The human protein MDDX28 is a putative RNA helicase and a nucleocytoplasmic shuttling protein also localized to the mitochondria. Its localization is novel among RNA helicases. We have studied its intracellular targeting signals and show that the first 20 amino acids of MDDX28 are necessary and sufficient for both mitochondrial import and nuclear export of the protein. Mutation of the five leucines in the sequence to alanines abolished the mitochondrial targeting signal as well as greatly reducing the nuclear export signal, indicating that these signal sequences are highly overlapping. Two short stretches of basic amino acids separated by 44 residues were both necessary and sufficient for full nuclear localization. However, they were not absolutely essential, because the protein was present in 7% of the nuclei when both signals were mutated. This indicates that MDDX28 contains another unidentified weak nuclear localization signal(s). Three basic domains in the N-terminal half of the protein and its RNA binding ability were essential for nucleolar localization as well as transcription-inhibition-dependent localization to nuclear subcompartments. Two of these basic domains were the same as those constituting the nuclear localization signal, suggesting that they are responsible for bringing the protein into the nucleus to the sites of RNA binding. Our results indicate that MDDX28 nucleocytoplasmic shuttling is dependent on the availability of nascent RNA.

MDDX28 (mitochondrial DEAD-box polypeptide 28) is a human putative RNA helicase that contains all the conserved DEAD-box motifs and has around 30% homology with other helicases (1). A mouse protein with 75% homology to MDDX28 has recently been cloned (2), and an incomplete sequence of the rat homologue is available (GenBankTM accession number AC094385.4). RNA helicases unwind double-stranded RNA using nucleoside triphosphates, mainly ATP, and in coupling the hydrolysis to the unwinding process (for a review, see Ref. 5). MDDX28 was recently shown to contain an RNA-dependent ATP-hydrolyzing activity (1). Endogenous MDDX28 is localized in the mitochondria and the nucleus, where it is also found in leukaemia bodies; PIC, protease inhibitor cocktail; PBS, phosphate buffered saline; PSP-1, paraspeckle protein-1; TRITC, tetramethylrhodamine isothiocyanate; Pipes, 1,4-piperazinediethanesulfonic acid.

**EXPERIMENTAL PROCEDURES**

Expression Constructs—The EGFP-N1 vector (Clontech) was used to make the wild type MDDX28-EGFP-N1 construct (1). In total, 36 deletion and mutation constructs were made (see Fig. 1 and Table I). These will be referred to by the missing amino acids (e.g. 32–139 is missing amino acids 2–139 or by the mutated amino acids (e.g. 330GAT → AAA, having mutated the amino acid sequence GAT at position 330–332 to AAA). Double deletions have been given serial names with two Δs in the

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The abbreviations used are: EGFP, enhanced green fluorescence protein; BS, basic stretch; FC, fibrillar centre; MTS, mitochondrial targeting signal; NES, nuclear export signal; NLS, nuclear localization signal; NoLS, nuclear localization signal; PML bodies, promyelocytic leukaemia bodies; PIC, protease inhibitor cocktail; PBS, phosphate buffered saline; PSP-1, paraspeckle protein-1; TRITC, tetramethylrhodamine isothiocyanate; Pipes, 1,4-piperazinediethanesulfonic acid.
Cell Culture and Immunofluorescence—COS-1 cells were grown on coverslips in Dulbecco’s modified Eagle’s medium (BioWhittaker) supplemented with 10% newborn calf serum (BioWhittaker) and 2 mM l-glutamine (Invitrogen). At appropriate times, the coverslips were washed briefly in PBS and fixed in 4% paraformaldehyde (Feraq) for 15 min (except for anti-FSP-1, 7 min). The cells were permeabilized in 0.5% Triton X-100 in PBS and blocked for 30 min in 3% bovine serum albumin (Sigma) in PBS. The samples were incubated with the primary antibody in 1% blocking solution at 4°C overnight, washed 3 times in PBS with 0.1% Triton X-100, and incubated for 1 h at room temperature with fluorescence-conjugated secondary antibody. When appropriate, the cells were counterstained with Hoechst 33258 (Sigma) before mounting. The samples were viewed with a Nikon Eclipse E400 micro- scope. The 100× primary immersion objective was used with a SPOT digital camera and combined with SPOT2 software (Diagnostic Instruments). Alternatively, confocal laser-scanning microscopy images were recorded on a TCS SP scanner mounted on a DM IRBE microscope (Leica Microsystems Heidelberg GmbH). The objective was a Plan Apo 63X/1.32 oil immersion lens. Laser (dual) excitation was done at 488 (or 476) and 546 nm; the main beam splitter was a dual band pass filter (Hamamatsu Photonics KK). Data acquisition and data analysis were controlled by means of the LCS software version 2.70 (Leica Microsystems Heidelberg GmbH). A series of 20 sections at an axial distance of 4.9 μm and with a strong zoom factor (7.5) was used as raw data for three-dimensional image reconstruction. A simulated fluorescence process shadow projection series (61 images at 3° rotational progression) was created from median-filtered raw data (kernel size 5 × 5 × 3) with the LCS software. The series was saved as an avi-file and compressed from 48 to 5.9 kilobytes with the AVIsoft software.

