The Newly Identified Human Nuclear Protein NXP-2 Possesses Three Distinct Domains, the Nuclear Matrix-binding, RNA-binding, and Coiled-coil Domains*

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Using a monoclonal antibody that recognizes a nuclear matrix protein, we selected a cDNA clone from a ∼gt11 human placenta cDNA library. This cDNA encoded a 939-amino acid protein designated nuclear matrix protein NXP-2. Northern blot analysis indicated that NXP-2 was expressed in various tissues at different levels. Forcibly expressed green fluorescent protein-tagged truncated mutants of NXP-2 as well as endogenous NXP-2 was localized in the nucleus and distributed to the nuclear matrix. NXP-2 was released from the nuclear matrix when RNase A was included in the buffer for nuclear matrix preparation. Mapping of functional domains was carried out using green fluorescent protein-tagged truncated mutants of NXP-2. The region of amino acids 326–353 was responsible for nuclear matrix binding and contained a cluster of hydrophobic amino acids that is similar to the nuclear matrix targeting signal of acute myeloleukemia protein. The central region (amino acids 500–591) was demonstrated to be required for RNA binding by Northern analysis, although NXP-2 lacked a known RNA binding motif. The region of amino acid residues 682–876 was predicted to have a coiled-coil structure. The RNA-binding, nuclear matrix-binding, and coiled-coil domains are structurally separated, suggesting that NXP-2 plays important roles in diverse nuclear functions, including RNA metabolism and maintenance of nuclear architecture.

The nuclear matrix is involved in the structural organization of chromatin and the integrity of the nucleus (1–3). In addition, DNA replication, RNA processing, and gene transcription have been suggested to be associated with the nuclear matrix. There are many reports of chromatin binding to the nuclear matrix during replication (4–10), as well as the enrichment of transcribed genes in this nuclear subcompartment (11–15). The DNA–nuclear matrix interaction seems to be mediated by chromatin-associated proteins such as topoisomerase II (3), matrix-associated binding proteins such as hnRNPU (16), and SATB1 (17). These proteins are capable of binding to A/T-rich DNA regions (3, 16, 17). It was reported that the hSWI/SNF protein complex involved in the remodeling of chromatin during gene activation could be associated with the nuclear matrix attachment region (18). The nuclear matrix may act as an active structure on which gene expression takes place (for review, see Ref. 9). On the basis of experimental observations, it has been suggested that actively transcribing nucleotide-protein complex is associated with the nuclear matrix (7, 19–22) and that posttranscriptional processing of nascent transcripts takes place in association with the nuclear matrix (23, 24). Recently, protein mass spectrometry of the interchromatin granule, a subfraction of the nuclear matrix, identified many RNA-binding proteins involved in RNA processing (25).

These results suggest that the nuclear matrix constitutes various dynamic nuclear substructures involved in diverse nuclear functions. To clarify the functional roles of the nuclear matrix, more protein components of nuclear matrix substructures should be identified and characterized. We carried out the immunoscreening of a human placenta cDNA library with antibodies to total nuclei or nuclear matrix proteins, and we isolated two clones coding for nuclear matrix proteins NXP-1 and NXP-2, which were composed of 631 and 939 amino acids, respectively. A data base analysis indicated that NXP-1 was identical to cohesin (26) but that NXP-2 was an unknown protein. In this article, we report that NXP-2 is a nuclear matrix-associated protein with RNA binding activity. The RNA binding activity and nuclear matrix binding of NXP-2 suggest a role in RNA processing. The sequence similarity with other nuclear RNA-binding proteins is low, indicating that NXP-2 might have a novel function in nuclear RNA metabolism.

EXPERIMENTAL PROCEDURES

Preparation of Monoclonal Antibody—A nuclear scaffold was prepared from rat liver as described by Paulson and Laemmli (27). The nuclear scaffold fraction (10 mg) was mixed with complete Freund’s adjuvant and injected into BALB/c mice six times at 2-week intervals. After the immunization, the spleen was excised, and hybridomas were prepared using conventional methods. One of the monoclonal antibodies, 4D11, recognized the nuclear matrix on the immunocytochemistry of rat 3Y1 and HeLa cells.

