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Toward understanding the function of Nuclear Myosin I

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I. Introduction

1. Myosins

Myosins are widely known as proteins responsible for muscle contraction. These molecules, however, have many more functions that have been only recently uncovered. Myosins comprise a very diverse superfamily with more than 20 different classes and they serve various functions where there is a need for movement or maintenance of tension.

Myosins are molecular machines capable of translating the energy stored in ATP to directed movement along an actin filament. All myosins share a similar structure that results from its mechanism of action; they consist of three parts: head (or motor domain), neck and tail. The motor domain binds actin and hydrolyzes ATP, and it is the most conserved part of the molecule. It changes its conformation dependending on whether it binds actin, ATP, or ADP and in this manner it is able to proceed unidirectionally through the myosin working cycle. The precise mechanism of the working cycle will be described later. The neck contains the binding sites for one or several light chains, usually calmodulins, which stiffen the neck and allow it to function as a lever arm translating the angular movements of the head to a linear movement of the tail. The tail is the most variable part of the molecule. It either binds to the cargo or allows dimerization (and in case of some myosins even polymerization) of the myosin molecules thus giving it the special properties needed to perform its specialized task in the cell.

The functions of myosins are multiple. Apart from the function in muscle cells, myosins move organelles and membrane appendages, regulate the tension of subcortical actin meshwork, contract the cleavage furrow in cytokinesis and regulate the tension of mechanoreceptors in inner ear just as examples.

2. Structure of myosins

The first high-resolution X-ray structure of myosin was solved in 1993 on the model of S1 fragment (head) of the chicken fast skeletal myosin (Rayment *et al* 1993, Fig. 1). Since then the high resolution structures of many other myosins in various conformations have been obtained and the basic architecture was found similar in all cases.

(a) Head

The myosin head domain contains the actin and nucleotide binding sites and it has the ATPase activity. This is the most evolutionary conserved part of the molecule and differ-

ences in the head sequence result in different biochemical properties of myosins, such as overall ATPase rate and occupancy of various stages of the myosin working cycle.

The myosin head consist of four large subdomains connected by rather flexible links called joints (Fig. 1). During the working cycle, the subdomains move relatively to each other and the conformational changes lead to the rotation of the most C-terminal domain, the converter domain, that is then translated into the swing of the neck.

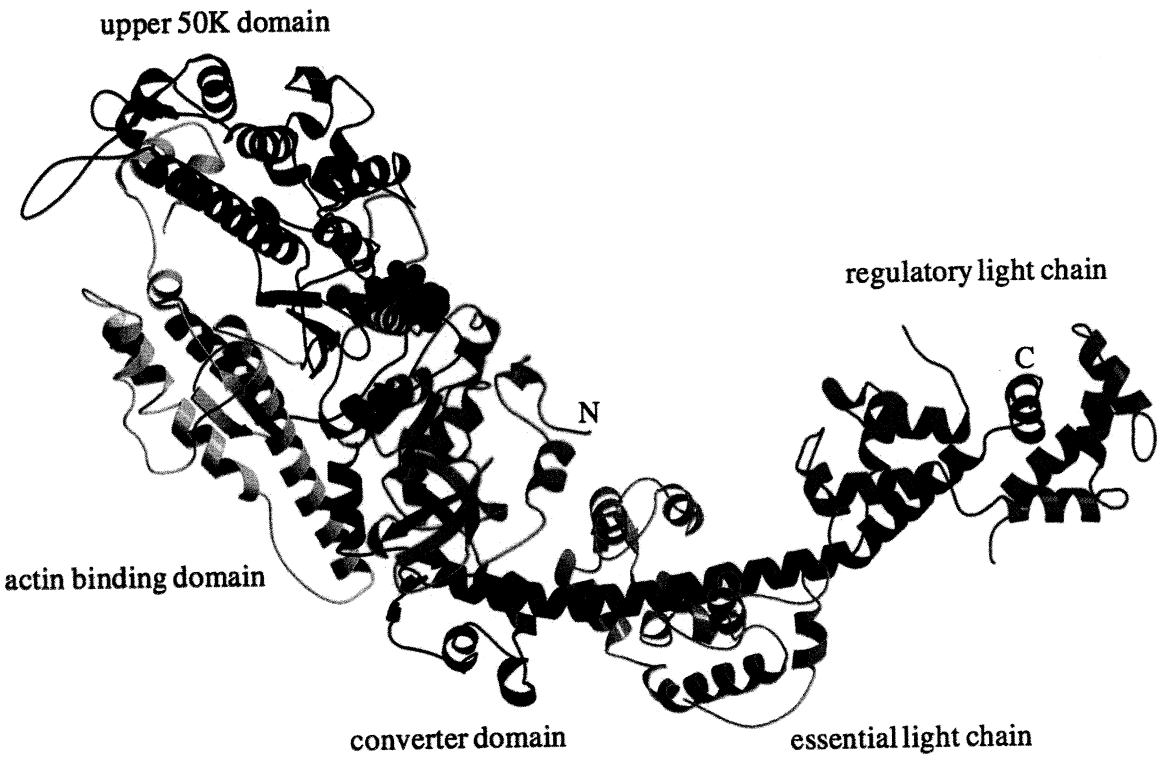

Fig. 1 Myosin structure. The myosin head consists of four subdomains: N-terminal subdomain (green), 50 kDa upper subdomain (red), 50 kDa lower subdomain (gray) and converter subdomain (blue). These subdomains are connected by highly conserved structures called joints. The two 50 kDa domains are separated by a deep cleft known as the 50 kDa cleft which divides the actin binding site to two parts and spreads to the γ-phosphate binding pocket. The converter subdomain continues as the α-helical neck (blue), here with two bound light chains (yellow and magenta). During the working cycle, the conformational changes in the motor domain lead to the rotation of the converter domain and swing of the neck. Picture from Holmes and Geeves (2000).

(b) Neck

The neck is an α-helical rod-like structure that binds the light chains, usually calmodulin. Binding of light chains gives the neck the stiffness needed to act as a lever arm to transfer the rotation of the converter domain to the movement of the tail. When the light chains are not attached the neck bends and the changes of the head are not efficiently transferred to the movement of the tail.

Light chains bind to IQ domains, motifs of consensus sequence IQxxxGRxxxR (Bahler and Rhoads 2002)

The length of the actin filament traversed by a single step and therefore velocity of the actin filament movement depends on the length of the neck and thus the number of the IQ motifs (Sakamoto *et al* 2003, Moore *et al* 2004). Calmodulins are signaling proteins that regulate many biochemical processes in response to the Ca^{2+} level. Calmodulin binds IQ domains, unlike most other calmodulin regulated proteins, in the absence of Ca^{2+} and releases the IQ domain when the concentration of Ca^{2+} raises (Bahler and Rhoads 2002).

(c) Tail

The tail is the most variable part of the molecule. It either binds to the cargo or allows dimerization (and in case of some myosins even polymerization) of the myosin molecules thus giving it the special properties needed to perform its specialized task in the cell.

The tail endows the myosin molecule with its specific properties. This is the most variable part of the molecule. In many myosins it contains sequences forming coiled-coil domains and thus allowing the myosins to dimerize. In other myosins it is believed to bind to the cargo being transported (Oliver *et al* 1999).

3. The myosin working cycle

The myosin working cycle was extensively characterized by kinetic analysis already 35 years ago (Lymn and Taylor 1971) reviewed in (Geeves and Holmes 1999). With gradually appearing structural data for myosins in various parts of the working cycle, it was recently possible to reconcile the structural with the kinetic data (Holmes *et al* 2004, Sweeney and Houdusse 2004, Fig. 2).

Myosin molecule without nucleotide binds strongly to actin filaments. This condition is called the rigor state after the "rigor mortis" – stiffness of the dead body. Addition of ATP dissociates the actomyosin complex, leading to the post-rigor state. The myosin molecule in this state rapidly changes conformation to the pre-power stroke state. This transition consists of the closure of the 50 kDa cleft and the rotation of the converter domain along with major movement of the lever arm, which is now in a position ready for the power stroke. At the same time the ATP binding site gets to the conformation in which ATP hydrolysis is possible and ATP is rapidly hydrolyzed. This assures the virtual irreversibility of this reaction while both products, ADP and phosphate Pi remain trapped in the active site. The myosin molecule

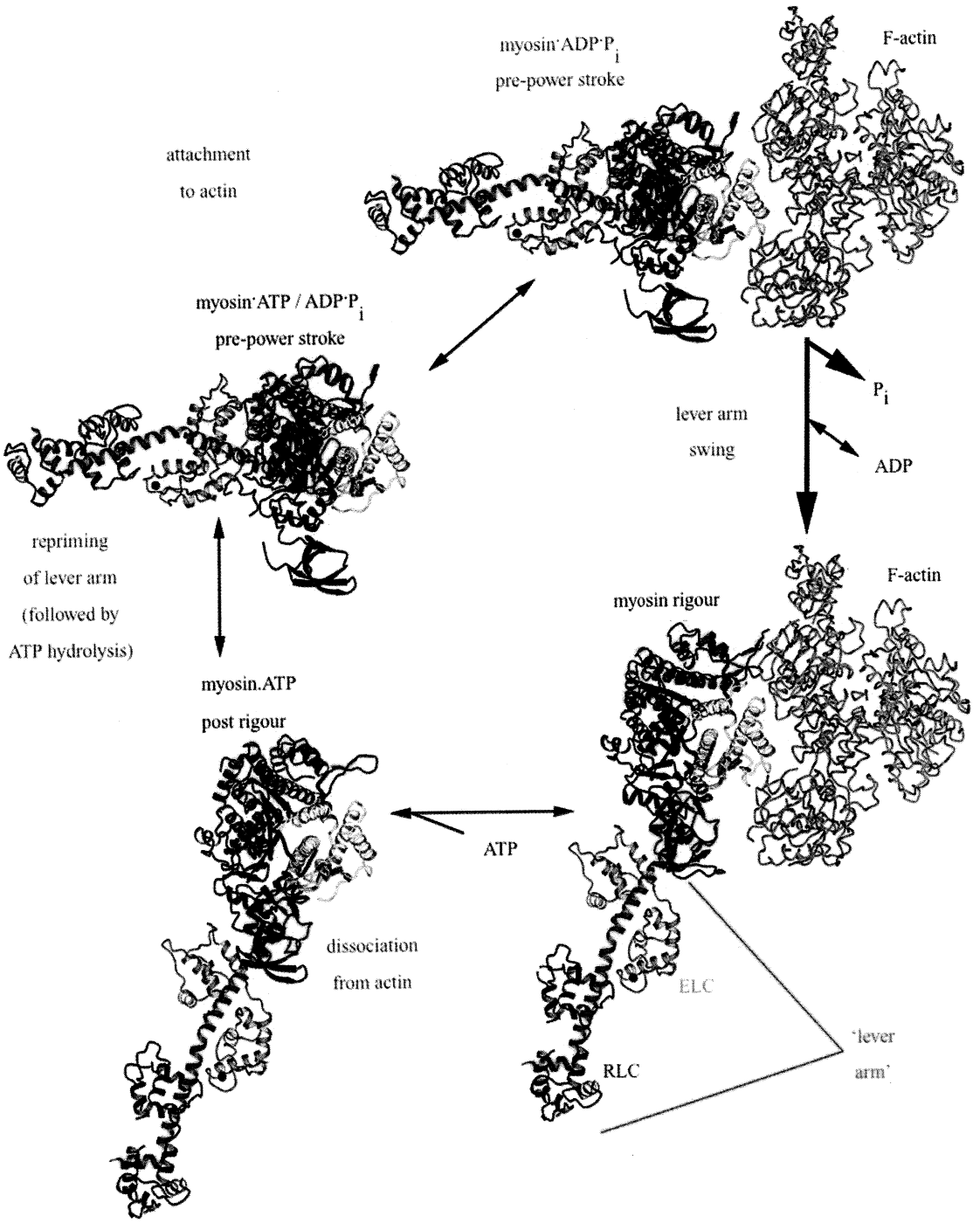

Fig. 2 The myosin working cycle. From Sweeney and Houdusse (2004).

is rather stable in this conformation, until it meets an actin filament. Myosin initially binds weakly to actin and this binding leads to change of conformation that opens a "back door" for Pi to leave the complex. A temperature-dependent isomerization step follows, resulting in strong actin binding and lever arm swing. This strong actin-binding, strong ADP-binding

state is characterized by intermediate position of the lever arm (between the pre-power stroke and rigor) and it serves as a strain sensing mechanism i.e. the ADP is not released until the strain is relieved. This mechanism is not present in the fast muscle myosins presumably as an adaptation for the speed of shortening. Further movement of the lever arm allows the release of ADP and the myosin is again in the rigor state.

While all myosins proceed through the same steps during the working cycle, the speeds of transitions vary widely. This allows the different myosins to be fine-tuned for their functions. One important determinant of the myosin properties is the relative amount of its cycle being strongly bound to actin. The ratio of occupancy of the strong states to the duration of the whole cycle is known as the duty ratio. For instance, the fast muscle myosins that work in large ensembles and must move quickly along the actin filaments, have very low duty ratio so that strongly bound myosin does not block the sliding movement. The slow muscle and non-muscle myosins, on the other hand, are optimized to keep tonus and they have much higher duty ratio (De La Cruz and Ostap 2004, Nyitrai and Geeves 2004).

4. Classification of myosins

The myosins are classified to at least 20 classes based on the sequence of the motor domain (Berg *et al* 2001, Foth *et al* 2006, Fig. 3). The structure of the tails is in good agreement with this classification (Korn 2000, Fig. 4). The best known and the first discovered are the muscle myosins representing class II. Class I contains simple monomeric myosins with short tail which usually binds directly to cargo. The rest of the myosin classes are numbered in the order of their discovery. Some myosin classes are widely phylogenetically expressed, whereas others have been identified only in a single organism. Budding yeast has only five myosin genes of the classes I, II and V, while human genome contains nearly 40 myosin genes from 12 classes (Tab. 1, Berg *et al* 2001).

Class	1	2	3	5	6	7	9	10	15	16	18	?	Together
No. of human genes	8	14	2	3	1	2	2	1	2	1	2	1	39

Tab.1 Classification of myosins in the human genome.

(a) Class II

Muscle myosin was discovered more than 60 years ago and it was the only known type of myosin for decades. For this reason the members of class II are also referred to as conventional myosins. They form complexes containing two myosin heavy chains and two

pairs of light chains. Each heavy chain has the prototypic motor domain, neck with two IQ motifs that bind two light chains, and the tail with coiled-coil forming sequence responsible for dimerization. Myosin II dimers associate at low ionic strength to form filaments that represent, along with actin filaments, the structural basis of muscle contraction. There are, however also non-muscle myosins II that play a role in contraction of cleavage furrow during cytokinesis and other processes that need the generation of tension within the cells. (For reviews see (Geeves and Holmes 2005, Craig and Woodhead 2006).

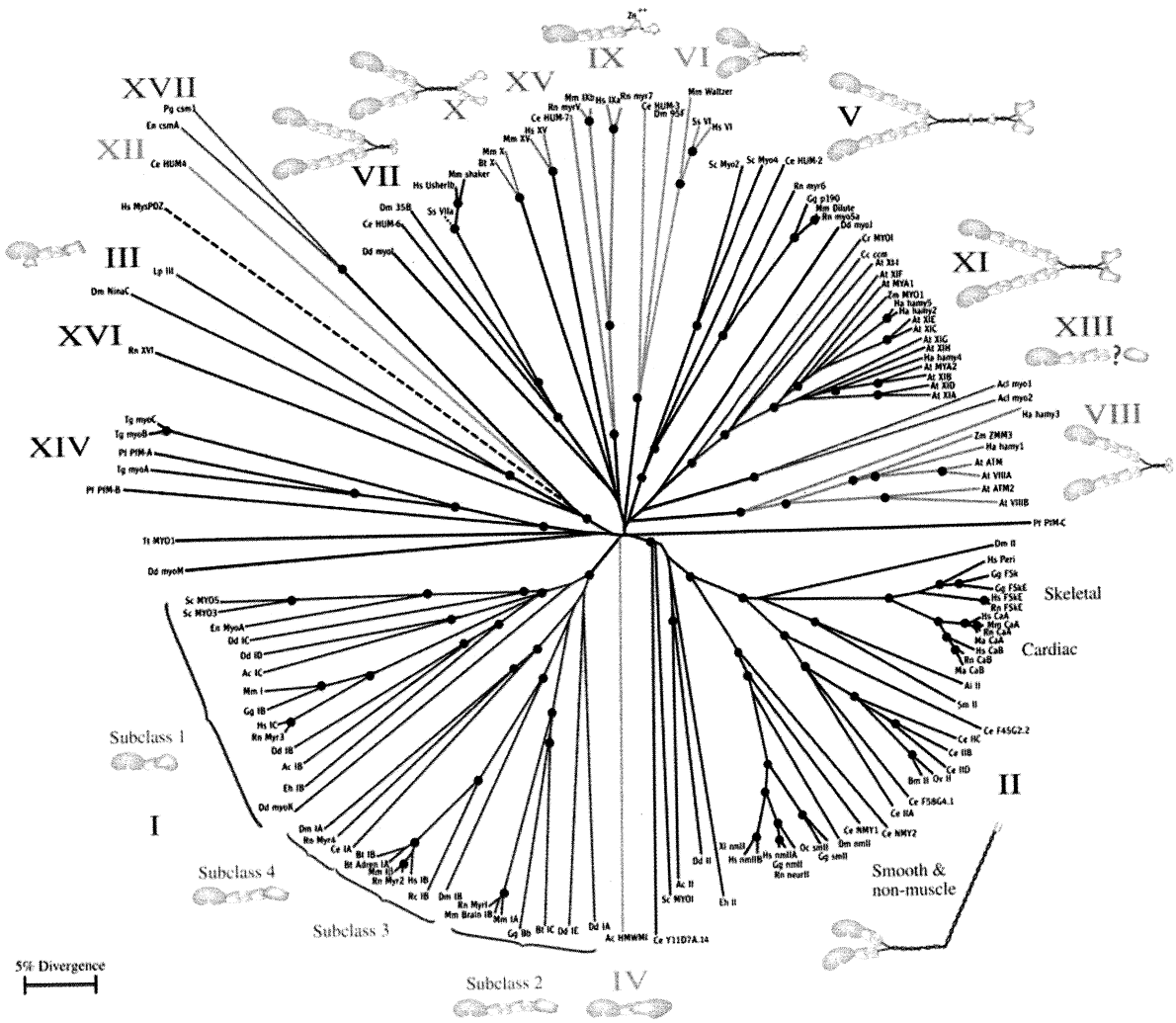

Fig. 3 An unrooted phylogenetic tree of myosin superfamily. The core motor domains were aligned. The black dots indicate nodes with >90% bootstrapping values. Picture from Hodge and Cope (2000).

(b) Class I

Class I myosins are short and monomeric molecules. Class I myosin in *Acanthamoeba castelanii* was the first discovered unconventional myosin (Pollard and Korn 1973). Their functions are very variable, for instance *in Dictyostelium discoideum* three class I myosins are implicated in the regulation of pseudopod formation by maintenance of tension in subcortical actin meshwork (Dai *et al* 1999, Falk *et al* 2003); Myo1a was shown to be essential for maintaining the structure and composition of the intestine brush border microvilli in mouse (Tyska *et al* 2005); class I myosins were also proposed to function in endocytosis – vesicle scission in budding yeast (Jonsdottir and Li 2004), and exocytosis in mouse (Bose *et al* 2004). The functions of the most studied myosin IC will be discussed later.

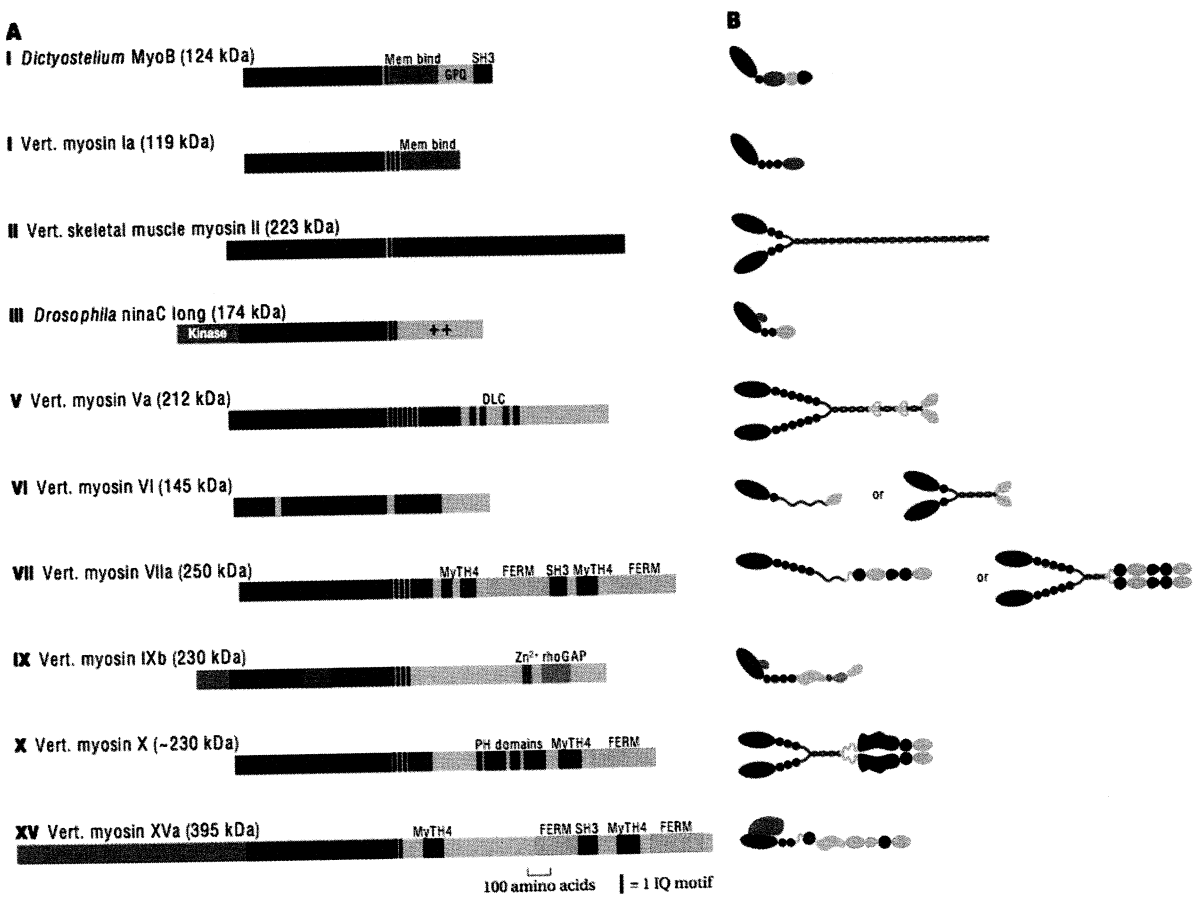

Fig. 4 Myosin superfamily. A) Sequences of various myosin heavy chains. Shown are examples from various myosin classes. Head domains are colored blue, IQ domains black, CC denotes coiled-coil, ++ positive charge domains. B) Schematic representation of the structure. From Krendel and Mooseker (2005).

