Microsphere Protein 1, Mi-2β, and RET Finger Protein Associate in the Nucleolus and Up-regulate Ribosomal Gene Transcription*

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The nucleolus is the site of ribosomal DNA (rDNA) transcription and ribosome production. In exploring the role of nucleolar protein MCRS1 (microsphere protein1)/MSP58 (58-kDa microsphere protein), we found that Mi-2β, a component of a nucleosome remodeling and deacetylase (NuRD) complex, RET finger protein (RFP), and upstream binding factor (UBF) were associated with MCRS1. Yeast two-hybrid assays revealed that MCRS1 bound to the RFP, and upstream binding factor (UBF) were associated with remodeling and deacetylase (NuRD) complex, RET finger protein (RFP), and ribosome production. In exploring the role of nucleolar protein MCRS1, Mi-2β, and RFP up-regulated transcription factor (RFP), and the rRNA transcription factor UBF in the nucleoli. We also found that MCRS1, Mi-2β, and RFP were associated with rDNA using a chromatin immunoprecipitation assay. Finally, we showed that MCRS1, Mi-2β, and RFP up-regulated transcriptional activity of the rDNA promoter and that ribosomal RNA transcription was repressed when MCRS1, Mi-2β, and RFP expression was reduced using siRNA. These results indicated that Mi-2β and RFP, known to be involved in transcriptional repression in the nucleus, co-localize with MCRS1 in the nucleolus and appear to activate the rRNA transcription.

The nucleolus is a subnuclear compartment in eukaryotic cells responsible for rRNA synthesis and ribosome biogenesis. In actively growing animal and plant cells, rRNA synthesis accounts for about 50–80% of total cellular RNA production (1). Human diploid cells contain about 400 ribosomal genes organized as tandem repeats at nucleolus organizer regions encoded on chromosomes 13, 14, 15, 21, and 22 (1, 2). rRNA gene transcription varies according to the demand for ribosome production and protein synthesis, and in yeast and murine cells, only 30–50% of the rRNA genes are in an open structure that facilitates active transcription (3, 4). Transcription of rRNA is extensively regulated by a large number of proteins such as growth factors, CBP (cAMP-response element-binding protein (CREB)-binding protein), Rb, p53, and Myc (5–10). In addition, growing evidence indicates roles for different histone modification and chromatin remodeling complexes in the coordinated regulation of rRNA gene transcription. Nucleolar remodeling complex (NoRC) is a well recognized chromatin remodeling complex involved in rRNA gene silencing and is known to cooperate with DNA methyltransferases, histone deacetylases, histone methytransferases, and TTF-I (transcription terminator factor) (11–14). In contrast, ribosomal DNA transactivation is associated with Tip60 histone acetyltransferase and histone acetylation (15, 16). However, the chromatin remodeling proteins that facilitate the establishment of the open state in rRNA genes remain to be characterized.

Mi-2 is a nuclear protein with chromatin remodeling activity and is the largest component of the nucleosome remodeling and deacetylase (NuRD) complex (17–20). The transcriptional repressive activity of Mi-2 is mediated by NuRD complex components, such as methyl CpG-binding protein MBD 2/3, histone deacetylases HDAC1/2, metastasis-associated protein MTA2/3, and RbAp46/48. NuRD complex and Mi-2 have been associated with the transcriptional repressive activities of KAP-1, RET finger protein (RFP), Trk69 (Tram-track), and RORγ (21–24). In addition, MTA-3, another component of the NuRD complex, was reported to repress Snail expression, and the estrogen-dependent transcriptional control of MTA3, Snail, and E-cadherin was associated with breast cancer progression (25). However, Mi-2 also plays roles in transactivation outside the NuRD complex. A transactivating role of dMi-2 was suggested in studies of Drosophila salivary glands that showed the localization of dMi-2 to chromatin puffs (26). We recently reported that Mi-2β possessed both transactivating and repressing subdomains (24). Furthermore, the study, based on a conditional Mi-2β knock-out mouse line, clearly showed that Mi-2β activated CD4 transcription in T cells by forming a complex with p300 histone acetyltransferase and the E box-binding protein HEB (27). Thus, Mi-2β appears to be involved in both transcriptional repression and activation through the formation of distinct protein complexes in the nucleus.

The RFP was originally identified as a fusion protein with RET-tyrosine kinase (28). RFP mRNA is strongly expressed in a variety of human and rodent tumor cell lines. In addition, its protein expression is detected strongly in male germ cells and relatively weak in peripheral and central neurons, hepatocytes, and adrenal chromaffin cells (28, 29). RFP contains a tripartite motif consisting of a RING finger, a B-box zinc finger, a coiled-coil domain that is involved in protein–protein associations, and an RFP domain. RFP is a nuclear protein and was reported to partially co-localize with promyelocytic leukemia protein and int-6 in the nucleus (30, 31). We found that RFP exhibited transcriptional with DNA methyltransferases, histone deacetylases, histone methyltransferases, and TTF-I (transcription terminator factor) (11–14).
repressive activity and that it was associated and co-localized with Enhancer of Polycomb 1 and Mi-2β in the nucleus (24, 32). We also showed that the subnuclear distribution of RFP was altered by small ubiquitin-like modifier modification by protein inhibitor of activated STAT (signal transducers and activators of transcription) (PIAS) proteins (33).

