Nuclear DNA helicase II (NDH II), also designated RNA helicase A, is a multifunctional protein involved in transcription, RNA processing, and transport. Here we report that NDH II binds to F-actin. NDH II was partially purified from HeLa nuclear extracts by ion-exchange chromatography on Bio-Rex 70 and DEAE-Sepharose. Upon gel-filtration chromatography on Sepharose 4B, partially purified NDH II resolved into two distinct peaks. The first NDH II peak, corresponding to the void volume of Sepharose 4B, displayed coelution with an abundant 42-kDa protein that was subsequently identified as actin. Several nuclear proteins such as RNA polymerase II, the U5 small nuclear ribonucleoprotein (RNP)-associated WD40 protein, and heterogeneous nuclear RNP proteins (hnRNPs) copurified with NDH II. However, only hnRNPs A1 and C were found together with NDH II and actin polymers during gel filtration. NDH II and hnRNP C from the HeLa nuclear extract coeluted with F-actin on Sepharose 4B in an RNase-resistant manner, whereas hnRNP A1 was nearly completely removed from F-actin-associated hnRNP complexes following RNA digestion. The association of NDH II and hnRNP C with F-actin was abolished by gelsolin, an F-actin-depolymerizing protein that fragments actin polymers into oligomers or monomers. Furthermore, NDH II co-immunoprecipitated with F-actin and hnRNP C, respectively.

In vitro translated NDH II coeluted with F-actin on Sepharose 4B, whereas no coelution with F-actin was observed for in vitro translated hnRNP A1 or C1. Binding to F-actin requires an intact C terminus of NDH II and most likely a native protein conformation. Electron microscopy indicated a close spatial proximity among NDH II, hnRNP C, and F-actin within the HeLa nucleus. These results suggest an important function of NDH II in mediating the attachment of hnRNP-mRPP RNP complexes to the actin nucleoskeleton for RNA processing, transport, or other actin-related processes.

Nuclear DNA helicase II (NDH II) is a nucleic-acid helicase that unwinds double-stranded DNA and RNA in a nucleotide-dependent manner (1–3). NDH II is highly conserved among man (4), cow (5), mouse (6), worm (7), and fruit fly (8). NDH II comprises two double-stranded RNA (dsRNA)-binding domains at the N terminus, a helicase catalytic domain in the central part, and a glycine-rich single-stranded nucleic acid-binding domain (RGG box) at the C terminus (9, 10). Sequence analysis revealed that NDH II contains seven helicase core motifs that are conserved among the DEX/D(H) helicase superfamily (4, 5). NDH II displays significant similarities to a group of yeast pre-mRNA splicing factors, including prp2, prp16, and prp22 (4). In general, nucleic-acid helicases may adopt an enzymatic mechanism that transforms energy from nucleotide hydrolysis into the mechanical work for protein translocation and/or disruption of nucleic acid duplexes (11). The nucleotide binding and hydrolysis by a helicase are governed by two Walker nucleotide-binding motifs (A and B), which have been originally defined from several ATPases, including F1-ATPase, adenylate kinase, myosin, and RecA (12). Recent crystallographic data support the presence of a RecA-type topology in a DEX(D/H) protein, which implies a common mechanism involving nucleic acid binding and protein conformational changes coupled with ATP hydrolysis (13). Although NDH II shares sequence homology with DEXH proteins within its core helicase motifs, the protein carries two additional nucleic acid-binding domains, i.e. the two N-terminal dsRNA-binding domains and a C-terminal RGG box. This domain arrangement is reminiscent of the architectural features of some nucleic acid-binding proteins such as heterogeneous nuclear ribonucleoproteins (hnRNPs), many of which contain multiple copies of the RNP motif at the N terminus and/or an RGG box at the C terminus (14).