Transfections into COS-1 cells were performed using the FuGENE 6 transfection reagent in 4:1 FuGENE6/plasmid ratio according to the Roche Applied Science protocol. Heterokaryon assays were performed as described earlier (1), except that treatment with cycloheximide was shortened in the assay of the TCP/NES constructs because the TCF11 protein has a half-life of less than 3 h.  

For RNase and DNase treatment of transfected cells, a method of Chiodi et al. (12) was followed. Briefly, 32–20-transfected cells that had been treated with actinomycin D for 2 h were washed with cold PBS and incubated for 10 min in Buffer A (100 mM NaCl, 300 mM sucrose, 10 mM Pipes, pH 6.8, 3 mM MgCl2, and 0.1% Triton X-100) containing protease inhibitors (PIC, 10 μg/ml apronin, 10 μg/ml leupeptin, 10 μg/ml antipain, 10 μg/ml chymostatin, 10 μg/ml phosphoramidon, 1 mM phenylmethylsulfonyl fluoride). Cells were incubated for 10 min in the same buffer made to 250 mM in ammonium sulfate and washed twice in Buffer A before treatment for 20 min at 37°C with 36 μg/ml of RNase A (Sigma) in Buffer A. Cells were then washed in PBS and fixed in ice-cold methanol.

Antibodies used were anti-SC35 (a generous gift of Dr. Kallan, University of Bergen), anti-p80 collin (a generous gift of Dr. Lamond, University of Dundee), anti-paraspeckle protein-1 (PSF-1, a generous gift of Dr. Fox, University of Dundee), and Hsp60 (Stressgen Biotechnologies). For anti-promyelocytic leukemia bodies (PML), the monoclonal antibody 5E10 (13) was used. TCF/NES was detected with anti-nr1 antibody (Santa Cruz Biotechnology). TRITC-, fluorescein isothiocyanate-, and Texas Red-conjugated secondary antibodies were used (DAKO, The Jackson Laboratories). All immunofluorescence studies were repeated at least twice, and all readings and quantifications were done blindly. Nucleolar, mitochondrial, and cytoplasmic localization were scored as present or not present, whereas nuclear localization was scored according to intensity of the localization as compared with the wild type.

Transcription Inhibition—Transiently transfected cells were grown for 20–24 h on coverslips before treatment with 10 μM actinomycin D (Sigma) for 2 h (unless otherwise stated). Cells were fixed as described above and viewed immediately in the fluorescence microscope.

