

Myeloid Leukemia Factor 1 Associates with a Novel Heterogeneous Nuclear Ribonucleoprotein U-like Molecule*

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Myeloid leukemia factor 1 (MLF1) is an oncoprotein associated with hemopoietic lineage commitment and acute myeloid leukemia. Here we show that Mlf1 associated with a novel binding partner, Mlf1-associated nuclear protein (Manp), a new heterogeneous nuclear ribonucleoprotein (hnRNP) family member, related to hnRNP-U. Manp localized exclusively in the nucleus and could redirect Mlf1 from the cytoplasm into the nucleus. The nuclear content of Mlf1 was also regulated by 14-3-3 binding to a canonical 14-3-3 binding motif within the N terminus of Mlf1. Significantly Mlf1 contains a functional nuclear export signal and localized primarily to the nuclei of hemopoietic cells. Mlf1 was capable of binding DNA, and microarray analysis revealed that it affected the expression of several genes, including transcription factors. In summary, this study reveals that Mlf1 translocates between nucleus and cytoplasm, associates with a novel hnRNP, and influences gene expression.

Myeloid leukemia factor 1 (*MLF1*)⁴ is a gene involved in hemopoietic lineage commitment and acute myeloid leukemia (1, 2). The gene was originally identified in a (3;5) translocation

associated with acute myeloid leukemia that generated an abnormal fusion with nucleophosmin (NPM), *i.e.* NPM-MLF1 (1). The t(3;5) is found mainly in the M6 erythroleukemic subtype of acute myeloid leukemia (3). Significantly the fusion protein NPM-MLF1 is almost exclusively nuclear (1).

The murine orthologue of *MLF1* was isolated independently as hemopoietic lineage switch 7 (*Hls7*), a gene up-regulated when J2E erythroleukemic cells spontaneously developed a monoblastoid appearance (2, 4). Upon reintroduction into parental J2E cells, *Hls7/Mlf1* induced a dramatic phenotypic change as the proerythroblastoid cells now displayed an immature, blast-like appearance (2). *Hls7/Mlf1* has also been shown to influence hemopoietic lineage commitment by altering the balance between erythroid and myeloid progenitors (2). In contrast with the nuclear localization of the NPM-MLF1 fusion protein, Mlf1 is located primarily in the cytoplasm with some punctate nuclear staining (1, 2).

Apart from a classical 14-3-3 binding site, Mlf1 has no recognizable motifs (1, 2, 5, 6). Therefore, yeast two-hybrid screens have been conducted to identify partner proteins that may elucidate the function of Mlf1. One molecule shown to bind Mlf1 was Mlf1 adaptor molecule (Madm), an adaptor protein involved in nuclear-cytoplasmic shuttling (7). Another Mlf1-binding protein is Mlf1-interacting protein/KSHV latent nuclear antigen-interacting protein (Mlf1IP/KLIP1), a novel nuclear molecule that may function as a transcriptional regulator (8, 9). Interestingly in *Drosophila*, Mlf1 associates with DNA replication-related element binding factor (DREF) (5), a DNA-binding protein that regulates genes involved in proliferation, including *E2f* (10). It has been suggested that “the DREF system may occupy an intersection in growth and differentiation signaling pathways” (11).

Recently we have shown that Mlf1 interferes with erythropoietin-induced differentiation by preventing cells from exiting the cell cycle (12). Mlf1 prevented p27^{KIP1} accumulation (12), which is essential for cell cycle arrest during erythroid terminal differentiation (13, 14). Mlf1 has also been reported to regulate the cell cycle via the CSN3/COP1 (COP9 signalosome complex subunit 3/constitutive photomorphogenesis protein 1 homologue) pathway that influences p53 levels (15).

In this study we examined two Mlf1-binding proteins identified in a yeast two-hybrid screen, *viz.* an uncharacterized nuclear protein we named Mlf1-associated nuclear protein (Manp) and 14-3-3ζ. Our results show that Mlf1 co-immunoprecipitated with Manp, and the two molecules co-localized in the nucleus. Manp is homologous to hnRNP-U and possesses a

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) DQ000354.

The amino acid sequence of this protein can be accessed through NCBI Protein Database under NCBI accession number AAY44301.

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⁴ The abbreviations used are: Mlf1, myeloid leukemia factor 1; hnRNP, heterogeneous nuclear ribonucleoprotein; SAP, SAF-A/B, Acinus, Pias; GST, glutathione S-transferase; GFP, green fluorescent protein; NES, nuclear export sequence; MEL, murine erythroleukemia; M1, murine myeloid leukemia; MSCV, murine stem cell virus; SPRY, SP1A kinase of *Dictyostelium*, ryanodine receptor; NPM, nucleophosmin; Hls7, hemopoietic lineage switch 7; Mlf1IP/KLIP1, Mlf1-interacting protein/KSHV latent nuclear antigen-interacting protein; DREF, DNA replication-related element binding factor; Madm, Mlf1 adaptor molecule; Manp, Mlf1-associated nuclear protein; Mdr1, multidrug resistance protein 1; MIBP1, c-Myc intron-binding protein 1; SAF-A, scaffold attachment factor-A; Ets-1, E26 avian leukemia oncogene 1; chURP, chicken hnRNP-U-like molecule.

Mlf1 Binds hnRNP-U-like Protein

DNA-binding SAP domain. Significantly in a subset of cells Mlf1 translocated to the nucleus when co-expressed with Manp and was able to bind DNA. 14-3-3 ζ played an important role in mediating retention in the cytoplasm. Collectively these data indicate that Mlf1 shuttles between the cytoplasm and the nucleus where it may influence gene expression.

EXPERIMENTAL PROCEDURES

Isolation of Manp cDNA Clones—An EML C.1 cDNA library in λ zapII (Stratagene, La Jolla, CA) (16) was screened for full-length Manp using the yeast two-hybrid fragment.

Production of Anti-Manp Antiserum—New Zealand White rabbits were immunized with 200 μ g of recombinant protein glutathione S-transferase-Manp (GST-Manp) three times at 4-week intervals. The resultant antisera were tested for specificity by immunoblotting of recombinant protein and protein lysates from cells transiently transfected with Manp.

Cloning, Expression, and Purification of Recombinant Protein Fragments—Expression clones encoding full-length Mlf1 and Manp as well as Mlf1 truncation mutants were constructed by cloning PCR-amplified fragments into pcDNA3(+) (Invitrogen) or pEGFP-C2 (Clontech) using primer-incorporated restriction sites. The Mlf1-S34A and Mlf1- Δ NES mutants were generated using PCR-based mutagenesis. 5' and 3' directional primers were produced incorporating the mutation/deletion: S34A5' (5'-CAT GAT GAG AAG TTT CGC TGA ACC TCT TG-3'), S34A3' (5'-CAA GAG GTT CAG CGA AAC TTC TCA TCA TG-3'), Δ NES5' (5'-AGG AGC CAA ATG GGC ATT CAT TTT G-3'), and Δ NES3' (5'-TTT GGC TCC TGT ATA CCA CTT C-3'). Each of these primers was used with an Mlf1 cDNA primer: either MLFBGL5' (5'-AGT GAG ATC TAT GTT CCG GAT GCT GAG CAG C-3') or MLFR13' (5'-GGA ATT CTT ATT TTT TGG TGA TTT TCA C-3'). Products incorporating each mutation/deletion were used to generate full-length Mlf1 cDNA containing the mutation/deletion. All PCRs were carried out with Pfu polymerase (Invitrogen). GST recombinant proteins (14-3-3 ζ and Manp) were prepared using the method of Smith and Johnson (17), i.e. full-length cDNA was cloned into pGEX 2T, expressed as recombinant protein in *Escherichia coli*, and then purified on glutathione-agarose.

Cell Culture, Transfection, and Infection of Cells—All cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum for COS7 cells or 5% fetal calf serum for J2E, murine erythroleukemia (MEL), and M1 monoblastoid cells (18–20). Cells were maintained in a humidified atmosphere at 37 °C with 5% CO₂. Cells were passaged at 90% confluency (COS7) or at a density of 5–8 \times 10⁵ cells/ml (J2E/MEL/M1). Differentiation of MEL cells was initiated with 5% Me₂SO, and differentiation of M1 cells was initiated with 1 μ g/ml leukemia inhibitory factor.

