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ARTICLE

Nuclear actin and myosin I are required for RNA polymerase I transcription

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The presence of actin and nuclear myosin I (NMI) in the nucleus suggests a role for these motor proteins in nuclear functions. We have investigated the role of actin and nuclear myosin I (NMI) in the transcription of ribosomal RNA genes (rDNA). Both proteins are associated with rDNA and are required for RNA polymerase I (Pol I) transcription. Microinjection of antibodies against actin or NMI, as well as short interfering RNA-mediated depletion of NMI, decreased Pol I transcription *in vivo*, whereas overexpression of NMI augmented pre-rRNA synthesis. *In vitro*, recombinant NMI activated Pol I transcription, and antibodies to NMI or actin inhibited Pol I transcription both on naked DNA and pre-assembled chromatin templates. Whereas actin associated with Pol I, NMI bound to Pol I through the transcription-initiation factor TIF-IA. The association with Pol I requires phosphorylation of TIF-IA at Ser 649 by RSK kinase, indicating a role for NMI in the growth-dependent regulation of rRNA synthesis.

Numerous reports suggest a role for actin in the nucleus, but until recently the functional significance of nuclear actin remained poorly understood. In the past few years, reports have linked actin to nuclear processes ranging from chromatin remodelling to transcription and splicing[1–5]. Actin and actin-related proteins have been found associated with SWI/SNF-like complexes, indicating that actin is involved in the regulation of chromatin structure. Actin usually works in conjunction with myosin motor proteins, and recent studies on energy-dependent movement of promyeolytic leukaemia (PML) nuclear bodies suggest that nuclear actin–myosin complexes mediate the dynamics of nuclear processes[6]. In support of this view, a type of myosin I (NMI) has been identified in the nucleus[7,8]. NMI is a monomeric, single-headed myosin that possesses a unique 16-amino-acid amino-terminal extension that is required for its nuclear localization. Remarkably, NMI has been found to co-localize and interact with RNA polymerase II (Pol II), and antibodies to NMI inhibit mRNA synthesis[8]. Moreover, treatment of cells with α-amanitin abolishes the co-localization of Pol II and NMI, suggesting a function for NMI in transcription[8].

Because of the conservation of basic transcriptional regulation mechanisms, the requirement of actin–myosin complexes would be expected not to be restricted to the transcription of mRNA-coding genes by Pol II, but to be used by all three classes of nuclear RNA polymerases. In support of this, both NMI and actin have been shown to localize in the nucleolus[7,9–11], suggesting that these motor proteins may be important for the transcription of rDNA by Pol I. Here we demonstrate that both actin and NMI have an essential function in Pol I transcription. Nucleolar transcription is decreased by depletion or inhibition of cellular NMI or actin, whereas pre-rRNA synthesis is augmented by NMI overexpression. Chromatin immunoprecipitation (ChIP) and co-immunoprecipitation assays reveal a physical association of actin and NMI both with rRNA genes and the Pol I transcription machinery. Whereas actin is bound to both initiating and elongating Pol I molecules, NMI interacts with the initiation-competent subpopulation of Pol I through TIF-IA, a basal transcription factor that is responsible for growth-dependent regulation of rRNA synthesis. These data demonstrate previously unknown, important roles of actin and NMI in the transcription of rDNA and provide insight into the role of these motor proteins in the transcription process.

RESULTS

Actin and NMI are required for cellular Pol I transcription

To investigate the role of nucleolar actin and NMI in rDNA transcription, endogenous actin and NMI were blocked by microinjecting antibodies to actin or NMI into the nuclei of HeLa cells, and pre-rRNA synthesis was monitored by indirect immunofluorescence with an anti-bromodeoxyuridine (BrU) antibody. Microinjection of antibodies against actin or NMI strongly decreased both the number and fluorescence intensity of Br-UTP-labelled nucleoli, indicating that pre-rRNA synthesis was impaired (Fig. 1a). Microinjection of dextran (control) or antibodies to vimentin or myosin II (data not shown) did not affect nucleolar transcription, underscoring the specificity of Pol I transcription inhibition by anti-actin and anti-NMI antibodies.

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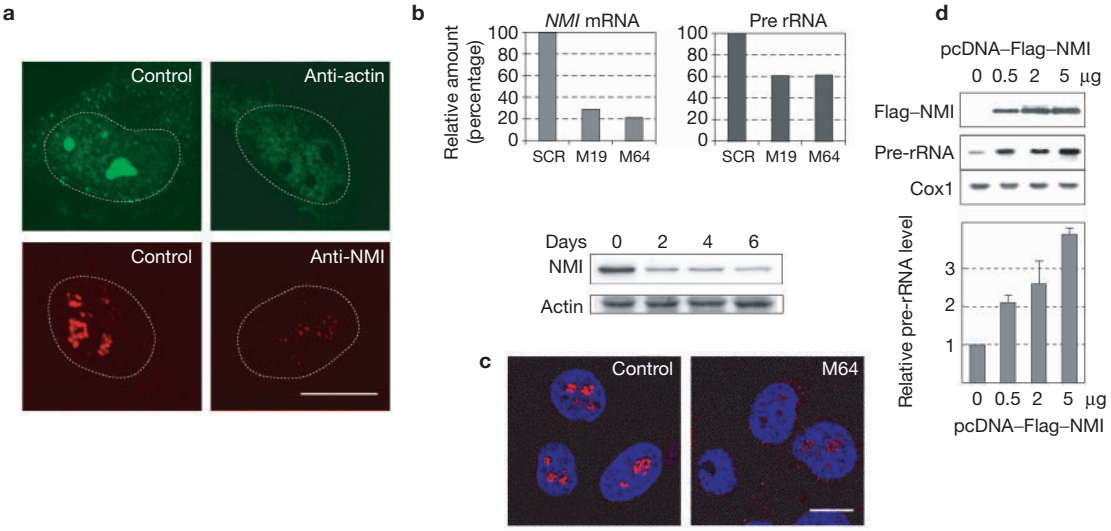

