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Nucleolar localization of murine nuclear DNA helicase II (RNA helicase A)

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SUMMARY

Nuclear DNA helicase II (NDH II) is a highly conserved member of the DEXH superfamily of eukaryotic helicases, whose physiological role is still unclear. To explore the function of NDH II, we studied the intracellular distribution of NDH II of different mammalian species by immunofluorescence and compared these findings with the known role of the *Drosophila* homologue MLE that is involved in sex-specific gene dosage compensation. NDH II displayed an apparent nucleolar localization in murine cells, whereas in cells from all other mammalian species examined so far the protein was confined to the nucleoplasm and apparently excluded from the nucleoli. The nucleolar localization of mouse NDH II strongly suggests a role in ribosomal RNA biosynthesis. Immunoelectron microscopic studies revealed that the mouse NDH II was found at the dense fibrillar components of the nucleoli, and a significant percentage of NDH II molecules colocalized with the RNA polymerase I (Pol I) transcription factor UBF (upstream binding factor). Additionally, the nucleolar localization of NDH II coincided with a preferential immunolabeling pattern of nascent transcripts with bromouridine (BrUMP). Furthermore, mouse NDH II redistributed in mitosis in a manner highly correlated with Pol I activity. Conditions leading to the inhibition of Pol I activity in the interphase decreased the amount of NDH II in the nucleoli that diffused into the nucleoplasm and the cytosol. Contrary to the effect of inhibiting rRNA synthesis, treatment of mouse cells with the translation inhibitor cycloheximide did not compromise the nucleolar localization of murine NDH II.

Key words: RNA and DNA helicase, RNA and DNA-dependent NTPase, BromoUTP, Regulation of rRNA biosynthesis, UBF

INTRODUCTION

NDH II is a ubiquitous DEXH nucleic acid helicase of higher eukaryotes that has been found in *Drosophila melanogaster* (Kuroda et al., 1991), *Caenorhabditis elegans* (Wilson et al., 1994), *Arabidopsis thaliana* (Wei et al., 1997), mouse (Lee et al., 1998), cow (Zhang et al., 1995), and man (Lee and Hurwitz, 1993). In all these species, the modular protein structure is highly conserved. NDH II contains two double-stranded RNA (dsRNA) binding domains (dsRBD) at the N terminus, an expanded DEIH helicase core in the middle, and an RGG-box at the C terminus (Gibson and Thompson, 1994; Zhang and Grosse, 1997).

Unusually, NDH II displays specialized intracellular functions within different species. For example, the *Drosphila* homologue of NDH II, the MLE (*maleless*) protein, is involved in X-chromosomal dosage compensation. MLE binds to the single X-chromosome of male flies at hundreds of discrete positions. Thereby MLE helps to increase the transcriptional level of the single male X-chromosome to that of the two X-chromosomes of females (Kuroda et al., 1991). Recently, MLE has been characterized as a nucleic acid helicase that unwinds both double-stranded DNA (dsDNA) and dsRNA (Lee et al., 1997). This finding supports the view that the enzymatic properties of NDH II are conserved from *Drosphila* to men.

Unlike the polytene chromosomes of *Drosophila*, where proteins involved in transcription, transcript package, and RNA processing can be visualized as discrete decorating bands, mammalian chromosomes are generally decondensed in interphase, which makes it more difficult to assess a possible function of a protein at a specific genetic locus. Human NDH II is found in the nucleoplasm and apparently excluded from the nucleolus (Zhang et al., 1999). The general nucleoplasmic immunostaining of human NDH II did not allow the identification of chromosome- or gene-specific functions. However, protein-protein interactions of human NDH II with RNA polymerase II (Anderson et al., 1998; Nakajima et al., 1997) as well as specific binding of NDH II to a retroviral constitutive RNA transport element (Tang et al., 1997) suggest both transcriptional and post-transcriptional functions of human NDH II that might be related to some class II genes. Here, we report that NDH II of murine cells was mainly found in the nucleolus, which provides evidence for a function during the expression of ribosomal RNA genes. Since the nucleolus is the most active region for the transcription of murine genes, NDH II seems to particularly decorate those parts of the chromosomes that display exceptionally high transcription

rates. Hence NDH II might act as a general transcription activator designed to facilitate ponderous transcriptional tasks.

MATERIALS AND METHODS

Antibodies

Polyclonal antibodies against bovine NDH II were developed by immunizing rabbits with purified bovine NDH II (Zhang et al., 1995). Rabbit antiserum against human RNA helicase A was kindly donated by Dr Jerard Hurwitz (Memorial Sloan-Kettering Cancer Center, New York). Human anti-UBF autoantiserum was kindly donated by Dr Ingrid Grummt (German Cancer Research Center, Heidelberg).

Immunofluorescence

Cells grown on coverslips were rinsed with PBS (10 mM sodium phosphate, pH 7.4, 140 mM NaCl, 3 mM KCl), fixed in 4% paraformaldehyde in PBS for 15 minutes and then permeabilized in 0.5% Triton X-100 in PBS for another 15 minutes. To reduce the non-specific background, cells were blocked by incubation with 5% bovine serum albumin (BSA) in PBS for 15 minutes. After washing with PBS, rabbit antiserum against RNA helicase A, 1/1000 diluted in PBS containing 0.5% BSA, was added. Incubation with the antigen-specific antibody was for 1 hour, followed by a PBS wash and incubation for another 1 hour with Cy3-labelled anti-rabbit IgG (Amersham-Pharmacia-Biotech, Freiburg, Germany) at a dilution of 1/400. Immunofluorescence was visualized with an Axiovert 150 fluorescence microscope (Zeiss, Jena, Germany) and photographed with 100-fold magnification.

In situ immunolabeling of nascent RNA transcripts

Bromouridine triphosphate (BrUTP) was utilized for detecting ongoing RNA synthesis in permeabilized mouse 3T3 cells by immunodetection with a monoclonal antibody against bromodeoxyuridine (BrdU) (Boehringer-Mannheim, Mannheim, Germany) (Jackson et al., 1993; Wansink et al., 1993). Cells on coverslips were incubated for 3 minute in glycerol buffer containing 20 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 0.5 mM EGTA and 25% glycerol and permeabilized by a 3 minute treatment with 0.05% Triton X-100 and 10 U/ml RNasin (Boehringer-Mannheim) in the same buffer. Run-on transcription assays in cells then proceeded for 20 minutes at room temperature in a buffer containing 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 0.5 mM EGTA, 100 mM KCl, 25% glycerol, 20 U/ml RNasin, and 0.5 mM each of ATP, CTP, GTP, and BrUTP. Then, the cells were rinsed three times with TBS (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 5 mM $MgCl_2$) plus 0.05% Triton X-100 and 5 U/ml RNasin. The samples were further processed for immunofluorescence as described above, i.e. fixation in 4% paraformaldehyde in PBS (10 mM sodium phosphate, pH 7.4, 140 mM NaCl, 3 mM KCl), treatment for further permeabilization by 0.5% Triton X-100 in PBS, blocking with 5% bovine serum albumin (BSA) and incubation with the BrdU mouse monoclonal antibody diluted at 1/50 and anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) (Boehringer-Mannheim) at a dilution of 1/60. For double-labeling of NDH II and nascent RNA transcripts, a rabbit antibody against human or bovine NDH II was included and visualized by a Cy3-labeled secondary antibody against rabbit IgG.