A homolog of NDH II in Drosophila, designated maleless protein (MLE), participates in X chromosome dosage compensation, a process that leads to a 2-fold increase in transcription from the single X chromosome in males compared with the two individual X chromosomes in females (8). Transcriptional enhancement of the male X chromosome is driven by the cooperative functions of male specific lethal (MSL) proteins, MLE, and regulatory RNAs (ROX) transcribed from the same X chromosome (15). MLE possibly mediates protein-nucleic acid interactions for the assembly of X chromosomal regulatory complexes (16). In addition to this sex-related function, MLE is required in both sexes for suppressing the aberrant splicing of the para-Nu channel pre-mRNA (17), which indicates a function of this protein in pre-mRNA processing. In mammals, NDH II is essential for embryonic development (18). NDH II stimulates transcription by an interaction with transcriptional coactivator CBP/p300 (19), the breast cancer-specific tumor suppressor protein BRCA1 (20), or RNA polymerase II (19, 21). NDH II also binds to the small nuclear ribonucleoprotein (snRNP)-associated protein for its recruitment to the RNA.
polymerase II holoenzyme (22). Furthermore, evidence exists that NDH II is bound to the promoter-proximal sequence for the translational response element (TAR), involved in the human immunodeficiency virus gene expression (24). The posttranscriptional functions of NDH II have been initially identified from its specific binding to the constitutive transport element of retroviral RNA (25). NDH II influences retroviral RNA splicing or transport, leading to an overall stimulation of the transcription level of retroviral RNAs (26). It has been found that NDH II carries signal sequences at the C terminus that facilitate its shuttling through the nuclear pore complexes (27). An involvement of NDH II in RNA transport has been supported by its physical interaction with the nuclear transport proteins HAP95 (28) and Tap (29).

In search for the cellular functions of NDH II, we have previously investigated the localization of this protein in different mammalian cell lines (30, 31). These studies have confirmed the close spatial relationship of NDH II to transcriptionally active loci, although NDH II is preferentially recruited to different gene types in different species, such as rDNA in the nucleolus of murine cells (31). Here we performed experiments to differentiate physical associations of NDH II with nuclear proteins involved in transcription, RNA processing, or transport. Surprisingly, this led to the finding that NDH II is a protein that directly binds to filamentous actin in the nucleus. NDH II was also found within hnRNP complexes attached to actin filaments. These results uncover an as yet unidentified function of NDH II, i.e. mediating the attachment of nuclear ribonucleoprotein complexes to actin filaments, which may be related to RNA processing, transport, or other actin-dependent functions in the nucleus (32).

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Rabbit antiserum against bovine NDH II was previously produced in our laboratory (5). Rabbit antiserum against human RNA helicase A was kindly donated by J. Hurwitz (Memorial Sloan-Kettering Cancer Center, New York). Mouse monoclonal antibodies against hnRNPs A1 (4B10) and C (4F4) were provided from (Howard Hughes Medical Institute, University of Pennsylvania School of Medicine, Philadelphia). A rabbit polyclonal antibody against the US snRNP-associated WD40 protein was a gift of R. Lührmann (Max-Planck-Institut für Biophysikalische Chemie, Göttingen, Germany). A rabbit polyclonal antibody raised against the N terminus of the large subunit of RNA helicase A was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A mouse monoclonal antibody against actin (2G2) was obtained from H. Hinsen (Department of Biochemical Cell Biology, University of Bielefeld, Bielefeld, Germany). The mouse monoclonal antibody C4 against actin was from Chemicon International, Inc. (Temecula, CA). The mouse monoclonal antibody AC-15 and a rabbit polyclonal antibody against actin were obtained from Sigma.

**Plasmids**—A pBluescript plasmid containing full-length NDH II cDNA was derived from a previously described screen of a human λ-ZAP cDNA library (10). This plasmid contains the human full-length NDH II open reading frame together with an 80-nucleotide 5′-untranslated region and a 314-nucleotide 3′-untranslated region. Plasmids containing cDNA of hnRNPs A1 (pBS01) and hnRNPs C1 (pHC12) were as described (33).

**Recombinant Proteins**—Human recombinant full-length NDH II proteins containing amino acids 1–1269 and NDH II deletion proteins containing amino acids 1–952, 313–1269, and 313–952, respectively, were constructed for baculovirus expression in insect cells (10). All these baculovirus recombinant proteins carried a 6×His tag at the N terminus. A bacterially expressed glutathione S-transferase fusion protein containing amino acids 955–1269 of NDH II was described (34).