Figure 1 Actin and NMI are required for rDNA transcription *in vivo*. (**a**) Microinjection of anti-actin or anti-NMI antibodies inhibits nucleolar transcription. Confocal images of transcription sites in HeLa nuclei microinjected with dextran (control), anti-actin or anti-NMI antibody as indicated. Br-UTP was incorporated in permeabilized cells for 10 min, and nascent BrU-labelled RNA was detected with an anti-BrdU antibody conjugated to Alexa Fluor 488 (green, top) or with an anti-BrdU monoclonal antibody and a Cy3-conjugated secondary antibody (red, bottom). Scale bar represents 10 µm. (**b**) RNAi-mediated knockdown of NMI inhibits pre-rRNA synthesis. Cells were transfected with scrambled control siRNAs (*SCR*) or either of two NMI-specific siRNAs (*M19*, *M64*). The relative expression of *NMI* mRNA and 45S pre-rRNA was measured using RT–qPCR. This experiment has been repeated four times with very consistent results. NMI depletion was monitored by western blot analysis two, four and six days after transfection. (**c**) RNAi-mediated depletion of NMI decreases nucleolar transcription. HeLa cells were transfected with 40 nM siRNAs complementary to *NMI* mRNA (*M19*, *M64*) or control siRNA (*SCR*). After six days, cells were permeabilized, incubated for 10 min with Br-UTP and nascent nucleolar RNA was visualized by immunolabelling with anti-BrdU antibody (red). Nuclei were stained with DAPI (blue). Scale bar represents 10 µm. (**d**) Overexpression of NMI augments pre-rRNA synthesis. HEK293T cells were transfected with increasing amounts of pcDNA–Flag–NMI as indicated. The expression of tagged NMI was assessed on immunoblots (top). The relative amount of 45S pre-rRNA and cytochrome *C* (*Cox 1*) mRNA was monitored by northern blot analysis (bottom). The bar diagram shows the average level of pre-rRNA from three independent experiments after quantitation in a PhosphorImager and normalization to cytochrome *C* mRNA.

The involvement of NMI in pre-rRNA synthesis was also demonstrated by RNAi-mediated knock-down of NMI expression. Two NMI-specific siRNAs were used that target different regions of *NMI* mRNA (M19 and M64). HeLa cells were transfected with either of the two NMI-specific or control siRNAs, and the cellular level of *NMI* mRNA and pre-rRNA was monitored by RT–qPCR. Expression of NMI-specific siRNA reduced the level of *NMI* mRNA by about 80% (Fig. 1b). The amount of 45S pre-rRNA decreased by about 40% compared with the control. rDNA transcription was also assessed by monitoring Br-UTP incorporation into nascent nucleolar RNA. Visualization of BrU-labelled nucleolar RNA by immunofluorescence revealed a roughly 30% decrease in nascent pre-rRNA levels after knock-down of NMI expression (Fig. 1c), supporting a role for NMI in rDNA transcription.

To examine whether overexpression of actin and NMI would affect cellular Pol I transcription, HEK293T cells were transfected with expression vectors encoding NMI or actin, and pre-rRNA synthesis as monitored by northern blot analysis. Overexpression of actin did not affect cellular pre-rRNA synthesis (data not shown), presumably because the levels of endogenous actin were too high. However, overexpression of NMI caused a dose-dependent increase in cellular pre-rRNA levels (Fig. 1d). Similarly, transcription of a co-transfected Pol I reporter plasmid was enhanced two-to-threefold (data not shown). These results demonstrate that NMI has an essential function in rDNA transcription by Pol I.

Both actin and NMI are required for Pol I transcription *in vitro*

To determine the molecular mechanisms that link actin and NMI to nucleolar activity, we examined the effect of either protein on Pol I transcription *in vitro*. The rationale of this approach was to learn whether actin and NMI have a direct role in initiation or elongation of Pol I transcription or whether their effect on pre-rRNA synthesis is indirect because of regulating other nuclear processes, such as chromatin remodelling or anchoring of active transcription complexes to the nucleoskeleton. A partially purified nuclear extract (DEAE-280 fraction) that contains Pol I and all essential transcription factors was pre-incubated with antibodies against actin or NMI before transcription was initiated by adding nucleotides and the rDNA template. Pre-incubation with anti-NMI antibodies abolished transcription both on naked DNA and pre-assembled chromatin, whereas antibodies to myosin II did not affect specific Pol I transcription (Fig. 2a). Moreover, addition of immunopurified NMI to a reconstituted transcription system that contains partially purified Pol I and basal transcription factors, but undetectable levels of endogenous NMI, enhanced Pol I transcription in a dose-dependent manner (Fig. 2b). At the maximal concentration (50 ng), a roughly sevenfold stimulation of Pol I transcription was observed (lane 3). This observation supports the *in vivo* data indicating that NMI functions in Pol I transcription and excludes the possibility that NMI enhances Pol I transcription through chromatin remodelling or anchoring the transcription apparatus to nuclear or nucleolar structures.

Inhibition of Pol I transcription was also observed if the extracts were pre-incubated with antibodies against actin. As with antibodies to NMI, antibodies to actin abolished Pol I transcription both on naked DNA and chromatin, whereas control antibodies (anti-Flag) did not affect transcriptional activity (Fig. 2c). There was no decrease in transcription if the antibody was heat-inactivated before being added to the reactions

