The κB and V(D)J Recombination Signal Sequence Binding Protein KRC Regulates Transcription of the Mouse Metastasis-associated Gene S100A4/mts1*

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A κB-like sequence, Sb, is integral to the composite enhancer located in the first intron of the metastasis-associated gene, S100A4/mts1. Oligonucleotides containing this sequence form three specific complexes with nuclear proteins prepared from S100A4/mts1-expressing CSML100 adenocarcinoma cells. Protein studies show that the Sb-interacting complexes include NF-κB/Rel proteins, p50-p50 and p50-p65 dimers. Additionally, the Sb sequence was bound by an unrelated 200-kDa protein, p200. Site-directed mutagenesis in conjunction with transient transfections indicate that p200, but not the NF-κB/Rel proteins, transactivates S100A4/mts1. To identify candidate genes for p200, double-stranded DNA probes containing multiple copies of Sb were used to screen a randomly primed λgt11 cDNA expression library made from CSML100 poly(A)† RNA. Two clones corresponding to the DNA-binding proteins KRC and AIf1 were identified. KRC encodes a large zinc finger protein that binds to the κB motif and to the signal sequences of V(D)J recombination. In vitro DNA binding assays using bacterially expressed KRC fusion proteins, demonstrate specific binding of KRC to the Sb sequence. In addition, introduction of KRC expression vectors into mammalian cells induces expression of S100A4/mts1 and reporter genes driven by S100A4/mts1 gene regulatory sequences. These data indicate that KRC positively regulates transcription of S100A4/mts1.

Mts1, also known as S100A4, is a member of the S100 family of small acidic Ca2+-binding proteins. The S100 proteins have been implicated in cell cycle progression, differentiation, metabolism, cancer development, and metastasis (reviewed in Refs. 1 and 2). Thirteen of the S100 genes are located as a cluster in the human chromosome region 1q21, where abnormalities are found in a number of malignancies, including breast cancer, lymphoma, and leukemia (3, 4). In a mouse myelomonocytic leukemia cell line, an insertional mutation of a metastatic gene, resulting in the aberrant activation of mts1, has been reported (5). In addition, the expression of a shorter mts1 transcript variant has been observed and proposed to be responsible for the etiology of disease progression in some human intraductal breast carcinomas (6, 7). The mechanism by which S100 proteins in general and mts1 in particular regulates the wide variety of growth functions attributed to them remains unclear. It has been proposed that physiological function of the S100 proteins is regulated via the interaction of target proteins or peptides, which increase the affinity of the S100 proteins to calcium. The target proteins for mts1 include the heavy chain of nonmuscle myosin (8), nonmuscle topomyosin (9), a 36-kDa microfibril-associated glycoprotein (10), and a 37-kDa protein (11).

In normal tissues, mts1 transcript was detected mainly in spleen, thymus, and bone marrow (12). Mts1 is also abundant in neutrophils, lymphocytes, and macrophages. However, mts1 transcripts or proteins were detected at high levels in a number of human tumors (13) and were more abundant in cell lines that possessed metastatic properties than in related nonmetastatic cell lines (12, 14). Clinically, the expression levels of mts1 were often associated with enhanced invasiveness of breast (15, 16), prostate (17), and colon carcinomas (18). Thus, elevated levels of mts1 show strong correlation with the proliferative potential and invasive and metastatic ability of cancers. Direct evidence demonstrating that mts1 plays a causal role in metastasis came from experimental elevation of mts1 by transfection of a benign rat epithelial cell line (19–21) and of src-transformed rat fibroblasts (22) that conferred in vitro cell invasion and promoted tumor progression. In animal models, mice harboring mts1 transgenes were more prone to metastasis. For example, the mouse GRS/A strain, characterized by a high incidence of mammary tumors that rarely metastasize, developed secondary tumors in the lungs when a mts1 transgene was introduced (23). A mts1 transgene coupled with the activated neu oncogene also formed metastatic mammary tumors in mice (24). On the other hand, expression of antisense RNA or hammerhead ribozyme directed against mts1 transcript suppressed or reversed metastatic phenotypes in tumor cells (25, 26). Thus, the investigation of the transcriptional regulation of mts1 may provide insights into the changes that occur in transcription-controlling mechanisms in carcinoma cells during tumor progression.