(c) Other Myosin Classes

Myosin V is uniquely adapted to transporting vesicles and other cargo along actin tracks in the cytoplasm. The cargo varies from synaptic vesicles to melanosomes in mammals and vacuoles to mRNA in the yeast (Provance and Mercer 1999). Myosin V has a globular tail domain that binds cargo through adaptor protein complexes. It contains a coiled-coil domain in its tail and forms dimers, and because each head stays firmly attached to the actin filament for larger part of the working cycle, at least one of the motor domains is attached to the actin filament during many steps. The myosin V head is also more likely to proceed in the working cycle and release actin when the strain is changed by the pull of the other head which binds more forward. These properties make myosin V processive (able to move long distances along the filament without dissociating). Furthermore, because of its long neck, myosin V takes 36 nm steps – the length of the actin filament helix twist. This way myosin V does not have to spiral and proceeds hand-over-hand along one side of the actin filament dragging the cargo through its flexible tail (for a review, see Sellers and Veigel 2006).

Myosin VI moves the actin filaments in the opposite direction than the other myosins i.e. to the pointed end. This is caused by the unique 50 amino acid insertion between the motor domain and the neck (Wells *et al* 1999). Mutation of this myosin leads to Snell's waltzer syndrome, which consists of deafness accompanied by waltzing and circling behavior, caused by progressive loss of cochlear hair cells (Avraham *et al* 1995).

Mutations in the myosin VII genes are also responsible for hereditary deafness in human and mice. In mouse, the mutations of the gene for myosin VIIA called *shaker1* cause deafness, hyperactivity and head tossing (Gibson *et al* 1995). The stereocilia of the hair cells are progressively disorganized. In human, the mutation leads to the Usher syndrome type 1B consisting of deafness and retinitis pigmentosa causing progressive blindness (Weil *et al* 1995).

The members of the classes VIII, XI, and XIII are found only in plants, where they are responsible for instance for cytoplasmic streaming – a dramatic movement of organelles along actin tracks (Shimmen and Yokota 2004).

5. Myosin IC

Myosin IC (Myo1c) is one of the most studied unconventional myosins. Due to the significant similarity of Myo1c and nuclear myosin I (NMI) most of the characteristics of Myo1c can also be applied to NMI.

(a) Properties of Myo1c

Myo1c motor domain has a low duty ratio and slow rate, therefore it seems to be optimized for tension maintenance (De La Cruz and Ostap 2004). Similarly to other class I myosins, Myo1c produces its working stroke in two steps (Batters *et al* 2004). The second step is accompanied by the release of ADP and completes only when the strain on myosin is relieved. Myo1c can therefore act as a strain-dependent crosslink, maintaining tension between actin and the structures bound to the myosin tail. Because of its low duty ratio, a single Myo1c molecule is not processive and there must be an ensemble of approximately 50 molecules to ensure processivity (De La Cruz and Ostap 2004).

The neck of Myo1c contains three IQ domains, the fourth partial IQ domain was described in bullfrog (Zhu *et al* 1996, Zhu *et al* 1998, Gillespie and Cyr 2002). Myo1c binds 3 calmodulins in the absence of Ca^{2+} and one of them is released in the presence of 0.1 mM Ca^{2+} causing complete loss of motor activity (Zhu *et al* 1996, Zhu *et al* 1998). On the other hand, Myo1c ATPase activity is increased in the presence of Ca^{2+}, presumably because calmodulin dissociation leads to softening of the neck "lever arm", and the catalytic circle can proceed without coupling to production of mechanical work.

The Myo1c neck binds to specific Myo1c receptors and is needed for proper localization of Myo1c in the hair cells (Cyr *et al* 2002). The receptor is most likely cadherin 23 and the interaction is blocked by excess calmodulin (Phillips *et al* 2006). Moreover, the neck contains many positively charged and hydrophobic residues and it was shown to contribute to the membrane binding by nonspecific interaction with anionic phospholipids. This effect is observable only in the presence of Ca^{2+} when at least one of the calmodulins is dissociated (Tang *et al* 2002, Hirono *et al* 2004). Therefore it seems that the level of Ca^{2+} determines whether Myo1c binds calmodulin or cadherin23/PHR1/anionic phospholipids.

The tail binds highly specifically the headgroup of an anionic phospholipid phosphatidylinositol-4,5-bisphosphate (PIP2) (Tang *et al* 2002, Hirono *et al* 2004, Hokanson and Ostap 2006) by a putative pleckstrin homology (PH) domain (Hokanson *et al* 2006). This interaction is needed for the proper localization of Myo1c to the lipid rafts in membranes (Hokanson *et al* 2006). The PIP2 content of different membrane domains varies and can be precisely regulated by the action of phospholipase C and various kinases and phosphatases. The localization of Myo1c on membranes might be controlled in this way.

6. Functions of Myo1c

(i) Inner ear hair cells adaptation motor

Myo1c is involved in adaptation of mechanotransduction in the inner ear. Mechanical stimuli like sound and movement are detected by hair cells in the inner ear (Fig. 5). The sensor of the hair cells is the hair bundle – a bunch of stereocilia at the apical surface of the hair cell. The stereocilia are connected by tip links – extracellular filaments bound to mechanically gated transmembrane ion channels. When the mechanical stimuli move the hair bundle back and forth, the tension of the tip links increases and decreases. This leads to opening and closing of the ion channels, and resulting electric signal is transmitted to the brain (Gillespie 2004). This system, however, must be able to adapt to prolonged changes of strain so that it is always properly tuned to sense rapid and weak signals. Indeed, during static excitatory stimulation the initially strong signal declines over ca. 20 ms and the hair bundle is ready to react strongly to any further displacement. On the contrary, when the hair bundle is negatively deflected the signal is decreased only for a short time and release of the force leads to a strong stimulation equivalent to a positive deflection (Gillespie and Cyr 2004).

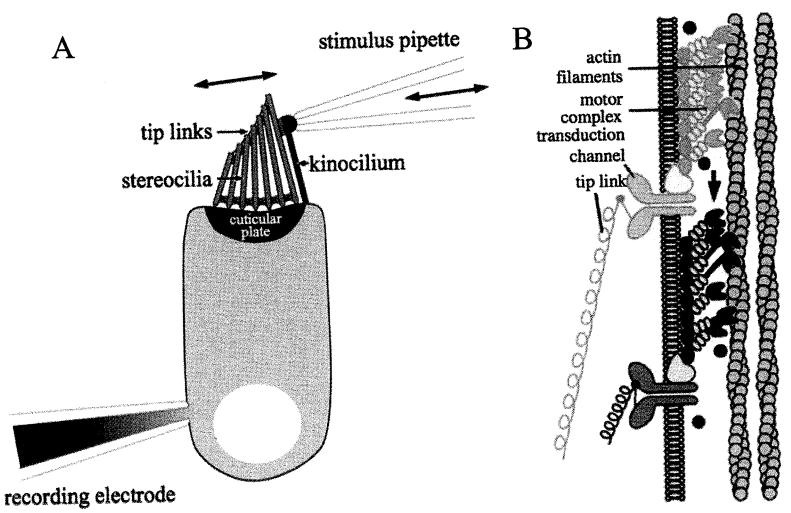

Fig 5. Hair cell. Hair cells detect movement in the inner ear sensory organs. A) The movement of the hair bundle on the top of the cell is transmitted to the tip links that in turn open the mechanically gated ion channels and action potential ensues. The hair cell can be stimulated artificially by moving the hair bundle and the action potential can be recorded. B) Myo1c maintains stable tension of the tip link. When the tension increases, the Myo1c assembly slips down along the actin filaments to achieve a new balance. When the tension decreases, myosins climb upwards. Picture from Gillespie (2004).

In a very elegant experiment Holt *et al* 2002 showed that Myo1c is needed for this adaptation. Block of Myo1c leads to loss of adaptation to both positive and negative stimuli. The authors hypothesize that Myo1c is connected in series between the transduction channel

and the actin filaments of the stereocilium. When the movement of the bundle increases the tension of the tip link (positive stimulus), the force overcomes the resistance of the myosin molecules and they slip along the actin bundle to a new position where the tension is optimal. On the other hand, when the hair bundle is deflected so that the tip link tension decreases (negative stimulus) the myosin molecules climb along the actin bundle until they reach the optimal tension.

(ii) Myo1c is involved in the glucose transport regulation

The muscle and adipose tissue cells increase their glucose uptake in response to the insulin signal. This is achieved by fusing membrane vesicles containing glucose transporter GLUT4 with the plasma membrane. When the signal abates, the GLUT4 is endocytosed and recycled. The exocytosis of GLUT4 vesicles is dependent on phosphatidylinositol 3-kinase (PI3K) activity and Myo1c is involved as well. Overexpression of Myo1c leads to increased GLUT4 exocytosis while siRNA knockdown or expression of a dominant negative tail construct decreases glucose transport (Bose *et al* 2002). When the PI3K is blocked by specific inhibitor, insulin signal leads to accumulation of GLUT4-containing vesicles underneath the plasma membrane. This effect could be overcome by Myo1c overexpression (Bose *et al* 2004). These and other data suggest that Myo1c is needed to tether the vesicles in the cortical actin meshwork and promote membrane fusion in some way. The authors observed dramatically intensified membrane ruffling after Myo1c overexpression. Increased concentration of Myo1c was found in the areas of ruffling, and the exocytosis after insulin signal took place predominantly in these areas (Bose *et al* 2004). The PI3K is able to phosphorylate PIP to PIP2 and therefore create more Myo1c membrane receptors. Myo1c is able to tether the assembling actin filaments to specific membrane areas containing actin nucleation/polymerization factors (Sokac *et al* 2006) and this could be the mechanism behind the increased ruffling.

(iii) Myo1c regulates the neuronal growth cone movement

The growth cone of the chicken dorsal root ganglia neurons is a very dynamic structure that continuously protrudes and retracts filopodia and lamellipodia in search of various axon guidance cues (Fig. 6) and the presence of several myosins including Myo1c was described there. Jay's laboratory used the chromophore-assisted laser inactivation (CALI) to study the involvement of motor proteins in the neuronal growth cone movement. The technique uses malachite green labeled antibodies to target a desired molecule and laser light is then used to topically activate the dye. The energy absorbed by the dye destroys bound pro-

teins without seriously affecting surrounding proteins (Jay 1988). CALI of Myo1c causes increased protrusion of lamellipodia and decreased retrograde actin flow, while CALI of non-muscle myosin II causes increased retraction (Wang *et al* 1996, Diefenbach *et al* 2002). The authors explain this by a clutch mechanism model, where Myo1c is responsible for the relative movement of membrane and actin filaments (Fig. 7).

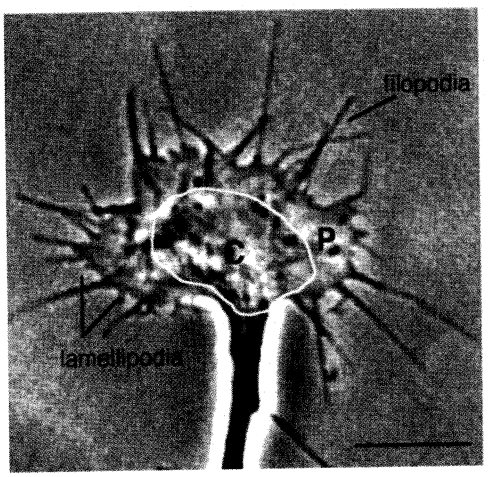

Fig. 6 The growth cone of chick dorsal neuronal ganglion. Central (C) and peripheral (P) domains are delineated. Fingerlike filopodia and veil-like lamellipodia dynamically protrude and retract in the periphery. Figure taken from Wang et al (2003).

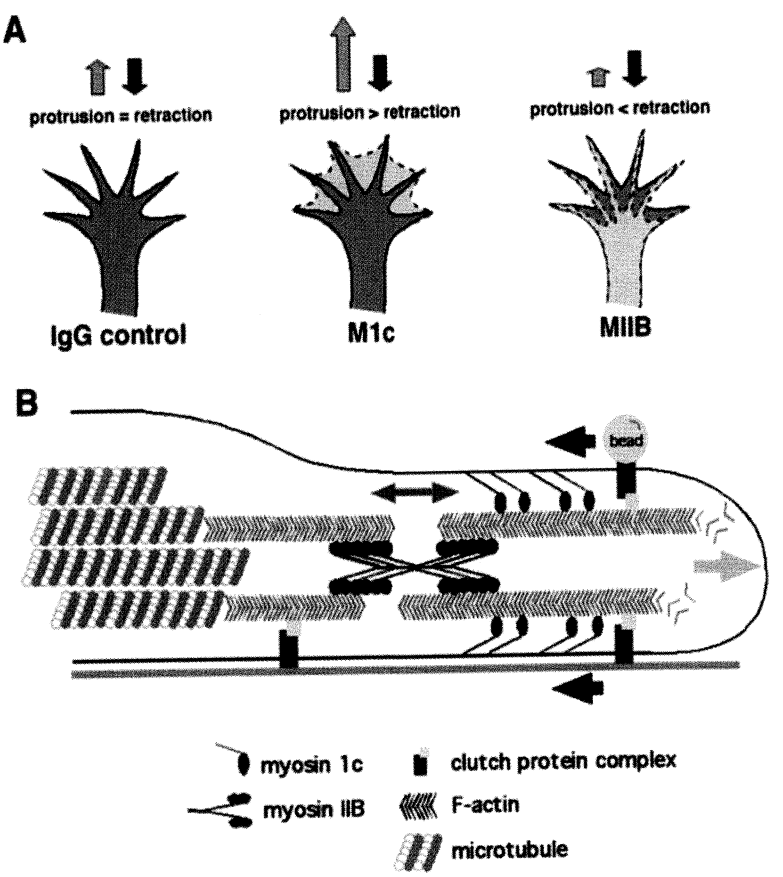

Fig. 7 A model of the function of myosins in lamellipodial dynamics. (A) In control IgG the CALI shows no effect on movement while the Myo1c targeted CALI causes lamellipodial protrusion and myosin II targeted CALI causes retraction. (B) Myo1c molecules move the membrane toward the plus end of the actin fibers that face the membrane in the direction of protrusion. When the actin filaments are fixed to the substrate by a clutch protein complex, protrusion ensues by the means of actin polymerization. When the actin filaments are not fixed to the substrate this movement leads to a retrograde actin flow, slowing the protrusion. Myosin II cross-links the actin filaments and increases the tension in the actin meshwork. This tension might be necessary for the leading edge progression. Figure taken from Diefenbach *et al* (2002).

By altering local Ca^{2+} concentration in different areas of the growth cone, which in turn modulates the activity of the myosins located there, the adhesion receptors can steer the direction of growth of the neurite (Wang *et al* 2003).

(iv) Myo1c is involved in regulation of sodium transport in kidney collecting duct cells

Overexpression of full-length Myo1c and deletion mutant lacking the motor domain leads to altered responses of the kidney collecting duct cells to stimulation by antidiuretic hormone and a diuretic Amilorid. The authors explain this by assumed perturbation of sodium channels transport to the membrane (Wagner *et al* 2005).

(a) Partners of Myo1c

The binding partners of Myo1c proved to be elusive. Myo1c was reported to interact with cadherin 23 (Siemens *et al* 2004, Phillips *et al* 2006) which was proposed to form the tip links, and with PHR1 (Etournay *et al* 2005), an integral membrane protein containing the pleckstrin homology (PH) domain. However, in neither case it was proven that the interaction is direct and especially in the case of PHR1, which also contains the IP3 binding PH domain, the interaction might be mediated via PIP2 in lipid rafts.

The interaction with phospholipids, on the other hand, is very well documented. The tail and neck of NMI was shown to bind to membrane phospholipids by several studies (Adams and Pollard 1989, Reizes *et al* 1994, Tang *et al* 2002, Hirono *et al* 2004, Hokanson and Ostap 2006).

7. *NMI*

NMI was first described in 1997 (Nowak *et al* 1997) and soon the gene was cloned and characterized. NMI mRNA originates from the same gene as Myo1c by alternative start of transcription and it differs from Myo1c mRNA only by a distinct 5' end exon. This exon codes a methionin and first six amino-acids of a 16 amino acid N-terminal sequence, the rest is coded by the next exon (Pestic-Dragovich *et al* 2000, Fig. 8).

This N-terminal sequence was proposed to be necessary for the nuclear translocation or retention based on immunofluorescence experiments with transfected tagged NMI and Myo1c but the precise mechanism of its function is still not known.

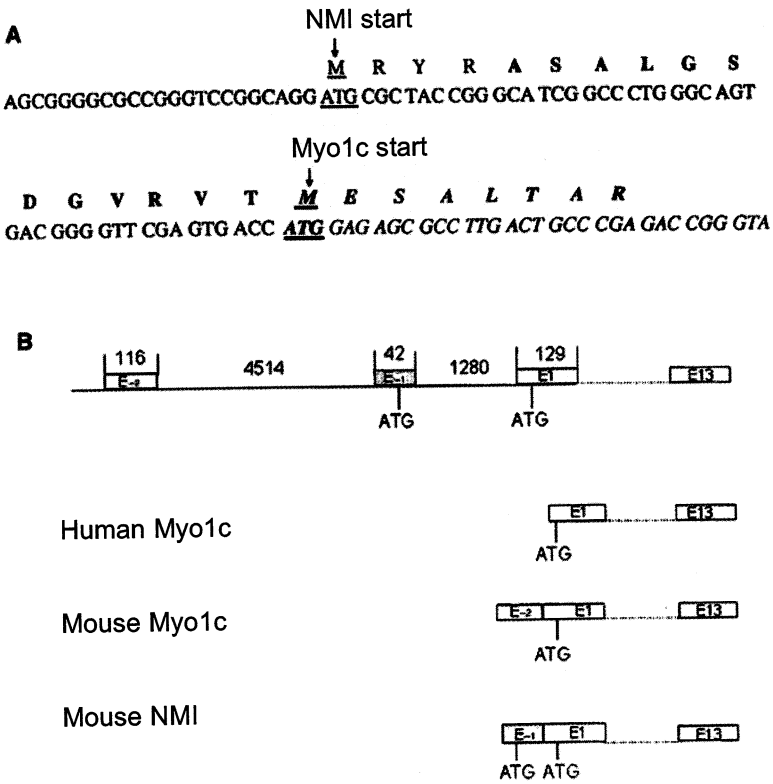

Fig. 8 The NMI gene. A) The NMI mRNA. The starting methionins for NMI and Myo1c are underlined and the Myo1c sequences are in italics. B) The structure of NMI gene in mouse and human. In human, the transcription of Myo1c starts with exon 1 while in mouse with exon -2. When the exon -1 is transcribed the translation starts from the first methionin and NMI is synthetized. Reproduced from Pestic-Dragovich et al. (2000).

(a) NMI in transcription

It was noted early on that NMI colocalizes with RNA polymerase I and II and with the sites of their transcription activity. As shown by confocal and electron microscopy, NMI is scattered in the nucleoplasm in foci containing also actin and Pol II. When the cells are treated with transcription inhibitors α-amanitin or actinomycin D, the colocalization is lost (Pestic-Dragovich *et al* 2000). In the nucleolus, NMI has been found in the dense fibrillar compartment (DFC), where the transcription of ribosomal genes takes place. Here it colocalizes with actin, Pol I and the sites of active transcription (Nowak *et al* 1997, Fomproix and Percipalle 2004).

At that time, it was important to find out whether NMI plays a role in transcription by Pol I and Pol II. In the first research paper, which is a part of this work, we have shown that NMI is indeed required for pol I transcription and that it is associated with Pol I through the basal transcription factor TIF-IA – a key regulator of Pol I transcription (Philimonenko *et al* 2004).

This claim is based on following evidence: NMI coimmunoprecipitates and copurifies with Pol I; RNAi knockdown of NMI or microinjections of anti-NMI antibodies decrease Pol I transcription in vivo while overexpression of NMI increases the transcription of ribosomal

genes; and in an in vitro transcription system, anti-NMI antibodies inhibit transcription by Pol I in a dose-dependent manner, while addition of purified NMI increases transcription. Moreover, we showed that NMI binds Pol I through the basal transcription factor TIF-IA which defines the initiation-competent subpopulation of Pol I and through which the rate of transcription initiation is regulated (Philimonenko *et al* 2004). NMI was also found on the promoter as well as the coding region of rDNA (Philimonenko *et al* 2004, Percipalle *et al* 2006) by chromatin immunoprecipitation (ChIP).

There are now convincing data suggesting an involvement of NMI also in transcription by Pol II. Hofmann *et al* 2006b show that NMI colocalizes and copurifies with Pol II, and antibodies to NMI inhibit, while NMI itself activates, Pol II transcription in vitro in a dose-dependent manner. In agreement with this, microinjections of anti-NMI antibodies decrease Pol II transcription in vivo. Interestingly, they show that antibodies to NMI do not inhibit formation of the Pol II pre-initiation complex but they block the formation of the first phosphodiester bond during transcription initiation.

However, there is not yet an agreement precisely in which step of transcription NMI is needed. The results from Pol II transcription system reconstituted from purified Pol II and transcription factors suggest that NMI is needed for the formation of the first phosphodiester bond (Hofmann *et al* 2006b), while the experiments with Pol I transcription in nuclear extracts indicate the involvement of NMI in the later steps (promoter escape or elongation) (Percipalle *et al* 2006).

(b) NMI and chromatin remodeling

It was recently described that approximately 10% of cellular NMI associates with the chromatin remodeling complex WSTF-SNF2h (Cavellán *et al* 2006, Percipalle *et al* 2006). Together with several other nuclear proteins, WSTF-SNF2h and NMI form a large 3 MDa complex referred to as B-WICH.

SNF2h is a member of the ISWI family of chromatin remodeling ATPases and its functions depend largely on the protein complex in which it is contained. For instance, it partners with TIP5 to form NoRC (nucleolar remodeling complex), a chromatin remodeling complex that sits on the promoters of unused ribosomal genes and inhibits their transcription (Santoro 2005). Together with WSTF (Williams syndrome transcription factor), SNF2h plays a role in DNA replication - it binds through WSTF to PCNA (proliferating-cell nuclear antigen) on replicated chromatin and prevents premature formation of heterochromatin and it

therefore allows the rebinding of factors that transmit the epigenetic state to the newly synthesized DNA (Poot *et al* 2005).

WSTF associates with both ISWI and SWI/SNF chromatin remodeling complexes. Together with SWI/SNF chromatin remodeling complex it is involved in regulation of vitamin-D coupled transcription (Kitagawa *et al* 2003). WSTF binds acetylated histones, mainly H3 acetylated at lysine 14, through its bromodomain and tethers the chromatin remodeling/modification complexes to the promoter of vitamin-D regulated genes (Fujiki *et al* 2005). Because WSTF associates with two different chromatin remodeling complexes, it was suggested that it acts as a platform tethering the complexes to chromatin (Kitagawa *et al* 2003).

NMI was shown to co-purify and co-immunoprecipitate with WSTF-SNF2h. The complex also contained RNAs: Pol I transcript 45S rRNA and Pol III transcripts 5S rRNA and 7SL RNA (part of signal recognition particle SRP). NMI was also found on rRNA, 5S and 7SL RNA genes by ChIP. Intriguingly, siRNA knockdown of WSTF decreases 5S and 7SL transcription (Cavellán *et al* 2006). Pol I transcription is inhibited by WSTF knockdown only on chromatin templates but not on naked DNA (Percipalle *et al* 2006). It was possible to immunoprecipitate Pol I with the WSTF-SNF2h-NMI complex, but only after in vivo crosslinking which indicates that the association of the complex with Pol I is relatively weak or very dynamic (Percipalle *et al* 2006).

