Expression of the Human Myotonic Dystrophy Kinase-related Cdc42-binding Kinase γ Is Regulated by Promoter DNA Methylation and Sp1 Binding

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Myotonic dystrophy kinase-related Cdc42 binding kinases (MRCKs) are family members most related to the myotonic dystrophy kinase (DMPK), RhoA-binding kinase (ROK), and citron kinase. Two highly conserved members, MRCKα and -β, have been previously identified and characterized. We now describe a novel isoform, MRCKγ, which is functionally and structurally related to members of this kinase family. We show these kinases to have marked similarities in their genomic organization, substrate phosphorylation, and catalytic autoinhibition. Unlike MRCKα and -β, which are expressed ubiquitously, MRCKγ mRNA was only expressed in heart and skeletal muscle. In cultured cells, MRCKγ showed differential expression with high levels of expression only in certain cell lines. DNA analysis showed that lack of expression is correlated with promoter DNA methylation. We have mapped the methylation sites in the MRCKγ promoter. Significantly, agents that suppressed DNA methylation caused increases in the expression of the kinase in low-expressing cells, further supporting the notion that promoter DNA methylation plays an important role in the expression of MRCKγ. Analysis of the MRCKγ promoter has also revealed two proximal Sp1 sites that are essential for transcriptional activity. We conclude that both promoter DNA methylation and Sp1 binding are important regulators for MRCKγ expression.

The Rho GTPases regulate cell morphology, cell growth, and cell polarity (1, 2). Numerous downstream effectors have been reported in the last decade; they comprise both kinases and non-kinases. Rho kinase ROK interacts specifically with the GTP-bound form of RhoA and organize actin bundling in cultured cells, whereas p21-activated kinases (PAKs) can phosphorylate and activate LIM kinases, which phosphorylate and inactivate myosin-targeting subunits of myosin phosphatase (9–11). MRCKs, resembling ROKs, can phosphorylate and activate LIM kinases, which phosphorylate and inactivate coflin, thereby facilitating actin polymerization events (12). At the cellular level, MRCK has been shown to be involved in regulating cell morphology by enhancing Cdc42-induced membrane extensions (8), and the dominant negative form of MRCKα can block nerve growth factor-induced neurite outgrowth in PC12 cells (13). The Drosophila autologue Genghis Khan (GEK) has also been implicated in actin polymerization events during development (14).

Two genes encoding different isoforms of MRCK have been reported in mammals (8). Both isoforms can exist as tetrameric forms through intermolecular interaction of their extended coiled-coil domains (15). A region in the distal coiled-coil region (CC2/3) was found to be essential for kinase inhibition, but the exact location has not been mapped previously. Activation of the kinase was observed upon binding of phorbol ester to the neighboring cysteine-rich domain, presumably by releasing the constraint of the inhibitory effect on the catalytic activity (15). Similar oligomeric structures with distinctive features have also been reported for the related Rho kinases ROKs (16, 17) and the myotonic dystrophy kinase (DMPK) (18). Both MRCKα and MRCKβ are ubiquitously expressed in various mammalian tissues and are abundantly expressed in all cell lines studied (18, 19). Interestingly, MRCKα is present as multiple species through differential splicing (20), mainly at an internal variable splice site, which is located between the inhibitory region and the phorbol-binding cysteine-rich domain. Whether or not...
these sequence diversities play any roles in MRCK function remains to be determined.

The characterization of yet another member, MRCKγ, has not been reported. We now describe the biochemical and functional characterization of MRCKγ. The expression of this kinase showed marked variation in some cell lines, and this is dependent on the methylation status of its promoter DNA as well as on Sp1 binding.

MATERIALS AND METHODS

Cell Culture, Transfection, and Cell Staining—HeLa, Hct116, and MRCK cells were cultured in minimum Eagle’s medium, MCF7 and MKN28 cells were grown in RPMI medium, and COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium. All media were supplemented with 10% fetal bovine serum, and cell cultures were maintained in humidified 5% CO2. Subconfluent plates cultured on culture dishes for 24 h were transfected with respective DNA constructs using LipoFectAMINE (Invitrogen) according to the recommended protocol. For immunostaining experiments, transfected cells were fixed with 4% paraformaldehyde and stained with anti-FLAG antibody (M2; Sigma).