**HeLa Nuclear Extract**—HeLa cells were grown in Dulbecco’s modified Eagle’s medium (C.C.Pro GmbH, Neustadt/ Weinstraße, Germany) supplemented with 10% fetal bovine serum (Invitrogen, Karlsruhe, Germany). Cells were harvested by centrifugation at 320 × g for 10 min. After being washed with ice-cold phosphate-buffered saline (10 mM sodium phosphate (pH 7.4), 140 mM NaCl, and 3 mM KCl) and recen-

**F-actin Sedimentation Assay**—F–actin was centrifuged by sedimentation assay as described in 20,000 × g for 30 min at 25 °C. Following protein precipitation in 10% trichloroacetic acid, the supernatant and pellet were both adjusted to the same volume with SDS-PAGE sample buffer (10 μl). Equal amounts (10 μl) of samples from the supernatant and pellet were electrophoresed on a 10% SDS-polyacrylamide gel, followed by silver staining or Western blotting. F–actin sedimentation assays were used to examine the effect of gelsolin (Sigma), which converts F–actin into shorter fragments or to actin monomers. In this experiment, 50 ng of gelsolin was added to the F–actin-containing solution in a volume of 50 μl (1.2 μg of F–actin, 20 mM Tris-HCl (pH 7.6), 0.15 mM KCl, 0.2 mM CaCl2, 0.2 mM ATP, and 1 mM dithiothreitol). After incubation for 30 min at room temperature, the samples were processed for the sedimentation assay as described.

**Immunoprecipitation**—Immunoprecipitations were performed for HeLa nuclear proteins collected from the void fractions of Sepharose 4B. The HeLa cell pellet (1 g) was used to prepare HeLa nuclear extracts. The nuclear extract (2 ml) was immediately loaded onto Sepharose 4B for gel-filtration chromatography. Fractions 10–12 of the void volume were pooled (3 ml) and then divided into two samples of equal volume. One was mixed with mouse IgG (control), and the other was mixed with mouse NDH II antiserum.
other was mixed with anti-actin (AC-15) or anti-hnRNP C (4F4) monoclonal antibody, both bound to protein A-Sepharose (50 μl; Calbiochem, Bad Soden, Germany). Nonspecific binding was blocked by 5% milk powder in binding buffer containing 50 mM Tris-HCl (pH 8.0), 25 mM NaCl, 0.1 mM EDTA, 0.2% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml pepstatin. The mixtures were shaken on ice for 2 h, followed by centrifugation at 10,000 × g for 1 min at 4 °C. The pellets were washed with a 10× bed volume of binding buffer and recentrifuged. This was repeated three times. Proteins were eluted by dissolving the pellet in 50 μl of SDS-PAGE sample buffer and heating at 95 °C for 5 min. Immunoprecipitated proteins were examined by Western blot analysis.

In Vitro Translation—In vitro transcription/translation of NDH II, hnRNPA1, and hnRNPC1 was performed with the TNT T7 or SP6 coupled reticulocyte lysate system (Promega). The reactions were performed using T7 RNA polymerase for the pBluescript plasmid encoding full-length NDH II, as described above, and for the plasmid (pBS01) encoding hnRNPA1 (35) or using SP6 RNA polymerase for the plasmid (pHC12) encoding hnRNPC1 (35). All translation products were radio-labeled by the incorporation of [35S]methionine.

Immunoelectron Microscopy—Post-embedding immunogold labeling of HeLa cells for electron microscopy was performed as described (30–31). Double immunogold labeling of HeLa cell ultrathin sections was performed with different combinations of mouse and rabbit primary antibodies against actin, NDH II, or hnRNPC, followed by incubation with a 5-nm gold-conjugated secondary antibody against mouse IgG and a 10-nm gold-conjugated secondary antibody against rabbit IgG (Plano). The mouse monoclonal antibodies against actin (2G2) and hnRNPC (4F4) and the rabbit polyclonal antibody against actin were diluted 1:10, rabbit antiserum against NDH II was diluted 1:100. All gold-conjugated secondary antibodies were diluted 1:100. An anti-mouse IgM secondary antibody was used for anti-actin monoclonal antibody 2G2.

Other Methods—In gel digestion of proteins by trypsin, isolation of individual tryptic peptides by reverse chromatography on Sephasil C18 2.1/10, and automated N-terminal amino acid sequencing were performed as described (34).