The mouse mts1 cDNA was isolated by differential expression of mts1 in highly metastatic mouse mammary adenocarcinoma cells and its nonmetastatic counterpart (27, 28). Subsequent cloning and sequencing showed that the mts1 gene consists of two protein coding exons and one small noncoding 5’ exon (29). The mts1 minimal promoter contains a “TATA” box...
KRC encodes a large zinc finger protein that binds to the c-fos expression cDNA library prepared from CSML100 cells. Two of those complexes were composed of sequence-specific complexes with nuclear proteins prepared from NIH/3T3 cells. Two of the complexes were composed of the NF-kB/Rel family of proteins: the p50/p50 homodimers and p50/p65 heterodimers (31). The largest DNA-protein complex was formed by a previously unidentified protein. This protein was named p200 because its molecular mass was estimated to be ~200 kDa by UV cross-linking experiments (31). DNA-protein interaction studies coupled with reporter gene assays showed that mutations preventing NF-κB binding had no effect on the mts1 natural enhancer activity, but mutations abolishing the binding of p200 led to the functional inactivation of this site in the mts1 first intron (31). p200, therefore, is a factor essential for the transcription regulation of mts1.

**EXPERIMENTAL PROCEDURES**

**Oligonucleotides, Plasmid Constructs, and Cell Cultures—Oligonucleotides** were chemically synthesized and purchased (Life Technologies, Inc.). The abbreviations used are: bp, base pair(s); kb, kilobase pair(s); EMSA, electrophoretic mobility shift assays; Mbp, maltose-binding protein.

The sequences of the oligonucleotides used in this study are listed below. Sh/forward (F), 5'-GGCCAGCTGTGGTGTTTCCCATC-3'; Sh/reverse (R), 5'-AAGTGAAAACCCCGTGGC-3'; mut2/F, 5'-GGCACGGCTGCTATTTTACACTT-3'; mut2/R, 5'-AAGTGAAAATAGC- 
AAGTGTAAAAATAGCT-3'; mut2/R, 5'-CCGGGCGCTCGACTCCAC-3'; E/B, 5'-CCGGGCCACTTGCCCTTCCAC-3'; E/B, GGGAGGGAATGGCCCTTCCAC-3'.

The parental reporter plasmid pLuc contains the luciferase gene under the control of the c-fos minimal promoter (37). Plasmid pLuc1 contains a 135-bp fragment including the mouse mts1 gene promoter and is mapped within a 41-bp region upstream of the luciferase gene.

The cellular factors that regulate the transcription of mts1 are largely unknown. One general approach to identify gene-specific transcription factors is to use the cis-acting DNA elements as ligands in functional or in DNA-protein interaction studies. The κB-related Sb element described above partially contributes to the activity of the composite enhancer in a mts1-expressing mouse adenocarcinoma CSML100 cell line (31). DNA-protein interaction analysis showed that Sb formed three sequence-specific complexes with nuclear proteins prepared from CSML100 cells. Two of those complexes were composed of the NF-κB/Rel family of proteins: the p50/p50 homodimers and p50/p65 heterodimers (31). The largest DNA-protein complex was formed by a previously unidentified protein. This protein was named p200 because its molecular mass was estimated to be ~200 kDa by UV cross-linking experiments (31). DNA-protein interaction studies coupled with reporter gene assays showed that mutations preventing NF-κB binding had no effect on the mts1 natural enhancer activity, but mutations abolishing the binding of p200 led to the functional inactivation of this site in the mts1 first intron (31). p200, therefore, is a factor essential for the transcription regulation of mts1.

