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

ORTHOLOGOUS PROTEINS WITH NINE C*XX*C 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 carboxyl-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 C*XX*C 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 nucleotide sequence(s) reported in this paper have been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AJ006591, Y12836, and AJ131564.

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[1] The abbreviations used are: UBF, upstream binding factor; EST, expressed sequence tag; CHO, Chinese hamster ovary; IF, 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.

A

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ggcacgagtgtcagcctccctggctgttagtaccttctttcccggagtcctggtccacgagttggatttactgctgtcgcgggtggg    87
cctcacgccattccctgtccctcggccccctgagtgagtccggtctcccggcgaaagtgagcgaggtttgcccggagcgcgcacgag   174
gggaaa  ATG CCT AAA AAA AAG ACT GGT GCG AGG AAG AAG GCT GAG AAC CGC CGA GAA CGT GAA AAA      240
        M   P   K   K   K   T   G   A   R   K   K   A   E   N   R   R   E   R   E   K     20
CAA CTA AGA GCA TCA AGA AGC ACT ATA GAT TTA GCT AAA CAT CCA TGT AAT GCC TCA ATG GAA TGT     306
Q   L   R   A   S   R   S   T   I   D   L   A   K   H   P   C   N   A   S   M   E   C     42
GAC AAG TGT CAG AGG CGG CAG AAG AAT AGA GCA TTT TGC TAC TTT TGT AAT TCT GTA CAG AAG TTA     372
D   K   C   Q   R   R   Q   K   N   R   A   F   C   Y   F   C   N   S   V   Q   K   L     64
CCA ATT TGT GCA CAG TGT GGG AAA ACA AAG TGC ATG ATG AAG TCT TCA GAC TGT GTC ATA AAG CAT     438
P   I   C   A   Q   C   G   K   T   K   C   M   M   K   S   S   D   C   V   I   K   H     86
GCT GGT GTA TAC AGT ACT GGC CTT GCA ATG GTG GGT GCA ATA TGT GAC TTC TGT GAA GCT TGG GTT     504
A   G   V   Y   S   T   G   L   A   M   V   G   A   I   C   D   F   C   E   A   W   V     108
TGC CAT GGG AGG AAG TGT CTC AGT ACA CAT GCC TGT GCC TGC CCT CTC ACT GAT GCT GAG TGT GTT     570
C   H   G   R   K   C   L   S   T   H   A   C   A   C   P   L   T   D   A   E   C   V     130
GAA TGT GAA CGA GGC GTG TGG GAC CAT GGA GGC AGA ATA TTC AGT TGT TCT TTT TGC CAT AAC TTT     636
E   C   E   R   G   V   W   D   H   G   G   R   I   F   S   C   S   F   C   H   N   F     152
CTC TGT GAA GAT GAT CAA TTT GAG CAT CAA GCC AGC TGC CAG GTT TTA GAG GCA GAA ACA TTT AAA     702
L   C   E   D   D   Q   F   E   H   Q   A   S   C   Q   V   L   E   A   E   T   F   K     174
TGT GTT TCA TGC AAT CGG CTT GGT CAG CAC TCA TGT CTC CGT TGT AAG GCT TGT TTC TGT GAT GAT     768
C   V   S   C   N   R   L   G   Q   H   S   C   L   R   C   K   A   C   F   C   D   D     196
CAT ACA AGG AGC AAA GTG TTT AAG CAA GAA AAA GGA AAA CAG CCT CCT TGT CCT AAA TGT GGG CAT     834
H   T   R   S   K   V   F   K   Q   E   K   G   K   Q   P   P   C   P   K   C   G   H     218
GAA ACT CAG GAG ACT AAG GAC CTT AGC ATG TCA ACA CGC TCC CTG AAA TTT GGC AGG CAG ACT GGA     900
E   T   Q   E   T   K   D   L   S   M   S   T   R   S   L   K   F   G   R   Q   T   G     240
GGT GAA GAG GGA GAT GGA GCT TCT GGG TAT GAT GCT TAT TGG AAG AAC CTT TCA TCT GAT AAG TAT     966
G   E   E   G   D   G   A   S   G   Y   D   A   Y   W   K   N   L   S   S   D   K   Y     262
GGT GAT ACC AGC TAC CAC GAT GAG GAG GAG GAT GAG TAT GAA GCA GAG GAT GAT GAA GAG GAA GAA    1032
G   D   T   S   Y   H   D   E   E   E   D   E   Y   E   A   E   D   D   E   E   E   E     284
GAT GAA GGC AGA AAG GAT TCA GAT ACT GAG TCA TCA GAT TTG TTT ACT AAT TTG AAT TTA GGA AGG    1098
D   E   G   R   K   D   S   D   T   E   S   S   D   L   F   T   N   L   N   L   G   R     306
ACC TAT GCT AGT GGC TAT GCT CAC TAT GAG GAA CAA GAG AAC TAG gggagctgctctggtggccgtgtgtga    1170
T   Y   A   S   G   Y   A   H   Y   E   E   Q   E   N   *                                 320
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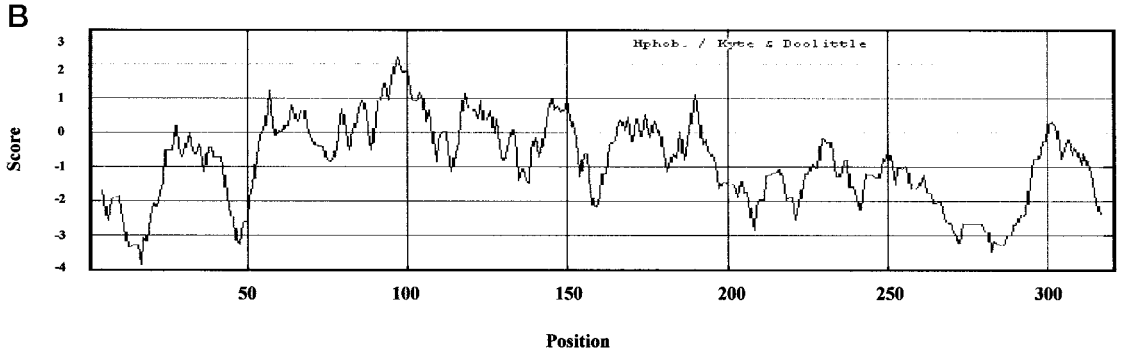
B



