Molecular Cloning of a Zinc Finger Autoantigen Transiently Associated with Interphase Nucleolus and Mitotic Centromeres and Midbodies

ORTHODOUS PROTEINS WITH NINE CXXC MOTIFS HIGHLY CONSERVED FROM NEMATODES TO HUMANS*

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We have cloned a novel human autoimmune antigen in a patient suffering from rheumatoid arthritis with high levels of antibodies to the nucleolus organizer regions. Initially the human autoimmune serum was used to select a cDNA of 317 amino acids from a hamster expression library. Using the hamster DNA as a probe, we isolated the human homologous cDNA of 320 amino acids. Human and hamster polypeptides share a 95% amino acid homology. The deduced 36-kDa protein contains a putative amino-terminal NLS signal, nine cysteine-X-X-cysteine motifs highly conserved, and a carboxy-terminal poly acidic region. Several homologous expressed sequence tags have been identified in data bases suggesting that orthologous proteins are present throughout evolution from worms to humans. A Drosophila expressed sequence tag was further completely sequenced for a full-length protein with 60% amino acid identity to the human homologue. Northern blot analysis revealed that this novel protein is widely distributed in human tissues with significantly higher expression levels in heart and skeletal muscle. Specific antibodies to the recombinant protein and transfection experiments demonstrated by immunofluorescence the localization of the protein predominantly but not exclusively to the nucleolus of interphase mammalian cells. In actinomycin D-treated cells the protein remains associated with the nucleolus but is not segregated, like other ribosomal factors such as upstream binding factor. In mitosis the protein was found to be associated with centromeres and concentrated at the midbody in cytokinesis. Transient distribution of this evolutionarily conserved zinc finger nucleolar autoantigen to the mitotic centromeres may provide the means for several aspects of cell cycle control and transcriptional regulation.

Antinuclear antibodies are a dominant feature in a number of rheumatoid diseases. These include systemic lupus erythematosus, mixed connective disease, polymyositis, scleroderma, and Sjögren’s syndrome (1–3). Examples of human autoantibodies directed against nuclear complexes are: (i) anti-RNP sera against specific components involved in the mechanism of processing heterogeneous nuclear RNA into messenger RNA (4) and (ii) anti-centromere sera directed against molecular components of the kinetochore and centromere regions (5). The nucleolus, the site of rDNA transcription and ribosome biogenesis has been described as a target organelle for autoimmune responses in humans, including components such as fibrillarin (6) and the upstream binding factor (UBF)1 for polymerase I (7, 8). Recent new studies have also defined mitotic and meiotic roles for the nucleolus in cell cycle control and gene regulation (9).

In humans, proteins containing zinc finger motifs (10) represent one of the largest known gene families (11). It has been estimated that up to 1% of all genes in the human genome may encode proteins with zinc finger domains (12). Although the structure of a large number of these molecules has been characterized (13, 14), conserved elements outside of the finger repeats are rare (15, 16). In light of their number, their widespread localization throughout the genome, and their putative role as transcriptional activators, as well as repressors (17), zinc finger factors form an attractive group of candidate disease genes.

We describe here NOA 36 (for Nucleolar Autoantigen), a novel human gene identified by a human anti-NOR serum (18, 19). Human, hamster, and fly cloned cDNAs showed a very high sequence homology to a number of ESTs found in several data bases. The deduced protein sequence significantly showed nine highly conserved CXXC motifs indicative of several putative zinc fingers. Immunolocalization of the protein in culture of mammalian cells indicated an association with the interphase nucleolus and a transient relocation to the mitotic centromeres and the midbody. The finding of this new centromere passenger component may contribute to the study of the role of the nucleolus in cell cycle control.