MCRS1 (microsphere protein 1)/MSP58 (58-kDa microsphere protein) and the related protein p78 were originally identified as proteins that interacted with nucleolar protein p120 and herpes simplex virus 1-infected cell protein 22 (ICP22) (34, 35). MCRS1 is a nucleolar protein that contains a bipartite nucleolar localization motif, a nucleolar localization motif, a coiled-coil domain, and a forkhead associated domain. Several functions for MCRS1 and its splice variant MCRS2 have been reported, including a transforming activity, nucleolar sequestration activity, and telomerase inhibition (36–39). MCRS1 and TOJ3, a protein with high structural similarity to MCRS1, exhibit transforming activity, whereas phosphatase and tensin homologue (PTEN) suppresses the transforming activity of MCRS1 (36, 38). Although these findings suggest that MCRS1 may have several distinct functions, its transcriptional role in addition, binding and stabilization of MSP58 by transcription factor STRA13 has been observed (40). Although these findings suggest that MCRS1 may have several distinct functions, its transcriptional role in the nucleolus has not yet been fully characterized.

In this report we investigated the role of MCRS1 in ribosomal gene transcription and its association and co-localization with Mi-2β and RFP in the nucleolus. Our results indicated that Mi-2β, RFP, and nucleolar protein MCRS1 were involved in ribosomal gene transcription and suggested that Mi-2 may be a candidate for the unidentified chromatin remodeling protein that establishes the euchromatin structure of ribosomal genes.

MATERIALS AND METHODS

Plasmids—MCRS1/MSP58 cDNA (GenBank™ accession number NM_006337) was obtained by PCR using a human testis cDNA library as a template and sequenced. The full-length MCRS1 was cloned in-frame into pEGFP-C1 plasmid (Clontech). The same product was obtained using a human placenta cDNA library as a template. The full-length MCRS1 was cloned in-frame into pEGFP-C1 plasmid (Clontech).

Human ribosomal DNA promoter spanning −483 to +377 bp with respect to the transcription initiation site (GenBank™ accession number U13369) (41) was amplified from genomic DNA isolated from human embryo kidney (HEK) 293 cells and checked by sequencing. The internal ribosome entry site (IRES) from the encephalomyocarditis virus was amplified from pIRES2-EGFP vector (Clontech) and checked by sequencing. The Kozak sequence of the pGL3-basic vector was optimized against the 19 carboxyl-terminal amino acids of MCRS1 (GenBank™ accession number NM_006337). Anti-FLAG (M2) monoclonal antibody was obtained from Sigma. Anti-upstream binding factor (UBF) monoclonal antibody was purchased from Santa Cruz Biotechnology. Anti-GFP polyclonal antibody was purchased from MBL (Japan). Alexa-conjugated secondary antibodies were purchased from Molecular Probes. Anti-MCRS1 polyclonal antibody was labeled with Alexa 594 using the Alexa Fluor 594 protein labeling kit (Molecular Probes).

Western Blotting and Co-immunoprecipitation Experiments—HEK 293 cells plated in 100-mm dishes were transfected with 4 µg of pFLAG-CMV2 expression vector or pEGFP-C1–MCRS1 expression vector using Lipofectamine PLUS (Invitrogen) according to the manufacturer’s instructions. After 48 h of incubation, cells were washed twice with ice-cold washing buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) and resuspended in 1 ml of hypotonic buffer (10 mM Tris-HCl, pH 7.5, 5 mM MgCl2, and 1 mM phenylmethylsulfonyl fluoride (PMSF)) containing Complete protease inhibitor mixture (Roche Diagnostics). The suspension was briefly sonicated and mixed with 160 µl of 2.5 M sucrose solution. After centrifugation at 1200 × g for 5 min at 4 °C, the pellet was lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 20 mM MgCl2, 0.5% Nonidet P-40, and 1 mM PMSF) containing Complete protease inhibitor mixture and centrifuged again at 12,000 × g for 25 min at 4 °C. The resultant supernatants were incubated with specific polyclonal antibodies or mouse anti-FLAG M2 antibody coupled with protein A-Sepharose or protein G-Sepharose (Sigma) for 5 h at 4 °C. Immunoprecipitation, electrophoretic separation, and Western blotting were performed as previously described (24). The nuclear/cytosol fractionation kit (Bio Vision) was used to prepare nuclear extracts to check the specificity of anti-MCRS1 antibody.

Yeast Two-hybrid Assay—The SfiI/Pmel fragment of the pcDNAV5-HisC-HA Mi-2β full was cloned in-frame into the SfiI/Smal site of the pcDNA5-V5-HisC plasmid. pcDNA5-V5-HisC-MCRS1 vector was transfected into HEK 293 cells using Lipofectamine PLUS (Invitrogen). Cells were grown in coverslips and washed twice with PBS (20 mM potassium phosphate, pH 7.4, 150 mM NaCl). To express FLAG-MCRS1, pcCMV-FLAG-MCRS1 vector was transfected into SW480 cells using Lipofectamine 2000 (Invitrogen). Cells were grown on coverslips and washed twice with PBS (20 mM potassium phosphate, pH 7.4, 150 mM NaCl). To express FLAG-MCRS1, pcCMV-FLAG-MCRS1 vector was transfected into SW480 cells using Lipofectamine 2000 (Invitrogen). Cells were fixed with methanol (5 min, −20 °C) and acetone (30 s, −20 °C), washed 3 times with PBS, and incubated in nuclease digestion buffer (66 mM Tris-HCl, pH 7.4, 0.66 mM MgCl2, 1 mM 2-mercaptoethanol, 100 units/liter deoxyribonuclease I) for 30 min at 37 °C. After washing 3 times with PBS, cells were blocked with 10% goat serum in PBS and incubated with primary antibodies (1:100 dilution for anti-MCRS1 polyclonal antibody, 1:1000 for anti-RFP polyclonal antibody, 1:2500 for anti-Mi-2 antibody, 1:200 for anti-UBF antibody, and 20 µg/ml for anti-FLAG antibody), again washed 3 times in PBS, and then stained with Alexa Fluor 488-conjugated anti-rabbit IgG antibody and an Alexa Fluor 594-conjugated anti-mouse IgG antibody. The Alexa 594-conjugated anti-MCRS1 antibody was diluted to 1:100 and used. To enhance the signal of the anti-MCRS1 antibody, Can Get Signal solution (Toyobo) was used. Slides were mounted in...
FIGURE 1. MCRS1 interacts with Mi-2 and RFP. A, FLAG-MCRS1 expression in HEK 293 cells. HEK 293 cells were transfected with FLAG or FLAG-MCRS1 expression vector. Whole cell lysates were immunoprecipitated with an anti-FLAG antibody (Ab) and analyzed by Western blotting (IB). B, co-immunoprecipitation (IP) of Mi-2 and RFP with FLAG-MCRS1. Lysates from HEK 293 cells transfected with FLAG or FLAG-MCRS1 were immunoprecipitated with an anti-FLAG antibody and then immunoblotted with anti-Mi-2 or anti-RFP antibody. Input represents 2% of the lysate for immunoprecipitation. C, co-immunoprecipitation of Mi-2 with GFP-MCRS1. Lysate from HEK 293 cells transfected with GFP-MCRS1 was immunoprecipitated with an anti-Mi-2 antibody and then immunoblotted with anti-GFP or anti-MCRS1 antibody. D, HEK 293 cell nuclear extracts were analyzed by Western blotting using anti-MCRS1 rabbit polyclonal antibody. Nuclear extract from HEK 293 cells transfected with FLAG-MCRS1 was used to identify the position of MCRS1. E, association of Mi-2 and UBF with endogenous MCRS1. HEK 293 cell whole cell lysates were immunoprecipitated with anti-MCRS1 polyclonal antibody and then immunoblotted using anti-Mi-2 and anti-UBF antibodies.
PermaFluor (Shandon) and observed using a confocal microscope (Olympus).

