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GAS41, a Highly Conserved Protein in Eukaryotic Nuclei, Binds to NuMA*

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The yeast two-hybrid system was used to identify binding partners of NuMA, a component of the nuclear matrix in interphase cells. By using the C-terminal half of NuMA as bait, a human cDNA sequence coding for a 223-amino acid protein with a non-helical N-terminal domain and a C-terminal α-helical portion was identified and fully sequenced. It was identical to GAS41, a sequence amplified in human gliomas. The sequence of the homologous *Drosophila* protein was established, and the alignment for GAS41 from nine different species showed that GAS41 is a general eukaryotic protein found in species as diverse as *Arabidopsis, Drosophila, Caenorhabditis elegans,* yeast, and man. Northern blot analysis showed a single transcript in eight human tissues. A polyclonal antibody to GAS41 showed a dotted staining pattern in interphase nuclei and a uniform distribution in mitotic cells. A GFP-GAS41 fusion protein displayed equivalent patterns. *In vitro* GAS41 bound to the C-terminal part of the rod region of NuMA, as shown by dot overlay and by surface plasmon resonance measurements. The K_d of the complex was 2×10^{-7} M. GAS41 is related to the AF-9 and ENL proteins, which are putative transcription factors found as fusion proteins in some acute leukemias. The NuMA/GAS41 interaction may provide a link between nuclear structure and gene expression.

The knowledge of proteins that form the nuclear skeletal structure in interphase is still incomplete. NuMA, the nuclear mitotic apparatus protein, is a protein that shows a striking change in localization during the cell cycle moving from the nuclear matrix in interphase to the spindle poles in mitotic cells (1). This change in location is accompanied by specific changes in phosphorylation of the NuMA molecule (2, 3) and presumably also by changes in the binding partners with which NuMA interacts. In interphase NuMA is a component of the nuclear matrix since it remains insoluble after DNase and high salt treatments of the nucleus. It is also resistant to extraction by non-ionic detergents, but it can be solubilized by high concentrations of urea (1, 4, 5). NuMA can bind specifically to DNA matrix regions (6). It also binds to the nonerythroid isoform of protein 4.1R located within the nucleus (7).

Transient overexpression of NuMA in HeLa cells results in regular nuclear networks that fill the nucleus, are stable to detergent extraction, and can be visualized by electron microscopy. Immunoelectron microscopy shows that these nuclear lattices are built from NuMA (8). In addition, we have shown that recombinant NuMA expressed in *Escherichia coli* can self-assemble *in vitro* under physiological salt conditions into a novel higher order structure, the multiarm oligomer. Computer modeling suggests that these oligomers could be the structural unit of the lattices seen in electron microscopy in the nuclei of cells transfected with NuMA (9). These lattices could possibly provide a framework where nuclear processes such as transcription take place.

The importance of NuMA in interphase as well as the fact that it plays an essential role in mitosis (4, 10–12) suggest that it would be of interest to characterize binding partners. We therefore used the yeast two-hybrid system (13) to screen a HeLa library for proteins that interact with NuMA. Our screen has revealed surprisingly that the protein product of a gene previously shown to be amplified in gliomas (GAS41) is a binding partner for NuMA. *GAS41* is amplified in 23% of glioblastomas and in 80% of grade I astrocytomas. *GAS41* was the first gene shown to be amplified in low grade glioma, and its definition showed that gene amplification is not limited to late events in tumor progression (14). Here we report that GAS41 is a highly conserved protein since homologs to the human form were found in *Arabidopsis*, *Drosophila*, *Caenorhabditis elegans,* and yeast. We further show that GAS41 is a nuclear protein giving a dotted pattern in interphase cells and is present in a variety of human tissues. The specific interaction of GAS41 with NuMA was confirmed *in vitro* by immunoprecipitation experiments and surface plasmon resonance binding assays.

EXPERIMENTAL PROCEDURES

RNA Preparation—mRNA was prepared using the messenger RNA isolation kit from Stratagene (La Jolla, CA). HeLa cells were homogenized in guanidinium isothiocyanate and β-mercaptoethanol. 3′-Polyadenylated mRNA was isolated using oligo(dT)-cellulose. *Drosophila melanogaster* embryo poly(A)$^+$ RNA was obtained from CLONTECH.

Construction of the pAD-GAL4 cDNA Library and Two-hybrid Screening—Starting from 5 μg of poly(A)$^+$ RNA, a cDNA library of HeLa cells was constructed in the phagemid pAD-GAL4 (GAL4 activation domain vector) using the HybriZAP two-hybrid cDNA Gigapack cloning kit (Stratagene) followed by mass excision. cDNA synthesis was oligo(dT)-primed. This library was used to identify novel components that interact with "bait" NuMA cDNA constructs. Different NuMA cDNA fragments obtained by PCR[1] amplification and *Eco*RI and *Sal*I treatment were ligated into the corresponding sites of pBD-GAL4 (GAL4 binding domain vector, Stratagene). PCR amplification was done

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ245746.

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[1] The abbreviations used are: PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; bp, base pair; kb, kilobase pair; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; GFP, green fluorescent protein.

TABLE I
Sequence of primers used for PCR amplification of pBD-GAL4 constructs
The restriction enzyme site within the primer sequence is underlined. The stop codon is shown in bold. Base pair positions refer to the sequence of Yang *et al.* (31).