Construction of Expression Vectors—A 997-bp DNA fragment covering the CpG islands and the putative Sp1 binding sites of the putative immunostaining experiments, transfected cells were fixed with 4% paraformaldehyde and stained with anti-FLAG antibody (M2; Sigma).

Expression of Constructs—Deletion constructs, forward primers (5’-GCT CCT ACT GAA GAC TAA AAC G-3’ and 5’-GGG GTA CCA TCT GCC AAA TGG GGG GCC GGC TCC CAT TGG CCG-3’; and 5’-CAT and pGEX-MRCK γKIM were obtained from a pGEX-vector containing the PCR product amplified from the full-length MRCK γKIM cDNA into their respective vectors digested with BamHI/SmaI. GST

Preparation of Whole Cell Extracts and Electromobility Shift Assays (EMSA)—Preparation of whole cell extracts from HeLa, MKN28, and COS-7 transfected cells was performed according to Manley et al. (22), and EMSA was performed essentially as described previously (23). The oligonucleotide used for EMSA was the consensus Sp1 binding site (underlined) in MRCKγ promoter (5’-CCG GGC GCG CCA CTG CTA AAG CAG AGG GA A-3’) as described previously. (8, 15).

Immunoprecipitation and Kinase Assays—Cos-7 cells expressing HA-MRCKγ-CAT or HA-MRCKγCAT alone or co-expressed with GST-MRCKγ-KIM were lysed in lysis buffer containing 25 mM HEPES, pH 7.3, 150 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 20 mM β-mercaptoethanol, 1 μM sodium vanadate, 5% glycerol, 0.5% Triton X-100, and 1× complete protease inhibitor mixture (Roche Applied Science), and immunoprecipitations were performed essentially as described (15) using either glutathione-Sepharose (Amersham Biosciences) or anti-HA antibody (12CA5; Roche Applied Science). Kinase assays with 10 μg of substrates (histone H1, myelin basic protein, GST fusion of myosin light chain-2 (MLC-2), or GST fusion of the phosphorylation inhibitory motif of myosin phosphatase MRCKγ) were carried out as described previously (15). The reactions were stopped by the addition of sample buffer, and the proteins were resolved by 11% SDS-PAGE, dried, and autoradiographed.

Western Blot Analysis—Protein expression was detected by Western immunoblotting. Cells were harvested in lysis buffer, and 100 μg of proteins were separated by 7.5% SDS-PAGE. MRCKα, -β, and -γ proteins were detected by specific antibodies raised against MRCKα, -β, and -γ, respectively.

In Vitro GTPase Binding Assay—GTP fusion proteins (50 ng) of MRCKα and MRCKγ containing the CRIK domain were resolved by SDS-PAGE and transblotted onto a polyvinylidene difluoride membrane. Immobilized proteins were then reconstituted for 3–4 h at 4°C in reconstituted buffer (phosphatase-buffered saline containing 1% bovine serum albumin, 0.1% Triton X-100, 0.5 mM MgCl2 and 5 mM dithio- 

Determination of Transcriptional Initiation Site—RT-PCR was performed using Hct116 poly(A)RNA to determine the transcriptional initiation site. Several primers close to the putative initiation site of MRCKγ were designed (including primer 1, 5’-GGA GCC GAC GGG GAGG-3’; primer 2, CCA GAT GAG GCG GCG CTG-3’; primer 3, 5’-CCC GCG CCA GAT GAC GGG GGC-3’; and primer 4, 5’-GCC CAT TC CGT CGG CCG-3’). These were synthesized to pair up with the reverse primer 5’-CAT CGT CAT CCG CGG-3’.