Electron microscopy

Mouse 3T3 cells grown on culture dishes were rinsed briefly with cold PBS and then immediately immersed in 4% paraformaldehyde in 0.1 M Sörensen phosphate buffer, pH 7.3, followed by detaching the cells into a centrifugation tube, where the collected cells were fixed for 6 hours at 4°C. After washing in 0.1 M Sörensen buffer for three times, the cell pellet was dehydrated in an ethanol series with gradually increasing concentrations from 30% to 75% and then embedded in LR White resin (Plano, Cardiff, UK). Post-embedding immunolabeling was then performed with the subsequently prepared ultra-thin sections, which were first blocked with 5% BSA in PBS for 1 hour and then incubated for 6 hours with rabbit anti-RNA helicase A antiserum and human anti-UBF autoantiserum, both at dilutions of 1/100, followed by an overnight incubation with gold-conjugated secondary antibodies against rabbit IgG and human IgG at dilutions of 1/100 (Plano). After immunolabeling, the samples were ultimately treated with an EDTA regressive staining developed for contrasting the ribonucleoprotein networks in cells (Bernhard, 1969).

Western blotting

HeLa and mouse 3T3 cells were grown as monolayers to ≈80% confluency in culture flasks (75 cm^2), than washed with cold PBS, and immediately scraped into Eppendorf tubes in 500 μl SDS-PAGE sampling buffer. The resulting cell lysates were heated to 95°C for 5 minutes, sonicated and briefly spun down at 15,000 ***g***. Supernatants were analyzed by western blotting with an antiserum against NDH II followed by enhanced chemiluminescence (ECL, Amersham-Pharmacia-Biotech, Freiburg, Germany).

Molecular cloning of the gene encoding mouse NDH II

A mouse spleen lambda phage ZAP II cDNA library (Stratagene, Amsterdam, The Netherlands) was initially screened by cDNA probes that were amplified by PCR from the middle of the human NDH II coding-frame (Zhang and Grosse, 1997). Clones with extensions towards both ends of the mouse NDH II gene were obtained by subsequent screening with cDNA probes designed from the 5′ or 3′ terminus of the initially identified mouse NDH II positive clones (Fig. 7A). All PCR probes were labeled with [α-^{32}P]dCTP using a random-priming method (Amersham-Pharmacia-Biotech). Screening of the cDNA library based on nucleic acid hybridization has been described before (Zhang and Grosse, 1997; Zhang et al., 1995).

RT-PCR analysis

Total RNA from mouse 3T3 cells was prepared by using the micro RNA isolation kit from Stratagene (Amsterdam, The Netherlands). Mouse 3T3 cells were denatured in the dish by 4 M guanidinium isothiocyanate and subsequently deproteinized by phenol/chloroform extraction. The nucleic acids were precipitated by an equal volume of isopropanol. For RT-PCR ≈2 μg of total RNA were used as a template for first-strand cDNA synthesis in a reaction volume of 50 μl containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM of each of the four dNTPs, 1 mM DTT, 40 U of RNasin, 0.5 μg of oligo$(dT)_{12\text{-}18}$ and 20 U of Superscript™ II reverse transcriptase (Life Technologies, Karlsruhe, Germany). After incubation at 42°C for 30 minutes, the reaction was stopped by heating to 95°C for 5 minutes. PCR synthesis was started by the addition of 100 pmol each of forward and reverse cDNA primers (Fig. 7A) in a volume of less than 2 μl and 1.25 U of *Taq* DNA polymerase (0.25 μl) (Life Technologies). PCR cycles included 30 rounds of sequential incubations at 94°C for 1 minute, 52°C for 2 minutes, and 72°C for 2 minutes, followed by a 10 minute extension at 72°C. RT-PCR products were analyzed by electrophoresis through 1% agarose in TAE buffer (40 mM Tris-acetate and 1 mM EDTA).

RESULTS

NDH II is a preferetially nucleolar protein in murine cells

The cellular localization of NDH II was examined in different mammalian species. Immunofluorescence revealed that NDH II from human HeLa cells and marsupial PtK_1 cells was found to be distributed throughout the nucleoplasm with an apparent negative staining of the areas corresponding to the nucleoli (Fig. 1A-A′, B-B′). Similar nucleoplasmic localization of NDH

II have also been found for other human cells, such as HEp-2 and 293 cells (data not shown) and for monkey TC-7 kidney cell (Zhang et al., 1995). Unexpectedly, immunofluorescence of NDH II from two murine cell lines, i.e. NIH 3T3 and p388-D1, a macrophage line (Dawe and Potter, 1957), revealed a preferentially nucleolar staining that was strikingly different from the nucleoplasmic localization observed in other mammalian cells (Fig. 1C-C′ and D-D′). In addition to the nucleolar localization, murine NDH II was also found diffusely distributed in the nucleoplasm; relative weak immunofluorescence signals were even found in the cytoplasm. These results suggest that the differing subnuclear localization of NDH II in two mammalian species mirrors a participation in the expression of different classes of genes, i.e. class II genes transcribed by RNA polymerase II (Pol II) in the nucleoplasm of human cells and class I ribosomal genes transcribed by RNA polymerase I (Pol I) in the nucleoli of mice.

NDH II was localized in dense fibrillar components of mouse nucleoli

We used immunoelectron microscopy to observe the nucleolar localization of NDH II in mouse 3T3 cell at the ultrastructural