**Chromatin Immunoprecipitation (ChIP) Assay**—After 48 h of incubation, HEK 293 cells plated in 100-mm dishes were fixed with 1% formaldehyde for 10 min at 37 °C and incubated with ice-cold glycine/PBS (125 mM glycine, 1 mM PMSF, and 1 μg/ml aprotinin) for 10 min at 4 °C. Cells were then washed twice with ice-cold PBS containing protease inhibitors (1 mM PMSF, 1 μg/ml aprotinin). After scraping and collecting cells by centrifugation at 500 × g for 3 min at 4 °C, cell pellets were resuspended in 300 μl of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, 1 mM PMSF, 1 μg/ml aprotinin) and incubated for 10 min on ice. Cell lysates were sonicated briefly 4 times on ice to shear the DNA and diluted with 2700 μl of ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl, 1 mM PMSF, 1 μg/ml aprotinin). Diluted cell suspensions were precleared with 80 μl of salmon sperm DNA/protein A-agarose (Upstate) for 1 h at 4 °C. After a brief centrifugation, supernatant fractions were immunoprecipitated overnight with specific antibodies or control normal rabbit immunoglobulin (Santa Cruz Biotechnology).

After washing with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), and LiCl buffer (0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Triton X-100), pellets were eluted twice with 150 μl of elution buffer (1% SDS, 0.1 M NaHCO3). Cross-links were then reversed by adding 12 μl of 5 M NaCl and incubated overnight at 65 °C, after which 10% of the purified DNA was amplified by PCR (30 cycles of 30 s at 94 °C, 30 s at 55 °C for 28 S ribosomal DNA, 57 °C for rDNA non-coding region, 60 °C for rDNA promoter, or 65 °C for GAPDH, and 30 s at 72 °C).

PCR primer sets used were: rDNA promoter 1 (forward primer, 5'-GACCCAGCCAGCTTCCTGCAGGAT-3'; reverse primer, 5'-AGGCGGC-GACCTCCTGGGCCG-3'), rDNA promoter 2 (forward primer, 5'-GCTCCGGTTGAGCGACCGGC-3'; reverse primer, 5'-AGGACGCGGAGGCCGCG-3'), 28 S RNA forward primer, 5'-GGGACCTCATGATAACAGTG-3'; reverse primer, 5'-CTTAACGGTTTCACGCC-3'), rDNA non-coding region 1 (forward primer, 5'-TGAAACGT-TTCTCCTGCTCCGTCC-3'; reverse primer, 5'-AGGACGATCAGCGGTTCCCCCTCC-3'), rDNA non-coding region 2 (forward primer, 5'-GAGGCCAACCTCATTGCCGCA-3'; reverse primer, 5'-GCTGTCCAGCTATTTTGAGAGGAGTGC-3'), and GAPDH (forward primer, 5'-GAAGTTGAAAGTCCGGAGTCAAA-3'; reverse primer, 5'-GAGATGATGGACCCCTGGTGCT-3').

**GAL4-based Ribosomal DNA Promoter-targeted Transcription Assay**—Mouse L cells were kindly provided by Dr. Kaibuchi at Nagoya University. HEK 293 cells and mouse L cells were cultured in 24-well tissue culture plates and co-transfected with 40 ng of GAL4-hrDNA promoter-IRES luciferase reporter plasmid, 40 ng of pRL-TK plasmid (Promega), and 320 ng of pcDNA3-GAL4BD Mi-2β, RFP, or MCRS1 expression plasmids with or without NoLS using Lipofectamine PLUS (Invitrogen). Cells were harvested 48 h after transfection, and luciferase assays were performed as previously described (32). Co-transfection with the pRL-TK plasmid was used to normalize luciferase values. Values were expressed as the means ± S.D. of at least three independent experiments.

**RNA-mediated Interference**—MCRS1 and Mi-2β siRNA and control siRNA (siControl non-targeting siRNA) were purchased from Dhamacon, and RFP siRNA was purchased from Qiagen. The siRNAs were transfected using X-tremeGENE siRNA transfection reagent (Roche Applied Science) according to the manufacturer’s instructions.