Production of J2E cell lines stably transfected with Mlf1 has been described in detail elsewhere (2). Here the Mlf1 mutant (Mlf1-S34A) was cloned into the pMSCV-neo retroviral vector. PA317 packaging cells were transfected with this construct, and viral supernatants were used to infect J2E cells. Individual clones were isolated from methylcellulose cultures (2).

For confocal microscopy, transient transfections of COS7 cells were performed on coverslips in 12-well plates. Cells were transfected with 0.42 μ g of DNA using 1.2 μ l of Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For immunoblotting, immunoprecipitations, and DNA binding assays COS7 cells were grown in 10-cm dishes. Following leptomycin B treatment, COS7 cells were transfected with Mlf1 for 20 h and then treated with either 10 μ g/ml leptomycin B or an equal volume of Me₂SO for 4 h.

pMSCV, pMSCV-Mlf1, and pMSCV-Mlf1-S34A constructs were transfected into the ψ 2 ecotropic retroviral packaging line for infection of hemopoietic progenitor cells from CBA mice (21). 8 \times 10⁵ fetal liver (day 12) cells were co-cultivated for 2 days with irradiated (3000 rads), virus-producing packaging cells in the presence of 10% WEHI 3B D⁻ conditioned medium and 50 μ g/ml transferrin. The cells were washed extensively, and then 2 \times 10⁴ cells/30-mm dish were cultured in Iscove's modified Dulbecco's medium containing 10% WEHI 3B D⁻, 0.9% methylcellulose, 18.75% fetal calf serum, 50 μ g/ml transferrin, and 1 mg/ml Geneticin in the presence or absence of 5 units/ml erythropoietin. Hemoglobin-positive burst-forming units-erythroid were visualized after benzidine staining (22).

Confocal Microscopy—Indirect immunofluorescence was performed on hemopoietic cells and transiently transfected COS7 cells fixed with methanol:acetone (1:1). Protein was detected either directly using green fluorescent protein (GFP) or using antisera against Mlf1 or Manp followed by anti-IgG Alexa Fluor 488/546 secondary antibody (Invitrogen). Coverslips were mounted in 50 mM Tris-HCl, pH 8.0, 2.5% 1,4-diazobicyclo-[2.2.2]-octane (Fluka, New South Wales, Australia) containing 0.00005% Hoechst 33258 (Calbiochem). Fluorescence was visualized on an MRC 1024 UV laser scanning confocal microscope (Bio-Rad). The cytoplasmic:nuclear ratio was determined using Comos software to analyze confocal images. Sample size was 20 \times 30 pixels (n > 300).

Immunoprecipitation and Immunoblotting—COS7 cells were transiently transfected with pcDNA3 constructs expressing genes of interest, using Lipofectamine 2000, followed by harvesting 24 h post-transfection. Protein lysates were prepared using 1% Nonidet P-40, 0.5% deoxycholic acid, 150 mM sodium chloride, 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 2 mM EGTA, 10 mM sodium fluoride, 1 mM sodium vanadate, 1 mM benzamide, 1 mM phenylmethylsulfonyl fluoride, and 1 μ g/ml aprotinin. Immunoprecipitations were performed with 0.5 mg of protein lysate with antibodies as indicated and protein G-Sepharose (Sigma). Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with specific antibodies before detection using enhanced chemiluminescence (Amersham Biosciences).

GST Pulldown Assays—Mlf1 protein was produced using the TNT[®] coupled reticulocyte lysate system (Promega, Madison, WI) according to the manufacturer's instructions. pcDNA3-Mlf1 (1 μ g) was incubated with 12.5 μ l of TNT rabbit reticulocyte lysate, 1 μ l of reaction buffer, 1 μ l of T7 polymerase, 1 mM amino acid mixture minus methionine, 1 μ Ci of [³⁵S]methionine, and 20 units of RNasin in a 25- μ l reaction at 30 °C for 90 min. Following incubation, analysis of the translation products was performed by SDS-PAGE. Equal amounts of protein were

A

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1 M E V K R L K V T E L R S E L Q R R R G L D S R G L K M D L A
1 ATGGAGGTGAAGCGGCTGAAGTGAACCGAGCTGCGGT CGGAGCTTCAGCGGGGGGCGCTGGATTGCGCGGGCTCAGAAATGGATCTGGCG
31 Q R L Q E A L D A E H L E D E A G V G G A G P G G A C K A E
91 CAGCGCTTTCAGGAGGCCTGGATGCTGAGATGCTGGAGGACAGGCGCGCTTGGTGGGGCCGGGCTGCRAGGCGGGAG
61 P R P V A A S G G T P G G D E E E E D D D E E E D E E A L L
181 CCTCGGCTGTGGCCGCT CGGGCGGAGGCCCGGGCGGGGACGAGGAGGACCGACGAGGAGGAGGACCGAGGAGGCGCGCTGCTT
91 E D E D E E P P P A Q A L G Q A A Q P P P P P P P P P E T S A M E
271 GAGGACGAGGACGAGGACRCCRCCTGCTAGGCTT TGGGCCAGGCCGCGACGCGCTC CGGAGCCCGGAGCGCT CAGCCATGGAG
121 A E S E A S D T P A E A T A G S G G V N G G E E H D N G K G
361 GCGGAGTCCGAGCTTCCGACACACCGGCGGAGGCCCGGCTGGTCCAGTGGGGTAARTGTTGGCGAAGACATGACACCGGCGAGGGG
151 E E D G E E P P P A R S G D E T P G S E A P G D K A V E E O G D D
451 GAGGAGACGGGCGGAGRACGGGAGCGGAGACGAGACTCCGGATCCGAGGACCGGGTACAGGCGCGTACAGGAGGAGGAGGATGAC
181 Q D S E K S K P A G S D G E R R G V K R Q R D E K D E H G R
541 CAGATAGTGAAGTCAARACGAGCAGGCTCAGATGTTGAGCGGGGGGTAARAGACAGCGGATGAGAAGGATGACATGGCCGA
211 A Y Y E F R E A Y H S R S K S P P P P P P P P P E E A K D E E E D
631 GCCTACTATGATTCGAGAGGAGGCTTCCACAGCCGCTCAAGTCCCCACCCCTCCGAGAGGAGGAGCAAGATGAGAGGAGGAGC
241 Q T L V N L D T Y T S D L H F Q I S K D R Y G G Q P L F S E
721 CAGACCTTGTGACCTGGACAGCTATACCTCGGATCCCTTTTCARATAGCAAGGCGATTTGGAGGCCAGCCGCTCTTCTCAGAA
271 K C P T L W F G A R S T Y G V T K G K V C T F E A K V T Q N L
811 AGTTCCTACCTTTGGTTGGGGCAGAGTACTTTGGAGTACCAAGGGGAAAGTATGCTTTGAGGCGAAGGATCTCAGATCTC
301 P M K E G C T E V S L L R V G W S V D P S C S Q L G E D E F
901 CCATGAGAGGAGGCTGACAGAGTCTCTCTCCTTAGAGTGGTGGTCTGTGATTTTCTGTTCACAGCTGGGAGAGGAGGATTC
331 S Y G F D G R G L K A E N G Q F E E F G Q R F D E K D E V I G
991 TCTACGGTTTTCAGGAGGAGGCTCAGGCGAGAAACCGGGCAGTTTGGAGAAATTTGGCCAGCTTTTGGGAGAGATGATGTTATGGC
361 C F A N F T E E V E L S F S K N G E D L G V A F R I S K E
1081 TGTTTTCTAARTTTGAGACTGAGAGAGTGTAGCTTCTCTCCAGATGAGGAGAGGACTTGGGTGTGGCTTCCGATTTAGCAGGAG
391 S L A D R A L L P H V L C K N C V V E L N F G Q K E E P P F
1171 TCTCGGAGATCGAGCCCTTCTACCCATGCTCTTGCAGAAATTTGTCGTAATTAACCTTTGTCAGAGGAGGAGCCCTTTTTC
421 P P P E E F V F I H A V P V E E R V R T A V P P K T I E E C
1261 CCACCCAGAGAGTGTGTCTCCATCTGCTGCTGCTGTTGAGGAGCGTGTGCGGACTGCGACTCCCCAGRCCATAGAGGAGTGT
451 E V I L M V G L P G S G K T Q W A L K Y A K D M P E R R Y N
1351 GAGGTGATTCGATGGTGGCTCTGAGATCTGGAAAGCCAGTGGGCACTGAAATTCGCAAGGACACCCCTGAGAGAGATACAT
481 V L G A E T V L Q M R M K G L E E P E M D P K S R D L L V
1441 GTTCTGGAGCTGAGACTGTTCTCAGCBAATGAGGATGAGGGACTTGGAGGACGAGAGATGGCCCAAAAGCCGAGACCTCTTAGT
511 Q Q A S Q C L S K L V Q I A S R S K R N F I L D Q C N V Y N
1531 CAGCAGCCTCCAGTCCCTTAGTAGCTGGTCCAGATTTGCTCAGATCAAGAGGAAATTTTATCTTGATCAGTGTARTGTGTACAC
541 S G Q R R K L L L F K T F S R K V V V V V P N E E D W K R R
1621 TCTGGCCAGCAGGAGTACTGCTGTTCAAGACTTCTCAGGAGGTTGGTGTAGTTGCTTCTAATGAGGAGGATTTGAGAGGAGG
571 L E L R K E V E G D D V P E S I M L E M K A N F S L P E K C
1711 CTGGAGTGGAGAGAGTGGAGGAGATGATGCTGAGTCTATTAATGCTGGAGATGAAAGCCACTTCTCTGCTGAGAGATGTC
601 D Y M D E V T Y G E L E K E E A Q P I V T K Y K E E A R K L
1801 GACTATGATGAGTGAATGATGAGGAGCTGGAARAGGAGGAGCTACCCATTTGTAACATACAGGAGGAGGAGCAGGAGGACTA
631 L P P S E K R T N R R N R N R N K R N R Q N R S R G Q G Y V G
1891 CTGCCCCCTCTGAGAGGTCARACCCCGGACRACCCGACRAGAGAAACCGGCRGACCGGAGCCGAGGCGGAGGCTATGTTGGCC
661 G Q R R R G Y D N R A Y G Q Q Y W G Q S T G S N R G G Y R N F Y D
1981 GGGCAGCCGCGAGCTACGACAGCCGGGCTACGGCCAGCAGTACTGGGGGCACTGGAACAGGAGGGGGCTACCGTATTTCTATGAT
691 R Y R G D Y E R F Y S R D Y E Y N R Y R D Y Y R Q Y N R D W
2071 CGATCAGAGGTCATATGACGATCTCAGCTGAGATATGATGACACAGATACAGGACTATTACAGCAATACACCGGGGRTTG
721 Q N Y Y Q H Q D D R D R Y R N Y Y G Y G Y R -
2161 CAGAACTACTACTACCACCACRACGAGGACGAGACCAGTACTACAGGATTTACTACGGTTACRAGGGGTATCGGTGA
    
