Isolation and characterization of a gene specifically expressed in different metastatic cells and whose deduced gene product has a high degree of homology to a Ca^{2+}-binding protein family

Alexander Ebralidze, Eugene Tulchinsky,[1] Mariam Grigorian, Anna Afanasyeva,[2] Viacheslav Senin,[2] Elena Revazova,[2] and Eugene Lukanidin

Engelhardt Institute of Molecular Biology and [1]Institute of Developmental Biology, USSR Academy of Sciences, Moscow; [2]National Cancer Research Center, USSR Academy of Medical Sciences, Moscow, USSR

The gene *mts1*, which is expressed specifically in metastatic cells, was isolated by molecular cloning coupled with differential DNA reassociation. Transcription of *mts1* was found not only in tumor cells, but also in normal cells; homologous RNA was detected only in spleen, thymus, bone marrow, and blood lymphocytes. DNA sequencing of *mts1* revealed an open reading frame containing information for a peptide of 101 amino acids, and the amino acid sequence suggested that the mts1 protein was identical to the previously isolated Ca^{2+}-binding mouse protein (Jackson-Grusby et al. 1987; Goto et al. 1988). Thus, the mts1 protein is a member of the calcium-modulated protein family, and our data indicate that mts1 is involved in regulating the metastatic behavior of tumor cells.

[*Key Words*: Mouse metastatic genes; transcription; *mts1*; Ca^{2+}-binding proteins]

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Tumor progression is thought to occur when variant cells having selective growth properties arise within a tumor cell population (Foulds 1975). One of the final stages of tumor progression is the appearance of the metastatic phenotype (Nicolson 1984). During metastasis, the tumor cells invade the blood vessels, survive against circulating host immune defenses, and then extravasate, implant, and grow at sites distant from the primary tumor (Nicolson 1982, 1987).

So far, little is known about the intrinsic mechanism involved in the metastatic cascade. It is likely that in some cases the augmented metastatic potential of certain tumor cells may be due to an increased expression of oncogenes, which normally are responsible for control of various cellular functions, including differentiation, proliferation, cell motility, and communication (Cairns 1981; Berger et al. 1988; Klein and Klein 1985). Recently, it has been shown that primary and established rodent fibroblasts transformed by activated oncogene sequences can form metastases (Vousden and Marshall 1984; Thorgiersson et al. 1985; Vousden et al. 1986; Storer et al. 1988); however, not all of the transformed cells expressing activated oncogenes are converted to the metastatic phenotype (Kris et al. 1985; Rotter et al. 1985). These observations indicate that oncogene transformation is not the only mechanism by which transformed cells evolve to the metastatic phenotype, and they imply that other genes can be responsible for the metastatic behavior of transformed cells.

In an attempt to identify such genes, we have been studying an animal model of the metastatic process. Two cell sublines obtained from a spontaneous mouse mammary carcinoma were used. One of the sublines (CSML-0) was derived from a tumor maintained by intramuscular passages and characterized by a low metastatic potential. Solitary lung metastases were detected in less than 10% of autopsied animals that were killed when in a moribund condition. A second, highly metastatic subline (CSML-100) was created by using the selective method of multiple successive subcutaneous transplantation of metastatic cells into the tail. The frequency of lung metastases by any route of inoculation was 100%. CSML-50 represents an intermediate stage of selection during the establishment of CSML-100. The frequency of lung metastases in this case was about 50% (Senin et al. 1983, 1984).

The purpose of the present study was to investigate whether the differential expression of genes in both of these sublines can be used to identify genes responsible for the metastatic behavior of CSML-100. Here we re-

port the results of studies on isolating *mts1*, the gene that is expressed specifically in metastatic cells, and we provide evidence that the product of this gene has a high homology with Ca^{2+}-binding proteins and thus is a member of the calcium-modulated protein family.