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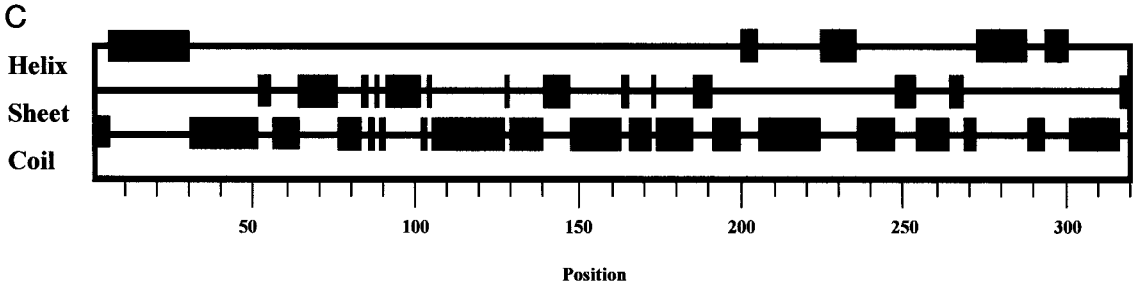

FIG. 1. **Sequence and hydrophobicity plot of human NOA 36.** *A*, nucleotide and deduced amino acid sequence of human *NOA 36* cDNA are shown. Eighteen cysteine residues forming putative zinc fingers of the C*X*X*C* type are indicated by *black shading*. A putative nuclear localization signal is *underlined* 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/GenBank™/EBI Data Base under the accession number AJ006591.