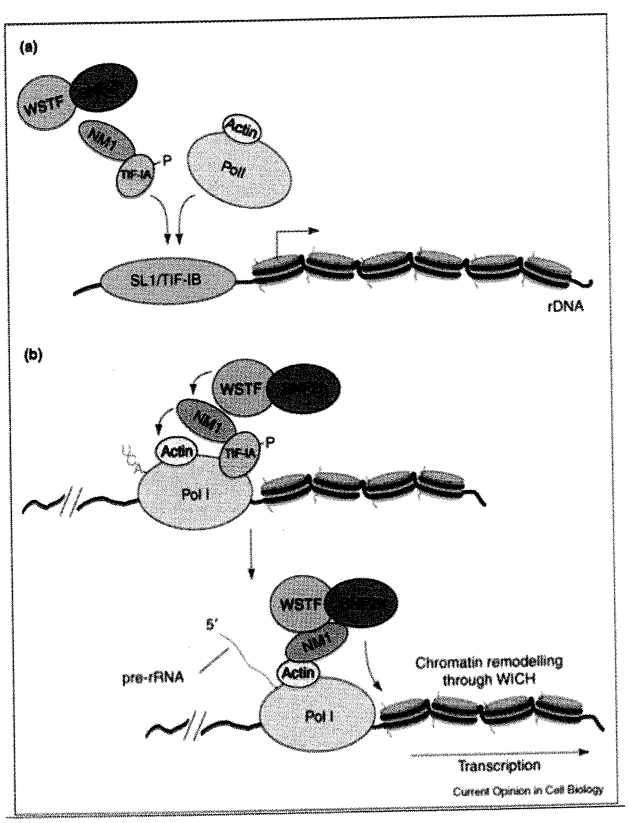

Fig. 9 A speculative model for the function of NM1 as part of B-WICH in rDNA transcription. (a) Pol I assembly on rDNA promoter and transcription initiation. (b) Promoter clearance and elongation of nascent rRNA transcripts. Following TIF-IA-mediated recruitment of NM1 onto the promoter to facilitate the assembly of the transcription competent pol I, B-WICH associates with the ribosomal gene through the interaction of NM1 with the pol I-associated actin. The actin–NM1 interaction might also be responsible for the displacement of TIF-IA. TIF-IA is only situated on the promoter and does not follow either actin-pol I or NM1 in the B-WICH complex along the ribosomal gene. By contrast, B-WICH is found both at the promoter and along the entire ribosomal gene, and it is possible that it functions in promoter clearance and elongation. The dynamic interaction between actin and NM1 might serve as a molecular platform to couple B-WICH to the ribosomal gene. Figure from Percipalle *et al* (2006).

It was suggested that the activation and repression of ribosomal genes could be achieved by similar mechanism. Ribosomal genes are repressed by NoRC where TIP5 binds to TTF1 (transcription termination factor 1) on rDNA and brings SNF2h, which mobilizes the chromatin, and histone deacetylases (HDAC) and DNA methyl transferases (DMTrs) that modify the chromatin (Santoro 2005). Conversely, WSTF could bring the SNF2h and associated factors to the rDNA to activate transcription and the recruitment of WSTF could be via NMI and the PolI bound actin (Grummt 2006, Percipalle and Farrants 2006, Fig. 9).

(c) Other proposed functions of NMI

There are other hypothetic models for the role of NMI in transcription. De Lanerolle and colleagues speculate that NMI could bind by its tail to the negatively charged DNA and interact with the Pol I bound actin to power elongation (de Lanerolle *et al* 2005, Hofmann *et al* 2006a). There is also a recent observation that chromosomal site after transcriptional activation translocates from the nuclear periphery to the interior, and this movement is perturbed by mutants of NMI and actin. The authors propose the possibility of direct involvement of actomyosin in the movement (Chuang *et al* 2006).

NMI was also implicated, along with actin, in accompanying the small ribosomal subunit (SSU) from the site of its assembly in the nucleolus to its export through the nuclear pore (Cisterna *et al* 2006). The authors were tracking the localization of the S6 protein, a component of SSU, in the nucleus by electron microscopy. They found NMI and actin associated with the SSU already in nucleolar granular compartment (GC) and onward up to the nuclear pore. When the antibodies against NMI or actin are delivered to the cells by permeabilization with lysophosphatidylcholine, the S6 incorporation into SSU is impaired and S6 accumulates on the way to the nucleolus. They were also able to immunoprecipitate NMI and actin with S6.

Interestingly, Myosin VI (Myo6) was recently found in the cell nucleus and it was shown to have very similar properties as NMI. I.e. it colocalizes with Pol II and newly transcribed mRNA in a transcription-dependent manner, coimmunoprecipitates with Pol II complex, it is present at the promoter and coding region of active genes, knockdown of Myo6 inhibits transcription of these genes, and antibodies to Myo6 reduce Pol II transcription in vitro (Vreugde *et al* 2006). As Myo6 move to the other direction than other myosins this raises the intriguing possibility that NMI and Myo6 can both regulate the process of transcription at the same time.

There is also the exciting possibility that NMI could perform its function bound to nuclear phospholipids. Phospholipids comprise roughly 10% of the nuclear volume and PIP2 is among them. Their form is not known although they do not seem to form bilayers (Irvine 2003). Association of these phospholipids with DNA (Kharakoz *et al* 1999) and nuclear speckles (Tabellini *et al* 2003) was described. Due to the known ability of NMI to bind PIP2, we suppose that NMI binds to these nuclear phospholipids and exerts its motor function in co-operation with nuclear actin (whichever form it has).

Taken together, the precise NMI N-terminal function of the nuclear myosin even after almost 10 years of research still remains a mystery. Here we try to contribute to the characterization of this elusive protein.

II. Aims

The objective of this study was to extend the knowledge of NMI characteristics and functions, confirm its role in transcription and characterize the NMI expression pattern on both single organism and phylogenetical level. Specifically, we aimed to answer following questions:

1. Is NMI involved in Pol I transcription?
2. Does the localization of NMI reflect the transcriptional activity of the cell?
3. Which part of the molecule is responsible for the nuclear localization?
4. What is the expression pattern in tissues?
5. How much evolutionary conserved is NMI?

III. Research papers

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

Philimonenko VV, Zhao J, Iben S, Dingova H, Kysela K, **Kahle M**, Zentgraf H, Hofmann WA, de Lanerolle P, Hozak P, Grummt I.

Nat Cell Biol. 2004 Dec;6(12):1165-72.

Nuclear distribution of actin and myosin I depends on transcriptional activity of the cell.

Kysela K, Philimonenko AA, Philimonenko VV, Janacek J, **Kahle M**, Hozak P.

Histochem Cell Biol. 2005 Nov;124(5):347-58.

The first two IQ domains are responsible for the nuclear localization of nuclear myosin I.

Kahle M, Dzijak R, Moško T, Přidalová J, Hozák P.

Manuscript.

Nuclear myosin is ubiquitously expressed and evolutionary conserved in vertebrates.

Kahle M, Pridalova J, Spacek M, Dzijak R, Hozak P.

Histochem Cell Biol. 2006 Sep 7; [Epub ahead of print]

ARTICLE

nature
cell biology

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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[6]Correspondence should be addressed to I.G. (e-mail: i.grummt@dkfz.de)

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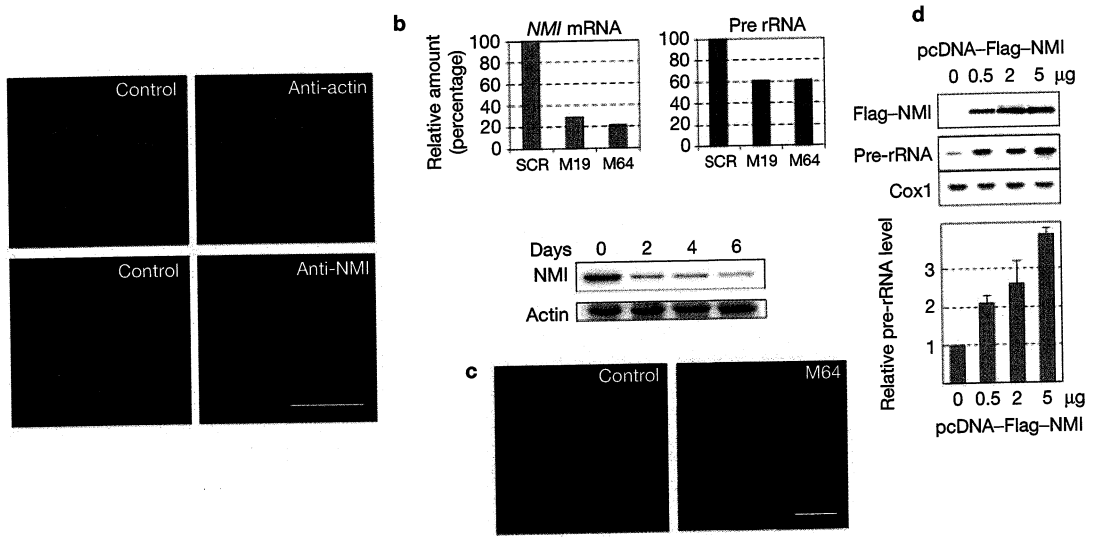

Figure 1 Actin and NMI are required for rDNA transcription *in vivo*. (**a**) Microinjection of anti-actin or anti-NMI antibodies inhibits nucleolar transcription. Confocal images of transcription sites in HeLa nuclei microinjected with dextran (control), anti-actin or anti-NMI antibody as indicated. 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

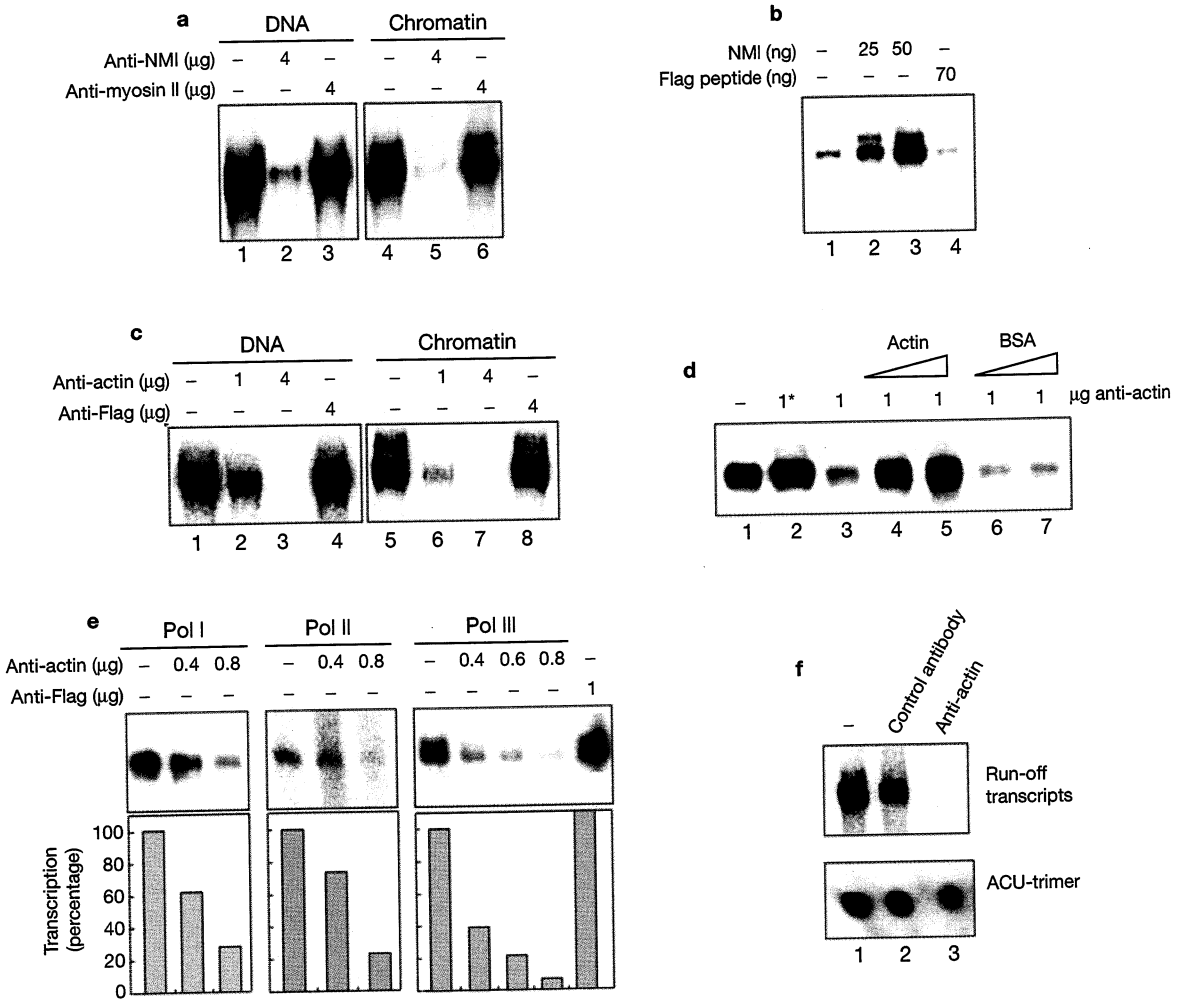

Figure 2 NMI and actin activate Pol I transcription. (**a**) Anti-NMI antibodies inhibit Pol I transcription *in vitro*. Transcription assays were conducted on either naked DNA (lanes 1–3) or preassembled chromatin (lanes 4–6). The DEAE-280 fraction was pre-incubated for 40 min with buffer (lanes 1, 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

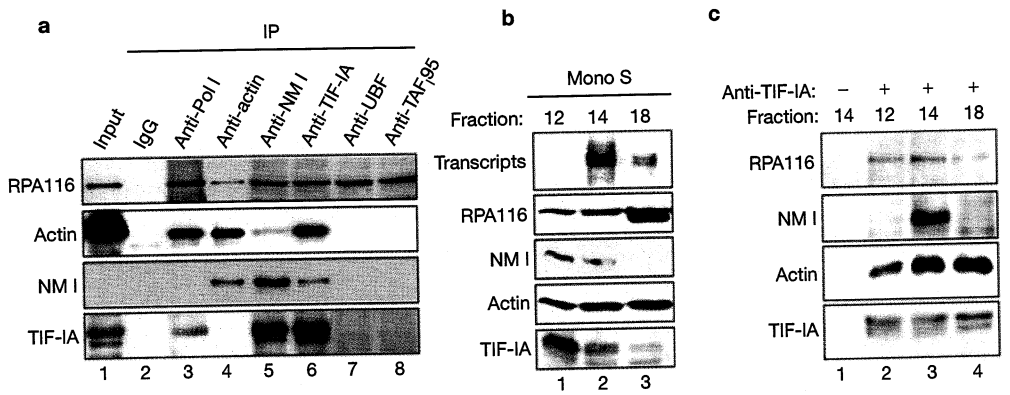

Figure 3 Actin and NMI are associated with the Pol I transcription machinery. (**a**) Co-immunoprecipitation of actin and NMI with Pol I and TIF-IA. Mouse IgGs and antibodies to Pol I (anti-RPA116), actin, NMI, TIF-IA, UBF and TIF-IB–SL1 (anti-TAF$_I$95) were immobilized on protein G agarose and incubated 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.

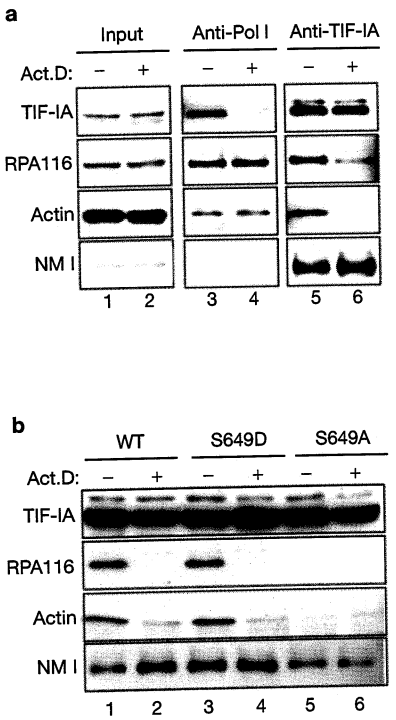

Figure 4 The association of NMI with the Pol I transcription apparatus requires phosphorylation of TIF-IA at Ser 649. (**a**) Actinomycin D treatment impairs the association of actin and NMI with the Pol I–TIF-IA complex. HeLa cells expressing Flag-tagged TIF-IA were cultured for 30 min in the absence (–) 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

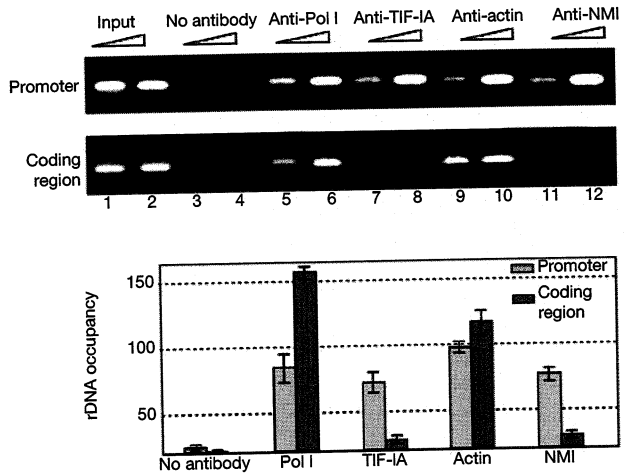

Figure 5 Actin and NMI are associated with rDNA. Crosslinked chromatin from U2-OS cells was immunoprecipitated with antibodies against Pol I (anti-RPA116), TIF-IA, actin and NMI as indicated. Input chromatin (0.02 and 0.06%) and immunoprecipitated DNA (1 and 3%) were amplified with primers specific to 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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ORIGINAL PAPER

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Nuclear distribution of actin and myosin I depends on transcriptional activity of the cell

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Abstract As previous studies suggested, nuclear myosin I (NMI) and actin have important roles in DNA transcription. In this study, we characterized the dynamics of these two proteins during transcriptional activation in phytohemagglutinin (PHA) stimulated human lymphocytes. The stimulation led to strong up-regulation of NMI both on the mRNA and protein level, while actin was relatively stably expressed. The intranuclear distribution of actin and NMI was evaluated using immunogold labeling. In nucleoli of resting cells, actin was localized predominantly to fibrillar centers (FCs), while NMI was located mainly to the dense fibrillar component (DFC). Upon stimulation, FCs remained the main site of actin localization, however, an accumulation of both actin and NMI in the DFC and in the granular component was observed. In the nucleoplasm of resting lymphocytes, both actin and NMI were localized mostly in condensed chromatin. Following stimulation, the majority of both proteins shifted towards the decondensed chromatin. In transcriptionally active cells, both actin and NMI colocalized with nucleoplasmic transcription sites. These results demonstrate that actin and NMI are compartmentalized in the nuclei where they can dynamically translocate depending on transcriptional activity of the cells.

Keywords Cell nucleus · Human lymphocytes · Actin · Nuclear myosin I · Transcription

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Abbreviations PHA: Phytohemagglutinin · FC: Fibrillar center · DFC: Dense fibrillar component · GC: Granular component · NMI: Nuclear myosin I · RT: Room temperature

Introduction

The presence of actin in the nucleus as well as its functions in various nuclear processes have been clearly established in the past few years (for review, see Rando et al. 2000; Olave et al. 2002; Pederson and Aebi 2002; Bettinger et al. 2004). Actin is known to be a part of the SWI/SNF-like BAF complex. It is implicated in the stimulation of ATPase activity of the Brg1 subunit, and in the association of this complex with nuclear matrix (Zhao et al. 1998). Furthermore, actin was shown to associate with small nuclear ribonucleoproteins, which have a major role in mRNA processing (Nakayasu and Ueda 1985; Kimura et al. 2000). Actin has also been found to form complexes with heterogeneous nuclear ribonucleoproteins (hnRNPs) that bind to mRNAs during their export from the nucleus (Percipalle et al. 2001; Percipalle et al. 2002; Kukalev et al. 2005). Moreover, the essential roles of actin in transcription by all RNA polymerases have been demonstrated recently (Fomproix and Percipalle 2004; Hofmann et al. 2004; Hu et al. 2004; Philimonenko et al. 2004).

On the other hand, nuclear myosin I (NMI) is the first molecular motor described in the cell nucleus (Nowak et al. 1997). The NMI interacts with RNA polymerase II (Pol II), and its function in Pol II transcription has been suggested (Pestic-Dragovich et al. 2000). In a recent study, Philimonenko et al. (2004) have shown the requirement of NMI for transcription of ribosomal genes, and its association with basal transcription factor TIF-IA. However, the intranuclear dynamics of actin and NMI has not yet been characterized.

It is accepted that the mammalian nucleus, despite the absence of intranuclear membranes, is organized

into functional domains or foci, where nuclear processes like DNA replication, transcription, RNA processing, and ribosome biogenesis take place (Hendzel and Bazett 1995; Jackson 2003). The nucleolus, the most morphologically defined subnuclear compartment, is the site of ribosomal RNA (rRNA) synthesis, rRNA processing, and assembly of ribosomal subunits. Each of these processes takes place in distinct nucleolar domains. Three morphologically distinct domains can be discriminated in the nucleolus at the electron microscopic level: fibrillar centers (FCs), surrounded with dense fibrillar component (DFC) and both together embedded in granular component (GC; for reviews, see Busch and Smetana 1970; Olson et al. 2000; Olson and Dundr 2005). The rDNA transcription occurs mostly at the border FC/DFC and in DFC (Hozák et al. 1994; Cmarko et al. 2000), while the accumulation, modification, and processing of ribosomal transcripts occur in the DFC; the later processing events followed by the assembly of ribosomal subunits take place in GC (Hozák et al. 1994; Scheer and Hock 1999; Mosgoeller et al. 2001). The number of nucleoli together with their relative size and configuration of individual compartments can vary greatly depending on the cell type and metabolic state of the cell (Busch and Smetana 1970; Olson et al. 2000). However, the nucleolus is not only the site of the biosynthesis and processing of RNA components of ribo-protein complexes, but has also an important role in regulating the activity of proteins involved in various aspects of cell cycle progression. For example, a sequestration in the nucleolus prevents proteins from reaching their targets in other nuclear compartments (Visintin and Amon 2000).

Apart from nucleoli, the interphase nucleus can be divided into two main compartments: chromatin compartment, consisting of all individual chromosome territories, and the interchromatin compartment, where several non-chromatin domains are localized (Cremer et al. 2004). The chromatin compartment consists of heterochromatic DNA that remains condensed throughout the cell cycle, whereas euchromatic DNA appears to be decondensed in interphase (Carmo-Fonseca 2002). Based upon ultrastructural mapping, the border zone between chromatin domains and interchromatin compartment, perichromatin region, is the compartment where most RNA synthesis and DNA replication take place (Fakan 1994; Puvion and Puvion-Dutilleul 1996; Spector 2003).

In this study, we revealed the dynamic properties of nuclear actin and NMI on the model of PHA-stimulated human lymphocytes. Upon stimulation, lymphocytes undergo rapid transcriptional activation accompanied by dramatic changes in nuclear and nucleolar morphology. Enlargement of nuclei, chromatin decondensation and activation of nucleoli are the characteristic signs of this process (Smetana and Potměšil 1968; Busch and Smetana 1970; Hozák et al. 1989). We showed that activation of human lymphocytes was accompanied by an increase in the expression of NMI mRNA and protein in the cells, whereas actin expression was relatively stable. We also demonstrated that in nucleoli of resting or PHA-stimulated lymphocytes, actin localized predominantly to FCs, while NMI localized mainly in the DFC. During transcriptional activation, actin was recruited from FCs to other nucleolar compartments, whereas NMI was accumulated in DFC where transcription of rDNA takes place. In the nucleoplasm, both actin and NMI relocalized from condensed chromatin to decondensed chromatin. Finally, nuclear actin and NMI colocalized with transcription sites in the nucleoplasm. These observations suggest new lines of connection between nuclear compartmentalization and regulation of functions for nuclear actin and NMI by protein sequestering.

