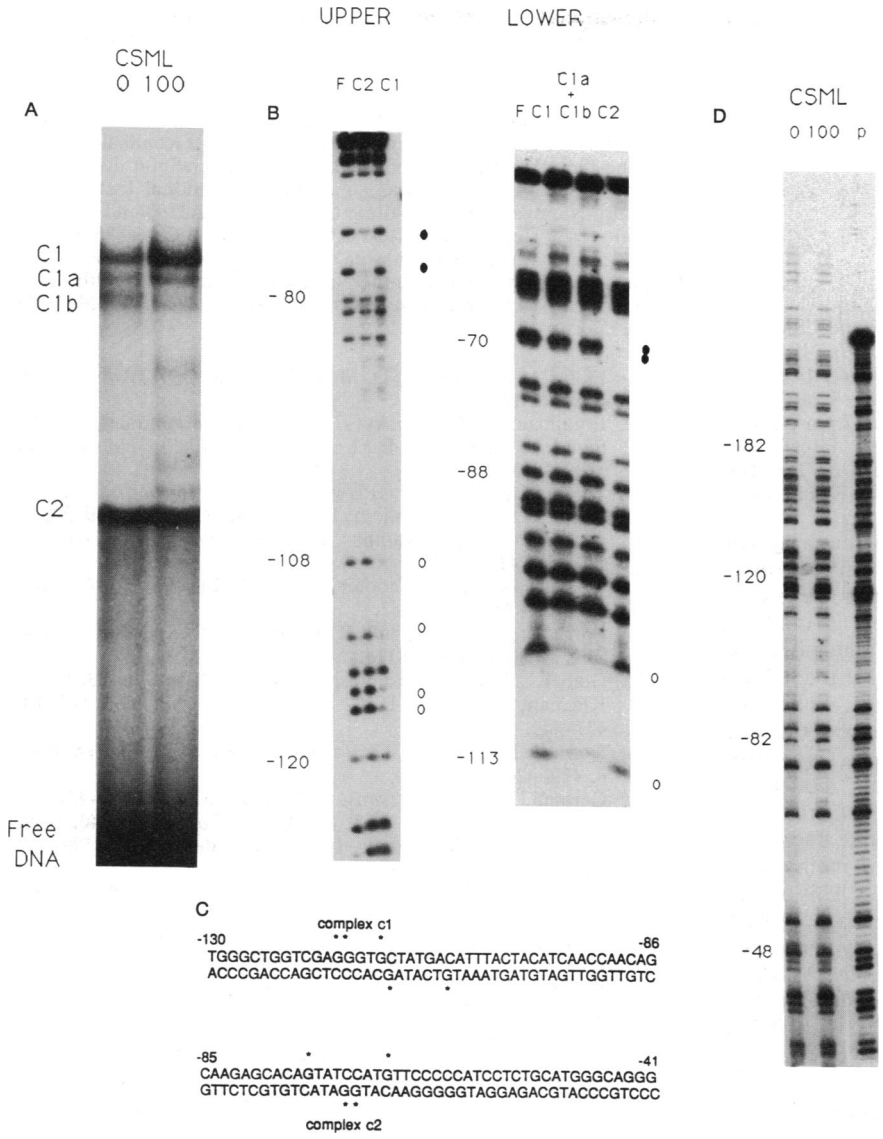


FIG. 2. Protein binding sites identified on the −41 to −130 region of the *mts1* gene. (*A*) Gel retardation analysis of the −41 to −130 DNA fragment after combining with nuclear extracts from CSML-0 cells or CSML-100 cells. C1 and C2 represent the two major complexes. C1a and C1b represent the two minor complexes identified. (*B*) Methylation interference analysis of the −41 to −130 DNA fragment. The fragment was labeled at the 5′ end for analysis of the upper strand (upper) and at the 3′ end for analysis of the lower strand (lower). After partial methylation of the DNA with dimethyl sulfate, it was incubated with nuclear extract from CSML-0 cells. Complexes and free DNA (F) were isolated by gel retardation electrophoresis. After cleavage at modified residues, the fractions were analyzed on a 10% polyacrylamide gel (for the upper strand experiment) or on a 6% polyacrylamide gel (for the lower strand experiment). Reduced bands are marked with open circles (complex C1) and filled circles (complex C2). (*C*) Summary of the methylation interference experiment. Guanosine residues involved in the formation of complexes C1 and C2 are marked with asterisks. (*D*) *In vivo* methylation interference experiment. The experiment was performed by using the ligation-mediated PCR method (20). No protein binding sites are seen in the upstream region of the mouse *mts1* gene. p, cloned plasmid DNA control.

p-1897CAT showed equal levels of promoter activity. No tissue-specific regulators were identified to bind the promoter region.

*In vitro* gel retardation assays and methylation interference analysis with extracts from CSML-100 and CSML-0 identified two major (C1 and C2) and two minor (C1a and C1b) DNA–protein complexes in the upstream region of the *mts1* gene. However, our transient expression data do not indicate these complexes as modulators of promoter activity. These complexes may be involved in the precise initiation of *mts1* transcription. The methylation of *mts1* may serve as an important mechanism of transcriptional control, since *mts1* is hypermethylated in cultured cells (Fig. 4*A*) where its expression is downregulated.

