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Identification and Characterization of the Ubiquitously Occurring Nuclear Matrix Protein NMP 238

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By systematic comparison of two-dimensional electrophoretic patterns of nuclear matrix proteins an ubiquitously occurring (common) nuclear matrix protein, termed NMP 238, was detected. Localization of the protein in isolated nuclear matrices and in nuclear and cytoplasmic regions of cells was determined by confocal immunofluorescence microscopy. N-terminal protein sequencing, mass spectrometry, and sequencing of a human EST cDNA clone showed identity of the protein with a nuclear protein, termed TIP49, of as yet uncertain function. Expression of the corresponding gene in diverse human and rat cells was confirmed by Northern blotting. The protein displays two nuclear localization signals. Sequence homologies indicate evolutionary related proteins in nematodes, yeast, and archaebacteria. Similarities to the AAA family of proteins and to a subgroup of chaperones suggest that the nuclear matrix protein may play a role in the assembly and ATP-dependent anchorage of proteins.

The nuclear matrix is conceived to constitute the intranuclear filamentous protein framework maintaining the structure of the interphase nucleus [for reviews, see (1, 2)]. In biochemical investigations the nuclear matrix has been defined as the insoluble material resisting treatment of isolated nuclei with detergents, DNase and high ionic strength salt solutions (3). Important nuclear processes have been found to proceed in tight association with the nuclear matrix. Data has been presented suggesting a role of the nuclear matrix, amongst others, in DNA replication, DNA transcription, RNA processing, RNA transport, steroid hormone action and signal transduction [for reviews, see (1, 2)].

The currently described ubiquitously occurring nuclear matrix protein, termed NMP 238, was detected in the course of systematic studies aimed to differentiate common from cell type-specific or cell state-dependent nuclear matrix proteins by two-dimensional gel electrophoresis (4–7). Sequence analyses of the cloned human cDNA revealed identity with a protein of as yet uncertain function which has been found in a nuclear multiprotein complex containing the TATA box binding protein (TBP) (8, 9). The present data shows that NMP 238 is an evolutionary conserved protein potentially mediating the assembly and interaction of nuclear proteins. This is of interest, since mechanisms involved in the constitution of the nuclear framework and its association with reacting complexes have not yet been unequivocally elucidated.

MATERIALS AND METHODS

Cells. The cultured human cells employed were HeLa S3 (ATCC CCL-2.2, cervix adenocarcinoma), Jurcat (ATCC TIB 152, acute T cell leukemia), KB (ATCC CCL-17, epidermoid carcinoma), K-562 (ATCC 45506, chronic myelogenous leukemia) and Caki-2 (ATCC HTB-47, kidney epithelial carcinoma) cells from the American type culture collection, TCL-598 (kidney epithelial tumor) and UAC (epithelial type human amniotic) cells. Leukocytes of healthy human donors were purified as described before (5). Human tissue samples were kindly provided by Dr. P. Obrist and Dr. C. Ensinger, University of Innsbruck, Austria.

Isolation and analysis of nuclear matrix proteins. Preparation of nuclear matrices, analysis of nuclear matrix proteins by high resolution two-dimensional electrophoresis, computer-assisted image analysis, sequencing of blotted proteins and mass analysis of peptides were performed as described previously (5, 7). In brief, isolated nuclei were pelleted through sucrose cushions, exposed to vanadyl ribonucleoside complexes, detergents, DNase I and high-ionic strength buffer, finally yielding the residual nuclear matrix. *In situ* prepared nuclear matrices were obtained from cells grown on glass slides for 24 h (10). Nuclear matrix proteins were resolved by two-

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Abbreviations used: hNMP238, human nuclear matrix protein 238; rNMP238, rat nuclear matrix protein 238; MALDI-TOF, matrix-assisted laser-desorption ionization-time-of-flight.

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank databases under Accession No. AJ010058.