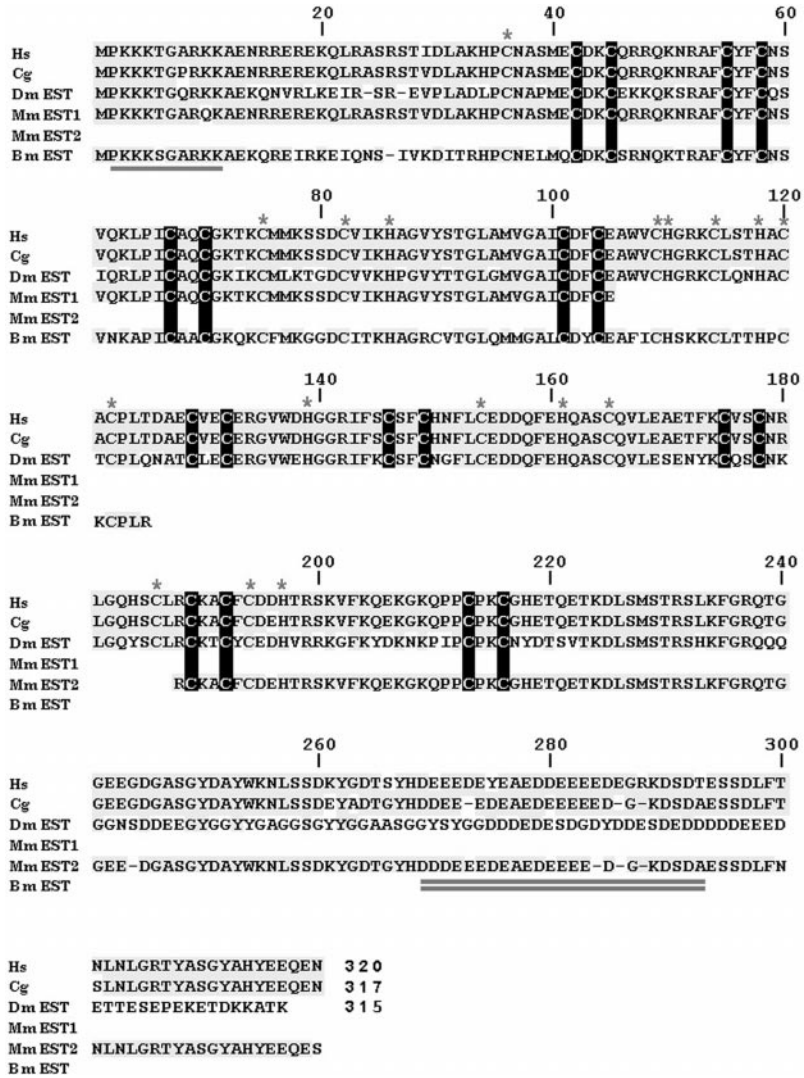

FIG. 2. **Amino acid sequence comparison of human, hamster, and *Drosophila* NOA 36 homologues with several ESTs.** The homology shared between NOA 36 orthologues is shaded in *gray*. Species are indicated by the following abbreviations: *Hs*, human (DDBJ/GenBank™/EBI accession number AJ006591); *Cg*, hamster (GenBank™ accession number Y12836); *Dm*, fly (GenBank™ accession number AJ131564); *Mm*, mouse (GenBank™ accession numbers AA796666 and AA240412); *Bm*, worm (GenBank™ accession number AA032099). Mouse and nematode EST sequences were translated to a reading frame encoding NOA 36. Translated amino acid sequences from several human ESTs at the data bases (GenBank™ accession number H82458 and N21079) matched exactly several regions of our cloned *NOA 36* cDNA. Conserved cysteines in C*XX*C motifs are *darkly shaded*. Other highly conserved cysteine (*C*) and histidine (*H*) residues, which may participate in putative zinc finger structures, are marked with *asterisks*. A putative NLS signal is *underlined*, and a *double underline* indicates the conserved poly acidic region.

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 spliceosome antigen.

Molecular Cloning and DNA Sequence Analysis—A λ ZAP cDNA expression library of CHO cells (Stratagene) was screened with a human autoimmune serum against the NOR region. This serum had previously been shown to recognize the ribosomal factor UBF as a major autoantigen (18, 19). Isolated *NOA 36* hamster clones were sequenced for a full-length cDNA by chain termination reaction (20) using the Sequenase 2.0 kit (U. S. Biochemical Corp.) (GenBank™ accession number for hamster *NOA 36* is Y12837). The hamster DNA *NOA 36* gene was used as a probe to select clones from a HeLa λ ZAP cDNA library (Stratagene) (GenBank™ accession number for human *NOA 36* is AJ006591). 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 (GenBank™ accession number AA978679) was obtained from Genome System and sequenced for a full-length *NOA 36* cDNA (our GenBank™ accession number AJ131564).

Northern Blots—The Northern transfers of RNAs derived from several human tissues were purchased from Invitrogen and CLONTECH 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 mM 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.

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 the specific NOA 36 band was cut out from the gel, eluted in PBS-0.1% SDS, dialyzed against PBS, and used for immunization. For immunoblots, the expressed NOA 36 polypeptide and protein extracts from HeLa and CHO culture cells were separated in 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 chloronaphthol 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

(CLONTECH) 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.