Materials and methods

Cells

Normal human lymphocytes were isolated from heparinized peripheral blood according to standard procedure (Boyum 1968) using Lymphoprep (Nycomed). Cells were grown at 37°C in a 5% CO_2 atmosphere in RPMI 1640 supplemented with 20% FBS (Gibco), 2 mM L-glutamine (Biotech), 1 U/ml penicillin (Sigma) and 1 μg/ml streptomycin (Sigma). For activation, cells were grown in the presence of 10 μg/ml PHA (Murex, HA15) for 24 or 48 h, at a final concentration of 1×10^6 cells/ml. Cell viability was tested by trypan blue staining.

Quantitative RT-PCR

Total RNA was isolated with TriReagent (Sigma) and reverse-transcribed using TaqMan reverse transcription reagents (Applied Biosystems) and random primers. Quantitative RT-PCR (qRT-PCR) was performed in Stratagene Mx3000p instrument using SYBR Green JumpStart Taq ReadyMix (Sigma) with following set of primers:

NMI 5′–GGGCAGGATGCGCTACC–3′ and 5′–AAGTTCTCCAGCAGCACGAAA–3′; GAPDH 5′–GTCGGAGTCAACGGATTTGG–3′ and 5′–AAAAG CAGCCCTGGTGACC–3′; β-actin 5′–AGGCACCAG GGCGTGAT–3′ and 5′–TCGCCCACATAGGAATC CTT–3′; β2–microglobulin 5′-TGCCGTGTGAGAA CCATGTGAC-3′ and 5′–GCGGCATCTTCAAAC CTCC–3′ and 18S rRNA 5′–CGTTCAGCCACCC-GAGATT–3′ and 5′–CGGACATCTAAGGGCATCA-CA–3′. The results were processed by $\Delta\Delta C_T$ method (Livak and Schmittgen 2001). β-actin, GAPDH, β^2-microglobulin and 18S rRNA were used as internal controls for normalization. For statistical evaluation, 18S rRNA was chosen as an internal control.

Immunoblotting

The lymphocytes were harvested at various times after stimulation with PHA and lysed in a lysis buffer (50 mM

Tris–HCl, 150 mM NaCl, 1 mM $MgCl_2$, 0.5% NP-40 and 1 mM DTT, pH 7.5) containing protease inhibitors (1 mM Pefabloc, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). After sonication, lysates were clarified by centrifugation at 14,000 *g*, and protein concentration was determined using the BCA method (Pierce). Fifteen microgram of protein was loaded per line. Samples were separated in 7% SDS-PAGE gels according to Laemmli (1970). Proteins were transferred to nitrocellulose membranes as described by Towbin et al. (1979) using a semi-dry blotting apparatus (Bio-Rad). The membranes were blocked with 5% nonfat dried milk in PBS supplemented with 0.05% Tween-20 and incubated with primary antibody for 1.5 h at RT. After washes, the membrane was incubated with peroxidase-conjugated goat anti-rabbit antibody (Bio-Rad), developed with ECL Femto (Pierce) substrate and exposed on X-ray film. The developed X-ray films were digitalized and the intensity of the signal was measured by densitometry (Gene Tools software, Syngene).

Electron microscopy

For morphological analysis, samples were fixed in 2.5% glutaraldehyde in Sörensen buffer (0.1 M sodium/potassium phosphate buffer, pH 7.3; SB) for 1 h, 4°C, post-fixed in 1% OsO_4 (2 h), dehydrated in ethanol series and propylenoxide, embedded in Epon-Durcupan and stained with uranyl acetate and lead citrate according to Reynolds (Hozák et al. 1989).

For immunoelectron microscopy, lymphocytes were fixed in 2% paraformaldehyde and 0.25% glutaraldehyde in SB for 1 h at 0°C, washed in two changes of SB (10 min each), and resuspended in 2% low melting point agarose (Sigma) in SB at 37°C. Cells were then spun down and the pellet was cut into small pieces. The pieces were dehydrated in series of ethanol solutions with increasing concentration of ethanol. The ethanol was then replaced in two steps by LR White resin (Sigma), polymerized under UV light for 48 h at 4°C and 80 nm sections were immunogold labeled according to standard procedures (Hozák et al. 1994). Finally, sections were contrasted with a saturated solution of uranyl acetate in water (4 min) and observed in Morgagni (F.E.I) electron microscope equipped with a CCD Mega View II camera (SIS, Germany). Following immunogold labeling, some grids were also stained with the EDTA regressive technique that preferentially contrasts RNP-containing structures (Bernhard 1969).

Labeling of nascent transcripts

For labeling of nascent transcripts with BrU, lymphocytes were resuspended in RPMI 1640 medium containing 20% FBS and 50 mM BrU (Sigma) for 15 min, rinsed in ice-cold PBS, fixed in 2% paraformaldehyde and 0.25% glutaraldehyde in SB, and processed for immunoelectron microscopy as described above.

Antibodies

Following antibodies were used: rabbit polyclonal anti-NMI (Nowak et al. 1997) diluted 1:50 for immunogold labeling, or rabbit polyclonal anti-NMI (Fomproix and Percipalle 2004) diluted 1:1,000 for immunoblots, mouse monoclonal anti-bromodeoxyuridine (EXBIO) diluted 1:50, mouse monoclonal anti-actin (Amersham N350), clone JLA20 (Lin 1981) at the dilution 1:200, and secondary gold-conjugated anti-IgG and IgM antibodies (Jackson).

Morphometry and quantification of the gold labeling

Volumes of nucleolar compartments were estimated on electron micrographs as described earlier (Hozák et al. 1989). Sections through the middle plane of nuclei with nucleoli were used for statistics and were referred to as nuclear/nucleolar profiles. Volumes of nuclei were estimated from nuclear profiles using following formula:

$$V = \frac{4}{3} \times \sqrt{\frac{x^3}{\pi}}$$

where x is the area of nuclear profile that was evaluated by using plug-in measure developed for the Ellipse program (ViDiTo, Slovakia). In order to quantify the number of gold particles and the area of nuclear compartments, digital images of 20 cells per experimental group were evaluated using LUCIA image-processing software (Laboratory Imaging Ltd.). The total number of the particles was also counted in different compartments of nuclei or nucleoli, both in resting and stimulated human lymphocytes. Multiple analysis of variance (MANOVA) and Scheffe's test were used for statistical evaluation of the results. The significance of colocalization of two kinds of immunogold labels was evaluated as described previously (Philimonenko et al. 2000). The colocalization in specific regions of interest was estimated using plugins Gold developed for the Ellipse program (ViDiTo, Slovakia; for details, see http://nucleus.biomed.cas.cz/gold).

Results

The levels of both NMI mRNA and protein raise strongly upon activation

We first examined whether the cellular levels of NMI and actin change upon transcriptional activation of PHA-stimulated human lymphocytes. On the level of NMI mRNA measured by qRT-PCR, we observed five-fold and six-fold increase after 24 or 48 h, respectively (Fig. 1a). The data were normalized to 18S rRNA, which is a preferred internal control for normalization of gene expression in human lymphocytes (Bas et al. 2004). The Western blot showed about 15-fold and 28-fold

increase in NMI protein levels after 24 and 48 h of stimulation, respectively, while the levels of actin remained unchanged (Fig. 1b). These results indicate that both NMI mRNA and protein levels are strongly up-regulated during lymphocytes activation.

Localization of actin and NMI in nucleoli

The distribution of actin and NMI in nuclear compartments was analyzed in detail using electron microscopy of immunogold-labeled ultrathin sections of unstimulated (Fig. 2a; 3a–d) and stimulated (24 or 48 h, Fig 2b, c) human lymphocytes. The nucleoli of resting cells have a typical ring-shaped structure (Smetana and Potměšil 1968) with one large FC surrounded by a thin layer of dense fibrillar component (DFC) and little GC. Upon activation of rDNA transcription, in stimulated lymphocytes, the number of FCs increases, they become smaller, while the volume of the DFC and GC increases.

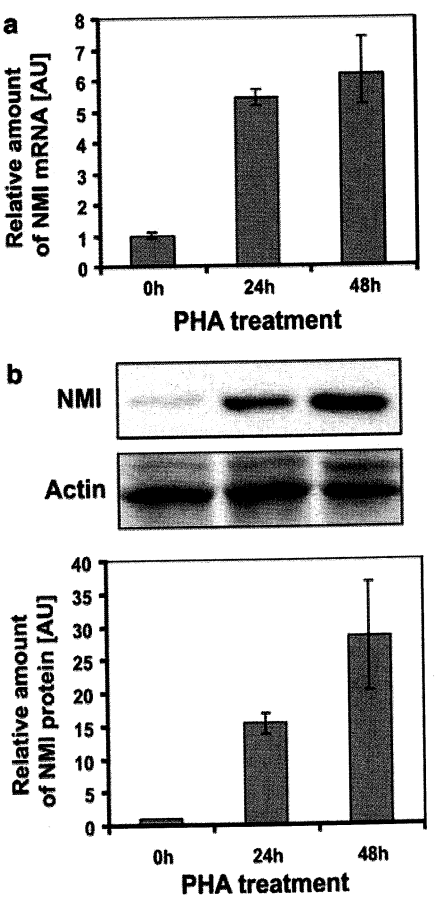

Fig. 1 NMI is strongly up-regulated during lymphocytes activation. **a** The relative expression of NMI mRNA after PHA-stimulation was measured using qRT-PCR. AU represents arbitrary units. 18S rRNA was used as internal control. **b** Western blot analysis of NMI levels 24 or 48 h after activation of lymphocytes by PHA. Fifteen microgram protein per line for each stage was loaded as controlled by Coomassie Brilliant Blue staining of the band corresponding to actin, and probed for NMI

Evaluation of actin labeling density in nucleolar components (Fig. 3e) demonstrated that actin was predominantly located in FCs (66.6 particles/μm^2) of resting human lymphocytes (Fig. 3a, b). The labeling density in the DFC and in the GC was significantly lower (11.9 particles/μm^2 and 7.3 particles/μm^2, respectively). During the activation of transcription in human lymphocytes, FCs remained the main site of actin localization, however, the density of actin labeling decreased. The labeling density in the DFC and GC slightly decreased during activation, however, the total amount of gold particles in these compartments increased because of the significant increase in the volume of the DFC and GC (see also the volumetric data below). The analysis of the nucleolar profiles showed that the total amount of actin in FCs decreased significantly ($P < 0.01$); at the same time, a significant increase in the amount of actin in CG was observed (Fig. 3g).

On the other hand, NMI labeling was the most abundant in the DFC (Fig. 3c, d) of the resting human lymphocytes (16.3 particles/μm^2; see Fig. 3f), i.e., in the nucleolar compartment where RNA polymerase I (Pol I) transcription takes place (for reviews, see Hozák et al. 1994; Scheer and Hock 1999; Huang 2002). NMI labeling density was lower in both the FCs (10.7 particles/μm^2) and the GC (10.5 particles/μm^2). As NMI is involved in transcription by Pol I, one can anticipate that the amount of NMI would increase in nucleoli during activation of transcription. Indeed, we observed on nucleolar profiles that NMI accumulated in the DFC as well as in the GC (Fig. 3h). Both changes were statistically significant ($P < 0.01$). The total amount of NMI in the nucleolus increased nearly three times in the course of stimulation. These results demonstrate a strong correlation between high transcriptional state and the amount of NMI in the nucleolus.

Extranucleolar localization of actin and NMI

The resting human lymphocytes have small, extremely compact nuclei with dense heterochromatin. However, within a few hours after activation, their nuclei increase in volume and areas occupied by decondensed chromatin enlarge (Zhao et al. 1998). Twenty-four hours after PHA-stimulation, visible signs of chromatin decondensation are seen (Fig. 2b). After two days of stimulation, cell chromatin was nearly entirely decondensed and only small clusters of condensed chromatin located almost exclusively along the nuclear membrane were visible (Hozák et al. 1989). The actin and NMI labeling was evaluated in condensed chromatin and in decondensed chromatin area (see Fig. 2a). In resting lymphocytes, both actin and NMI were located predominantly in condensed chromatin (Fig. 4a, c). In stimulated lymphocytes, a distinct increase of actin and NMI labeling was seen in decondensed chromatin area (Fig. 4b, d). Quantification of gold labeling in unstimulated lymphocytes showed that actin and NMI labeling density in condensed chromatin was two–

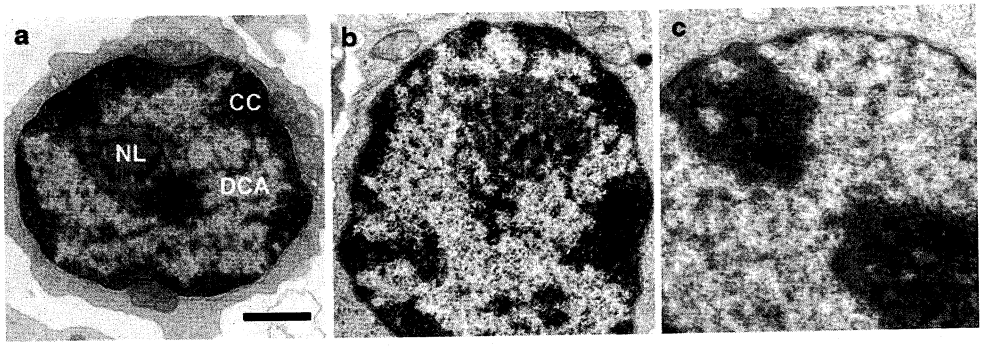

Fig. 2 Ultrastructure of resting and PHA-activated human lymphocytes. **a** An electron micrograph of a nucleus of resting human lymphocyte. Nucleolus (*NL*) has a typical ring-shaped structure. Condensed chromatin (*CC*) locates at the periphery of the nucleus and around the nucleolus. Decondensed chromatin area (*DCA*) occupies the central part. *Bar* 1 μm. **b** The nucleus of 24 h stimulated human lymphocyte. Note the enlargement of the area occupied by decondensed chromatin and increase in the number of FCs in the nucleolus. **c** An electron micrograph showing nucleus of 48 h stimulated human lymphocyte with fully decondensed chromatin (only some small clusters of condensed chromatin remain along nuclear membrane). The nucleolus is fully developed with many FCs

three times higher than in decondensed chromatin area (Fig. 5a, b). A significant increase ($P < 0.01$) of NMI labeling density in condensed chromatin was observed after activation. At the same time, the volume occupied by this compartment dramatically decreased and the total amount of NMI in condensed chromatin dropped in the course of activation. The analysis of nuclear profiles demonstrated a significant shift of actin and NMI from condensed chromatin to decondensed chromatin area (Fig. 5c, d) during the stimulation period. These results show that actin and NMI redistribute dynamically within the nuclei in response to activation of transcription.

Actin and NMI colocalize with transcription sites

The results described above demonstrate that the distribution of actin and NMI in individual nuclear compartments is connected with the level of cell metabolism and changes upon transcriptional activation. While functional compartments in the nucleolus are morphologically well-defined, most processes in the nucleoplasm take place in foci that are not connected with morphologically obvious structure. We therefore analyzed whether actin and NMI are concentrated in foci or distributed uniformly over the extranucleolar area. In resting cells, actin labeling was not clustered in contrast to transcriptionally active cells where actin formed clusters up to 50 nm in diameter ($P < 0.01$). On the contrary, NMI was always found in clusters with 30–50 nm in diameter ($P < 0.01$).

As both actin and NMI are essential for transcription, we evaluated whether their distribution in the cell nucleus is connected with the positioning of extranucleolar transcription sites. Nascent transcripts were labeled in vivo with bromouridine (BrU, 50 mM for 15 min). Double immunogold labeling was performed to evaluate the colocalization of actin (Fig. 6a) or NMI (Fig. 6b) with nascent transcripts in 24 h PHA-stimulated human lymphocytes. For better distinguishing of condensed chromatin and perichromatin regions, some sections were after double labeling of actin (Fig. 6c) or NMI (Fig. 6d) together with nascent transcripts stained with the EDTA regressive technique (Bernhard 1969). Because most extranucleolar transcripts are concentrated in foci with a mean diameter of ~80 nm (Jackson et al. 1998), we evaluated particles that are up to 150 nm of each other. A statistical analysis (Philimonenko et al. 2000) showed that colocalization of actin and transcription sites was highly significant at distances ranging from 30 to 100 nm between particles ($P < 0.01$) in both resting and stimulated human lymphocytes (see Table 1). NMI significantly colocalized with extranucleolar transcription sites at distances ranging from 100 to 150 nm ($P < 0.05$) only in PHA-stimulated lymphocytes. These results demonstrate that the changes in the distribution of actin and NMI in the nucleoplasm are connected, at least partly, with their role in transcription and with the dynamics of transcription sites in the course of lymphocytes activation.

Volumetric changes of nuclear compartments

To assess quantitatively the dynamics of the nuclear compartments during lymphocytes activation, we measured the volume of nuclei and that of nucleolar compartments. The total volume of nuclei significantly increased during lymphocyte activation: 1.5-times after 24 h, and 3-times after 48 h (see Fig. 7a). In nucleoli, the FCs become smaller in the course of activation, however this decrease in the size of individual FCs is compensated by the increase of the FCs number (Hozák et al. 1989). Quantitative analysis (Fig. 7b) showed that the total volume of FCs per cell did not change significantly during the first 24 h, comprising 0.08 μm^3 in resting cells and 0.11μm^3 after 24 h of stimulation. Later, 48 h after stimulation, the total volume of FCs extremely increased (0.69 μm^3). In contrast, the volume of DFC increased dramatically already during the first 24 h of stimulation: from 0.13 μm^3 in resting cells to 0.5 μm^3 in 24 h PHA-stimulated lymphocytes. Moreover, after 48 h, the volume of DFC increased nearly 30 times compared to the volume of DFC in resting cells and a similar trend was observed for GC.

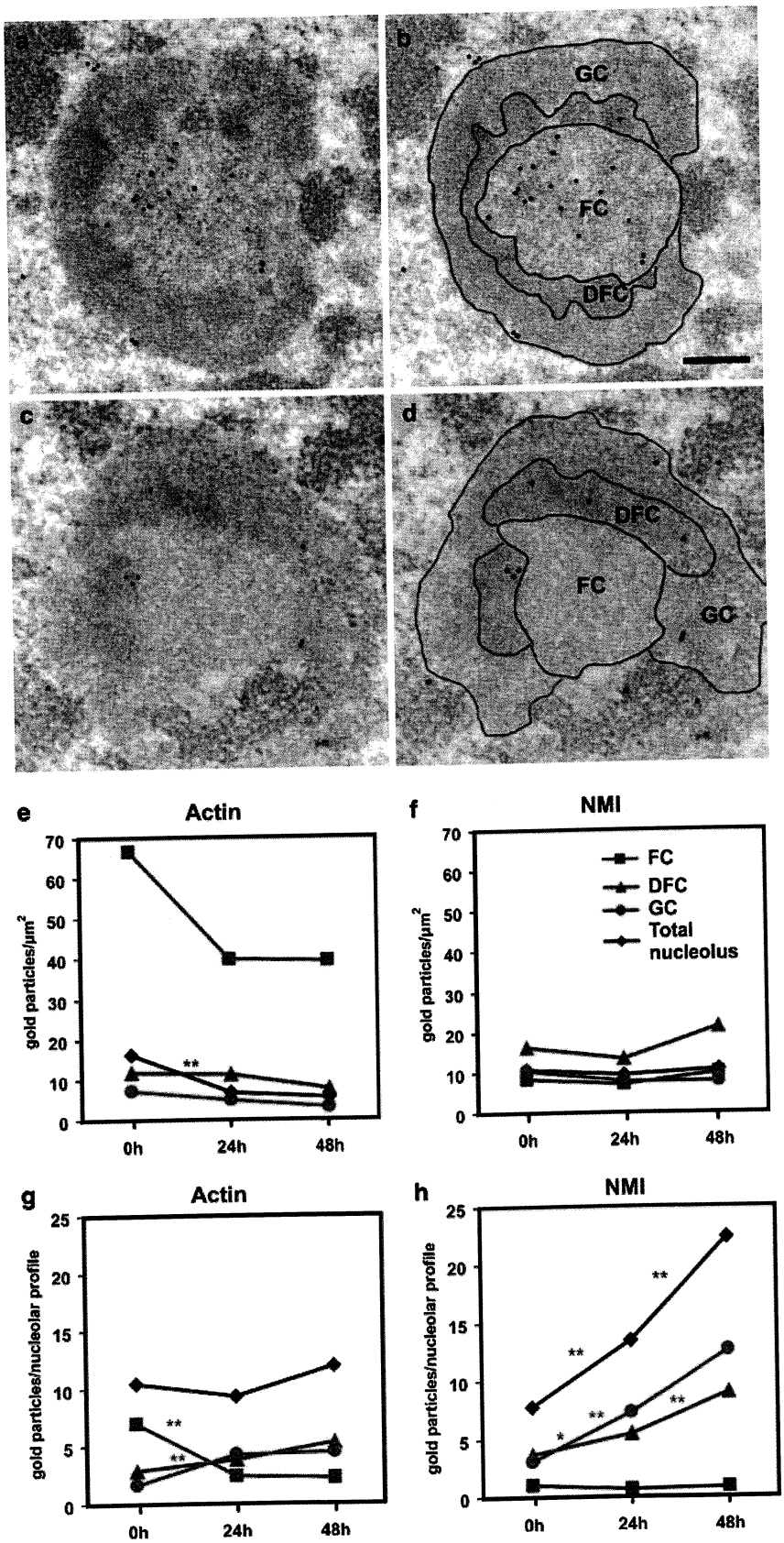

Fig. 3 Ultrastructural localization of actin and NMI in nucleoli and quantification of immunogold labeling. **a** An electron micrograph of resting human lymphocyte nucleolus labeled with anti-actin antibody (12 nm gold particles). **b** The same image as **a**, but the nucleolar compartments are outlined. Fibrillar centers (*FC*), DFC, and granular component (*GC*) are marked. Gold particles are highlighted in *red color*. FC is highly positive for actin. *Bar* 200 nm. **c** An electron micrograph of resting human lymphocyte nucleolus labeled with anti-NMI antibody (12 nm gold particles). **d** The same image as (C), the nucleolar components are outlined and gold particles are highlighted as in B. NMI is localized predominantly in DFC. **e** Actin labeling density in nucleolar compartments of resting (0 h) and PHA-stimulated (24 and 48 h) human lymphocytes. Labeling density is expressed in gold particles/μm^2; ** ($P < 0.01$), * ($P < 0.05$). The corresponding legend for graphs (**e**–**h**) as in F. **f** The same as (**e**) for NMI labeling. **g** The number of gold particles labeling actin on nuclear profile in different nucleolar compartments of resting (0 h) and PHA-stimulated (24 and 48 h) human lymphocytes. **h** The same as (**g**) for NMI labeling

