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A Human Common Nuclear Matrix Protein Homologous to Eukaryotic Translation Initiation Factor 4A

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Amino acid sequencing and mass spectrometry revealed identity of a human nuclear matrix protein, termed hNMP 265, with a predicted protein of gene KIAA0111. Two-dimensional electrophoresis and Northern hybridization showed the protein to ubiquitously occur in various human cell types. Exhibiting DEAD-box motifs characteristic for RNA helicases, hNMP 265 is highly similar to the human initiation factors eIF4A-I and -II. On the other hand, hNMP 265 greatly differs from the initiation factors by a N-terminal sequence rich in charged amino acids. Sequence searches and alignments indicate proteins related to hNMP 265 in other eukaryotes. Chimeras between hNMP 265 and green fluorescence protein or hapten appeared as speckles in extranucleolar regions in the nucleus, but not in the cytoplasm. Experiments with tagged deletion mutants indicated that the N-terminal amino acid sequence is necessary for nuclear localization. A putative role of hNMP 265 in pre-mRNA processing is discussed.

In the cytoplasmic compartment of eukaryotic cells initiation of protein synthesis is regulated by several translation initiation factors (eIFs) that are involved in the binding of the 40S and 60S ribosomal subunits to a messenger RNA molecule. Factor eIF4A with RNA helicase activity is required for melting of intramolecular secondary structures within the mRNA [for review, see (1)]. eIF4A—more abundant than ribosomes [for review, see (2)]—is a member of the DEAD-box family of proteins (3). These are characterized by eight motifs, with motif I responsible for ATP binding, motif II for ATPase activity and coupling to helicase, motif III for helicase and motif VI for RNA binding activity [for reviews, see (4, 5)]. Most of the DEAD-box proteins are presumed ATP-dependent helicases that are involved in RNA synthesis, RNA processing and RNA transport. Being highly dependent on the presence of specific factors and substrates, helicase activity has only been documented for some of the known DEAD-box proteins (5).

The filamentous protein framework maintaining the structure of the interphase nucleus has been designated as nuclear matrix [for reviews, see (6, 7)]. In technical terms the nuclear matrix has been described as the insoluble material that resists exposure of isolated nuclei to nucleases, detergents and highly concentrated salt solutions (8). Basic nuclear processes have been reported to proceed in tight association with the nuclear matrix. Thus, the nuclear matrix has been shown to play a role in DNA replication, RNA synthesis, RNA processing, RNA transport, steroid hormone action and signal transduction (6, 7).

In this communication a human nuclear matrix protein (hNMP) is described that is highly similar to the translation initiation factor eIF4A. hNMP 265 was detected in the course of our systematic studies on nuclear matrix proteins (9–14). Contrasting from eIF-4A by its terminal amino acid sequence, the eIF4A-like protein was found localized in the nonnucleolar nuclear region of the cell, and thus divergent from a related yeast protein, Fal1p, known for a role in nucleolar processing of pre-ribosomal RNA (15). Using tagged deletion mutants the influence of the N-terminus of hNMP 265 on its intracellular localization was investigated.

MATERIALS AND METHODS

Cells. Leukocytes of healthy human donors were purified, and human HeLa S3 (ATCC CCL-2.2, cervix adenocarcinoma) cells cultured as described previously (10). Human tissue samples were from

Abbreviations used: GFP, green fluorescence protein; HA, hapten; hNMP, human nuclear matrix protein; MALDI-TOF, matrix-assisted laser-desorption ionization-time-of-flight.

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Preparation of nuclear matrices. Nuclear matrices were prepared from human cells and tissues as described (12). In brief, isolated human nuclei were pelleted through sucrose cushions, exposed to detergents and vanadyl ribonucleoside complexes, sheared in a Potter homogenizer, incubated with DNase I and high-ionic strength buffer, finally yielding the nuclear matrix pellet. Alternatively, nuclear matrices were *in situ* prepared from cells grown on glass slides (16).