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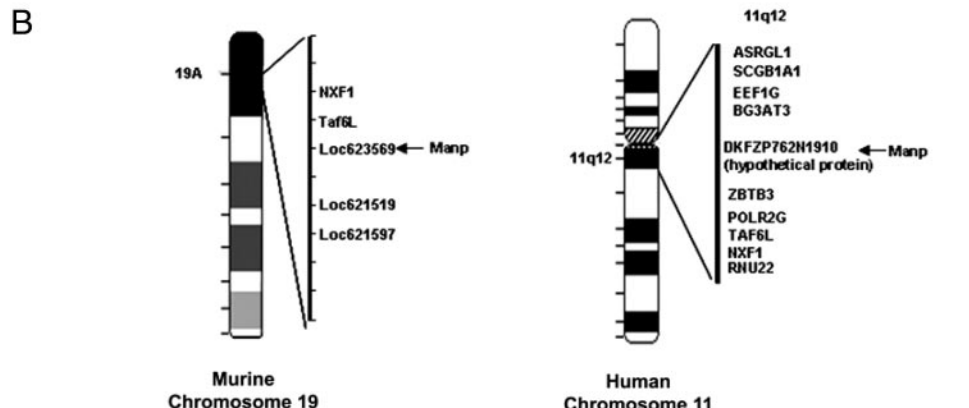


FIGURE 1. Analysis of Manp sequence. A, nucleotide sequence of murine Manp and the predicted amino acid sequence. B, localization of Manp to murine chromosome 19 and human chromosome 11q12 showing the approximate location of neighboring genes.

incubated with 1 ng of GST or GST·14-3-3ζ in protein lysis buffer at 4 °C for 2 h. Proteins were washed four times, separated by SDS-PAGE, and then transferred to polyvinylidene difluoride membranes.

DNA Binding Assay—DNA binding assays were performed using a modification of the method of Leaw *et al.* (23). Cell lysates (1 mg) from COS7 cells transiently transfected with pcDNA3·Mlf1 in DNA binding buffer (10 mM Tris-Cl, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Nonidet P-40, 50 mM

sodium fluoride, 0.1 mM sodium vanadate, 1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride) were applied to double-stranded calf thymus DNA-cellulose (Sigma) that had been previously blocked with bovine serum albumin (10 mg) in DNA binding buffer. Following overnight incubation at 4 °C, DNA-cellulose was washed extensively before elution with a stepwise gradient of KCl (0–1000 mM in DNA binding buffer). Eluted fractions were separated by SDS-PAGE, and bound proteins were detected by immunoblotting.

Luciferase Assays—Reporter assays were carried out using the Dual-Luciferase® reporter assay system (Promega). COS7 cells were transfected with pGL3-basic containing ~1 kb upstream of the murine multidrug resistance protein 1 (*Mdr1*) or *c-Myc* intron-binding protein 1 (*MIBP1*) transcriptional start site and pcDNA3 containing *Mlf1* and/or *Manp*. TK promoter activity was used to normalize for transfection efficiency, and empty pcDNA3 vector was used to normalize the effects of Mlf1 and/or Manp. Lysates were analyzed using a FLU-Ostar OPTIMA plate reader (BMG Labtech, Offenburg, Germany). Each experiment was performed at least three times.

Microarray—RNA was prepared from J-MSCV and J-Mlf1 cell lines using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Biotinylated cRNA was prepared from 2 μg of total RNA and hybridized to Affymetrix oligonucleotide microarrays, MG-U74Av2 (Affymetrix, Santa Clara, CA), following the manufacturer's protocols. Array images were reduced to intensity values for each probe using Affymetrix MicroArray Suite version 5.0. Changes in gene expression determined by microarray analysis were then checked by real time PCR or quantitative PCR. To date 70% of changes have been confirmed with no false positives identified.

RESULTS

Mlf1 Associates with Nuclear Protein Manp—A yeast two-hybrid screen to isolate binding partners of Mlf1 identified sev-

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Mlf1 Binds hnRNP-U-like Protein

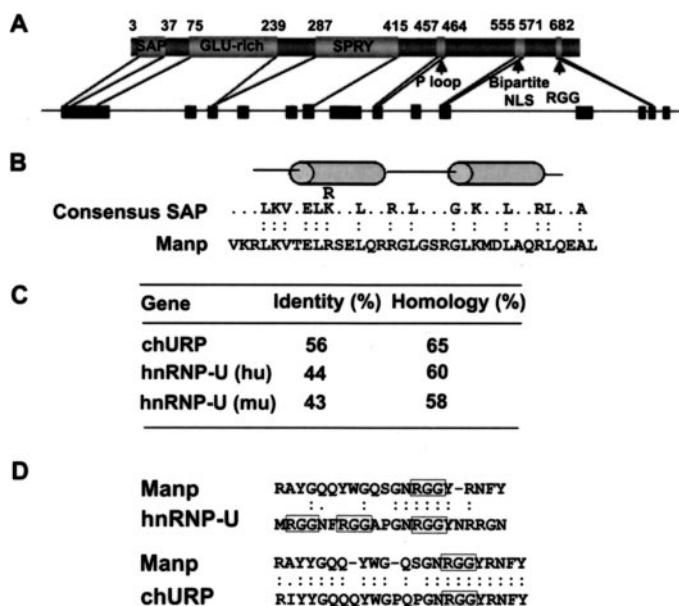


FIGURE 2. Manp domains. *A*, schematic representation of the predicted domains in Manp. The genomic organization is shown below. *B*, schematic representation of secondary structure of a SAP module (two amphipathic helices) and alignment of consensus SAP domain (60) with the predicted SAP domain of Manp. *C*, Manp identity and homology with chURP (gi3822553) (30), human (*hu*) hnRNP-U (gi6226894) (61), and murine (*mu*) hnRNP-U (gi7949051) (62). Values were obtained using BLASTP (NCBI). *D*, alignment of Manp with the RGG box of hnRNP-U and chURP. RGG motifs are boxed. NLS, nuclear localization signal. All sequences were obtained from the NCBI database.