PCR primer	Sequence 5′ → 3′	Restriction enzyme	Base pair position 5′	Base pair position 3′
27	GCATGAATTCATGAGACGGCTGAAGAAGCAGCTTGCTG	*Eco*RI	802	829
33	GCATGAATTCATGACACTCCACGCCACCCGGGGGGCTG	*Eco*RI	157	184
35	GCATGAATTCCAGGTGGCAACTGATGCTTTAAAGAGCCG	*Eco*RI	5257	5285
38	GGCATGTCGAC**TT**AGTGCTTTGCCTTGCCCTTGGCTCGAG	*Sal*I	6501	6476
39	GGCATGTCGAC**TT**AGAATTTGCCCAGGTCTCGAAGCTGCTGG	*Sal*I	5256	5229
53	GCATGAATTCCTGCAAGAGGCACTGGCTCATGCCCTGAC	*Eco*RI	3298	3326
54	GGCATGTCGAC**TT**ACTTGGCCAACTCCTGGTCCTTGCCTTCC	*Sal*I	3360	3333
55	GGCATGTCGAC**TT**ACTGGAACTGTGGGGTCTGCAGGATATCAC	*Sal*I	801	773

using Vent polymerase (New England Biolabs, Schwalbach, Germany), which has 3′ to 5′ proofreading exonuclease activity. NuMA clones λ1 and λ2 (5), or full-length cDNA, were used as template. Primer pairs for the different two-hybrid bait constructs (Fig. 1) and the amino acid residues in which they result were as follows: N (33, 54; 1–1068), C (53, 38; 1048–2115), H (33, 55; 1–215), T (35, 38; 1701–2115), Ncoil (27, 54; 216–1068), and Ccoil (53, 39; 1048–1700) (see Table I). To screen for interacting proteins the *Saccharomyces cerevisiae* strain YRG-2 (Stratagene) was sequentially transformed with the bait (NuMA-cDNA) and the HeLa library containing phagemids using the LiAc method. Clones were examined for transcriptional activation of reporter genes *his3* and β-galactosidase (filter lift) indicating interaction between bait/binding domain and library/activation domain constructs. Only clones meeting all standard two-hybrid specificity tests were considered as positive. These tests included absence of an interaction between the target construct and p53 and pLaminC-negative control constructs and the inability of colonies containing the target construct alone, or target and bait vector without insert, to pass His^- and β-galactosidase assays. Positive clones were partially sequenced.

5′-Rapid Amplification of cDNA Ends (5′-RACE)—5′-RACE experiments were performed using the Marathon cDNA amplification kit (CLONTECH) and HeLa messenger RNA.

Northern Blots—A PCR-amplified 1055-bp DNA fragment (bp 210–1264) of the GAS41 cDNA coding sequence was radiolabeled with [α-^{32}P]dCTP using a random hexamer labeling kit (Quick Prime, Amersham Pharmacia Biotech), and the probe was purified with a Qiaquick nucleotide removal kit (Qiagen, Hilden, Germany). The probe supplemented with 100 μg of salmon sperm DNA was hybridized with a multiple tissue Northern blot (CLONTECH, Palo Alto, CA) containing 2 μg of purified poly(A)$^+$ RNA from various human tissues in ExpressHyb hybridization solution (CLONTECH) for 1 h at 68 °C. The blot was then extensively washed following the manufacturer's recommendations and subjected to autoradiography at −70 °C. A human β-actin cDNA probe was used as control.

GFP Plasmid Construction—First strand cDNA synthesis was initiated using the oligo(dT) anchor primer of the 5′–3′ RACE kit from Roche Molecular Biochemicals. After converting mRNA into cDNA GAS41-cDNA was amplified using the gene-specific primer AGATCTATATGTTCAAGAGAATGGCCG (*Bgl*II site underlined) and the PCR anchor primer GACCACGCGTATCGATGTCGAC, which includes a *Sal*I site. The amplified product was cloned into the *Bgl*II and *Sal*I site of the pEGFP-C2 vector (CLONTECH). Immunocytochemistry and transient transfection used standard techniques (4, 15). Cells were examined 24 h post-transfection.

Antibodies—Monoclonal NuMA SPN3 antibody (4, 5) was used as undiluted hybridoma supernatant. The rabbit GAS41 antibody was raised by injecting a peptide covering residues 206–222 of GAS41 coupled to ovalbumin. The antibody was affinity-purified using the peptide coupled to EAH-Sepharose 4B with the linker m-maleimidobenzoyl-*N*-hydroxy-sulfosuccinimide ester by the N-terminal cysteine residue. Anti-GFP rabbit antiserum was from Molecular Probes, (Eugene, OR).

Expression and Purification of λ1a and λ2b Constructs—SOB medium (20 g of tryptone, 5 g of yeast extract, 0.5 g of NaCl, 0.186 g of KCl, and 20 mM $MgCl_2$ per liter H_2O) containing ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml) was inoculated with a single recombinant *E. coli* BL21(DE3)pLysS colony that contained the appropriate plasmid and grown overnight at 37 °C with shaking. This culture was used to inoculate 400 ml of SOB medium with antibiotics. The culture was grown at 37 °C with shaking until it reached an absorbance at 600 nm of 0.5. Expression was initiated by adding isopropyl-1-thio-β-D-galactopyranoside to a concentration of 1 mM. After about 4 h, cells were harvested by centrifugation. The purification of the soluble recombinant constructs λ1a and λ2b was as described in Ref. 9.