Results

Cloning of cDNAs corresponding to mRNAs that are expressed differentially in the CSML-100 and CSML-0 cell lines

To examine the differences in gene expression in metastatic and nonmetastatic tumor lines, we used DNA cloning and colony hybridization techniques. Molecular cloning was coupled to differential DNA reassociation to yield a library highly enriched with cDNA molecules specific for metastatic and nonmetastatic cells. Double-stranded cDNA (ds-cDNA) made from CSML-100 poly(A) RNA was cross-hybridized with a 200-fold excess of a driver CSML-0 poly(A) RNA. To increase the rate of the cDNA/mRNA reassociation reaction, we used the phenol-emulsion reassociation technique (PERT) of Kohne et al. (1977). Most of the cDNA in such conditions hybridized with homologous mRNAs, and less than 5% of cDNA renatured and formed ds-cDNA molecules. These molecules were ligated to the arms of a λgt10 vector DNA and, after packaging in a phage coat, were used to infect *Escherichia coli*. A λgt10 cDNA library of $\sim 1 \times 10^4$ clones was established from poly(A)$^+$ mRNAs of CSML-100.

After plating the cDNA library, two sets of replica filters were prepared for in situ hybridization. These were screened differentially with cDNA probes derived from mRNAs of CSML-100 and CSML-0 cell lines. After comparing the resulting pairs of autoradiographs, 1×10^3 clones were selected for further screening on the basis that they gave a high signal after hybridization with the CSML-100-derived probes and a low or zero signal with the CSML-0 probes. Further hybridization analysis indicated that the cDNA inserts could be classified into three groups. The largest group, representing about 90% of the clones, hybridized equally well with both probes. The second group (about 8%) hybridized more intensively with the homologous CSML-100 probes as compared to hybridization with heterologous probes. The third group of the clones (less than 1%) hybridized only with the homologous probes. One clone of the latter group, designated as clone mts1, was taken for further analysis.

To determine whether the cloned cDNA represented mRNA sequences, we performed Northern blot analysis on mRNAs isolated from CSML-100 and CSML-0 cells. The results presented in Figure 1 confirmed that the DNA insert in clone mts1 hybridized only to mRNAs isolated from CSML-100 cells. This probe detected a single 0.55-kb transcript.

These data indicate that we have isolated a unique DNA sequence (clone mts1) that is expressed specifically in metastatic cells.

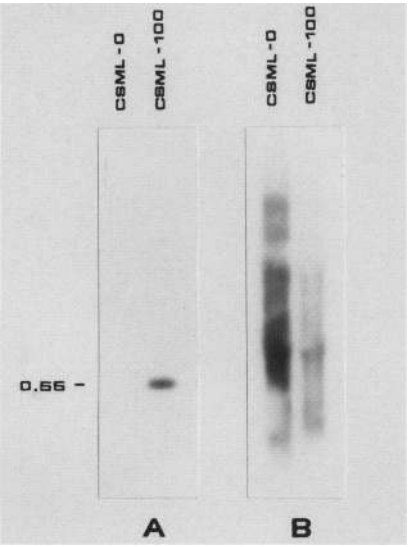

Figure 1. Detection of a 0.55-kb transcript in the CSML-100 cell line. (*A*) A Northern blot of total RNA (20 μg/lane) isolated from the mouse CSML-100 and CSML-0 cell lines was analyzed by hybridization to the ^{32}P-labeled probe of clone mts1. (*B*) The same filter, after the ^{32}P-label was washed out, was rehybridized to the ^{32}P-labeled cDNA made from CSML-100 poly (A)$^+$ RNA.

DNA insert of clone mts1 *represents a single-copy gene*

Enzymatic digests of genomic DNA from normal mouse liver and the CSML-100 cell line were probed with ^{32}P-labeled *mts1* DNA. In both cases, a *Bam*HI fragment of 14 kb and an *Eco*RI fragment of 4.4 kb were detected (Fig. 2A). The patterns obtained with the two DNA preparations were indistinguishable in location and band intensity. Hybridization of the restriction fragments of genomic DNA from a variety of other species (human, pig, rat, chicken) indicated the presence of a gene whose sequences cross-hybridize with the mts1 probe (Fig. 2B).

Transcription of mts1 *sequences in tumor and normal cells*

Tumor strains and cell lines used in the present work were checked for their metastatic ability. All transformed cell lines described in the literature as metastatic expressed the metastatic phenotype except one, the melanoma B-16 cell line (Table 1). The phenotype of this tumor strain apparently has changed during many sequential passages in mice, with a resultant loss of metastatic capability.