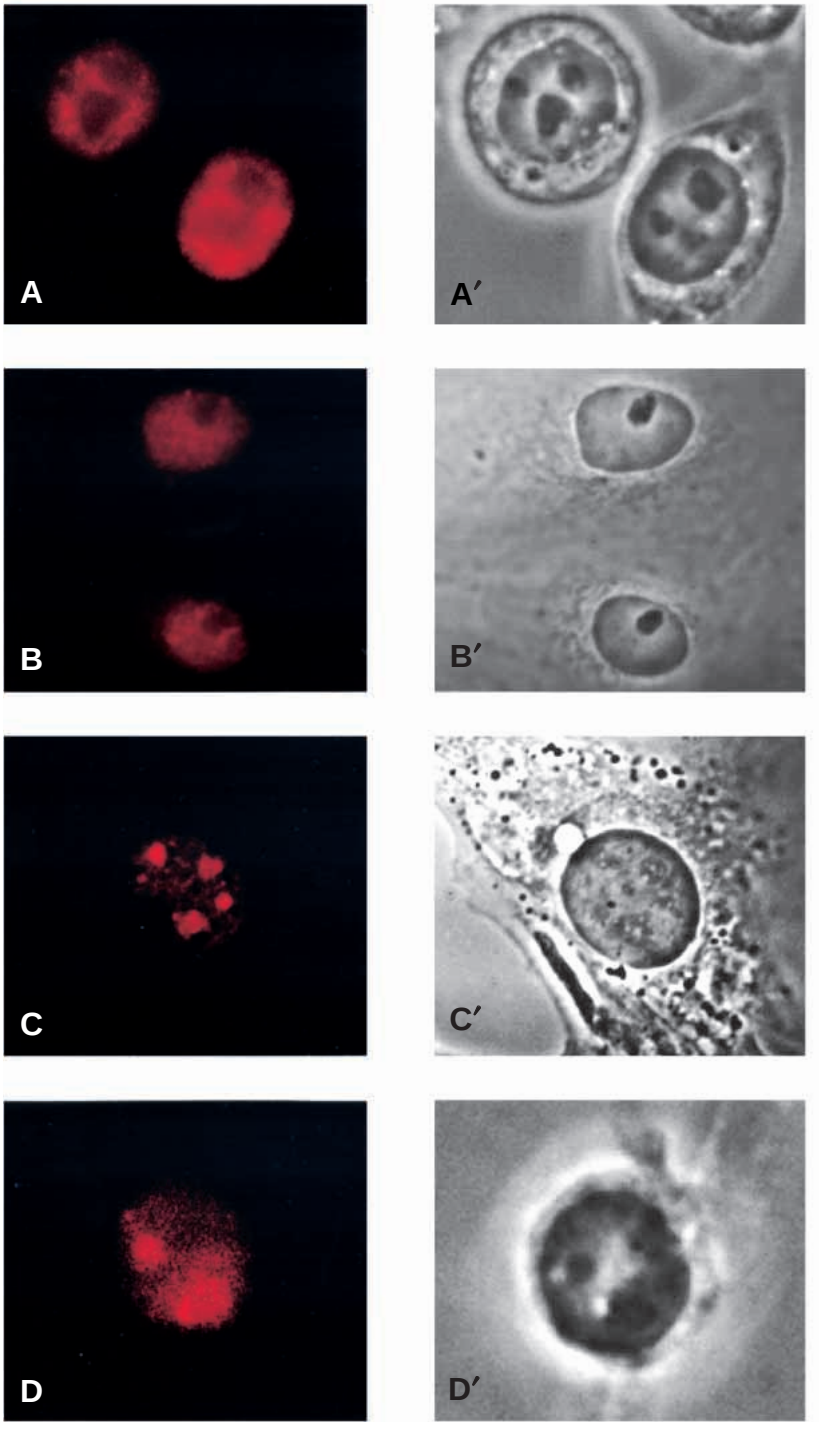


Fig. 1. Immunofluorescence of NDH II from different mammalian cells. Intracellular localization of NDH II in different species was examined by immunofluorescence with antiserum against RNA helicase A. NDH II was localized in the nucleoplasm of human HeLa (A) and PtK_1 (B) and in the nucleolus of murine NIH 3T3 (C) and murine p388D1 macrophages (D). Phase contrasts are shown in A′, B′, C′ and D′.

level. Electron microscopic studies of the nucleoli allows one to distinguish three major components, i.e. the fibrillar centers (FCs), the dense fibrillar components (DFCs), and the granular components (GCs). The lightly stained FCs contain rDNA chromatin in interphase which corresponds to the secondary constrictions of mitotic chromosomes. Pol I, which is responsible for the synthesis of pre-ribosomal RNAs, is localized in FCs (Scheer and Rose, 1984). DFCs, on the other hand, display apparently higher electron densities and surround the FCs. In DFCs, nascent ribosomal transcripts (Dundr and Raska, 1993; Raska et al., 1995) and Pol I transcription factors like UBF (Roussel et al., 1993) and nucleolin (Escande et al., 1985) are situated. Granular components (GCs) surround the DFCs in the rest of the nucleolar regions. GCs may correspond to pre-ribosomal particles. Ribosomal RNA synthesis and maturation all occur at these three morphologically distinguishable subregions. Mouse NDH II, identified by immunogold labeling (10 nm), was predominantly found in the DFCs (Fig. 2), suggesting a function in rRNA synthesis, packaging, or early processing. In the DFCs mouse NDH II was frequently found to accompany the Pol I transcription factor UBF, as shown by immunolabeling with 40 nm gold particles (Fig. 2). Colocalization of mouse NDH II and UBF suggests that loading of NDH II onto ribosomal RNA may be a co-transcriptional event occurring in the vicinity of or even mediated by the Pol I transcription complex. As compared with its enrichment in DFCs, only small amounts of NDH II molecules were found in FCs and GCs (Fig. 2).

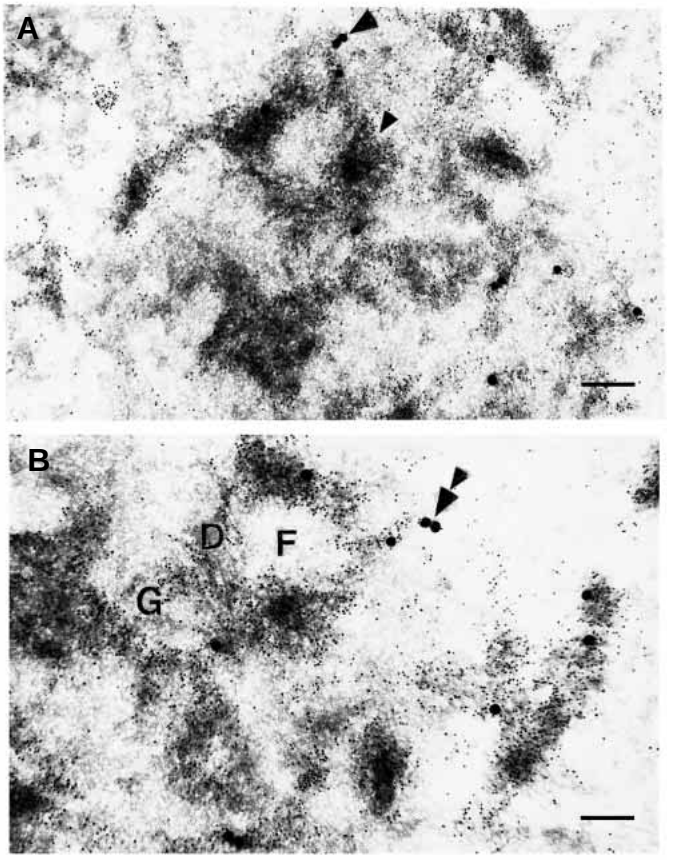


Fig. 2. Immunogold labeling and electron microscopy of NDH II and UBF in mouse nucleoli. Post-embedding immunolabeling of mouse 3T3 cells was performed with ultra-thin sections by co-incubation with rabbit antiserum against RNA helicase A and human autoantiserum against UBF. NDH II was identified by a secondary antibody against rabbit IgG that was coupled with 10 nm gold particles and UBF was detected with a secondary antibody against human IgG coupled with 40 nm gold particles. Electron micrographs at two magnifications, i.e. 22,500-fold (A) and 33,000-fold (B), are shown. The small arrows in the figure indicate 10 nm gold particles corresponding to NDH II and the larger arrowheads indicate 40 nm gold particles corresponding to UBF. Double arrowheads indicate colocalization of NDH II and UBF. Bars: 400 nm (A); 250 nm (B). F stands for fibrillar centers (FCs), D for dense fibrillar components (DFCs) and G for granular components (GCs).