Immunoprecipitation—Transiently transfected COS-1 cells were cultured in 10-cm tissue culture dishes. The constructs were tested for equal transfection efficiency with direct immunofluorescence. One day post-transfection, 40 μl of [3H]uridine (50 μCi/ml) was added to the cultures, and incubation continued overnight. The cells were washed in PBS and
incubated on ice in radioimmune precipitation assay buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing PIC. The cells were scraped off, homogenized, and centrifuged briefly at full speed in a bench-top centrifuge to remove debris. The extracts were precleared with 20 μl of M280 Dynabeads coated with sheep-anti-rabbit IgG (Dynal) for 1 h at 4 °C. Dynabeads were conjugated with 1.5 μg of GFP antibody (Santa Cruz Biotechnology) and incubated for 2 h at 4 °C. Conjugated beads were then added to precleared extracts and incubated at room temperature for 2 h. The beads were washed 3 times in PBS, suspended in 50 μl of PBS and mixed with Pico Aqua liquid scintillation mixture (Packard). The samples were counted in a Packard 1600TR liquid scintillation analyzer. The counting was adjusted for total protein of each sample as measured with the Bio-Rad assay. Background (EGFP-N1 vector only) was subtracted. The experiment was repeated three times.

Statistics—The distribution of the number of punctate structures containing SC35 and MDDX28 was compared in ∼100 cells with a two-tailed independent sample t test using the SPSS software package.

RESULTS

Mitochondrial Targeting Signal (MTS)—Both endogenous MDDX28 and protein fused to EGFP are localized to the mitochondria of all cells, implying that it may be constantly needed in the organelle, e.g., in connection with the continuous mitochondrial transcription (1). We had previously predicted that a MTS was located at amino acids 3–18 of MDDX28 (1) because this sequence has the MTS characteristics consisting of 16 amino acids, potentially forming an amphiphilic α-helix with a net positive charge. To determine whether this was the MTS sequence, MDDX28 lacking the first 20 amino acids (Δ2–20) was cloned into the EGFP-N1 vector. When this construct was transfected into COS-1 cells the protein was no longer expressed in the mitochondria (see Fig. 2A), confirming that the first 20 amino acids of MDDX28 are necessary for the mitochondrial localization. To find out whether this sequence alone is sufficient for mitochondrial localization, a construct coding only for the first 44 amino acids of MDDX28 was made (Δ44–540), tagged with EGFP-N1, and transfected into COS-1 cells. The corresponding fusion protein had only mitochondrial localization (Fig. 2A), showing the sufficiency of the sequence for mitochondrial localization. Only after prolonged incubation of the transfected cells (48 h) did the Δ44–540 protein start to appear in the cytoplasm, possibly due to saturation of the mitochondrial import (data not shown).

Right orientation insertion of the 20 N-terminal amino acids of MDDX28 into the transcription factor TCF11 (TCF/NES-R, Fig. 1) was sufficient for mitochondrial localization of the otherwise nuclear-localized protein (Fig. 2A). The mitochondrial localization of this fusion protein is less visible in Fig. 2B, b, possibly due to the treatment with protein synthesis inhibitor. Wrong orientation of the insert (TCF/NES-W) did not result in mitochondrial localization (Fig. 2A). This shows that not only the sequence but also its correct orientation is necessary for mitochondrial transport.

NES—We have previously shown that when MDDX28-transfected cells are treated with actinomycin D the protein is effectively exported from the nucleus (1). The protein does not reappear in the nucleus during transcription inhibition, suggesting that its re-entry into the nucleus is blocked, e.g., by masking of the nuclear localization signal (NLSs) by binding proteins or by phosphorylation. To search for NESs in MDDX28, the nuclear localization of proteins encoded by several single deletion constructs (Fig. 1) was assessed after 8–24 h of actinomycin D treatment. Whereas Δ149–274, Δ276–540, and Δ520–540 as well as the wild type protein were successfully exported from the nucleus, the Δ2–20 and Δ2–139 mutants remained in the nucleus of all cells even after 24 h of treatment (Fig 2C, graph a). We also noticed that, unlike the wild type protein, which localized to the nucleus in about half of the transfected cells, the Δ2–20 and Δ2–139 proteins as well as the Δ520–540 protein localized to the nucleus in all transfected cells.