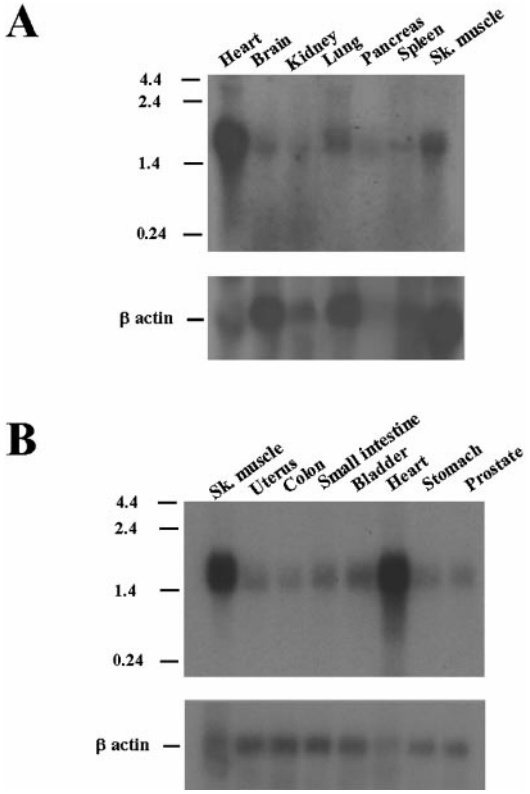

FIG. 3. **Transcriptional analysis of *NOA 36* from human tissues.** Northern blots of total mRNA from several tissues were hybridized successively with the *NOA 36* cDNA probe or an actin control probe. A unique transcript of 1.8 kilobases was detected in all RNA analyzed, although a higher expression in heart and skeletal (*Sk.*) muscle tissues was detected. Note the similar finding by the hybridization assay resulting from the analysis of human mRNAs provided from different manufacturers (Invitrogen (*A*) and CLONTECH (*B*)).

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 NH_2-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. 1*B*) 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. 1*C*). 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 C*XX*C 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