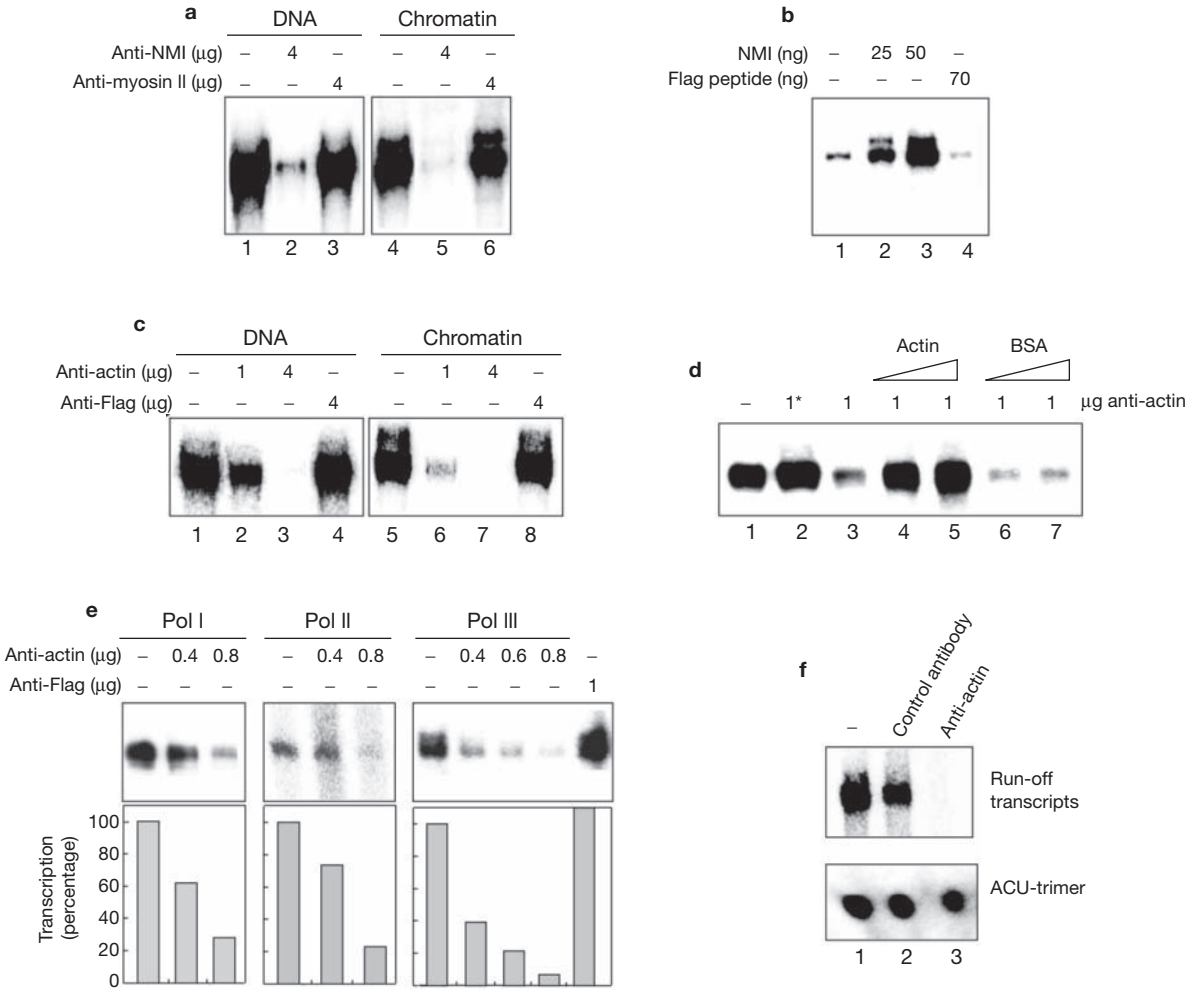

Figure 2 NMI and actin activate Pol I transcription. (**a**) Anti-NMI antibodies inhibit Pol I transcription *in vitro*. Transcription assays were conducted on either naked DNA (lanes 1–3) or preassembled chromatin (lanes 4–6). The DEAE-280 fraction was pre-incubated for 40 min with buffer (lanes 1, 4), 4 μg of anti–NMI (lanes 2, 5) or 4 μg of anti–myosin II antibody (lanes 3, 6) before transcription was initiated. (**b**) NMI activates Pol I transcription in a reconstituted system. Assays containing partially purified Pol I, TIF-IA–TIF-IC, TIF-IB, recombinant UBF and TTFΔN185 were pre-incubated for 20 min with the indicated amounts of recombinant NMI or 70 ng of Flag peptide before transcription was initiated. (**c**) Anti-actin antibody inhibits Pol I transcription. Assays containing either naked DNA (lanes 1–4) or preassembled chromatin (lanes 5–8) were pre-incubated for 40 min with buffer (lanes 1, 5), 1 or 4 μg of anti–actin antibody (lanes 2, 3, 6 and 7), or 4 μg of anti-Flag antibody (lanes 4, 8) before transcription was initiated. (**d**) Exogenous actin overcomes anti-actin-mediated inhibition of Pol I transcription. Transcription assays contained no antibody (lane 1), 1 μg of heat-inactivated (5 min, 95 °C) anti-actin (lane 2), or 1 μg of anti-actin antibody (lanes 3–7) plus 150 or 500 ng of exogenous actin (lanes 4, 5) or bovine serum albumin (lanes 6, 7). (**e**) Actin is required for transcription by all three classes of nuclear RNA polymerases. Nuclear extract was pre-incubated for 40 min with anti-actin antibodies before transcription was initiated by adding nucleotides and template for Pol I (pMr170-BH), Pol II (pMLc$_2$AT/*Sma*I) and Pol III (pBh5S). (**f**) Abortive transcription-initiation assay. The DEAE-280 fraction was preincubated for 40 min with antibody before the template, ATP and CTP were added, and incubation was continued for 20 min (lane 3). Half of the reactions were supplemented with GTP and α-^{32}P-UTP to synthesize full-length transcripts (top); the other half was supplemented with α-^{32}P-UTP to synthesize ACU trimers (bottom). Reactions contained no antibody (lane 1), 1 μg of heat-inactivated anti-actin antibody (lane 2) or 1 μg of anti-actin antibody.

(Fig. 2d, lane 2). Notably, exogenous actin rescued transcriptional activity of the reactions that were treated with actin antibodies (lanes 4, 5), demonstrating the specificity of antibody-mediated inhibition of transcription. Antibodies to actin also inhibited transcription by Pol II and Pol III in a concentration-dependent manner (Fig. 2e). This result supports previous studies that have associated nuclear actin with transcription by RNA polymerase II[12–15] and suggests an indispensable function for actin in transcription by all three classes of nuclear RNA polymerases.