EXPERIMENTAL PROCEDURES

Culture Cell Lines and Reagents—CHO (CCL-61), HeLa (CCL-2.2), and 3T3 (CCL-92) cells were obtained from the American Type Culture Collection. Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. For ribosomal tran-

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† The abbreviations used are: UBF, upstream binding factor; EST, expressed sequence tag; CHO, Chinese hamster ovary; IP, immunofluorescence; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; GFP, green fluorescence protein; DAPI, 4,6-diamidino-2-phenylindole; NLS, nuclear localization signal; NOR, nucleolar organizer region.
FIG. 1. Sequence and hydrophobicity plot of human NOA 36. A, nucleotide and deduced amino acid sequence of human NOA 36 cDNA are shown. Eighteen cysteine residues forming putative zinc fingers of the CXXC type are indicated by black shading. A putative nuclear localization signal is underlined and a poly acidic domain is double underlined. The termination codon is indicated by an asterisk, and the in-frame non-sense codons in the upstream flanking region and a polyadenylation signal are shown in bold letters.

B, hydropathy plot of NOA 36 using the algorithm of Kyte and Doolittle (44). C, schematic representation of secondary structural elements of human NOA 36, analyzed using the GOR IV Program (45), showing α helix, β sheet, and putative coil regions. The nucleotide sequence of human NOA 36 cDNA has been submitted to DDBJ/GenBankTM/EBI Data Base under the accession number AJ006591.
scription inhibition, CHO cells at 75% confluence were treated with actinomycin D (0.02 µg/ml) for 4–8 h prior to the IF analysis. Human anti-NOR autoimmune serum has been described in a previous report to react at the electron microscopy level with the dense fibrillar component of nucleoli and NOR regions of mitotic chromosomes (18). Anti-SM (a gift of Ian Mattaj, EMBL, Germany) is a monoclonal to a major component of nucleoli and NOR regions of mitotic chromosomes (18). An- ti-SM is Y12837). The hamster DNA probe was matched exactly several regions of our cloned NOA 36 cDNA. The probe was used as a control probe. 

Expression of NOA 36 and Antibody Production—Truncated hamster NOA 36 polypeptide of 226 amino acids (residues 39–265) was expressed in the pET 3a system in BL21 (DE3) Escherichia coli cells. Protein expression was induced by isopropyl-β-D-thiogalactopyranoside (0.5 mM), and inclusion bodies were partially isolated under denaturing conditions. Proteins from inclusion bodies were resolved in a 10% SDS-PAGE and transferred to nitrocellulose membranes by routine methods (21). As primary antibodies, rabbit anti-NOA 36, human anti-NOR autoimmune serum, rabbit anti-UBF serum, or rabbit preimmune were used at appropriate dilutions. Peroxidase-labeled secondary antibodies (Roche Diagnostics) were used as the second-stage reagent and chlonaphol was used to develop the blots.

Immunofluorescence and Transfection—For indirect immunofluorescence staining, cells grown on coverslips were washed with PBS and fixed in cold methanol for 10 min at −20 °C. The cells were then washed with PBS and incubated with primary antibody diluted in PBS (anti-NOA 36 at 1:400 dilution) at 37 °C for 45 min. Cells were then washed with PBS for 30 min at room temperature and incubated with fluorescein/rhodamine-labeled secondary antibody at 37 °C for 45 min. After the second antibody staining, the cells were washed twice in PBS and mounted in PBS-glycerol containing Hoechst 33342 at 0.1 µg/ml. In some experiments, double IF analysis was done using human anti-NOR serum (dilution 1:200) for UBF or mouse anti-SM (dilution 1:300) as primary antibody together with rabbit anti-NOA 36 (Fig. 5). For transient transfection, human NOA 36 full-length cDNA was fused by polymerase chain reaction upstream of GFP on vector pGFP-N1

**Fig. 2. Amino acid sequence comparison of human, hamster, and Drosophila NOA 36 homologues with several ESTs.** The homology shared between NOA 36 orthologues is shaded in gray. Species are indicated by the following abbreviations: Hs, human (DDBJ/GenBankTM/EBI accession number A006591); Cg, hamster (GenBankTM accession number Y12837); Mm, mouse (GenBankTM accession number AA766666 and AA240412); Bm, worm (GenBankTM accession number Y12836). The homology shared among several human tissues was purchased from Invitrogen and CLONTECH Labrador ESTs (GenBankTM accession number AJ131564). The cDNA sequences were analyzed using the data base at the National Center for Biotechnology Information (Bethesda, MD) and the BLAST and PROSITE programs were used to search sequence data bases. A Drosophila EST (GenBankTM accession number AA978679) was obtained from Genome System and sequenced for a full-length cDNA (our GenBankTM accession number AJ131564).