To test whether amino acids 1–20 were sufficient to enable nuclear export, they were inserted in both directions into the N-terminal end of the short version of the nuclear-localized transcription factor TCF11 (TCF/NES-R and -W, Fig. 1). The nuclear export ability of the two constructs was examined in an interspecies heterokaryon assay. Untransfected NIH/3T3 mouse cells were fused to COS-1 cells that had been transfected with the TCF/NES constructs. The cells were treated with 50 μg/ml cycloheximide for 1 h before and for 1 h after the fusion before fixation with paraformaldehyde. Therefore, all fusion protein present in the nucleus of NIH/3T3 cells would have originated in the transfected COS-1 cells. To distinguish the nuclei derived from mouse and monkey cells, the cells were counterstained with Hoechst 33258 after fixation. The TCF/NES-R protein was seen in the nucleus of NIH/3T3 cells (Fig. 2B, a–c) in numerous heterokaryons on every coverslip examined, indicating that the inserted sequence was active as an export signal. Six coverslips were scanned in the heterokaryon assay for TCF/NES-W, and only one NIH/3T3 nucleus containing TCF11 was found (data not shown). Thus, TCF/NES-W was largely incapable of nucleocytoplasmic shuttling, indicating...
that NES in the wrong orientation was not active as an export signal.

A leucine-rich sequence is located at amino acids 9–18. To test whether the leucine residues were necessary for the nuclear export, all five were mutated to alanine (9FLSLVTRL → AFSAVTRAAA, NES-5). After 24 h of actinomycin D treatment of cells transfected with NES-5, the protein was seen in the nucleus of 70% of cells transfected with the NES-5 construct.
Nucleolar localization (No) is indicated with +H11001+/H9004+). Localization in the nucleus (N) is described as heterokaryon assay (Fig. 2). Export of the NES-5 mutant was further established using a back mechanism. Such a mechanism has been suggested, e.g. for protein X of hepatitis B virus that is imported into the nucleus by binding to the NLS-carrying I

The amino acid sequence in the basic domains is shown except for the fourth domain (BS-4), which is shown either with the wild type sequence or as a deletion (Δ). Localization in the nucleus (N) is described as ++ as in wild type, + greatly reduced intensity as compared to wild type. Nucleolar localization (No) is indicated with + (localization) or − (no localization).

### TABLE I

| Construct | BS-1 (49–54) | BS-2 (100–104) | BS-3 (244–247) | BS-4 (520–523) | N | No |
|-----------|--------------|----------------|----------------|----------------|---------|---|
| Wild type | RQSR | RR | RR | RR | ++ | + |
| Δ 520–540 | RQSR | RR | RR | RR | Δ | + |
| NLS-1     | RQSSA | AA | AA | RR | Δ | + |
| NLS-2     | RQSSA | AA | AA | RR | Δ | + |
| NLS-3     | RQSSA | AA | AA | RR | Δ | + |
| NLS-1/Δ   | RQSSA | AA | AA | RR | Δ | + |
| NLS-2/Δ   | RQSSA | AA | AA | RR | Δ | + |
| NLS-3/Δ   | RQSSA | AA | AA | RR | Δ | + |
| NLS-1/3   | RQSSA | AA | AA | RR | Δ | + |
| NLS-2/3   | RQSSA | AA | AA | RR | Δ | + |
| NLS-1/2/3 | RQSSA | AA | AA | RR | Δ | + |
| NLS-1/2/3/Δ | RQSSA | AA | AA | RR | Δ | + |
were differently affected by inhibition of transcription. After 8–24 h of treatment, NLS-1- and NLS-2-transfected cells showed a greatly reduced presence of MDDX28 in the nucleus. This reduction was not significantly different from that observed for the wild type protein (data not shown). This indicated that actinomycin D could inactivate the two major NLSs of MDDX28.

**Nucleolar Localization Signal (NoLS)—**The wild type MDDX28 localized to the nucleolus in up to 20% of transfected cells, depending on time after transfection (1). To look for the NoLS, the nucleolar localization of three deletion constructs (∆2–139, A149–274, and ∆276–540) spanning the whole protein was studied. All the constructs resulted in proteins that lacked nucleolar localization (Fig. 2C). Because the deletions of the constructs do not overlap, we suggest that the NoLS is composed of two or more sequences, which are all essential. Alternatively, the nucleolar localization depends on the enzymatic activity of the protein. Large parts of the conserved enzymatic domain had been deleted from the two constructs, the encoded fusion proteins of which no longer localized to the nucleolus.