eral molecules, including 14-3-3 ζ and two novel proteins, Madm (7) and Manp. Using the fragment of *Manp* obtained from the yeast two-hybrid screen, a full-length cDNA was then isolated. The nucleotide and predicted amino acid sequences of *Manp* are displayed in Fig. 1A. The gene localized to murine chromosome 19 and 11q12 in humans (Fig. 1B). Interestingly a number of genes involved in transcription are clustered within this syntenic region. *MANP* is described as a hypothetical protein on 11q12 and is located in the vicinity of several genes associated with transcriptional regulation, e.g. *POLR2G* (polymerase RNA II (DNA-directed) polypeptide G), *TAF6L* (TAF-like RNA polymerase II), and *NXF1* (nuclear RNA export factor 1) (24–26). Furthermore it has been reported that chromosomal abnormalities at 11q12 can result in “anemia with excessive blasts in transformation” (data available from Mitelman Database of Chromosome Aberrations in Cancer, cgap.nci.nih.gov/Chromosomes/Mitelman).

Analysis of the amino acid sequence revealed that Manp has several discrete domains (Fig. 2A). The N terminus contains a SAP domain (Fig. 2, A and B), which is present in numerous nuclear proteins and has DNA binding properties (27), whereas the C terminus has a bipartite nuclear localization signal. The presence of the SAP domain and nuclear localization sequence strongly indicates that Manp is a nuclear protein. Other domains in Manp include a SPRY region and a P loop; SPRY domains are found in several proteins, but the function of this domain has yet to be elucidated (28), whereas P loops are involved in ATP binding (29).

Manp has significant homology with hnRNP-U-like molecules (Fig. 2C) and is most probably the murine orthologue of

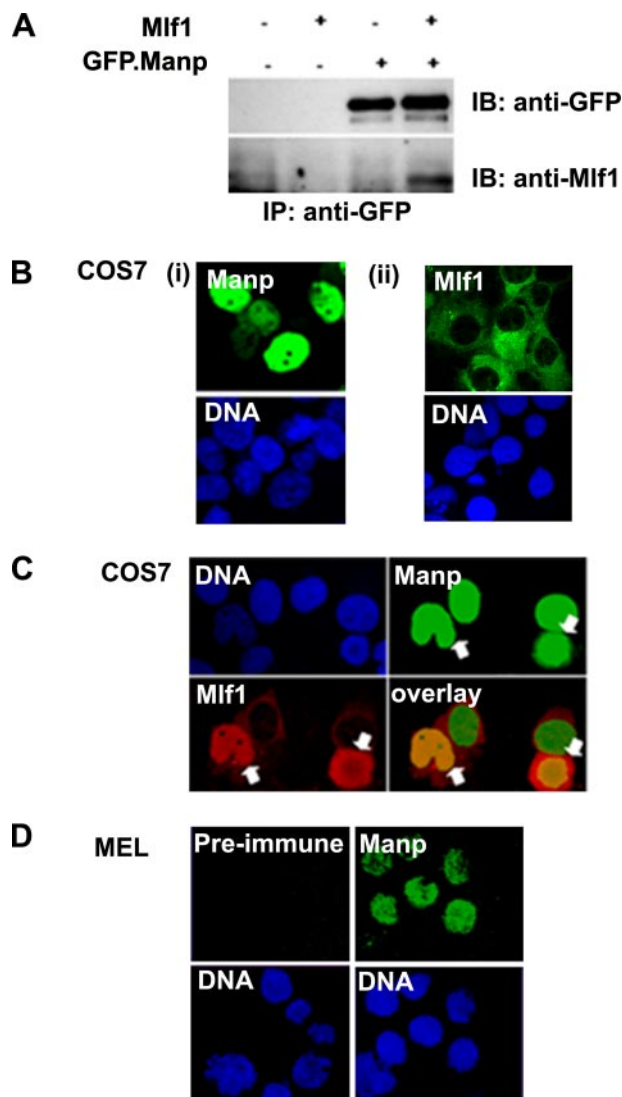


FIGURE 3. Association of Mlf1 with Manp. *A*, lysates from COS7 cells transfected with *Mlf1* and *GFP-Manp* were immunoprecipitated (IP) with anti-GFP antibodies and immunoblotted (IB) with antibodies against GFP or Mlf1. *B*, COS7 cells were transfected with (i) *GFP-Manp* or (ii) *Mlf1* and visualized by confocal microscopy. *C*, COS7 cells were co-transfected with *GFP-Manp* and *Mlf1* and then visualized using confocal microscopy. Arrows indicate co-localization of Mlf1 and Manp in the nucleus, which was counterstained with Hoechst 33258. *D*, cytocentrifuge preparations of MEL cells incubated with either preimmune or Manp antisera visualized by confocal microscopy. Nuclei were counterstained with Hoechst 33258.

the chicken protein chicken hnRNP-U-like molecule (chURP) (30). The protein also contains a potential RNA-binding RGG motif (Fig. 2D). Because hnRNP-U, also known as scaffold attachment factor-A (SAF-A) has been implicated in the regulation of numerous aspects of gene expression (31–33), Manp may also play a role in transcript production and processing.

To confirm the yeast two-hybrid interaction between Mlf1 and Manp, COS7 cells were co-transfected with constructs expressing these molecules. Immunoprecipitation of Manp followed by immunoblotting with anti-Mlf1 antibodies demonstrated that the two proteins did indeed interact (Fig. 3A). Confocal microscopy revealed that exogenous Manp localized almost exclusively in the nucleus of transiently transfected COS7 cells (Fig. 3B, *i*). As described previously (1, 2), Mlf1 was found in the cytoplasm of

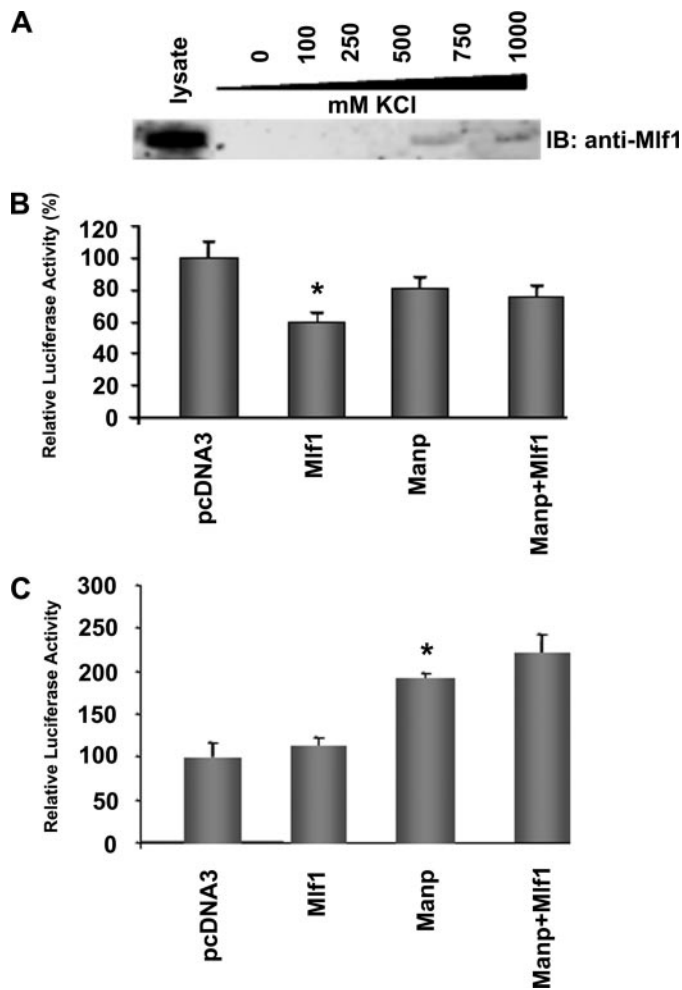


FIGURE 4. Mlf1 binds DNA. *A*, Mlf1 bound to calf thymus DNA-cellulose was eluted with increasing concentrations of KCl. An aliquot of each eluted fraction was separated electrophoretically, and bound proteins were detected by immunoblotting (*IB*). *B*, luciferase reporter assays for the *MIBP1* promoter were performed in the presence of Mlf1 and/or Manp. The normalized values are expressed as a percentage of control (pcDNA3). *C*, luciferase reporter assays for the *Mdr1* promoter were performed in the presence of Mlf1 and/or Manp. The normalized values are expressed as a percentage of control (pcDNA3). Error bars represent mean \pm S.D. ($n = 3$) with * representing $p < 0.01$.