We investigated the correlation of transcription of *mts1* and the metastatic phenotype of different transformed and normal cells. Northern blot analysis was conducted using the DNA of clone mts1 as a probe. The probe hybridized to the unique 550-base transcript in all the metastatic cell lines analyzed except HMC-Lr. The levels of *mts1* gene expression in both HMC-Lr and nonmetastatic HMC-0 lines were very low (Fig. 3A). The intensity of hybridization was different for each cell line,

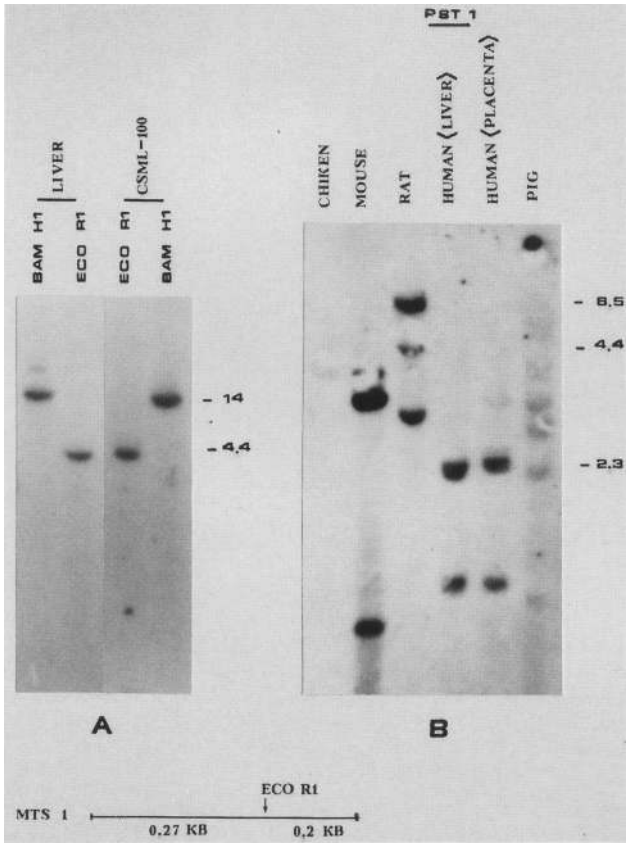

Figure 2. Gel transfer hybridization analysis of DNA from different species. Total DNA was digested with endonucleases, electrophoresed in a 0.8% agarose gel, transferred to nitrocellulose, and probed with a 0.27-kb ^{32}P-labeled *Eco*RI fragment of clone mts1 (*A*). The 0.27-kb fragment is shown in the schematic diagram of the mts1 clone at the bottom of the figure. (*B*) Hybridization with ^{32}P-labeled DNA of the mts1 clone.

with the strongest hybridization found to RNAs isolated from CSML-100 (Fig. 3B).

The expression of *mts1* depended on the conditions of cell growth. We found that, after five passages in vitro, the level of *mts1*-specific RNA in the $T36_c$ cell line derived from mice bearing T36 embryocarcinoma was much lower than the expression of these RNAs in the parental tumor (Fig. 3A). The RNA isolated from most nonmetastatic tumors did not hybridize to the ^{32}P-labeled mts1 probe (Fig. 3A).

We then asked whether the expression of *mts1* DNA was specific for transformed cells. RNAs were isolated from different organs of A/Sn mice. Northern blot analysis was performed and 550-base mRNAs were detected only in spleen, thymus, bone marrow, and bloodstream lymphocytes (Fig. 4). The amount of RNA expressed in these organs calculated from the intensity of hybridization was low compared with the amount of mRNA in the CSML-100 cell line. It is interesting that hybridized RNA was found only in the so-called 'lymphoid organs' or in lymphocytes.

The influence of DNA methylation on the expression of *mts1* was investigated. The CSML-0 cell line was treated with 5-azacytidine and total RNA was analyzed by Northern blotting with ^{32}P-labeled probes of clone mts1. RNA isolated from the treated and untreated CSML-0 cell lines did not hybridize with the mts1 clone (data not shown). These results suggest that *mts1* transcription is not regulated at the level of DNA methylation.