Transcription-dependent nucleolar localization of murine NDH II

Murine NDH II displayed a dynamic relocalization under conditions leading to transcription inhibition. As compared with its normal nucleolar staining (Fig. 3A), NDH II was significantly released from the nucleoli of 3T3 cells after

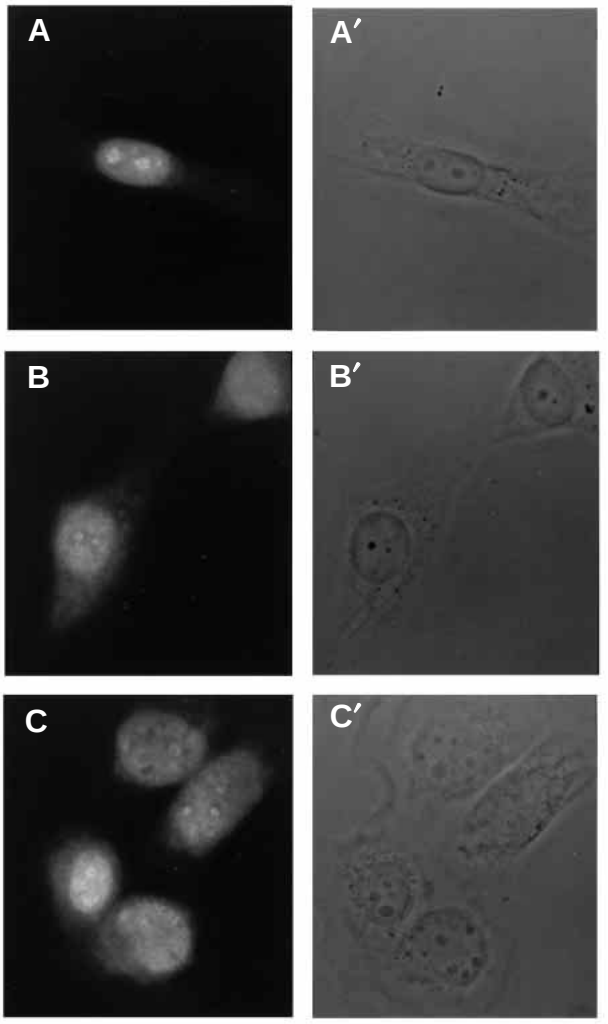


Fig. 3. Nucleolar localization of mouse NDH II under the condition of transcriptional inhibition. Immunofluorescence of NDH II was performed with mouse 3T3 cells cultured under normal conditions (A), treated with 5 μg/ml actinomycin D for 3.5 hours (B), and heat-shocked by incubation at 42°C for 3.5 hours (C). Images of phase-contrast microscopy are presented in A′, B′ and C′.

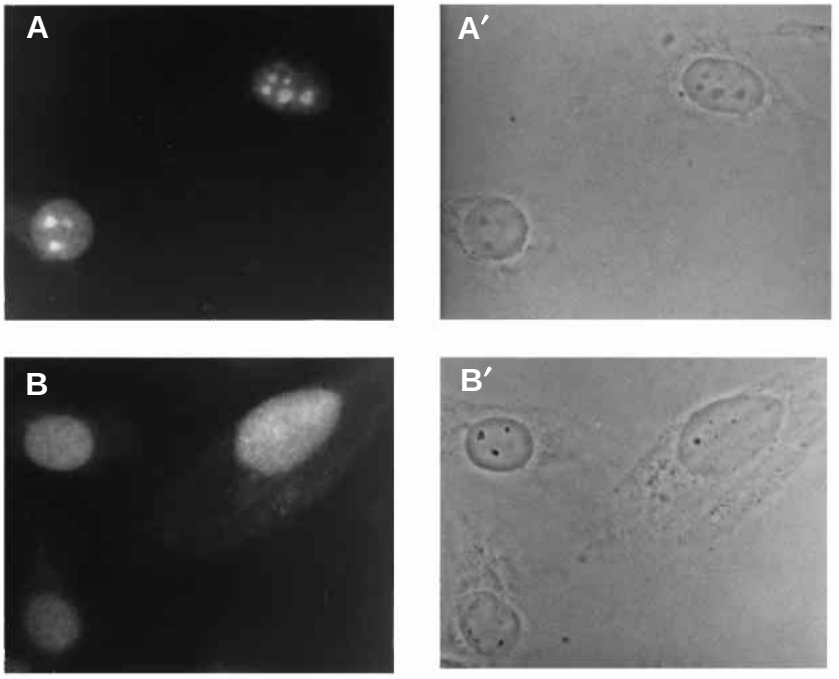


Fig. 4. Immunofluorescence of NDH II in mouse 3T3 cells in the presence of cycloheximide. NDH II was observed by immunofluorescence in mouse 3T3 cells treated with 20 μg/ml cycloheximide for 3.5 hours (A), or 20 μg/ml cycloheximide plus 5 μg/ml actinomycin D (B). Phase-contrast images of mouse 3T3 cell are shown in A′ and B′.

treatment with actinomycin D (Fig. 3B) or by exerting thermal stress (Fig. 3C). Under these conditions, the decreased immunofluorescence of NDH II in the nucleoli was accompanied by increased and diffused NDH II signals in the nucleoplasm and cytoplasm, indicating a redistribution of NDH II caused by transcription inhibition in the nucleoli. In all these cases the nucleolar exclusion correlated with diminished nucleolar structures and decreased nucleolar contrasts (Fig. 3A′,B′ and C′). Nucleolar exclusion following transcription inhibition resulted in NDH II-depleted regions at the periphery of the nucleoli (Fig. 3B and C).

Nucleolar localization of NDH II in the presence of cycloheximide

Cycloheximide is a specific inhibitor of protein synthesis. Mouse 3T3 cells were treated with cycloheximide for 3.5 hours, and then observed for immunofluorescence of NDH II. After this treatment NDH II remained concentrated in the nucleoli, with some cells even displaying enhanced immunofluorescence (Fig. 4A and A′). This might reflect an even increased concentration of nucleolar NDH II in response to the inhibition of protein biosynthesis. However, when cycloheximide was delivered together with actinomycin D, nucleolar NDH II immunostaining decreased with a correspondingly increasing signal in the nucleoplasm and the cytoplasm (Fig. 4B and B′).

The dynamic redistribution of mouse NDH II during mitosis correlated with ribosomal RNA synthesis

While the results presented above were obtained with interphase cells, the correlation between the localization of mouse NDH II and sites of ribosomal RNA synthesis was also apparent during mitosis. In prophase, murine NDH II remained associated with the initially condensed chromosomes at nucleolar organizer regions (NORs) (Fig. 5A). BrUMP immunosignals were also visible at NORs, indicating persistent transcription at this stage of the cell cycle (Fig. 5B). When the mitotic cells progressed into metaphase, the majority of NDH II diffused into the periphery of the highly condensed chromosomes and into the cytoplasm (Fig. 5D) whereas Pol I-catalyzed transcription at the NORs went down until it was no longer detectable (Fig. 5E). In early telophase, the nuclear transcription activity resumed, together with the occurrence of gradually decondensed chromosomes. In mouse telophase nuclei, the main transcription activity was found at NORs (Fig. 5H). Concomitantly NDH II was found to be locally concentrated at NORs to an extent that correlated with the resuming Pol I activity (Fig. 5G). Finally, when murine cells progressed into interphase, double-immunofluorescence of murine NDH II and BrUTP-RNAs revealed a coincidence of the relatively strong nucleolar NDH II staining and the predominant ribosomal RNA synthesis (Fig. 5J and K). DAPI-stainings are also presented to show the condensation state of chromosomes during the cell cycle (Fig. 5C,F,I and L).

