Nuclear myosin I acts in concert with polymeric actin to drive RNA polymerase I transcription

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Actin is associated with all three nuclear RNA polymerases and acts in concert with nuclear myosin I (NM1) to drive transcription. Practically nothing is known regarding the state of actin and the functional interplay of actin and NM1 in transcription. Here we show that actin and NM1 act in concert to promote RNA polymerase I (Pol I) transcription. Drugs that prevent actin polymerization or inhibit myosin function inhibit Pol I transcription in vivo and in vitro. Mutants that stabilize the polymeric state actin are tightly associated with Pol I and activate transcription, whereas a polymerization-deficient mutant does not bind to Pol I and does not promote rDNA transcription. Consistent with nuclear actin and myosin synergizing in transcription activation, NM1 mutants that lack specific functions, such as binding to ATP, actin, or calmodulin, are incapable of associating with Pol I and rDNA. The results show that actin polymerization and the motor function of NM1 are required for association with the Pol I transcription machinery and transcription activation. These observations provide insights into the cooperative action of actin and myosin in the nucleus and reveal an actomyosin-based mechanism in transcription.

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scription in vivo (Pestic-Dragovich et al. 2000), and antibody-mediated depletion of either actin or NM1 inhibited transcription in vitro [Hofmann et al. 2004, Philimonenko et al. 2004]. Actin has been shown to associate with Pol I, regardless of whether or not it is engaged in transcription. NM1, on the other hand, is recruited to the Pol I transcription machinery by the transcription initiation factor TIF-IA, a factor that is bound to the initiation-competent subpopulation of Pol I [Philimonenko et al. 2004].

Although these studies have demonstrated the importance of nuclear actin and myosin for transcription by all three classes of nuclear RNA polymerases, the role of these motor proteins in transcription and other chromatin-based processes remains unclear. The challenge now is to understand the mechanisms that underlie the role of both proteins in transcription. Practically nothing is known regarding the state of actin and the interplay of actin and NM1 in the transcription process. It has been suggested that actin and NM1 may trigger a conformational switch of Pol I that is required for the transition of the initiation complex into the elongation phase. Alternatively, actin and NM1 may act as a motor that facilitates the movement of the transcription machinery [de Lanerolle et al. 2005]. These hypotheses, however, have never been experimentally validated. We still do not know whether the two proteins work together, whether ATP binding and hydrolysis by actin and myosin is required for transcription, and whether monomeric, polymeric, or “unconventional” forms of actin promote transcription. In this study we take the step toward deciphering the functional interplay of actin and NM1 in transcription. We show that polymeric actin cooperates with NM1 in transcription activation and NM1 functions as an actin-based molecular motor that powers Pol I transcription.

Results

Polymeric actin is required for transcription

Current models of nuclear actin function are speculative, with most discussions on the subject proposing that nuclear actin functions as a monomer or as short oligomers. To examine whether transcription activation depends on the state of actin, we treated cells with drugs that affect actin polymerization and monitored pre-rRNA synthesis by RT–qPCR. As shown in Figure 1A, phalloidin and jasplakinolide, drugs that favor the assembly of actin filaments, did not inhibit Pol I transcription. However, cytochalasin D and latrunculin B, drugs that inhibit actin polymerization, strongly decreased pre-rRNA synthesis. A similar result was obtained when transcriptional activity was monitored in vitro. Again, phalloidin and jasplakinolide did not affect run-off transcription in nuclear extracts, while transcription was inhibited in the presence of cytochalasin D and latrunculin B in a concentration-dependent manner [Fig. 1B]. In add-back experiments, purified actin was capable of restoring Pol I transcription in nuclear extracts that were incubated with anti-actin antibody [Fig. 1C, lanes 1–4]. This rescue of transcriptional activity did not occur in the presence of latrunculin B [Fig. 1C, lane 5], consistent with polymeric rather than monomeric actin promoting transcription.