Luciferase Reporter Assay—Hct116 cells were transfected with various MRCKγ luciferase reporter gene constructs, and cells were harvested for luciferase assays using the single luciferase assay system (Promega). pXJ40-HA-Sp1 was also co-transfected with pGL3-2 to detect the effect of Sp1. 500 ng of the promoter reporter construct was added to 100 ng of the promoter–β-galactosidase reporter (pCMV-β-galactosidase) construct was also included to monitor transfection efficiency.

Preparation of Genomic DNA and Methylation and Sp1 Binding

Preparation of Genomic DNA and MrcbC Digestion—The genomic DNA from various cell lines was prepared using a DNeasy® tissue kit (Qiagen) according to the manufacturer’s instructions. Genomic DNA were cleaved with MrcbC (New England Biolabs) in a final reaction volume of 25 μl at 37°C for 1 h. The heat-inactivated cleavage mixture (2.5 μl) was used for PCR using primers 5’-GCT TGA ACG CAC TAA TAT GTC-3’ and 5’-CTG CTC TGG CAC TGT CTA TGG-3’.
FIG. 1. Genomic and sequence organization of human MRCK. A, DNA and deduced amino acid sequences of human MRCK. The bolded letters represent the exon-intron boundaries. The amino acid sequence is represented in order from the N terminus to the C terminus, and the kinase domain, kinase inhibitory motif, cysteine-rich domain, pleckstrin homology domain, Citron homology domain, and Cdc42/Rac-interactive binding motif are highlighted. B, genomic structure of the human MRCK gene. In the top panel, the exons are boxed and numbered, and introns known to be above 1 kb are shown. Lower panel, protein domain arrangement of MRCK organized from deduced amino acid sequence. Corresponding exons and amino acid sequences in the subdomains are linked by dotted lines. Subdomain arrangements for MRCK/H9251 and -/H9252 were also shown for comparison. N, N-terminal region; C, CRIB motif; CC, coiled-coil domain; V, internal variable region; CR, cysteine-rich C1 domain; PH, pleckstrin homology domain; CNH, Citron homology domain.
Regulation of MRCK\(\gamma\) by DNA Methylation and Sp1 Binding

RESULTS

MRCK\(\gamma\) Is a Novel Member of the MRCK Serine/Thrreonine Kinase Family—We have previously reported two members, MRCK\(\alpha\) and MRCK\(\beta\), in this class of Rho GTPase-binding serine/threonine kinases. Sequences of a related kinase have been reported in the databases (e.g. accession number XM_290516), but hitherto, no reports of its biochemical and functional characterization have been reported. We have now isolated and characterized the human cDNA of this novel kinase. The sequence codes for a new member of the MRCK family, which we have termed MRCK\(\gamma\) (Fig. 1A). The organization of domains in MRCK\(\gamma\) is similar to the other MRCKs, and comparisons with MRCK\(\alpha\) revealed the following sequence identity (in percent) in the various domains: kinase (73%), cysteine-rich (54%), pleckstrin homology (47%), Citron homology (42%), and CRIB (53%). The coiled-coil domain in MRCK\(\gamma\) is much shorter (mainly in the proximal coiled-coil 1 (CC1) region) and more diverse (29%). Within the coiled-coil domain, there is a highly conserved motif (66% identical to MRCK\(\alpha\)), termed the KIM because of its unique property of interacting with, and inhibiting activity of, the kinase (Figs. 1, A and B, and 2A) (see also Ref. 15). MRCK\(\gamma\) has a molecular mass of about 160 kDa, when compared with 180 kDa for the other known family members, the main difference being due to the shorter CC1 domain. Phylogenetic analysis of the MRCK family showing its relationship to the mammalian and invertebrate counterparts is presented in Fig. 1C. The gene is located at chromosome 11q13. Analysis of the genomic sequence reveals the coding sequence to reside within 36 exons (Fig. 1, A and B) with an organization that is markedly similar to that of MRCK\(\alpha\) (19) and MRCK\(\beta\) (Ref. 20 and data not shown). However, the overall size of the gene is only about 30 kb, which is far more compact than the 250 kb reported for MRCK\(\alpha\) and the 130 kb reported for MRCK\(\beta\).