To investigate the mechanism underlying actin-mediated activation of Pol I transcription, abortive transcription-initiation assays were performed. These assays take advantage of the fact that functional initiation complexes can cycle short RNA products specific to the transcriptional start site when provided with limited nucleotide substrates. In the experiment in Fig. 2f, the DEAE-280 fraction was incubated with the DNA template in the presence of ATP and CTP (the first two nucleotides of mouse pre-rRNA) to form initiated complexes. One half of the reaction was supplemented with GTP and α-^{32}P-UTP, and transcription was allowed to proceed for 1 h. The other half was supplemented with only α-^{32}P-UTP. In this case, Pol I cannot progress into the elongation phase because the forth nucleotide of pre-rRNA (GTP) is not present. Instead, the trinucleotide pppApCpU is released from the ternary complex and repeated abortive initiation (but not elongation) takes place. Consistent

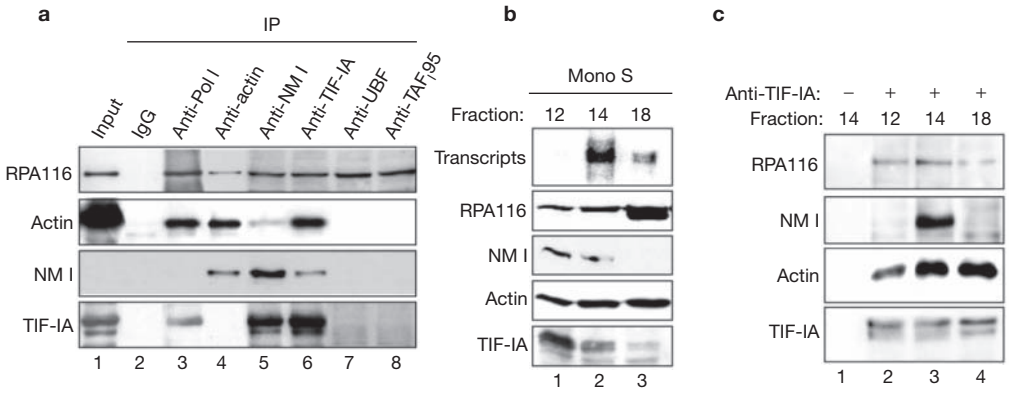

Figure 3 Actin and NMI are associated with the Pol I transcription machinery. (**a**) Co-immunoprecipitation of actin and NMI with Pol I and TIF-IA. Mouse IgGs and antibodies to Pol I (anti-RPA116), actin, NMI, TIF-IA, UBF and TIF-IB–SL1 (anti-TAF$_I$95) were immobilized on protein G agarose and incubated with the DEAE-280 fraction. Twenty percent of the input (lane 1) and 50% of co-precipitated Pol I (RPA116), actin, NMI and TIF-IA were analysed by western blotting. (**b**) Co-fractionation of NMI and actin with Pol I. Pol I was fractionated by chromatography on a MonoS FPLC column as described[21] and individual fractions (numbers 12, 14 and 18) were assayed for activity in a reconstituted transcription system (top, lanes 1–3). The western blots below show the level of Pol I (RPA116), NMI, actin and TIF-IA in the individual fractions. (**c**) Association of NMI with the Pol I–TIF-IA complex is required for Pol I transcription. Three fractions (numbers 12, 14 and 18) from the MonoS column were immunoprecipitated with anti-TIF-IA antibodies and co-precipitated Pol I (RPA116), NMI and actin were analysed by western blotting.

with the data in Fig. 2c, d, the anti-actin antibody inhibits the synthesis of run-off transcripts (Fig. 2f, top). Notably, the formation of abortive trinucleotides was not affected (bottom). This result reveals that actin is critical in a step subsequent to transcription initiation, presumably promoter clearance or transcription elongation.

Actin and NMI are associated with the Pol I transcription machinery

Next, we determined whether actin and NMI proteins are physically associated with the Pol I transcription machinery. Pol I, actin or NMI were immunoprecipitated from the DEAE-280 fraction and co-precipitated proteins were monitored by western blot analysis (Fig. 3a). A notable amount of actin co-precipitated with Pol I (lane 3). Similarly, Pol I (RPA116) co-precipitated with actin (lane 4), indicating a physical association of actin with Pol I. NMI, whose concentration was too low to be detected in the DEAE-280 fraction (input, lane 1) or the Pol I immunoprecipitate (lane 3), was strongly enriched by immunoprecipitation with anti-actin antibodies (lane 4). Notably, both actin and Pol I were found to co-precipitate with NMI (lane 5). This indicates that both actin and NMI are associated with the Pol I transcription machinery. Previous work has shown that the majority of cellular Pol I is transcriptionally inactive. Even in cells that exhibit a high pre-rRNA synthetic activity, only a fraction of Pol I is capable of assembling into productive initiation complexes that direct rDNA transcription[16–18]. Initiation-competent Pol I is associated with TIF-IA, a basal transcription-initiation factor that mediates growth-dependent regulation of Pol I transcription[19]. TIF-IA interacts with both Pol I and the TBP-containing selectivity factor TIF-IB/SL1, and these interactions are required to assemble initiation complexes at the rDNA promoter[18,20]. To examine which component (or components) of the Pol I transcription apparatus associates with actin and NMI, we precipitated Pol I, actin, NMI, TIF-IA, UBF and TAF$_I$95 from the DEAE-280 fraction, and probed the immunoprecipitates for the presence of actin and NMI. Consistent with TIF-IA being associated with a fraction of Pol I, antibodies to TIF-IA co-precipitated Pol I (Fig. 3a, lane 6). Importantly, the immunoprecipitate also contained notable amounts of actin and NMI, indicating that actin and NMI are associated with the fraction of Pol I that is decorated with TIF-IA. In contrast, neither actin nor NMI were found in the precipitates with anti-TAF$_I$95 or anti-UBF antibodies (lanes 7, 8).