Figure 1. Inhibition of actin polymerization decreases Pol I transcription. (A) Drugs that inhibit actin polymerization impair Pol I transcription in vivo. RNA was isolated from HEK293T cells treated for 2 h with dimethylsulphoxide (DMSO), phalloidin [Pha., 1 µM], jasplakinolide [Jasp., 1 µM], cytochalasin D [Cyto.D, 2 µM], or latrunculin B [Lat.B, 2 µM]. Pre-rRNA synthesis was measured by RT–qPCR using GAPDH mRNA as internal control. (B) Cytochalasin D and latrunculin B inhibit Pol I transcription in vitro. Nuclear extracts were preincubated for 30 min at 30°C with DMSO, phalloidin [Pha.], jasplakinolide [Jasp.], cytochalasin D [Cyto.D], or latrunculin B [Lat.B] before transcription was started. (C) Latrunculin B abolishes actin-mediated rescue of Pol I transcription. Nuclear extract was preincubated for 30 min either with buffer [lanes 1,2] or with 1 µg of anti-actin [Ac74] antibody [lanes 3–5] before recombinant Flag-tagged actin [5 µg] were added. The assay in lane 5 contained 10 µM latrunculin B [Lat.B] before transcription was started. (D) Cofilin inhibits Pol I transcription in vitro. [Top panel] Transmission assays were conducted after preincubation of nuclear extracts for 30 min at 30°C with the indicated amounts of purified profilin or cofilin. The Coomassie-stained polyacrylamide gel in the middle panel shows the amounts of added profilin and cofilin. [Bottom panel] To assay the effect of profilin and cofilin on actin polymerization, FM3A cell lysate was incubated with increasing amounts of profilin or cofilin, and the level of F-actin was monitored by ultracentrifugation and Western blot analysis of pelleted actin using Ac74 antibody.
To provide further evidence for the involvement of polymeric actin in Pol I transcription, we assayed profilin and coflin, proteins that regulate actin polymerization, in the cell-free transcription system. Profilins are small proteins that bind to monomeric actin and promote growth of actin filaments. Coflin, on the other hand, belongs to a family of actin-binding proteins that sever and depolymerize actin filaments [Paavilainen et al. 2004]. To examine the effect of profilin or coflin on transcriptional activity, we preincubated nuclear extract with increasing amounts of the two nuclear actin-binding proteins before transcription was started. In nuclear extracts, G-actin readily polymerizes into filaments [McDonald et al. 2006], and therefore exogenous profilin did not affect Pol I transcription in the nuclear extract [Fig. 1D, lanes 3–5]. In contrast, coflin inhibited transcription in a dose-dependent manner [Fig. 1D, lanes 6–8]. Notably, transcription inhibition correlated with the actin-depolymerizing activity of recombinant coflin [Fig. 1D, bottom panel, lanes 6–8], supporting the view that actin polymerization is required for Pol I transcription.

To elucidate the involvement of polymeric actin in transcription, we assayed several actin mutants for their capability to overcome antibody-induced inhibition of transcription. These mutants have been generated by the Treisman group [Posern et al. 2002, 2004] and shown to either stabilize F-actin (S14C, G15S, and V159N) or not to be incorporated into actin filaments (R62D). In the experiment in Figure 2A, nuclear extract was first preincubated with anti-actin antibody before recombinant wild-type or mutant actin was added and transcription was started. Transcription was restored by wild-type actin and mutants S14C, G15S, and V159N, all of which have been shown to stabilize F-actin [Posern et al. 2002, 2004]. In contrast, R62D, a mutant that does not incorporate into actin filaments, was incapable of rescuing transcriptional activity, supporting the view that polymeric actin powers transcription.

Given that the association of actin with RNA polymerase is important for transcription, polymeric rather than monomeric actin should be bound to Pol I. To test this, we monitored the association of wild-type and mutant actin with the Pol I transcription machinery. Immunoprecipitation assays revealed that all actin mutants that rescues in vivo transcription coprecipitated with TIF-IA and Pol I; i.e., they were associated with transcribing Pol I [Fig. 2B]. Importantly, the polymerization-deficient mutant R62D neither interacted with TIF-IA nor with Pol I, underscoring the link between actin polymerization, association with Pol I, and transcriptional activation.