The protein product of mts1 *is very similar to calcium-binding proteins*

The nature of the *mts1* gene product was identified by comparing its amino acid sequences deduced from the nucleotide sequences in the Protein Sequence Database (Fig. 5A). The amino acid sequence suggested that the mts1 protein was identical to the previously isolated mouse Ca^{2+}-binding protein (Jackson-Grusby et al. 1987; Goto et al. 1988).

Homology was also found with several rat proteins belonging to the family of S-100 related peptides. Especially high homology was found for two rat proteins—p9Ka (Barraclough et al. 1987) and 42A (Masiakowski and Shooter 1988). The only difference between mts1 and these two rat proteins is the substitution of the two amino acid residues. Valine and serine residues at positions 58 and 60 in the mts1 protein are replaced by leucine and asparagine residues in rat proteins.

A 55% identity in the amino acid sequences was found in Ca^{2+}-binding domains between the S-100 protein and the product of the *mts1* gene. An especially high homology was found in the loop part of the domain (66%). A homology between the analogous part of the rat intestinal Ca^{2+}-binding protein and the *mts1* gene product was 48% and 63%, respectively (Fig. 5B). The hydrophobicity profiles of S-100, ICaBP, and mts1 were also compared (data not shown). All three profiles are very similar and are in good agreement with the data presented in Masiakowski and Shooter (1988). These data indicate that the mts1 protein is homologous with Ca^{2+}-binding proteins, not only in their amino acid sequences but also in the structural organization of the protein molecules.

Discussion

In the experiments presented here, a gene that is expressed differentially in tumor metastasis has been identified. PERT was used to enrich specific DNA sequences (Kohne et al. 1977) and the cDNA synthesized on poly(A)$^+$ RNA from metastatic cells was hybridized in the presence of excess mRNA obtained from nonmetastatic cells. Under these conditions, the bulk of cDNA molecules hybridized with mRNAs and less than 5% of cDNA was used to reconstruct the recombinant library. Thus, this method increases the relative amount of specific cDNA molecules and simplifies the screening procedure.

We found that the level of *mts1* expression correlates with the metastatic potential of tumor cells—the cells

Table 1. *Metastatic potential of analyzed tumors and tumor cell lines*

Tumors and cell lines[a]	Spontaneous metastases	Target organs
Mammary carcinosarcoma		
CSML-0	low metastatic[b]	lung
CSML-50	50%	lung
CSML-100	high metastatic[c]	lung
Mammary solid carcinoma		
HMC-0	low metastatic	liver[d]
HMC-Lr	high metastatic	liver[d]
Teratocarcinoma cell line		
$PCC4_c$-B	nonmetastatic	—
$PCC4_c$-P	nonmetastatic	—
$PCC4_c$-107	nonmetastatic	—
C12–	nonmetastatic	—
Embryocarcinoma, T-36	50%	lymph node
Cell line derived from T-36, $T\text{-}36_c$	50%	lymph node
Embryocarcinoma, LMEC	high metastatic	lymph node
Teratocarcinoma, T-9	low metastatic	lymph node
Colon adenocarcinoma, Acatol	nonmetastatic	—
Melanoma, B-16	low metastatic	lung
Lung carcinoma, RL-67	high metastatic	lung[d]
Lewis lung carcinoma, LLC	high metastatic	lung

[a] $PCC4_c$-B, $PCC4_c$-P, and $PCC4_c$-107 are cell lines derived from PCC4-Blangy, PCC4-Pasteur, and PCC4-107 teratocarcinomas.
[b] Low metastatic indicates 20% of injected mice give rise to solitary metastases.
[c] High metastatic indicates 100% of multiple metastases in target organs.
[d] Metastases in other organs.

that have a high level of *mts1* expression demonstrate a high degree of metastasis, and the cells that do not express *mts1* or express it at a low level either do not metastasize or have a low metastatic potential (CSML-50). The one exception we did find was HMC-Lr, a highly metastatic tumor in which *mts1* is expressed at a low level. Perhaps another regulatory mechanism operates in this particular tumor to convert the cells to the metastatic phenotype.