Fractionation of Pol I on a MonoS fast performance liquid chromatography (FPLC) column separates the majority of cellular Pol I from the subpopulation of Pol I that is associated with TIF-IA, and therefore is capable of forming productive transcription-initiation complexes[21]. Individual column fractions contain different Pol I moieties that differ in their capability to specifically transcribe rDNA templates. In the experiment in Fig. 3b, three MonoS FPLC column fractions (numbers 12, 14 and 18) were assayed both for their transcriptional activity and the presence of Pol I, NMI, actin and TIF-IA. Consistent with previous results, transcriptional activity did not coincide with the amount of Pol I — that is, transcription peaked in fraction 14 and was weak in fraction 18, even though fraction 18 contained much more Pol I than fraction 14. Actin was present in all fractions that contain Pol I, whereas NMI and TIF-IA co-fractionated with early eluting, transcriptionally active Pol I and was not detected in fraction 18.

A plausible explanation for the presence of NMI in TIF-IA-containing fractions is that the association of both NMI and TIF-IA is necessary for Pol I transcription. We investigated this possibility by precipitating TIF-IA from the column fractions and probing similar amounts of precipitated TIF-IA for Pol I, actin and NMI. Pol I was associated with actin and TIF-IA in all three fractions (Fig. 3c). Most importantly, the amount of NMI associated with TIF-IA was greatest in fraction 14 — the fraction with the highest transcriptional activity. No NMI was associated with TIF-IA in fractions 12 and 18, which support little or no transcription. This indicates that the subpopulation of Pol I that is decorated with TIF-IA and NMI, but not the bulk of Pol I, is capable of assembling into productive initiation complexes and directing initiation and elongation of Pol I transcription.

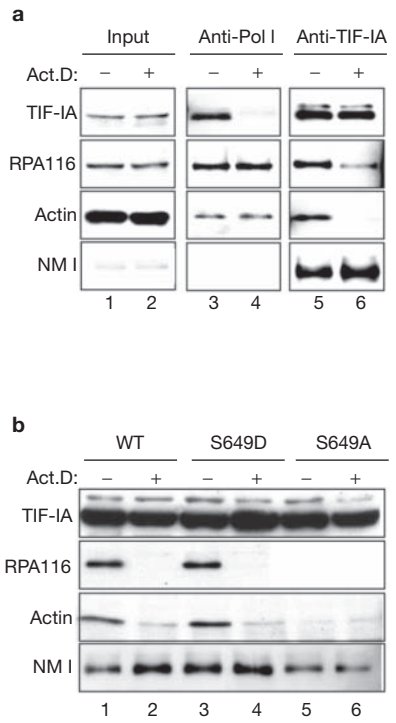

Figure 4 The association of NMI with the Pol I transcription apparatus requires phosphorylation of TIF-IA at Ser 649. (**a**) Actinomycin D treatment impairs the association of actin and NMI with the Pol I–TIF-IA complex. HeLa cells expressing Flag-tagged TIF-IA were cultured for 30 min in the absence (–) or presence (+) of 50 ng ml^{-1} of actinomycin D (Act. D). Pol I and TIF-IA were precipitated with anti-RPA116 (lanes 3, 4) or anti-Flag (lanes 5, 6) antibodies. Ten percent of precipitated TIF-IA and Pol I (RPA116), and 50% of co-immunoprecipitated Pol I, TIF-IA, actin and NM I (lanes 3-6) were compared with the amount of the respective proteins present in nuclear lysates (Input). (**b**) Phosphorylation of TIF-IA at Ser 649 and ongoing transcription are required for association of TIF-IA with Pol I but not with NMI. HEK293T cells were transfected with expression vectors encoding Flag-tagged wild-type or mutant TIF-IA (TIF-IAS649D and TIF-IAS649A). Forty-eight hours after transfection, cells were treated for 30 min with actinomycin D (50 ng ml^{-1}), lysed and TIF-IA was immunoprecipitated with anti-Flag antibodies. TIF-IA, Pol I, actin and NMI in the immunocomplexes were analysed by western blotting.

The association of NMI with the Pol I machinery requires phosphorylation of TIF-IA at Ser 649

To examine whether the association of actin and NMI with the initiation-competent fraction of Pol I requires ongoing transcription, HeLa cells expressing Flag-tagged TIF-IA were incubated for 30 min with actinomycin D (50 ng ml^{-1}) to selectively inhibit Pol I transcription[22]. Then, Pol I or TIF-IA were immunoprecipitated and co-precipitated proteins were visualized on immunoblots (Fig. 4a). The amount of TIF-IA associated with Pol I was markedly reduced after actinomycin D treatment, indicating that the formation of the Pol I–TIF-IA complex depends on ongoing transcription. Actin, however, remained associated with Pol I, regardless of whether or not Pol I transcription was inhibited (lane 4). This indicates that the association of actin with Pol I does not require the presence of TIF-IA. Consistent with this, less Pol I co-precipitated with TIF-IA from actinomycin D-treated cells, and the amount of actin dropped to undetectable levels (lane 6). In contrast, similar amounts of NMI were associated with TIF-IA, regardless of whether or not transcription was inhibited (lanes 5, 6). Together, this experiment shows that: first, the formation of the Pol I–TIF-IA complex depends on ongoing transcription; second, NMI associates with the Pol I–TIF-IA complex by interacting with TIF-IA; third, NMI remains associated with TIF-IA, regardless of whether or not transcription is occurring; and fourth, actin remains bound to Pol I, but not to TIF-IA, after transcription is inhibited by actinomycin D treatment. Thus, although both actin and NMI are required for Pol I transcription, they seem to have distinct functions.

We have previously shown that the interaction of TIF-IA with Pol I, and hence transcription-initiation complex formation, depends on phosphorylation at specific serine residues of TIF-IA. In particular, phosphorylation at Ser 649 by ribosomal S6 kinase (RSK) is essential for transcriptional activity[23]. Replacing Ser 649 with alanine (TIF-IA-S649A) abolished TIF-IA activity, whereas substitution with aspartic acid (TIF-IA-S649D) enhanced Pol I transcription. To test whether phosphorylation at this essential serine residue would affect the interaction with Pol I, NMI or actin, TIF-IA was precipitated from cells overexpressing either Flag-tagged wild-type or mutant TIF-IA, and co-precipitated Pol I, actin and NMI were analysed on immunoblots. Pol I, actin and NMI co-precipitated with wild-type TIF-IA and TIF-IA-S649D, both of which are transcriptionally active (Fig. 4b, lanes 1, 3). No association with Pol I and actin could be detected with the inactive mutant TIF-IA-S649A (lane 5), indicating that a negative charge at Ser 649 is required for the interaction of TIF-IA with Pol I. Similarly, treatment of cells with low doses of actinomycin D abolished the interaction of TIF-IA with Pol I and reduced co-precipitated actin to background levels (lanes 2, 4). Notably, NMI remained bound to TIF-IA, regardless of whether or not TIF-IA was phosphorylated at Ser 649. Together, these data demonstrate that phosphorylation of TIF-IA at Ser 649 is required for the formation of the Pol I–TIF-IA complex; the association of NMI with TIF-IA does not depend on phosphorylation of Ser 649; and the interaction of the TIF-IA–NMI complex with Pol I is required for recruiting Pol I to the rDNA promoter and formation of a productive initiation complex.