Protein analysis. Proteins were resolved by high resolution 2-dimensional polyacrylamide gel electrophoresis, and the patterns of silver-stained spots analyzed by computer-assisted image analysis (9–12). Peptides formed by hydrolysis of blotted proteins were separated by RP-HPLC on a 1.0 × 250 mm Vydac C-18 column (Hewlett-Packard, Waldbronn, Germany), using the Hewlett-Packard 1090 HPLC Series II system. Amino acid sequencing was performed with the Hewlett-Packard G1005A system. Peptide-mass fingerprinting of tryptic digests was carried out on the Hewlett-Packard G2025A MALDI-TOF/MS instrument (10).

Database searches and alignments. Sequence identity searches with the TBLASTN and BLASTP programs (17) were performed in databases at the National Center for Biotechnology Information (NCBI, (Bethesda, MD). For peptide-mass fingerprinting the MS-Fit program was employed, an option of UCSF Mass Spectrometry Facility (U.S.A.) at http://rafael.ucsf.edu. Mass data were analyzed as described, using the PAWS program (10). *Xenopus laevis* XeIF-4AIII (xNMP 265) and *Caenorhabditis elegans* (cNMP 265) were found deposited in GenBank under Accession Numbers 2443810 and 2773184, respectively, and yeast FAL1 in the SwissProt protein sequence data bank under Q12099. Protein sequences were aligned using the MULTALIN program at http://www.toulouse.inra.fr/multalin.html/ (11). For prediction of NLS the programs PSORT and PSORT II were used at http://psort.nibb.ac.jp/ (18, 19).

Preparation and expression of fusion constructs. The human cDNA clone of gene KIAA0111 was a kind gift of Dr. T. Nagase, Kazuka DNA Research Institute, Chiba, Japan (GenBank Accession No. D21853) (20). The complete coding region for hNMP 265 was amplified by PCR using recombinant *Pfu* DNA Polymerase (Stratagene). Primers P1 (5′-ACTGAGCTCTCAGAATCATGGCGACCAC-3′), P2 (5′-GCGGATCCTCAGATAAGATCAGCAAC-3′), P3 (5′-GCGGATCCGATAAGATCAGCAACGTTC-3′) and P4 (5′-ACTGAGCTCTCAGAATCATGACTAAAGTGGAAT-3′) generated restriction sites for enzymes *Sac*I and *Bam*HI (underlined) at the beginning and end of the coding sequence. Restriction fragments of amplified hNMP 265 cDNA with primer pairs P1/P2 and P1/P3 were inserted in frame into the green fluorescent protein expression vectors pEGFP-C1 and pEGFP-N3 (Clontech, Palo Alto, CA), resulting in protein chimeras GFP-hNMP 265 and hNMP 265-GFP consisting of full length hNMP 265 fused to the C and N termini of GFP, respectively.

For construction of the mutant protein chimera (N23C411) lacking the N-terminal 22 aa of hNMP 265, the cDNA was amplified with primer pair P1/P2 and inserted in frame into pEGFP-C1. pGFP-hNMP 265 was cut with *Eco*RI and *Bam*HI, blunted and ligated yielding the mutant protein chimera (N1C27) with the N-terminal 27 aa of hNMP 265. Furthermore, inserting P1/P2 amplified hNMP 265 cDNA in an expression vector, the protein chimera HA-hNMP265 was obtained with the hapten epitope fused to the N terminus of hNMP 265. The hapten epitope tagged vector pTL1-HA0 was obtained from Dr. E. E. Wanker, Max-Planck Institute for Molecular Genetics, Berlin, Germany. For correct in frame fusion it was treated with *Pst*I, with the Klenow fragment and ligated. HeLa S3 cells were transiently transfected with lipofect Tfx-20 (Promega) according to the manufacturer's recommendations.