transiently transfected COS7 cells with limited punctate nuclear staining (Fig. 3*B*, *ii*). Significantly in a portion of the cells expressing both proteins ($\approx 15\%$), Mlf1 translocated completely from the cytoplasm to the nucleus and displayed diffuse nuclear staining (Fig. 3*C*, *arrows*). Quantification of the Mlf1 nuclear:cytoplasmic content showed that this redistribution of Mlf1 in the presence of Manp was statistically significant ($p < 0.001$) by analysis of variance. This observation indicates that Manp can redirect Mlf1 into the nucleus. Importantly antibodies raised against Manp detected the endogenous protein in the nuclei of MEL cells (Fig. 3*D*). These data demonstrate that Mlf1 associates with a novel hnRNP family member in the nucleus.

Mlf1 Binds DNA and Influences Gene Expression—Because Mlf1 relocated to the nucleus when co-transfected with Manp, we examined its ability to bind DNA. To this end, DNA was incubated with lysates from COS7 cells transiently transfected with Mlf1 in the presence of varying concentration of KCl; proteins associated with DNA were then identified by immuno-

TABLE 1

Genes differentially regulated by microarray in the presence of Mlf1

Accession number	Gene	Change	Function
		%	
AB031037	<i>TBR2</i>	370	Transcription
U28423	<i>p58</i>	140	Transcription
AF077659	<i>HIPK2</i>	-100	Transcription
X95316	<i>USF1</i>	-100	Transcription
U80821	<i>C1</i>	-120	Transcription
AF084480	<i>wbscr9</i>	-150	Transcription
Y15907	<i>MIBP1</i>	-210	Transcription
U63387	<i>MPc2</i>	-280	Transcription
AI882555	<i>ets-1</i>	-320	Transcription
U20159	<i>SLP-76</i>	330	Signaling
D78354	<i>TRA1</i>	-200	Signaling
U66873	<i>PLA2V</i>	-290	Signaling
D37837	L-plastin	-330	Cytoskeleton
X66449	Calcyclin	-340	Differentiation
AB027125	<i>AKR1C13</i>	-200	Enzyme
U48896	<i>UGT</i>	-300	Enzyme
AF055638	<i>CR6</i>	-200	Growth arrest
D38218	<i>RyR3</i>	-190	Transporter
M60348	<i>MDR1</i>	600	Transporter
U19271	<i>DEC205</i>	350	Receptor, endocytic
AA793993	<i>DPPA4</i>	-210	Developmental

blotting with specific antibodies. Fig. 4*A* shows that Mlf1 could attach to DNA; under similar experimental conditions c-Myc bound DNA at KCl concentrations between 500 and 750 mM (data not shown). These results show that Mlf1 can translocate to the nucleus and can bind DNA.

Microarrays were then used to identify genes that might be affected by Mlf1. Table 1 shows that transcripts from a number of genes were altered 2-fold or more by exogenous Mlf1; the mRNA levels of some genes increased, whereas others decreased. This experiment revealed that several genes that regulate gene transcription were influenced by Mlf1, *e.g.* *USF1* (upstream transcription factor 1), *MIBP1*, and E26 avian leukemia oncogene 1 (*Ets-1*). Notably Mlf1 suppressed calcyclin and *CR6* (cytokine response protein), genes associated with differentiation and growth arrest; these observations are consistent with the ability of Mlf1 to inhibit erythroid terminal differentiation and prevent cell cycle arrest (2, 12). Unexpectedly the *Mdr1* transporter was markedly elevated in Mlf1-expressing cells. These data show that Mlf1 affected expression of a variety of genes, including a number of transcription factors.

As Mlf1 was able to bind DNA and influence gene expression, we wished to determine whether it could influence transcription. To test this proposition luciferase reporter assays were established using the *MIBP1* and *Mdr1* gene promoters. The *MIBP1* and *Mdr1* genes were chosen because they were down-regulated (210%) or up-regulated (600%), respectively, by Mlf1 in microarray analyses (Table 1) and by quantitative PCR (data not shown); additionally the *Mdr1* promoter has been well characterized (34). The effects of Manp on these promoters were also evaluated as hnRNP-U has been shown to affect transcription (31–33). Fig. 4*B* shows that the *MIBP1* promoter was reproducibly inhibited by Mlf1, consistent with the decrease in *MIBP1* transcripts (Table 1). However, no additive effects were observed with Manp. In contrast Mlf1 had no effect on *Mdr1* promoter activity, whereas Manp induced a 2-fold rise in transcriptional activity (Fig. 4*C*). Together these data demonstrate that Mlf1 and Manp could influence gene expression. Although the effects of Mlf1 and Manp on the *MIBP1* and *Mdr1*

Mlf1 Binds hnRNP-U-like Protein

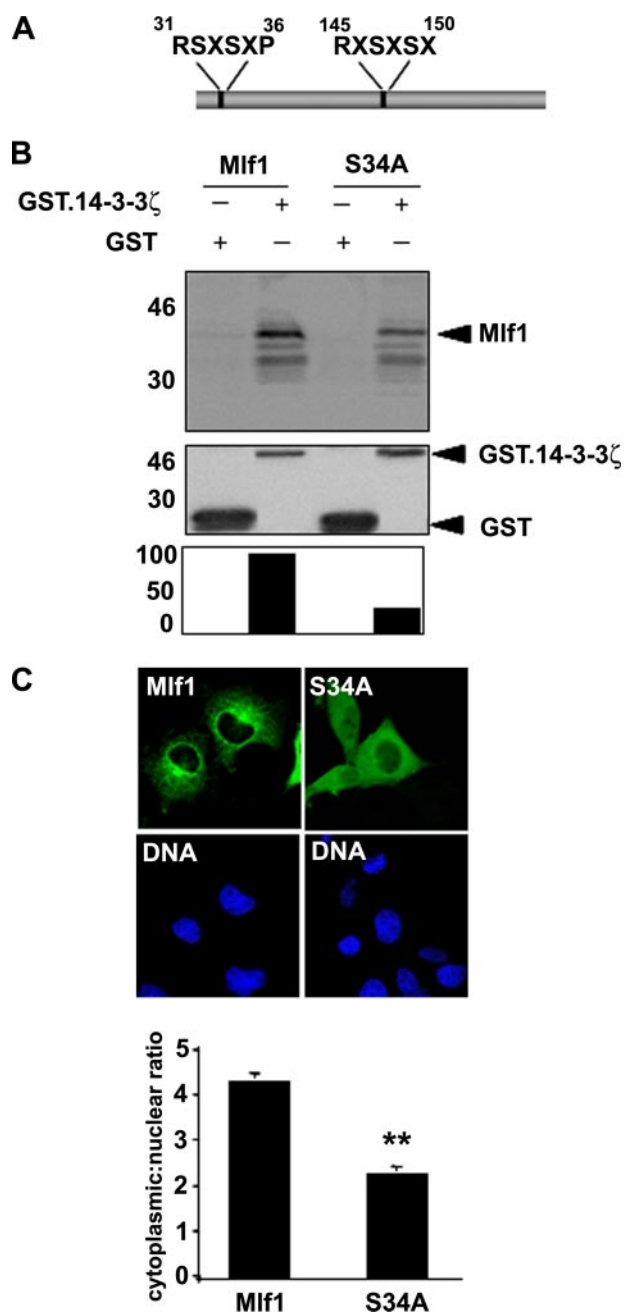


FIGURE 5. 14-3-3 binding affects Mlf1 subcellular localization. *A*, schematic representation of Mlf1 protein indicating canonical (RSXSXP) and non-canonical (RXSXSX) 14-3-3 binding sites. *B*, *in vitro* pull-down assays of [³⁵S]methionine-labeled Mlf1 with purified GST-14-3-3ζ and GST bound to glutathione-agarose (nonspecific binding control). *C*, COS7 cells transfected with Mlf1 and S34A were incubated with anti-Mlf1 antisera and visualized by confocal microscopy. The cytoplasmic:nuclear ratio of Mlf1 was determined. Error bars represent means ± S.E. with ** representing $p < 0.001$ ($n > 120$).

promoters were modest, it is possible that these proteins may have a greater effect on other regulatory regions of these genes; alternatively, Manp may modulate chromatin assembly as seen with some hnRNP family members (35, 36).