**Northern Blots**—The Northern transfers of RNAs derived from several human tissues were purchased from Invitrogen and CLONTECH Labrador and used according to the recommendations of the manufacturers. The DNA template used in the preparation of the radiolabeled probe was a 427–1423-nucleotide fragment of human NOA 36 cDNA. The probe was used for hybridization at 60 °C overnight in 5 × SSC, 5 × Denhardt’s, 0.1% SDS, 20 µm sodium phosphate, 1 mm EDTA, and 100 µg/ml salmon sperm DNA. Washes were twice in 2 × SSC 0.1% SDS at 60 °C and once in 0.1 × SSC 0.1% SDS at 60 °C. A β-actin cDNA was used as a control probe.
to generate NOA 36-GFP, and the DNA constructs were purified over Qiagen columns (Qiagen Inc.). CHO-transfected cells seeded onto coverslips were grown for 24–48 h before being fixed in acetone for 5 min at −20 °C and mounted in PBS-glycerol for GFP autofluorescence observation. A Zeiss Axiophot microscope equipped with a 63 × NA 1.3 oil-immersion objective was used routinely. Images were taken using T-max Kodax 400 ASA film.

RESULTS AND DISCUSSION

Cloning and Evolution of NOA 36 Genes—Human autoimmune anti-NOR sera have previously been found to stain nucleolar organizer regions and to serve for the cloning and immunological characterization of UBF, the ribosomal transcription factor for RNA polymerase I (7, 8, 19). Here we have extended our previous studies with a human anti-NOR serum by cloning and sequencing a hamster cDNA encoding a novel protein of 317 amino acids. This probe was further used to select human homologue cDNAs from a HeLa λ ZAP expression library. The largest human cDNA isolated (Fig. 1) showed an initial ATG preceded by several stop codons in the same frame, suggesting that the complete open reading frame has been identified (GenBank™ accession number AJ006591). This was also correlated with several orthologous EST sequences found in various data bases where the first methionine residue is at the same position as in the human and hamster cDNAs characterized. The complete human open reading frame has 320 amino acid residues for a predicted molecular mass of 36,260 daltons. The analysis of the novel sequence showed a potential nuclear localization signal (PKKK—RKK) at the NH2-terminal region, highly conserved cysteines and histidines residues for several putative zinc fingers, and a poly acidic region at the C-terminal. These domains are widely found in regulatory proteins that have been shown to be implicated in transcription control and/or regulation (22) and nucleolar localization (23, 24, 25). Analysis of the hydrophilic/hydrophobic profile of the protein (Fig. 1B) indicates a predominant hydrophobic part of the molecule between amino acids 50 and 200, and secondary structure analysis predicts that this protein is primarily a coiled coil (Fig. 1C). Data base searches using PROSITE, BLAST, and FASTA homology indicate that NOA 36 shares homology with a number of ESTs sequenced from various species, from nematodes to mammals (Fig. 2). As shown, NOA 36 shares significantly nine CXXC motifs for four putative zinc fingers of the C2-C2 type and high internal sequence similarity to those ESTs. Complete sequence analysis of the Drosophila EST identified (GenBank™ accession number AA978679) served to us to characterize the full-length of the fly cDNA (our GenBank™ accession number...
The homology between the human NOA 36 deduced amino acid sequence and other NOA 36 proteins is as follows: 95% identity with hamster cDNA, 98.1% with mouse EST1, 90.2% with mouse EST2, 60.7% with Drosophila EST, and 63.2% with the nematode Brugia malayi EST. The high conservation throughout the protein sequence suggests that these might be
orthologues (26–28). No sequence similarity to NOA 36 was found in yeast proteome, for which the whole genome has been sequenced. This is not surprising because, although almost equal fractions of the human disease genes had regions of significant similarity to nematode and to yeast proteins, a recent study (27) identified true orthologues in the complete yeast proteome for only 20% of human proteins.