Biochemical Characterization, Expression, and Cellular Localization of MRCK\(\gamma\)—The marked homology of the kinase domain of MRCK\(\gamma\) with those of MRCK\(\alpha\)/\(\beta\) and Rho kinases ROK inferred similar substrate specificity. A GST kinase domain fusion protein of MRCK\(\gamma\) was tested for this. Like MRCK\(\alpha\), MRCK\(\gamma\) kinase phosphorylated the known substrates, MLCK-2 and a fragment from the myosin binding subunit of myosin phosphatase MB585 (GTP; Ref. 2A) but not histone H1 nor myelin basic protein. The conserved KIM motif of MRCK\(\gamma\) (amino acid residues 677–765 in CC2/3) also bound the kinase domain of MRCK\(\alpha\) and MRCK\(\beta\) and inhibited their catalytic activity (Fig. 2A). These results are consistent with MRCK\(\gamma\) sharing similar substrate specificity and conserved auto-inhibitory mechanism with the other MRCK kinases.

The CRIB domain responsible for Cdc42/Rac1 interaction in MRCK\(\gamma\) is far less conserved when compared with other MRCK counterparts (Fig. 2B). We therefore tested the binding of GST-MRCK\(\gamma\)-CRIB to RhoA, Rac1, Cdc42 as well as the related TC10 GTPase. Although this CRIB domain can bind Cdc42, it binds more strongly to TC10 (Fig. 2B). It binds Rac1 weakly but not RhoA (data not shown). It seems that MRCK\(\gamma\) differs from the other MRCK isoforms in that it may interact preferentially with Rho GTPases other than Cdc42 and Rac1. Apart from TC10, the identity of any other GTPase binding and their physiological roles remain to be determined.

Northern blot analysis of MRCK\(\gamma\) revealed that a 6-kb message was expressed in human heart and skeletal muscle (Fig. 2C). By immunological analysis, the protein was found to be also highly expressed in a number of cell lines, including MKN28 cells (Fig. 3A). RT-PCR of MKN28 cells showed a differential splicing event at the internal variable region within exons 21–23 (Fig. 1B), with the major product containing exons 21/22/23 and minor products containing alternative exons 21/22a/23 or 22/23 alone (Fig. 2D). Extensive splicing events in this region have been documented for MRCK\(\alpha\) (20), and MRCK\(\gamma\) has adopted a similar but simpler processing. However, other splicing events occurring at the CRIB domain and C-terminal of MRCK\(\alpha\) were not observed with MRCK\(\gamma\). When expressed in HeLa cells, FLAG-tagged MRCK\(\gamma\) was mainly cytoplasmic with a higher density at the leading edges (Fig. 2E).

Distinctive Expression Pattern of MRCK\(\gamma\) in Various Cell Lines Determined by Promoter Activity—We used specific antibodies to the different isoforms of MRCKs to evaluate their relative expression in the soluble fractions derived from a variety of human and mammalian cell lines. Although the protein expression of MRCK\(\alpha\) and MRCK\(\beta\) was ubiquitous, that of MRCK\(\gamma\) was more restricted. High expression was detected in MKN28, HCT116, and MCF7 cells but not in HeLa, MRC5, and COS-7 cells in the panel of mammalian cells tested (Fig. 3A). RT-PCR analysis of MRCK\(\gamma\) mRNA expression (Fig. 3B) showed this to be correlated with protein expression, high and poor transcription being responsible for the high and very low protein expression of MRCK\(\gamma\) in MKN28 and HeLa cells, respectively. We then investigated whether promoter DNA methylation, a key regulator in transcriptional control, contributed to the variation in transcription observed in these cells. Genomic DNA isolated from the low-expressing HeLa and MRCK5 cells (but not from high expressers MKN28 and Hct116 cells) was extremely sensitive to restriction enzyme cleavage by the methylation-requiring nuclease MbrBC at the putative promoter region of MRCK\(\gamma\) and resulted in failure to obtain an
intact PCR product within this region, indicating hypermethylation in the presumed promoter of these cells (Fig. 3C) (see also Refs. 24 and 25). From the pattern of expression and the characteristics of the putative promoter, we conclude that MRCKγ expression is regulated by transcription and is likely to be under the control of promoter DNA methylation.