Mlf1 Subcellular Localization Is Affected by 14-3-3ζ—We sought to examine mechanisms that might regulate Mlf1 nuclear-cytoplasmic shuttling because the protein is located primarily in the cytoplasm of transiently transfected COS7 cells but relocated to the nucleus when co-expressed with Manp (Fig.

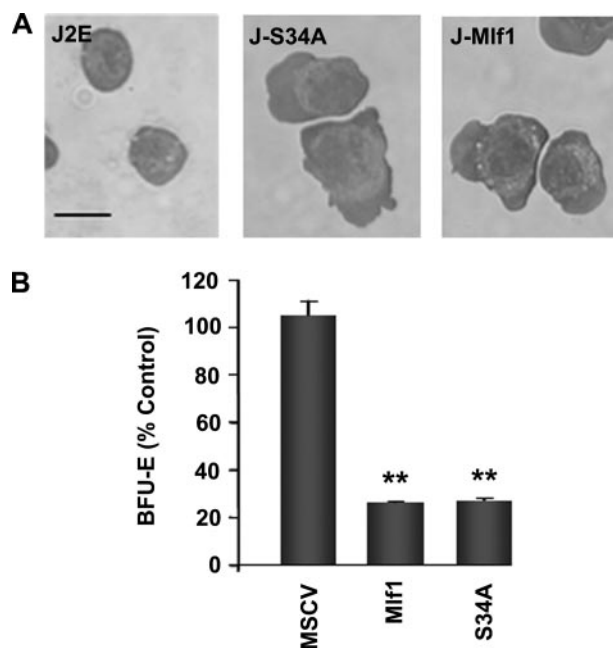


FIGURE 6. S34A does not affect biological effects of Mlf1. *A*, Wright-Giemsa-stained cytocentrifuge preparations of J2E cells or J2E cells stably transfected with Mlf1 (J-Mlf1) or the Mlf1-S34A mutant (J-S34A). The bar represents 10 μm. *B*, fetal liver cells were co-cultivated with packaging cell lines expressing either MSCV, MSCV-Mlf1 (Mlf1), or MSCV-Mlf1-S34A (S34A) retroviruses and then plated in methylcellulose in the presence of erythropoietin. Each result is the mean ± S.D. with ** representing $p < 0.001$ ($n = 3$). BFU-E, burst forming unit-erythroid.

3C). Significantly Mlf1 bears a classic RSXSXP 14-3-3 binding motif (amino acids 31–36) as well as a non-consensus RXSXSX motif (amino acids 145–150) (Fig. 5A); moreover a yeast two-hybrid screen identified 14-3-3ζ as an Mlf1-interacting protein. To determine the impact of 14-3-3ζ binding on Mlf1 subcellular localization, the serine residue in the classic 14-3-3 motif was mutated (S34A). Fig. 5B shows that mutation of this site resulted in an appreciable reduction (≈70%) in Mlf1 binding to 14-3-3ζ. The S34A mutation also resulted in an increase in the nuclear content of Mlf1; whereas wild type Mlf1 localized primarily in the cytoplasm of transiently transfected COS7 cells, the S34A mutant showed increased levels of Mlf1 in the nucleus (Fig. 5C). As a consequence the cytoplasmic:nuclear ratio decreased (Fig. 5C). These data indicate that the association of Mlf1 with 14-3-3ζ influences its subcellular compartmentalization.

Among the most significant biological effects of Mlf1 are its ability to (i) alter the phenotype of J2E erythroleukemic cells and (ii) suppress growth of normal erythroid progenitors (2, 12). J2E cells do not express Mlf1, and expression of exogenous Mlf1 induces a remarkable change from a proerythroblastoid to monoblastoid appearance (2). To determine whether altering the subcellular localization of Mlf1 influenced this phenotypic change, the S34A mutant of Mlf1 was introduced into J2E cells via retroviral transduction. Strikingly each of the clones ectopically expressing the mutated Mlf1 also altered their phenotype (Fig. 6A). Like cells expressing wild type Mlf1, these stably transfected J2E cells no longer resembled proerythroblasts; instead they displayed a blastlike morphology. Moreover the S34A mutant did not prevent the Mlf1-induced reduction of

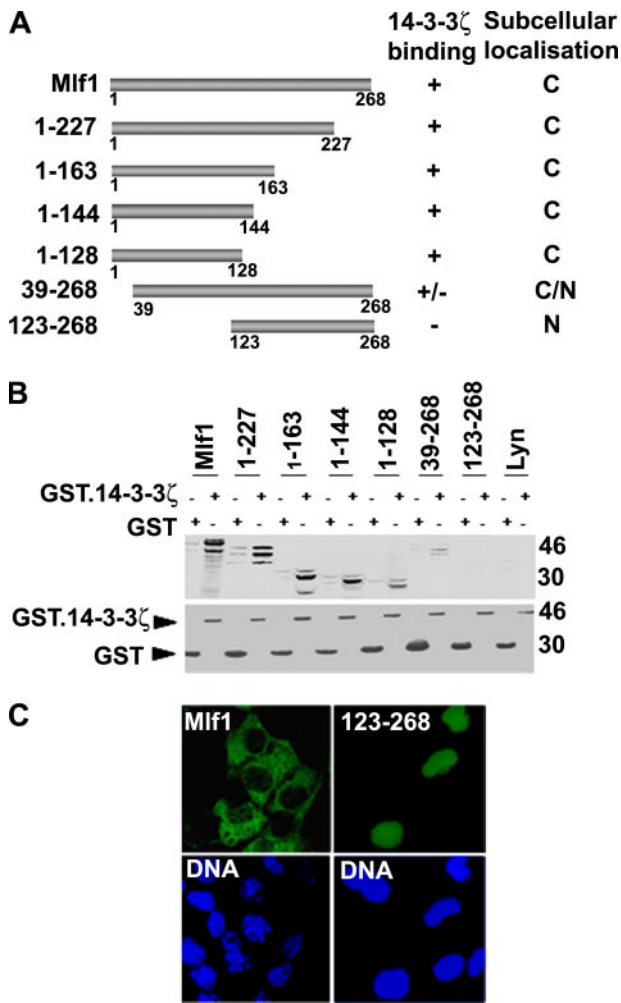


FIGURE 7. 14-3-3 ζ binds to the N terminus of Mif1. *A*, Mif1 deletion constructs were generated in pcDNA3. *B*, *in vitro* pull-down assays were performed using [³⁵S]methionine-labeled truncated proteins incubated with purified GST-14-3-3 ζ and GST bound to glutathione-agarose (nonspecific binding control). *C*, COS7 cells were transfected with full-length Mif1 or deletion mutant 123–268, incubated with anti-Mif1 antisera, and visualized by confocal microscopy. C, cytoplasm; N, nucleus.

erythroid progenitors (burst-forming units-erythroid) in methylcellulose colony assays (Fig. 6*B*). It was concluded that the S34A mutation altered Mif1 retention in the cytoplasm of COS7 cells but did not affect the biological consequences associated with elevated Mif1 expression.