RESULTS

Identification of F-actin-linked NDH II and hnRNPs—In view of the current suggestions that NDH II may be involved in transcription by its interaction with the RNA polymerase II holoenzyme and in RNA processing or transport because of its specific binding to regulatory sequences of retroviral RNAs, we examined the association of NDH II with other nuclear proteins of human cells. The already established method for the purification of NDH II (1) was adapted to follow NDH II-copurifying proteins during column chromatography on Bio-Rex 70 and DEAE-Sepharose, followed by gel filtration on Sepharose 4B to determine the apparent size of NDH II-containing complexes in comparison with other nuclear components (Fig. 1A). A silver-stained gel of the partially purified proteins revealed the copurification and enrichment of a prominent protein with a molecular mass of ≈42 kDa (Fig. 1B, arrow). Western blotting revealed that NDH II eluted from Bio-Rex 70 at 300 mM NaCl and from DEAE-Sepharose at 320 mM NaCl (Fig. 1C). Simultaneously, we followed the elution of RNA polymerase II, hnRNPs A1 and C, and the U5 snRNP, associated WD40 protein (35). All these proteins could be detected in fractions that contained partially purified NDH II after chromatography on Bio-Rex 70 and DEAE-Sepharose (Fig. 1D). Unexpectedly, when fractionated by gel filtration, NDH II displayed two elution peaks. The earlier peak eluted with the void volume, corresponding to a molecular mass of ≈10^7 Da. The second peak corresponded to a molecular mass of oligomeric or monomeric NDH II (Fig. 2A). The SDS-PAGE was subsequently performed to examine proteins that coeluted with NDH II on Sepharose-4B. Silver staining revealed an abundant 42-kDa protein that came off with fractions containing NDH II eluting with the void volume (Fig. 2B). A gradual enrichment of this 42-kDa protein during purification of NDH II could also be seen (see Fig. 1B). This 42-kDa protein was subjected to an in-gel trypsin digestion procedure, followed by the purification of tryptic products by reverse-phase high pressure liquid chromatography and N-terminal amino acid sequencing of the separated oligopeptides. From three tryptic peptides, we obtained the partial amino acid sequences AGFAGDDAPR, DSYVG, DEAQSK, and GYSPFTTAER. These sequences correspond to human β-actin (GenBankTM/EBI Data Bank accession number P02570) from amino acids 19 to 28, 51 to 61, and 197 to 206, respectively. Directed by this result, Western blot analysis with a mouse monoclonal antibody against β-actin was performed. This confirmed that the 42-kDa protein was indeed β-actin (Fig. 2C). The exclusion of 42-kDa β-actin from Sepharose 4B indicated a polymeric form, i.e. F-actin. Other nuclear proteins that accompanied NDH II on Bio-Rex 70 and DEAE-Sepharose were also examined for their elution positions on Sepharose 4B. Although ion-exchange column chromatography may lead to an enrichment of ribonucleases that causes the degradation of ribonucleoprotein complexes, a fraction of hnRNPs A1 and C remained excluded from Sepharose 4B together with NDH II and actin filaments, indicating a potential physical association between them (Fig. 2, E and F). In contrast, the elution of RNA polymerase II and the WD40 protein did not coincide with actin filaments under the same conditions (Fig. 2, D and G).

Examination of Proteins Associated with F-actin by Gel-filtration Chromatography of Nuclear Extracts—Because F-actin coeluted with NDH II and hnRNPs on Sepharose 4B, we hypothesized that similar complexes could be revealed from HeLa nuclear extracts. To examine this possibility, we directly loaded HeLa nuclear extract onto Sepharose 4B for gel-filtration chromatography. As shown by silver staining and Western blotting, actin from the nuclear extract eluted as two distinct peaks on Sepharose 4B (Fig. 3, A and B), with the first one excluded in the void volume, similar to that shown in Fig. 2, and probably related to F-actin. As estimated by Western blotting, the elution of the majority of actin (~90%) was at positions corresponding to a low molecular mass, suggesting a partially depolymerized or monomeric form (G-actin). Actin contained within the void volume coeluted with NDH II, hnRNPA1, and hnRNPC, whereas most of the RNA polymerase II holoenzyme eluted after the void volume (Fig. 3A). Differences could be seen between the broad elution pattern of hnRNPA1 and the mainly early elution of hnRNPC on Sepharose-4B. In this respect, elution of NDH II was more similar to that of hnRNPA1 compared with hnRNPC. To further resolve a possible physical association with polymerized actin, we treated nuclear extract with high amounts of RNase A (1 mg/ml) prior to gel filtration. After this procedure, limited amounts of NDH II and hnRNPC remained excluded from the columns (fractions 10–12) (Fig. 3B) and coeluted with F-actin. In contrast, only spurious amounts of hnRNPA1 eluted in the void volume after RNase treatment (Fig. 3B). RNase digestion also changed the elution pattern of the RNA polymerase II holoenzyme (Fig. 3B), which became distributed from fractions 14 to 24. Although these experiments did not show an apparent association of NDH II and RNA polymerase II, complex formation between these two partners should not be excluded because this has been reported elsewhere (19) and was also reproduced by us (data not shown). The lack of evidence for an association of RNA polymerase II with NDH II from gel-filtration studies might be due to the fact that most of the NDH II molecules are involved in hnRNP binding, whereas a lesser amount is part of the transcriptional complex. Once hnRNPs have been formed, RNA polymerase is no longer associated with these complexes and may not be detectable by this assay.