**Construction and Screening of CSML100 agt11 cDNA Expression Library—agt11 cDNA expression library was constructed using poly(A)+ RNA isolated from CSML100 cells, and a cDNA synthesis kit according to the manufacturer's instruction (Amersham Pharmacia Biotech). Random hexanucleotide primers were used for the synthesis of the first-strand cDNA. After the addition of EcoRI/NotI adapters, cDNAs were ligated to EcoRI-digested agt11 arms (Stratagene) and packaged in vitro (Gigapack III gold packaging extracts, Stratagene). To prepare the probe, two complementary single-stranded oligonucleotides, Sb/F and Sb/R (sequences as shown in the above section), were annealed to form a double-stranded Sb oligonucleotide. Sb oligonucleotide was phosphorylated with T4 polynucleotide kinase and [γ-32P]ATP on both ends and then concatenated by T4 DNA ligase (High DNA polymerase). Plasmid DNAs were prepared with the Magic Maxiprep DNA purification system (Promega Corp.) and were used directly for sequencing with Sequenase (Amersham Pharmacia Biotech). DNA homology searches were performed with the BLAST program from NCBI (39). Multiple sequence alignments were performed with the program DIALIGN version 2.1 (40, 41), accessed through the Internet site and annotated manually.

**Recombiant Proteins, Nuclear Extracts, and Antisera—** The KRC fusion proteins Mbp/Rea and KRC/ZA1 and a polyclonal antisera raised against Mbp/Rea have been described (42–44). Nuclear extracts were isolated from CSML100 cells according to the method of Dignam et al. (45). Antisera raised against KRC/ZA1 in rabbits was produced with the BLAST program from NCBI (39). Multiple sequence alignments were performed with the program DIALIGN version 2.1 (40, 41), accessed through the Internet site and annotated manually.

**Electrophoretic Mobility Shift Assays and Gel Supershift Assays—** Electrophoretic mobility shift assays were performed as described (31, 42). Briefly, Sb oligonucleotide labeled with [32P]dCTP and Klenow (0.2 ng, ~5000 cpmp) was incubated with proteins for 25 min in 25 μl of binding reaction buffer in the presence of poly(dI-dC) (1 μg). For DNA competition experiments, a 100-fold molar excess of double-stranded oligonucleotides Sb, E, and mut2 or 5 μg of poly(dI-dC) was supplementary to the binding reaction. The gel supershift assays were conducted using KRC antibodies and Sp1 antibodies as a negative control. To eliminate nonspecific binding of the KRC antisera to the probe, the antisera were preincubated with sonicated salmon sperm DNA (10 μg, 1:10 dilution in buffer D). Diluted KRC antibodies (5 μl) or Sp1 antibodies (1 μl) were added 15 min after the incubation of binding reactions, and incubation was continued for another 10 min before gel loading.

**Southern Blot and Northern Blot Analyses—** DNA was digested with EcoRI, and Southern blot analysis was performed using a KRC 3΄ cDNA probe (44). Northern blot analysis was performed using a [32P]-labeled exon II-mts1-specific probe (55) and a [32P]-labeled glycerolaldehyde 3-phosphate dehydrogenase probe (35).

**Luciferase Assay—** Reporter plasmid DNA (1 μg) was transfected into semi-confluent tissue cultures using LipofetAMINE PLUS according to the manufacturer’s protocol. NIH/3T3 and five independent NIH/3T3-
KRC+ clones were used in each experiment. Forty-eight h after transfection, luciferase assays were performed using the luciferase reporter system (Promega Corp.) and a luminometer (Berthold, model LB9501). The luciferase activity was normalized with the SV40-controlled β-galactosidase activity using the Galacton-PLUS substrate system (Tropix, Inc.) (0.25 µg of pCDH110, Promega Corp.), which was co-injected in all experiments. The normalized luciferase activity of the plasmid pLUC in parental NIH/3T3 cells was tentatively assigned as 1.