Cloning and Sequence Analysis—A cDNA clone, H4, was cloned by conventional immunoscreening with 4D11 of 6 × 10⁶ independent clones of a ∼gt11 human placenta cDNA library. Immunoscreening was performed as described previously (26). cDNA inserts released by EcoRI digestion were subcloned into pBluescript II KS(+) vector (Stratagene) and further analyzed by sequencing. A homology search was performed using BLAST programs against the GenBank™ and European Molecular Biology Laboratory data bases. Although no homologous gene had been reported previously, H4 was identical with a part of a cDNA clone registered under the name KIAA0136.

KIAA0136 (GenBank™ accession no. D50926) was one of the new genes obtained by analysis of randomly sampled full-length cDNA
 clones from a human immature myeloid cell line, KG1. For further study, we used KIAA0136 obtained from Dr. Nobuo Nomura (Kazusa DNA Research Institute, Kisarazu, Japan). This clone consists of 4197 nucleotides and contains an open reading frame encoding a protein of 939 amino acid residues at nucleotide position 36. We named the protein encoded by KIAA0136 NXP-2. The deduced amino acid sequence was subjected to a Sequence Motif Search on the GenomeNet Data Base Service and the prediction program of coiled-coil developed by Lupas et al. (28).

**Cell Culture and Immunostaining—**HeLa and transfected cells were maintained in F-12 medium containing 10% fetal calf serum at 37 °C under the CO₂. For immunostaining, HeLa cells were grown on coverslips and cultivated for 2 days. The coverslips were washed twice with PBS. Subsequently, the cells were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature, permeabilized with 0.5% Nonidet P-40 in PBS for 30 min, and blocked in PBS containing 1.5% fetal calf serum. Immunostaining was carried out with 4D11, followed by incubation with secondary fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Cappel Research, Organon Teknika Corp., Durham, NC). For immunostaining the nucleolus, mouse anti-human nucleoli monoclonal antibody (Chemicon International Inc.) and rhodamine-labeled goat anti-mouse IgM (Cappel Research, Organon Teknika Corp.) were used.

**Nuclear Matrix Preparation in Situ—**High salt isolation of nuclear matrix was conducted as described below. For **in situ** extraction, cells grown on coverslips were washed three times with ice-cold PBS. After a wash with PBS, cells were extracted with cytoskeleton extraction buffer (CSK buffer containing 250 mM ammonium sulfate) for 40 min at 4 °C. This treatment removed DNA digestion buffer (CSK buffer containing 0.2 mM EDTA and 10 mM Tris, pH 8.0, 1 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, and 1 mM EDTA supplemented with leupeptin, aprotinin, and pepstatin (1 μg/ml each), 1 μM phenylmethylsulfonyl fluoride, 1 μM dithiothreitol, 20 mM vanadyl ribonucleoside complex, and 0.5% (v/v) Triton X-100). After 3 min at 4 °C, the cytosolic soluble proteins were removed. Extraction buffer (CSK buffer containing 250 mM ammonium sulfate) was added next, and the mixture was incubated for 5 min at 4 °C to remove nuclear soluble proteins. Chromatin was solubilized with DNA digestion buffer (CSK buffer containing 0.2–0.5 unit/ml RNase-free DNase I) for 40 min at 37 °C. This treatment removed DNA and histone from the nuclei. The samples were washed with the digestion buffer and then fixed with 4% paraformaldehyde in PBS for 15 min. To digest the nuclear RNA, RNase A (1 unit/μl) was added as a substitute for 20 μM vanadyl ribonucleoside complex in the CSK, extraction, and digestion buffers.