Although the S34A mutation reduced the Mif1/14-3-3 ζ association, binding of the two proteins was not eliminated altogether (Fig. 5*B*). This indicated that other structural features, in addition to the RSXSXP motif, promoted the association with 14-3-3 ζ . The non-consensus RXSXSX binding motif was considered a potential association domain; however, mutation of serine residue 149 (S149A) within this motif failed to influence binding (data not shown). A series of deletion mutations were then designed in an attempt to define additional binding regions (Fig. 7*A*). Deletion to the C terminus of Mif1 had little impact on the interaction with 14-3-3 ζ (Fig. 7, *A* and *B*). In contrast, removal of the N-terminal 39 amino acids, which included the RSXSXP motif (amino acids 39–268), reduced binding significantly (Fig. 7*B*). The association between Mif1 and 14-3-3 ζ was extin-

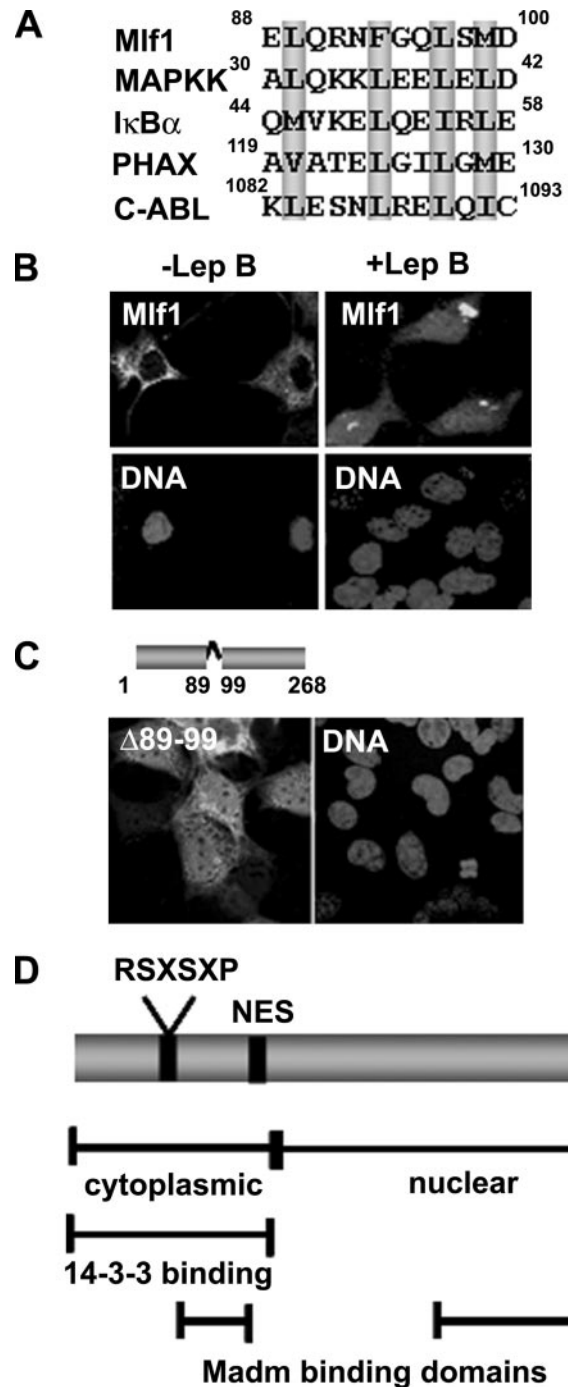


FIGURE 8. Mif1 has a functional NES. *A*, alignment of the potential NES of Mif1 (amino acids 88–100) with other known NESs. *B*, COS7 cells were transfected with Mif1 and then treated with leptomyacin B (*Lep B*). The cells were then incubated with anti-Mif1 antisera and visualized by confocal microscopy. *C*, Mif1 deletion mutant (Δ 89–99) lacking the NES was transfected into COS7 cells, incubated with anti-Mif1 antisera, and visualized by confocal microscopy. *D*, schematic representation of Mif1 binding domains.

guished with the 123–268 construct, which lacks the N-terminal 123 amino acids. These data indicate that 14-3-3 ζ associated with the RSXSXP motif and regions within the N terminus of Mif1. Importantly elimination of 14-3-3 binding resulted in almost complete nuclear localization of Mif1 (Fig. 7, *A* and *C*), supporting the notion that 14-3-3 binding plays a significant role in excluding Mif1 from the nucleus.

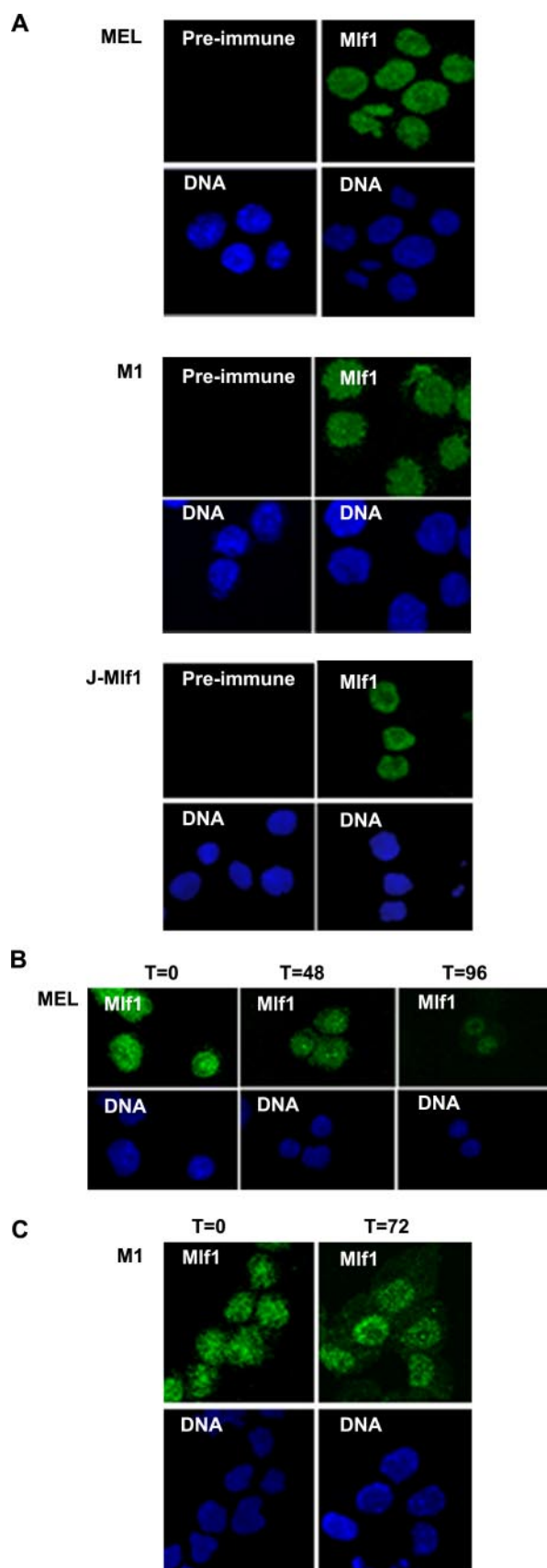


FIGURE 9. Nuclear localization of Mlf1 in hemopoietic cells. A, cytocentrifuge preparations of MEL, M1, and J-Mlf1 cells incubated with anti-Mlf1 antisera and visualized by confocal microscopy. Images using preimmune or anti-Mlf1 antisera were obtained using identical settings. Nuclei were

Nuclear Shuttling of Mlf1—As the subcellular distribution of Mlf1 was affected by Manp and 14-3-3 ζ , the amino acid sequence of Mlf1 was examined for motifs that might influence nuclear localization. Close scrutiny of the Mlf1 sequence revealed a potential nuclear export signal (NES) between residues 88 and 100. The classical NES pattern is LX_{2,3}(L)X_{2,3}LX(L) where (L) can either be Leu, Ile, Val, Phe, or Met (37). Fig. 8A shows an alignment of the putative NES of Mlf1 with other functional NESs (37). As leptomycin B inhibits nuclear export (38), this compound was added to transiently transfected COS7 cells, and the effects on Mlf1 subcellular localization were determined. Fig. 8B shows a significant accumulation of Mlf1 in the nucleus after leptomycin B treatment. Similarly deletion of the NES resulted in much more Mlf1 being retained within the nucleus (Fig. 8C). These observations indicate that Mlf1 possesses a functional NES.

The various binding domains of Mlf1 are summarized in Fig. 8D. The N terminus of the molecule contains the 14-3-3 binding region and an NES; these domains promote cytoplasmic localization. Conversely the C terminus of Mlf1 localized the protein to the nucleus. Interestingly two portions of the molecule have been shown to bind Madm (7).