We first addressed the question whether function of MDDX28 was important for the localization of the protein. Drawing on the experience with the eIF4A protein (17–18), we mutated MDDX28 in three of the conserved motifs, 330GAT → AAA (SAT in eIF4A), 286DEAD → DQAD, and 481YIHR → YIQQ. The corresponding residues in the eIF4A protein affected the helicase function, the ATP hydrolysis, and the RNA binding, respectively. Cells transfected with the DEAD and GAT mutants retained the nucleolar localization of the protein (Fig. 2D). Interestingly, the mutation of the DEAD-box resulted also in cytoplasmic but not mitochondrial localization (Fig. 2D).

Mutation of the YIHR motif completely inhibited the nucleolar localization of the fusion protein (Fig. 2D), suggesting its dependence on the RNA binding ability of the protein. Double-mutated MDDX28 (DM1, 286DEAD → DQAD; 481YIHR → YIQQ) had the same localization as 286DEAD → DQAD, except that it did not localize to the nucleolus (Fig. 2D). To test whether the 481YIHR → YIQQ mutation affected the RNA binding of MDDX28, the hybrid protein was immunoprecipitated from transiently transfected COS-1 cells that had been cultured overnight in the presence of [14C]uridine. The results were normalized to the total amount of protein in each precipitate, and the background (activity pulled down with EGFP-precipitated recombinant proteins derived from various expression constructs detected by immunoprecipitation. Transfected COS-1 cells were incubated with 40 μl of [14C]uridine (50 μCi/ml) overnight. The fusion proteins were immunoprecipitated using anti-GFP antibody (Santa Cruz Biotechnology) bound to sheep-anti-rabbit IgG-coated M280 Dynabeads (Dynal) as described under “Experimental Procedures.”

**Fig. 3. RNA binding of MDDX28.** A, RNA binding of recombinant proteins derived from various expression constructs detected by immunoprecipitation. Transfected COS-1 cells were incubated with 40 μl of [14C]uridine (50 μCi/ml) overnight. The fusion proteins were immunoprecipitated using anti-GFP antibody (Santa Cruz Biotechnology) bound to sheep-anti-rabbit IgG-coated M280 Dynabeads (Dynal) as described under “Experimental Procedures.” B, dependence of nucleolar localization (loc.) of MDDX28 on RNA binding. Cells transfected with the ∆2–20 construct were treated with actinomycin D for 2 h, washed with cold PBS, and incubated for 10 min in Buffer A that contains 0.1% Triton X-100. Cells were incubated for 10 min in the same buffer made to 250 mM ammonium sulfate (Amm.Sulf.) and washed twice in Buffer A before treatment for 20 min at 37°C with 36 μg/ml of RNase A (Sigma) in Buffer A. Cells were then washed in PBS and fixed in ice-cold methanol. The experiment was repeated twice. The samples were counted blindly for nucleolar localization of MDDX28. C, nucleolar localization of MDDX28 in RNase A-treated (a) and control sample (b). Wt, wild type.

Thus, we have shown that the nucleolar localization of MDDX28 depends on the RNA binding of the YIHR motif at amino acids 481–484. However, because proteins encoded by the ∆2–139 and ∆A149–274 constructs did not localize to the nucleolus although these deletions leave the YIHR box intact, other parts of the protein must also contribute to its nucleolar localization. The most common NoLS identified to date consists of a cluster of basic amino acids (20–21) that we tested whether mutations of basic regions BS 1–4 could affect the nucleolar localization. Protein encoded by the NLS-1, NLS-2,
and NLS-3 constructs were all excluded from the nucleolus in transiently transfected cells (Fig. 2D), suggesting that in addition to the RNA binding ability, the basic regions of the protein are necessary for its nucleolar localization.