**Northern blot analysis—**For Northern analysis, an MTN blot filter (CLONTECH) containing 2 μg/lane poly(A)⁺ RNA from human heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas was prehybridized at 65 °C for 30 min in ExpressHyb hybridization buffer (supplied by CLONTECH), hybridized for 1 h at 65 °C with radiolabeled H4 (2 × 10⁶ cpm/ml) in the same buffer, and washed with 2 × SSC containing 0.1% SDS twice for 30 min each, followed by two washes with 0.1 × SSC containing 0.1% SDS for 40 min each at 50 °C, as described in the manufacturer’s instructions. Hybridized sequences were identified by autoradiography for 18 h.

**Plasmids and Constructs—**The GFP-tagged expression vector used in this study was the phGFP(105) series, encoding a GFP mutant that has increased brightness (30). phGFP-NXP-2-(25–939) was obtained by introducing a fragment excised from KIAA0136 with SpeI and DraI into the blunt-ended EcoRI site of the phGFP(105)-C1 vector. NXP-2-(25–939) was excised from phGFP-NXP-2-(25–939) with SalI. NXP-2-(25–939) was generated as follows and introduced into the pGEX-4T series of vectors to adjust the frame of the amino acid sequence. NXP-2-(25–307) and NXP-2-(307–959) were excised from phGFP-NXP-2-(25–939) with PstI. NXP-2-(589–689) was generated by PCR using phGFP(105)-NXP-2-(25–939) as a template and NXP-2-(307–591) as a reverse primer. NXP-2-(307–591) was excised from phGFP-NXP-2-(307–591) with HindIII. GFP-NXP-2-(500–939) was excised from phGFP-NXP-2-(307–939) with HindIII and A/BII. NXP-2-(352–500) was excised from phGFP-NXP-2-(307–959) with EcoRI and SalI. For the production of GFP nuclear localization signal (NLS)-tagged proteins, phGFP(105)-NLS-C1 was created, in which the NLS signal sequence from the trunucated SV40 DNA is inserted between the GFP coding sequence and the C1-type mitochondrial sequence. phGFP(105)-NLS-C1 was used for the production of truncated mutants phGFP-NLS-NXP-2-(589–689), phGFP-NLS-NXP-2-(688–939), phGFP-NLS-NXP-2-(307–591), phGFP-NLS-NXP-2-(500–939), phGFP-NLS-NXP-2-(500–591), and phGFP-NLS-NXP-2-(500–353), and phGFP-NLS-NXP-2-(326–500) was excised from phGFP-NLS-NXP-2-(307–591) with SalI and XhoI. phGFP-NXP-2-(589–689) was excised from phGFP-NXP-2-(589–689) with SalI and XhoI. phGFP-NXP-2-(352–500) was excised from phGFP-NXP-2-(307–591) with HindIII. NXP-2-(326–500) was excised from phGFP-NXP-2-(307–591) with BMHI and EcoRI. phGST-NXP-2-(500–591) was obtained by self-ligation of phGFP-NXP-2-(307–591), which removes the 5′ fragment by HindIII digestion.

**Electroporation—**HeLa cells were harvested and suspended in a concentration of 3 × 10⁵ cells/ml in PBS. Electroporation was performed in 1 ml of 30% glycerol (250 mM at 50 molar units, pulse time, 10 ms) using an Electro Cell Manipulator (BTX Electroporation System, San Diego, CA) at room temperature. Cells were then incubated in a complete medium for 16 h at 37 °C and washed three times with PBS. They were subjected to **in situ** nuclear matrix treatment or fixation with 4% paraformaldehyde in PBS for 15 min at room temperature for microscopy. After the nuclear matrix treatment, the cell nuclei containing fluorescence were counted for the calculation of expression and retention efficiencies. Western blot analysis with anti-GST antibody was carried out as described previously (26). For Northwestern analysis, the membranes were blocked overnight at room temperature in Northwestern blocking solution and then washed with 2 × SSC containing 0.1% SDS for 40 min each at 50 °C, as described in the manufacturer’s instructions. Hybridized sequences were visualized by autoradiography for 18 h.