Expression and Purification of GST and GST-GAS41—GAS41 was expressed as a C-terminal fusion with glutathione *S*-transferase (GST). GAS41 was amplified by PCR. The following primers were used: 5′-dGGATCCATGGCCGAATTTGGGCCTGACTCC (sense) and 5′-dCTCGAGTTATATGTCTTTTGCTTGGTCATC (antisense) (restriction sites are underlined). The product was cloned into the *Bam*HI and *Xho*I sites of pGEX-KT expression vector. A single *E. coli* colony BL21 DE3(pLysS) transformed with the construct was used to inoculate 2× YTG (2× YT + 2% glucose) with the antibiotics chloramphenicol (34 μg/ml) and ampicillin (100 μg/ml). Cultures were grown at 28 °C to an absorbance at 600 nm of 0.6. Then cultures were transferred to 14 °C, and expression was induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside. After expression for ~20 h cells were harvested and resuspended in PBS with protease inhibitors (Complete Mini, EDTA-free, Roche Molecular Biochemicals). After sonication Triton X-100 was added to 1%. The extract was incubated for 30 min at 4 °C with rotation and then centrifuged for 15 min at 12,000 × *g* at 4 °C. The supernatant was filtered (0.45 μm) and mixed with equilibrated glutathione-Sepharose 4B (Amersham Pharmacia Biotech). After 1 h incubation at room temperature the matrix was extensively washed with PBS, and the GST fusion protein was eluted with 10 mM glutathione in 50 mM Tris-HCl, pH 8. Fractions containing the fusion protein were pooled and dialyzed against the buffer of interest. GST protein was expressed in pGEX-KT and purified under standard conditions following the protocol supplied by Amersham Pharmacia Biotech.

Gel Electrophoresis and Western Blot Analysis Were by Standard Procedures—SDS polyacrylamide gel electrophoresis was on 12.5% acrylamide gels. Proteins were transferred to nitrocellulose using the semi-dry transfer procedure (5).

Ligand Interaction Studies—To determine dissociation constants (K_d) surface plasmon resonance studies were performed using the BIAcore 2000 apparatus (BIAcore, Uppsala, Sweden). Purified NuMA constructs λ1a and λ2b were immobilized on a CM5 sensor chip via their primary amino groups following the manufacturer's instructions. Both proteins were coupled at a density of 9000 resonance units. For all further experiments, PBS (137 mM NaCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 2.7 mM KCl, pH 7.3) was used as running buffer at a flow rate of 20 μl/min. 0.5% SDS, 10 mM NaOH was used to regenerate the sensor surface after each experimental cycle (16). Purified GST-GAS41 fusion protein was used at concentrations ranging from 50 to 1000 nM, and GST as a control to monitor nonspecific binding. Protein concentrations were determined by the Bradford assay (17). The rate constants were determined with the BIAcore evaluation software 3.0, assuming first order kinetics (1:1 interaction).

RESULTS

Identification of a Binding Partner for NuMA in the Yeast Two-hybrid Assay—The two-hybrid system was used to screen for proteins that interact with NuMA. We used the fusion protein GAL4-BD/NuMA-C (amino acid residues 1048–2115 of NuMA) as a bait in the pBDGAL4 vector (Fig. 1*A*) and a human HeLa cDNA library in λHybriZAP fused to the GAL4 activation domain in pADGAL4. ~5 × 10^6 cotransformants were screened. Around 30 colonies grew on agar lacking histidine and of those 5 colonies expressed *lacZ*. Here we focus on the interaction of one clone (clone X) and NuMA-C in the two-hybrid assay. This was specific (Fig. 1*B*), since neither the GAL4-BD NuMA-C hybrid interacted with the unfused GAL4-

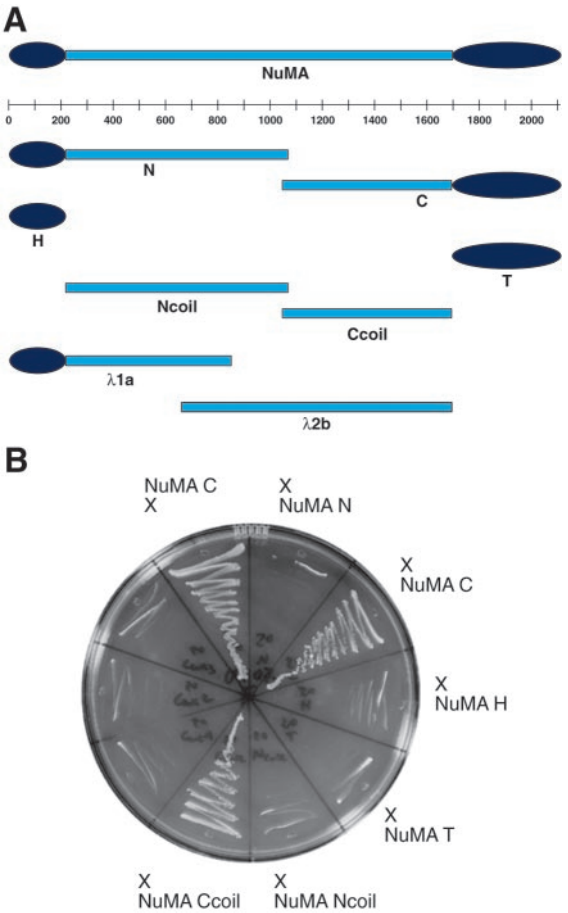

FIG. 1. *A, top,* secondary structure of NuMA indicating the long coiled-coil region flanked by non-helical head and tail domains. *Middle,* constructs used for the two hybrid system (*N, C, H, T, NcoIl,* and *CcoIl*). *Bottom,* constructs used for the *in vitro* binding assay (*λ1a* and *λ2b*). The amino acid sequence numbers for NuMA are shown in the *2nd line,* and the exact limits of each of the constructs are given in the text. *B,* specific interaction between NuMA and GAS41 observed by the yeast two-hybrid system. The bait protein (the NuMA construct) was fused to the GAL4 DNA-binding domain (pBD vector). The interacting protein that was fused to the GAL4 activation domain (pAD vector) activates the transcription of the *HIS3* and *lacZ* genes when the probe and interactor associate physically. Constructs in the pAD vector are always in the *top line,* and constructs in the pBD vector are always in the *bottom line*. Note that clone X interacted strongly with constructs containing the C-terminal part of the coiled-coil rod domain but did not react with the H, T, N, or NcoIl constructs. Cloning X into the pBD vector and NuMA C into the pAD vector also revealed strong interaction. The unlabeled parts of this figure are not relevant to this study.