RESULTS

Molecular Cloning Experiment Identified KRC as a Candidate Gene for p200—To isolate candidate genes that encode p200, a Agt11 cDNA library was constructed with poly(A)+ RNA prepared from mouse adenocarcinoma CSML100 cells. The library contained ~1.7 million clones, of which ~60% were recombinants. Protein filters prepared from the library were screened with [32P]-labeled DNAs containing multiple copies of the mts1 enhancer motif Sb. Two positive clones, λ1000 and λ1200, were obtained. The CDNA insert of these clones were amplified by the polymerase chain reaction using Agt11 forward and reverse primers that flank the cloning site and were subsequently subcloned into the pGEM-7Zf plasmid vector. The inserts of λ1000 and λ1200 were 942 base pairs and 1272 base pairs, respectively. The nucleotide sequences of both clones were completely determined on both strands. Sequence homology searches of these sequences performed using the BLAST program from NCBI show that both clones were derived from previously known genes, Alf1 (47) and KRC (35, 36). The insert of λ1000 corresponded to nucleotides 1195 to 2136 of mouse Alf1 cDNA (accession number X64840). Alf1 is a helix-loop-helix transcription factor identified by expression cloning using the Akv murine leukemia virus enhancer (47). The fusion protein produced by λ1000 contains the carboxyl portion of Alf from amino acids 378 to 688, including the helix-loop-helix DNA binding motif specific for the NCAGNTGN version of the E-box. An E-box motif flanking the Sb sequence is present in the DNA probe used to screen the expression library. The insert of λ1200 corresponds to nucleotides 5265 to 6536 of mouse KRC cDNA (36) and directs the production of amino acids 1516 to 1940 of KRC, which includes a pair of zinc fingers, an acidic domain, a serine-rich region, and two Ser-Pro-Xaa-Arg sequences. Zinc fingers are well known DNA binding motifs. Ser-Pro-Xaa-Arg motifs have been suggested to form β-turn 1 structures for nonspecific DNA binding at the minor grooves (48). Both cDNAs isolated from the CSML100 expression library, therefore, encode protein structures capable of specific DNA binding.

The DNA-binding protein KRC (for kxB binding and recognition component of the V(D)J recombinase) is a member of a family of large zinc finger proteins that bind to the kxB or related motifs (reviewed in Ref. 36). Each protein in this family contains two DNA binding domains that are separated widely apart. Fusion proteins of KRC have been shown to have dual DNA binding specificities for the kxB motif and the signal sequences of V(D)J recombination (42, 44). The calculated molecular mass of the large open reading frame of KRC is 246,905 Da. The molecular cloning experiment reported here together with previous results showing KRC to bind the kxB motif identify KRC as a candidate gene for the Sb-binding protein p200.

KRC Fusion Proteins Bind Specifically to Sb—To test the hypothesis that p200 is a gene product of KRC, we initially examined the physical interaction between KRC fusion proteins and Sb DNA by electrophoretic mobility shift assays. The fusion protein Mbp/Rc490 harbors the carboxyl DNA binding domain of KRC from amino acids 1630 to 2119 fused to maltose-binding protein, Mbp (42). A major DNA-protein complex was observed when Mbp/Rc490 (~1 µg) was incubated with the [32P]Sb oligonucleotide (Fig. 1A, lane 1). A minor faster migrating species, probably a complex of Mbp/Rc490 monomer and Sb, was also observed. Previously, we showed that Mbp/Rc490 mainly binds DNA as dimers, tetramers, and multiples of tetramers (42). The specificity of Mbp/Rc490 for Sb was demonstrated by adding unlabeled DNA oligonucleotides in competition experiments. Because Mbp/Rc490 also bound the kxB motif (35, 42), we also competed Sb binding with unlabeled kxB oligonucleotides. The presence of a 100-fold excess of the unlabeled Sb (Fig. 1A, lane 2) or kxB DNA (Fig. 1A, lane 3) competed away the binding of Mbp/Rc490 to [32P]labeled Sb efficiently, whereas an irrelevant DNA poly(dI-dC) (~5 µg) competed insignificantly (Fig. 1A, lane 5). Sb binding specificity of Mbp/Rc490 was further illustrated by mutating designated nucleotides of the Sb site in the oligonucleotide. In the oligonucleotide mut2, four nucleotides within the Sb site that were crucial for protein binding and for the potency of the mts1 enhancer were replaced (31). No complexes were observed when [32P]-mut2 DNA was used as a ligand in the binding reaction (Fig. 1A, lanes 7 and 8). In addition, a 100-fold excess of mut2 DNA did not compete significantly with the binding of Mbp/Rc490 to [32P]Sb DNA (Fig. 1A, lane 4). The ability of the KRC fusion protein Mbp/Rc490 to bind specifically to Sb supports the hypothesis that KRC encodes p200.