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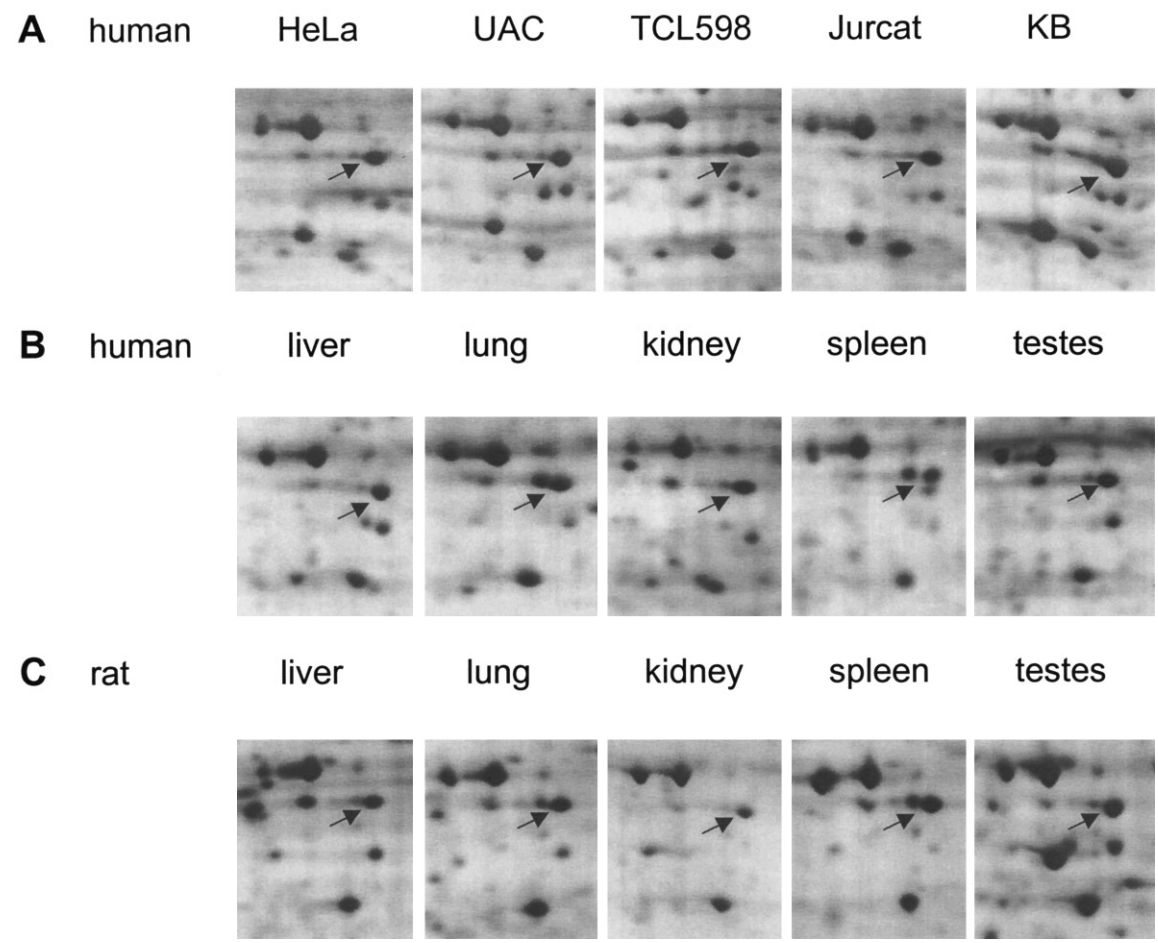


FIG. 1. Identification of human and rat NMP 238 as common nuclear-matrix proteins. 2D-electropherograms of nuclear matrix proteins. Comparable regions (p*I* 6.30–6.60, 45–65 kDa) of individual gels are presented. The area depicted is indicated in Fig. 2A. Other conditions as described under Materials and Methods. (A) Human cell lines: HeLa, adenocarcinoma; UAC, amnion; TCL598, kidney epithelial tumor; Jurcat, T cell leukemia; KB, mouth epidermoid carcinoma. (B) Human tissues and (C) rat tissues, as indicated. Arrows mark the positions of spot hNMP 238 and rNMP 238, respectively.

dimensional electrophoresis and blotted onto poly(vinylidene difluoride) membranes. For N-terminal amino acid sequencing of blotted proteins by Edman degradation the Hewlett–Packard G1005A protein-sequencing system was used. Peptide-mass fingerprinting of tryptic digests was carried out on the Hewlett–Packard G2025A MALDI-TOF/MS instrument.