AD, nor did the GAL4-AD-X hybrid interact with the unfused GAL4-BD (data not shown). Fusing the original bait (NuMA-C) into the pAD vector and the target protein into the pBD vector also gave a positive result (Fig. 1*B*). The same clone was found in a second independent screen. This led to the isolation of a cDNA clone with a length of 652 base pairs. This clone X showed an open reading frame of 50 amino acids and a long 3′-translated region that included a polyadenylation signal and the poly(A) tail. The interaction of clone X with the other NuMA constructs shown in Fig. 1*A* was also tested. Clone X interacted strongly with the C-terminal part of the coiled-coil rod domain (amino acid residues 1048–1700). Thus it interacted with the NuMA C and the NuMA CcoIl constructs but did not interact with the N, H, T, or NcoIl constructs (Fig. 1*B*). The data from the two-hybrid assay show that the C-terminal 50 amino acids predicted by the clone X sequence bind to residues 1048–1700 of NuMA.

Since the HeLa library was oligo(dT)-primed, we used the

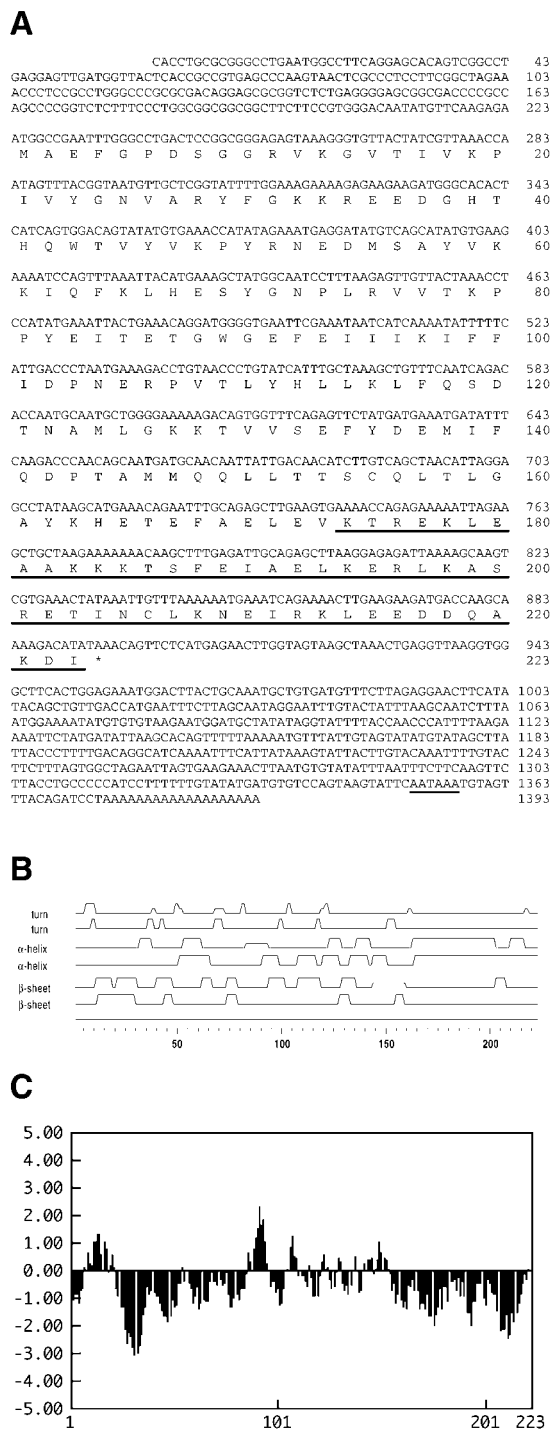

FIG. 2. *A,* the nucleotide and corresponding amino acid sequence of human GAS41. The translational stop codon is marked by an *asterisk.* The *underlined* protein sequence indicates the residues where the cDNA was fused to the GAL4 activation domain (the original clone from the two-hybrid screen). The polyadenylation signal in the 3′-translated region is *underlined.* These sequence data have been submitted to the EMBL data base under accession number AJ245746. *B,* secondary structure of GAS41. Predictions were made using the Chou-Fasman algorithm (28) with the overall probability procedure introduced by Nishikawa (29) (*top*) or the Garnier-Osguthorpe-Robson algorithm (20) of the Wisconsin Package GCG (*bottom*). *C,* hydrophobicity blot according to Kyte and Doolittle (30) with a window size of 10 residues.