**Ribosomal RNA Expression Analysis**—Total cellular RNA was isolated using the RNasy Mini kit (Qiagen), and residual DNA was digested using RNase-free DNase (Qiagen). RNA was reverse-transcribed with MultiScribe reverse transcriptase (Applied Biosystems) for 30 min at 48°C. The resultant cDNA was amplified by PCR for 12–40 cycles of 30 s at 96 °C, 30 s at 55 °C for 28 S ribosomal RNA and 47 S/45 S ribosomal RNA or 65 °C for GAPDH, and 30 s at 72°C.

**RESULTS**

**MCRS1 Interacts with Mi-2 and RFP**—MCRS1 is a nucleolar protein that contains a bipartite nuclear localization motif, a nucleolar localization motif, a coiled-coil domain, and a forkhead-associated domain. MCRS1 is involved in the nucleolar sequestration of Daxx and was recently reported to have transforming activity (38, 39). In a survey of MCRS1-interacting proteins by immunoprecipitation of lysates from HEK 293 cells transfected with FLAG-MCRS1 (Fig. 1A), we repeatedly identified a 220-kDa protein that co-precipitated with FLAG-MCRS1. Because 220 kDa was consistent with the molecular mass of Mi-2 and
mass spectrometric analysis of nucleolar proteins suggested the presence of Mi-2β (CHD4) in the nucleolus (44), we investigated whether Mi-2 is associated with FLAG-MCRS1. As shown in Fig. 1B, we found that Mi-2 co-immunoprecipitated with FLAG-MCRS1. We then examined the possibility that RFP also interacts with MCRS1, as we had previously reported an association between Mi-2 and RFP (24). Immunoprecipitation experiments revealed an association between RFP and MCRS1 (Fig. 1B). To further confirm the association of MCRS1 and Mi-2, we next performed reciprocal immunoprecipitation by using anti-Mi-2 rabbit polyclonal antibody. GFP-MCRS1 was used to avoid the superimposition of the immunoglobulin band on that of MCRS1. Fig. 1C shows that GFP-MCRS1 co-immunoprecipitated with endogenous Mi-2.

To characterize proteins that are associated with endogenous MCRS1, we raised an anti-MCRS1 polyclonal antibody and confirmed its specificity (Fig. 1D). As shown in Fig. 1E, Mi-2 was associated with endogenous MCRS1 in HEK 293 cells. Nucleolar transcription factor UBF also co-immunoprecipitated with endogenous MCRS1 (Fig. 1E). However, we could not investigate the association between RFP and endogenous MCRS1, as the RFP band coincided with the anti-MCRS1 rabbit polyclonal antibody band used for immunoprecipitation.

We next performed a yeast two-hybrid assay to map the binding domains. We performed PCR on human testis and placenta cDNA libraries to obtain full-length MCRS1/MSP58 cDNA (GenBank™ accession number NM_006337). We cloned the full-length MCRS1 cDNA into the pACT2 vector and constructed pAS2–1 vectors that carried different regions of Mi-2β/H9252 and RFP (Fig. 2A). Yeast strain Y190 was then transformed with the pAS2–1 and pACT2 constructs, and interactions between full-length MCRS1 and Mi-2β or RFP fragments were assayed both by growth on selective medium lacking histidine, tryptophan, and leucine with 40 mM 3-amino-1,2,4-triazole and by β-galactosidase activity.
As shown in Fig. 2B, full-length MCRS1 was specifically associated with the ATPase/helicase region of Mi-2β. Deletion constructs of MCRS1 (Fig. 2A) mapped the central region (MCRS1–2) as the region that interacted both with the ATPase/helicase region and with the full length of Mi-2β. The association between the full-length Mi-2β and the full-length MCRS1 was relatively weak in this assay. We speculate that transcriptional repressive activity of the full-length Mi-2β and steric constraint of the large GAL4 fusion protein may affect the expression of yeast histidine and β-galactosidase genes.

Full-length MCRS1 and its central region (MCRS1–2) also interacted with both full-length RFP and its coiled-coil region (Fig. 2B). Although the RFP coiled-coil region (RFP-2) interacted with both MCRS1–2 and the carboxyl-terminal region of MCRS1 (MCRS1–3), full-length RFP interacted with only MCRS1–2. Taken together, we concluded that MCRS1 interacted with Mi-2β and RFP, with the central region of MCRS1 acting as the binding site for the ATPase/helicase region of Mi-2β and the coiled-coil region of RFP.

**MCRS1, Mi-2β, and RFP Co-localize in the Nucleolus**—Although MCRS1 localizes exclusively to the nucleolus (34), endogenous Mi-2 and RFP are distributed throughout the nucleoplasm in a fine granular pattern or as variously sized dot-like structures. To explore the possibility that some of these domain-like structures represented the nucleolar distribution of Mi-2 and RFP, we transfected human colorectal adenocarcinoma SW480 cells with the pFLAG-MCRS1 expression plasmid and checked for the co-localization of RFP and Mi-2 with MCRS1 in the nucleolus. In SW480 cells, Mi-2 and RFP appeared to co-localize with MCRS1 at the nucleolus, as revealed by the superimposition of confocal images (Fig. 3A). We also examined the localization of RFP, Mi-2, and MCRS1 in HeLa cells and obtained the same results (data not shown).

We then investigated the endogenous distribution patterns of MCRS1, Mi-2, and RFP and assessed their co-localization in the nucleolus. Immunofluorescence analysis of SW480 cells using anti-RFP or anti-Mi-2 polyclonal antibodies together with Alexa 594-conjugated-anti-Mi-2 antibody clearly showed the co-localization of these proteins in nucleoli (Fig. 3B). The distribution of RFP, Mi-2β, and MCRS1 showed a relatively large fusing dot-like structure or a circular distribution of small fusing dot-like structure in the nucleoli of SW480 cells.