In an attempt to determine if Mbp/Rc490 may influence the Sb binding of CSML100 nuclear extracts, we performed electrophoretic mobility shift assays using both protein sources. CSML100 nuclear extracts alone formed three specific complexes when incubated with the [32P]Sb oligonucleotide (Fig. 1B, lane 2). The slowest migrating complex was formed by p200, and the other two complexes were displaced by the Mbp/Rc490 complex, whereas the p200 complex remained (Fig. 1B, lane 3). In addition, enhanced DNA binding of p200 and Mbp/Rc490 were observed when CSML100 nuclear extracts and fusion proteins were used.
in combination than when used separately (compare lanes 1 and 2 with lane 3 in Fig. 1). The data may imply that p200 and Mbp/Rc490 may enhance DNA binding of each other and that the DNA-protein complex formed by p200 is more stable than those formed by the NF-κB.

Interaction of KRC Antisera with the Complex Formed by Sb and p200—Other KRC family members have been reported to bind κB-like DNA sequences. However, according to the cloning experiment, we view KRC as the best candidate gene for p200. To test this hypothesis, we employed a gel supershift assay using KRC antibodies. KRC is a large zinc finger protein with composite DNA-protein interaction domains (Ref. 36; Fig. 2A). KRC contains two DNA binding structures, ZAS1 and ZAS2, that are separated widely apart. Two KRC fusion proteins were used to generate polyclonal antisera in rabbits (Fig. 2A). The fusion protein KRC/ZAS1 contains 407 residues including the ZAS1 domain near the N terminus of KRC from amino acids 77 to 483 fused to vector-encoded thioredoxin domain, His-tag and S-tag (44). Polyclonal antibodies Ab/N were raised against N-terminal portion of KRC, led to the formation of gel-supershifted complexes (Fig. 2C, lanes 3 and 6). Thus the antibody/antigen pairs were specific, did not cross-react, and were effective in gel supershift assays.

To determine if p200 is related to KRC immunologically, gel supershift assays were performed by supplementing binding reactions of CSML100 nuclear extracts and [32P]Sb with KRC antibodies. The specific interaction of antibodies with a DNA-binding protein will further retard the mobility of that DNA-protein complex. The addition of Ab/N resulted in the formation of a further retarded complex in the KRC/ZAS1-Sb binding reaction (Fig. 2C, lane 3) but not in the Mbp/Rc490-Sb binding reaction (Fig. 2C, lane 5). Similar activity was observed for Ab/C with Mbp/Rc490-specific complexes (Fig. 2C, lanes 3 and 6). The antibody/antigen pairs were specific, did not cross-react, and were effective in gel supershift assays.
fusion proteins and Sb DNA in conjunction with gel supershift assays of CSML100 nuclear extracts and KRC antisera suggest that p200 is a gene product of KRC. To determine if KRC regulates transcription of endogenous mts1, we examined the stable levels of mts1 transcripts and the activity of mts1 reporter genes in cells harboring KRC expression constructs. Plasmid pKRC/N6 is derived from the mammalian expression vector pEBVHIS and contains a ~7.2-kb KRC cDNA under the control of the Rous sarcoma virus long terminal repeat. The Rous sarcoma virus long terminal repeat directs the expression of genes in many cell types. pKRC/N6 was long-term transfected into mouse NIH/3T3 fibroblast cells. The largest ~9.5-kb KRC transcripts have been identified mainly in lymphoid and neuronal tissues (35, 44), and NIH/3T3 express low levels of KRC. Long-term pKRC/N6 transfectants, NIH/3T3-KRC, were selected for by culturing in medium containing hygromycin (~100 μg/ml). The expression vector pEBVHIS confers hygromycin resistance to mammalian cells. Subsequently, EcoRI digests of genomic DNA prepared from independent hygromycin-resistant clones were subjected to Southern blot analyses using a KRC 3′ cDNA fragment radiolabeled by random priming as a hybridization probe. The hybridization patterns of three representative NIH/3T3-KRC clones are shown in Fig. 4A. Positive clones from the hygromycin-resistant colonies displayed an additional 2-kb EcoRI hybridization fragment, anticipated from integration of pKRC/N6 and readily distinguishable from the 3.5-kb EcoRI fragment derived from the endogenous KRC gene (Fig. 4A). Subsequently, Northern blot analysis revealed that the steady state of mts1 mRNA was ~3- to 5-fold higher in NIH/3T3-KRC cells than in NIH/3T3 cells (Fig. 4B). The lower panel shows the hybridization pattern of the filter to a glyceraldehyde 3-phosphate dehydrogenase (G3PDH) probe as a control (Fig. 4B). The increased level of mts1 transcripts in NIH/3T3-KRC cells suggests that KRC transactivates mts1.