5′-RACE technique to obtain the sequence 5′ to the new cDNA. The full-length cDNA was amplified, cloned, and sequenced. The nucleotide and corresponding amino acid sequences are shown in Fig. 2*A*. DNA sequencing revealed a nucleotide se-

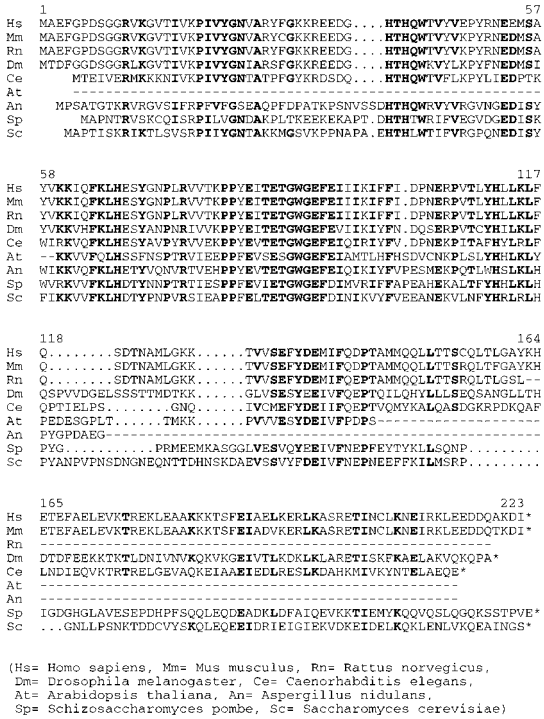

FIG. 3. **Alignment of the amino acid sequences of GAS41 from 9 different species, showing that the protein is conserved from yeast to man.** The human sequence and the *D. melanogaster* DNA sequences are from this study. Open reading frames or ESTs from other species were found in the data bases. For *R. norvegicus*, *A. thaliana,* and *A. nidulans* only partial sequences are available. Missing parts of the sequence are indicated by *dashes.* Gaps introduced to optimize the alignments are indicated by *dots. Bold type* is used to identify residues that are identical in at least 7 of the 9 sequences or where all, or all except one or two of the known sequences, show identical residues. The amino acid numbers for the human sequence are shown at the *top.*

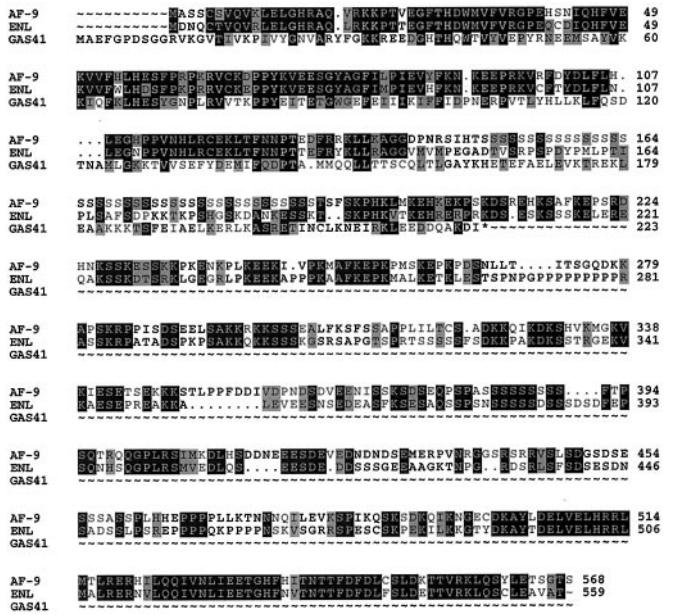

FIG. 4. **Alignment of human AF-9, ENL, and GAS41.** Identical amino acid residues are shown in *black boxes* and similar residues in *gray boxes.* Note that GAS41 corresponds only to the first 208 or 206 residues of the longer proteins AF-9 and ENL (568 or 559 residues total length).

quence of 1393 bases that contains a single open reading frame. There are two ATG codons at nucleotides 212 and 224. We consider that initiation is more likely to occur at the second ATG codon since experiments using site-directed mutagenesis have confirmed the importance of G^{+4} for initiation of translation in higher eukaryotes (18). Moreover the N-terminal alignment of the human sequence with that of other species (Fig. 3) is in favor of the second ATG. An in-frame stop codon upstream is found at position 80. The open reading frame is followed by a 498-base 3′-untranslated region with a consensus sequence for polyadenylation (AATAAA) at positions 1352–1357 and a TTTTGT sequence at positions 1322–1327 characteristic of early response genes (19). The amino acid sequence predicts a protein of 223 amino acids with a calculated molecular mass of 26 kDa and an isoelectric point of 6.39.

In the data base we found a 507-bp *D. melanogaster* 5 prime EST sequence (AA441282) from the Berkeley Drosophila Project, which showed homology to the N-terminal 170 amino acid residues of the human sequence. 3′-RACE was used to obtain the C-terminal part of the sequence for the *Drosophila* homolog. The 5′ sequence was confirmed by 5′-RACE. The *Drosophila* protein sequence has 227 amino acid residues and is shown in Fig. 3.

GenBank™ Homology Searches and Protein Sequence Analysis Identify the Protein as the GAS41 Gene Product—Homology searches of the cDNA were performed using BLAST algorithms. A BLASTN search revealed strong homologies to open reading frames in *C. elegans* (Z77667), *S. cerevisiae* (Z71383 and Z69382), and *Schizosaccharomyces pombe* (Z69795.1). EST sequences for *Mus musculus* (AA529582 and AA086873) and partial EST sequences for *Rattus norvegicus* (AI031024 and AI012982) were also found. So were sequences for *Arabidopsis thaliana* (H76547) and *Aspergillus nidulans* (AA787444). An alignment of all nine sequences is shown in Fig. 3. The human and *Drosophila* protein sequences show 61% identity and 70% similarity, whereas the human and the *C. elegans* sequences show 48% identity and 59% similarity. The GenBank™ search also showed that the cloned human sequence was identical to *GAS41*, a gene shown by Fischer *et al*. (14) to be amplified in some human gliomas.