Regulation of Expression of MRCKγ by DNA Methylation—We first attempted to determine the transcriptional initiation site for MRCKγ. The use of either conventional primer extension assay of mRNA from MNK28 cells or PCR from human cap-site cDNA libraries failed to produce recognizable products. This failure to detect the start site may possibly stem from low mRNA expression in the available human tissues or as a result of the very GC-rich region around this site (Fig. 4B). We therefore used PCR primers to tentatively identify this site. As the primer 5'-CCA GGT GAG GGC CCG CTG-3' (Fig. 4A, primer 2) gives more intense PCR product than 5'-CGG CGC CCA GGT GAG GGC-3' (primer 3 with common sequence underlined) from cDNA derived from poly(A)−RNA of MNK28 cells, we infer that the former primer encompasses the start site. This site is located 171 bp upstream of the ATG translation start codon (Fig. 4B).

Fig. 2. Biochemical characterization and cellular localization of MRCKγ. A, a, substrate specificity of MRCKα-CAT and MRCKγ-CAT. Kinase assays with histone H1 (H1), myelin basic protein (MBP), GST myosin light chain (GST-MLC-2), and GST-PIM were used as substrates, which were marked with asterisks. b, the inhibitory effects of MRCKγ-KIM on the catalytic activities of MRCKα and MRCKγ. HA-MRCKα-CAT and HA-MRCKγ-CAT constructs were expressed alone (lanes 1 and 3) or co-expressed with GST-MRCKα-KIM construct (lanes 2 and 4). Immunoprecipitations were carried out using anti-HA antibody (lanes 1 and 3) or glutathione-Sepharose beads (lanes 2 and 4), and the immunoprecipitates recovered were assayed for kinase activity using GST-MLC-2 as substrate. Blotted filters were also immunostained with anti-HA and anti-GST antibodies after autoradiography. c, KIM motif alignment. KIM motifs of various MRCKs from human and Drosophila were aligned with the Clustal method (DNASTAR). Conserved residues are boxed in black, and the numbers indicate the positions of residues. B, sequence alignment of the CRIB sequences of human hMRCKα, hMRCKβ, and hMRCKγ, Fugu fMRCKα, Drosophila GEK, and Caenorhabditis elegans ceMRCK was performed as described in the legend for Fig. 1. The bottom panels show the in vitro GTPase binding assay of MRCKα and MRCKγ. Purified GST fusion proteins containing the CRIB domain of MRCKα and MRCKγ immobilized on polyvinylidene difluoride filters were assayed for binding with [γ-32P]GTP-labeled Cdc42 (left panel) or [γ-32P]GTP-labeled TC10 (right panel). C, Northern blot analysis of MRCKγ. The mRNA blot was purchased from Clontech and probed with a 3-kb DNA fragment from the 3′ end of human MRCKγ. S, Muscle, smooth muscle. D, analysis of the internal splice site in MRCKγ. A primer pair specific for covering the internal splice site in the human sequence was used to amplify total cDNA prepared from MNK28 cells. For a control, a primer pair specific for sequence in the C-terminal region was used to amplify the same cDNA. The marker lane (Mr) is shown in bp. The numbered boxes represent exons present in the corresponding bands as revealed by DNA sequencing. The bottom panel represents the deduced amino acid sequence of exons involved in alternate splicing at the internal splice site. E, HeLa cells were stained with anti-FLAG antibody for detecting the expressed MRCKγ. The arrows show MRCKγ localized mainly at the cytoplasm, with higher density at the leading edges.
inhibit DNA methylation, a concomitant increase in MRCK significantly, when HeLa cells were treated with agents that specific antibodies against MRCK—Essential for Transcriptional Activity of MRCK/ H9253 antibodies against the CRIB domain of human MRCK/ H9253—were also amplified using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene primers as control. (Left panel shows the undiluted RNA sample, whereas the right panel shows the 10\(^{-6}\) diluted RNA sample. C, genomic DNA from various human cell lines was incubated in the presence or absence of nuclease McrBC, and a PCR reaction of the uncleaved and cleaved DNA was performed with specific primers spanning the promoter region and the putative CpG islands.