Transcription Inhibition-dependent Nuclear Localization—We have reported earlier that the nuclear localization of MDDX28 depends on ongoing polymerase II transcription (1). When testing the effect of RNA polymerase II inhibition on the nuclear localization of protein encoded by the MDDX28 constructs we also noticed marked changes in the nuclear phenotype of cells transfected with the Δ2–20, Δ520–540, or the wild-type construct as well as of the endogenous MDDX28. Shortly after the addition of actinomycin D, these proteins were seen in punctate structures in the nucleoplasm and in caps at the nucleolar periphery (Fig. 4A, a, d, and g; data not shown for Δ520–540, the wild type construct or endogenous protein). The same effect was obtained with other polymerase II inhibitors (α-amanitin and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; data not shown). This suggests that the protein accumulates in subcompartments of the nucleus during transcription inhibition. The protein disappeared from the punctate structures after 4 h of actinomycin D treatment in cells transfected with the wild-type and Δ520–540 constructs as a result of the protein being exported from the nucleus (data not shown). Therefore, this phenomenon was not observed in the previous study where the shortest time studied was 4 h post-initiation of actinomycin D treatment (1). The Δ2–20 protein has lost the NES and is, therefore, not exported from the nucleus. Accordingly, it was still localized in the punctate structures after 24 h of actinomycin D treatment. Interestingly, localization to the punctate structures during transcription inhibition was not observed in cells transfected with constructs not encoding protein localized to the nucleolus, including the YIHR mutant and the NLS-1, NLS-2, and NLS-3 mutants (data not shown).

We have studied whether MDDX28 in punctate nuclear structures colocalizes with other known nuclear bodies (PML bodies, splicing speckles, Cajal bodies (coiled bodies), and paraspeckles) by staining actinomycin D-treated cells with antibodies against PML (Fig. 4A, b), SC35 (Fig. 4A, e), p80-coilin (Fig. 4A, h), and PSP-1 (Fig. 4A, k). Only the results for Δ2–20 are shown, because its localization to the punctate structures is not affected by nuclear export, and therefore, the intensity of the structures remains stronger. The same results were obtained for the wild type protein (data not shown). The punctate nucleoplasmsic structures did not co-localize with PML, p80-coilin, or PSP-1; however, they were found to be adjacent to (but did not colocalize with) the SC35 protein detected in conventional immunofluorescence studies (Fig. 4A, f). This was further confirmed with confocal microscopy (Fig. 4B, a–e). SC35 speckles are bigger than the punctate structures containing MDDX28. However, the number of these two structures in transcription-arrested cells did not show significantly different distribution (p > 0.422, Fig. 5).

MDDX28 also formed caps at the nucleolar periphery in cells treated with actinomycin D. These were found to partially co-localize with PSP-1 (Fig. 4A, l) and to be adjacent to p80-coilin (Fig. 4A, i), as observed with conventional immunofluorescence microscopy. The adjacent colocalization with p80-coilin was further confirmed with confocal microscopy (Fig. 4B, f–j).

DISCUSSION

The subcellular localization of the MDDX28 protein is novel for RNA helicases. MDDX28 is a nuclear shuttling protein also found in the nuclear subcompartments and in the mitochondria. We have studied the signals that direct the protein to these various localizations.

Most mitochondrial proteins are encoded in the nucleus, synthesized in the cytoplasm, and transported into the mitochondria either post- or co-translationally (for a review, see Ref. 22). No consensus sequence has been found for mitochondrial localization; however, sequences with shared characteristics have been described (23). The first 20 amino acids of MDDX28 have such MTS characteristics, and these were found...

FIG. 4. Co-localization of MDDX28 punctate nuclear structures with known nuclear bodies. COS-1 cells were grown on coverslips and transfected with Δ2–20. One day post-transfection, 10 μg/ml actinomycin D was added to the cultures for 2 h. A, the cells were fixed and stained with PML (b, SC35 (e), p80-coilin (h), and PSP-1 (k). c, f, i, and l are merged pictures showing the co-localization. Primary magnification, 100 ×. Only the nuclei are shown. B, transcription inhibited Δ2–20 transfected cells (green) stained with SC35 (a–e) (red) and p80-coilin (f–j) (red) were viewed with confocal laser microscopy. Red and green images were merged. Five consecutive focal planes of the same cells are shown, with 0.2-μm step size. Primary magnification, 63 ×. Only the nuclei are shown. Arrows indicate adjacent localization.
to be both necessary and sufficient for mitochondrial localization of the protein in deletion and domain-swapping experiments. The EGFP protein and the transcription factor TCF11 gained mitochondrial localization when fused to the MTS sequence of MDDX28, whereas full-length MDDX28 lacking only the MTS did not localize to the mitochondria. The orientation of the sequence was found to be important, because only MTS inserted in the right orientation resulted in mitochondrial localization of the otherwise nuclear-localized TCF11. Furthermore, the five leucine residues of the sequence were essential for the mitochondrial localization.