Our data suggest that *mts1* is involved in regulating the metastatic behavior of tumor cells. An increase in the transcription of some genes or in the expression of some proteins suggests that these genes may participate in the processes of tumor growth and metastases. For instance, a high level of *ras* gene expression in NIH-3T3 cells transformed with an activated c-Ha-*ras* oncogene correlates with the metastatic potential of these cells in nude mice (Bernstein and Weinberg 1985; Thorgeirsson et al. 1985).

Recently Collard and co-workers (1987) have shown that fusion of nonmetastatic T-lymphoma cells with normal T lymphocytes results in the formation of hybrid T cells with a high metastatic potential. Therefore, it is relevant to note that we found transcription of *mts1* not only in tumor, but also in normal cells. Homologous RNA was found only in spleen, thymus, bone marrow, and blood lymphocytes. Thus, the finding that *mts1* gene expression correlates with the metastatic potential of tumor cells expressed specifically in normal blood cells suggests that properties peculiar to some normal cells can contribute to formation of the metastatic phenotype.

The data on the amount of mRNA corresponding to *mts1* or its analogs pEL98 and 18A2 are rather conflicting. Jackson-Grusby and co-workers (1987) have found a low but detectable level of the 18A2 gene expression in kidney and a very low amount of mRNA in thymus. In contrast, Goto's (1988) as well as our data do not show any detectable signals on Northern blot analysis of mRNA from kidney. In addition, we observed a low level of *mts1* mRNA expression in thymus. The amounts of mRNA corresponding to the pEL98 and 18A2 genes in primary embryo fibroblasts were also different. This discrepancy may be due to different mouse strains used in the experiments. However, the ability of the mouse probe to cross-hybridize with DNA sequences from human and other species points to the conservation of *mts1* sequences.

Several mechanisms have been proposed for regulation of gene expression, including a different reorganization of the cell genome (Yokota et al. 1986). Restriction enzyme digests of genomic DNA from the normal mouse liver and the CSML-100 cell line hybridized to the mts1 probe reveal only one fragment in each digest, which suggests that *mts1* is a single-copy gene. Furthermore, identical hybridization patterns in both the normal liver and CSML-100 digests indicate that the high expression of *mts1* in metastatic cells does not result from a gene translocation as well as from a gene amplification event, because the intensities of hybridization signals appear to be the same.

DNA methylation can also be an important mechanism regulating gene expression. The presence of 5-methylcytidine in the 5′ promoter regions of genes is associated with the inactivation of transcription. The content of 5-methylcytidine in tumors has been found to be