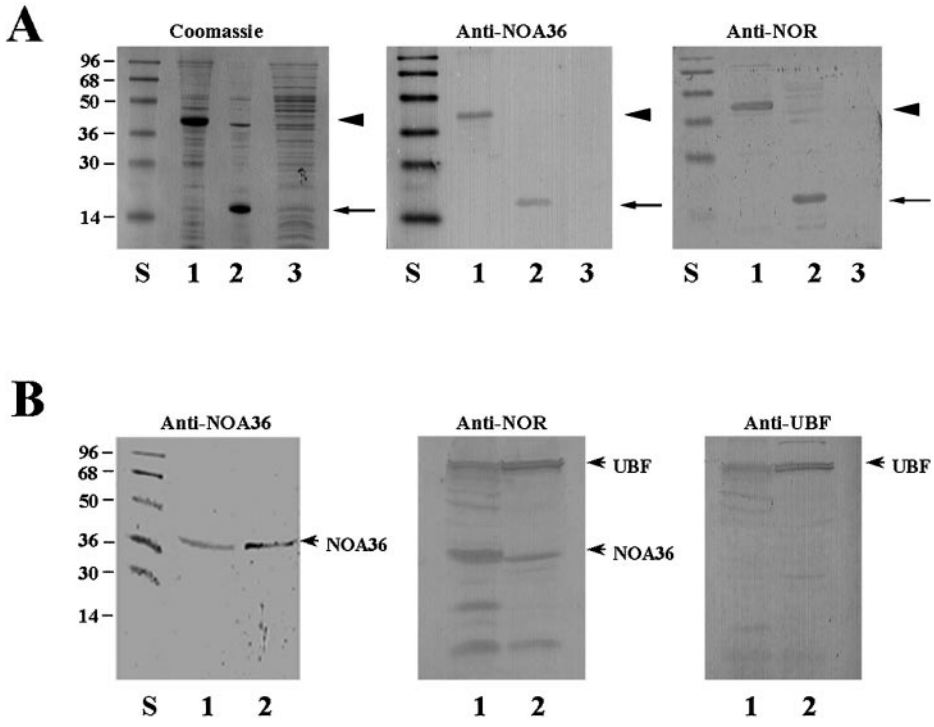

FIG. 4. **Immunoblot analysis of NOA 36.** *A*, recombinant NOA 36 polypeptides are recognized by human autoimmune anti-NOR serum. Full-length (*lane 1*) and NH_2-terminal (residues 1–154) (*lane 2*) of NOA 36 were isolated from inclusion bodies, as shown by Coomassie Blue staining. *Lane 3* represents a protein extract control from wild type *E. coli.* The blots were proved with a human autoantibody to NOR and with polyclonal anti-NOA 36 serum generated in rabbits as indicated. *B*, immunoblots of NOA 36 in mammalian cell extracts. Whole proteins from HeLa (*lane 1*) and CHO (*lane 2*) were transferred and proved with anti-recombinant NOA 36 or human autoimmune anti-NOR. A band of approximately 38 kDa detected by the NOA 36 antibody is also observed with human anti-NOR (*arrows*). The major autoantigen recognized by the human autoimmune anti-NOR serum is a double band for UBF at 90–92 kDa, as shown by a blot with specific rabbit anti-UBF antiserum. Molecular weight mass are indicated in kilodaltons.