Database searches and alignments. Sequence identity searches with the TBLASTN and BLASTP programs (11) were performed in databases at the National Center for Biotechnology Information (NCBI) (Bethesda, MD). The PROPSEARCH program (12) was used at http://www.expasy.ch/. Alignments were made, using MULTALIGN, multiple alignment version 5.3.3. of the Institute of Biology and Chemistry of Proteins, Lyon, France at http://www.toulouse.inra.fr/multalin.html (13). For prediction of NLS the program PSORT version 6.4 was used at http://psort.nibb.ac.jp/ (14). The dendrogram of the phylogenetic tree was constructed by distance analysis, using the PILEUP and DISTANCE programs of the GCG software package (Program Manual for the Wisconsin Package, Version 8, Genetics Computer Group, Madison, WI). The Kimura protein distance correction of the DISTANCE program was used. Distances are based on estimated number of substitutions per 100 amino acids. The BESTFIT program used for calculation of identities and similarities was part of the GCG package.

DNA sequencing and in vitro expression. Human EST clones No. 591284 (IMAGp998B211430) and No. 591307 (IMAGp998C201430) with NCBI/Genbank accession numbers AA158977 and AA158998, respectively) originated from a pancreas cDNA library (#937208, Stratagene). The clones were obtained from the German Human Genome Project Resource Center (Berlin, Germany) (15). Both strands of cDNA clone 591284 were sequenced. *In vitro* transcription and translation were performed in the presence of [^{35}S]methionine in 50 μl volume according to the manufacturer's protocol for the TNT coupled reticulocyte lysate system (Promega, Madison, WI). The EST cDNA construct was inserted between the EcoRI and XhoI sites of pBS SK- phagemid (16). For comigration experiments 3 μl of the reaction mixture were added to 50 μg nuclear matrix protein samples prior to 2D electrophoresis. Synthesized [^{35}S]methionine-labeled proteins were detected by autoradiography.

Northern blot analyses. Poly(A)$^+$ RNA was isolated, using the mRNA-Isolation-Kit of Boehringer Mannheim, Germany. Two-microgram samples were fractionated on 1.2% agarose gels containing 2.2 M formaldehyde, and transferred to positively charged nylon membranes (Amersham, UK). Human normalized RNA dot blots were obtained from Clontech, USA. Gel-purified [^{32}P]-random-labeled hNMP 238 cDNA probes were employed for hybridization.