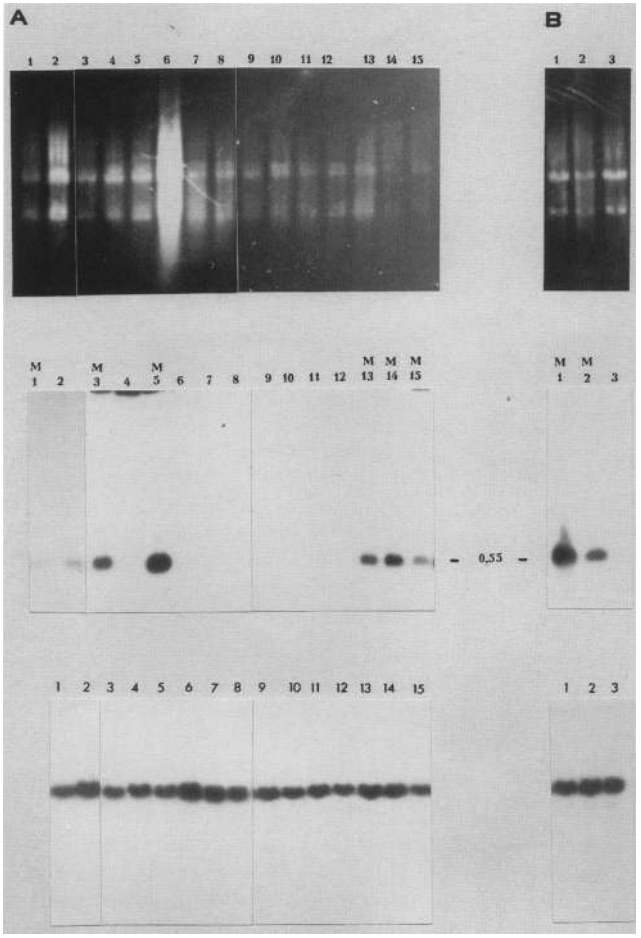

Figure 3. Northern analysis of total RNA from different metastatic and nonmetastatic mouse tumors and cell lines using *mts1* DNA as a probe. The numbers indicate different tumors and cell lines. (*A*) Three days autoradiography: (lane *1*) HMC-Lr; (lane *2*) HMC-0; (lane *3*) RL-67; (lane *4*) B-16; (lane *5*) LLC; (lane *6*) Acatol; (lane *7*) C12; (lane *8*) $PCC4_c$-B; (lane *9*) $PCC4_c$-P; (lane *10*) $PCC4_c$-107; (lane *11*) PCC4 107; (lane *12*) T9; (lane *13*) LMEC; (lane *14*) T-36; (lane *15*) T-36_cl. (*B*) Twelve hours autoradiography: (lane *1*) CSML-100; (lane *2*) CSML-50; (lane *3*) CSML-0. M indicates metastatic tumors. Ethidium bromide staining of the agarose gels is shown at the top of the figure. The amount of RNA loaded per gel lane was 20 μg. Hybridization with the actin probe is shown at the bottom of the figure.

lower than in normal cells (Feinberg and Vogelstein 1983). The treatment of nonmetastatic cells with 5-azacytidine makes the cells metastatic (Olsson and Forchhammer 1984; Litepto and Kerbel 1987). In the present study, we have found that *mts1* transcription does not depend on DNA methylation. The *mts1*-specific RNA cannot be detected in nonmetastatic cells before and after 5-azacytidine treatment.

DNA sequencing of *mts1* revealed two ORFs containing the information for peptides composed of 38 and 101 amino acids. However, the Kozak consensus sequences (Kozak 1984) flanking the translational start were found only for the large peptide (101 amino acids). The start region of the short peptide (38 amino acids) does not contain such sequences, and probably the ORF for this peptide is not functional.

Comparison of the amino acid sequences of the *mts1* product revealed homology with the family of Ca^{2+}-binding proteins (CaBPs). A common structural organization has been postulated for the Ca^{2+}-binding site of the large CaBPs family by Szebenyi et al. (1981). These authors describe each calcium-binding site as being organized into a structure referred to as an EF hand and composed of a loop with two helical structures, one on either side. Sixteen amino acids in the sequences made of 29 residues are essential for the formation of a calcium-binding EF hand. Kretsinger (1976) has predicted that intracellular calcium-modulated proteins contain an EF hand and, vice versa, any protein with an EF hand is calcium-modulated. The structure of the *mts1* protein product is shown in Figure 5B, and the sequences of some calcium-binding proteins are aligned with those of an EF hand. Although the mts1 protein has a 55% homology with the S-100 protein, all the residues present in the EF hand meet the proposed requirements. The two loops (residues 21–23 and 63–74) are remarkably conserved. In contrast, many differences are found in the linker regions and in the helices at positions where any amino acid may appear. On the whole, the structure of the *mts1* protein product reported here fits in well with the EF hand theory, thus, identifying it as a member of the calcium-modulated protein family.

Neoplastic transformation is known to alter the expression pattern for the various members of this Ca^{2+} modulated protein family. For example, S-100 protein has been found in human melanoma cells (Gaynor et al. 1980), and in rodent fibroblasts transformed with RSV or SV40 viruses the amount of calmodulin is twice the normal level (Chatouleas et al. 1981). Oncomodulin, another Ca^{2+}-binding protein, is expressed in significant