Gelsolin-severed Actin Filaments Associate with NDH II and hnRNPC—To confirm the association of NDH II and hnRNPC C-
with F-actin, we treated the nuclear proteins eluted in the void volume of Sepharose 4B with gelsolin, an F-actin-depolymerizing protein that converts actin filaments into actin oligomers or monomers. As shown, the coelution profiles of NDH II, hnRNP C, and actin on Sepharose 4B did not change significantly upon rechromatography on the same column (Fig. 4A, panel a). However, if the void fractions collected from the first chromatography was treated with gelsolin, a delayed elution of actin occurred, indicating a partial conversion of F-actin to G-actin (Fig. 4A, panel b). Importantly, this was found to be accompanied by a concomitantly altered elution of NDH II and hnRNP C (Fig. 4A, panel b), supporting the view that the observed coelution of these proteins in the void volume was due to an association with F-actin. Similarly, we added gelsolin to F-actin that was obtained from partially purified fractions of NDH II. Using an F-actin sedimentation assay, we found that F-actin without gelsolin treatment was sedimented to the bottom of the centrifugation tubes by ultracentrifugation (Fig. 4B, panel a). However, fragmentation of F-actin by gelsolin released actin to the supernatant (Fig. 4B, panel a). Most importantly, NDH II (Fig. 4B, panel b) and hnRNP C (panel c) were found to be co-sedimented with F-actin by ultracentrifugation, whereas gelsolin treatment shifted them, together with actin, to the supernatant (panels b and c). Taken together, these results support the physical association among F-actin, NDH II, and hnRNP C.

Co-immunoprecipitation of NDH II with F-actin and hnRNP Complexes—Here, immunoprecipitations were performed to obtain further evidence for a physical association among F-actin, NDH II, and hnRNP C. In these experiments, we focused on the nuclear proteins that initially coeluted in the void volume of Sepharose 4B. HeLa nuclear extracts were chromatographed on Sepharose 4B, and the fractions of the void volume were collected for immunoprecipitation with a mouse mono-
clonal antibody against actin or hnRNP C. Co-immunoprecipitation of actin with NDH II and hnRNP C from the excluded fractions could be seen, which was not observed if a mouse IgG control antibody was used for the immunoprecipitation (Fig. 5A). Co-immunoprecipitation between NDH II and hnRNP C was also observed when a mouse monoclonal antibody (4F4) against hnRNP C was used for immunoprecipitation (Fig. 5B).

Importantly, significant amounts of NDH II co-immunoprecipitated with hnRNP C also after treatment of the immunoprecipitates with RNase. Using [35S]methionine-labeled, in vitro translated hnRNP C1 as a protein probe for the far-Western blot assay, we found that the C terminus of NDH II, where the RGG box is located, gave rise to weak binding to hnRNP C (data not shown). This seems to suggest a weak but direct interaction between NDH II and hnRNP C1, which might, however, be enhanced by their concomitant binding to RNA.

**NDH II Directly Interacts with F-actin In Vitro**—We performed additional experiments to differentiate between direct protein-protein interactions between F-actin and NDH II or hnRNPs, respectively. For this purpose, the binding capacity of baculovirus-expressed full-length NDH II for F-actin was examined on Sepharose 4B. This revealed the coelution of NDH II with F-actin in the void volume of this column (Fig. 6A). The coelution of NDH II and actin was abolished when gelsolin was added prior to chromatography on Sepharose 4B (Fig. 6B). These experiments were repeated using in vitro translated full-length NDH II, which revealed a similar F-actin-dependent elution of NDH II on Sepharose 4B, i.e. NDH II coeluted with F-actin in the void volume of the column, whereas gelsolin delayed the elution due to the dissolution of F-actin (Fig. 7, A and B). However, neither in vitro translated full-length hnRNP A1 (Fig. 7C) nor hnRNP C1 (Fig. 7D), after incubation with actin, eluted at the positions corresponding to F-actin on Sepharose 4B in the absence of gelsolin (Fig. 7E). These experiments excluded the possibility that hnRNP A1 or C1 directly binds to F-actin.