Confirmation of homology between human and mouse NDH II

The prevalent nucleolar localization of mouse NDH II provided morphological evidence for a function in ribosomal RNA synthesis and processing. However, this function of NDH II seems to be restricted to mice, since in all other mammalian cells tested so far, the protein was confined to the nucleoplasm and apparently excluded from the nucleolus. The different localization of murine and human NDH II may be caused by different protein structures. Western blot experiments of both human HeLa and mouse 3T3 cell extracts revealed significantly different molecular masses of ≈140 kDa in humans and ≈150 kDa in mice (Fig. 6). Thus murine NDH II was considerably bigger than the other mammalian homologues. To find the reasons for this difference, we cloned and sequenced the cDNA encoding mouse NDH II (Fig. 7A). After finishing this work, a full-length cDNA sequence for mouse RNA helicase A was

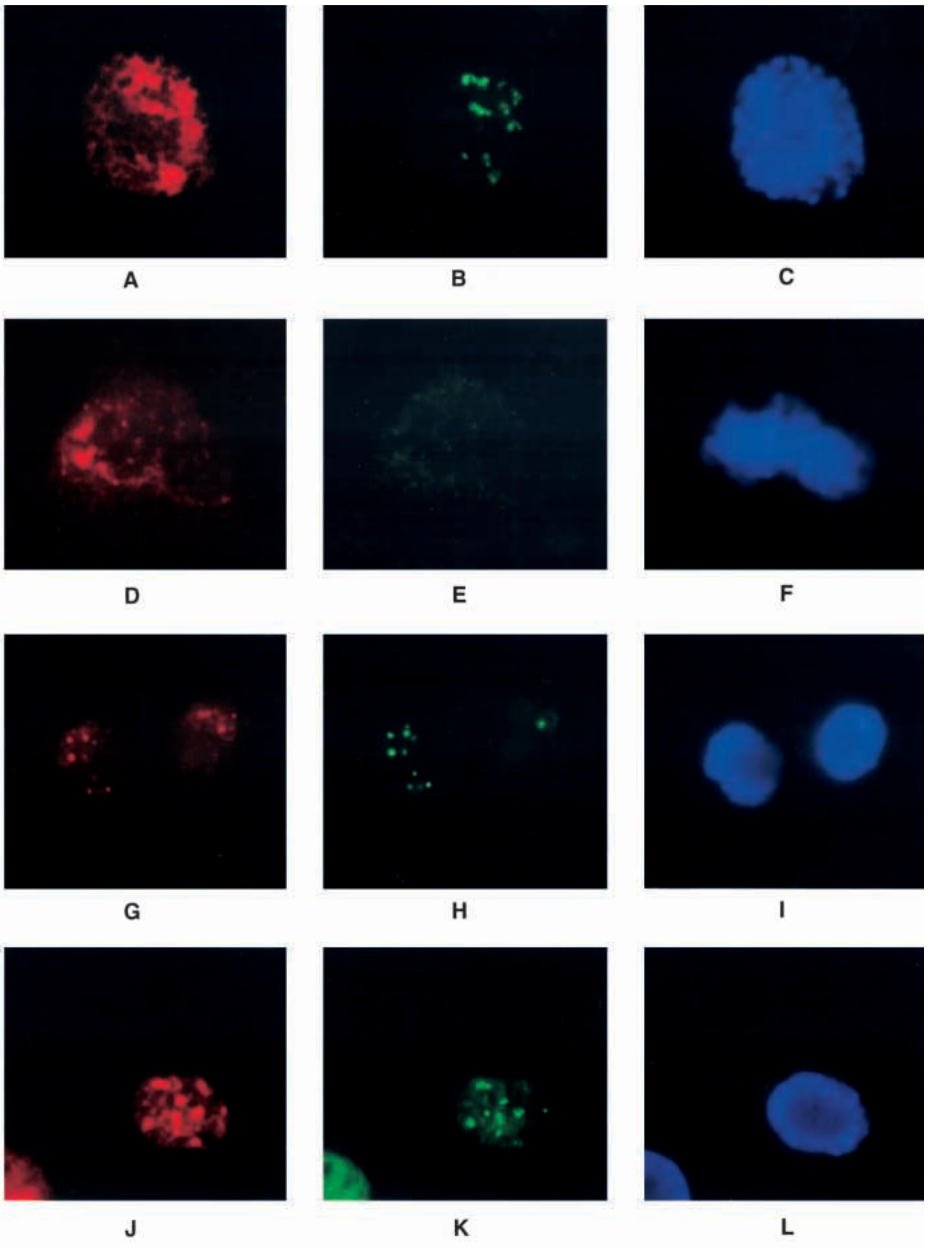


Fig. 5. Double-immunofluorescence studies of NDH II and nascent RNA transcripts in mitotic mouse 3T3 cells. Mouse 3T3 cells were blocked at interphase by incubation with 2 mM thymidine for 14-16 hours. After release from the growth arrest by adding fresh medium the cells were grown for another 9-10 hours to enrich them at mitosis. The cells were then proceeded for run-on transcription and visualized by double-immunofluorescence with antiserum against human RNA helicase A and a monoclonal antibody against BrdU. The results are presented for mouse 3T3 cells at prophase (A-C), metaphase (D-F) and telophase (G-I), and interphase (J-L). A, D, G and J show the immunofluorescence of NDH II, B, E, H and K show the immunofluorescence of BrUMP, C, F, I and L show DAPI stainings.

published (Lee et al., 1998) that was identical to our sequence for murine NDH II. Full-length mouse NDH II has a calculated molecular mass of 149,488 Da, which is consistent with the experimentally determined molecular mass of NDH II from mouse 3T3 cells (see Fig. 6). Mouse, human, and bovine NDH II display a 91% identity of their amino acid sequences.

There was no evidence for alternatively spliced NDH II in mouse 3T3 cells. A nested RT-PCR analysis gave products of different lengths that covered the full-length mouse NDH II gene entirely, including a flanking sequence of 27 nucleotides upstream of the first ATG codon and 112 nucleotides downstream of the stop codon (TAA) (Fig. 7A). All RT-PCR products displayed the expected lengths that were deduced from the sequence of the spleen cDNA, making it unlikely that further isoforms of NDH II exist (Fig. 7B).

We further performed sequence comparisons of NDH II from different species (Fig. 8). The amino acid sequences within the two N-terminal dsRBDs (I and II), the nuclear localization signals (NLSs), the seven DEXH helicase motifs (I, IIa, IIb, III, IV, V and VI) and its extension up to the C-terminal RGG-box are strictly conserved among mouse, man and cow (Fig. 8A). Some non-conserved amino acids were identified, which most frequently appeared within the region between dsRBD I and dsRBD II (aa 100-150), where the amino acid identity locally decreased to 87%. It remains to be seen whether these variations have functional consequences. An alignment of mouse NDH II with *Drosophila* MLE and its homologue from *C. elegans* revealed 52% and 43% identity, respectively. Despite this stronger divergence the amino acids within dsRBD I and II, the DEIH helicase core, and the C-terminal extension are highly conserved (Fig. 8B). The NLSs of the mammalian enzymes were not found in NDH II from *Drosophila* and *C. elegans.* Different from other species, NDH II of *C. elegans* carries an 103 aa long N-terminal extension of a still unknown function.