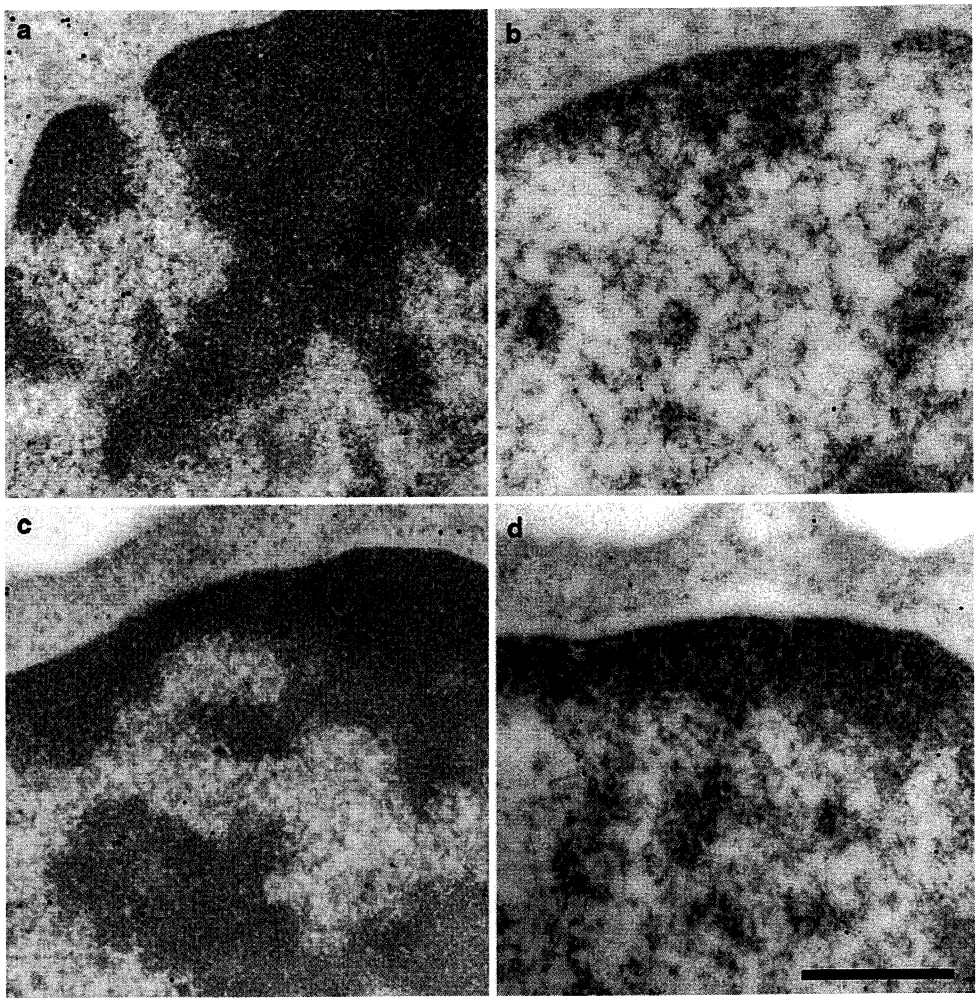

Fig. 4 Ultrastructural localization of actin and NMI in the nucleoplasm changes during transcriptional activation. Electron micrographs of resting (**a**, **c**) and 24 h PHA-stimulated (**b**, **d**) human lymphocytes. **a** and **b** actin labeling, 12 nm gold particles. **c** and **d** NMI labeling, 12 nm gold particles. *Bar* 500 nm

Discussion

Previous studies demonstrated important roles of actin in various nuclear functions such as nucleocytoplasmic transport, chromatin remodeling, transcription, and splicing (for reviews, see Rando et al. 2000; Olave et al. 2002; Pederson and Aebi 2002; Bettinger et al. 2004). Recently, it has been shown that actin is indispensable for transcription by Pol I, II, and III (Fomproix and Percipalle 2004; Hofmann et al. 2004; Hu et al. 2004; Philimonenko et al. 2004) and NMI has been found to participate in transcription by Pol I and II (Pestic-Dragovich et al. 2000; Philimonenko et al. 2004). However, a detailed study of actin and NMI localization in nuclear compartments, as well as their dynamics upon changes in transcriptional activity of the cells is still lacking. The principal aim of this paper was therefore to characterize the intranuclear dynamics of actin and nuclear myosin I on a physiological cellular model of transcriptional activation.

The experimental model of stimulated human lymphocytes was chosen for two reasons. Firstly, it provides an excellent system to study nuclear changes during transcriptional activation. Secondly, the model allows us to use cells without the possible effects of long-term cultivation. For characterization of actin and NMI dynamics in the course of activation, we used a combination of molecular and in situ approaches. Cellular levels of actin and NMI were estimated using Western blot and qRT PCR, while the analysis of gold-immunolabeling on ultrathin sections allowed us to study the dynamics of their translocation relative to distinct nuclear compartments. The distribution of gold particles was quantified on nuclear sections through the middle plane of the nucleus/nucleolus, referred to as nuclear/nucleolar profiles. Given that the accessibility of the epitopes to the antibodies does not change significantly upon lymphocytes stimulation, the changes in labeling density (number of gold particles per square micrometer) reflect changes in the concentration of respective protein in a nuclear compartment. The number of gold particles in a nuclear profile reflects then the changes in the total amount of the protein.

We have demonstrated that NMI was up-regulated both on the mRNA and protein level following PHA-stimulation of resting lymphocytes. The increase of protein level was remarkably higher than that of

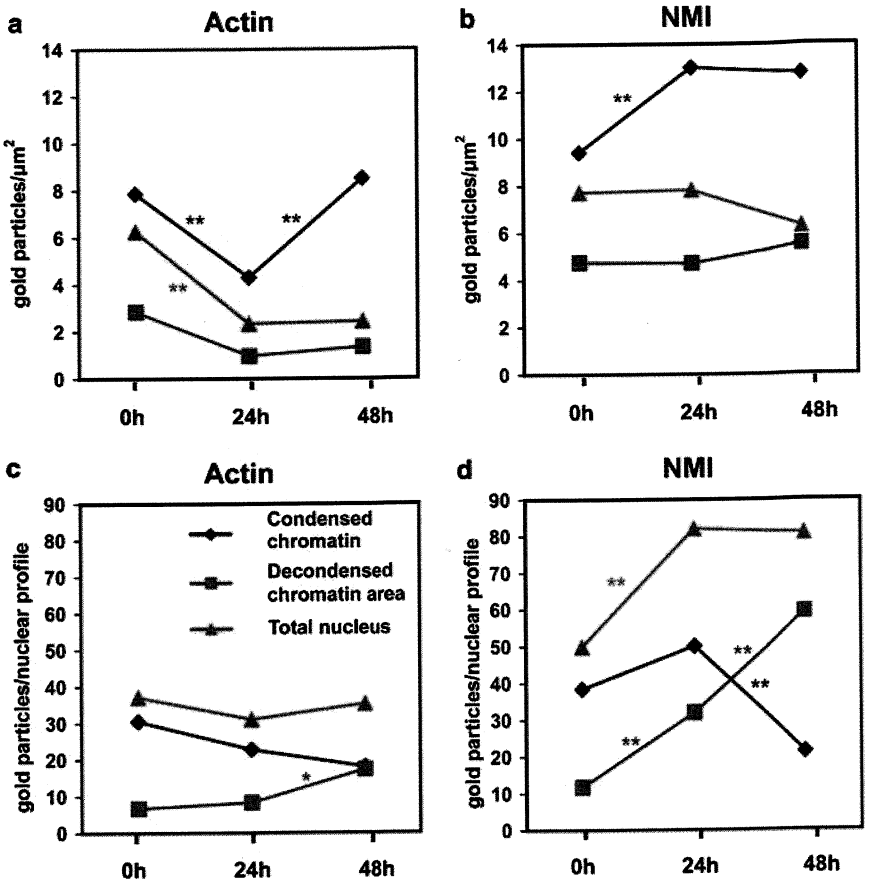

Fig. 5 Quantification of actin and NMI labeling in the nucleoplasm. **a** Actin labeling density in the nucleoplasm of resting (0 h) and PHA-stimulated (24 and 48 h) human lymphocytes. Labeling density is expressed in gold particles/ μm^2 ; **($P < 0.01$), *($P < 0.05$). The corresponding legend for graphs (**a–d**) as in **C**. **b** The same as **a** for NMI labeling. **c** The number of gold particles labeling actin on nuclear profile in condensed chromatin and decondensed chromatin area of the resting (0 h) and stimulated (24 and 48 h) human lymphocytes. **d** The same as (**c**) for NMI labeling

mRNA (15-fold and 5-fold after 24 h of stimulation, respectively). This suggests that the up-regulation of NMI involves several mechanisms at different levels. Apparently, both transcription and translation of NMI were activated; decreased levels of mRNA and/or protein degradation might also be involved. The ultrastructural results go in line with the biochemical data. The analysis of immunogold labeling on nuclear profiles revealed significant increase of the total amount of NMI in the nucleoplasm and in nucleoli upon PHA-stimulation. These results are in agreement with previously demonstrated role of NMI in transcription by Pol I and II (Pestic-Dragovich et al. 2000; Philimonenko et al. 2004), and they support the hypothesis about the importance of NMI in the transcription.

In contrast, the protein level of actin in human lymphocytes during transcriptional activation was rather stable, as shown by both Western blot and quantification of immunogold labeling. This is an expected result, as actin is known to be stably expressed and is commonly used as an internal standard for quantitative determination of mRNA of interest (Suzuki et al. 2000). Moreover, two nuclear export sequences were found in actin molecule, which are responsible for shuttling of actin between the nucleus and the cytoplasm, suggesting a steady-state level of the actin in the nucleus (Wada et al. 1998).

Compartmentalization of proteins within discrete nuclear/nucleolar domains generally reflects the compartmentalization of nuclear/nucleolar metabolism. We therefore assessed the dynamics of NMI and actin within individual nuclear compartments. NMI was preferentially localized in the DFC which is the site of rDNA transcription. The localization of NMI and its accumulation in DFC during transcriptional activation is in agreement with its role in the synthesis of rRNA, and/or it may also indicate the involvement of NMI in the early processing events of rRNA precursors. Importantly, NMI is associated with transcription factor TIF-IA, a basal transcription factor that confers initiation competence to Pol I and mediates the growth-dependent regulation of rRNA synthesis (Philimonenko et al. 2004). Thus, the regulatory role of NMI in activation of ribosomal genes transcription in stimulated human lymphocytes cannot be excluded.

A small pool of actin in nucleoli is present in the DFC together with NMI, consistent with recently reported function of actin in transcription by Pol I, and with the known localization of rDNA transcription in DFC (Philimonenko et al. 2004). However, the main site of actin localization in nucleoli are the FCs, the labeling density there being the highest in unstimulated lymphocytes and decreasing in the course of transcriptional activation. The FCs are known to be the storage sites for Pol I and other components of Pol I transcription

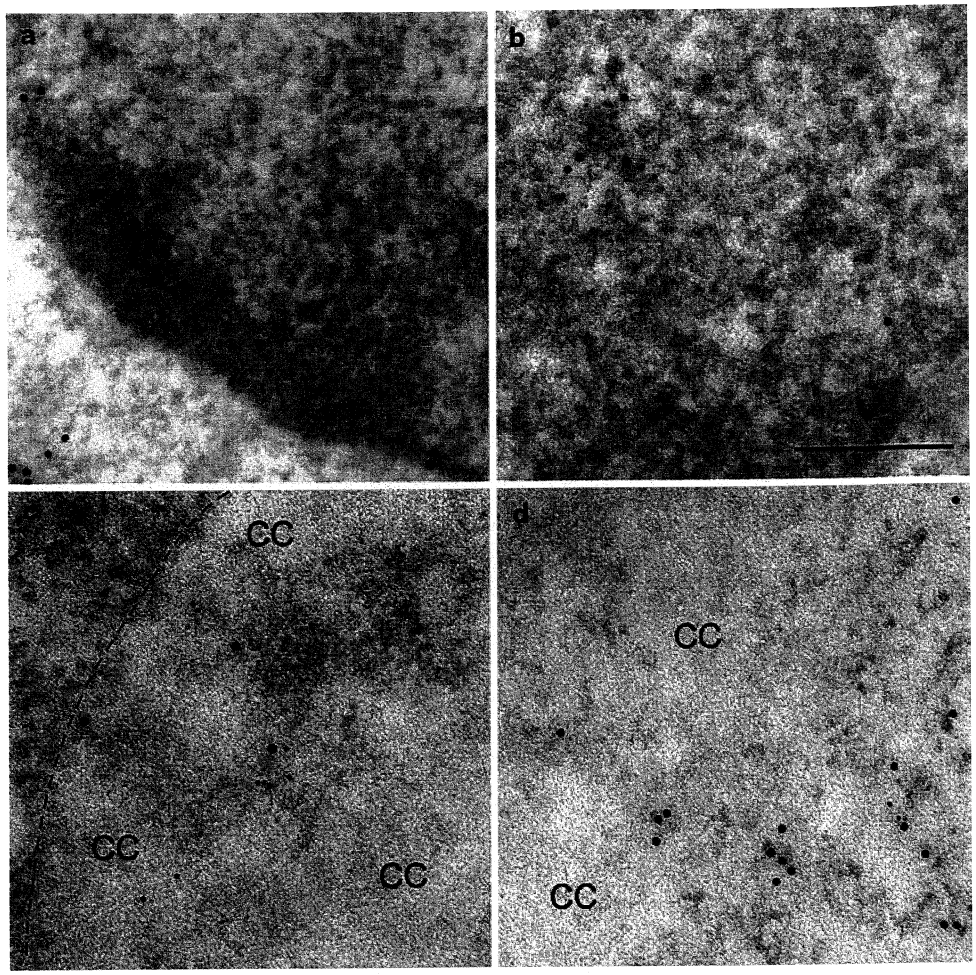

Fig. 6 Actin and NMI colocalize with transcription sites. Electron micrographs of 24 h PHA-stimulated human lymphocytes. **a** Double immunogold labeling with anti-actin (12 nm gold particles) and anti-BrdU antibody (6 nm gold particles). **b** Double immunogold labeling with anti-NMI (10 nm gold particles) and anti-BrdU antibody (6 nm gold particles). *Bar* 200 nm. (**c**, **d**) The same as (**a**, **b**) but after the preferential contrasting of RNP-containing structures, CC. *Dash line* marks the border between the cytoplasm and the nucleus

Table 1 Colocalization of actin and NMI with non-nucleolar transcription sites in resting (0 h) and active (24 h) stimulated human lymphocytes

	Gold particles		Statistical significance of colocalization	
	12 nm	6 nm	30–100 nm	100–150 nm
Resting cells	Actin	Transcription sites	$P<0.01$	NS
	NMI	Transcription sites	NS	NS
Active cells	Actin	Transcription sites	$P<0.01$	$P<0.05$
	NMI	Transcription sites	NS	$P<0.05$

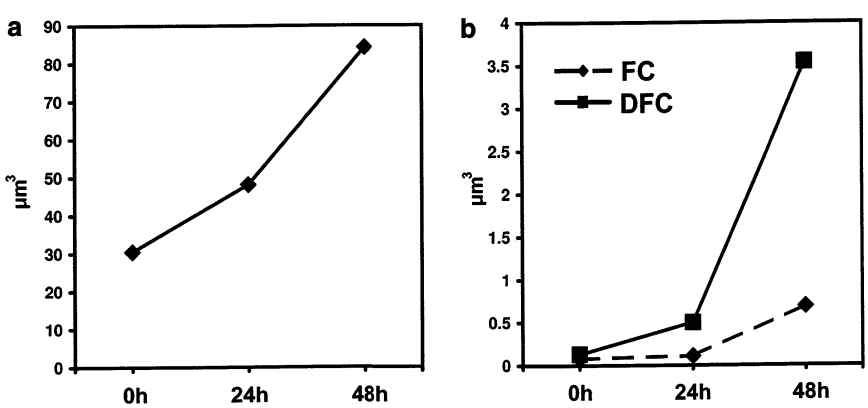

Fig. 7 Quantification of total nuclear volume (**a**) and the volumes of distinct nucleolar compartments (**b**) in the course of lymphocytes activation

machinery such as UBF, SL-1 and topoisomerase I (Jordan et al. 1996; Roussel et al. 1996; Mosgoeller et al. 1998). Actin was shown to be associated with Pol I regardless of Pol I transcriptional state (Philimonenko et al. 2004). Therefore, it is possible that a significant part of actin in FCs is associated with inactive Pol I. Moreover, the described dynamics of actin suggests that it is accumulated in FCs in inactive cells and it is then recruited to sites of function upon transcriptional activation, possibly together with Pol I. Furthermore, recent studies have revealed a role for the nucleolus in regulating the activity of proteins involved in various aspects of cell-cycle progression (Visintin and Amon 2000). Sequestration in the nucleolus prevents proteins from reaching their targets in other nuclear compartments. Remarkably, nucleolar accumulation of actin in FCs described here, seems to be closely related to growth arrest of human lymphocytes. Interestingly, expression of actin mutants with abnormal cellular localization induced a decrease in the proliferative potential of the cell (Wada et al. 1998). This might suggest a correlation between the proper actin localization and the metabolic state of the cell. It was shown earlier that FCs could serve as principal elements in forming not only active nucleoli, but also the nucleoskeletal filaments radiating from FCs towards nuclear periphery thus contributing to the organization of nuclear interior (Hozák et al. 1994; for a review, see Schwarzacher and Mosgoeller 2000). Actin, a classical structural protein, can also contribute to this structural role of the FCs in forming these nucleoskeletal structures; however, the binding partners have yet to be determined.

In the nucleoplasm, actin and NMI behave in a similar manner. In resting cells, actin and NMI are both concentrated predominantly in condensed chromatin domains and they are less abundant in regions containing decondensed chromatin. The suggestion that condensed chromatin may contain higher amount of actin has been made previously (Rungger et al. 1979) based upon microinjecting of anti-actin antibodies into the nuclei of *Xenopus* oocytes that blocked chromosome condensation. Furthermore, it has been demonstrated previously that actin as well as actin-related protein (ARP) BAF53 are the components of the SWI/SNF-like BAF remodeling complex (Zhao et al. 1998). ARPs and actin isoforms appear to have evolved from a common ancestor and they compose a branch of the actin family within a superfamily of proteins that have ATPase activity (Rando et al. 2000). Our data showing that actin is enriched in condensed chromatin are also consistent with earlier observations that revealed an association of actin-related protein ARP4 with centric heterochromatin and a colocalization with hetechromatin protein HP1 in Drosophila (Frankel et al. 1997).

Although a role of NMI in chromatin remodeling has not yet been demonstrated, it is also enriched in condensed chromatin in unstimulated lymphocytes. The transcriptional activation of human lymphocytes leads to relocalization of both actin and NMI to decondensed chromatin area. We show here that clusters of both actin and NMI colocalize with transcription sites in the nucleoplasm of activated human lymphocytes, which is in agreement with the importance of both proteins for transcription. Apparently, heterochromatin can store large amounts of actin and NMI that are after stimulation redistributed to nuclear compartments with active DNA transcription. Interestingly, while actin is detected in transcription sites in both unstimulated and stimulated lymphocytes, NMI did not significantly colocalize with transcription sites in resting cells, where transcription levels are very low. This points to more dynamic nature of NMI association with the sites of RNA synthesis. Possibly, the amount of NMI in the sites with only basal transcription is too low to be detected by immunolabeling. The elevated labeling intensity upon stimulation raises an intriguing possibility that NMI plays a role also in regulatory events during transcriptional activation.

To obtain a complete picture of intranuclear dynamics of actin and NMI, we quantified the changes in the volume of the nuclei and that of nucleolar compartments upon PHA-stimulation of human lymphocytes. In agreement with previous studies (Steen and Lindmo 1979; Setterfield et al. 1983), the volume of lymphocytes nuclei increased about three times during 48 h of stimulation. Taking into account the fact that the proportion between the volumes occupied by condensed and decondensed chromatin shifts dramatically towards decondensed chromatin at the same time, the tendency of both actin and NMI accumulation in this compartment becomes even more pronounced. In nucleoli, the total volume of the FCs did not change significantly during the first 24 h after stimulation. This is in agreement with previous studies which showed that initial stages of nucleolar activations in lymphocytes are accompanied with changes in the shape of FCs and with their fragmentation, but not with an increase in volume (Hozák et al. 1989). The volume of FCs then increased during the following 24 h. This can be explained by S-phase progression that results in doubling the number of nucleolar organizers which are known to carry a permanent load of rDNA-attached proteins (Zatsepina et al. 1988; Sirri et al. 1999). The most dramatic augmentation in the volume was observed in DFC, the nucleolar compartment where transcription of ribosomal genes takes place (Hozák 1995; Cmarko et al. 2000). Again, this result stresses the trend of NMI accumulation in the DFC during transcriptional activation.

To summarize, we showed here for the first time that nuclear actin and NMI associate with distinct nuclear compartments. Transcriptional activation leads to relocalization of both proteins to compartments with active transcription. Protein sequestration in the nucleolus and in the condensed chromatin is apparently involved in regulation of actin and NMI dynamics. Further studies are needed to understand actin and NMI functions in gene expression in the context of nuclear architecture.

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The first two IQ domains are responsible for the nuclear localization of nuclear myosin I

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Abstract

Nuclear myosin I (NMI), one of the myosin isoforms that is localized in the nucleus, was shown to be necessary for transcription. NMI is closely related to mainly cytoplasmic myosin IC (Myo1c) from which differs only by its 16 amino acid N-terminal extension. The N-terminal extension was reported to be responsible for the nuclear localization. Here we show that translocation of vector expressed NMI and Myo1c to the nucleus is reduced by addition of FLAG, V5, and EGFP tags and that both myosins translocate to the nucleus to the same extent. After mitosis, NMI does not get to the newly formed nucleus bound to chromatin but it is transported there after the nuclear envelope formation. Deletion mutant analysis showed that the two first IQ domains of the neck are sufficient to direct the fusion protein to the nucleus. As this part of the molecule binds calmodulin in the absence of Ca^{2+}, we hypothesize that calmodulin binding could regulate the NMI nuclear transport.

Introduction

Nuclear myosin I (**NMI**) is a single-headed member of the myosin superfamily of molecular motors. It is localized in the cell nucleus and participates along with nuclear actin in transcription (for reviews, see Grummt 2006, Hofmann et al 2006, Percipalle and Farrants 2006). NMI arises from the same gene as the cytoplasmic Myosin IC (**Myo1c**) from which it differs only by 16 amino acid N-terminal extension (Pestic-Dragovich et al. 2000). Myo1c is located mainly in the cell periphery and at the plasma membrane in the leading edges of the motile cells and it is involved in very diverse processes such as exocytosis, movement of transmembrane channels and neuronal growth cone steering (Bose et al. 2004, Gillespie 2004, Diefenbach et al. 2002). Both myosins share the same structure with three major domains. The head binds actin and has the ATPase activity, the neck is able to bind three molecules of the calcium sensor calmodulin through which the myosin activity is regulated, and the tail binds anionic phospholipids (Barylko et al 2005, Hokanson et

al. 2006, Hokanson and Ostap 2006). The NMI N-terminal domain was proposed to cause the nuclear translocation of NMI (Pestic-Dragovich et al. 2000). Puzzlingly, this extension as well as the whole molecule does not contain any known nuclear localizing sequence (**NLS**) according to the NLSdb (Nair et al. 2003), and addition of the NMI N-terminal extension is not sufficient to translocate a cytoplasmic protein to the nucleus (R. Dzijak, unpublished results).