Nuclear Localization of Mlf1 in Hemopoietic Cells—Having established that Mlf1 could translocate to the nucleus in transiently transfected COS7 cells, we wished to determine the subcellular localization of Mlf1 in hemopoietic cells. Antibodies were raised against the protein, and confocal microscopy was used to define Mlf1 compartmentalization in immature blood cells. Data presented in Fig. 9A show that endogenous Mlf1 was located mainly in the nuclei of MEL cells and M1 monoblastoid cells. Furthermore exogenous Mlf1 expressed in J2E cells was primarily nuclear (Fig. 9A). Although the nuclear content of Mlf1 was retained as MEL cells underwent nuclear condensation during terminal differentiation, an increase in cytoplasmic Mlf1 was detected as the cells matured (Fig. 9B). The protein content of Mlf1 also decreased during MEL cell differentiation (Fig. 9B) in agreement with previous observations that *Mlf1* transcripts decreased (2). Mlf1 was retained in the nucleus of M1 cells as they developed into macrophages; however, an increasing proportion of Mlf1 appeared in the cytoplasm of mature cells (Fig. 9C). These observations contrast markedly with the predominantly cytoplasmic localization of exogenous Mlf1 in COS7 cells (1, 2) and NIH3T3 cells (data not shown). It appears that the subcellular localization of Mlf1 differs between hemopoietic and non-hemopoietic cells with the molecule residing preferentially in the nuclei of hemopoietic cells.

DISCUSSION

Mlf1 is an oncoprotein that can influence erythroid/myeloid lineage commitment, cell cycle exit, and differentiation (2, 12, 15); dysregulation of MLF1 can result in acute myeloid leukemia (1). Previous studies have reported that Mlf1 is located almost entirely in the cytoplasm (1, 2); data presented in this study demonstrate that Mlf1 possesses a functional NES and

counterstained with Hoechst 33258. MEL cells (B) and M1 cells (C) were induced to differentiate, then incubated with anti-Mlf1 antisera, and examined by confocal microscopy.

localized primarily in the nuclei of hemopoietic cells. Furthermore Mlf1 was capable of binding DNA and could modulate gene expression. Other reports have shown that Mlf1 associates with transcriptional regulators DREF and Mlf1IP/KLIP1 (5, 8, 9). Collectively these data suggest that Mlf1 plays a role in the nuclei of immature blood cells; it is noteworthy that several oncoproteins involved in leukemias have transcriptional regulatory roles (39).

A yeast two-hybrid screen identified Manp as a novel nuclear partner protein for Mlf1. Manp is a new member of the hnRNP family and is most probably the murine orthologue of chURP (30). Although the precise function of chURP has yet to be described, hnRNP-U/SAF-A plays an important role in regulating gene expression (31–33, 40). hnRNP-U/SAF-A is involved in RNA processing, DNA binding, and transcriptional regulation (27, 32, 41). The SAP domain present in hnRNP-U/SAF-A has been shown to bind to AT-rich regions associated with transcriptionally active chromatin (42–44). hnRNP-U is also part of a regulatory complex required for efficient transcription initiated by RNA polymerase II (33) and has been shown to exert transcriptional control over specific genes (31, 32, 40). Thus, Manp may also play a role in regulating gene expression within the nucleus; consistent with this possible function, Manp was able to enhance transcription, albeit modestly, of the *Mdr1* promoter.

Importantly Manp increased the nuclear content of Mlf1 in transiently transfected COS7 cells. As Manp is found in the nuclei of hemopoietic cells, it may be involved in redirecting Mlf1 into the nuclei of immature blood cells. It is intriguing to note that chromosomal abnormalities at 11q12, the locus to which Manp maps, can result in anemia with excess blasts in transformation; these clinical features are remarkably similar to NPM-MLF1-induced erythroleukemias (45). One possibility is that Mlf1 and Manp act in concert to induce leukemias if dysregulated. Other hnRNP family members have recently been linked to cancer; hnRNP-U has been shown to regulate the β -transducin repeat-containing protein ubiquitin ligase (40), which is implicated in colorectal cancer (46), whereas hnRNP-K has been shown to regulate the proto-oncogene *c-Myc* (47).

The amount of Mlf1 in the nucleus is governed, at least in part, by 14-3-3 ζ binding. Members of the 14-3-3 family are involved in the regulation of diverse cellular functions often by sequestering proteins from their site of action, e.g. the proapoptotic protein BAD (B-cell CLL/lymphoma 2 agonist of cell death) (48), transcription factors FKHRL1 (forkhead in rhabdomyosarcoma-like 1) (49) and NF-AT (nuclear factor of activated T-cells) (50), and the cell cycle protein phosphatase *cdc25C* (cell division cycle 25 homolog C) (51). The canonical RSXSXP motif in Mlf1, conserved among human, mice, and *Drosophila* (1, 2, 5, 6), plays an important role in regulating Mlf1 compartmentalization as mutation of the motif increased levels of Mlf1 in the nucleus. However, like a number of proteins, additional molecular structures at the N terminus of Mlf1 were required for complete association with 14-3-3 ζ (52–54).

Our data indicate that Mlf1 may reside in different subcellular compartments, and the model proposed in Fig 10 suggests that Mlf1 shuttles between the nucleus and cytoplasm. The

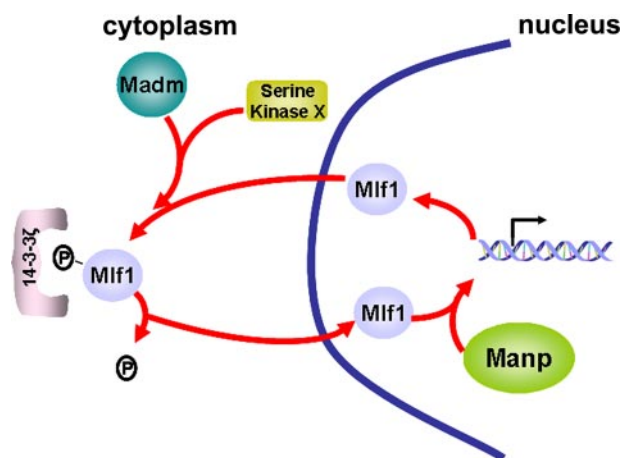


FIGURE 10. Model for regulation of Mlf1 shuttling between the nucleus and the cytoplasm.

Mlf1-binding adaptor protein Madm recruits a serine kinase that phosphorylates the RSXSXP motif of Mlf1 (7); thus, it mediates phosphorylation of Mlf1 and retention by 14-3-3 ζ in the cytoplasm. Conversely Manp promotes translocation of Mlf1 into the nucleus where it may function as a transcriptional regulator. Sequestration in the cytoplasm by 14-3-3 ζ is likely, therefore, to modulate nuclear activities of Mlf1. Significantly the leukemogenic fusion protein NPM-MLF1 does not bind 14-3-3 ζ (7) and is found almost exclusively in the nucleus (1).

Ectopic expression of Mlf1 in J2E cells altered the expression of several genes. A surprising number of transcriptional regulators were influenced by Mlf1, including *Ets-1* and *MIBP1*. Members of the *Ets* family have been strongly implicated in the control of erythroid proliferation and differentiation (55, 56). *MIBP1* is a molecule known to regulate *c-Myc* levels (57), influencing the growth and maturation of immature red blood cells (20, 58). Strikingly Mlf1 up-regulated *Mdr1* 6-fold. The gene for this transporter is highly expressed in hemopoietic stem cells (59), and the marked increase induced by Mlf1 coincided with the dedifferentiation of J2E cells toward a more blast-like phenotype (2).

Mlf1 appears to be a multifunctional protein (2, 12, 15). Several pieces of evidence presented in this study indicate that Mlf1 exerts some of its effects within the nucleus: (i) removal of the 14-3-3 binding region increased nuclear localization of Mlf1, (ii) Mlf1 has a functional NES, (iii) Mlf1 is primarily nuclear in hemopoietic cells, (iv) Mlf1 is capable of binding DNA, (v) Mlf1 associates with nuclear proteins, including Manp, and (vi) Mlf1 alters gene expression. How Mlf1 influences gene expression is not clear; its effects may be direct through DNA binding or indirect via interactions with nuclear partners, such as Manp.

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