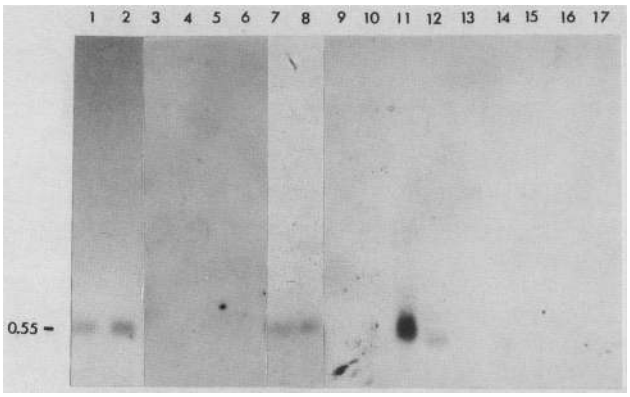

Figure 4. Northern blot analysis of total RNA from different organs of A/Sn mice using *mts1* DNA as a probe. The lane numbers indicate different organs: (lane *1*) bone marrow; (lane *2*) lymphocytes; (lane *3*) female liver; (lane *4*) male liver; (lane *5*) female brain; (lane *6*) male brain; (lane *7*) male spleen; (lane *8*) female spleen; (lane *9*) male salivary glands; (lane *10*) female salivary glands; (lane *11*) male thymus; (lane *12*) female thymus; (lane *13*) female heart; (lane *14*) female lung; (lane *15*) male kidney; (lane *16*) testis; (lane *17*) ovary. Seven days autoradiography.

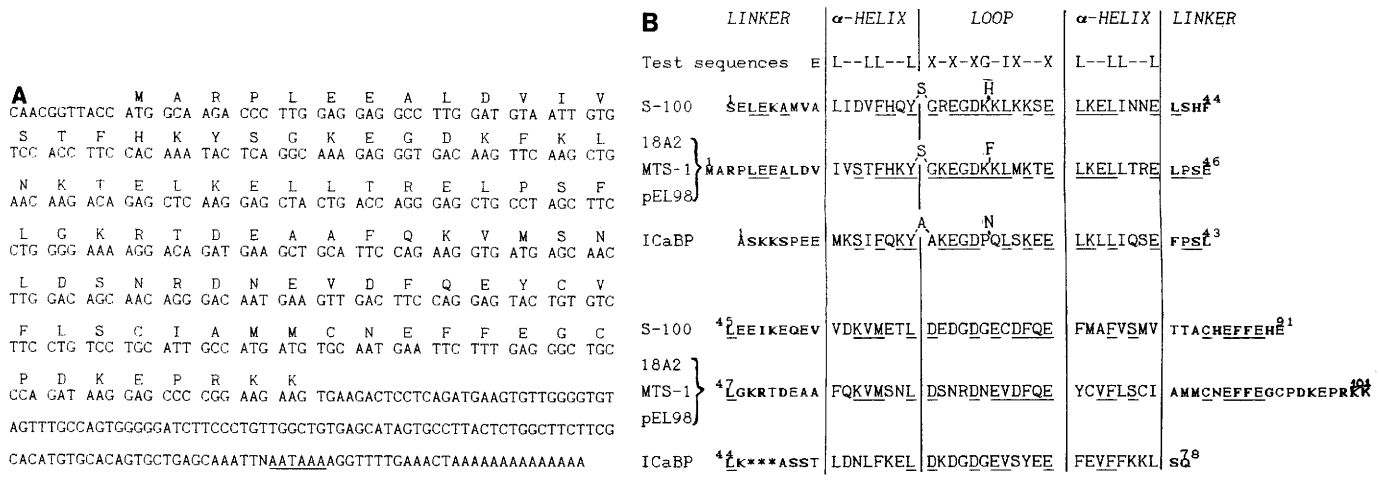

Figure 5. (*A*) DNA sequence of *mts1* cDNA and the deduced amino acid sequence. The putative polyadenylation signal is underlined. (*B*) The homology of amino acid sequences in the EF hand region of some calcium-binding proteins. The test sequence represents residues suggested to be critical to the EF hand structure (Kretsinger 1976). (X) An oxygen-containing residue (D, E, N, Q, S, T); (L) a hydrophobic residue (L, V, T, K, H); (G) glycine; (E) glutamic acid; (–) any amino acid appears in this position.

chemically induced rat hepatomas, but is not found in normal adult rat liver (Gillen et al. 1987). mRNA corresponding to the protein pEL98 is presented in large amounts in both chemically transformed and activated oncogene-transformed cell lines (Goto et al. 1988). *mts1* is also expressed both in normal and transformed cells, but its level of expression in tumor cells is higher than in normal cells. Thus, this gene may have a significant diagnostic potential for metastatic disease; however, the delineation of its possible role in metastases awaits transfection of the full-length gene.