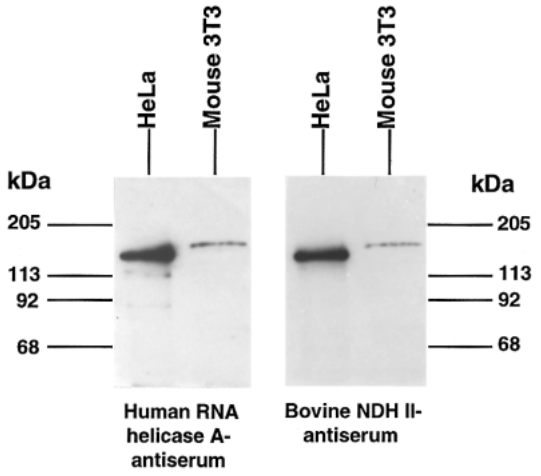


Fig. 6. Western blot analysis of human and mouse NDH II. HeLa and mouse 3T3 whole cell extracts (0.5 μl) were electrophoresed through a 10% SDS-polyacrylamide gel and transferred to a Hybond-nitrocellulose membrane (Amersham). The immunoblots were developed with antiserum to human RNA helicase A (A) and bovine NDH II (B). A dilution of 1/1000 was used for both antisera.

An apparent feature of mouse NDH II is its unusually extended RGG-box. The RGG-box of NDH II from all species contains tandemly arranged arginine (R)-glycine (G)-rich sequence repeats that are regularly spaced between aromatic amino acids. The invariance of the conserved -Y-$(RG)_n$-Y-sequence but the increased number of RG-rich repeats in murine NDH II suggests that the length of the RGG module may constitute a factor in determining the subnuclear localization.

DISCUSSION

NDH II belongs to the DEXD/H superfamily of nucleic acid helicases whose members participate in various genetic activities ranging from transcription, RNA processing, RNA transport to translation (for reviews see Gorbalenya et al., 1989; Linder et al., 1989; Wassarman and Steitz, 1991; Bork and Koonin, 1993; Kadare and Haenni, 1997; Eisen and Lucchesi, 1998; Luking et al., 1998). Beside their catalytic activities individual members of the DEXD/H helicase family possess different substrate-targeting domains that may control

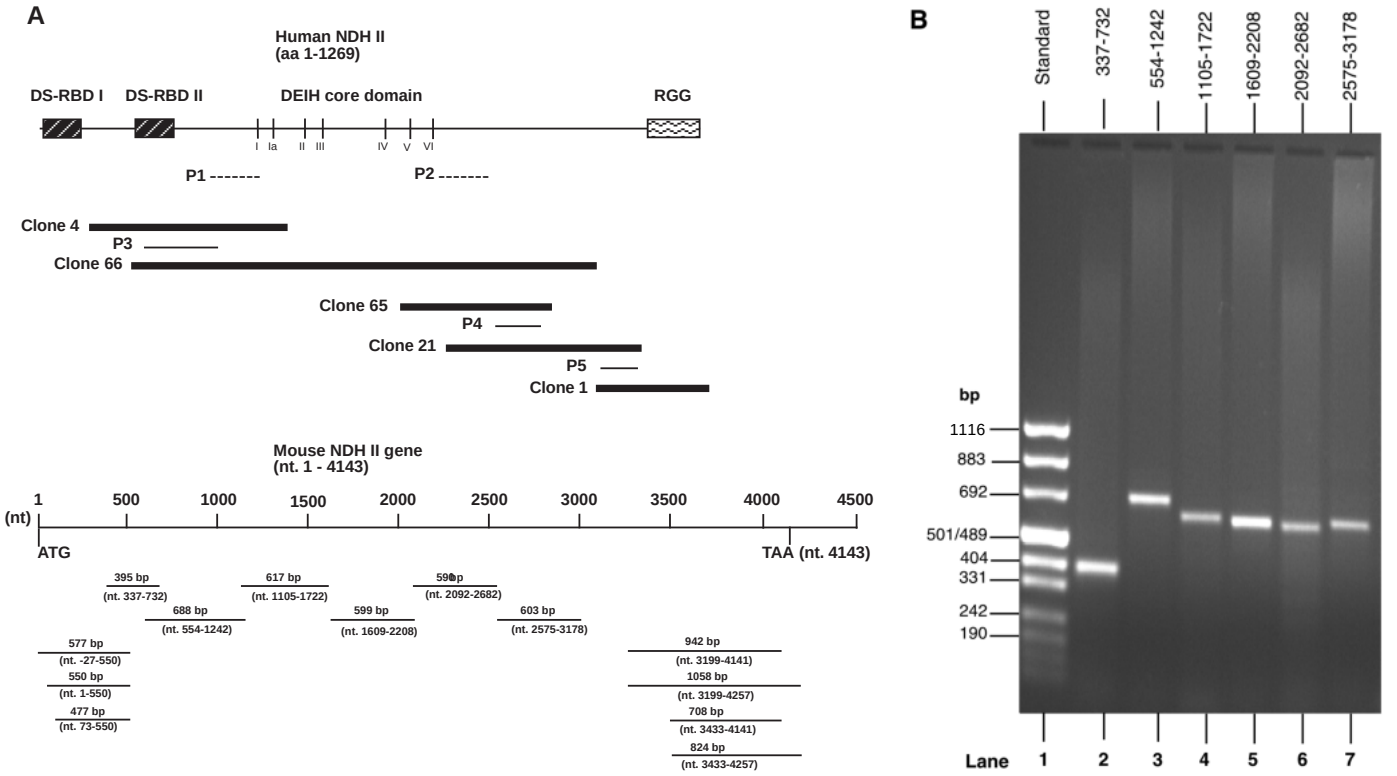
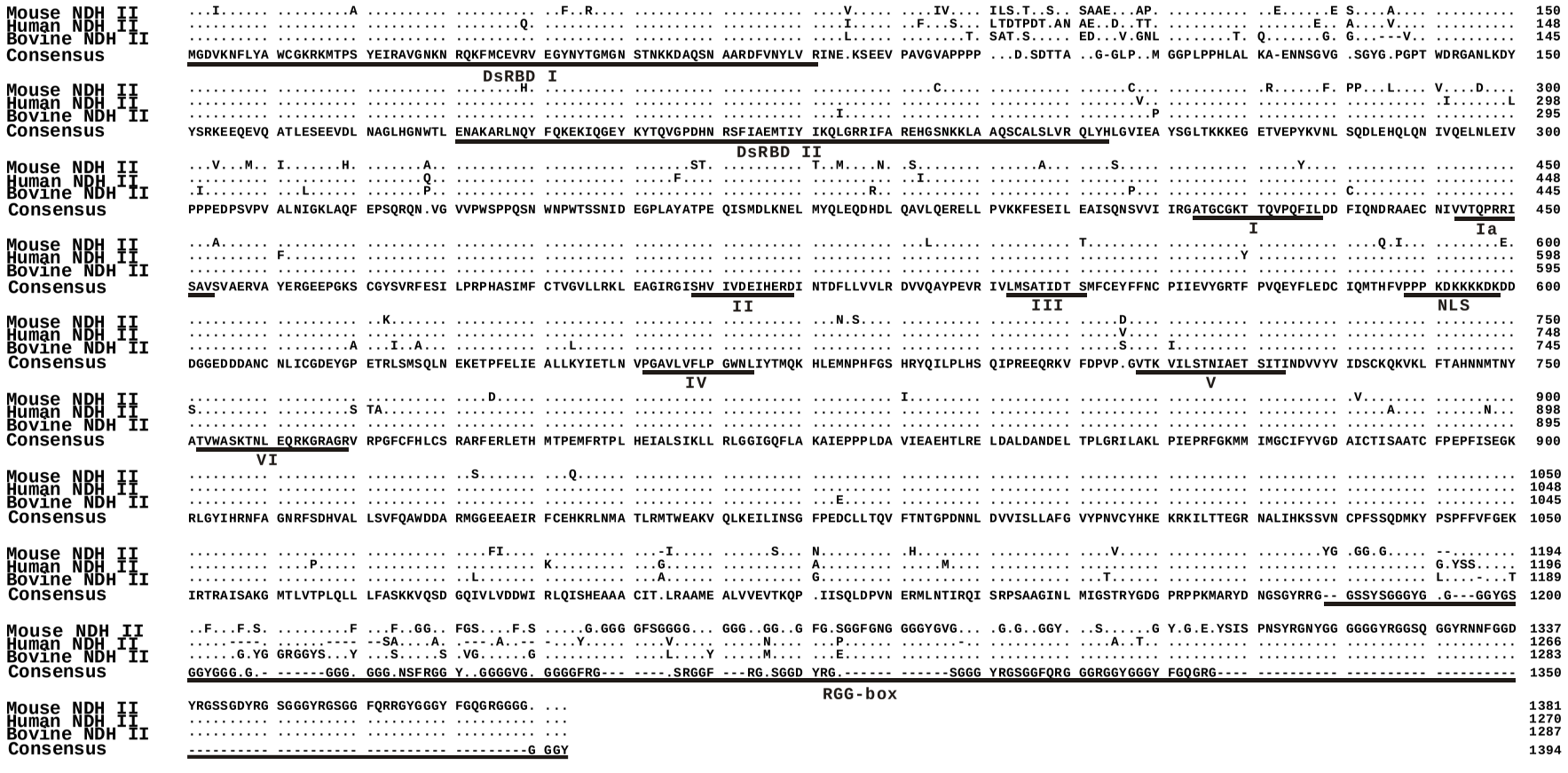
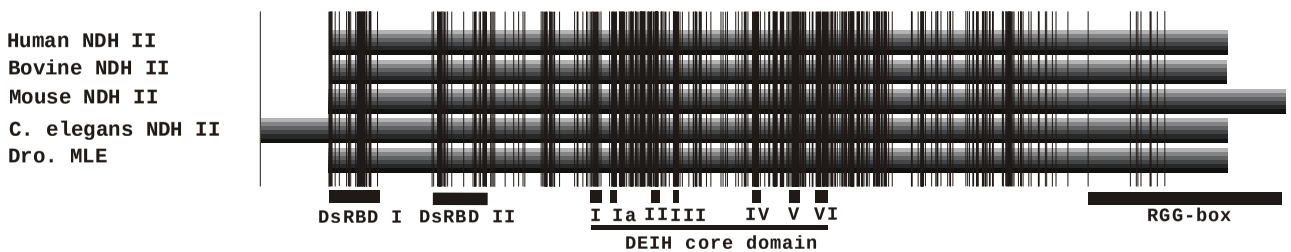