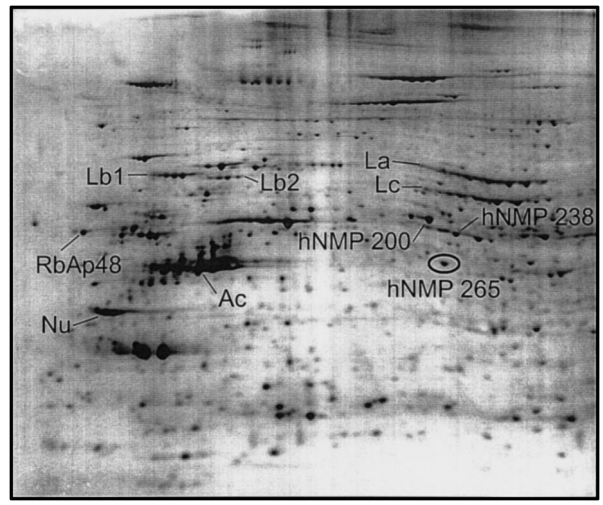

FIG. 1. Position of hNMP 265 in the 2-D electrophoretic pattern of human liver nuclear matrix proteins. hNMP 200, hNMP 238, hNMP 265, RbAp48 (retinoblastoma-binding protein p48), reassembling common nuclear matrix proteins; La, Lb1, Lb2, Lc, nuclear lamins A, B1, B2, C; Nu, numatrin (B23); Ac, Actin.

Immunofluorescence microscopy. Cells grown on glass slides were fixed in 2% formaldehyde in PBS at 20°C for 30 min, and exposed to 50 mM ammonium chloride, 0.1% Triton X-100 in PBS. For detection of HA-tagged proteins, cells were incubated with a monoclonal mouse anti-HA antibody (Boehringer Mannheim, clone 12CA5, 0.4 $\mu g/\mu l$) diluted 1:200 in PBS. The second antibody was goat anti-mouse IgG conjugated to BODIPY FL (Molecular Probes). To stain for DNA, cells were incubated with RNase A and propidiumiodide, and mounted with Mowiol (Calbiochem, U.S.A.). Expressed tagged proteins were assayed in the Bio-Rad MRC 600 confocal microscope and the Nikon inverted Eclipse TE300 microscope with a TE-FM Epi-Fluorescence attachment.

Northern blot analyses. Human normalized RNA dot blots were obtained from Clontech, U.S.A. For other experiments, poly(A)$^+$ RNA was isolated, fractionated on 1.2% agarose gels, and blotted onto nylon membranes (11). Gel-purified [^{32}P]-random-labeled hNMP 265 cDNA probes were employed for hybridization. Images were obtained within linear intensities of 0-750 counts, using a phosphor imager (Molecular Dynamics, U.S.A.).

RESULTS

Identification of hNMP 265 as a common nuclear matrix protein. Two-dimensional electrophoresis of nuclear matrix proteins isolated from various human cells and tissues revealed a prominent spot, situated at 47 kDa, p*I* 6.40. Figure 1 shows the position of the silver-stained protein within the pattern of human liver nuclear matrix proteins. The protein, termed hNMP 265, was found in all cell types investigated and accordingly classified as common nuclear matrix protein. Since computer-assisted matching of gels has been exemplified before (9–12) the data relevant to hNMP 265 are not presented in this communication. A rNMP 265-termed protein, comigrating with the currently presented human protein (not shown) has been demonstrated before as ubiquitously occurring in nu-

clear matrices of rat cells (9). Figure 1 also depicts the position of some other human nuclear matrix proteins previously described (11, 12, 14).

Molecular definition of human NMP 265. N-terminal sequencing of blotted hNMP 265 from human leukocytes failed, suggesting that the N-terminal amino acid was blocked. Edman degradation of two peptides isolated by reverse-phase HPLC from tryptic digests yielded the sequences (R/K)LD(D/Y)X(Q/S)HVV(V/A)G and (R/K)EAN(P/G/F)TV(K/S) (E/A/S)M(S/H). BLAST-program searches revealed identity with the predicted coding sequence of an unidentified human gene, termed KIAA0111 (GenBank Accession No. D21853), isolated from the human male myeloblast cell line KG-1 (20).

The identity of hNMP 265 with the product of gene KIAA0111 was finally proven by MALDI-TOF analysis. The masses of nine from a total of thirteen tryptic peptides of hNMP 265 corresponded to masses calculated from the KIAA0111 sequence (data not shown). Within the 0.06% mass-deviation limit, 27% of the predicted primary protein sequence was covered between positions 52 and 387 of a total of 411 amino acids.

Tentative rat and mouse consensus sequences TC47799 and TC34245, created by assembling ESTs into virtual transcripts (TIGR, http://www.tigr.org/), and mouse EST sequences (GenBank Accession Nos. AA240922, W55838, AA022139, and AA153238) indicate 96% identity of rodent amino acid sequence with human NMP 265 sequence, thereby also explaining the observed co-migration of rat and human proteins on 2D gels (not shown).