We subsequently attempted to delineate the region of NDH II responsible for F-actin binding. The experiments were performed with baculovirus-expressed NDH II deletion variants (Fig. 8A) by studying co-chromatography of actin and NDH II on Sepharose 4B. NDH II(1–952) with a C-terminal deletion exhibited weaker binding to F-actin than NDH II(313–1269) with an N-terminal deletion (Fig. 8B). Elution in the void volume was barely detectable for NDH II(313–1269), which contains only the central DEHX domain of NDH II (Fig. 8B). In the absence of actin, NDH II(313–1269) ran exclusively at
positions apart from the void volume of the column (Fig. 8C). This in turn supports the view that binding to F-actin is the only reason for the earlier eluted NDH II-(313–1269) as shown in Fig. 8B. Although the C-terminal region of NDH II sustained efficient binding to F-actin, a glutathione S-transferase fusion protein containing only amino acids 953–1269 of NDH II displayed no apparent affinity for F-actin (Fig. 8D). This suggests that the extreme C terminus of NDH II alone is not sufficient for stable binding of this protein to F-actin. Most likely, NDH II requires the full-length sequence to achieve a proper conformation for efficient binding to F-actin.

In Vitro Interaction between NDH II and hnRNP C1—The observed co-immunoprecipitation of hnRNP C with NDH II indicated a possible physical interaction between them (see Fig. 5). Here, this issue was examined with respect to the physical association of actin with NDH II and hnRNP C, respectively. In vitro translated hnRNP C1 was mixed with a mouse monoclonal antibody against actin or with the same antibody after preincubation with actin or with both actin and purified 6-His-tagged NDH II baculovirus protein. Samples from the immunoprecipitations were examined for the presence of 35S-labeled hnRNP C1 in vitro translation products by SDS-PAGE and autoradiography. As shown, hnRNP C1 could be precipitated only by the anti-actin monoclonal antibody that had been preincubated with both actin and NDH II, whereas no detectable hnRNP C was precipitated by the anti-actin antibody precoated with actin but not together with NDH II (Fig. 9). These experiments suggest an important role for NDH II in mediating the physical linkage of hnRNP C (or hnRNP C1-associated hnRNP complexes) to actin filaments.

Co-localization of NDH II with F-actin and hnRNP Complexes in Vivo—To analyze a possible physiological significance of the observed interaction of NDH II with F-actin and hnRNP complexes, we further examined their in vivo localization by double immunogold labeling and electron microscopy. For the immunolabeling of nuclear actin, we used a rabbit polyclonal antibody against actin and mouse monoclonal antibody 2G2, which has been shown to specifically recognize actin in the nucleus (36). Both antibodies were used for immunofluorescence studies, which revealed satisfactory actin signals from the HeLa cell nucleus, especially from the nuclear periphery (data not shown). Double immunogold labeling was thus performed by combining one of these two anti-actin antibodies with polyclonal antibodies against NDH II or with a mouse monoclonal antibody against hnRNP C (4F4). As observed, nuclear actin filaments were frequently co-localized with NDH II inside the nucleus (Fig. 10A) or at the nuclear pore (Fig. 10B). Similarly, we also identified hnRNP complexes associated with hnRNP C in the vicinity of nuclear actin filaments (Fig. 10C), yet much smaller amounts of hnRNP C were found to be co-localized with actin filaments near the nuclear envelope (Fig. 10D). Furthermore, a partial co-localization of NDH II and hnRNP C in the nucleus could also be seen (Fig. 10E).
DISCUSSION

The results from this work support the conclusion that NDH II binds to filamentous actin. To our knowledge, NDH II is the first nucleic-acid helicase shown to directly bind to F-actin. At present, the physiological consequences of F-actin binding of NDH II remain unclear. NDH II was previously suggested to be a pre-mRNA/mRNA-binding protein on the basis of its nuclear localization, which shared similarities with the diffuse distribution of hnRNP A1, but was apparently different from the speckled pattern observed for the RNA-splicing factor Sc35 (30). In agreement with this conclusion, NDH II was shown here to be associated with hnRNP complexes because hnRNPs A1 and C were partially copurified. Importantly, NDH II and hnRNP C seemed to be more closely associated with actin filaments compared with hnRNP A1. After RNase digestion, a “protected” association with F-actin, as indicated by the coelu-
tion in the void volume of Sepharose 4B, was seen for NDH II and hnRNP C, but not for hnRNP A1. Despite the close proximity of hnRNP C to actin filaments, direct interactions between these two proteins could not be established using in vitro translated hnRNP C and purified actin in gel-filtration studies or immunoprecipitation, which, however, revealed binding of NDH II to F-actin.