Because nucleolar protein UBF co-immunoprecipitated with MCRS1 using HEK 293 cell lysates (Fig. 1D), we further investigated whether MCRS1, Mi-2, and RFP also co-localized with UBF. Fig. 4 shows that MCRS1, RFP, and MCRS1 co-localized with UBF in the nucleoli of SW480 cells. It has been shown that UBF localizes to the dense fibrillar component and at the periphery of the fibrillar centers in the nucleoli, where active ribosomal DNA transcription occurs by associating with RNA polymerase I and by inducing the chromatin remodeling (1, 45, 46). Co-localization of MCRS1, Mi-2, and RFP with UBF in the nucleoli suggested that these proteins may be associated with the regulation of nucleolar activity and ribosomal gene transcription.

**MCRS1, Mi-2, and RFP Are Associated with Human rDNA**—To investigate the recruitment of MCRS1, Mi-2, and RFP to the ribosomal DNA regions, ChIP assays were performed. Ribosomal genes are aligned as tandem repeats at nucleolar organizer regions on chromosomes 13, 14, 15, 21, and 22 (2). Each ribosomal DNA repeating unit is about 43 kilobases in length, with about 13 kilobases of 47 S ribosomal RNA transcribed from each unit (Fig. 5A). We designed two sets of primers within the promoter region, one set of primers within the 28 S ribosomal RNA coding region and two sets of primers within the 3′ non-coding region (Fig. 5A). As shown in Fig. 5B, Mi-2 and RFP were associated with both the promoter regions and the 28 S rRNA coding region. In addition, the proteins also bound to the 3′ non-coding regions examined. These results suggested that Mi-2 and RFP bound to the entire rDNA region. The distribution profiles of Mi-2 and RFP matched the proposed distribution of UBF, an essential component of the RNA polymerase I initiation complex, which is distributed throughout the ribosomal gene repeats rather than restricted to the promoter region (47). However, MCRS1 was associated with both the promoter regions and the 28 S rRNA coding region but not with the 3′ non-coding regions (Fig. 5C). This suggested that the co-operative role of MCRS1 with Mi-2 and RFP may be restricted to the modulation of rRNA transcription and that MCRS1 is not associated with Mi-2 and RFP in 3′-non-coding regions. However, because Mi-2 and RFP are associated with the entire ribosomal gene repeat, these proteins might also be involved in the modulation of chromatin structure, like UBF.

**MCRS1, Mi-2β, and RFP Activate rRNA Promoter Activity**—The co-localization of MCRS1, Mi-2, and RFP with rRNA transcription factor UBF in the nucleolus together with the association of MCRS1, Mi-2, and RFP with ribosomal gene repeats strongly suggested that these proteins are involved in ribosomal gene transcription. To investigate this hypothesis, we cloned the human rRNA promoter region spanning from −483 to +377 bp and constructed the pGL3-GAL4-hrDNAP-IRES-Luc luciferase vector (Fig. 6A). To minimize a luciferase gene expression driven by RNA polymerase II, the Kozak sequence of the pGL3 vector was replaced by the IRES sequence as previously described (42, 48). It is known that the human rRNA promoter is not transcribed by mouse pol I transcription machinery and vice versa (49). By taking advantage of the species specificity of pol I transcription, Ghoshal et al. (42) demonstrated the pol I-specific transcription of the luciferase reporter construct that contained the human rRNA promoter region (from −410 to +314) (42). We confirmed the pol I-specific transcription of our pGL3-GAL4-hrDNAP-IRES-Luc luciferase vector by comparing the luciferase activities in human HEK 293 cells and mouse L cells. Fig. 6B shows that the transcriptional activity of the pGL3-GAL4-hrDNAP-IRES-Luc luciferase vector is markedly lower in mouse L cells...
than in human HEK 293 cells, demonstrating the pol I-specific transcription of the pGL3-GAL4-hrDNAP-IRES-Luc vector.

Transcriptional activity of the rRNA promoter was assessed by the transient transfection of GAL4-DBD-fused MCRS1, Mi-2β/H9252, or RFP constructs with pGL3-GAL4-hrDNAP-IRES-Luc luciferase plasmid into HEK 293 cells. As shown in Fig. 6C, Mi-2β strongly transactivated rRNA promoter activity by ~400%, whereas RFP and MCRS1 transactivated rRNA promoter activity by ~200%. We have previously shown that Mi-2β and RFP repressed the RNA polymerase II-driven luciferase activity by about 70% in the nucleus (24, 32). Thus, our results indicated that Mi-2β and RFP have distinct transcriptional activities in the nucleus and the nucleolus.

To further assess the transcriptional activity of each region of MCRS1, Mi-2β, and RFP, we constructed a series of GAL4-DBD-NoLS-fused deletion constructs (Fig. 6D). The NoLS was used to ensure nucleolar localizations of the deletion constructs. As shown in Fig. 6E, both the central region of MCRS1 (i.e. the Mi-2β and RFP binding site) and the carboxyl-terminal region that contains the forkhead-associated domain transactivated the rRNA promoter. In contrast, the amino-terminal region of MCRS1 showed no such transactivating activity. We recently reported that the amino-terminal region of Mi-2β had strong transactivating activity, whereas the carboxyl-terminal region of Mi-2β had repressing activity in the nucleus (24). Surprisingly, assessment of transcriptional activities of Mi-2β deletion constructs revealed that the repressive activity of the carboxyl-terminal region was completely lost, such that all three regions examined transactivated the rRNA promoter (Fig. 6F). We speculate that the difference of associating proteins between nucleolar pol I transcription machinery and nuclear pol II transcription machinery may alter the transcriptional activity of Mi-2β and its carboxyl-terminal region.

We next investigated the transcriptional activities of RFP deletion constructs. Fig. 6G demonstrates that the coiled-coil region of RFP (i.e.
FIGURE 6. Transactivating activities of MCRS1, Mi-2β, and RFP on the rDNA promoter region. 