Figure 2 depicts the aa sequence of hNMP 265, derived from the nucleotide sequence of gene KIAA0111 (20). The sequence is 66% and 71% identical with those of the human initiation factors eIF4A-I (21) and eIF4A-II (22), respectively. The aligning sequences are shown in Fig. 2. A cDNA sequence differing from that of KIAA0111 by only two bases has been deposited as that of an initiation factor 4A (IF4A)-like protein (SwissProt Accession No. P38919).

The eight DEAD-box motifs in hNMP 265 (Fig. 2) correspond to those of ATP-dependent RNA helicases (5). The influence of small differences in motifs I, V, and VI on enzymatic activity is considered under Discussion.

A database search revealed homologues to hNMP 265 in various eukaryotic organisms down to yeast, indicating the existence of evolutionary conserved, structurally related proteins. Identities and similarities with the human protein were 94% and 95% in the *X. laevis*, 87% and 95% in the *C. elegans* and 61% and 81% in the yeast protein.

Remarkably, the N-terminal region of hNMP 265 significantly differed from those of the human initiation factors IF4A-I and IF4A-II (Fig. 2). Figure 3A shows an underlined sequence of hNMP 265, extending from position 14 to 22. It contains both, a cluster of the basic amino acids lysine (K) and arginine (R), and a cluster of the acidic amino acids glutamic (E) and aspartic acid (D). A comparably charged region does not exist in initiation factors of human or other origin (Fig. 3B). However, related, charged sequences were found in hNMP 265-like proteins of other eukaryotes, as shown for proteins of *X. laevis* and *C. elegans* (Fig. 3A). Remarkably, in FAL1p, a IF4A-like protein of yeast, the majority of basic amino acids were differently positioned than in the hNMP 265-like proteins.

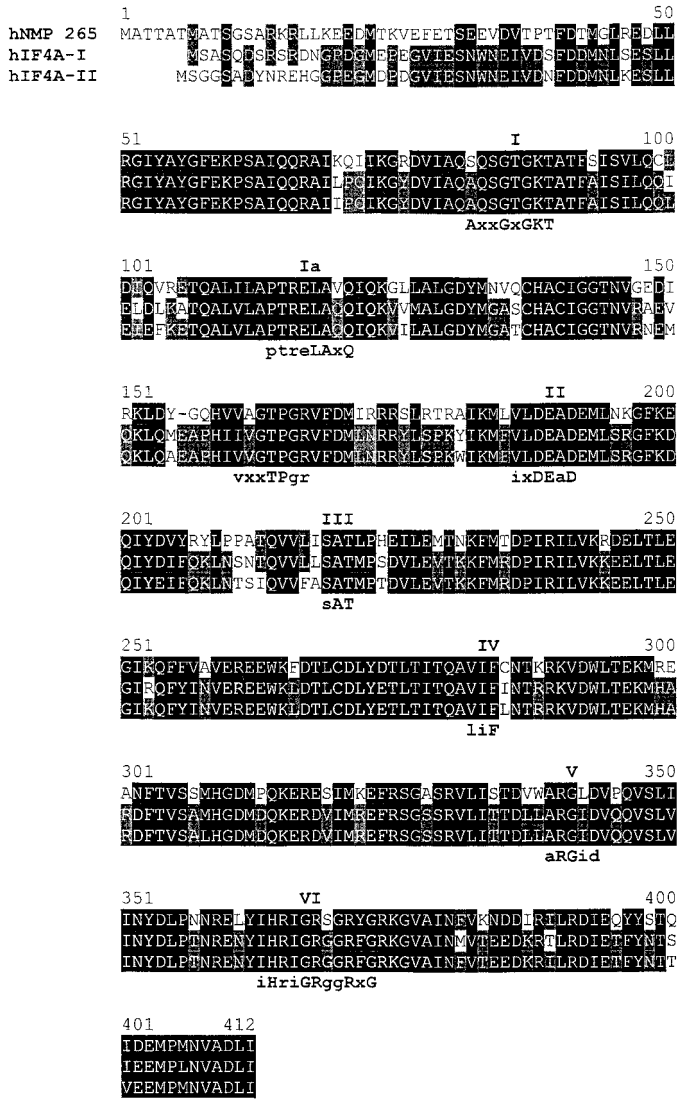

FIG. 2. Sequence of hNMP 265 compared to those of human eIFA4-I and eIF4A-II. Similarities: 50–90% in gray, >90% in black boxes. Consensus sequences below the aligned sequences represent the eight conserved motifs characteristic of DEAD-box proteins. The hNMP 265 sequence was deduced from GenBank Accession No. D21853; the eIF4A-I and eIF4A-II sequences were deduced from SwissProt Accession Nos. P04765 and Q14240, respectively.