**RESULTS—**To clarify the structural components of the cell nucleus, the nuclear matrix was prepared and used as an immunogen to generate monoclonal antibodies. One of the monoclonal antibodies, 4D11, was used to screen a human placenta cDNA expression library. A positive clone, H4 (1.9 kb), was isolated from 6 × 10⁶ colonies. Upon sequence analysis, H4 was found to correspond to part of a cDNA clone called KIAA0136. KIAA0136 contained 41.5 kb of cDNA sequence derived from the human immature myeloid cell line KG1. An open reading frame encoding a protein of 939 amino acid residues was found starting at nucleotide position 36. We searched for homologous cDNA species by performing a BLAST homology search against the GenBank data base, and we found a mouse partial cDNA 55% homologous to human KIAA0136 in the protein coding region. No other eukaryotic homolog has been identified yet.
Northern blot analysis with the KIAA0136 cDNA probe identified a mRNA band of 4.4 kb that was particularly abundant in the heart, placenta, and skeletal muscle and was also expressed in several other tissues, but not in kidney tissue (Fig. 1). The size was consistent with that of KIAA0136. To analyze the 5′ sequence of the NXP-2 transcript, we carried out a 5′ rapid amplification of cDNA ends assay with mRNA prepared from HeLa cells. There was no clone containing more of the upstream sequence than KIAA0136. We analyzed the genomic sequence obtained from the data base to identify the 5′-untranslated region of the NXP-2 gene. There was no methionine codon in-frame with the NXP-2 coding sequence within 400 bp upstream of the first methionine codon of KIAA0136. On the other hand, we found a predicted CCAAT box and GC box, but no TATA box, within 150 bp upstream of the first methionine codon of KIAA0136. These results suggest that KIAA0136 encodes the whole NXP-2 protein sequence.

NXP-2 was found to be distributed throughout the nucleus, with the exception of the nucleolus, upon indirect immunofluorescence microscopy with a monoclonal antibody, 4D11 (Fig. 2A). The subnuclear distribution of endogenous NXP-2 was further characterized in 3Y1 and HeLa cells. NXP-2 was retained in the nuclear matrix and distributed throughout the nuclear regions (Fig. 2B). Thus, NXP-2 is a nuclear matrix protein. Release of NXP-2 from the nuclear matrix into the supernatant was apparently detected when 1 unit/μl RNase A was added as a substitute for 20 munits vanadyl ribonucleoside complex to the CSK, extraction, and DNase I digestion buffers (Fig. 2D). RNA probably supports the binding of NXP-2 to nuclear matrix.

To narrow down the nuclear matrix-associated region, several truncated mutants of NXP-2 (GFP-NXP-2-(25–939), GFP-NXP-2-(307–939), GFP-NLS-NXP-2-(307–939), and GFP-NLS-NLS-NXP-2-(589–939)) were constructed. Two truncated mutants, GFP-NLS-NXP-2-(307–939), and GFP-NLS-NLS-NXP-2-(589–939) were associated with the nuclear matrix (Fig. 5B). GFP-NLS-NLS-NXP-2-(326–353) remained in the matrix even in the presence of RNase, indicating that this region of NXP-2 associates with the nuclear matrix via protein-protein, but not protein-RNA, interaction. On the other hand, GFP-NLS-NXP-2-(326–500) was released from the nuclear matrix by RNase treatment, suggesting that the region for binding was masked in the large segment of NXP-2.(326–500). These results indicate that the domains of NXP-2 for nuclear localization and for nuclear matrix attachment are functionally distinct.

The above-mentioned results showed that NXP-2 is associated with the nuclear matrix, depending on RNA. Hence, we were prompted to assess the RNA binding activity of human NXP-2. For this purpose, GST fusion proteins of nearly full-length and various truncated NXP-2 proteins were expressed in E. coli and used for in vitro RNA binding assays (Fig. 6). When the expressed proteins were soluble (GST-NXP-2-(25–
human placenta Agt11 cDNA expression library. The obtained clone, KIAA0136, was deduced to encode a protein of 939 amino acids designated NXP-2 in this study. Based on Northern blot analysis, NXP-2 is expressed in several tissues, skeletal muscle, placenta, and heart but is undetectable in kidney. It is unlikely to be a constitutive factor for the maintenance of general RNA metabolism. The biological function of NXP-2 is presently unknown.