If polymeric actin interacts with Pol I and is required for transcription activation, rDNA transcription and Pol I occupancy should be increased if the F-actin stabilizing mutants were overexpressed. Indeed, overexpression of S14C and V159N markedly activated Pol I transcription, whereas the nonpolymerizable mutant R62D did not increase transcriptional activity [Fig. 2C]. Chromatin immunoprecipitation (ChIP) assays revealed an increased binding of Pol I to rDNA after overexpression of actin, and Pol I association with rDNA was enhanced when S14C, the mutant that stabilizes F-actin, was overexpressed [Fig. 2D]. Again, the nonpolymerizable mutant R62D did not affect Pol I occupancy on rDNA. The strik-
ing correlation between actin-driven transcriptional activation and the polymerization competence of actin demonstrates that polymeric actin interacts with Pol I, and this interaction is required for transcription.

**Actin and NM1 work together to support Pol I transcription**

Previous studies have demonstrated that antibodies to actin inhibit transcription in cell-free transcription assays, and adding back exogenous actin partially restored transcriptional activity [Hofmann et al. 2004, Philimonenko et al. 2004]. To provide further evidence for actin and NM1 working hand in hand to support transcription, we inhibited Pol I transcription in nuclear extracts by an anti-actin antibody that recognizes the N terminus [amino acids 2–15] of actin, and assayed transcriptional activity after adding back purified actin, NM1, or both. Transcription inhibition by anti-actin antibody was partially overcome by exogenous actin, whereas NM1 alone did not increase transcriptional activity [Fig. 3A]. If both actin and NM1 were added to the reactions, complete transcriptional rescue was achieved. This result suggests that both proteins cooperate in transcription activation.

Myosin is a molecular motor that generates force in a unidirectional manner relative to filamentous actin through hydrolysis of ATP. This is a cyclic process in which ATP reduces the affinity for actin, whereas the hydrolysis product ADP traps the myosin–actin intermediate. To investigate whether ATP/ADP-dependent modulation of actin–myosin interaction is required for the association of actin and NM1 with the Pol I transcription apparatus, we performed communoprecipitation experiments in the absence or presence of ATP using extract from cells overexpressing V5-tagged NM1. Consistent with previous results showing physical association of NM1 and actin with the Pol I/TIF-IA complex, significant amounts of actin and NM1 were coprecipitated with TIF-IA or Pol I [Fig. 3B, lanes 2–7]. The interaction of NM1 and actin with the Pol I/TIF-IA complex was not affected by treating the lysates with RNase or ethidium bromide, demonstrating that the association of both proteins with the Pol I transcription machinery was not mediated by nucleic acids [Supplemental Fig. S2].

Significantly, in the presence of ATP the association of actin with the transcription machinery was prevented and the association of NM1 with Pol I was reduced [Fig. 3B, lanes 5,10], indicating that ATP binding and hydrolysis leads to dissociation of the actin–NM1 complex and to detachment of actin from the Pol I/TIF-IA complex. In contrast, the complex consisting of Pol I, TIF-IA, actin, and NM1 remained preserved in the presence of ADP [Fig. 3B, lanes 4,9], consistent with ADP trapping myosin in the actomyosin ATPase cycle. In the presence of ATP–γS, an ATP analog that is hydrolyzed 500 times more slowly than ATP, and therefore keeps myosin in the weakly bound state, a reduction of actin and NM1 binding to TIF-IA/Pol I was observed [Fig. 3B, lanes 5,10]. This implies that the actomyosin ATPase cycle governs the dynamic association of NM1 and actin with the Pol I transcription apparatus.

Next, we examined the effect of 2,3-butanedione 2-monoxime (BDM), a widely used inhibitor of non-muscle myosins, on Pol I transcription. BDM shifts the equilibrium between two actomyosin states toward a weakly bound form and therefore interferes with actin dynamics [Yarrow et al. 2003]. Consistent with force generation being required for transcription, pre-rRNA synthesis was decreased by BDM treatment [Fig. 3C]. Inhibition of Pol I transcription by BDM was also observed in cell-free transcription assays [Fig. 3D], demonstrating that inhibition of rDNA transcription was not caused by pleiotropic effects of BDM on cell proliferation, but that the dynamics and functional interplay of the actin–NM1 complex is required for transcription activation.