Actin and NMI are associated with rDNA

If NMI and actin have a direct role in Pol I transcription, they should be associated with rDNA. To assay for rDNA occupancy of NMI and actin, chromatin immunoprecipitation (ChIP) experiments were performed. The immunoprecipitated DNA was analysed by PCR using primers that amplify either the 5′-terminal part of the rDNA transcription unit (including the promoter) or part of the 28S rRNA coding sequence (Fig. 5). As expected, Pol I occupied both the 5′-terminal part of rDNA (including the promoter) and the 28S rRNA coding region (lanes 5, 6). The distribution of actin was identical to that of Pol I (lanes 9, 10). In contrast, both NMI and TIF-IA associated with the rDNA promoter, but not with the coding region (lanes 7, 8, 11 and 12). This observation is consistent with the immunoprecipitation data that demonstrated a physical association of NMI with TIF-IA and implies that NMI, like TIF-IA, has a role in early steps of transcription, whereas actin seems to be required for later steps, presumably transcription elongation.

DISCUSSION

As actin is a highly abundant cellular protein and is the major component of the cytoskeleton, the physiological significance of its occurrence in nuclear preparations could be questioned. However, the discovery that actin is a component of the mammalian chromatin-remodelling

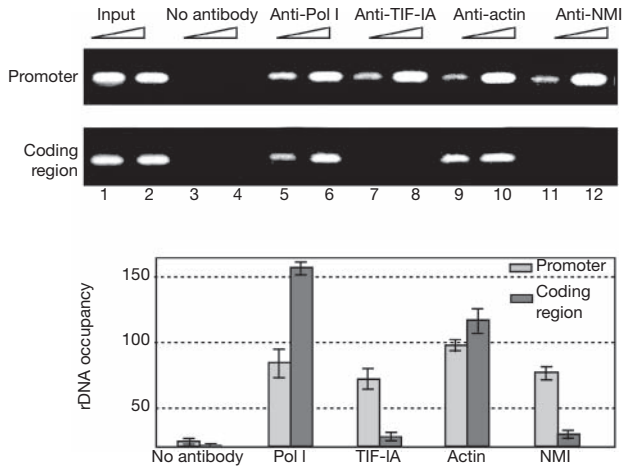

Figure 5 Actin and NMI are associated with rDNA. Crosslinked chromatin from U2-OS cells was immunoprecipitated with antibodies against Pol I (anti-RPA116), TIF-IA, actin and NMI as indicated. Input chromatin (0.02 and 0.06%) and immunoprecipitated DNA (1 and 3%) were amplified with primers specific to the 5′-terminal region of human rDNA or the 28S rRNA coding sequence. PCR products were visualized on ethidium-bromide-stained agarose gels. The diagram shows the quantitative measurement of immunoprecipitated DNA by RT–PCR. The relative enrichment of rDNA was determined by calculating the ratio of rDNA present in the immunoprecipitates compared with rDNA in the input chromatin, and normalizing the data to control reactions containing no antibodies. Bars represent average values from two independent experiments.

BRG-associated factor (BAF) complex[24] has spurred renewed interest in the functions of nuclear actin. A number of previous reports provide circumstantial evidence of a role for actin in transcription. Briefly, actin co-purifies with Pol II and is required for specific transcription initiation by Pol II[12,25]. Microinjection of anti-actin antibodies or actin-binding proteins inhibits transcription of protein-coding genes in lampbrush chromosomes[13]. Recent studies have demonstrated that actin is required for initiation complex formation and transcription by Pol II[14]. In addition, newly synthesized RNA molecules are associated with actin in the nuclear matrix[26,27], actin is associated with a specific subset of hnRNP A/B-type proteins[28] and actin aggregates co-localize with transcription sites in early mouse embryos[29]. Similarly, the interaction between actin and an RNA-binding protein (hsr) is required for transcription on *Chironomus tentans* lampbrush chromosomes[15,30]. However, it was previously unknown whether actin, presumably in conjunction with actin-interacting proteins, has a role in transcription by Pol I and Pol III. In this study we have shown that actin also has a direct role in transcription by Pol I. Actin is associated with Pol I in the presence or absence of ongoing transcription, occupies both the promoter and the coding region of the rDNA transcription unit and anti-actin antibodies inhibit rDNA transcription. Importantly, anti-actin antibodies reduce Pol II and Pol III transcription to a similar extent as Pol I. This suggests that actin has similar roles in transcription by all three classes of nuclear RNA polymerases.

The identification of myosin I in nucleoli of HeLa cells[7] raised the intriguing possibility that cytoskeletal proteins are also important in the structural organization and function of nucleoli. Indeed, our data reveal an essential role for both actin and NMI in rDNA transcription. Microinjection of anti-actin and anti-NMI antibodies significantly reduced nucleolar transcription in cultured HeLa cells, indicating the requirement of both actin and NMI for transcription *in vivo*. Similarly, depletion of NMI by siRNA notably inhibited pre-rRNA synthesis. In cell-free transcription assays, antibodies to actin or NMI abolished transcription on both naked DNA templates and pre-assembled chromatin. Transcription inhibition by antibodies to actin could be overcome by addition of exogenous actin in a dose-dependent manner. Moreover, *in vitro* transcription assays with partially purified Pol I revealed that both actin and NMI are needed for maximal activity. Lastly, overexpression of NMI in mammalian cells augmented the synthesis of 45S pre-rRNA. Consistent with this, recombinant NMI stimulated Pol I transcription in a reconstituted *in vitro* transcription system.