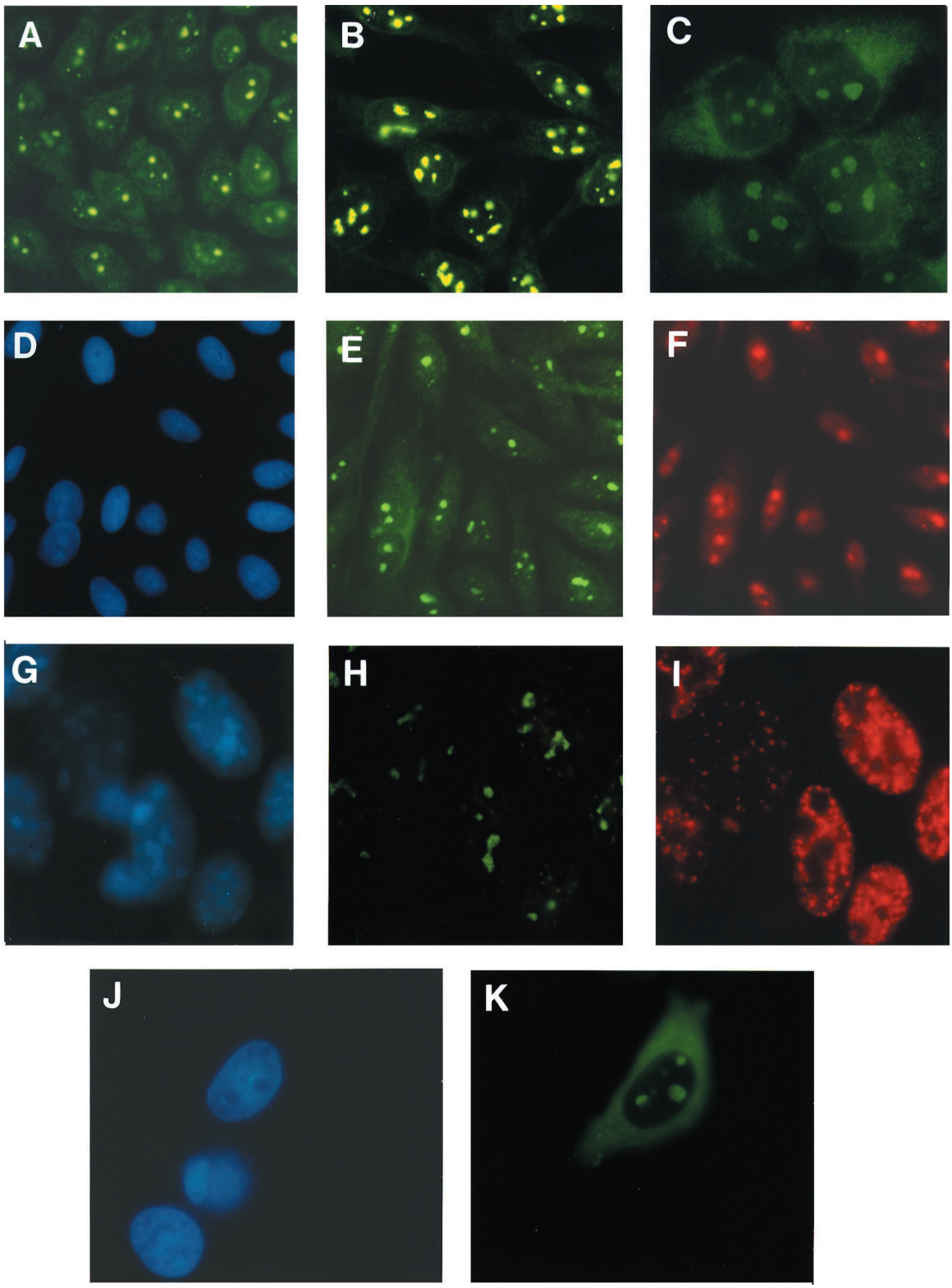

FIG. 5. **Immunofluorescence localization of NOA 36 human autoantigen.** Staining for NOA 36 in CHO (*A*), 3T3 (*B*), and HeLa (*C*) culture cells is predominantly at the nucleolus with diffuse labeling also at the cytoplasm. Distribution of NOA 36 at the nucleolar region was studied by double IF staining of CHO cells with rabbit anti-NOA 36 (*E*)- and human anti-NOR (*F*)-specific antibodies. NOA 36 does not co-localize with fluorescence speckles for splicing factors as shown by double IF with anti-NOA 36 (*H*) and anti-SM (*I*). In addition, CHO cells transiently transfected with human *NOA 36*-GFP were visualized directly by GFP-green fluorescence showing again a nucleolar pattern (*K*). High cytoplasmic staining observed in transfected cells most probably is due to overexpression of the transfected plasmid, although specific binding sites in the cytoplasm for NOA 36 could not be ruled out. Blue DAPI nuclear staining is also shown in several cases (*D*, *G*, *J*). The original magnification was × 100.

AJ131564 for *Dm* in Fig. 2). 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