A mutation of the conserved DEAD-box (286DEAD → DQAD) resulted in protein localized to the nucleus and to the cytoplasm but not to the mitochondria. Due to the cytoplasmic localization of the 286DEAD-mutated protein it was not possible to test the shuttling ability of the protein in a heterokaryon assay. The DEAD-box is a Walker B motif involved in ATP hydrolysis and is highly conserved among RNA helicases. The 286DEAD → DQAD mutation has been shown to affect the ATP hydrolysis and not ATP binding to RNA helicases (17, 24). Furthermore, it has been shown that ATP hydrolysis causes conformational changes of the eIF4A helicase and decreases its affinity for single-stranded RNA 40-fold (25). Based on the similarity of the core domain of eIF4A and MDDX28 and the finding that a double-mutated MDDX28 (DM1, 286DEAD→DQAD; 481YIHR→YIQQ) has the same localization as 286DEAD→DQAD, we propose that in addition to the MTS, the conformational change caused by ATP hydrolysis is necessary for successful mitochondrial localization of MDDX28. This change of conformation may not be needed for ΔA44–540, where nearly the whole MDDX28 is deleted. Transport of another RNA helicase, An3, is significantly affected by mutation of the DEAD motif (An3 E389Q; Ref. 26), indicating that similar mechanism may apply to the transport of both proteins.

In addition to functioning as a MTS, the first 20 amino acids of MDDX28 were found to be necessary and sufficient for nuclear export of the protein in deletion- and domain-swapping experiments. Transcription factor TCF11 was exported from the nucleus when fused to the NES sequence of MDDX28, whereas full-length MDDX28 lacking only the NES sequence was not exported from the nucleus. NES in the right orientation was much more efficient for nuclear export than NES in the wrong orientation.

Leucine-rich NES is the best-studied type of nuclear export signal (for a review, see Ref. 27). Proteins with leucine-rich NES as well as with NES rich in other hydrophobic amino acids are exported via the CRM1 pathway, which is sensitive to leptomycin B (27–30). We have shown earlier that leptomycin B does not significantly alter the nuclear localization of MDDX28 (1), suggesting that the protein does not contain a classical leucine-rich NES. The 20 N-terminal amino acids of MDDX28 include 5 leucine residues. Although mutation of the leucine residues to alanine decreased the nuclear export of the resulting protein, it was not completely inhibited. This suggests that residues other than the N-terminal leucines of MDDX28 are also important for the nuclear export. Furthermore, the result presented here shows that the MTS and NES depend at least in part on the same amino acids within the sequence. Co-localization of these two signals has to our knowledge not been described before.

Most nuclear-localized proteins have been shown to rely on signal-mediated import (31). Therefore, we expect MDDX28 to contain a NES for active nuclear import. This is further supported by the findings that MDDX28 is localized to the nucleus in about 50% of the cells, whereas it would be expected to localize to the nucleus of all cells if its nuclear import was passive. This further suggests that the protein is only temporarily needed in the nucleus or as a nuclear-cytoplasmic shuttling protein and that there is a mechanism for controlling its nuclear localization.

The most common NES sequence is a small cluster of basic amino acids. Other types of NES include the M9 sequence (38 amino acids long), which also mediates nuclear export, and the bipartite NES, made of two small clusters of basic amino acids separated by 9–12 residues, (31) or NES made of two or more mono- or bipartite basic domains separated by up to hundreds of amino acids (15, 32). We found that two clusters of basic amino acids, separated by 44 residues were necessary and sufficient for full nuclear localization of the protein in mutation and domain-swapping experiments. Additional unidentified NES was also shown to contribute to the nuclear localization. Partial nuclear import was obtained with a series of deletion constructs where one or more basic clusters had been deleted or mutated. This is in concordance with the findings for proteins with two independent NES, where both signals need to be deleted to fully inhibit the nuclear import (15, 33–34).