at position \(-45\). Multiple GC-rich Sp1 binding sites were found in the 1-kb region of the promoter; in particular, a tandem sequence GCCCGCCCGCCG with consensus to Sp1/EGR1 was used to locate important regions for gene transcription. Deletion of \(5'386\) bp (from \(-844\) to \(-357\); Fig. 5A, pGL3-1 to pGL3-4) caused a 20–55% decrease in transcriptional activity, suggesting a putative positive regulatory site(s) within this region. However, no matches to any known transcription factor binding sites are detectable using the BIMAS Proscan program. Further deletion from \(-357\) to \(-60\) (Fig. 5A, pGL3-5) resulted in almost complete loss of transcriptional activity. A number of Sp1 binding sites, especially a tandem sequence with Sp1/EGR1 consensus, were present between the CpG island and CAAT box, suggesting that these may be the important positive regulators for the MRCK\(y\) promoter. Furthermore, truncation of the GC-rich 3' end sequence (Fig. 5A, pGL3-Δ3') resulted in significant increases in transcription activity, indicating that this GC-rich region may form secondary structures that negatively regulate transcription. However, deletion mutants of the putative inverted CAAT box (Fig. 5A, pGL3-ΔBssHIII and pGL3-3A/BssHIII) did not show any significant effects, and the identity and importance of this CAAT sequence remains to be investigated.

To determine whether the putative Sp1 binding sites are capable of interacting with their cognate binding partner, a 205-bp fragment (nucleotides \(-264\) to \(-60\)) encompassing the multiple Sp1 binding sites was analyzed by EMSA. This revealed a pattern of major DNA-binding proteins resembling that obtained with the control lysate from COS-7 cells overexpressing Sp1 (Fig. 5B). To confirm the identity of the binding proteins, a specific oligonucleotide corresponding to one of the tandem Sp1-binding repeat sequences was used for the gel shift assay. Again, major binding resembling that of control Sp1 lysate was detectable in both MKN28 and HeLa cell lysates (Fig. 5B). In addition, both major binding proteins corresponding to Sp1 could be supershifted in the presence of Sp1 antibody; this is a further confirmation that Sp1 is the major DNA-binding protein to this region of the promoter. Moreover, overexpressing Sp1 further increased the promoter activity of MRCK\(y\) (Fig. 5C), strongly supporting the notion that this transcription factor has a positive regulatory role for the expression of MRCK\(y\).

**DISCUSSION**

Here we describe a novel human kinase, MRCK\(y\) kinase, that shows a marked similarity to the related MRCK\(z\) and MRCK\(β\). This includes the number and the arrangement of the functional domains, the genomic structure, and splicing events. Internal splice sites in the variable region of MRCK are also conserved in MRCK\(y\), but the splicing events in MRCK\(y\) are far less extensive and generate fewer splice products. The MRCK\(y\) gene, however, does not contain tandem CRIB domains as reported for MRCK\(α\). The kinase domain of MRCK\(y\) shares 73% identity with that of MRCK\(z\) and MRCK\(β\) and has similar substrate specificity. All members of the MRCK family, resembling ROK, are capable of phosphorylating both MLC-2 and myosin phosphatase regulatory subunit (10–11), unlike Citron kinase, which acts on MLC-2 but not myosin phosphatase (26). Furthermore, the coiled-coil domains of MRCKs that resemble ROK, are capable of phosphorylating both MLC-2 and myosin phosphatase regulatory subunit (10–11), unlike Citron kinase, which acts on MLC-2 but not myosin phosphatase (26). Furthermore, the coiled-coil domains of MRCKs that are less conserved (29%) contain within their CC2/3 regions a highly homologous sequence that is known to exhibit kinase auto-inhibition (15). We show this conserved sequence in MRCK\(y\), the KIM, to be able to directly interact with and inhibit the MRCK kinase domain. It appears that MRCK\(y\) and the other MRCK members utilize a similar mechanism in regulating its catalytic activity.