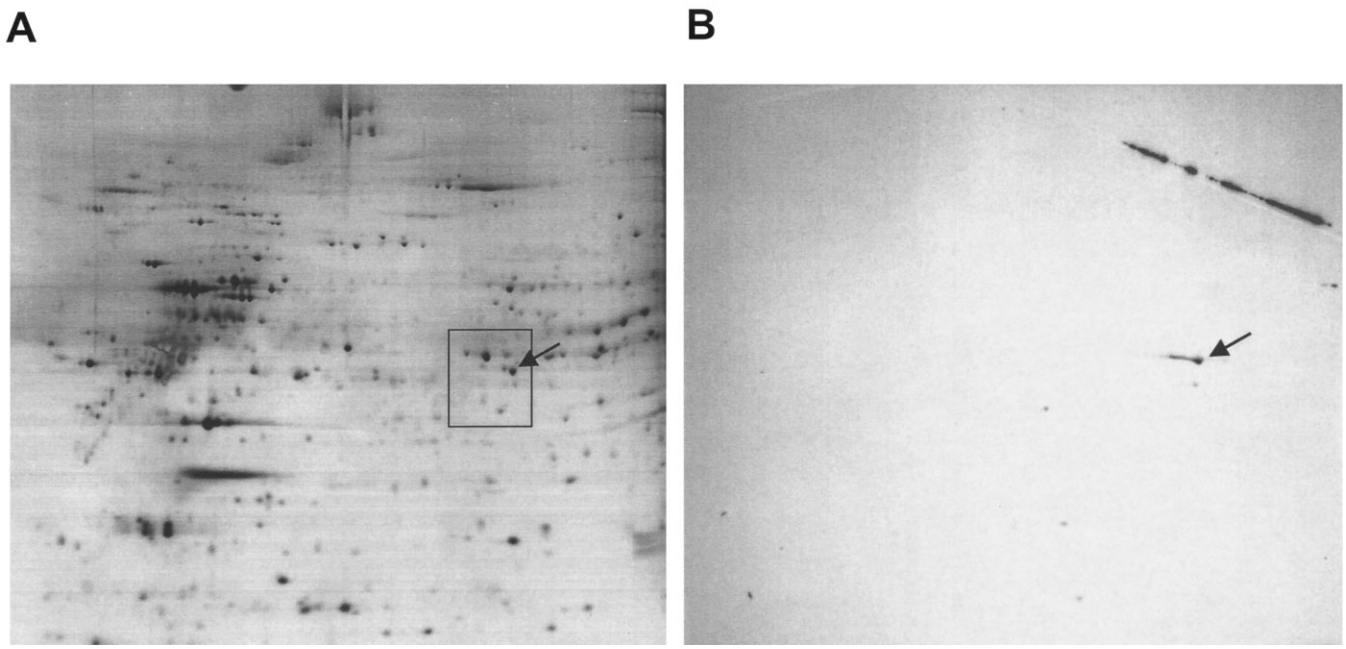


FIG. 2. Co-migration of hNMP 238 and the expressed cDNA-encoded protein. 2D electrophoresis of a mixture of the synthesized [^{35}S]-labeled protein and nuclear matrix proteins of human leukocytes. *In vitro* transcription and translation of the protein encoded in EST clone 591284 as described in Materials and Methods. (A) Silver staining; (B) autoradiography. Arrows indicate the position of hNMP 238. In rectangle, area depicted in Fig. 1.

Images were obtained within linear intensities of 0-750 counts, using a phosphor imager (Molecular Dynamics, CA).

Immunofluorescence microscopy. Cells grown on glass slides and *in situ* prepared nuclear matrices were fixed in 2% formaldehyde in PBS at 20°C for 30 min, and exposed to 50 mM ammonium sulfate, 0.1% Triton X-100 in PBS. The affinity-purified rabbit anti-rat-TIP49 antibody (9) was 1:100 diluted with 2% BSA in PBS, and the secondary goat anti-rabbit-IgG antibody conjugated to Texas Red (Jackson ImmunoResearch Laboratories, West Grove, PA) was 1:150 diluted with PBS. DNA was stained with Hoechst 33258 (Calbiochem, La Jolla, CA) and cells and matrices mounted with Mowiol (17). The Nikon inverted microscope Eclipse TE300 with a TE-FM Epi-Fluorescence attachment and the Bio-Rad MRC 600 confocal microscope were used.

RESULTS

Identification of NMP 238 as common nuclear matrix protein. To search for ubiquitously occurring (common) nuclear matrix proteins, nuclear matrices were prepared from various types of human cells and tissues. After 2-dimensional protein electrophoresis and silver staining, the resulting images were analyzed with the help of the BioImage Investigator software program. As shown in Figures 1A and 1B a spot termed human nuclear matrix protein 238 (hNMP 238) prominently appeared at 54 kDa, p*I* 6.50 in samples isolated from diverse tissues and cell lines. Since the protein was, in addition, found in the nuclear matrix of other human normal and cancer tissues and cells (not shown), it was classified as common nuclear matrix protein.