A, schematic representation of the pcDNA-V5-GAL4BD-MCRS1/Mi-2β/RFP construct and pGL3-GAL4-hrDNAP-IRES-Luc reporter plasmid. A segment of the rDNA gene with promoter activity (−483 to +377 bp with respect to the transcriptional start site) was amplified and cloned into the modified pGL3 luciferase vector. IRES, internal ribosomal entry site of the encephalomyocarditis virus. B, pol I-dependent transcription of pGL3-GAL4-hrDNAP-IRES-Luc reporter. pGL3-GAL4-hrDNAP-IRES-Luc reporter plasmid (RLU1: firefly) and the internal control plasmid pRL-TK (RLU2: Renilla) were co-transfected into HEK 293 cells or L cells. After 36 h, both firefly and Renilla luciferase activities were measured. The table represents the RLU1 and RLU2 luciferase activities in HEK 293 cells and L cells. A lower panel shows the graphical representation of the relative ratio of RLU1 to RLU2 activity in HEK 293 cells and L cells. Each value represents a result of three independent experiments. Error bars indicate S.D. of each value. RLU, relative luminescence units. C, MCRS1, Mi-2β, and RFP transactivate the rDNA promoter. pGL3-GAL4-hrDNAP-IRES-Luc plasmid was co-transfected with pcDNA3-V5-GAL4-DBD effector constructs that express GAL4-DBD fused with MCRS1, Mi-2β, or RFP. Luciferase activity in cells transfected with control plasmid expressing GAL4-DBD alone was set at 100%, and luciferase activities in cells transfected with the indicated plasmids were expressed as average percentages of the control value. Each value represents a result of three independent experiments. Error bars indicate the S.D. of each value. BD, binding domain. D–G, transcriptional activity of each region of MCRS1 (D), Mi-2β (E), and RFP (G) on the rDNA promoter region. The indicated regions of MCRS1, Mi-2β, and RFP were cloned into the pcDNA3-V5-GAL4-DBD effector constructs. The luciferase activity of each construct was assayed as described above. NLS, nuclear localization signal; NoLS, nucleolar localization signal; BD, binding domain.
the Mi-2β and MCRS1 binding site) transactivated the rRNA promoter, whereas the RFP domain region had weak transactivating activity. In contrast, the RING finger B-box region showed no transactivation function. These results suggested that the repressing functions of RFP and Mi-2β subdomains were not observed in the nucleolus and that MC RS1, Mi-2β, and RFP are involved in the transactivation of ribosomal gene transcription.

siRNA-mediated Down-regulation of MC RS1, Mi-2β, and RFP Inhibited rRNA Production

To determine the effect of MC RS1, Mi-2β, and RFP on rRNA production in vivo, we treated HeLa cells with siRNA against the respective genes. Treatment of HeLa cells with MC RS1, Mi-2β, and RFP siRNA resulted in significant reductions of the respective mRNAs and proteins compared with treatment with the control siRNA (Fig. 7A). We further confirmed that siRNAs used specifically repressed their target molecule and did not affect the expressions of other molecules such as MC RS1, Mi-2β, RFP, and UBF (Fig. 7A).

Finally, we assessed the amounts of rRNA in HeLa cells treated with the specific siRNAs. Human rRNA is transcribed as a primary 47 S precursor and is subsequently processed to generate mature 28 S, 18 S, and 5.8 S through the activities of different RNA-processing enzymes (Fig. 7B). PCR primer sets specific for 5′ETS-18 S rRNA region and 28 S rRNA region were designed to measure the amounts of 47 S/45 S rRNA and subtot al rRNA, except for 18 S and 5.8 S, respectively (Fig. 7B). Reverse transcription-PCR experiments demonstrated that 28 S rRNA region production was inhibited by 24–43% when HeLa cells were treated with MC RS1, Mi-2β, or RFP siRNAs (Fig. 7, C and E). The earlier processed 47 S/45 S rRNA production was more significantly reduced by 52–59% (Fig. 7, D and E). These results agreed with the transactivating activities of MC RS1, Mi-2β, and RFP for the rRNA promoter as shown in Fig. 6 and indicated that MC RS1, Mi-2β, and RFP are involved in the up-regulation of ribosomal gene transcription in vivo.

DISCUSSION

Transcriptional Function and Expression Profile of MC RS1 in the Nucleolus

MC RS1 is a nucleolar protein that is highly expressed in microspherules within the nucleolus. The numbers of microspherules and the size and density of the dense fibrillar centers in the nucleolus are associated with increased nucleolar activity and the production of pre-

FIGURE 7. Effects of siRNA-mediated down-regulation of MC RS1, Mi-2β, and RFP on rDNA transcription in vivo. A, down-regulation of MC RS1, Mi-2β, and RFP by specific siRNA. HeLa cells were transfected with specific siRNA, and expression of MC RS1, Mi-2β, RFP, and UBF was assayed by both reverse transcription (RT)-PCR and Western blotting. si, small interfering. B, schematic representation of pre ribosomal RNA processing pathway. 47 S rRNA is processed to generate 18 S, 5.8 S, and 28 S. The primer sets used for reverse transcription-PCR analysis are indicated. ETS, external transcribed spacer; ITS, internal transcribed spacer. C–D, HeLa cells were transfected with control siRNA or specific siRNA for MC RS1, Mi-2β, and RFP. At 48 h after transfection the expressions of 28 S ribosomal RNA that reflects subtotal rRNA (C) and 47 S/45 S ribosomal RNA (D) were analyzed by reverse transcription-PCR. GAPDH was amplified as a control. Samples were analyzed at indicated cycle numbers as shown on the right side of the panel. The numbers below the panel correspond to the intensity of each product as measured by WinROOF software. E, relative intensity of ribosomal RNA bands of cells treated with specific siRNAs. Amounts of 47 S/45 S ribosomal RNA at 37 cycles and 28 S ribosomal RNA region at 15 cycles were assayed as described above and normalized according to the intensity of the GAPDH amplified product. Results are expressed as average percentages of the value obtained from control siRNA-treated cells. Each value represents a result of three independent experiments. Error bars indicate S.D.
ribosomal RNA and pre-rRNP particles. Although the finding that UBF, RNA polymerase I, fibrillarin, and p130 along with MCRS1 localized to microspherules suggested an association between MCRS1 and ribosomal gene transcription or ribosomal subunit maturation (34, 50–53), the transcriptional roles of MCRS1 have not yet been elucidated.