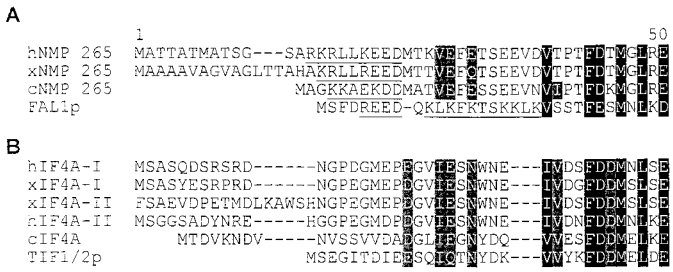

FIG. 3. N-terminal sequence analysis of (A) eIF4A-like proteins and (B) eIF4A-type translation initiation factors. Regions rich in charged basic and acidic amino acids, putatively responsible for nuclear localization, are underlined. h, human; x, *Xenopus laevis;* c, *Caenorhabditis elegans;* FAL1p, TIF1p and TIF2p, yeast proteins.

Ubiquitous transcription of the hNMP 265 gene. Northern blotting experiments were performed to test presence of messenger RNA for hNMP 265 in various cell types. Human normalized RNA dot blots containing poly(A)$^+$ RNA samples of 40 distinct human tissues and cells, including 6 of fetal origin, were hybridized with [^{32}P]-labeled cDNA of human hNMP 265. All of the blotted spots interacted with the labeled cDNA, thus further confirming the ubiquitous expression of the hNMP 265 gene (not shown). In addition, poly(A)$^+$ RNA was extracted from three types of cultured human cells. After electrophoresis and blotting, the [^{32}P]-labeled probe detected RNA of ~1.7 kb length (Fig. 4), correlating with the full-length of hNMP 265 mRNA (Fig. 2).

Intracellular localization of hNMP 265. To determine the intracellular localization of hNMP 265, green

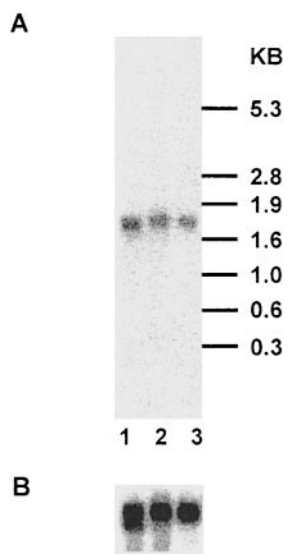

FIG. 4. hNMP 265 mRNA detected by Northern blotting. Poly(A)$^+$ RNA extracted from human HeLa S3 (lane 1), K-562 (lane 2) and Caki-2 cells (lane 3) was separated on 1.2% agarose gels. (A) Hybridization with a hNMP 265 [^{32}P]-cDNA probe revealed an ~1.7 kb mRNA in all samples. (B) Control hybridization with ubiquitin [^{32}P]-cDNA.