Our data suggest that actin and NMI function in concert to drive Pol I transcription. However, both proteins are involved in distinct steps of the transcription process. Anti-actin antibodies inhibit the synthesis of full-length transcripts but not the formation of ACU trimers — the first nucleotides of mouse pre-rRNA — indicating that actin exerts its effects on Pol I transcription at a post-initiation step. This is consistent with immunoprecipitation experiments that show actin associated with Pol I, regardless of whether or not it is engaged in transcription. Moreover, actin, like Pol I, is present both at the 5′-terminal part of the rDNA transcription unit and the transcribed region. In contrast, NMI occupies the 5′-terminal part of rDNA that harbours the promoter and the transcription start site. Importantly, NMI associates with the Pol I transcription machinery by binding to TIF-IA, a basal transcription factor that confers initiation competence to Pol I and mediates growth-dependent regulation of rRNA synthesis[19]. Phosphorylation of TIF-IA regulates the formation of the Pol I–TIF-IA complex and subsequent transcription initiation[18,20,31]. The interaction between NMI and TIF-IA does not depend on the activity of TIF-IA or on active Pol I transcription. This indicates that binding of NMI to TIF-IA precedes the association of TIF-IA with Pol I and suggests a role for NMI in the assembly of productive Pol I initiation complexes. In a subsequent step, Pol I is recruited to the initiation complex through its interaction with TIF-IA, which may facilitate the interaction of actin bound to Pol I and NMI associated with TIF-IA. We favour a model in which the association of the NMI–TIF-IA complex with Pol I triggers a conformational change within Pol I, and this structural change is required for efficient transcription. This model is supported by previous studies demonstrating that TIF-IA dissociates from Pol I at early steps of elongation[32,33], and the reversible formation and disruption of the Pol I–TIF-IA complex represents a molecular target for regulation of pre-rRNA synthesis. We propose that a nucleolar actin–NMI complex may facilitate this switch, possibly in concert with a supramolecular structure that leads to correct positioning of rRNA genes at distinct functional zones within nucleoli. Although further studies are needed to understand the function of actin and NMI in transcription, our data demonstrate that actin and NMI are indispensable for transcription by RNA polymerase I. □

METHODS

Cells, transfections and RNA analysis. HeLa and U2-OS cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% or 10% fetal calf serum (Invitrogen, Carlsbad, CA). To selectively inhibit Pol II transcription, cells were treated for 30 min with 50 ng ml^{-1} actinomycin D (Sigma, St Louis, MO). To transiently overexpress NMI, 5×10^5 HEK293T cells were transfected with different amounts of pcDNA3.1–Flag–NMI, RNA was isolated after 48 h and

pre-rRNA was analysed on northern blots, by hybridization to antisense RNA complementary to the first 155 nucleotides of unprocessed 45S pre-RNA.

Antibodies. The following antibodies were used in this study: anti-NMI[7], anti-RPA[34], anti-TIF-IA[21], anti-actin (C4; Sigma) and anti-BrdU (Boehringer Mannheim, Mannheim, Germany). Anti-mouse IgGs and secondary antibodies were from Jackson Immunoresearch Laboratories (West Grove, PA).

Microinjection of antibodies and detection of nucleolar transcription by immunofluorescence. HeLa cells were grown on CELLocate microgrid coverslips (Eppendorf, Hamburg, Germany). About 50 nuclei were microinjected with 40 pl of antibody (2–3 μg μl^{-1}) using a MMO-202ND micromanipulator and an IM-30 microinjector (Narishige, Tokyo, Japan) mounted on an ECLIPSE TE300 inverted microscope (Nikon, Tokyo, Japan). Forty minutes after injection, cells were permeabilized for 6 min on ice with 1 mg ml^{-1} saponin in PBS and nascent pre-rRNA was labelled with Br-UTP (10 min, 35 °C). After treatment with 4% paraformaldehyde (20 min, 4 °C) and 0.5% Triton X-100 (10 min, room temperature), BrU-labelled RNA was visualized either directly using an Alexa Fluor 488-conjugated monoclonal anti-BrdU antibody (1:20 dilution), or with a monoclonal anti-BrdU antibody (5 μg ml^{-1}) followed by a Cy3-conjugated donkey anti-mouse IgG antibody. Nucleolar transcription was quantified by capturing digital images with a CCD camera attached to a Leica DMRXA fluorescence microscope.

siRNA-directed gene silencing and RT–qPCR. siRNAs were transcribed *in vitro* using the Silencer siRNA Construction Kit (Ambion, Austin, TX). HeLa cells were transfected with Lipofectamine 2000 (Invitrogen) and 40 nM siRNAs. Two different siRNAs were used: M19, which targets nucleotides 558–578 of *NM1* mRNA (5′-AAGTACATGGATGTGCAGTTT-3′) and M64, which targets nucleotides 1,355–1,385 (5′-AACCCGTCCAGTATTTCAACA-3′). A scrambled sequence was used as a control (5′-UCGUUGCAGGAUAUGUAGUUU-3′). The effect of NMI depletion on rDNA transcription was assessed either by measuring the immunofluorescence intensity after Br-UTP incorporation or by RT–qPCR with primers to 45S rRNA. Cellular RNA was isolated with TriReagent (Sigma) and 100 ng of RNA were reverse-transcribed with TaqMan Reverse Transcription Reagents (Applied Biosystems, Issaquah, WA) using random primers. RT–qPCR was performed in an ABI Prism 7000 instrument using SYBR Green PCR Master Mix (both Applied Biosystems), primers that amplify *NMI* mRNA (5′-GGGCAGGATGCGCTACC-3′ and 5′-TGAGCGCACTCTCCATGGT-3′) and 45S pre-rRNA (5′-CTCCGTTATGGTAGCGCTGC-3′ and 5′-GCGGAACCCTCGCTTCTC-3′). Probes to actin, GADPH and 18S rRNA were used to normalize the amount of loaded RNA. In parallel, cellular NMI levels were estimated by western blot analysis.