To determine if KRC transactivates mts1 via its intronic enhancer, mts1 reporter constructs were transiently transfected into NIH/3T3 and NIH/3T3-KRC cells. Recently, we further delineated the gene regulatory elements of mts1 to be within a 135-bp fragment from nucleotides 782 to 916 of the first intron. This fragment contains several cis-acting regulatory elements starting with the Sb element at the 5′ end (Fig. 4C). Plasmid peLUC1 contains the mts1 regulatory DNA upstream of the c-fos promoter linked to the luciferase gene. When introduced into NIH/3T3 cells, the mts1 gene regulatory DNA in peLUC1 confers an enhancer activity ~5-fold higher than the parental vector pfLUC (Fig. 4C). The contribution of the Sb site to the expression of mts1 is further demonstrated by transfection experiments using peLUC2, in which the Sb site in peLUC1 is replaced by the mut2 sequence. In EMSA, mut2 DNA neither forms complexes with nuclear extracts of CSML100 cells (31) nor with the KRC fusion protein Mbp/Rc490 (Fig. 1A, lanes 7 and 8). In reporter gene assays, the mut2 mutation resulted in a decrease of the mts1 enhancer activity to 32–37% that of the wild type levels in CSML100 cells (31). Similarly, the introduction of the mut2 mutation into the mts1 enhancer also decreased the enhancer activity in NIH/3T3 cells. The reduction, however, is more subtle here as peLUC2 retained ~80% of the activity of peLUC1. The data suggest that KRC may be limiting in NIH/3T3 cells. To test this hypothesis, peLUC1 was introduced into NIH/3T3-KRC cells. The luciferase activity of peLUC1 was ~2.8-fold higher in NIH/3T3-KRC than in NIH/3T3 cells (Fig. 4C). This level of induction of the mts1 reporter gene is similar to that of the endogenous mts1 in NIH/3T3-KRC (Fig. 4B). On the other hand, less significant induction was observed when peLUC2 was introduced into NIH/3T3-KRC cells. Together, the data suggest that Sb contributes partially to the enhancer activity of the mts1 enhancer in NIH/3T3 cells and KRC transactivates mts1 via the Sb site.