Fig. 7. Molecular cloning of the gene encoding mouse NDH II. (A) A mouse spleen cDNA library was initially screened with two human NDH II cDNA probes, P_1 and P_2, which led to the identification of cDNA clones #66 and clone #65. Clones that included the N- and C-termini were obtained by screening the same library with homologous mouse NDH II probes. P_3 was synthesized from the N-terminal part of clone #66 (nt 555 to 1077), which led to the identification of clone #4 (nt 256 to 1357). P_4 was derived from the C-terminal part of clone #65 (nt 2576 to 2935) and led to the identification of clone #21 (nt 2324 to 3528). Finally, P_5 was synthesized from the C-terminal part of clone #21 (nt 3198 to 3520) and used for the identification of clone # 1 (nt 3162 to 3891). (B) NDH II cDNA from mouse 3T3 cells was analyzed by nested RT-PCR assays, with primers designed from the mouse NDH II cDNA. Positions of the expected RT-PCR products were indicated for the first nucleotide of the forward PCR primer and the last nucleotide of the reverse PCR primer, as indicated (see A). Lane 1, DNA molecular mass standard composed of mixed *Ms* I-digested pUC19 and *Dra*I plus *Hin*dIII-digested pUC57 products. Lane 2 to 7, the six NDH II RT-PCR products covering nucleotide 337 to 3178 in the middle of mouse NDH II coding frame (see A).

A


Mouse NDH II
Human NDH II
Bovine NDH II
Consensus
DsRBD I
DsRBD II
I
Ia
II
III
IV
V
VI
NLS
RGG-box

B


Human NDH II
Bovine NDH II
Mouse NDH II
C. elegans NDH II
Dro. MLE
DsRBD I
DsRBD II
I Ia II III
IV V VI
RGG-box
DEIH core domain

the substrate specificity and thereby define the function, such as transcription of a specific class of genes. In agreement with this view, many DEXD/H helicases are known to be involved in transcription and post-transcriptional processing of either pre-messenger RNAs or pre-ribosomal RNAs. Notwithstanding, a few members of DEXD/H helicases have more general functions and are involved in the nucleic acid metabolism of both class I and II genes. For example, human RNA helicase p68 translocates from the nucleoplasm to the nucleolus in a cell-cycle dependent manner (Iggo and Lane, 1989). The An3 protein from *Xenopus* is found in the nucleolus of oocytes depending on specific stages of development (Gururajan et al., 1994). Also NDH II seems to belong to these more generally acting helicases, since in human cells it is found in the nucleoplasm, where it is associated with pre-mRNAs and mRNAs during transcription, processing, and transport (Zhang et al., 1999). In contrast, in murine cells NDH II is mainly situated in the nucleoli. There is a precedence for a species-dependent localization, since Werner's syndrome helicase has been identified as a nucleolar enzyme in human cells, whereas in murine cells it was confined to the nucleoplasm and apparently excluded from the nucleolus (Marciniak et al., 1998).