![Figure 3. Actin and NM1 cooperate in Pol I transcription activation.](image)
The motor function of NM1 is required for Pol I transcription activation

To function as a molecular motor, myosin has to interact with actin, hydrolyze ATP, and bind to calmodulin via its C-terminal IQ motifs. To decipher the function of NM1 in Pol I transcription, we generated a set of mutants where functionally important protein domains were deleted—e.g., the internal neck region [NM1-ΔC] or the C-terminal tail [NM1-ΔC]—or where specific myosin functions, such as ATP binding [NM1-G126S, NM1-K127Q] or the interaction with actin [NM1-RK605AA], were impaired by single or double amino acid substitutions [Fig. 4A]. To examine the association of wild-type and mutant NM1 with the transcription apparatus, we precipitated TIF-IA and Pol I from HEK293T cells expressing V5-tagged wild-type or mutant NM1 and monitored coprecipitated NM1 on immunoblots. As shown in Figure 4B, similar amounts of V5-tagged wild-type and mutant NM1 were coprecipitated with cellular TIF-IA, indicating that none of the mutations affected the interaction of NM1 with TIF-IA. Wild-type NM1 and NM1-ΔC, the mutant that lacks the C-terminal tail, were efficiently coprecipitated with Pol I [Fig. 4B, right panel]. This demonstrates that the positively charged tail domain of NM1 is not required for binding to the transcription apparatus. In contrast, mutants NM1-G126S, NM1-K127Q, NM1-RK605AA, and NM1-ΔIQ, all of which have impaired motor activity, did not interact with Pol I. Thus, essential myosin functions are required for NM1 binding to Pol I.

Given that NM1 promotes transcription and that the motor activity is required for association with Pol I, then more Pol I should be associated with rDNA if NM1 was overexpressed. Conversely, overexpression of the motor-deficient mutants should decrease Pol I binding to rDNA. Indeed, overexpression of wild-type NM1 caused a significant increase in Pol I occupancy along the rDNA repeats, whereas overexpression of mutants NM1-G126S, NM1-RK605AA, and NM1-ΔIQ strongly decreased the level of Pol I in the pre-rRNA coding region, but not at the rDNA promoter [Fig. 4C]. This result suggests that the actin-dependent motor activity of NM1 is required for transcription elongation but not for the recruitment of Pol I to rDNA. The tail domain, on the other hand, appears to serve a role in earlier steps of transcription, because mutant NM1-ΔC reduced the level of Pol I both at the promoter and the transcribed region. Together, these results reveal that a functional actomyosin motor is associated with the Pol I transcription machinery, and the motor activity of NM1 is required both for binding to Pol I and for rDNA occupancy of the transcription apparatus.

Actin and NM1 are associated with transcribed and nontranscribed rDNA sequences

Previous ChIP analyses have shown that actin covers the entire rDNA transcription unit—i.e., the promoter and the transcribed region—whereas NM1 was exclusively associated with the rDNA promoter [Philimonenko et al. 2004]. However, in a subsequent study using a different antibody, NM1 was also associated with the pre-rRNA coding region [Percipalle et al. 2006]. This apparent dis-
crepancy suggested that NM1 undergoes a structural change during transition from the initiation into the elongation phase. In support of this, a newly generated monoclonal antibody (α-NM1#26) raised against the N-terminal domain of NM1 (amino acids 1–16) precipitated NM1 both in the promoter and the transcribed region, while another antibody (α-NM1#39) recognized only NM1 bound to the promoter [Supplemental Fig. S4]. To examine whether the distribution of actin and NM1 along the rDNA repeat is similar to that of Pol I, we established a cell line expressing V5-tagged NM1 and compared rDNA occupancy of Pol I, TIF-IA, actin, and NM1-V5 by ChIP using primers that amplify the 5′-terminal part of rDNA including the transcription start site, the transcribed region (18S and 28S rRNA sequences), and sequences within the intergenic spacer (IGS) separating rDNA repeats [Fig. 5A]. As expected, TIF-IA was exclusively associated with 5′-terminal sequences including the rDNA promoter. Pol I [RPA116] occupied the rDNA promoter and the pre-rRNA coding region, but was absent in the IGS. Surprisingly, however, actin and NM1 covered the entire rDNA repeat, including the rDNA promoter, the transcribed region, and the intergenic spacer, the occupancy at the IGS being consistently higher than at the pre-rRNA coding region. ChIP analysis of the NM1 mutants revealed that the tailless mutant NM1-ΔC bound stronger than wild-type NM1 to all rDNA regions. Binding of the motor-deficient mutants (NM1-G126S, NM1-RK605AA, NM1-ΔIQ), on the other hand, was severely impaired [Fig. 5B]. These results are consistent with the data from the coimmunoprecipitation experiments, demonstrating that the motor activity is required for the interaction of NM1 with both Pol I and rDNA.