**DISCUSSION**

Our analysis suggests KRC encodes a product that regulates transcription of mts1 in metastatic cells via the interaction with the Sb site, a κB-like motif, of the composite enhancer in the first intron of mts1. This Sb-binding protein, p200, was initially identified and characterized in the mts1-expressing adenocarcinoma cell line CSML100 (31). Using 32P-labeled Sb DNA as a ligand to screen a cDNA expression library prepared from CSML100 poly(A)+ RNA, we isolated a clone that corresponds to the carboxyl portion of a large zinc finger protein KRC, including a pair of C_{3}H_{2} zinc fingers. Previously, we showed that KRC fusion proteins containing this zinc finger pair bind specifically to the κB motif (35, 42). In this study, we show that KRC fusion proteins also interact specifically with Sb DNA. Additionally, we show that KRC positively regulates endogenous mts1 as well as reporter genes whose expression is under the control of the mts1 gene enhancer present in the first intron.

Recent analyses show that unlike most introns that are biologically inert, the first intron of mts1 is involved in important biological functions. The first intron regulates the expression of the host gene, and several positive and negative gene regulatory elements have been localized in this region. In addition to those protein binding sites, computer alignment (DI-ALIGN2 program) shows significant general conservation among the first intron sequences of the mts1 genes in human (49), mouse (33), and rat (5, 51) (Fig. 5), suggesting that other elements involved in transcription regulation or in DNA dynamics may exist. So far, three regulatory elements essential for the transcription regulation of murine mts1 have been described. One of the regulatory elements is a methylation-dependent AP1 binding site. Protein binding to the AP1 site is dependent on the resultant generation of a novel inhibitory AP1 binding site by DNA methylation in mouse (32). Of interest, this may explain the observation that methylation of the 5′ region of the mts1 gene correlated with the repression of mts1. DNA methylation has also been suggested to impose develop-
mental stage-specific regulation of mts1 in human and rat (52–54). However, this AP1 binding site is absent in the corresponding regions in the human and rat mts1 genes, despite generally good conservation of the sequence of the flanking regions (Fig. 5). Other elements are likely present in the human and rat mts1 gene for regulating gene expression by DNA methylation.

The other two regulatory elements, Sa and Sb, are located close together between nucleotides +788 to +841 (31). As shown in Fig. 5, the Sa/Sb sequence is conserved in human, mouse, and rat and is followed by the most conserved region in the sequence alignment (Fig. 5). A data base search of the gene sequence of mouse mts1 revealed that this region harbors a sequence of 51 nucleotides that completely matches a minisatellite sequence in rat (31). The Sa sequence is located at the 5’ end of the minisatellite sequence and is similar to the core sequence of hypervariable tandem-repeated minisatellites (31, 34). The Sa sequence also resembles the χ sequence of Escherichia coli, which is often associated with recombination hotspots and provides binding sites for recombination proteins. Minisatellite DNA stimulates homologous recombination in mammalian cells (55, 56).

The regulatory Sb element is 9 to 17 nucleotides upstream of Sa (Fig. 5). The Sb sequence is conserved, although there is a single nucleotide G to T transversion of the first nucleotide in human and a three-nucleotide insertion in rat compared with the composition of mouse Sb. The evolutionarily conservation of Sb suggests that Sb plays an important function. Sb is a target for KRC, which is a large protein of ~250 kDa. The proximity of Sa and Sb and the large size of KRC suggest that KRC may be in contact with Sa-binding proteins. Of interest, KRC was cloned by the interaction of its expressed products with the signal sequences of V(D)J recombination and, therefore, has been suggested to be involved in DNA rearrangement (35). It is provocative to suggest that the first intron of mts1 is not only involved in transcription regulation but also represents a dynamic genetic region of DNA recombination leading to deregulation of mts1 and metastasis. So far, no direct evidence to support DNA recombination mediated by the minisatellite DNA has occurred in the first intron of mts1, although an insertion of an intracisternal A-particle element ~600 bp upstream has been reported in a mouse myelomonocytic leukemia cell line (5).

The identification of Sb-binding proteins in vitro has led to the identification of transcription factors that regulate transcription of the mts1 gene. Mouse Sb interacts with three proteins, p50, p65, and p200, but human Sb only interacts with p200 (31). Here, we have shown that the binding of p200 to Sb is more stable when compared with the binding of NF-κBs (Fig. 1B). Previously, we have shown that specific mutations introduced into the mouse Sb sequences that abolish NF-κB binding have no significant effect on the mts1 enhancer activity (31). Furthermore, overexpression of p50 inhibits the mts1 enhancer in transient transfection experiments.3 Together, the data suggest KRC and NF-κBs may play distinct roles in regulating the expression of mts1, although both proteins can bind to the Sb site.