Presently it remains difficult to explain why NDH II displays species-dependent intracellular localizations. The highly conserved NDH II homologue MLE from *Drosophila* decorates the single X-chromosome of males and enhances its transcriptional level to that of the two female chromosomes (Kuroda et al., 1991). This function of MLE is part of the gene dosage compensation mechanism that is necessary because of the imbalanced number of X-chromosomes between males and females (Bashaw and Baker, 1996). Since *Drosophila* rRNA genes are also situated on the X-chromosome (Glover, 1991), their expression should be regulated by a dosage compensation system. Dosage compensation in humans and mice is achieved by the random inactivation of one of the female X-chromosomes, a process completely unrelated to that of *Drosophila* (Heard et al., 1997). However, if the basic function of MLE has been conserved through evolution (the extremely high conservation of MLE and NDH II speaks in favor of it), the altered location of NDH II in mouse cells may reflect a gene dosage balance activity that has been specifically adapted for the needs of the murine genome. Murine NDH II may therefore be recruited to the nucleoli to ensure a high rDNA transcription level that must be attained in accordance with the cellular requirements of this species.

The restricted localization of NDH II in murine nucleoli allowed a morphological study of its potential functions. Ultrastructural observations by immunoelectron microscopy revealed abundant amounts of NDH II molecules accumulated within the dense fibrillar components (DFCs) of the murine nucleolus, while it was hardly identified within fibrillar centers (FCs) or granular components (GCs). So far DFCs have been considered to be the nucleolar sites where nascent rRNAs are produced and undergo early processing for further maturation. After synthesis and early processing, rRNAs gradually move from DFCs to GCs where they are assembled into pre-ribosomes. There are also other nucleolar proteins within the DFCs including the Pol I transcription factor UBF (upstream binding factor), nucleolin, nucleolar snRNPs, the B23 protein, and fibrillarin (for review see Shaw and Jordan, 1995). The initial step of ribosomal transcription is represented by the binding of UBF to the upstream promoter and the core promoter elements, which subsequently recruit transcription initiation factors (TIFs) to initiate transcription by Pol I (Sollner-Webb and Mougey, 1991). Immunolabeling studies identified UBF at some specific sites of the DFCs as well as at the surface of FCs (Roussel et al., 1993), which may serve as markers for active rRNA production. Immunodetection of freshly transcribed rRNA revealed the most active regions at the boundary between DFCs and FCs (Dundr and Raska, 1993; Raska et al., 1995). Unfortunately, these data contrast with another line of evidence where Pol I is mainly found in FCs (Scheer and Rose, 1984) and where significant levels of rRNA were also attributed to FCs by in situ RNA hybridization (Thiry, 1992). The latter observations led to the idea that FCs may be the sites of rRNA transcription while DFCs are the sites where pre-rRNAs are accumulated (Thiry and Goessens, 1991). Our observation that NDH II was concentrated within DFCs rather than FCs argues against the possibility that FCs may be the predominant rDNA transcription sites, at least if NDH II participates directly in the biosynthesis of rRNAs (see below). Therefore, we favor an already proposed different model that considers FCs to be the storage sites for inactive Pol I molecules. Active Pol I may be engaged in rRNA synthesis at the periphery of FCs, where it binds to the nucleolar matrix to thread the rDNA template through the fixed Pol I transcription complex. From this the nascent transcripts may be radially extruded into the surrounding DFCs for further processing and maturation (Hozak et al, 1994; Shaw et al., 1995). The observed co-localization of NDH II and UBF apparently supports this scenario. An unrelated overlap between NDH II and UBF seems unlikely since some of the co-localization studies provided a view of UBF surrounded by NDH II and followed as a trail by NDH II at the borders between DFCs and FCs, where active rRNA genes are most likely situated. Hence, the helicase seems to be directly involved in Pol I-catalyzed transcription processes, possibly via its binding to single-stranded DNA of the open complex (Zhang et al., 1999). Then it may become redirected to nascent transcripts to mediate post-transcriptional processing events. These functional diversities correlate well with the biochemical observation of NDH II as being both a DNA and an RNA binding protein (Zhang et al., 1999) as well as its functional

Fig. 8. Multiple alignment of NDH II genes from different species. (A) The computer program GeneWorks (IntelliGenetics, Geel, Belgium) for Macintosh was used for multiple alignments of NDH II genes from *Homo sapiens*, *Bos taurus*, and *Mus musculus.* Sequence consensus was deduced from a 50% cut-off of identical amino acids. Only amino acids diverging from the consensus sequence are shown. Conserved domains are underlined including the N-terminal dsRBD I and II, the central DEXD/H helicase core motifs (I, Ia, II, III, IV, V and VI), the nuclear localization signal (NLS) and the RGG-box. (B) Cartoon depiction on the alignment of NDH IIs from men, cow, mouse, fruit fly, and worm. Vertical bars indicate identical amino acids shared among all five proteins. Database accession numbers for NDH II genes are Y10658 for human NDH II, X82829 for bovine NDH II, U91922 for mouse NDH II, P24785 for Drosophila MLE (Dro. MLE) and Z50071 for *Caenorhabditis elegans* NDH II *(C. elegans* NDH II). The full-length sequence of mouse NDH II was confirmed by this work.

interaction with transcriptional co-activators of class II genes (Nakajima et al., 1997; Anderson et al., 1998).

The mechanism of nucleolar targeting of murine NDH II is still an open question. In comparison to its homologs, the most striking feature of murine NDH is its unusually extended RGG-box at the C terminus. RGG-boxes are single-stranded nucleic acid binding domains that increase the affinity for nucleic acids (Dreyfuss et al., 1993). Interestingly, a specific nucleolar localization can be correlated with a long RGG repeat. This long repeat, which is also called GAR (glycine and arginine-rich), is found with several nucleolar proteins, such as GAR1 (Girard et al., 1992), GAR2 (Gulli et al., 1995), nucleolin (Schmidt-Zachmann and Nigg, 1993), fibrillarin (Aris and Blobel, 1991), and the herpes simplex virus regulatory protein ICP 27 (Mears et al., 1995). The elongated RGG-box of murine NDH II has not only been found in our study but has also been described by Lee et al. (1998), indicating a significant difference between the human and bovine enzyme on the one hand and murine NDH II on the other. Hence, the elongated RGG- or GAR-box may be one domain that is necessary for a nucleolar retention. Furthermore, nucleolar retention may dynamically depend on an ongoing ribosomal transcription activity (Hernandez-Verdun, 1991). This is supported by the finding that NDH II was found in the nucleolus in a manner that highly correlated with active rRNA synthesis. During mitosis, NDH II gradually left the nucleolar organizers (NORs) when chromosomes started to condense and rRNA synthesis ceased between prophase and metaphase. NDH II returned to NORs at telophase when chromosomes decondensed and Pol I-catalyzed transcription resumed. When murine cells were subjected to treatment with actinomycin D, a drug that mainly blocks rRNA formation (Sobell, 1985), NDH II neither returned to the telophase nucleolus nor was it retained in the interphase nucleolus. Alternatively, protein shuttling into the nucleolus may be achieved by specific associations with other nucleolar proteins, such as nucleolin (Bouvet et al., 1998) and B23 (Li, 1997). Presently the possibility cannot be excluded that murine NDH II may have a murine-specific protein partner that mediates its nucleolar localization. Further experiments, e.g. microinjections of murine and human NDH II or plasmid transfections to study the reciprocal exchange of the helicase between human and murine cells should be helpful to resolve the mechanism of the murine-specific subnuclear localization of NDH II.

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