The association of actin and NM1 with rDNA does not depend on ongoing transcription

The finding that significant amounts of actin and NM1 were associated with the intergenic spacer was unexpected, because the requirement of both proteins in Pol I transcription suggested that actin and NM1 would be exclusively associated with transcribed regions. To determine whether actin and NM1 are also associated with silent rRNA genes, we digested DNA from ChIP assays with HpaII before PCR amplification. This approach distinguishes active copies that are unmethylated, and therefore sensitive to HpaII digestion, from silent ones that are methylated and resistant to HpaII cleavage [Santoro et al. 2002]. As shown in Figure 6A, 50%–60% of rRNA genes are unmethylated—i.e. transcriptionally active—whereas 40%–50% are epigenetically silenced. Pol I and UBF were associated with active, unmethylated rRNA genes, while TIP5, a subunit of the silencing complex NoRC, was exclusively bound to the promoter of silent genes. Notably, actin and NM1 occupied both methylated and unmethylated rDNA repeats, indicating that they are associated with both active and silent rRNA genes.

Previous studies suggested that NM1 functions as an actin-based auxiliary motor that powers transcription [for review, see de Lanerolle et al. 2005]. The finding that actin and NM1 are associated with the intergenic spacer and are present both at active and at silent rRNA genes questions the idea that a kind of actomyosin motor traver-
els with Pol I during transcription. If rDNA association of actin and NM1 required ongoing transcription, then inhibition of Pol I transcription should decrease the level of Pol I, NM1, and act in rDNA. To test this, we inhibited Pol I transcription by treating cells with low doses of actinomycin D and monitored pre-rRNA levels as well as rDNA occupancy of Pol I, NM1, and act in the absence or presence of actinomycin D. Consistent with actinomycin D inhibiting Pol I transcription, the level of Pol I in the transcribed region was strongly decreased [Fig. 6B]. Strikingly, actinomycin D treatment did not affect the association of actin and NM1 along the rRNA genes, demonstrating that the association of actin and NM1 with rDNA does not depend on active transcription. This observation suggests that actin and NM1 are anchored to chromatin, providing a molecular track that supports Pol I movement.

**Discussion**

Actin is a constituitive component of all three classes of nuclear RNA polymerases and plays an essential role in transcription. Nuclear actin has been shown to form filaments that are distinct from those in the cytoplasm (Stuven et al. 2003) and several studies have implicated nuclear actin and myosin in transcriptional regulation (de Lanerolle et al. 2005; Grummt 2006). However, little is known about the role of these motor proteins in the transcription process, their functional interplay, and their regulation. Moreover, there are controversial views regarding the role of monomeric G-actin or some kind of polymeric F-actin in transcription. In most cell types, the concentration of actin in the nucleus is much lower than in the cytoplasm, i.e., too low for an actin-based filament system (Stuven et al. 2003). Nevertheless, a recent study convincingly demonstrated the existence of polymeric forms of actin in the nucleus (McDonald et al. 2006). Using fluorescence recovery after photobleaching (FRAP), both rapidly and slowly moving populations of nuclear actin were observed that correspond to monomeric and polymeric forms. The polymers in the nucleus are highly dynamic and inherently different from cytoplasmic actin filaments, and therefore it seems unlikely that their conformation is that of classical F-actin. Indeed, nuclear actin does not form long F-actin filaments, but can assume shorter, potentially novel conformations that are distinct from those found in conventional actin filaments in the cytoplasm (Pederson and Aebi 2002, 2005; Jockusch et al. 2006). This suggests that the plasticity of the actin molecule may facilitate its diverse functions in the nucleus.

In this study, we provide experimental evidence that efficient transcription requires filamentous or some kind of unconventional oligomeric or polymeric actin. Drugs that prevent de novo actin oligomerization, such as cytochalasin D and latrunculin B, reduced transcription both in vivo and in vitro. Moreover, cofilin, which severs and depolymerizes actin filaments, strongly inhibited Pol I transcription. Finally, actin mutants that stabilize F-actin interact more strongly with the Pol I transcription machinery, enhance the occupancy of Pol I at rDNA, and activate transcription. In contrast, a polymerization-defective actin mutant (R62D) does not bind to Pol I and does not promote transcription. Together, these data provide strong evidence that actin-mediated transcription activation is linked to its ability to form polymers, a finding that is in accord with a recent study demonstrating that nuclear N-WASP (Neuronal Wiskott-Aldrich syndrome protein) regulates transcription through its ability to promote actin polymerization (Wu et al. 2006).