The nuclear staining of NXP-2 detected by the anti-NXP-2 antibody 4D11 was diffuse and remained after nuclear matrix treatment. We also found that exogenously expressed NXP-2 was tightly associated with the nuclear matrix because detergent, DNase, and high salt treatment of the nucleus did not release it from the matrix. Binding of NXP-2 to the nuclear matrix was diminished after RNase treatment, indicating that attachment to the matrix was dependent on RNA.

We showed in this study that the nuclear matrix targeting of NXP-2 is mediated by its central domain (amino acid residues 307–591), which is dependent on the presence of nuclear RNA. This region was further analyzed with truncated mutants. A region for nuclear matrix binding independent of RNA was found at amino acid residues 326–353. This region contains a cluster of highly hydrophobic amino acids, MGVGVVH (Fig. 8). A hydrophobic sequence responsible for nuclear matrix binding was also reported in the nuclear matrix targeting signal of acute myeloleukemia protein (32, 33). The central region of the nuclear matrix targeting signal of acute myeloleukemia protein is V(T/S)SIGIGIGIGMS, which is conserved among several vertebrate species. Such hydrophobic motifs were not reported for other nuclear matrix targeting signals, although a number of nuclear matrix targeting signals have been identified in diverse proteins (34, 35).

We reported in this study that NXP-2 is an RNA-binding protein. hnRNPA1, a typical RNA-binding protein, binds to poly(A), poly(U), poly(G), poly(C), poly(A,U), and poly(A,U,G). On the other hand, NXP-2 binds to ribonucleotide homopolymers, with a preference for poly(U), but not to poly(C), in contrast to hnRNPA1. Nuclear hnRNPC binds to poly(U), but not to poly(A) or poly(C) (36). To our knowledge, NXP-2 is a unique mammalian nuclear protein that is expressed in specific tissues and has specific RNA binding. hnRNPA1 is known to be a nuclear matrix protein responsible for stabilization of mRNA, suggesting that the binding to the nuclear matrix plays a part in the stabilization process (37). The finding that NXP-2 is also able to bind the nuclear matrix supports the hypothesis that this type of RNA-binding protein may have roles in the control of posttranscriptional processes through RNA binding as reported for most of the hnRNPs.

Binding to a mixed polymer containing A, G, and U was mediated by region 500–591, but not by region 307–326 or 326–500. The binding of region 307–591 to poly(A,G,U) was more intense than that of region 500–591. These results suggest that the peptide is too small to fold the entire active structure on the blotting membrane or that region 307–500 facilitates the RNA binding activity of region 500–591. The RNA binding and nuclear matrix binding functions appear to be distinct because different regions were responsible for the activities. It is unclear at present how nuclear RNA regulates nuclear matrix binding of NXP-2 in vivo.

To follow up the biological function of NXP-2, it will now be essential to identify its in vivo RNA targets. Furthermore, it is also required to determine with what types of nuclear matrix proteins and RNA-binding proteins NXP-2 interacts in vivo to form a functional RNA-protein complex. Various structural studies on a number of known RNA-binding proteins and splicing factors have identified several functional domains respon-
sible for the interaction with RNA or proteins. One highly conserved domain (RBD, RRM, or RNP-CS) has been intensively studied as an independent functional motif for specific RNA binding (38–41). Another conserved domain, the RGG box, has initially been identified as a RNA-binding domain of hnRNP-U. KH domains, first identified in heterogeneous nuclear ribonucleoprotein K, have several repeats of GXXG consensus sequences (42, 43). The central region 500–591 of NXP-2, which we have demonstrated to be required for RNA binding (Fig. 8), has no such domains and motifs. Region 500–591 contains three sets of serine dipeptide and arginine dipeptide sequences, two RRLS sequences, and one RRHLS sequence. An acidic amino acid-rich sequence is located at positions 571–582.