There is an ample evidence for involvement of NMI in transcription by RNA polymerase I and II (**Pol I** and **Pol II**). NMI colocalizes with both polymerases and with sites of their active transcription. NMI also coimmunoprecipitates and co-purifies with both Pol I and Pol II complexes and was found by chromatin immunoprecipitation on the promoter as well as the coding region of rDNA. RNAi knockdown of NMI or microinjections of anti-NMI antibodies decrease transcription by both polymerases in vivo. In an in vitro transcription system, anti-NMI antibodies inhibit transcription by both polymerases in a dose dependent manner, while addition of purified NMI increases transcription (Philimonenko et al. 2004; Hofmann et al. 2006). NMI was shown to bind Pol I through the basal transcription factor TIF-IA which defines the initiation-competent subpopulation of Pol I and through which the rate of transcription initiation is regulated (Philimonenko et al. 2004).

It is still not clear precisely at which step NMI is needed during transcription. While the data from Pol II transcription suggest that NMI is needed for the formation of the first phosphodiester bond (Hofmann et al. 2006), experiments with Pol I system indicate the involvement of NMI in the later steps (promoter escape or elongation) (Percipalle et al. 2006).

Interestingly, there are reports suggesting the role of NMI in chromatin remodeling. NMI is associated with the chromatin remodeling complex WSTF-SNF2h and might therefore recruit this complex to the actively transcribed genes (Percipalle et al. 2006).

In summary, NMI is an important protein involved in transcription. However, the mechanism of NMI nuclear import and retention is not known. Here we show by expressing tagged full-length myosins and deletion mutants that the neck region

comprising the first two IQ domains is responsible for the nuclear translocation of NMI.

Materials and Methods

Antibodies

We used an antibody directed to the NMI specific N-terminus (M3567, Sigma), anti-lamin B antibody (M-20, Santa Cruz Biotechnology), and antibody to V5 tag (Serotec) and to Flag (Sigma).

Cells and transfections

U2OS osteosarcoma cells and NIH-3T3 cells were grown in DMEM with 10 % fetal calf serum in 5 % CO_2, 37°C, humidified atmosphere. The U2OS cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol, polyethyleneimine according to (Boussif et al. 1995), or with calcium-phosphate as described (Jordan et al. 1996), and stained with antibodies 48 h later.

Immunofluorescence microscopy

Cells grown on coverslips were fixed with freshly prepared 3% formaldehyde for 10 min., permeabilized with 0.1 % Triton X-100 in PBS for 10 min., incubated with antibody against NMI (16 µg/ml) and V5-tag (1 µg/ml) for 1 h at RT, and with FITC-conjugated goat anti rabbit secondary antibody (Jackson ImmunoResearch). Coverslips were mounted with Mowiol (Sigma) containing DABCO (Sigma) as an antifading agent, and 0.1 µg/ml DAPI (Sigma), and observed with confocal microscope.

Cell cycle synchronization

To synchronize, U2OS cells were treated with nocodazole (400 ng/ml) or aphidicolin (1 µg/ml) for 16 hours, washed and then fixed in indicated time points. 3T3 cells were synchronized by mitotic shake-off to poly-lysine coated coverslips. For flow cytometric analysis, the cells were fixed in 70% ethanol, treated with 100 µg/ml ribonuclease, and stained with 50 µg/ml propidium iodide.

Plasmid DNA preparation

Full length NMI cDNA was ligated into EGFP-C3 vector (GFP-NMI) or into EGFP-N3 (NMI-GFP, Clontech). GFP-HDL (aa 712 to 1044), GFP-T (aa 793 to 1044), GFP-HDL-Δ853 (aa 712 to 853), and GFP-IQ-1,2 (aa 712 to 770) were constructed from GFP-NMI using standard cloning methods.

Results and Discussion

Localization of overexpressed myosin constructs does not depend on the presence of the N-terminal sequence

During the testing of our tagged NMI constructs we noticed varied degree of their nuclear localization. To reconcile these observations, we transfected U2OS cells with a panel of selected constructs by three different transfection methods (Lipofectamine 2000, polyethyleneimine, and calcium phosphate) and observed the cells 48 h later with immunofluorescence microscopy. We categorized the transfected cells into three categories – cells with predominantly nuclear localization of the signal, cells where the intensity of the signal was approximately equal in the nucleus and in the cytoplasm, and cells with predominantly cytoplasmic signal excluding nucleus (Fig. 1). While both mouse and human NMI had predominantly nuclear localization in 80 % of the cells, V5/His tagged NMI and Myo1c predominated in the nucleus only in 50 %, and EGFP and FLAG tagged myosins only in less than 20 % of the cells. The results from Lipofectamine 2000 transfected cells are shown and there were no differences in the cells transfected by different methods (not shown).

These data indicate that the nuclear localization of the fusion proteins does not depend on the presence of the N-terminal sequence but rather it is influenced by the kind of the attached tag. In all cases, the myosin molecules were tagged at their C-terminus, i.e. the end of the basic, phospholipid-binding tail, and all of the tags decreased the nuclear localization of myosin. It is possible that the C-terminal part of the tail is needed for a proper nuclear localization in some way and that the attached tags might interfere with its function. The strong inhibitory effect of FLAG might be caused by its acidic nature interfering with the basic tail while EGFP is quite bulky and might interfere in that way. The finding that there is no difference in nuclear localization of tagged NMI and Myo1c, which we observed repeatedly with different

cell lines and transfection methods, is in disagreement with published data (Pestic-Dragovich et al. 2000) reporting nuclear localization of the FLAG-tagged NMI and only cytoplasmic localization of the FLAG-tagged Myo1c overexpressed in NIH-3T3 cells.

Taken together, these data suggest that modifications of the C-terminus of the molecule interfere with the nuclear translocation mechanism. However, the transport mechanism does not seem to be selective for NMI, and Myo1c is transported with similar efficiency. How much endogenous Myo1c is located in the nucleus in the cells under normal conditions and by which mechanism different distribution of NMI and Myo1c is achieved remain to be elucidated.

The localization of V5 tagged myosin I is cell cycle dependent

To explain the variability of nuclear localization of the overexpressed myosin in the cell population, we hypothesized that the localization depends on the cell cycle phase. To test this, we transfected U2OS cells with V5/His tagged NMI or Myo1c, synchronized the cells the next day with either nocodazole or aphidicolin, and assessed the nuclear localization of the constructs at several time points after the release from the block (Fig. 2).

The cells treated with nocodazole are synchronized on G_2/M boundary and they continue with mitosis after washout. We observed the lowest presence of both NMI and Myo1c in the nuclei at the first time point after mitosis (2h) and subsequently a steady increase of the nuclear localization (Fig. 2a and b).

Aphidicolin stalls the cell cycle in S phase and after washout the cells finish S phase and continue with G_2 and mitosis (Fig. 2e). The amount of both myosins in the nuclei reached the minimum after 17 h (Fig. 2c and d) when most cells underwent mitosis and entered the G_1 phase (Fig. 2e). There were no significant differences between NMI and Myo1c in these experiments.

The most likely explanation of these results is that V5 tagged myosin is not present in the newly forming nucleus after mitosis and it is transported there slowly during interphase.

The endogenous NMI is transported to the nucleus after mitosis

To asses whether endogenous NMI is enclosed to the newly formed nucleus after mitosis bound to chromatin or whether it is not present in the newly formed nucleus and it is imported there later in a similar manner as the tagged and overexpressed NMI, we immuno-stained the cells directly after mitosis. Mitotic 3T3 cells were shaken-off the dish to poly-lysine coated coverslips, allowed to attach for 15 min, and stained with antibodies to NMI and lamin B. In the mitotic cells, NMI stays in the cytoplasm outside the condensed chromosomes (Fig. 3a). In the cells with formed nuclear lamina recognizable by the lamin B staining, most of the cells contained NMI predominantly outside of the nucleus (Fig. 3b).

These findings are consistent with the hypothesis that the endogenous NMI is not present in the nucleus after mitosis and must be imported there after the nucleus is formed. The nuclear import has to be rapid because in unsynchronized culture, the cells with endogenous NMI located in the cytoplasm are very rare. We hypothesize that the tagged myosins are imported to the nucleus by the same mechanism as the endogenous one but the epitope tags hinder and slow down the process. The mechanism by which the epitope tags interfere with the nuclear import is not clear and need to be addressed by further research.

The first two IQ domains are sufficient to transport the fusion protein to the nucleus

To asses which part of the molecule is responsible for the nuclear transport of myosin, we constructed a series of EGFP-tagged deletion constructs of NMI (Fig. 4a) and tested their localization after transient transfection in U2-OS cells.

Although both N-terminally (GFP-NMI) and C-terminally (NMI-GFP) tagged NMI localized preferentially outside the nucleus, there was some, albeit weaker than cytoplasmic, nuclear signal too. Similar situation resulted from overexpression of tailless myosin (TLL-GFP). On the other hand, the constructs containing only the head (Head-GFP) or tail (GFP-T) without the neck region localized exclusively to the cytoplasm. All other constructs that do not contain the motor domain but contain at least part of the neck localized strongly to the nucleus (Fig. 4b).

While the constructs containing the whole neck and tail (GFP-HDL) or the neck and part of the tail (GFP-HDL-Δ853) localized to the nucleoplasm and excluded nucleoli with the exception of fibrillar centers in the manner similar to the endogenous NMI, the construct with whole tail deleted (GFP-IQ12) accumulated in the nucleoli. We conclude that the neck region containing the first two IQ motifs is responsible for the translocation of the myosin to the nucleus while the tail is needed for the proper nuclear localization, possibly through the phospholipid binding domain which it contains.

The area of the first two IQ domains contains a cluster of basic residues that resembles NLS although it does not have a canonic NLS sequence. It could serve as an NLS and bind directly to the nuclear transport machinery or it could interact with some other protein that would be responsible for the nuclear transport. The identical area of the second IQ domain of Myo1c also binds to receptors in the inner ear hair-cells, probably to cadherin 23 (Cdh23) (Cyr et al. 2002; Phillips et al. 2006), and to phospholipids (Tang et al. 2002; Phillips et al. 2006). These interactions are disrupted by calmodulin binding (Phillips et al. 2006). This open an interesting possibility that calmodulin binding could regulate the nuclear translocation of NMI.

In this work we have focused on the mechanism of nuclear translocation of NMI. We show that NMI is transported to the nucleus after formation of the nuclear envelope and that even relatively minor modifications of the molecule with epitope tags lead to significant disruption of the process. Moreover, we demonstrate that the NMI N-terminal domain is not essential for the nuclear localization while the first two IQ domains are sufficient for the nuclear transport. This brings some interesting questions. Does the neck area contain a genuine NLS or does it get to the nucleus by piggybacking some other protein? What is the role of the N-terminal domain? Does it facilitate the nuclear import of the full-length myosin or is it rather involved in NMI retention in the nucleus? These questions need to be addressed experimentally.

Figures

Fig. 1 The localization of overexpressed NMI depends on the kind of epitope tag. U2OS cells were transfected with expression vectors coding variously tagged NMI and Myo1c. The cells were fixed and stained with antibodies to NMI for human

and mouse untagged NMI; V5 and Flag in case of tagged myosins. EGFP was observed directly. The cells were observed in fluorescence microscope and manually categorized to three groups: cells with predominantly nuclear localization of the signal (nucleus brighter than cytoplasm), cells with equal distribution in nucleus and cytoplasm, and cells with predominantly cytoplasmic localization of the signal. Numbers of counted cells are stated above the bars.

Fig. 2 Tagged NMI is transported to the nucleus during interphase. U2OS cells were transfected with V5-tagged NMI or Myo1c and synchronized with nocodazole or aphidicolin. Cells were fixed at the indicated time points, stained with anti-V5 antibody and categorized according to the predominating location of the signal. **a, b** The amount of both V5-tagged NMI and Myo1c was gradually increasing during 10 h after release from the nocodazole mitotic block. **c, d** When the cells were synchronized in S-phase by aphidicolin, the share of the cells with predominantly nuclear localization decreased when the cells underwent mitosis. **e** Flow cytometric analysis of the cell cycle progression of the cells in **c** and **d**.

Fig. 3 The endogenous NMI is localized outside of the nucleus immediately after mitosis. 3T3 cells obtained by mitotic shake-off were stained with antibodies to NMI and lamin B. NMI is localized clearly outside the condensed chromosomes (a) as well as the newly formed nucleus (b). Bar, 10 μm.

Fig. 4 The first two IQ domains are responsible for the translocation of NMI to the nucleus. (a) EGFP-tagged full-length NMI and various truncated NMI constructs were expressed in U2OS cells. (b) EGFP (green) and DRAQ5 to stain chromatin (red) are shown. The first two IQ domains are sufficient to direct the fusion protein to the nucleus. Bar, 20 μm.

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Fig. 1

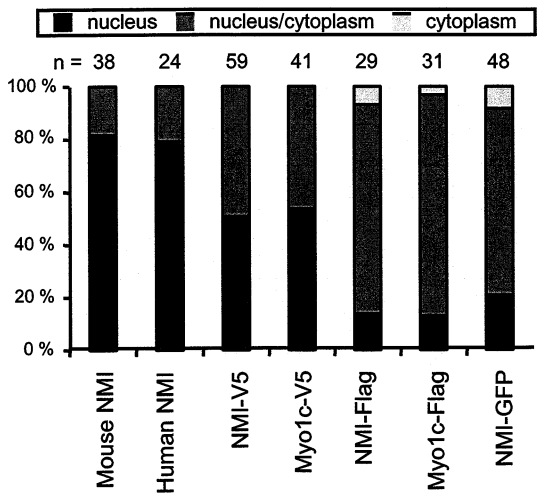
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Fig. 2

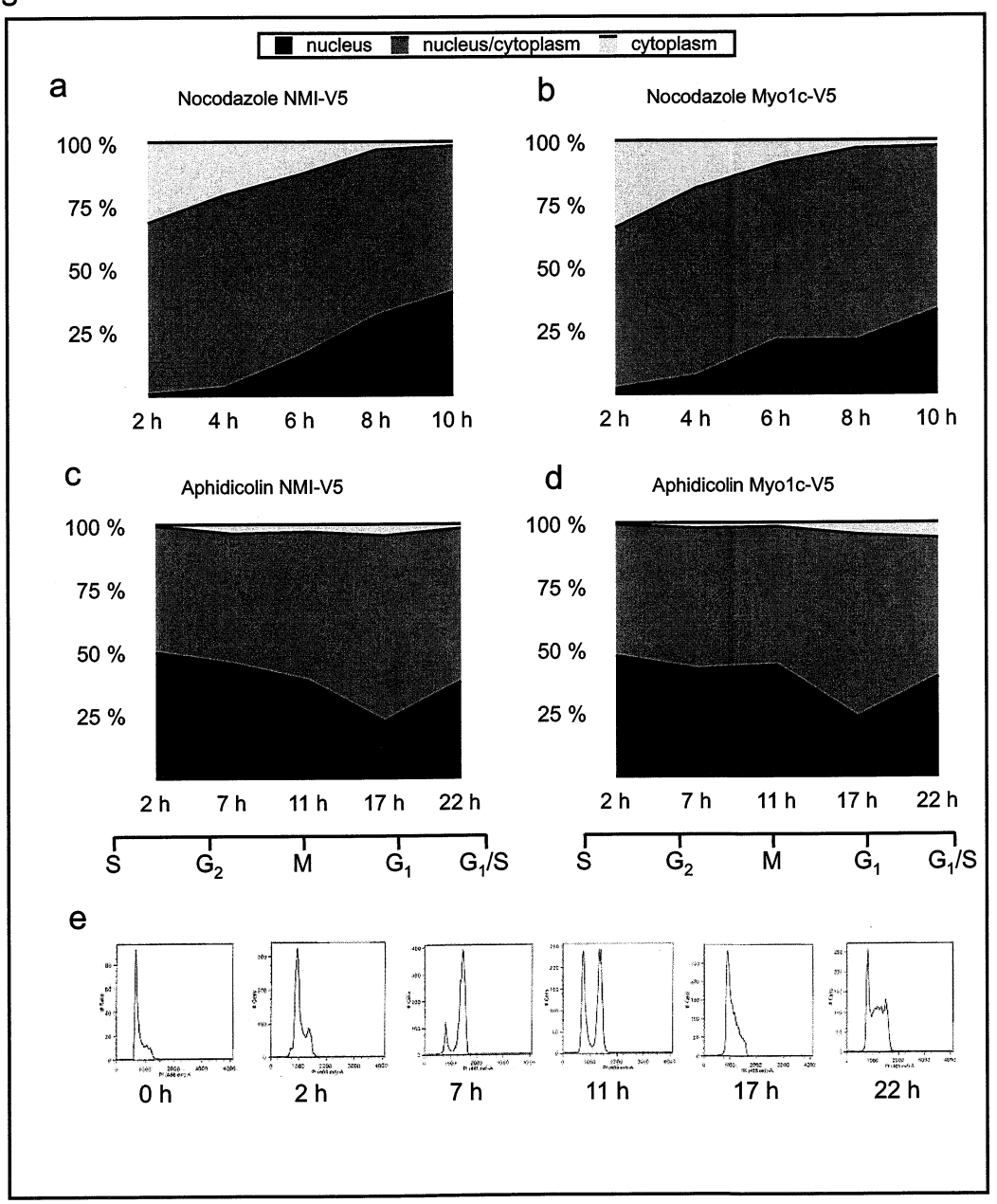
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Fig. 3

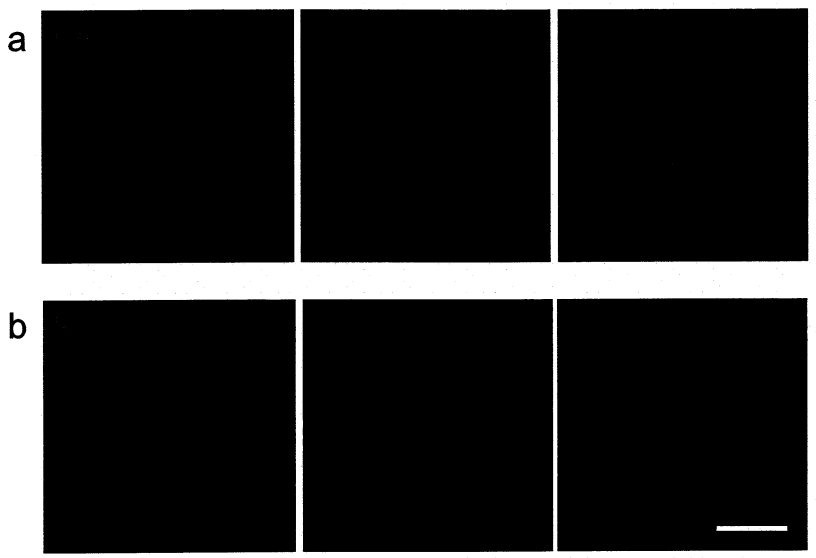
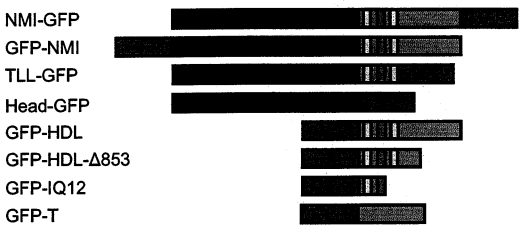


Fig. 4

a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GFP-NMI
TLL-GFP
Head-GFP
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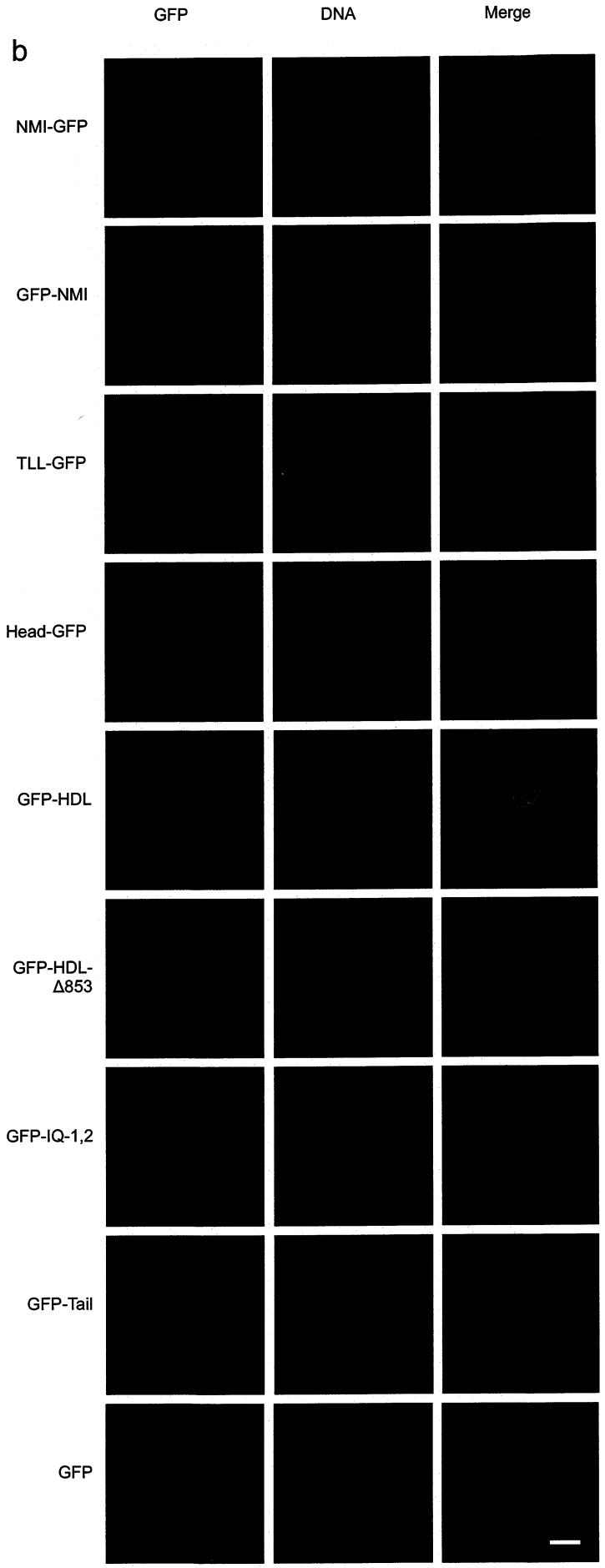
GFP-HDL-Δ853
GFP-IQ12
GFP-T

b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GFP-IQ-1,2
GFP-Tail
GFP

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ORIGINAL PAPER

Nuclear myosin is ubiquitously expressed and evolutionary conserved in vertebrates

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Abstract Nuclear myosin I (NMI) is a single-headed member of myosin superfamily localized in the cell nucleus which participates along with nuclear actin in transcription and chromatin remodeling. We demonstrate that NMI is present in cell nuclei of all mouse tissues examined except for cells in terminal stages of spermiogenesis. Quantitative PCR and western blots demonstrate that the expression of NMI in tissues varies with the highest levels in the lungs. The expression of NMI is lower in serum-starved cells and it increases after serum stimulation. The lifespan of NMI is longer than 16 h as determined by cycloheximide translation block. A homologous protein is expressed in human, chicken, *Xenopus*, and zebrafish as shown by RACE analysis. The analysis of genomic sequences indicates that almost identical homologous NMI genes are expressed in mammals, and similar NMI genes in vertebrates.