KRC belongs to a emerging family of proteins that bind to κB-related motifs. The κB binding specificity and the large sizes of these proteins led to our previous speculation that a family member may encode p200 species (31). The efficacy of

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3 M. A. Cohn and E. M. Tulchinsky, unpublished observations.
FIG. 5. Conserved DNA elements in the 5' end of the human, mouse, and rat mts1 genes. Sequence alignment was performed with the computer program DIALIGN release 2.1 (39, 40). Only uppercase letters are considered to be aligned. The numbers underneath the alignment reflect degrees of similarity, with 10 being the highest. Exon sequences are shown in bold type, splice acceptor or splice donor dinucleotides are shown in italic type, TATA boxes in the mts1 promoters and the gene regulatory elements reported are shown in boxes, and the E-box immediately upstream of Sb is underlined. Exon 1b involved in alternative splicing was reported in mts1 transcripts of human only (6, 7). Sequences were retrieved from GenBank under the following accession numbers: human, Z33457; mouse, X16094; and rat, U94663 and X06916. The rat sequence was compiled from two sequences: U94663 for the 5'9 sequence and X06916 for the 3'9 sequence. Because the two rat sequences did not overlap, a gap of ~96 nucleotides in the rat sequence was left blank in the alignment.
the KRC antibodies in gel supershift assay as well as the ability of a KRC expression construct to activate endogenous mts1 and mts1 reporter genes support KRC as the gene encoding p200. The identification of KRC as a transcriptional regulator of mts1 allows the investigation of how changes in the expression of transcription factors may promote the expression of mts1 in metastatic cancer cells.

Here we present results showing that the expression of endogenous mts1 and mts1 reporter genes were induced in NIH/3T3 cells long term-transfected with a KRC expression vector pKRC/N6. Transfection experiments were also performed in CSML100 cells. However, no significant differences in expression were observed when mts1 reporter genes were co-transfected with pKRC/N6 in transient transfection experiments (data not shown). The inability of the pKRC/N6 to affect the expression of mts1 reporters may be due to surplus levels of endogenous KRC in CSML100 cells or inefficient expression of pKRC/N6 in transient transfection. Experiments are under way to study the regulation of mts1 in CSML100 cells harboring an antisense construct for KRC transcripts.

The KRC gene has more than seven exons with a full-size transcript of ~9.5 kb encoding 2282 amino acids (36). Multiple KRC transcripts can be generated by alternative RNA splicing events, which result in smaller protein isoforms (44). In addition to the largest gene product, smaller KRC isoforms of 2129 amino acids, 1702 amino acids, and 651 amino acids may be produced (44). Potentially, those KRC proteins of 2282, 2129, and 1702 residues are large, contain one or more Sb-DNA binding structures, and therefore, are candidates for p200. At this stage, we have not determined which KRC isoform(s) interact with the mts1 enhancer and how these KRC isoforms orchestrate the transcription of mts1 in CSML100 cells. Currently studies are under way to characterize the KRC transcripts and proteins in CSML100 and NIH/3T3 cells.

Members of the KRC family of genes have previously been shown to activate transcription of target genes. A representative member Mbp1 activated the transcription of the human immunodeficiency virus enhancer element, which contains two κB motifs (57). The mouse counterpart, αα-CRYBP1, has also been implicated in the activation of transcription because the introduction of an αα-CRYBP1 antisense construct has a significant inhibitory effect on the activity of a reporter gene under the control of the αα-CRYBP1 binding site (58). Recently, another family member, MIBP1, has been shown to mediate the expression of the somatostatin receptor type II gene in the brain (50). The results presented here provide the first evidence that KRC is a transcription activator and defines mts1 as one of the target genes of KRC.

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