Expression of NOA 36 in Human Tissues—The molecular characterization of NOA 36 was begun by examining its mRNA levels in various human tissues. Two mRNA blots (Invitrogen, CLONTECH) were probed with radiolabeled NOA 36 cDNA probe. As shown in Fig. 3, A and B, the NOA 36 transcript is expressed as a unique species of 1.8 kilobases in all tissues examined. However it is noteworthy that the expression varied substantially between tissues, being clearly higher in heart and skeletal muscle. The same result was obtained when hamster mRNAs from various tissues were also analyzed (data not shown). Although the significance of the variation in the level of NOA 36 expression in heart and skeletal muscle is at present unknown, one possibility is that it may be related to higher protein synthesis and/or protein metabolism requirements in those tissues. A detailed analysis of the cysteine residues organization in NOA 36 primary sequence indicated that the new protein shares putative zinc finger motifs at residues 42–109 and 129–193 with the consensus sequence, \( \ldots \cdot CX_3C \ldots \cdot CX_2C \ldots \cdot CX_2C \ldots \cdot CX_5CNNNC \ldots \cdot \), described previously for the steroid hormone receptor family. Furthermore, it is noteworthy that the cysteine-rich motifs found in NOA 36 are of the GATA CXXC type family (29, 30). Based on these structural concordances, it appears that the zinc-binding motifs of NOA 36 may be compatible with a nucleic acid-binding function, although, as demonstrated for other transcription factors containing CXXC motifs, an alternative protein-protein interaction for those domains could not be ruled out.

NOA 36 Is a Human Nucleolar Autoantigen—The human autoimmune serum used in this study served initially to select cDNAs from a hamster expression library. Therefore the serum was expected to contain autoantibodies directed to the nucleolar NOA 36 autoantigen. Western blot using recombinant polypeptides and immunofluorescence analysis with specific
anti-NOA 36 serum allowed us to demonstrate that NOA 36 is a truly human autoantigen. Specifically, the human autoimmune anti-NOR serum reacts by immunoblots with the full-length and with a NH2-terminal polypeptide of human NOA 36 (Fig. 4A, right), as does a rabbit polyclonal anti-NOA 36 generated against a recombinant form of the autoantigen (Fig. 4A, center). Furthermore, using protein extracts from HeLa and CHO cells, we were able to detect, among the putative autoantigens recognized by the human anti-NOR serum, a 38-kDa band (Fig. 4B, center), which is similar in molecular weight to that recognized by the anti-NOA 36 in hamster and human cells (Fig. 4B, left). We have observed that the native NOA 36 polypeptide runs more slowly than expected in SDS-PAGE gels (40–38 kDa), probably due to a posttranslational modification in the molecule. Note that, as demonstrated previously (19), the main reactivity of the human autoimmune anti-NOR serum used in this study was against the UBF transcription factor for RNA polymerase I (90–92-kDa polypeptides), as shown by specific anti-UBF serum (Fig. 4B, right).

The observation that NOA 36 is a human autoantigen raises interesting questions about its putative linkage with specific autoimmune responses in humans. The generation of autoantibodies and subsequent tissue deposition of immune complexes is thought to trigger the pathogenic consequences of
systemic autoimmune diseases. Although the origin of autoimmune diseases is not yet known, several hypotheses, including the processing of cellular antigen during apoptosis, have been put forward and clues have been found (31–33). With that in mind, studies to identify the human autoantibody(ies) in the NOA 36 polypeptide and the distribution of NOA 36 on in vitro induced apoptosis in culture cells are in progress. Furthermore, future studies with additional anti-NOR sera will be interesting to pursue, to clarify the significance of this new autoantigen in the specific NOR autoimmune response in humans.