Keywords Nuclear myosin I · Myosin 1c · Transcription · Chromatin · Tissue expression

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Introduction

Nuclear myosin I (NMI) is a single headed, monomeric member of the myosin superfamily closely related to myosin-IC (Myo1c) from which it differs only by 16 amino acid N-terminal extension (Pestic-Dragovich et al. 2000). The mechanism of nuclear import and retention is not known; however, the N-terminal extension was proposed to be responsible for its nuclear localization (Pestic-Dragovich et al. 2000). In mouse, the mRNA for NMI arises by alternative start of transcription from the same gene as the cytoplasmic Myo1c located on 11th chromosome. mRNA for NMI differs only by a distinct exon on its 5′ end which codes an alternative start of translation and 6 out of 16 extra amino acids (Pestic-Dragovich et al. 2000).

The role of NMI in transcription by RNA polymerase I (Pol I) is well established. In nucleoli, NMI was found in foci that colocalized with sites of active transcription (Fomproix and Percipalle 2004). Moreover, Pol I transcription is decreased by depletion of NMI in vivo by antibody microinjections or siRNA, whereas overexpression of NMI increases pre-rRNA synthesis in a dose dependent manner. In vitro, addition of recombinant NMI activates Pol I transcription, while antibodies to NMI inhibit Pol I transcription on both naked DNA and pre-assembled chromatin templates (Philimonenko et al. 2004). Hinting at molecular mechanism of its function, NMI was shown to bind Pol I through the basal transcription factor TIF-IA which is bound to the initiation-competent subpopulation of Pol I and through which the rate of transcription initiation is regulated (Philimonenko et al. 2004). Interestingly, while antibodies against NMI block synthesis of full-length transcripts in an in vitro transcription system, the

formation of short three nucleotide abortive transcripts is not impaired, suggesting a role of NMI in the steps subsequent to transcription initiation (Percipalle et al. 2006). There is also some evidence that NMI plays a role in transcription by RNA Polymerase II (Pol II). NMI colocalizes and co-immunoprecipitates with Pol II, and in vitro immunodepletion of NMI inhibits transcription by Pol II (Pestic-Dragovich et al. 2000).

Recently, a link between NMI and chromatin remodeling has been established. NMI associates with WICH—a chromatin remodeling complex containing WSTF and SNF2h. NMI together with core proteins of WICH are situated along the entire ribosomal gene (Percipalle et al. 2006) and also at some RNA polymerase III transcribed genes such as 5S rRNA and 7SL but not at tRNA genes (Cavellan et al. 2006). Antibodies against WSTF reduce rDNA transcription on preassembled chromatin but not on naked DNA in vitro and RNAi mediated knockdown of WSTF reduce rDNA transcription in vivo. These data indicate that WICH might be recruited by NMI to facilitate transcription on chromatin (Percipalle et al. 2006). Interestingly, it was recently reported that mutants of actin and NMI are able to inhibit repositioning of transcriptionally activated chromatin site from the nuclear periphery to the interior (Chuang et al. 2006).

In summary, important roles of NMI in DNA transcription and chromatin remodeling are clearly emerging (for reviews, see de Lanerolle et al. 2005; Grummt 2006; and Percipalle and Farrants 2006), however, the molecular mechanisms of NMI action are still little understood. It is known from earlier studies that in PHA-stimulated lymphocytes, the levels of both NMI mRNA and protein raised strongly upon stimulation of the lymphocytes and onset of transcription (Kyselá et al. 2005), suggesting that the NMI expression correlates with the cell metabolism. In this paper, we extended these studies on NMI expression and show that NMI is ubiquitously expressed and located in nuclei of all mouse tissues, with the highest expression in lungs. NMI is regulated in dependence on proliferative status of the cells and it is a relatively stable protein with a lifespan between 16 and 48 h. Almost identical homologous NMI genes are expressed in mammals, and similar NMI genes in vertebrates.

Materials and methods

Antibodies

NMI was detected using rabbit polyclonal antibody directed to N-terminal part of NMI (M3456, Sigma). Polyclonal antibody R2652 against the tail of Myo1c was a gift from Peter G. Gillespie, Oregon Hearing Research Center and Vollum Institute. Antibodies against β-actin (A2066) and γ-tubulin (T6557) were purchased from Sigma; antibody against GAPDH (clone 6G5) is available from Acris.

Cells and serum activation

NIH 3T3 and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics in humidified 5% CO_2/air, 37°C environment. The protocol for serum activation experiments was essentially as described (Iyer et al. 1999). Briefly, NIH 3T3 cells were grown on six-well plates. When they reached approximately 50% confluence they were washed two times with phosphate buffered saline (PBS), and low serum medium (0.1% FCS) was added to the plates. The cells did not show morphological signs of apoptosis under these conditions. After 48 h, the medium was replaced with a fresh medium containing 10% FCS. Cells were harvested before the addition of serum (time 0) and after 2, 4, 8, and 16 h of serum stimulation.

Animals

Laboratory mice strain C53/Bl6, four males and three females, 10–16 weeks old were killed by cervical dislocation. Samples of the small intestine, pancreas, brain, kidney, skin, heart muscle, testis/ovary, striated muscle, spleen, liver, and lungs intended for RNA isolation were stored in RNAlater storage solution (Sigma), parts of the tissues intended for subsequent cryostat slicing were frozen in liquid nitrogen. Experiments were performed according to the national and local regulations for use of experimental animals.

Immunofluorescence microscopy

For immunostaining, 6 μm cryostat sections were mounted onto glass slides coated with poly-L-lysine, and fixed 15 min in absolute ethanol at room temperature. Sections were then permeabilized in 0.2% Triton X-100 in PBS for 10 min, incubated with antibody against NMI (16 μg/ml, 1 h at RT), and with FITC-conjugated goat anti rabbit secondary antibody (Jackson ImmunoResearch). Coverslips were mounted with Mowiol (Sigma) containing DABCO (Sigma) as an antifading agent, 0.5 μg/ml propidium iodide, and 0.1 μg/ml DAPI (Sigma), and observed with confocal microscope Leica TCS SP.

Immunoblots

Tissues and cells for the experiments on Figs. 2 and 5 were homogenized in SDS lysis buffer (60 mM Tris pH 6.8, 10% glycerol, 2% SDS), lysates were cleared by centrifugation and total protein content was measured using BCA assay (Pierce). In other experiments, cultured cells were lysed in lysis buffer (20 mM Tris pH 7.4, 200 mM NaCl, 1% Triton X-100, 2 mM EDTA, 10 mM EGTA, 10 mM K_2HPO_4, 1 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml pepstatin), briefly sonicated, and cleared by centrifugation. Protein content was measured by Bradford assay (Biorad). Equal amounts of protein were loaded on 8% gel, proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. The blots were incubated with antibodies, horseradish peroxidase conjugated secondary antibodies (Bio-Rad), and the signal was visualized by ECL (Pierce). The two myosin isoforms were separated according to (Rossini et al. 1995) with following modifications. We used a 0.75 mm thick midigel format (Hoefer SE 600 Ruby), 4% stacking gel (15 mm high) and 8% resolving gel. Both gels contained 30% glycerol. Electrophoresis was run for 14 h with maximum power 3 W, maximum voltage 350 V, and with cooling to 8°C by circulator bath.

RNA isolation and RT-qPCR

Total RNA from cells and tissues was isolated with TriReagent (Sigma) according to manufacturer's protocol. The cells were lysed on plates. Concentration of RNA was measured by spectrophotometry and the integrity of RNA was checked on denaturing agarose gel. A total of 100 ng of RNA was reverse-transcribed with random hexamers as primers using TaqMan Reverse Transcription Reagents (Applied Biosystems). Real-time qPCR was performed in ABI Prism 7300 instrument using SYBR Green PCR Master Mix (all Applied Biosystems). The following primers were used: mouse NMI CGGCAGGATGCGCTACC and TCAAGGCGCTCTCCATGG, mouse ß-actin GCCC TGAGGCTCTTTTCCA and TGCCACAGGATTCC ATACCC, and mouse GAPDH GGAAGGGCTCAT GACCACAG and GCCATCCACAGTCTTCTGGG. Data were evaluated with 2-ΔΔ CT method (Livak and Schmittgen 2001) using control genes as described in Results.

RACE

RLM-RACE (RNA ligase-mediated rapid amplification of cDNA ends) was performed according to the protocol developed by Invitrogen with minor modifications. Briefly, total RNA was first treated with Shrimp Alkaline Phosphatase (SAP) to remove 5′ phosphates from all uncapped RNAs. RNA was then extracted by phenol:chloroform and treated with Tobacco Acid Pyrophosphatase to remove the cap, yielding 5′ phosphate. After phenol:chloroform extraction, a special RNA adapter oligonucleotide was ligated to 5′ phosphorylated RNAs by T4 RNA Ligase. RNA was extracted once more and reverse-transcribed with random hexamers as primers using TaqMan Reverse Transcription Reagents (Applied Biosystems). PCR was performed as above with touchdown protocol. (Annealing temperature went down every five cycles from 70° to 68°, 65°, and then remained 63° during the rest of amplification.) Primers were designed to align with homologues of Myo1c in the respective species: human MYO1C (GenBank NM_033375) GTGTCCGC CACGGCAAACAGG and CCGTGCGCAGTGCTC GGTACAC, chicken MYO1C (GenBank NM_0010062 20) TCACTGGTGAAGTTCTCAAGCAGG, *Xenopus laevis* MYO1C (GenBank BC044718) TCTTTATATG GGTTGACAGAGACC and GCTTCACTTGTATA GTTTTCCAACAG, and *Danio rerio* Myo1c homologues GenBank XM_690832 ATGAAAGCGGCTT CACTGTTGTGG and GenBank XM_689729 GAC-AAAATCCTGCACACCCACCC. PCR products were separated on 3% agarose gel, bands were excided, isolated by Zymoclean Gel DNA Recovery Kit (Zymo Research), and sequenced on ABI Prism 3100 (Applied Biosystems).

Sequence searches, alignment, and construction of phylogenetic trees

The sequences similar to NMI were found in the GenBank and Ensembl databases using TBLASTN (Altschul et al. 1997). Genscan (Burge and Karlin 1997) was used for gene predictions in genomic DNA. Sequences were aligned with ClustalW and manually refined. The phylogenetic trees were constructed and bootstrap tested by minimum evolution method using molecular evolutionary genetics analysis software MEGA version 3.1 (Kumar et al. 2004).

Results

NMI is ubiquitously expressed and its amount in tissues varies

Different levels of NMI expression in tissues could suggest certain tissue-specific functions. We have therefore

screened eleven mouse organs: small intestine, pancreas, brain, kidney, skin, heart muscle, testis, striated muscle, spleen, liver, and lungs by immunofluorescence microscopy (Fig. 1). We observed nuclear staining in all cell types examined but no obvious differences in signal intensity were observed in nuclei of various cell types. There was only one exception—cells in the latest stages of spermiogenesis which were completely negative.

We also examined the expression of NMI in these tissues by Western blot (Fig. 2b). Intriguingly, the amount of NMI in lungs was many times higher than in any other tissue. Relatively high levels of NMI were also found in the small intestine, kidney, skin, heart, testis, spleen, and liver. Low levels were detected in pancreas, brain, and skeletal muscle.

Next, we compared the tissue levels of NMI and cytoplasmic Myo1c. Since Myo1c differs from NMI only by missing 16 amino-acid epitope, there is no antibody available that would recognize Myo1c specifically. We used therefore an antibody directed against the tail of Myo1c recognizing both Myo1c and NMI. The tissue levels in this case were similar to NMI, however, the signal in lungs is not so prominent and there is relatively stronger signal in intestine, pancreas, kidney, heart, and liver. We were also able to separate the two myosin isoforms in 3T3 cells and liver tissue by SDS-PAGE. Two distinct bands were distinguished when stained by the antibody recognizing Myo1c tail while the anti-NMI antibody recognized only the upper band. In both 3T3 cells and mouse liver sample (same as in Fig. 2b) the NMI band was about two to three times weaker than Myo1c band (data not shown).

To quantify the NMI mRNA, we performed real-time RT-qPCR in the same tissues as above. The primers were designed to anneal to 5′ region unique to NMI and therefore they detected NMI mRNA specifically. The amounts of NMI mRNA are shown relatively to total RNA in samples. We found the highest levels of NMI mRNA in lungs, medium in heart, testis, and spleen, and low in other tissues (Fig. 2a).

The expression of NMI is stimulated by serum

To test the connection of NMI expression with cell proliferation, we measured the response of NIH 3T3 cells to serum. The cells were first starved in 0.1% serum medium for 48 h and then stimulated by addition of medium containing 10% serum. The amount of mRNA for NMI was then measured by RT-qPCR (Fig. 3a). Results were normalized to total RNA. The levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin mRNAs are shown for comparison. Actin mRNA was found previously in a similar experiment not to change relatively to total mRNA levels (Iyer et al. 1999) so it can represent an "average" transcript. The amount of mRNA for NMI as well as for actin and GAPDH halved during serum starvation but increased during 2–4 h after serum stimulation, and then slightly decreased. NMI mRNA was activated by serum more than the housekeeping genes' mRNA. Additional RT-qPCR experiments showed that NMI expression rose gradually during the first two hours of serum activation (data not shown). However, on the protein level, the amounts of NMI relatively to total protein content did not change significantly as shown by Western blot (Fig. 3b).

The lifespan of NMI is longer than 16 h

To assess the stability of NMI, we treated NIH 3T3 cells with protein synthesis inhibitor cycloheximide at concentration 2 μg/ml for 16 h and observed the levels of NMI. This concentration of cycloheximide was chosen because half of this concentration was reported to completely block translation in Chinese Hamster Lung cells (Yildirim and Whish 1997) and it did not cause morphological signs of apoptosis. The levels of NMI did not change significantly during 16 h while the amount of control tubulin already started to decrease (Fig. 4). This experiment was repeated twice with similar results.

The specific N-terminal sequence of NMI is identical in mammals and conserved in vertebrates

Information on NMI phylogenetic conservation could suggest how fundamental is the role of NMI in transcription. To test this, we examined the expression of NMI in representatives of various animal classes, namely in human, mouse, chicken, *Xenopus laevis*, zebrafish, and *C. elegans*. Using immunoblot, we found a signal with the mobility corresponding to NMI in human (HeLa) and mouse (NIH-3T3) cell lines, mouse liver, chicken lungs, but not in chicken liver, *Xenopus* lungs, zebrafish, and *C. elegans* (Fig. 5). In the *Xenopus* liver, we found a band with slightly higher mobility than other samples. In chicken, these results can be explained by weak binding of the antibody to varied epitope, which is compensated by a high expression of NMI in lungs. In *Xenopus* there is no signal in lungs and slightly smaller protein is detected in the liver, therefore we cannot exclude that the antibody does not recognize *Xenopus* NMI, but cross-reacts with another protein in the liver extract.

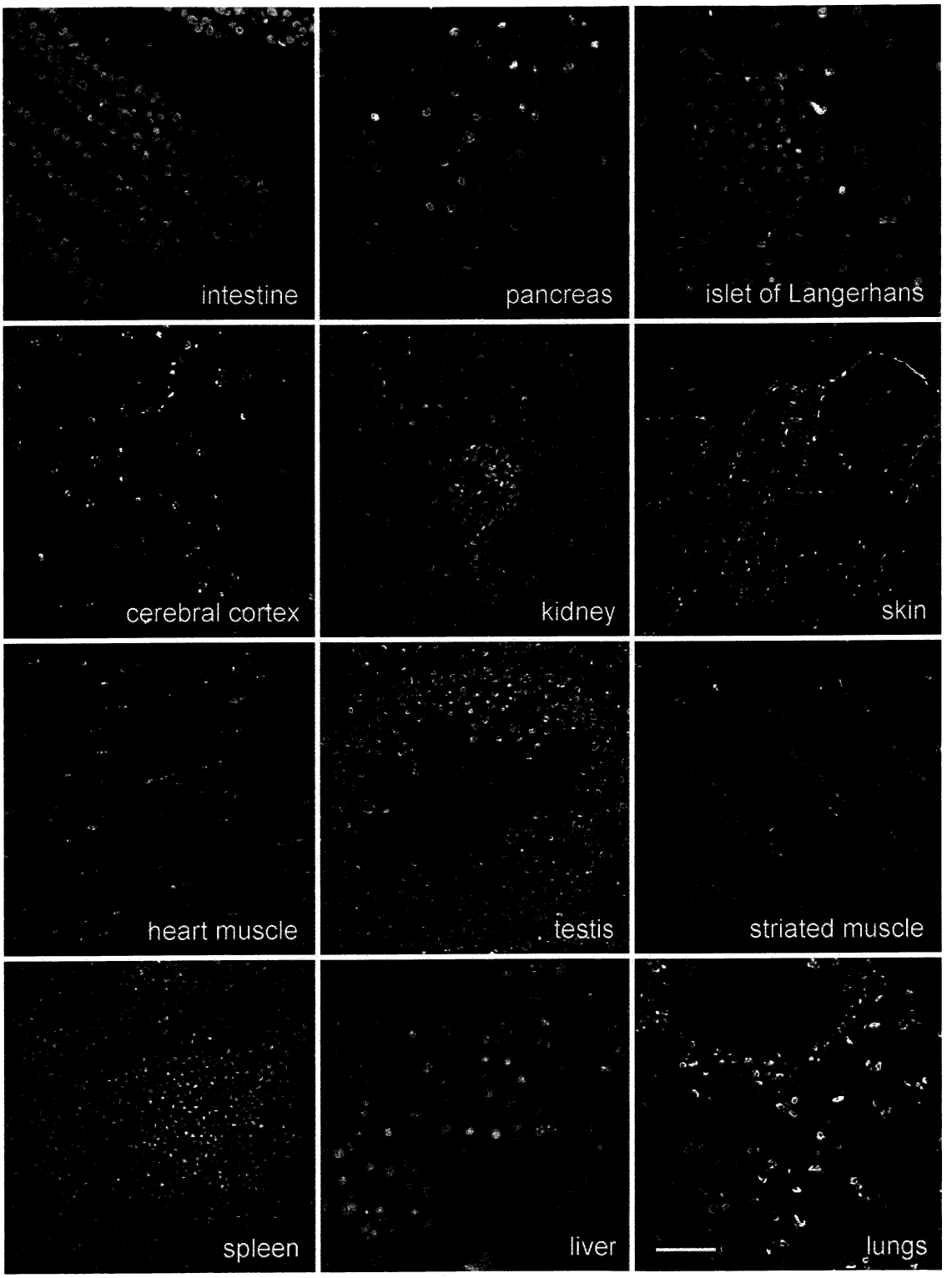

Fig. 1 NMI is present in nuclei of all mouse tissues. Confocal immunofluorescence microscopy on cryosections shows that NMI is present in nuclei of all tissues tested with one exception—there is no signal in cells in latest phases of spermiogenesis. *Bar* 50 μm

In the next step, we analyzed the NMI sequences in the genomic databases of various species. In every model animal, we selected one or two genes homologous to mouse Myo1c by similarity search and then searched in approximately 10–15 kb upstream region of the genomic sequence for an intron coding for a sequence similar to the NMI N-terminus. Expression of a sequence identical to mouse NMI N-terminus was

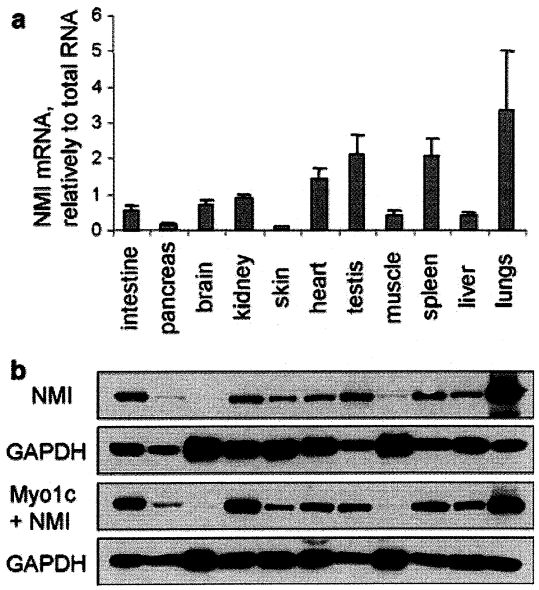

Fig. 2 Expression of NMI varies in mouse tissues. **a** Total RNA was isolated from mouse tissues and identical amounts were analyzed by quantitative RT-PCR. Measurements were repeated with samples from five to eight animals except for skin where only one sample was of sufficient quality, and lungs where samples from two animals were taken only after finding of the high expression on the blot. Averages and standard errors are shown. **b** Western blot of NMI and Myo1c in the same tissues. A total of 65 μg of proteins were loaded, β-actin serves as loading control

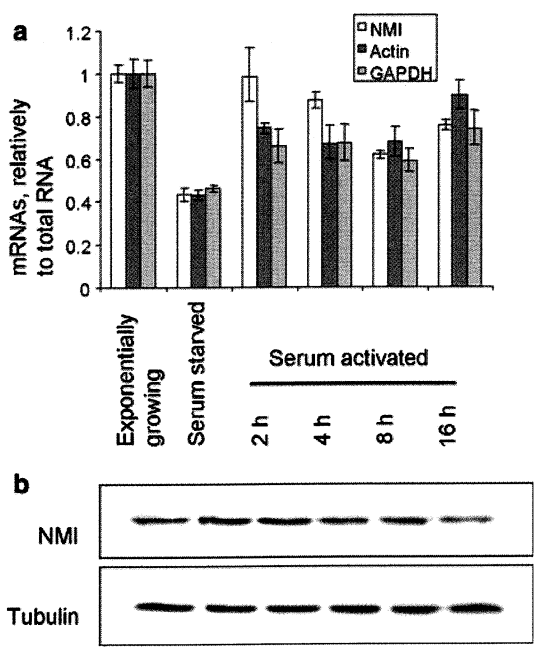

Fig. 3 Transcription of NMI raises during serum activation. NIH 3T3 cells growing in medium with 10% serum were subjected to 0.1% serum for 48 h and then returned to 10% serum medium. **a** RT-qPCR quantification of mRNA for NMI, actin, and GAPDH. Samples were run in duplicates, and standard deviations are indicated. **b** Western blot analysis of NMI expression. Identical amounts of protein were loaded; tubulin serves as a loading control

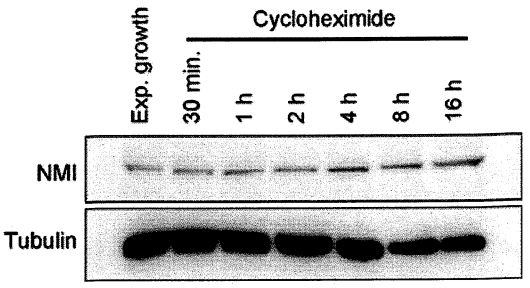

Fig. 4 NMI is not significantly degraded during 16 h of translation block. Western blot analysis of 3T3 cells treated by 2 μg/ml cycloheximide and harvested in indicated time points

predicted by gene prediction software in the genome of human, dog, cow, and rat. Furthermore, similar NMI N-terminal sequences were found in a genome of chicken (50% identity), *Xenopus tropicalis* (60% identity), and zebrafish (41% identity). We have also found N-terminal sequences 35% identical to mammalian ones in the genome of pufferfish (*Fugu rubipes*) and *Tetraodon nigroviridis*. In contrast, genomic analysis identified no sequences similar to NMI N-terminus in urochordate *Ciona intestinalis*, *Drosophila*, or *C. elegans* (Fig. 6; for a complete phylogenetic tree, see Fig. 7).