Given that actin usually works in conjunction with myosin motor proteins, and all myosins use actin as a track along which to move, it is not surprising that a form of myosin I (NM1) is also present in the nucleus. This finding, along with the observation that both actin and NM1 colocalize at sites of active transcription (Kysela et al. 2005) and are associated with RNA polymerases (Kahle et al. 2007), suggests a close link between nuclear actin, myosin, and transcriptional activity. Depletion or inhibition of actin or NM1 decreased transcription in vivo and in vitro, indicating that both proteins contribute in important ways to the transcription process. The association of actin and myosin with rDNA and the Pol I transcription apparatus requires the motor function of NM1. Mutants that are deficient in ATPase
NM1 and actin in Pol I transcription

activity or actin binding did not interact with Pol I and their association with rDNA was greatly impaired. Significantly, the association of actin and NM1 with Pol I was abolished in the presence of ATP and stabilized by ADP, an observation that implicates that actin and myosin function by the same mechanism in both the nucleus and cytoplasm, supporting the view that nuclear actomyosin complexes act as a molecular motor that facilitates transcription. These results, together with previous findings demonstrating that different anti-NM1 antibodies do or do not recognize NM1 in the transcribed region [Philimonenko et al. 2004; Percipalle et al. 2006], indicate that NM1 in the initiation complex has a different conformation from NM1 functioning in transcription elongation, and suggest that the interaction of NM1 with actin in the initiation complex may trigger a conformational change that favors the transition of Pol I from the initiation into the elongation phase. Consistent with the high dynamics of nuclear actin [McDonald et al. 2006], transient binding and detachment of NM1 from the actin–polymerase complex may power the sliding of RNA polymerase relative to DNA. It has been suggested that by anchoring NM1 to DNA and actin to RNA polymerase, an auxiliary motor is generated that works in concert with nuclear RNA polymerases to facilitate transcription [de Lanerolle et al. 2005]. In this model, NM1 binds the DNA backbone through its positively charged tail domain, while the head interacts with actin bound to RNA polymerase. However, our data showing that deletion of the tail did not affect binding of NM1 to Pol I and rDNA indicate that the tail is very likely not responsible for tethering NM1 to DNA.

In resting human lymphocytes, actin and NM1 are concentrated in condensed chromatin, indicating that a significant fraction of condensed actin and myosin is bound to heterochromatin [Kysela et al. 2005], raising the possibility that, beside playing a role in transcriptional activation, actin and NM1 may serve a function in the establishment or maintenance of heterochromatin. This is consistent with a recent immunofluorescence microscopy study showing that a specific chromosomal site translocates from the nuclear periphery to the interior after transcription activation [Chuang et al. 2006]. Chromosome movement was blocked by BDM and eliminated by a polymerization-defective actin mutant, suggesting that long-range chromatin movement depends on F-actin and myosin. Our data showing that actin and NM1 are not only associated with actively transcribed rDNA repeats, but also with intergenic spacer sequences, is consistent with nuclear actin and myosin serving functions in the nucleolus beside powering transcription.

Materials and methods

Chemicals, antibodies, and plasmids

Actinomycin D, BDM, phalloloidin, jasplakinolide, latrunculin B, and cytochalasin D were purchased from Calbiochem. Antibodies against TIF-IA [Bodem et al. 2000] and RPA116 [Seither and Grummt 1996] have been described. The human autoimmune serum SS7299 against Pol I was obtained from a scleroderma patient. Antibodies against the Flag epitope [M2] and the V5 epitope, anti-actin antibodies [Ac74 and Ac40], and anti-NM1 antibodies were from Sigma-Aldrich. Anti-mouse and anti-rabbit antibodies conjugated to horseradish peroxidase were from Jackson ImmunoResearch Laboratories, Inc. cDNAs encoding wild-type and mutant NM1 were tagged at the 3′-end with sequences encoding the V5 epitope peptide and cloned into pcDNA3.1 [Invitrogen]. NM1 mutants were generated by site-directed mutagenesis and deletion PCR. Expression vectors encoding Flag-actin and the respective point mutants (Posern et al. 2002, 2004) were provided by R. Treisman.