Computer analysis with an algorithm developed by Lupas et al. (28) showed the existence of a coiled-coil domain between amino acid residues 682 and 876 (Fig. 8D). Coiled-coil structures are found in some structural proteins, e.g. myosin, and in some leucine zipper DNA-binding proteins. Several nuclear matrix proteins, including lamin and nuclear mitotic apparatus protein (NuMA), contain coiled-coil structures that consist of a central rod domain flanked by globular terminal domains.

The RNA-dependent attachment to the nuclear matrix sug-
gests the possibility that the association of NXP-2 with the core filaments of the nuclear matrix is mediated by RNA. Nuclear matrix core filaments have been visualized by electron microscopy. It was assumed that the filaments were associated with various RNA-binding proteins, but their actual protein compositions are unknown (8, 44). Further study will be required to determine if other members of the large family of RNA-binding proteins are constituents of nuclear matrix.

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Fig. 7. Northwestern blot analysis of NXP-2 deletion mutants. The GST fusions of various NXP-2 deletion mutants were resolved by 10% SDS-PAGE and transferred to nitrocellulose membrane. A–D: lane 1, hnRNPA1; lane 2, NXP-2-(25–939); lane 3, NXP-2-(25–307); lane 4, NXP-2-(307–591); lane 5, NXP-2-(589–689); lane 6, NXP-2-(688–939); and lane 7, GST. NXP-2-(25–939) (lane 2) and NXP-2-(307–591) (lane 4) were obtained as insoluble pellets in E. coli; E and F: lane 1, hnRNPA1; lane 2, NXP-2-(307–591); lane 3, NXP-2-(307–326); lane 4, NXP-2-(326–500); lane 5, NXP-2-(500–591); and lane 6, GST. A and E, Western analysis using anti-GST antibody. B–D and F, Northwestern analyses using labeled RNA probes. B, poly(A); C, poly(A,U); D, poly(A,G,U); F, poly(A,G,U). The membranes were exposed to Kodak x-ray film overnight. Bands corresponding to the predicted sizes of the GST fusion proteins are indicated in A (GST-hnRNPA1, arrow next to lane 1; NXP-2-(25–307), closed circle next to lane 3; NXP-2-(307–591), open circle next to lane 4; NXP-2-(589–689), closed circle next to lane 5; NXP-2-(688–939), closed circle next to lane 6; GST, closed circle next to lane 7). NXP-2-(25–307) (lane 2) showed no significant band because of heavy degradation. The positions of the white arrow and white open circle in B–D are identical to those of hnRNPA1 and NXP-2-(307–591), respectively, as revealed by immunoblotting. Positions of GST-hnRNPA1 (arrow next to lane 1), NXP-2-(307–591) (open circle next to lane 2), NXP-2-(307–326) (closed circle next to lane 3), NXP-2-(326–500) (closed circle next to lane 4), NXP-2-(500–591) (open square next to lane 5), and GST (closed circle next to lane 6) are indicated in F. Positions of molecular mass markers are indicated together with the sizes (in kDa). The positions of the arrow, white open circle, and open square in F are identical to those of hnRNPA1, NXP-2-(307–591), and NXP-2-(500–591), respectively, as revealed by immunoblotting.

Fig. 8. Domain structure and amino acid sequences of NXP-2. A, diagram of the functional domains of NXP-2. The amino acid sequence (1–939) was deduced from the nucleotides of the cDNA (KIAA0136). By motif search, a bipartite type of nuclear localization signal (542–558) and coiled-coil domain (682–876) were found. B, amino acid sequence of the nuclear matrix-binding domain (region B in A). The hydrophobic region is underlined. C, amino acid sequence (500–591) of the RNA-binding domain (region C in A). The putative bipartite NLS is underlined. D, prediction of the coiled-coil domain. The amino acid sequence was analyzed using a coiled-coil domain prediction program.
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