To determine the cellular localization of NOA 36, specific antibodies were used by IF in several mammalian cell lines. In interphase cells, anti-NOA 36 sera produced a clear nucleolar staining in all cell lines analyzed, including CHO, 3T3, and HeLa (Fig. 5, A–C). As shown, the nucleolar staining was high and similar in rodent cells and significantly less intense in HeLa cells. The explanation for this could be that the immunogen used to generate the specific anti NOA 36 serum was a truncated hamster recombinant protein (see “Experimental Procedures”). High cytoplasmic staining above the background signals observed with preimmune serum was also unequivocally found in all the cell lines tested; this may also represent specific localization of the autoantigen. A similar pattern of NOA 36 fluorescence was seen with cells that had been fixed with either methanol, acetone, and formaldehyde, although more diffuse nuclear staining was evident with formaldehyde-fixed cells (data not shown). The nucleolar localization of the novel autoantigen was demonstrated by double IF analysis with UBF a genuine nucleolar factor (7, 18, 19). Double staining with anti-UBF and NOA 36 sera demonstrated the colocalization of both proteins at the nucleolus (Fig. 5, D–F). However, we observed a NOA 36 fluorescence pattern dispersed throughout most of the nucleoli structure, whereas UBF has been described in previous reports to be located at the dense fibrillar component (18). As a control, double staining with anti-SM serum for splicing factors (Fig. 5, G–J) excluded NOA 36 for nuclear compartments other than the nucleoli. In an attempt to demonstrate further the cellular localization of NOA 36, we transfected human NOA 36 cDNA into a hamster (CHO) cell line. The GFP was fused in-frame to the carboxyl terminus of the NOA 36 human full-length cDNA. NOA 36-GFP fluorescence was also found in the nucleolus, which confirmed the IF results and the specificity of the anti-NOA 36 serum (Fig. 5, J and K). The strong staining also observed at the cyttoplasm of transfected cells may indicate overexpression of the transfected construct, although specific localization of a significant amount of NOA 36 at the cyttoplasm could not be ruled out, as suggested by the indirect IF results (Fig. 5, A–C).

To investigate further the association of NOA 36 with the nucleolus, we performed IF studies in culture cells treated with wortmannin (CHO) cells. The GFP was fused in-frame to the carboxyl terminus of the NOA 36 polypeptide and the distribution of NOA 36 on in vitro transcription inhibition (see details in Fig. 6, A–C). This was corroborated by double fluorescence staining with a CREST serum to centromere proteins (data not shown). Furthermore, in nocodazole-treated cells the NOA 36 autoantigen remains attached to the centromere in blocked condensed chromosomes (data not shown). One stage further in the cell cycle, in cytokinesis, NOA 36 has returned to the reformed nucleolus, although staining was also concentrated within the intracellular bridge at either side of the midbody (Fig. 7h). It is well known that centromere/kinetochore assembly is a cell cycle-dependent process that is based on the temporary interaction of multiple components of the complex. This conclusion has been supported by the discovery of a subset of kinetochore proteins that bind transiently to the kinetochore as it matures. Among these proteins are CENP-F (37), CENP-E (38), p34<sup>cdc2</sup> (39), dynein (40), and mitotic spindle checkpoint proteins (41). Now, NOA 36 can be added to the increasing number of proteins that move to the centromere and to the intracellular bridge at telophase (42).

The transient localization of NOA 36 at the nucleolus and mitotic centromeres could be related to some of the new roles postulated for the nucleolar dynamic. Localization of a nucleolar component to heterochromatic structures (centromeres and telomeres) is a known phenomenon (9, 43). Recent studies have demonstrated the convergence of silencing of heterochromatic regions and nucleolar proteins and support the idea that the nucleolus and silenced heterochromatin may in fact be privileged regulatory sites of gene expression. If NOA 36 as a passenger protein re-locating from interphase nucleoli to mitotic centromeres, performs a function in cell cycle regulatory mechanisms, this is a role that could now be investigated.

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