In vitro transcription assays

Nuclear extracts were prepared from exponentially growing FM3A cells and transcription assays were performed as described [Schnapp and Grummt 1996]. Transcription reactions (25 µL) contained 50 ng of template DNA [pMr600/EcoR I] and 30–50 µg of nuclear extract proteins in 12 mM Tris-HCl [pH 8.0]; 0.1 mM EDTA; 5 mM MgCl2; 80 mM KCl; 10 mM creatine phosphate; 12% (v/v) glycerol; 0.66 mM ATP, GTP, and CTP; 12.5 µM UTP, and 0.5 µCi [α-32P]UTP [5000 Ci/mmole]. After incubation for 60 min at 30°C, RNA was analyzed on nondeaturing 4.5% polyacrylamide gels. To examine the effect of actin and NM1 on transcription activity, 0.1–1 µg of antibodies were preincubated with nuclear extracts for 30 min at room temperature before the template and nucleotides were added.

ChIP assays

HEK293T cells were fixed for 15 min at room temperature with 1% formaldehyde and lysed in 200 µL of buffer containing 50 mM Tris-HCl [pH 8.0], 10 mM EDTA, and 1% SDS. After sonication to yield DNA fragments of 0.2–0.5 kb, lysates were cleared by centrifugation, diluted 10-fold with ChIP buffer [12.5 mM Tris-HCl at pH 8.0, 200 mM NaCl, 1% Triton X-100], and precleared with protein A/G-Sepharose blocked with BSA and sonicated salmon sperm DNA. After washing, immunoprecipitated DNA was isolated by adding 100 µL of 10% (v/v) chlex (Bio-Rad) and quantified by real-time PCR. The relative enrichment of rDNA was determined by calculating the ratio of rDNA present in the immunoprecipitates to rDNA in the input chromatin. Data were normalized to control reactions without antibodies or with IgGs. To monitor CpG methylation of rDNA, we digested immunoprecipitated DNA with HpaII before PCR amplification (Sanctoro et al. 2002).

RNA analysis

RNA [500 ng] from HEK293T cells was reverse-transcribed using random primers, and 45S pre-rRNA was quantified by real-time PCR using primers that amplify a fragment from +353 to +549 of the human rDNA (forward, 5′-GGAGTGGGGGGTGG-3′; reverse, 5′-GGGGAGAGGAGCAGACGAG-3′). Data were normalized to the level of GAPDH mRNA.

Coimmunoprecipitation assays

HEK293T cells overexpressing Flag-tagged actin or V5-tagged NM1 were lysed in IP buffer (20 mM Tris-HCl at pH 7.4, 200 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100, “Complete” protease inhibitor cocktail [Roche], cleared by centrifugation (16,000g, 30 min), and incubated overnight at 4°C
with anti-Pol I or anti-TIF-IA antibodies bound to protein A/G agarose. The immunoprecipitates were washed with IP buffer and precipitated proteins were subjected to SDS-PAGE and Western blotting.

**Protein purification**

Flag-tagged actin or NM1 was immunopurified from HEK293T cells with M2-agarose, eluted with the Flag peptide [200 μg/μL], and dialyzed against 20 mM Tris-HCl [pH 8.0], 0.2 mM EDTA, 5 mM MgCl₂, 100 mM KCl, and 20% (v/v) glycerol. Cofilin was expressed in bacteria and purified by chromatography on DEAE-, CM- and SP-Sepharose and reactive Red-120 column (Sigma-Aldrich). Purified profilin was kindly provided by B. Jockusch. The quantity and purity of proteins were estimated on silver-stained SDS-PAGE gels.

**Actin polymerization assay**

To determine the level of F-actin, FM3A cells were lysed, cleared by centrifugation (16,000g, 30 min), and incubated with profilin or cofilin for 30 min at 30°C in the presence of 1 mM ATP. After centrifugation (90,000g, 30 min), actin in the cell lysate or the pellet fraction was monitored by immunoblotting.

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