Materials and methods

Tumors and tumor cells

CSML-0 and CSML-100, as well as HMC-0 and HMC-Lr, are tumor lines established from spontaneous mammary adenocarcinomas of A/Sn mice (Senin et al. 1983, 1984). T-9 as well as T-36 and its variant LMEC are coupled sublines of two original tumors. The latter were induced by ectopic transplantation of 6–7 days' gestation syngeneic embryos to CBA/J and A/Sn mice (Senin et al. 1984).

Tumor cells were trypsinized, rinsed, and suspended in sterile Hanks' salt solution. A total of 1×10^6 cells in 0.3 ml of Hanks' solution were injected subcutaneously into each 8- to 10-week-old A/Sn mouse. Mice were examined weekly for the appearance of tumors. The resultant tumors were excised and used for DNA and RNA preparations.

Cell lines, $PCC4_c$-P, $PCC4_c$-B, and PCC4-$_c$-107 were derived from PCC4-Blangy, PCC4-Pasteur, and PCC4-107 teratocarcinomas, respectively. All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). Cells were passed weekly.

Metastatic activity

Metastatic activity was determined upon intramuscular injection of 1×10^6 tumor cells per tumor cell line in 10–15 mice. The mice were killed 4–5 weeks after tumor inoculation, and the number of lung metastases was counted. Nonmetastatic cell lines were defined as cell lines that did not result in visible metastases. Highly metastatic lines under the same conditions gave rise to multiple metastases in target organs of each mice.

Nucleic acid purification and analysis

Procedures for the preparation, analysis, and recombination in vitro of plasmid DNA were as described in Maniatis et al. (1982). RNAs were prepared from different tumor cells and normal cells according to the procedure described by Chomczynski and Sacchi (1987). Gel electrophoresis, RNA blotting to nylon membrane filters, and hybridization with nick-translated DNA probes were as in Grigorian et al. (1985). Total DNA was prepared from cells according to Maniatis et al. (1982). Southern blots were performed using 10 μg of genomic DNA extracted from mouse liver, CSML-100 cells, human placenta and liver, rat liver, pig liver, and chicken liver. DNAs were digested with *Bam*HI, *Eco*RI, and *Pst*I endonucleases. Following electrophoresis in a 0.8% agarose gel, the DNA was transferred onto a nylon membrane (Hybond N, Amersham). The filter was prehybridized and hybridized following a standard procedure (Maniatis et al. 1982). The nucleotide sequences of *mts1* were determined using the chemical degradation procedure of Maxam and Gilbert (1980).

cDNA/RNA solution reassociation, construction, and screening of cDNA libraries

The PERT reaction (Kohne et al. 1977) employed cDNA synthesized with the aid of reverse transcriptase on poly(A) mRNAs (2 μg) from the CSML-100 cell line and an excess of driver poly(A) mRNA (50 μg) isolated from the CSML-0 cell line. The cDNA was heated at 100°C for 5 min, cooled on ice, and added to the final reaction volume of 1 ml in a 10-ml glass centrifuge tube in 7% phenol (adjusted to pH 7.6 with 0.1 M Tris-HCl, 1.25 M NaCl, 120 mM sodium phosphate buffer, pH 6.8). The tube was shaken for 7 days at 25°C. After hybridization, the mixture was extracted twice with chloroform, then dialyzed against 10 mM Tris-HCl (pH 7.5), 1 mM EDTA to remove excess salts, and then precipitated with ethanol. The PERT-reassociated ds-cDNA was mixed with λgt10 arms DNA and 1 μl of T4 ligase (80

U/ml), and the mixture was packaged in vitro into bacteriophage particles that were used for *E. coli* infection. The library was screened differentially with radioactively labeled first-strand products of the reverse transcriptase reaction using 1 μg of poly(A)$^+$ mRNAs from the CSML-100 and CSML-0 cell lines.

Sequence homologies

The homologous domains of the *mts1* product were aligned using the protein alignment program PROTAN, followed by inspection.

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A Ebralidze, E Tulchinsky, M Grigorian, et al.

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