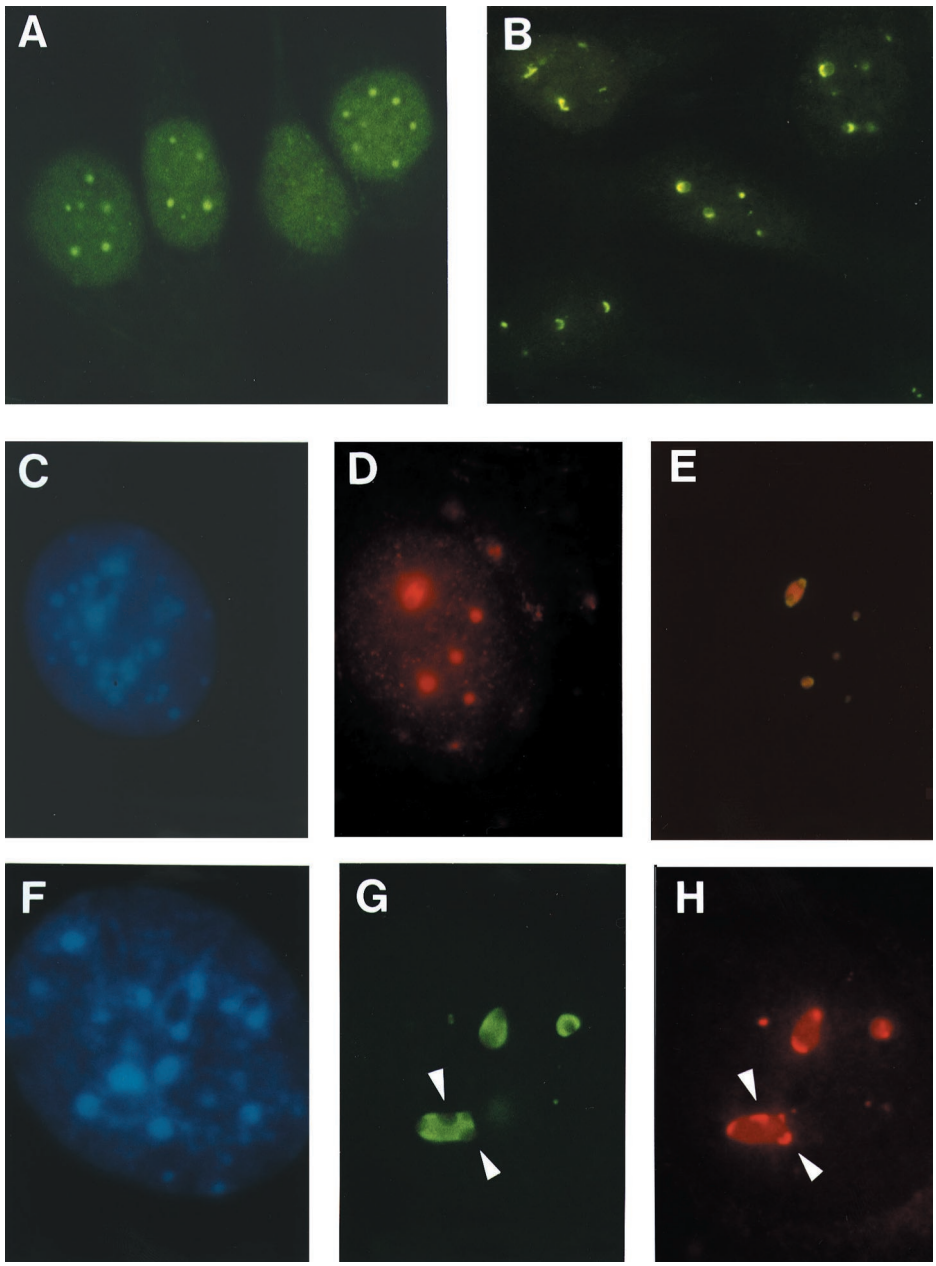

FIG. 6. **Immunofluorescence analysis of NOA 36 in actinomycin D-treated cells.** Inhibition of RNA polymerase I transcription by actinomycin D in CHO cells produced the phenomenon of nucleolus segregation, as shown by anti-UBF fluorescence staining (*B*). The drug treatment apparently reduced the staining at the nucleolus of NOA 36 (*A*) and clearly modified its localization from the whole nucleolar area to the area excluding the nucleolar caps occupied by UBF (*C–H*). Double staining with anti-NOA 36 (*D*, *G*) and anti-UBF (*E*, *H*) sera show clear separation of the subnucleolar structures occupied by both autoantigens, after nucleolar segregation induced by ribosomal transcription inhibition. DAPI staining for DNA is also shown (*C*, *F*). Original magnification was × 100.

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 proved 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, ...CX_2C. . . . CX_2C. . . . .CX_5C. . . $CX_2CNNNNC$. . . . , 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 C*XX*C 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 C*XX*C 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