The N-terminal part of GAS41 seems to be highly conserved between different species (Fig. 3) with the region corresponding to residues 80 and 94 of the human sequence being especially well conserved. Secondary structure prediction rules (20) show that the C-terminal 60 amino acids are essentially α-helical (Fig. 2*B*). This region shows a heptad repeat pattern characteristic for the formation of hydrophobic interactions and the formation of homo- or heterodimers. The frequency of the acidic amino acid glutamate and of the basic amino acid lysine is about twice the frequency in an average protein. An enrichment of acidic amino acids (27%) is found within the C-terminal 60 amino acids. There are no clearly identifiable nuclear localization sequence motifs in the sequence, but we note a basic highly conserved motif KK or RK at position 60 followed by another highly conserved basic motif FKLH at position 64. Together these motifs represent a potential nuclear localization signal. A number of protein kinase recognition sequence motifs are also present as follows: for example a potential phosphorylation site, ^{183}KKKT^{186} and/or ^{184}KKTS^{187} for cAMP-dependent protein kinase (consensus (R/K)*XX*(S/T) (21)) at the beginning of the α-helical region, a casein kinase II phosphorylation site ^{186}TSFE^{189} (consensus (S/T)*XX*(D/E) (22)) in the same region, and a tyrosine kinase phosphorylation site ^{51}RNEEMSAY^{58} (consensus (R/K)*XX*(D/E)*XXX*Y (23)) in the N-terminal region.

The GAS41 sequence (223 residues) can also be aligned with the N-terminal portions of the AF-9 (568 residues) and ENL proteins (559 residues) (Fig. 4 (14)). GAS41 and ENL show 24% identity and 35% similarity, whereas GAS41 and AF-9 show 21% identity and 30% similarity. Between residues 32 and 89

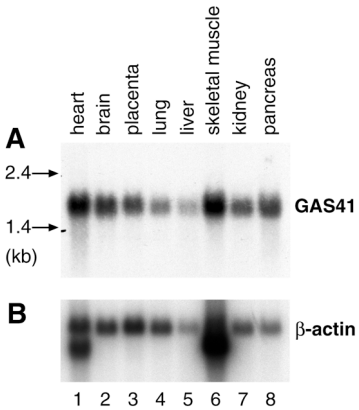

FIG. 5. **Human multiple tissue Northern blot containing 2 μg per lane of poly(A)$^+$ RNA probed sequentially with [^{32}P]dCTP-labeled GAS41 probe and a control β-actin cDNA probe provided by the manufacturer.** *A,* Northern blot analysis of transcripts encoding GAS41 from eight different human tissues shows a single band. Size (in kb) of the molecular markers are indicated at the *left. B,* Northern blot using a β-actin cDNA control. In both heart and skeletal muscle there are two isoforms of β-actin RNA, a 2- and a 1.6–1.8-kb form due to hybridization to either the α or γ form of actin.

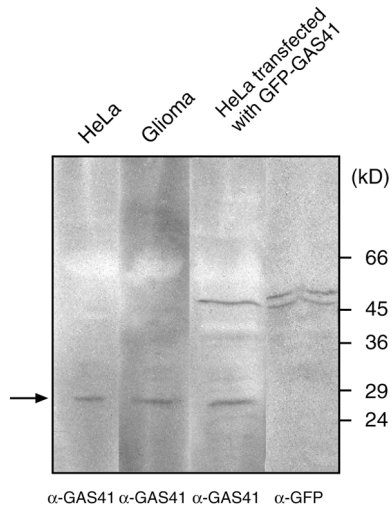

FIG. 6. **Polyclonal antibody directed against a C-terminal peptide of GAS41 detects a band of 26 kDa (*arrow*) in HeLa and glioma cell lines and a band of ~52 kDa in HeLa cells transfected with the GFP-GAS41 fusion protein.**

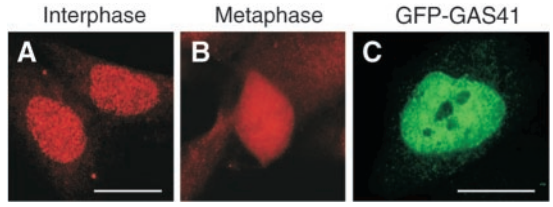

FIG. 7. *A* and *B,* immunofluorescence microscopy of GAS41 with the rabbit peptide antibody on the glioma cell line U333CG/343MG. *A,* in interphase GAS41 is found in a punctate distribution throughout the nucleoplasm except for the nucleolus. *B,* in metaphase GAS41 is found distributed throughout the mitotic cell. *C,* full-length GAS41 cDNA was cloned into a GFP vector. HeLa cells transfected with this GFP construct showed a punctate pattern in the nuclei of interphase cells. *Bars,* 5 μm.

GAS41 shows 39% identity and 59% similarity to the human AF-9 protein and 37% identity and 61% similarity to the human ENL protein (Fig. 4). AF-9 and ENL share 56% identity and 68% similarity, with the highest homology located to 140 residues at the N terminus and 67 residues at the C terminus. AF-9 and ENL are thought to belong to a new class of transcription factors (24).