Methylation at CpG residues has been shown to play a role in gene expression. When enzyme-deficient rodent cell lines were treated with 5-azacytidine, a potent demethylating agent, they were strongly reactivated to wild type (23). Doerfler *et al.* (24) demonstrated that the sequence-specific methylation of three 5′-CCGG-3′ sequences in the E2A promoter of adenovirus type 2 inactivates this promoter. In several cases the methylation patterns of 5′ flanking regions

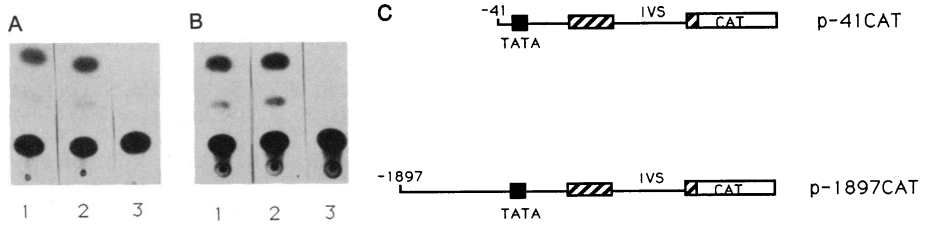


FIG. 3. CAT constructs with different lengths of the *mts1* 5′ flanking DNA reveal no cis-acting elements important for promoter activity. (*A*) Levels of CAT activity are approximately equal when CSML-100 cells are transfected with two constructs containing either 41 nucleotides upstream of the *mts1* transcription start site (p-41CAT; lane 1) or 1897 nucleotides upstream of the transcription start site (p-1897CAT; lane 2). Lane 3, mock transfection. (*B*) The same results as those described above were obtained in CSML-0 cells. Lane 1, p-41CAT; lane 2, p-1897CAT; lane 3, mock transfection. (*C*) Diagramatic representation of the two constructs used in the above experiment. IVS, intervening sequence.

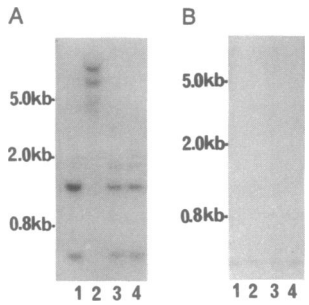


FIG. 4. Methylation pattern of *mts1* in CSML-0 and CSML-100 cells. (*A*) DNA samples were digested with *Msp* I or *Hpa* II, run on a 1% agarose gel, transferred to nylon membranes, and probed with a *mts1*-specific probe. Lane 1, *Msp* I digest of DNA isolated from CSML-0 cells; lane 2, *Hpa* II digest of DNA isolated from CSML-0 cells; lane 3, *Msp* I digest of DNA isolated from CSML-100 cells; lane 4, *Hpa* II digest of DNA isolated from CSML-100 cells. (*B*) The same filter was probed with a calcyclin-specific probe. kb, Kilobases.

correlate with gene expression. Examples include the globin genes (25), the vitellogenin gene (26), and certain X chromosome genes such as the phosphoglycerate kinase gene (27).

The above-mentioned cases may represent a mode of transcriptional regulation in which positive regulators that bind to 5′ flanking regions of a gene recognize methylated CpG residues as a mutation in their binding sites. As a result, these regulators are not able to bind their sites, and the level of transcription of the genes decreases. This model is referred to as the "direct" model (28). The "indirect" model proposed by Boyes and Bird suggests that a specific factor exists that binds only methylated DNA. Once bound, the factor inhibits transcription of the gene (28). The methyl-CpG binding protein may represent such a factor (29). This protein complexes with a variety of DNA sequences when they are methylated at CpG residues. Therefore, it may serve as a negative regulator that recognizes methylated DNA in general rather than recognizing specific DNA sequences. It is possible that in the progression of the CSML-0 cell line toward its metastatic counterpart, CSML-100, an inactivation of the normal methylation system may occur. A multicomponent methylation system (composed of genes encoding the methylase, transcription factors that regulate the gene, etc.) could easily be inactivated by mutational events that occur during tumor progression, resulting in hypomethylation of the *mts1* gene. This could then lead to the increased expression of *mts1* seen in many tumors in the later stages of progression (2).

The idea that known carcinogenic agents may affect DNA methylation is not new. It has been proposed that DNA-damaging agents can change gene expression by altering the pattern of DNA methylation (30). Ultraviolet light has been shown to induce hypomethylation and gene activation (31). Conversely, it has been suggested that ethyl methanesulfonate may introduce methylation at sites that are normally nonmethylated, thereby inactivating certain genes (32).

Therefore, evidence exists that certain carcinogenic agents may disrupt the normal methylation pattern of the genome, thereby inactivating and activating genes whose regulation may be important in maintaining the nonmetastatic phenotype. *mts1* is expressed in activated macrophages, T-cells, lymphoidal tissue, and embryonic tissue. These cell and tissue types all exhibit motility. The *mts1* gene may code for a protein that is somehow involved in the process of motility. Normally, the methylation pattern of this gene downregulates its expression in other tissue and cell types. However, when the cells are exposed to some carcinogenic agent, some of their DNA may undergo demethylation. As a result of demethylation, the *mts1* gene is overexpressed, and its product may than aid in the invasive capacity of the cells, leading to the metastatic phenotype.

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