In this report we examined the role of MCRS1/MSP58 in the transactivation of the ribosomal genes. MCRS1 was associated and co-localized with RFP and chromatin remodeling protein Mi-2β/H9252 in the nucleolus. MCRS1, Mi-2β, and RFP up-regulated ribosomal gene promoter activity and co-localized with UBF, an essential component of the RNA polymerase I initiation complex and a transactivator of RNA polymerase I-mediated transcription. In addition, down-regulation of MCRS1, Mi-2β, and RFP by siRNA treatment inhibited the rRNA production. These findings support the view that MCRS1, Mi-2β, and RFP are involved in RNA polymerase I-mediated transcriptional activation.

**Transactivating Role of Mi-2β and RFP in the Nucleolus**—Mi-2β is a major component of the well characterized NuRD complex. Mi-2β forms a complex with histone deacetylase HDAC1, methyl CpG-binding protein MBD2/3, metastasis-associated protein MTA 1/2/3, and histone-binding protein RbAp 48/46 and is involved in transcriptional repression in the nucleus. In a previous report we found that RFP acted as a transcriptional co-repressor that was associated with Polycomb-group protein, Enhancer of Polycomb 1, and Mi-2β in the nucleus (24, 32).

We first speculated that MCRS1, Mi-2β, and RFP formed a repressive complex in the nucleolus, as proposed in the nucleus. To our surprise, a transcriptional assay using a ribosomal gene promoter revealed that Mi-2β and RFP exhibited transactivating activities in the nucleolus. In addition, the strong repressive activities of both the Mi-2β carboxyl-terminal region and the RFP coiled-coil region were lost, with significant transactivation of the ribosomal gene promoter observed.
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Ronald J. Howell*1,2,4,21,22, RFP, and UBF in the nucleolus. Thus, it is possible that Mi-2β can facilitate the formation of an active chromatin structure in the nucleolus by remodeling chromatin and associating with histone acetyltransferases such as Tip60 or p300.

Our study investigated the transcriptional function of nucleolar protein MCRS1/MSP58 and its interaction with Mi-2β and RFP. We identified the Mi-2β as a candidate molecule for the chromatin remodeling protein that is involved in the formation of open chromatin structures that facilitate active rRNA gene transcription. Our results contribute to the understanding of active chromatin structure formation at ribosomal gene repeats in the nucleolus.