The physical vicinity of NDH II and hnRNP C at the F-actin attachment sites of hnRNP complexes may provide an important clue to the physiological relevance of F-actin binding of NDH II. Metazoan hnRNPs represent a group of RNA-binding proteins that package heterogeneous nuclear RNAs after their synthesis and subsequently function in RNA splicing and transport (14). Unlike hnRNP A1, which carries the M9 transport signal at the C terminus for nuclear RNA export (37), hnRNP C is retained in the nucleus due to a nuclear retention signal (38). It has been shown that hnRNP C preferentially binds to poly(pyrimidine-rich) sequences, such as those from the introns of pre-mRNAs or from some small nuclear RNA species (39). Assuming that NDH II is a heterogeneous nuclear RNA-binding protein, it may recognize specific RNA sequences near the pre-mRNA-binding site of hnRNP C and carry the hnRNP complexes to the actin nucleoskeleton. Efficient RNA splicing may potentially depend upon the attachment of hnRNP complexes to actin filaments. This may create a proteinaceous scaffold for protecting RNA-splicing intermediates that are transiently sensitive to RNase attacks and thereby confine pre-mRNA to a subnuclear compartment enriched with RNA-splicing factors or provide local bridges to facilitate the assembly of the spliceosome. Indeed, there is some evidence for the involvement of F-actin in RNA splicing, such as the anchoring of actively transcribed RNA to nuclear actin filaments (40), the enrichment of snRNPs within the nuclear matrix (where actin filaments are abundantly found) (41), the tight association of a structural protein of the actin cytoskeleton with the RNA-splicing machinery (42), and the subnuclear localization of actin adjacent to the spliceosomes (43).

Co-localization of F-actin with hnRNP complexes was shown in the nucleus of HeLa cells by electron microscopy using immunogold-labeled actin, hnRNP C, and NDH II. Similarly, actin filaments in the nucleus were previously observed by immunoelectron microscopy in frog oocytes (44) and dorsal root ganglia sensory neurons (45). In accordance with the co-localization of F-actin and hnRNP complexes, there has been earlier evidence indicating the attachment of RNP-like high density particles to actin fibers isolated from the nucleus of some Am-
phibian species (46). Recently, nuclear filamentous structures have been recognized as important for roles in the transcriptional and post-transcriptional fate of Balbiani ring RNAs from Chironomus tentans (47). A report in this line of studies suggested the binding of actin to the hnRNP Hrp36, which was incorporated into Balbiani ring pre-messenger ribonucleoproteins (mRNPs) during transcription and associated with mature Balbiani ring RNAs for transport to the cytosol (48).

Although actin monomers with a molecular mass of ~42 kDa may be small molecules to pass through the nuclear pores by free diffusion, two nuclear export signals have been previously identified in actin’s amino acid sequence (49). Although actin by itself has no affinity for RNA, it cannot be excluded that monomeric actin, or even actin filaments, may participate in the packaging of hnRNP complexes by binding to a specific type of hnRNP. Recently, some studies have addressed the involvement of actin in the nuclear export of human immunodeficiency virus RNA (50, 51). Similar to these results, we observed here that nuclear actin filaments were localized in the vicinity of the nuclear pore complexes. This may provide some hints for a transport function of nuclear actin. There is growing evidence for a function of NDH II as an RNA-shuttling protein (25–29).

NDH II Interacts with F-actin

Fig. 8. Deletion of NDH II affects binding to F-actin. A, shown is a schematic presentation of partial deletions of NDH II. B, NDH II-1–952, NDH II-(313–1269), and NDH II-(313–952) were expressed from recombinant baculoviruses (11) and subsequently examined for association with F-actin as described in the legend to Fig. 6. C, for comparison, a chromatogram of NDH II-(313–1269) on Sepharose 4B in the absence of actin is shown. D, NDH II-(953–1269), a glutathione S-transferase fusion protein containing the extreme C terminus of NDH II that was expressed and purified from Escherichia coli (11), was chromatographed with actin on Sepharose 4B. Eluted proteins were visualized by silver staining after SDS-PAGE. L represents 5% of the amount of the sample prior to loading onto Sepharose 4B. Note that all numbers in parentheses after NDH II indicate the positions of amino acids of the full-length protein. DS-RBD, double-stranded RNA-binding domain.