In a previous study we have reported about the finding of common nuclear matrix proteins in rat tissues (4). One of the spots was located in the same position as hNMP 238 in the 2D gels. Upon electrophoresis of a mixture of human and rat nuclear matrix samples the spots comigrated (not shown). Figures 1B and 1C allow comparison of the human and rat spot patterns. While some spots differed depending on the cell type and species of origin, the appearance and position of hNMP 238 and rat nuclear matrix protein 238 (rNMP 238)

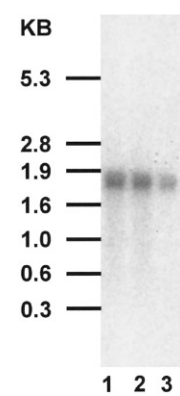


FIG. 3. Expression of the hNMP 238 gene determined by Northern blotting. Poly(A)$^+$ RNA extracted from HeLa S3 (lane 1), K-562 (lane 2) and Caki-2 cells (lane 3) was separated on 1.2% agarose gels. Hybridization with a ^{32}P-labeled hNMP 238 cDNA probe revealed a ~1.8-kb mRNA in all samples.

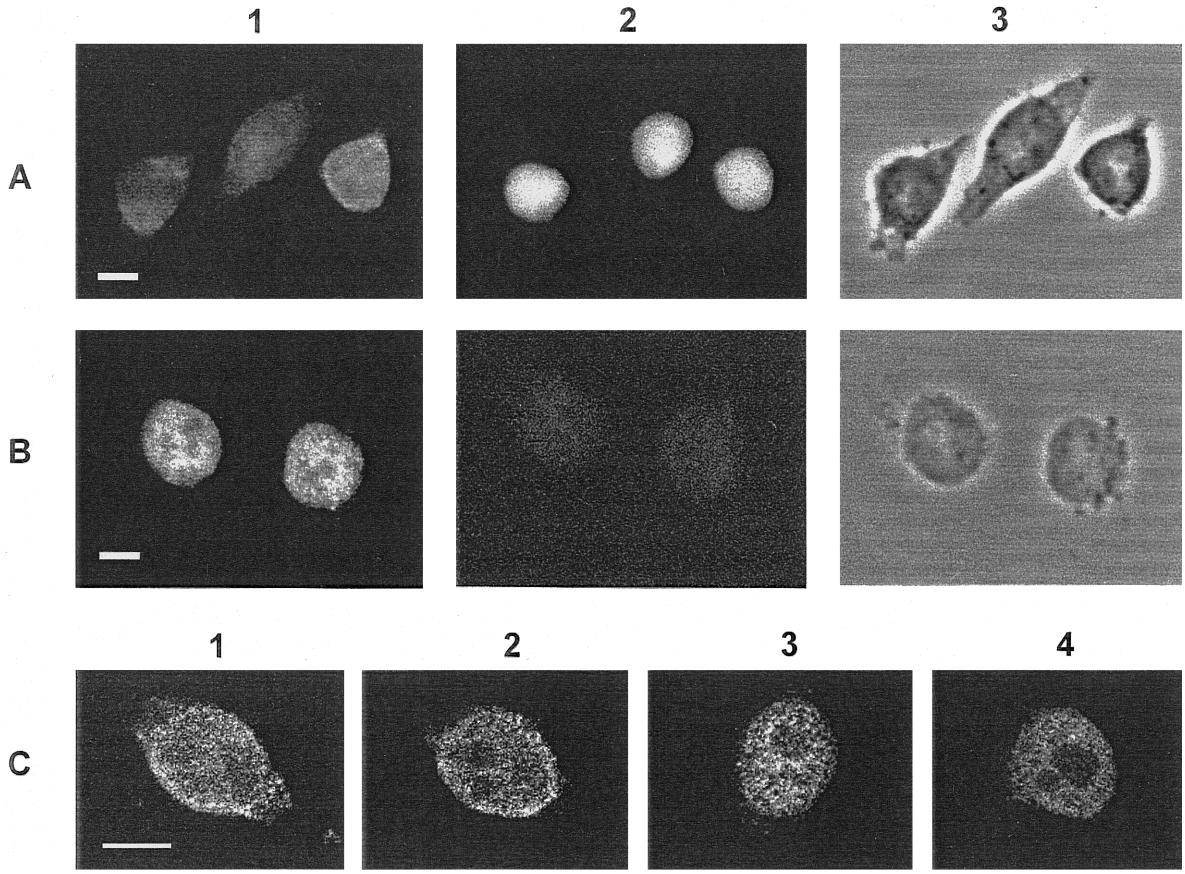


FIG. 4. Intracellular localization of hNMP 238 in HeLa S3 cell by immunofluorescence microscopy. Double-staining, using polyclonal anti-TIP49 antibody for hNMP 238, and Hoechst 33258 for DNA detection. A-1,2,3, C-1,2, whole cells. B-1,2,3, C-3,4, *in situ* prepared nuclear matrices. A-1, B-1, C-1,2,3,4, hNMP 238 staining. A-2, B-2, DNA staining. A-3, B-3, phase-contrast microscopy. C-1,2,3,4, laser scanning confocal microscopy. Bars, 10 μm.

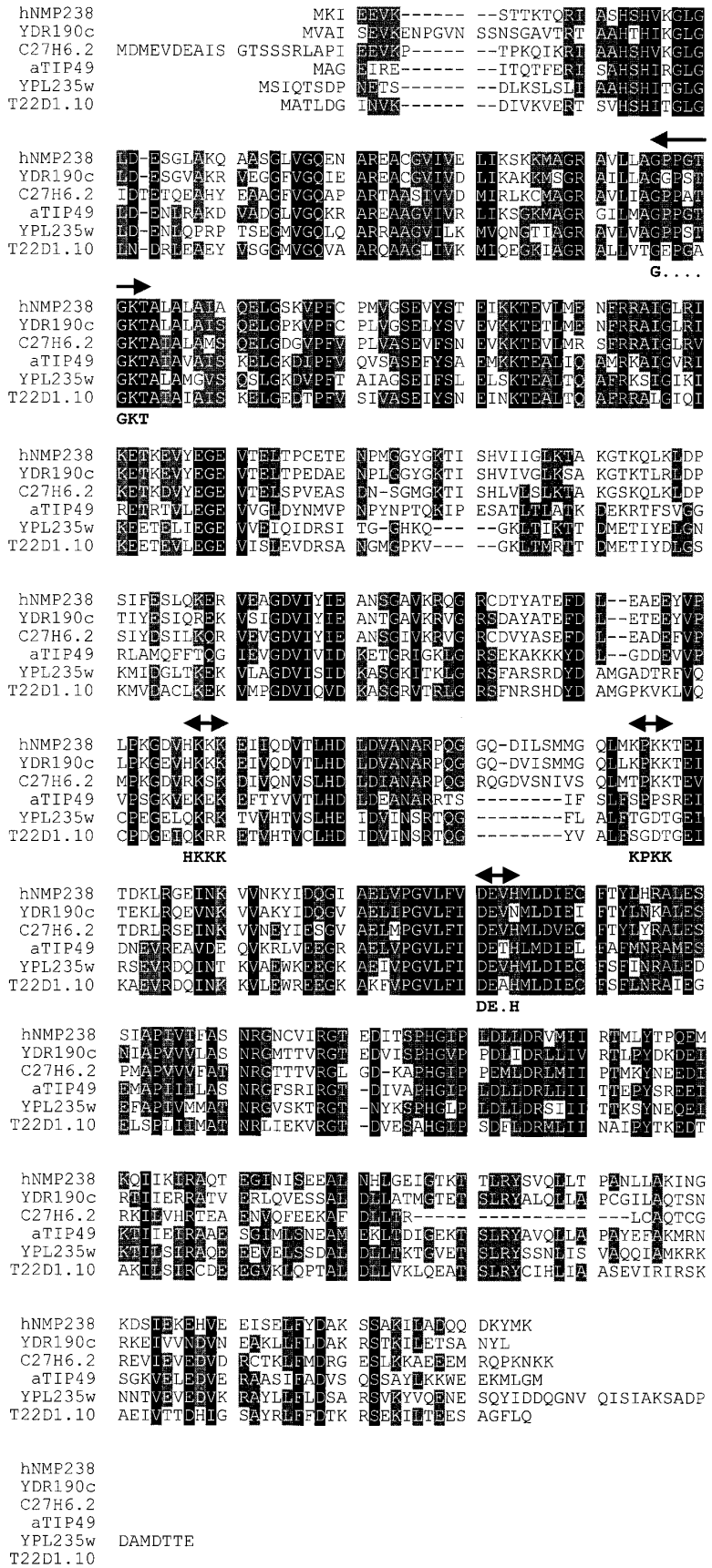
were identical. This may be interpreted as signifying a protein of basic function.