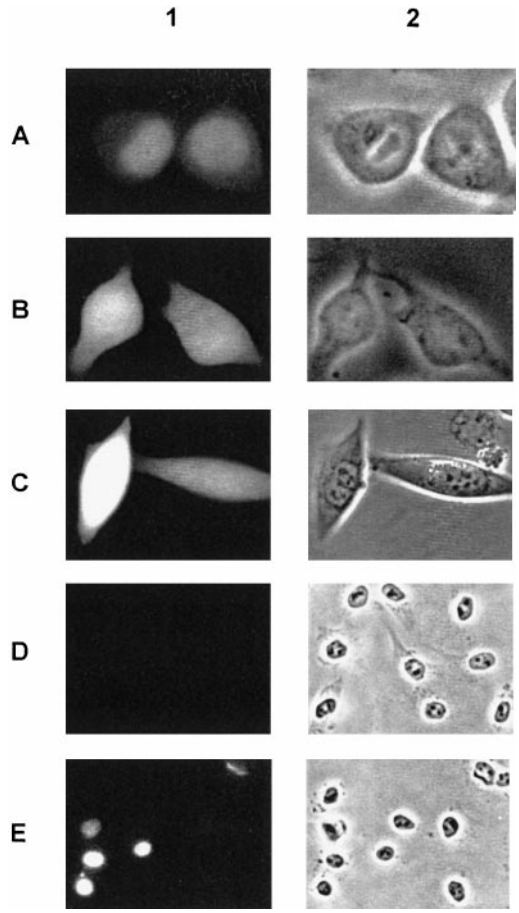

FIG. 5. Intracellular localization of hNMP 265 and effect of the N-terminal aa sequence. (A–C) Whole HeLa S3 cells. (D, E) Nuclear matrices *in situ* prepared from transfected cells. 1, fluorescence microscopy. 2, phase contrast microscopy. A and E, transfection with GFP-hNMP 265. B and D, control, transfection with pEGFP-C1. C, transfection with a mutant of GFP-hNMP 265 deficient of the first N-terminal 21 amino acids of hNMP 265, rich in charged amino acids.

fluorescent protein-tagged and hapten-tagged hNMP 265 were transiently expressed in HeLa cells. Formation of the fusion proteins was confirmed by immunoblotting (not shown).

Immunofluorescence microscopy of whole cells revealed that the GFP-hNMP 265 chimera was predominantly localized in the nuclear region (Figs. 5A and 6B). Similar results were obtained with the C-terminally tagged protein hNMP 265-GFP (not shown) and with the N-terminally hapten-tagged protein HA-hNMP265 (Fig. 6A). In contrast, control cells solely expressing GFP displayed dispersed staining of whole cells (Fig. 5B).

In nuclear matrices isolated from pGFP-hNMP 265-transfected cells the protein chimera was detected (Fig. 5E), while no signal was recognized in control nuclear matrices isolated from pGFP-transfected cells (Fig. 5D). These data demonstrate that localization of the

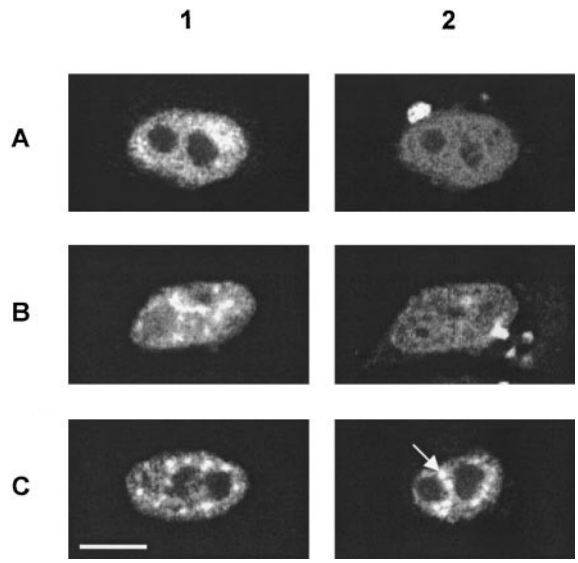

FIG. 6. Intranuclear localization of hNMP 265. Laser scanning confocal fluorescence microscopy. A, B, whole cells. C-1, nuclear matrices isolated 24 h posttransfection; C-2, nuclear matrices isolated 48 h post transfection. A-1, immunostained hapten-tagged hNMP 265 (HA-hNMP 265); B-1, C-1, C-2, GFP-tagged hNMP 265 (GFP-hNMP 265) expressed in HeLa S3 cells. A-2, B-2, propidium iodide staining for DNA, corresponding to A-1, B-1. Bar, 10 μm.

tagged proteins in the nucleus and in the nuclear matrix depended on the hNMP 265 moiety of the protein chimera.