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REFERENCES

1. Raska, I., Kobeha, K., Malinsky, J., Fiderova, H., and Masata, M. (2004) Biol. Cell 96, 579–594
2. Henderson, A. S., Warburton, D., and Atwood, K. C. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 3394–3398
3. Conconi, A., Widmer, R. M., Kolmer, T., and Sogo, J. M. (1989) Cell 57, 753–761
4. Sandmeyer, L. J., French, S., Odheimer, Y., Zheng, W. L., Gallo, C. M., Beyer, A. L., and Smith, J. S. (2002) EMBO J. 21, 4959–4968
5. Pelletier, G., Stefanovsky, V. Y., Faubalder, M., Hirschler-Laszkiewicz, I., Savard, J., Rothblum, L. I., Cote, J., and Moss, T. (2000) Mol. Cell 6, 1059–1066
6. Moss, T., and Stefanovsky, V. Y. (2002) Cell 109, 545–548
7. Zhai, W., and Comai, L. (2000) Mol. Cell. Biol. 20, 5930–5938
8. Araki, A., Ku, S., Bidderstreike, K., Bierhoff, H., Shiono, C., Fathi, K., Fahlen, S., Hybring, P., Soderberg, Ö., Grummt, I., Larsson, L. G., and Wright, A. P. (2005) Nat. Cell Biol. 7, 303–310
9. Grandori, C., Gomez-Roman, N., Felton-Edkins, Z. A., Ngouenet, C., Galloway, D. A., Eisenman, R. N., and White, R. J. (2005) Nat. Cell Biol. 7, 311–318
10. Greul, S. S., Li, L., Orian, A., Eisenman, R. N., and Bar, A. B. (2005) Nat. Cell Biol. 7, 295–302
11. Strohner, R., Nemet, A., Nightingale, K. P., Grummt, I., Becker, P. B., and Langst, G. (2004) Mol. Cell. Biol. 24, 1791–1798
12. Santoro, R. I., Li, and Grummt, I. (2002) Nat. Genet. 32, 393–396
13. Zhou, Y., Santoro, R., and Grummt, I. (2002) EMBO J. 21, 4632–4640
14. Strohner, R., Nemet, A., Jansa, P., Hofmann-Rohrer, U., Santoro, R., Langst, G., and Grummt, I. (2001) EMBO J. 20, 4892–4900
15. Hirschler-Laszkiewicz, I., Cavanaugh, A., Hu, Q., Catania, J., Avantaggiati, M. L., and Rothblum, L. I. (2001) Nucleic Acids Res. 29, 4114–4124
16. Halkidou, K., Logan, I. R., Cook, S., Neal, D. E., and Robson, C. N. (2004) Nucleic Acids Res. 32, 1654–1665
17. Zhang, Y., LeRoy, G., Seelig, H. P., Lane, W. S., and Reinberg, D. (1998) Cell 95, 279–289
18. Xue, Y., Wong, J., Moreno, G. T., Young, M. K., Cote, J., and Wang, W. (1998) Mol. Cell Biol. 18, 851–861
19. Tong, J. K., Haasig, C. A., Schuettler, G. R., Kingston, R. E., and Schreiber, S. L. (1998) Nature 395, 917–921
20. Wade, P. A., Jones, P. L., Vermaak, D., and Wolffe, A. P. (1998) Curr. Biol. 8, 843–846
21. Schultz, D. C., Friedman, J. R., and Rauscher, F. J., 3rd. (2001) Genes Dev. 15, 428–443
22. Johnson, D. R., Lovett, J. M., Hirsch, M., Xie, F., and Chen, J. D. (2004) Biochem. Biophys. Res. Commun. 318, 714–718
23. Murnawsky, C. M., Brehm, A., Badenhorst, P., Lowe, N., Becker, P. B., and Travers, A. A. (2001) EMBO Rep. 2, 1089–1094
24. Shimono, Y., Murakami, K., Kawai, K., Wade, P. A., Shimokata, K., and Takahashi, M. (2003) J. Biol. Chem. 278, 51638–51645
25. Fujita, N., Jaye, D. L., Kajita, M., Geigerman, C., Moreno, C. S., and Wade, P. A. (2003) Cell 113, 207–219
26. Hirose, F., Ohshima, N., Kwon, E. J., Yoshida, H., and Yamaguchi, M. (2002) Mol. Cell Biol. 22, 5182–5193
27. Williams, C. J., Naito, T., Arco, P. G., Seavitt, J. R., Cashman, S. M., De Souza, B., Qi, X., Keabl, P., Von Andrian, U. H., and Georgopoulos, K. (2004) Immunity 20, 719–733
28. Takahashi, M., Inaguma, Y., Hiai, H., and Hirose, F. (1998) Mol. Cell Biol. 8, 1853–1856
29. Yediel, G., Nagarakka, T., Ishiwashi, N., Naka, N., Ishiwashi, T., Sakata, K., and Takahashi, M. (1999) Patol. Int. 49, 881–886
30. Morris Desbois, C., Boccard, V., Reynaud, C., and Jalainot, P. (1999) J. Cell Sci. 112, 3331–3342
31. Gao, T., Duprez, E., Borden, K. L., Freemont, P. S., and Etilin, L. D. (1998) J. Cell Sci. 111, 1319–1329
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32. Shimono, Y., Murakami, H., Hasegawa, Y., and Takahashi, M. (2000) J. Biol. Chem. 275, 39411–39419
33. Matsuura, T., Shimono, Y., Kawai, K., Murakami, H., Urano, T., Niwa, Y., Goto, H., and Takahashi, M. (2005) Exp. Cell Res. 308, 65–77
34. Ren, Y., Busch, R. K., Perlaky, L., and Busch, H. (1998) Eur. J. Biochem. 253, 734–742
35. Bruni, R., and Roszman, B. (1998) J. Virol. 72, 8525–8531
36. Bader, A. G., Schneider, M. L., Bister, K., and Hartl, M. (2001) Oncogene 20, 7524–7535
37. Song, H., Li, Y., Chen, G., Xing, Z., Zhao, J., Yokoyama, K. K., Li, T., and Zhao, M. (2004) Biochem. Biophys. Res. Commun. 316, 1116–1123
38. Okumura, K., Zhao, M., Depinho, R. A., Furnari, F. B., and Cavenee, W. K. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 2703–2706
39. Lin, D. Y., and Shih, H. M. (2002) J. Biol. Chem. 277, 25446–25456
40. Ivanova, A. V., Ivanov, S. V., and Lerman, M. L. (2005) Cell. Mol. Life Sci. 62, 471–484
41. Miesfeld, R., and Arnheim, N. (1982) Nucleic Acids Res. 10, 3933–3949
42. Ghoshal, K., Majumder, S., Datta, J., Motiwala, T., Bai, S., Sharma, S. M., Frankel, W., and Jacob, S. T. (2004) J. Biol. Chem. 279, 6783–6793
43. Wade, P. A., Gegenone, A., Jones, P. L., Ballestar, E., Aubry, F., and Wolfe, A. P. (1999) Nat. Genet. 23, 62–66
44. Staub, F., Fiziev, P., Rosenthal, A., and Hinzmann, B. (2004) Bioessays 26, 567–581
45. Grummt, I. (2003) Genes Dev. 17, 1691–1702
46. Roussel, P., Andre, C., Masson, C., Geraud, G., and Hernandez-Verdun, D. (1993) J. Cell Sci. 104, 327–337
47. O’Sullivan, A. C., Sullivan, G. J., and McStay, B. (2002) Mol. Cell. Biol. 22, 657–668
48. Palmer, T. D., Miller, A. D., Reeder, R. H., and McStay, B. (1993) Nucleic Acids Res. 21, 3451–3457
49. Heiz, J., and Grummt, I. (1995) Curr. Opin. Genet. Dev. 5, 652–656
50. Rendon, M. C., Bolivar, J., Ortiz, M., and Valdiliva, M. M. (1994) Cell Struct. Funct. 19, 153–158
51. Jimenez-Garcia, L. F., Rothblum, L. I., Busch, H., and Ochs, R. L. (1989) Biol. Cell 65, 239–246
52. Pai, C. Y., Chen, H. K., Shea, H. L., and Yeh, N. H. (1995) J. Cell Sci. 108, 1911–1920
53. Ochs, R. L., Lischwe, M. A., Spohn, W. H., and Busch, H. (1985) Biol. Cell 54, 123–133
54. Lawrence, R. J., Earley, K., Pontes, O., Silva, M., Chen, Z. J., Neves, N., Viegas, W., and Pikaard, C. S. (2004) Mol. Cell 13, 599–609
55. Chen, D., Belmont, A. S., and Huang, S. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 15106–15111