Surprisingly, whereas protein encoded by ΔA7 is cytoplasmic, ΔNLS-1/2-encoded protein is diffusely localized in nucleus and cytoplasm. Both constructs contain EGFP fused to parts of MDDX28 lacking functional NESs. This discrepancy can only be explained with the size difference between the two constructs. Together with EGFP, the size of the protein encoded by
ΔΔ7 is ~54 kDa, whereas the size of the ΔNLS-1/2 protein is 35 kDa, apparently allowing passive nuclear import of the protein in the absence of intracellular targeting signal.

Little is known about the signal(s) that directs proteins to the nucleolus, although several proteins have been described with NLS consisting of clusters of basic amino acids (20–21). Even less is known about the mechanisms by which proteins are routed to the nucleolus, and no nuclear transport proteins have yet been identified. It has been suggested that domains that are necessary and sufficient for nuclear localization act as retention signals rather than targeting signals (35). MDDX28 was not identified as a nucleolar protein in a recent proteomic analysis of the nucleolus (36). We have shown, however, that the protein localizes to the nucleolus in a part of the cells, suggesting that the protein cycles between the nucleolus and other parts of the nucleus (1). We now show that the ability of MDDX28 to bind RNA is essential for the nucleolar localization of the protein, because mutation of the conserved YIHR motif completely inhibited the nuclear localization as well as impaired the RNA binding ability of the protein. Furthermore, RNase A treatment of transfected cells significantly decreased the nucleolar localization.

In addition to the RNA binding ability, the three clusters of basic residues that are found in the N-terminal half of MDDX28 (BS 1–3) were shown to be independently necessary for the nucleolar localization. Two of these domains also confer nuclear localization of the protein, indicating that they are responsible not only for bringing the protein into the nucleus but also to the sites of RNA binding. The basic regions are hydrophilic and likely to be on the surface of the protein. They are, thus, possible sites of interaction of MDDX28 with other proteins or protein complexes, suggesting that both RNA and protein binding are needed for nucleolar targeting or retention of the protein in the nucleolus. Identification of proteins or protein-RNA complexes interacting with the basic regions of MDDX8 may reveal the function of the protein.

The nuclear localization of wild type MDDX28 is diffuse in untreated cells. When transcription inhibitors are added for 2 h, the protein relocates to punctate nucleoplastic structures and to caps at the nucleolar periphery. We have found that only those MDDX28 constructs whose protein can localize to the nucleolus have the capability to form these structures, suggesting that the processes depend on the same mechanisms. This may be a combination of RNA and protein interactions.

MDDX28 in punctate nucleoplastic structures were adjacent to the splicing factor SC35, which upon transcription inhibition relocates to interchromatin granule clusters (37), normally associated with transcription of highly active genes (for a review, see Ref. 38). The organization of the nucleolus itself changes upon transcription inhibition; the compartments of the nucleolus disperse, and a necklace of tandemly organized rDNA and transcription of rRNA forms at the nucleolar periphery (39). After transcription, inhibition MDDX28 localized to the parts of the nucleolus that correspond to the necklace. There it partially co-localized with PSP-1 and was adjacent to p80-coilin, both of which have earlier been shown to relocate to nucleolar caps upon inhibition of transcription (40). The function of these proteins in the nucleolar caps is not yet known, and it remains to be resolved whether they are functionally related. At present the co-localization study serves only to identify the subnuclear compartments in which MDDX28 is trapped upon transcription inhibition. We have not studied whether MDDX28 interacts directly with the proteins with which it was shown to co-localize, and no other MDDX28-interacting proteins have yet been identified.

In conclusion, we have mapped the intracellular targeting signals of the MDDX28 protein. The conservation of signals for dual or triple purposes is interesting in terms of evolution as well as in understanding the control and function of MDDX28.

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