To test whether these N-terminal sequences are really expressed in the predicted form, we performed a 5′ RLM-RACE analysis in selected species. The results are summarized in the Table 1. We confirmed that a sequence corresponding to NMI 16 amino-acid N-terminus is expressed in human, mouse, chicken, and zebrafish. We have not detected the expression in *Xenopus laevis*, but an expression of sequence coding for 63% similar peptide was described (GenBank AAH44718).

Discussion

Since the discovery of NMI in 1997, several studies showed that it has important functions in transcription (for reviews, see de Lanerolle et al. 2005; Grummt 2006; Percipalle and Farrants 2006). However, all data obtained so far have dealt with cell cultures and there is no information on tissue-specific expression or phylogenetic distribution of NMI. This paper aims to fulfill these gaps and provides also additional data on NMI concerning the protein lifespan and the connection with overall cell metabolism and proliferation.

The tissue distribution of NMI is interesting because it might answer the question whether NMI serves a tissue-specific or general role in transcription. These data exist for Myo1c and one can compare them conveniently

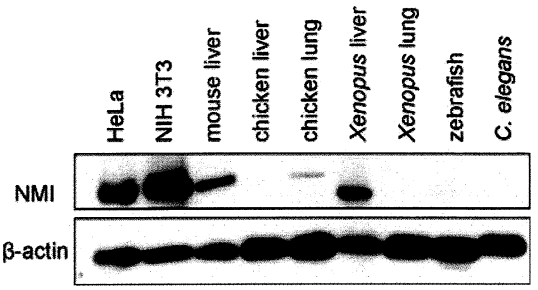

Fig. 5 NMI in various species as detected by western blot. Total protein (50 μg) extracted from indicated tissues of different organisms were separated by western blot and tested by polyclonal antibody to NMI. β-actin serves as loading control

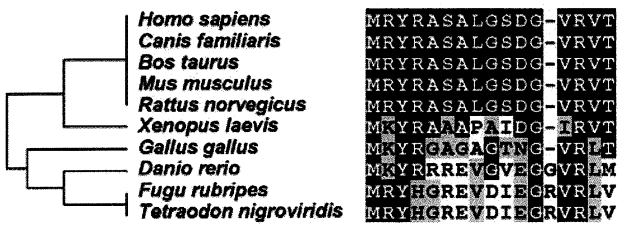

Fig. 6 NMI is conserved in mammals and expressed in vertebrates. Alignment of NMI N-terminal sequences from organisms where it was found to be expressed. Residues with *black background* denote identity with consensus, *gray background* marks conservative substitution. The tree on the left shows inferred relations between sequences

with those on NMI. Wagner et al. (1992) assayed the distribution of Myo1c in mouse tissues by western blot finding the highest levels in lungs, adrenal gland, and stomach followed by spleen, heart, and esophagus. This study was complemented by Crozet et al. (1997) who compared similar selection of mouse tissues by Northern blot and found also the highest levels in lungs followed by kidney, spleen, heart, and testis. Similar experiments were performed in rat, and the highest expression of Myo1c was observed again in lung, followed by heart and kidney both by Northern (Sherr et al. 1993) and Western blot (Ruppert et al. 1995). In the data of Yanai et al. (2005) who assayed 12 healthy human tissues by Affymetrix GeneChip, the expression of Myo1c was again highest in lungs, followed by prostate, heart, and kidney. As these studies were not designed to distinguish between Myo1c and NMI, they show cumulative levels of both myosin isoforms. In general, our results on the "total" myosin are in agreement with these literature data. In addition, we were able to show the expression of the nuclear isoform separately. If we take in account that in liver NMI comprises one-quarter to one-third of the "total" pool, we can estimate the ratio of both isoforms also in other organs. Strikingly, in lung, NMI seems to comprise the large majority of the "total" pool. This suggests some organ-specific functions of the NMI in lung.

There is a discrepancy between the observation of similar levels of NMI in the nuclei of different cell types by immunofluorescence microscopy and varied expression detected by quantitative PCR and western blot. The tissues differ in the amount of cytoplasmic and extracellular protein and thus in the ratio of NMI to total protein. For example, high amounts of actin and muscle myosin are present in striated muscle while there is much less cytoplasmic and extracellular protein in lung tissue—this increases the fraction of nuclear proteins relative to the total protein. However, the many-fold higher expression of NMI in lungs cannot be explained by this mechanism alone as other nuclear proteins such as Pol II, TATA binding protein (TBP), lamin A and C, and nuclear DNA helicase II do not show such markedly high expression in lungs (data not shown). In addition, the immunofluorescence confocal microscopy has a limited quantitative ability and it is therefore not possible to measure differences between various tissues or cell types, unless they are dramatic like in the latest stages of spermiogenesis.

Because NMI plays a role in transcription it is important to asses how tightly is NMI expression regulated in connection with overall transcriptional activity of the cell. We used therefore a convenient model of NIH-3T3 cells subjected to serum starvation and metabolically reactivated following serum addition. During serum starvation, the cells enter G_0 phase, and after the addition of serum they reenter the cell cycle while increasing overall transcription. The levels of NMI after serum activation peak after 2–4 h, and the transcription of NMI is increased approximately by 30% when compared to an "average" gene like actin or GAPDH. This is a relatively weak response considering that several fold change is not uncommon for other highly regulated genes under these conditions (Iyer et al. 1999). It is, however, stronger response than that of Pol II or TATA-box binding protein (TBP) that respond in a similar fashion as actin (database supplementary to Iyer et al. 1999). It seems, therefore, that the biochemical activity of NMI is not regulated chiefly on the level of expression but by other mechanisms like calmodulin binding/dissociation (Zhu et al. 1998), or phosphorylation (Gillespie and Cyr 2004; Williams and Coluccio 1995).

In order to further characterize the dynamic properties of NMI, the stability of NMI was assessed. Because the treatment with cycloheximide inhibits cytosolic protein synthesis, any observed decrease in NMI presence should reflect mainly NMI degradation. No significant change in cellular NMI levels was observed following 16 h of cycloheximide treatment which indicates that NMI has a lifespan not shorter than 16 h.

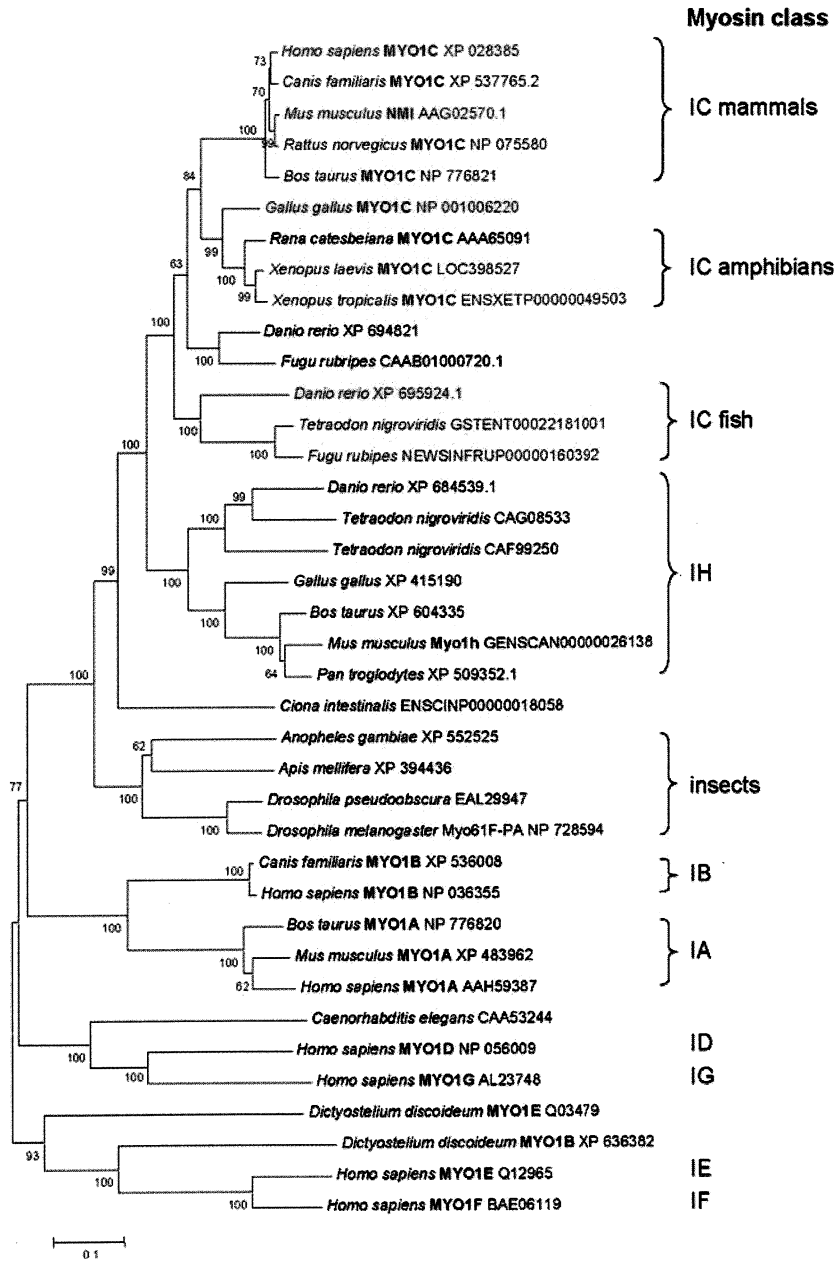

Fig. 7 An unrooted phylogenetic tree of putative Myo1c homologs. Sequences similar to Myo1c were found by BLAST in protein, nucleotide, and genomic databases. Sequences from different organisms most similar to Myo1c were selected and some members of other mammalian myosin I subclasses were added for comparison. Alignment was made by ClustalW, and the tree was constructed and bootstrap tested by minimum evolution algorithm. This tree served to choose candidates for further search for the sequences similar to the NMI N-terminal. The genes in green were shown to express a sequence similar to N-terminal sequence by RACE, and in red the genes with an exon coding for a similar sequence as found by analysis of genomic sequence. In *Rana catesbeiana*, we did not perform RACE and the genome is not sequenced, therefore we were not able to find the N-terminus. In fish, there are two distinct homologs of Myo1c that probably arose by genome duplication in teleost fish lineage (Jaillon et al. 2004). Only one of these, however, contains the NMI N-terminal sequence. We have found the N-terminus neither in *Ciona intestinalis* nor in other more distant organisms genomic sequence. *Scale bar* shows evolutionary distance in number of replacements per amino acid. *Numbers in branch points* denote bootstrapping values

In our previous study, we have shown that NMI reaches its minimum level after 48 h after siRNA inhibition in HeLa cells (Philimonenko et al. 2004). Taken together, we can estimate the lifespan of NMI molecules between 16 and 48 h. This result is consistent with half-life prediction of about 30 h based on the "N-end rule" (Gonda et al. 1989).

We found the N-terminal sequence of NMI to be identical in mammals and the rest of the protein is also conserved in mammals with minimum of 95% similarity.

Table 1 Results of 5′ RACE of myosin IC genes

Species	NMI	Myo1c
Mouse	EB724369	EB724364-8
Human	EB724371-3	–
Xenopus laevis	–	EB724387
Chicken	EB724384, EB724386	EB724385
Danio rerio	EB724379	EB724375-8

GenBank accession numbers are stated where the RACE matched with database sequences of NMI and Myo1c

The N-terminal peptide is also conserved in all vertebrates tested but we have not found it in urochordate *Ciona intestinalis* or any other more distant species. This means that NMI N-terminal sequence appears to be a specific achievement of vertebrate evolution.

Altogether, NMI is evolutionary conserved in vertebrates, it has a lifespan between 16 and 48 h, it is ubiquitously expressed in tissues but with varying intensity, and cellular mRNA levels of NMI are connected with the level of cellular metabolism. These results suggest that NMI is universally needed in vertebrate cells and it could have some tissue specific functions especially in lungs. Further studies are needed to elucidate the molecular mechanisms underlying these characteristic features of NMI.

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IV. Discussion

1. NMI in Pol I transcription

Identification of a myosin I in the nucleus have led to hypotheses of its possible roles in nuclear processes and later studies indeed found NMI to be associated with RNA polymerases and suggested the involvement of NMI in transcription (Pestic-Dragovich *et al* 2000, Fomproix and Percipalle 2004). We have extended these studies and confirmed by functional assays the involvement of NMI in transcription. In vivo, microinjections of anti-NMI antibodies or siRNA knock-down of NMI decreased Pol I transcription, while overexpression of NMI increased the transcription of the ribosomal genes. In an in vitro transcription system, antibodies to NMI decreased Pol I transcription on both naked DNA and preassembled chromatin, while addition of purified NMI increased transcription. We have also shown that NMI does not bind directly to the Pol I complex. Instead, it associates with the polymerase through the transcription initiation factor TIF-IA (Philimonenko *et al* 2004). TIF-IA is a Pol I general transcription factor which is essential for initiation of transcription by Pol I and through which rDNA transcription is regulated. Only the Pol I complexes with associated TIF-IA are able of transcription initiation and those are only the minority of the cellular Pol I pool. TIF-IA serves as a link between the Pol I and TIF-IB/SL1-UBF complex which is sitting on the rDNA promoter and whose role is to position Pol I to the transcriptional start site (Grummt 2003). The association of TIF-IA with Pol I requires phosphorylation of TIF-IA at Ser 649 by RSK kinase, which is the target of the MAPK signaling pathway activated by mitogenic signals. The association with TIF-IA and the fact that actin is stably associated with Pol I complex might indicate a role for NMI in the growth-dependent regulation of rRNA synthesis.

It was reported before that actin associates with Pol II (Smith *et al* 1979, Egly *et al* 1984, Scheer *et al* 1984, Hofmann *et al* 2006b) and Pol III (Hu *et al* 2004), for a review, see Miralles and Visa (2006). We found that actin is attached to the polymerase complex very strongly and antibodies to actin inhibit transcription by Pol I (Philimonenko *et al* 2004) as was also found for Pol II (Hofmann *et al* 2004). NMI is also involved in the initiation of transcription by Pol II although no Pol II general transcription factors were identified as binding partners of NMI yet (Hofmann *et al* 2006b).

These and other data suggest an intriguing possibility that actin and myosin could act at as a motor during the transcription initiation of Pol I (and possibly other RNA polymerases

as well). Taken together, the role of NMI in transcription clearly emerges. Further studies are needed, however, to elucidate the precise mechanism of NMI action.

2. Dynamics of NMI during the activation of transcription

We show that the levels of NMI raise strongly upon activation of lymphocytes (Kysela *et al* 2005) as well as serum activation of NIH-3T3 cells (Kahle *et al* 2006) and these findings could mean that more NMI is needed to support the increased rate of transcription.

In activated lymphocytes, the amount of NMI increases in both condensed and decondensed chromatin during the first 24 hours as shown by immuno-electron microscopy. After 48 hours, when most of the chromatin decondenses, NMI is located predominantly in the decondensed chromatin. This could suggest a role of NMI in decondensing of the chromatin or perhaps in the first stages of transcription that take place on not yet fully decondensed chromatin. In the nucleolus, NMI is located in all compartments but predominantly in the DFC where transcription of rDNA takes place. When the lymphocytes are activated, the amounts of NMI raise in both in DFC and in GC, where the ribosomal RNA is loaded with proteins and where the ribosomal subunits mature (Kysela *et al* 2005). The finding of NMI in DFC is consistent with its role in transcription while the movement to GC could mean that NMI could play some role even in the later steps such as ribosomal subunits assembly and maturation. Indeed, there have been already first reports of the association of NMI with ribosomal subunits. NMI along with actin was shown to accompany the small ribosomal subunit from the site of its origin in GC to the nuclear pore complex by electron microscopy, and in vivo incorporation of antibodies to NMI or actin led to accumulation of the small ribosomal subunits in the nucleoplasm (Cisterna *et al* 2006). Similar results have been obtained independently by the Percipalle group (P. Percipalle, personal communication). In conclusion, on the structural level NMI seem to be involved in the processes of transcription and ribosome biogenesis. Now the precise biochemical mechanism of its involvement remains to be elucidated.

3. Nuclear translocation of NMI

Our results suggest that the presence of the N-terminal domain is not solely responsible for nuclear localization of NMI. When tagged myosins are overexpressed we do not see the difference between the distribution of the tagged NMI and Myo1c and the situation is similar with all tags we tested (FLAG, V5-6His, EGFP). This is in discord with previous finding that in HeLa cells FLAG-tagged NMI localizes to the nucleus while FLAG-tagged Myo1c

does not (Pestic-Dragovich *et al* 2000). We tested several cell lines repeatedly and even have our construct fully sequenced to ascertain ourselves about this finding.

The localization of the endogenous myosins is not so easy to reconcile. The antibody recognizing specifically NMI by its N-terminus stains almost exclusively nucleus. As a Myo1c specific antibody is not available we had to use the anti-tail antibody that detects both Myo1c and NMI. This antibody stains nucleus, cytoplasm, and cytoplasmic membrane structures and it is impossible to decide whether the nuclear signal is completely NMI or there is some Myo1c in the nucleus too. We succeeded in separating the two isoforms from the NIH-3T3 whole-cell lysate on the SDS-PAGE gel (Kahle *et al* 2006) and we found that NMI is approximately one-half to one-third as abundant as Myo1c. This would suggest that most of the nuclear signal is caused by NMI. The mechanism by which endogenous NMI is retained in the nucleus and endogenous Myo1c in the cytoplasm while tagged myosins are distributed between these compartments is unknown and will be subject of our further research.

The exact mechanism of NMI nuclear translocation is puzzling. The NMI molecule does not contain a classical nuclear localization sequence (NLS) but there is a cluster of basic residues in the neck area, which could theoretically serve this role. Another possibility would be that NMI gets to the nucleus by "piggybacking mechanism" bound to some other protein. This possibility is however less likely because there were significant efforts by us and other groups to identify Myo1c binding partners that were not fruitful (Barylko *et al* 2005, Gillespie 2004). Moreover, the area of the second IQ domain binds to the hair-cell receptors, probably cadherin 23 (Cyr *et al* 2002, Phillips *et al* 2006) and to PIP2 (Tang *et al* 2002, Phillips *et al* 2006). Calmodulin competes with PIP2 and Cdh23 for the binding site (Phillips *et al* 2006). The same domain seems to be responsible for nuclear translocation in our data. It would be interesting to find out whether the nuclear translocation is in some way influenced by the level of intracellular Ca^{2+} and hence calmodulin binding. The exact mechanism of the NMI nuclear translocation remains to be elucidated experimentally.

4. Tissue expression of NMI

We found NMI to be expressed in all mouse cell types tested except for latest stages of spermatogenesis. This is consistent with the involvement of NMI in transcription. By quantitative RT-PCR and western blot we determined the expression of NMI in tissues to be variable, and by far the highest expression was found in lungs (Kahle *et al* 2006). This in our opinion hints at possible tissue-specific function of NMI in lungs. Probably the best way to test this hypothesis would be to make the gene knockout mouse lacking the first 16 amino ac-

ids of NMI. Attempts to knockdown the whole Myo1c gene would probably cause embryonic lethality owning to the Myo1c ubiquity and important functions. A failed attempt to knockout Myo1c was already mentioned in the literature (Gillespie 2004).

5. Evolutionary conservation of NMI

The evolutionary conservation of NMI is interesting because it could reflect how fundamental is its role in transcription. We found the N-terminal extension to be identical in all mammals where the sequence could be obtained. The rest of the protein, Myo1c, is conserved in mammals with at least 95 % similarity. The N-terminal sequence homologs were present in all vertebrate sequences tested but we were not able to find it in chordate *Ciona intestinalis* or in any other lower eukaryote (Kahle *et al* 2006). These findings mean that NMI and probably also the function it serves are specific achievement of evolution of vertebrates and it is not universal for eukaryotic transcription.

V. Summary and conclusions

Nuclear myosin I is a monomeric actin-based molecular motor located in the nucleus which is involved in transcription. A very similar Myo1c, located mainly in the cytoplasm and plasma membrane, has important functions in many diverse physiological and biochemical processes. We aimed to extend our knowledge of the properties of NMI and its functions in nuclear metabolism. Our findings presented in this work can be summarized as follows:

1. **NMI is necessary for transcription by Pol I.** We have shown that NMI coimmunoprecipitates and co-purifies with both Pol I and was found by chromatin immunoprecipitation on the promoter of rDNA. RNAi knockdown of NMI or microinjections of anti-NMI antibodies decrease Pol I transcription in vivo. In an in vitro transcription system, anti-NMI antibodies inhibit transcription in a dose dependent manner, while addition of purified NMI increases transcription. NMI was shown to bind Pol I through the basal transcription factor TIF-IA which defines the initiation-competent subpopulation of Pol I and through which the rate of transcription initiation is regulated.
2. **Transcriptional activation leads to relocalization of NMI to the sites of active transcription.** We have shown, mainly by immunoelectron microscopy and statistical analysis that during the activation of lymphocytes the level of NMI increases and it is redistributed to the sites of active Pol I and Pol II transcription.
3. **The first two IQ domains are sufficient to direct the fusion constructs to the nucleus.** Using the set of truncated NMI mutants we identified the part of the molecule which was responsible for the NMI nuclear localization and shown it was the calmodulin-binding neck.
4. **NMI is expressed in all mouse tissues with highest expression in the lungs.** We have shown by immunofluorescence microscopy, western blot and qPCR that NMI is present in the nuclei of all mouse tissues except for the latest stadia of spermatogenesis and that the levels vary with the highest expression in lungs.
5. **The NMI N-terminal domain is identical in mammals and conserved in vertebrates.** We searched for the sequences similar to the NMI N-terminal domain in the expressed and genomic sequence databases and found it in all vertebrates where sufficient genomic data were available. Moreover, we confirmed the expression of this domain by RACE in selected species. We did not find a similar sequence in any more distant species than vertebrates.

VI. Prospects

In near future we plan to address the following questions:

1. **Adressing the nuclear import of NMI.** Does the NMI neck contain a not-yet-described NLS? If so, which importin does it bind to be transported to the nucleus? Or is NMI transported to the nucleus bound to some other carrier protein which in turn binds importin? These questions will be addressed by co-immunoprecipitations with tagged NMI neck to find its binding partners. We may also map the neck with point mutagenesis to determine which residues are needed for proper nuclear transport.

2. **Questioning the indispensability of NMI for actively transcribing cells.** The mouse cells lacking the exon coding for the NMI N-terminal domain will be produced and the phenotype will be assessed. Specifically, we will be interested in the amount and localization of Myo1c located in the nucleus and the level of basal and stimulated transcription. If there will be significant changes, we will try to rescue them by expressing various NMI mutants and truncated constructs.

3. **Determining NMI importance for tissue/organ functional integrity.** We will attempt to produce the NMI knockout mice to evaluate the phenotype on the tissue/organism level. We will use the Cre/*lox* system where a part of the gene that is intended to be removed is flanked by the *loxP* sites. This part is highly efficiently and accurately removed in cells where the Cre recombinase is expressed. The gene for the Cre recobinase can be introduced in the genome under tissue specific promoter enabling a tissue-specific knockouts. This system is especially useful in cases where the general knockout is embryonically lethal. Especially interesting would be the lung pathology as this is the site with by far the highest NMI expression.

4. **Specifying the molecular role of NMI in transcription.** In vitro transcription/remodeling assays can be employed. We plan to use the nuclear lysates from NMI knockout cells to avoid the tedious production of purified reconstituted transcription system devoid of NMI. In such a system, the influence of NMI addition in various steps of transcription initiation/elongation can be assessed.

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