Molecular definition of human NMP 238. N-terminal sequencing of blotted hNMP 238 of human leukocytes revealed the amino acid sequence MKIEEVK. Database searches indicated two human EST cDNA clones encoding the above sequence (clones 591284 and 591307). Complete analysis of both strands of clone 591284 revealed a DNA sequence identical to that of the recently described human TIP49 sequence (9) (not shown). The fact that the human protein of 456 aa differed from the corresponding rat protein (8) by only one amino acid, while the nucleotide sequence differed by 9.7%, may be taken as indicative for an evolutionary highly conserved protein. Both, the calculated molecular weight (50 kDa) and the p*I* (6.02) of the human protein were close to the experimentally determined values for spot hNMP 238 (Fig. 2A).

To test whether clone 591284 encoded protein hNMP 238, the cDNA was *in vitro* expressed by transcription and translation. Comigration experiments were performed with samples consisting of the synthesized radiolabeled protein and of nuclear matrix proteins. Figure 2A shows the silver-stained nuclear matrix protein spots of human leukocytes, and Fig. 2B the gene product, as detected by autoradiography. Colocalization of the labeled and of the stained spots in the 2D gels confirmed the identity of the recombinant protein with hNMP 238.

Finally, the identity of hNMP 238 was proven by MALDI-TOF analysis. The masses of 17 tryptic pep-

FIG. 5. Homologies between human NMP 238 and proteins of other organisms. aa sequences of hNMP 238 derived from cDNA clone 59248 and (9) were aligned with those of yeast proteins YDR190c and YPL235w (PIR locus S52698 and S61029), of *Caenorhabditis elegans* proteins C27H6.2 (EMBL Accession No. Z81042) and T22D1.10 (Genbank Accession No. AF039052), and of *Archaeoglobus fulgidus* aTIP49 protein (Genbank Accession No. AE000977). Similarities: 50–90% in gray, >90% in black boxes. Walker A motifs (Gx4GKT) for ATP/GTP-binding, Walker B motifs (DExH) for ATPase/GTPase activity, and nuclear localization signals (NLSs) (HKKK, KPKK) are indicated by arrow-lines.


hNMP238
YDR190c
C27H6.2
aTIP49
YPL235w
T22D1.10
G....
GKT
HKKK
KPKK
DE.H

tides of hNMP 238 corresponded to masses calculated from the coding sequence of clone 591284 (data not shown). Within the 0.2% mass-deviation limit, 49% of the primary protein sequence was covered.

Ubiquitous transcription of the hNMP 238 gene. To test for *in vivo* expression of the encoded gene, poly(A)$^+$ RNA was isolated from three cell lines and separated by electrophoresis. Using radiolabeled hNMP 238 cDNA for hybridization, a ~1.8-kb RNA was detected in each of the samples, thereby demonstrating that hNMP 238 cDNA encoded the full-length sequence of hNMP 238 mRNA (Fig. 3). Furthermore, employing human normalized RNA dot blots, the mRNA was detected in poly(A)$^+$ RNA samples of 40 distinct human tissues and cells, including 6 of fetal origin (not shown). This accorded with the ubiquitous presence of the protein, demonstrated before by 2D protein electrophoresis.