The CRIB motif was originally characterized as a Cdc42/Rac-interactive motif (6, 7). The CRIB motif of ACK, WASP and, to
a certain extent, MRCKα and MRCKβ has been shown to interact specifically with Cdc42. The CRIB motif of MRCKγ binds only weakly to Cdc42 and even more weakly to Rac1, suggesting that it may interact specifically with some other Rho GTPases. Indeed, we have demonstrated here that it has greater binding to TC10, another highly related Rho GTPase. The biological significance of this interaction and the possible interaction with some other yet unidentified GTPases require further investigations.

As deduced from phylogenetic analysis of the CRIB domain from various MRCKs (27), MRCKγ has been suggested to be the older of the mammalian MRCK kinases. MRCKγ shows an overall divergence from the other members, with marked differences in the coiled-coil domain as well as moderate differences in other regions. This divergence and its more compact genomic makeup lend support to the hypothesis that MRCKγ has an earlier evolutionary history than its mammalian counterparts.

MRCKγ expression is restricted, being detectable in heart...
and skeletal muscle but not other human tissues tested, unlike the other MRCKs, the expression of which is ubiquitous. The expression pattern of MRCK\(_{1}/\text{H}9253\) in the various cultured cells is also peculiar. Whereas high expression was found in some cells such as MKN28, MCF7, and HCT116 cells, only very low expression was detectable in other cells such as HeLa, MRC-5, and COS-7 cells. Most significantly, this pattern of expression can be correlated with the sensitivity of the cellular genomic DNA to cleavage by the methylation-sensitive nuclease McrBC in the putative promoter region of MRCK\(_{1}/\text{H}9253\). This is consistent with promoter DNA methylation playing a role in the regulation of MRCK\(_{1}/\text{H}9253\) expression. This notion finds further support from the subsequent mapping and identification of a CpG island in the MRCK\(_{1}/\text{H}9253\) promoter. Moreover, MRCK\(_{1}/\text{H}9253\) expression was increased in HeLa cells when these were treated with agents that interfere with DNA methylation. The 5'-untranslated region is extremely GC-rich (89%) and can also impose a negative regulation as its deletion resulted in significant increases in promoter activity. This unique feature of being extremely GC-rich may also explain the failure to identify the transcriptional initiation site by conventional methods such as primer extension as it is possible that the secondary structure formed in this region may perturb the extension reaction, which was optimally carried out at lower temperatures.

Analysis of the MRCK\(_{1}/\text{H}9253\) promoter has also revealed the importance of the multiple Sp1 binding sites. Sp1 is a general transcriptional activator in many gene promoters, although it has also been amply documented to be involved in gene repression through its expression, protein modifications, and interaction with other transcriptional components (28–30). Here we report a positive regulatory role of Sp1 binding in the activation of the MRCK\(_{1}/\text{H}9253\) promoter. Interestingly, these multiple Sp1 sites are in close proximity to the CpG island, and it is possible that the Sp1 binding may well be influenced by the neighboring methylation event. Such a prospect has been documented for the human Leukosialin gene, in which methylation of the pro-
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MRCK-related kinases play a role in filopodia formation and motility (8), and the Drosophila GEK counterpart has been shown to participate in actin polymerization events in development (9). Like other mammalian MRCKs, MRCKy is a cytosolic kinase enriched in the leading edges of cultured cells. However, its expression is restricted, being detectable in heart and skeletal muscle but not other human tissues. Our studies on its promoter have shown transcription of MRCKy to be subject to both negative and positive regulation. Given the role of the MRCK family in actin-myosin events underlying crucial cellular events and its presence only in muscle-containing tissues, MRCKy certainly merits further investigations, particularly in relation to the other mammalian MRCKs. It may well be that deliberate changes in the transcription activity of MRCKy at some stage play a role in signaling for dynamic cellular events such as cell polarity and cell migration (1, 2).

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J. Biol. Chem. 2004, 279:34156-34164.
doi: 10.1074/jbc.M405252200 originally published online June 11, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M405252200

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