GAS41 Is Present in a Variety of Human Tissues and in Cell Lines—The distribution of transcripts of GAS41 in a variety of human tissues was examined by Northern blot hybridization analysis using as a probe the 1055-bp fragment starting in the 5′-translated region at base number 210 and ending in the 3′-untranslated region at base 1264. We detected a single ~1.7-kb mRNA transcript corresponding to GAS41 in all eight tissues tested (Fig. 5*A*). When normalized using the β-actin probe as control, equivalent amounts of GAS41 seemed to be present in the eight tissues listed in Fig. 5.

To see if GAS41 is expressed in human cell lines, immunoblots were performed using an affinity-purified rabbit antibody to GAS41. The antibody was raised against the C-terminal peptide ^{206}CLKNEIRKLEEDDQAKD^{222} and affinity-purified on the peptide coupled to bovine serum albumin. An immunoreactive band with an apparent molecular mass of ~26 kDa was detected in Western blots of whole cell extracts of HeLa and glioma cells (Fig. 6). In HeLa cells transiently transfected with the GFP-GAS41 construct the affinity purified antibody detected the endogenous GAS41 and an additional band around 52 kDa which probably corresponds to the GFP-GAS41 fusion protein. When the same cell extract was tested with a GFP antibody, the 52-kDa protein was also found. However in addition a second slightly larger polypeptide that might correspond to a phosphorylated form of the fusion protein was also detected by immunoblotting (Fig. 6).

The subcellular localization of GAS41 was determined by immunofluorescence microscopy using the rabbit GAS41 antibody. In interphase cells GAS41 was found in dots throughout the nucleoplasm with the exception of the nucleolus (Fig. 7*A*). In metaphase GAS41 was found distributed uniformly throughout the mitotic cell but did not specifically bind to the chromosomes (Fig. 7*B*).

The nuclear localization of GAS41 was confirmed by monitoring HeLa cells transfected with the GFP-GAS41 construct (Fig. 7*C*). Again, the GFP fusion protein was distributed in a punctate form in the nucleoplasm of interphase cells, and the nucleoli were not stained.

Interaction between GAS41 and NuMA: Immunoprecipitation Experiments, Dot Overlay Assays, and Surface Plasmon Resonance Experiments—To determine whether GAS41 and NuMA interact *in vivo*, coimmunoprecipitation using nuclear extracts from HeLa cells was performed. NuMA was precipitated using a polyclonal antibody directed against the head region. Subsequent detection with anti-GAS41 polyclonal antibody and an F_c-specific second antibody showed a band in the expected range of 26 kDa, which was not detected in the control without first antibody (data not shown). This suggests that GAS41 and NuMA are contained in the same protein complex *in vivo*. The relatively low efficiency of coimmunoprecipitation suggests that only a fraction of these molecules associate together *in vivo*.

An interaction between GAS41 and NuMA was verified *in vitro* using dot overlay assays (Fig. 8). GAS41 was expressed as a GST fusion protein in *E. coli*. The NuMA N-terminal construct (λ1a, residues 1–851) and the construct including the C-terminal part of the rod (λ2b, residues 670–1,700) were soluble when expressed in *E. coli* and purified to homogeneity as described earlier (5). These constructs were spotted onto nitrocellulose and incubated with either the purified GST-GAS41 fusion protein or with GST. Binding was monitored by treating the dot blots with the GST-specific antibody. The results show that the GST-GAS41 fusion protein bound to the λ2b construct but not to the λ1a construct (Fig. 8*A*). SDS gel electrophoresis of the protein preparations used in the dot blot

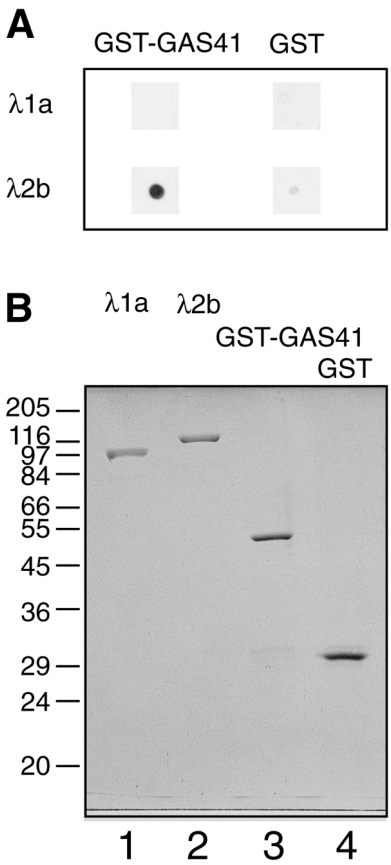

FIG. 8. **Interaction of NuMA with GAS41 in dot overlays.** *A,* the purified NuMA λ1a and NuMA λ2b fragments were dotted on to nitrocellulose and are indicated at the *left*. The GST-GAS41 and GST proteins were added as probes and are indicated at the *top*. The GST antibody was employed to monitor protein interactions. Note the strong interaction of GST-GAS41 and λ2b, and the lack of interaction of GST-GAS41 and λ1a. *B,* Coomassie-stained gel to show the purity of the NuMA constructs λ1a and λ2b and of the GST-GAS41 and GST constructs.