Intracellular localization of hNMP 238. By immunofluorescence microscopy, hNMP 238 was found to be mainly localized in the nuclear region of the cell (Fig. 4, A-1, C-1,2), as reported before by Makino *et al.* (9). However, the protein was also present in the cytoplasm, which was confirmed by immunoblot analysis of cytosol preparations (not shown). Confocal microscopy revealed punctuate signals in the nucleoplasm of whole cells and, as expected, in *in situ* prepared nuclear matrices (Fig. 4, C-3,4), with nucleolar exclusion.

hNMP 238-related proteins. Analysis with the TBLASTN program revealed great similarities between hNMP 238 and putative full-length proteins of as yet unknown function, encoded in *Caenorhabditis elegans*, yeast and bacterial DNA. The alignments of hNMP 238 with proteins of other organisms are shown in Fig. 5. The dendrogram indicates a family of homologous proteins, the identities of aa sequences between hNMP 238 and the above proteins ranging from 39 to 70 per cent (Fig. 6). A subclass consisting of the archaebacterial protein aTIP49, the *C. elegans* protein T22D1.10 and of yeast protein YPL235w apparently diverged early during evolution. The existence of a mammalian homologue to members of that subclass is indicated by the finding of corresponding human and mouse EST clones (not shown). Figure 5 also shows the position of Walker A and B motifs and of two nuclear localization signals in the hNMP 238 sequence.

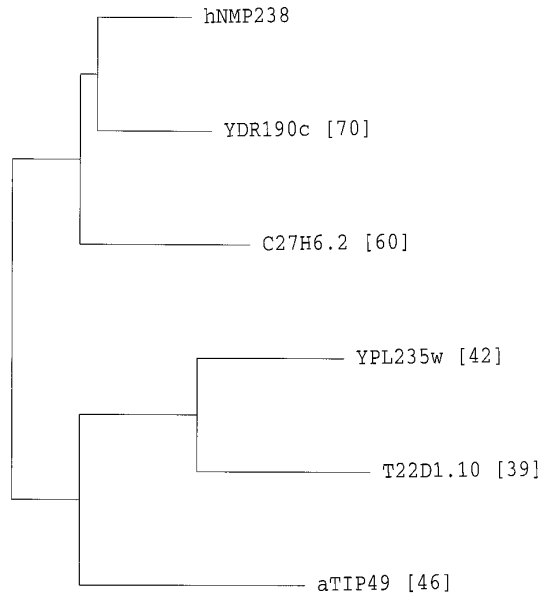


FIG. 6. Phylogenetic tree of hNMP 238 and related proteins. Percent identity with hNMP 238 in brackets. Alignments as described under Materials and Methods.

DISCUSSION

As regards the potential function of nuclear matrix protein hNMP 238, the Walker A (Gx4GKT) and Walker B (DExH) motifs (18) characteristic for ATP binding and A/GTPase sites are of interest (Fig. 5). By these and other criteria, hNMP 238 relates to a group termed as AAA family or, alternatively, as CAD family of proteins, AAA designating "ATPases associated with diverse cellular activities" (19) and CAD "conserved ATPase domain" (20). Members of these families have, amongst others, been suggested to be involved in ATP-dependent anchorage of proteins (for review, see (21)).

Furthermore, hNMP 238 was classified as being related to subunits of T-complex protein 1 (TCP-1)-containing cytosolic chaperones [for review, see (22)]. This resulted, with 99.6% reliability, from comparison of hNMP 238 with seven distinct subunits (23–29) in the PROPSEARCH program. Neglecting the order of aa residues, the program uses 144 other properties to detect structural and functional homologues. In the present context it is of interest that the subunit TCP-1 gamma has been reported as also being localized in the nuclear matrix (30). Remarkably, both hNMP 238 and TCP-1 gamma contain the same NLS motif (HKKK) (Fig. 5), which is not present in other TCP-1 subunits. Moreover, both proteins have been described as occurring in 800-kDa complexes (9, 30). As regards to their function, molecular chaperons are considered to assist folding and oligomeric assembly of proteins in ATP-dependent reactions [for review, see (22)]. Since mechanisms for the assembly of a nuclear framework have not yet been elucidated, the characteristics of the presently described common nuclear matrix protein are of particular interest.

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