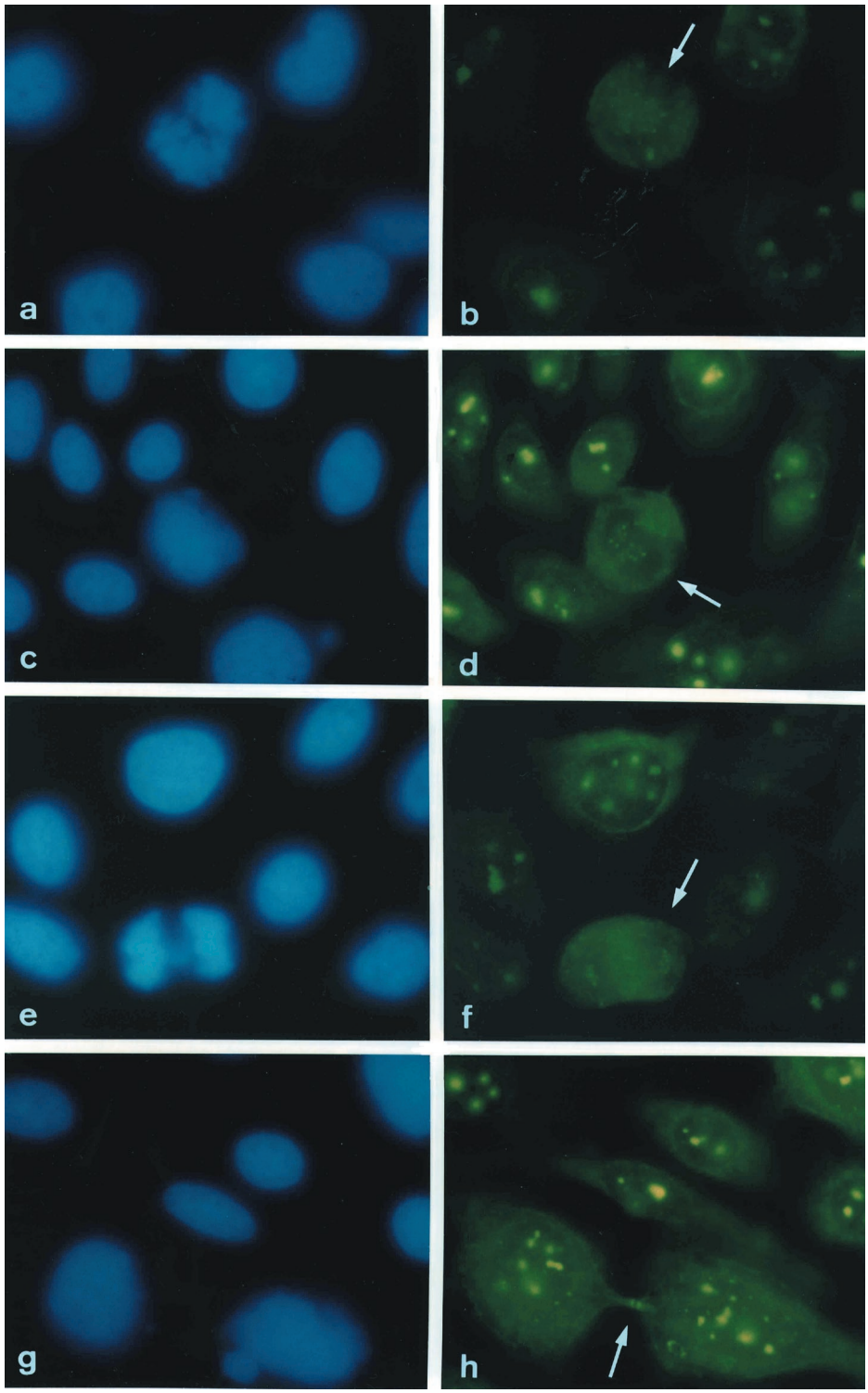

FIG. 7. **Immunofluorescence localization of NOA 36 autoantigen during mitosis.** Rabbit polyclonal serum generated against a recombinant hamster NOA 36 truncated polypeptide shows staining on CHO mitotic centromeres on prometaphase (*b*), metaphase (*d*), and anaphase (*f*) cells. In cytokinesis the autoantigen is distributed into the reformed nucleoli and to the midbody (*arrow* in *h*). DAPI staining for DNA is also shown (*a*, *c*, *e*, *g*). Original magnification was × 100.

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 NH_2-terminal polypeptide of human NOA 36 (Fig. 4*A*, *right*), as does a rabbit polyclonal anti-NOA 36 generated against a recombinant form of the autoantigen (Fig. 4*A*, 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. 4*B*, *center*), which is similar in molecular weight to that recognized by the anti-*NOA 36* in hamster and human cells (Fig. 4*B*, *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. 4*B*, *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 hypothesis, 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 autoepitope(s) 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 co-localization 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–I*) 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 in hamster (CHO) cells. 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 cytoplasm of transfected cells may indicate overexpression of the transfected construct, although specific localization of a significant amount of NOA 36 at the cytoplasm 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 actinomycin D for ribosomal transcription inhibition. This drug produces nucleolar segregation of the formless fibrillar, granular areas and chromatin in compact nucleoli. As expected and as shown in Fig. 6, the UBF factor moves from the dense fibrillar centers to the edges of the nucleolus (Fig. 6*B*). This fibrillar cap observed at the nucleoli in actinomycin D-treated cells has been shown previously to contain RNA polymerase I, fibrillarin, and UBF (6, 19). Surprisingly, NOA 36 fluorescence staining at the nucleolus was apparently diminished after the drug treatment (Fig. 6*A*) and was concentrated at the center of the nucleolus in regions where UBF was excluded after transcription inhibition (see details in Fig. 6, *C–H*). This unambiguously indicates that NOA 36 remains associated with the nucleolus but is not co-localized with UBF after the drug treatment. The IF localization of NOA 36 is similar to that described for other nucleolar components characterized previously (34) and suggests that NOA 36 is probably associated with the granular component of the nucleolus, although this still has to be demonstrated by a detailed immunoelectron microscopy analysis. Alltogether these immunolocalization analysis let us to speculate that NOA 36 participates as a component of a heterogeneous antigen complex at the nucleolus responsible for some rare (NOR) autoimmune responses in humans (33, 36).

In mitotic cells, the immunolocalization of NOA 36 surprisingly reveals its association with the centromeres of chromosomes during the mitotic cell cycle (Fig. 7). Staining of different stages of mitosis showed several fluorescence dots at the tips of moving chromosomes, a typical feature of the centromere/kinetochore region (Fig. 7, *a–f*). 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. 7*h*). 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^{cdc2} (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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