Immunoprecipitation assays. Pol I and basal transcription factors were enriched by chromatography of nuclear extracts from exponentially growing FM3A cells on a DEAE-Sepharose CL-6B column[35]. Proteins required for Pol I transcription were eluted with buffer AM (20 mM Tris-HCl at pH 7.9, 0.2 mM EDTA, 0.5 mM dithioerythritol, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 20% glycerol) containing 280 mM KCl (AM-280). This fraction (DEAE-280) exhibits a fivefold higher Pol I transcriptional activity than unfractionated extracts. Immunoprecipitations were performed with 100 μg of the DEAE-fraction or nuclear extracts, or 1 mg of total lysates of HeLa cells overexpressing Flag-tagged TIF-IA. To analyse the interaction of actin and NMI with Pol I and TIF-IA, cells were lysed in immunoprecipitation buffer (50 mM Tris-HCl at pH 7.4, 2 mM EDTA, 150 mM NaCl and 1% Triton X-100), cleared by centrifugation at 10,000*g* for 30 min, and the supernatants incubated for 4 h at 4 °C with the respective antibodies and protein A/G agarose. After washing in immunoprecipitation buffer, precipitated proteins were analysed on immunoblots.

ChIP assays. 3×10^5 U2-OS cells were fixed for 10 min with 1% formaldehyde and lysed in hypotonic buffer (20 mM Tris-HCl at pH 7.4, 10 mM NaCl and 0.5 mM PMSF). Nuclei were lysed in 200 μl of buffer containing 50 mM Tris-HCl at pH 8.0, 10 mM EDTA and 1% SDS. After sonication to yield DNA fragments of 0.5–1 kilobase (kb), lysates were cleared by centrifugation at 10,000*g* for 30 min, diluted fourfold with ChIP buffer (12.5 mM Tris-HCl at pH 8.0, 200 mM NaCl and 1.25% Triton X-100) and pre-cleared with protein G agarose at 4 °C for 45 min. For each immunoprecipitation, 10 μg of the respective antibodies and 10 μl of protein G agarose were incubated with lysate in the presence of sonicated salmon sperm DNA at 4 °C overnight. After elution and reversion of crosslinks by heating for 6 h at 65 °C, 1% and 3% of purified DNA were amplified by PCR with 30 cycles in the presence of 1.5 mM $MgCl_2$, 200 μM dNTPs and 10 pmoles of primers (rDNA promoter forward (-46/-26), 5′-GGTATATCTTTCGCTCCGAG-3′ and rDNA promoter reverse (+13/+22), 5′-AGCGACAGGTCGCCAGAGGA-3′); and 28S rRNA forward, 5′-CGACGACCCATTCGAACGTCT-3′ and 28S rRNA reverse, 5′-CTCTCCGGAATCGAACCCTGA-3′). PCR products were separated by electrophoresis on 2% agarose gels and stained with ethidium bromide. RT–PCR was performed in triplicate using a LightCycler (Roche, Basel Switzerland) and the SYBR Green detection system. The relative enrichment of rDNA was determined by calculating the ratio of rDNA present in the immunoprecipitates compared with rDNA in the input chromatin, and normalizing the data to control reactions containing no antibodies or control IgGs.

***In vitro* transcription assays.** Nuclear extracts were prepared from exponentially growing FM3A cells and transcription assays were performed as described[35]. The template was pMr170-BH[36], a ribosomal minigene construct representing a fusion between a murine 5′-proximal rDNA fragment (from –170 to +292) and a 3′-terminal rDNA fragment containing two transcription terminator elements. The template was used either as naked DNA or *in vitro*-assembled chromatin. For chromatin reconstitution, 11 μl of cytoplasmic extracts from 0–90 min *Drosophila melanogaster* embryos, 200 ng of plasmid DNA, 3 mM ATP, 30 mM creatine phosphate and 1 μg ml^{-1} creatine kinase were incubated in 40 μl reactions for 6 h as described[37]. Transcription reactions (25 μl) contained 10–20 ng of template, 5 μl of the DEAE-280 fraction (15 μg of total protein) and 30 ng of recombinant termination factor TTFΔN185 in 12 mM Tris-HCl at pH 7.9, 5 mM $MgCl_2$, 0.12 mM EDTA, 80 mM KCl, 10 mM creatine phosphate, 0.5 mM dithioerythritol, 12% glycerol, 0.66 mM each of ATP, CTP and GTP, 12.5 μM UTP and 1 μCi of α-^{32}P-UTP (800 Ci mmol^{-1}). Alternatively, a reconstituted transcription system was used[35] containing 3–5 μl of partially purified Pol I, 3 μl of TIF-IA–TIF-IC (QS-300 fraction), 2.5 μl of immunopurified TIF-IB and 10 ng of recombinant Flag-tagged UBF. After incubation for 60 min at 30 °C, RNA was isolated and analysed on 4.5% polyacrylamide gels. To examine the effect of actin and NMI on transcription activity, 1–4 μg of antibodies were added to the DEAE-280 fraction and incubated for 40 min at room temperature before addition of the template and nucleotides. NMI was immunopurified from HEK293T cells overexpressing C-terminally Flag-tagged NMI. Cells were lysed in 50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 0.1 mM PMSF and 1 mM dithiothreitol. NMI was bound to M2-agarose (Sigma) and eluted with 300 μg ml^{-1} of Flag peptide in buffer AM-300 containing 0.1% NP-40.

Abortive transcription-initiation assay. To assemble pre-initiation complexes, the DEAE-280 fraction was preincubated for 40 min at 30 °C with anti-actin antibodies. For initiation complex formation, template DNA and 0.5 mM ATP and CTP were added and incubation was continued for 20 min. Abortive transcripts were synthesized after addition of 1 μCi of α-^{32}P-UTP during a 45-min incubation period. The samples were treated for 15 min with alkaline phosphatase (1.5 U) to resolve labelled mononucleotides from ACU trimers. Abortive transcripts were extracted with phenol–chloroform, lyophilized and analysed on a 25% polyacrylamide sequencing gel.

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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