In order to investigate whether the N-terminal region of hNMP 265 affected the intracellular localization of the protein, a deletion mutant (N23C411) was employed extending from aa position 23 to 411 of the original intact molecule. Figure 5C shows that the mutant protein, devoid of the sequence rich in charged amino acids, was not confined to the nucleus. Like expressed GFP (Fig. 5B), it was distributed over the entire cellular region. The GFP-tagged mutant peptide (N1C27) consisting of the 27 N-terminal aa of hNMP 265 was also distributed throughout the cell (not shown). It thus appears that the N-terminal amino acid sequence of hNMP 265 is necessary, but not sufficient for restriction of the protein to the nucleus.

Confocal microscopy revealed diffuse to punctuate nuclear staining of hapten-tagged hNMP 265 in the non-nucleolar region of the nucleus (Fig. 6A). Tracking GFP-tagged hNMP 265, punctuate signals were seen in the nucleoplasm of whole cells and in nuclear matrices *in situ* prepared from transfected cells (Figs. 6B and 6C). Apparently, fluorescent protein spots were preferentially localized at perinucleolar sites (Fig. 6C).

DISCUSSION

The present study shows that the common nuclear matrix protein hNMP 265 occurring in all human cell types investigated is identical with a predicted protein of unknown function encoded in gene KIAA0111 (20). The amino acid sequence of the protein is highly similar to the sequence of the human initiation factors eIF4A-I and -II. These factors are involved in binding of mRNA to the ribosomal 43S preinitiation complex and exhibit RNA duplex unwinding activity (1).

The DEAD-box motifs suggest an ATP-dependent RNA helicase activity of hNMP 265. The deviations observed in some of the DEAD-box motifs of hNMP 265 would not be expected to abrogate that enzymatic activity. Thus, the yeast proteins Dbp5p and Prp5p show helicase (23) and RNA stimulated ATPase activities (24), respectively, though having serine instead of alanine in the ATPase motif (motif I), as has hNMP 265. Likewise, several DEAD-box proteins have isoleucine substituted for leucine in motif V (5), and Fal1p has glycine substituted for serine in motif VI (15). In our experiments polyribonucleotide-stimulated ATPase activity of recombinant hNMP 265 was observed, while ATP-dependent RNA helicase activity was not detectable (not shown). However, unknown regulatory factors might be required for enzymatic activity, as, e.g., factor eIF4B for helicase activity of eIF4A (21, 25–27).

The eIF4A- and hNMP 265-like yeast protein Fal1p which is predominantly localized in the nucleolar region has been reported to be essential for processing of pre-ribosomal RNA (21). Therefore, hNMP 265 presently found localized in the non-nucleolar region of the nucleus, might putatively be involved in processing of pre-mRNA. Indeed, many heterogeneous nuclear ribonucleoproteins (hnRNPs) and splicing factors, which play a role in the processing and splicing of pre-mRNA, have been identified as essential constituents of the nuclear matrix (6, 7, 10, 28, 29). Thus, a hNMP 265-mediated RNA helicase activity might be required for RNA processing at that intranuclear site.

Using various categories characteristic for nuclear localization [for review, see (30)], the PSORT II program predicted cytoplasmic localization for hNMP 265. However, immunofluorescence microscopy of whole cells and isolated nuclear matrices, and the presence of the protein in nuclear matrix preparations indicate nuclear localization of hNMP 265. Experiments with the tagged deletion mutant demonstrated that the 22 N-terminal amino acids confined hNMP 265 to the nucleus. If deleted, the protein was distributed in both, the nuclear and cytoplasmic regions. Judging from the molecular mass (approximately 70 kDa) the GFP-tagged deletion mutant would not be expected to diffuse passively into the nucleus. The N-terminal region of hNMP 265 might thus be involved in nuclear retardation, rather than nuclear import of the protein.

Comparing hNMP 265 and hNMP 265-like proteins with the ribosomal translation initiation factors eIF4A and TIF1/2p, the N-terminal regions imposed as most divergent (Fig. 3), in contrast to the main portion of the

molecules with conserved DEAD-box motifs (Fig. 2). Apparently, a region rich in charged amino acids positioned close to the N-terminus of the proteins is characteristic for the group of hNMP 265-like proteins (Fig. 3A). Remarkably, in the yeast protein Fal1p of nucleolar localization the relative position of the major basic amino acids in the N-terminal region differed from that of the other hNMP 265-like proteins. It remains further to be investigated whether these peculiarities of intranuclear localization depend on specificities of the charged region.

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