Fig. 9. In vitro translated hnRNP C co-immunoprecipitates with actin in the presence of NDH II. Equal amounts of in vitro translated hnRNP C were mixed with agarose beads that were coupled to anti-actin monoclonal antibody (mAb) AC-15 (lane 2) or to the same antibody in the presence of actin (5 μg) (lane 3) or in the presence of both actin (5 μg) and a 6-His-tagged full-length NDH II baculovirus protein (0.3 μg) (lane 4). After immunoprecipitation, 35S-labeled hnRNP C was detected by SDS-PAGE and autoradiography. Lane 1 represents the input of the in vitro translated hnRNP C product.
We found that NDH II localized not only adjacent to actin filaments of the inner nuclear regions, but also close to the nuclear periphery or directly at the nuclear pores. This makes it reasonable to speculate that the transport of nuclear RNA is mediated by the binding of NDH II to actin filaments. Probably, NDH II remains associated with mature mRNPs after the processing of pre-mRNAs has been finished and maintains the attachment of mRNPs to actin filaments. This may be important, for example, for the movement of RNP complexes toward the nuclear envelope or the docking of mRNPs to the nuclear pore complexes during transit into the cytosol.

Anchorage of RNA to cytoskeletons such as actin microfilaments and microtubules has been known to be an important strategy for achieving the asymmetric distribution and translation of mRNA during development or cell differentiation (52). For these purposes, a special group of RNA-binding proteins promote specific binding to the specific stem-loop structures (zipcode RNA sequence) at the 3′-untranslated region of the mRNAs and mediate their delivery to the actin cytoskeleton or microtubules (53). In budding yeast, the mating-type switch after cell division is regulated by the delivery of Ash1 mRNA. The Ash1 protein encodes a repressor of transcription of the HO endonuclease gene. The transport of Ash1 mRNA from the mother cells to the distal end of the daughter cells is based on the myosin-driven movement of the Ash1 mRNP along the actin microfilaments (54). Alternatively, RNA may move along the microtubules, as in the transport of the bicoid RNP particles to the anterior pole of the oocyte in Drosophila. This process is mediated by the Staufen protein, which contains five copies of dsRNA-binding domains to anchor bicoid at its 3′-untranslated region to the microtubules (55). A microtubule-mediated RNA localization has been also found for hnRNPs. hnRNP A2 contains two RNP motifs and selectively binds to the RNA trafficking sequence of myelin mRNA in oligodendrocytes, which supports its movement along the microtubules to the distal myelin compartment (56). Due to its direct binding to actin filaments, NDH II seems to share functional similarities with those cytosolic mRNA-localizing proteins that serve as a bridge between mRNAs and the cytoskeleton, although the association of NDH II with hnRNP complexes suggests functions in the nucleus rather than the cytoplasm. However, as discussed above, NDH II may also fulfill functions in RNA transport and subsequently accompany the mature RNAs into the cytoplasm. Most importantly, NDH II contains two N-terminal dsRNA-binding domains that specifically bind to dsRNA as Drosophila Staufen protein (9). Hence, there might be the possibility that NDH II decodes and binds to the zipcode sequence of mRNAs to mediate an anchorage of RNA to actin filaments in the nucleus and perhaps also the cytosol of human cells.

Because nucleic-acid helicases commonly share two Walker nucleotide-binding motifs with the actin-based motor protein myosin, they are believed to utilize a similar mechanism for coupling of nucleotide hydrolysis with protein translocation...
and unwinding of the nucleic acid duplex (57). Alternatively, the energy transduction mechanism of a nucleic-acid helicase may also lead to the displacement of protein-protein and/or protein-nucleic acid interaction (58). The importance of the latter types of helicase functions has been recognized for the structural rearrangement of ribonucleoprotein complexes, e.g. the spliceosome (59), or RNA translocation through the nuclear pores, where multiple protein-protein interactions may occur between an mRNA-associated RNA helicase and the nuclear pore complexes (60). We believe that the identified F-actin binding of NDH II may imply a novel type of helicase mechanism whereby a protein conformational change induced by nucleic-acid-dependent nucleotide hydrolysis becomes coupled with an altered interaction with F-actin. Whether this may result in disruption of protein-protein interaction or even movement of NDH II along the actin filament track is currently being investigated in our laboratory.

Acknowledgments—We thank A. Willittor for assistance in amino acid microsequencing and R. Smith for critically reading the manuscript.

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J. Biol. Chem. 2002, 277:843-853.
doi: 10.1074/jbc.M109393200 originally published online October 30, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M109393200

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