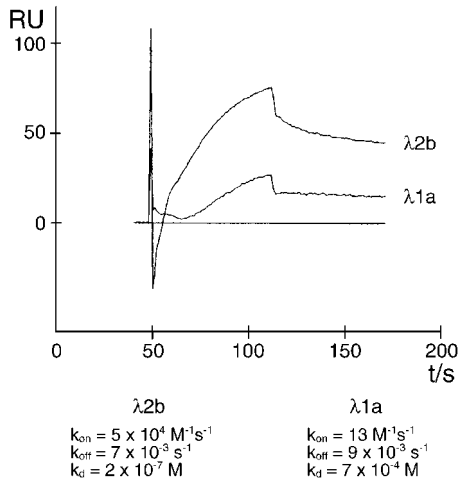

FIG. 9. **Surface plasmon resonance measurement of the NuMA-GAS41 interaction.** Purified NuMA constructs λ2b and λ1a were immobilized on the sensor chip and probed with GST-GAS41 (50–1000 nM). GST was used at equal concentrations to monitor unspecific background. Graph represents results were concentrations of 250 nM were used. Values for k_{on}, k_{off}, and K_d in terms of resonance units were calculated for values from 50, 250, and 1000 nM and are presented in the *table below*. The data shows that the ratio of λ2b:λ1a binding to GST GAS41 is ~3500:1.

assays show that there were no contaminating proteins present that might have mediated this interaction (Fig. 8*B*). Since GST alone did not bind to the λ2b construct, our results imply a direct binding of GAS41 to the C-terminal part of the coiled-coil rod domain of NuMA.

Finally, we analyzed the kinetic properties of the complex formation between NuMA and GAS41 by surface plasmon resonance measurements. The two different NuMA constructs λ1a and λ2b (see Fig. 1*A*) were immobilized on the sensor chip surface. Binding and dissociation of either GST-GAS41 fusion protein or GST were monitored in terms of relative units (Fig. 9). When the purified GST-GAS41 fusion protein was used, binding and dissociation was easily monitored in the range of 50–1000 nM GST-GAS41. The corresponding curves obtained with the same concentrations of GST were subtracted. The data shown in Fig. 9 were used to calculate dissociation kinetics, yielding a k_{off} of 7×10^{-3} s^{-1}. The k_{on} value was in the range of 5×10^4 M^{-1} s^{-1}, with the exact value depending on the fitting method used. Thus, the dissociation constant of the complex is $K_d = 2 \times 10^{-7}$ M. In the case of λ1a, binding was barely detectable and a factor of 3500 less compared with λ2b (Fig. 9).

DISCUSSION

A two-hybrid screen using the NuMA C-terminal half as bait in the yeast two-hybrid system led to a human cDNA which by sequence was shown to correspond to GAS41. GAS41 specifically interacts with the C-terminal part of the central coiled-coil region of NuMA (residues 1048–1700). The C-terminal 50 residues of GAS41 were necessary for NuMA binding in the two-hybrid system. Dot overlays provided independent proof that the C-terminal part of the coiled-coil rod region of NuMA binds to GAS41 since construct λ2b, covering residues 670–1700, showed a strong reaction, whereas the N-terminal construct λ1a covering residues 1–851 did not. Independent evidence for the interaction between NuMA and GAS41, as well as the kinetic constants for association and dissociation of the NuMA-GAS41 complex were provided by surface plasmon resonance studies (Fig. 9). The NuMA fragment λ2b bound 3500 times more strongly to GAS41 than did λ1a. The calculated dissociation constant of the NuMA-GAS41 complex is 2×10^{-7} M, and this is in the range commonly found for the interaction of cytoskeletal components (25).

GAS41 is highly conserved among species as diverse as fungi, yeast, plants, and man (Fig. 3). Thus GAS41 is a general eukaryotic nuclear protein. This provides a handle to examine directly the function of GAS41, for instance by making knockouts in mice or *C. elegans*.

Northern blot analysis showed that the mRNA for GAS41 was expressed in all eight human tissues that were tested. Immunohistochemistry with GAS41-specific antibodies showed a nuclear-specific localization in interphase cells. Interphase nuclei were stained in a punctate fashion (Fig. 7*A*). An independent determination of the cellular localization of GAS41 used cells transfected with a GFP-GAS41 construct. In interphase cells again a punctate nuclear distribution of GAS41 was seen both in fixed cells assayed by immunofluorescence microscopy and in living cells analyzed for the distribution of the GFP construct (Fig. 7*C*). Further experiments using methods with higher resolution (for example 4Pi microscopy) are needed to see if GAS41 colocalizes to a subfraction of NuMA dots reported for instance with the NuMA 705 antibody (8). In mitotic cells GAS41 and NuMA have different localizations. GAS41 is found throughout the cell (Fig. 7*B*), and NuMA is associated with spindle poles (1, 4).

The sequence homology of GAS41 with the human AF-9 and ENL proteins is intriguing (Fig. 4 and Ref. 14). *AF-9* is found fused to the *ALL-1* gene in leukemias with t(9:11) transloca-

tions, and *ENL* is found fused to the *ALL-1* gene in leukemias with t(11:19) translocations. The fact that AF-9 and ENL have extensive sequence homology as well as several shared sequence motifs (*e.g.* a nuclear targeting sequence, serine-rich domains and stretches rich in proline or in basic amino acids) has led to the suggestion that the functional activities of the proteins coded for by *AF-9* and *ENL* are related and are important in leukemia (24). Many genes associated with translocations in acute leukemia code for transcription factors (26) leaving open the question whether *AF-9* and *ENL* might also code for such proteins (*cf.* Ref. 24). An artificially constructed gene which fused the *AF-9* gene to the mouse MII (*ALL-1*) gene by homologous recombination has been shown to cause acute leukemia in chimeric mice (27). GAS41 is shorter than AF-9 and ENL, and in contrast to these two proteins lacks a typical DNA-binding domain for transcriptional activation (Fig. 4). However, GAS41 could activate transcription together with a second protein that contains a DNA-binding domain.

We speculate that the interaction between NuMA and GAS41 shown here may provide a direct link between nuclear architecture and gene expression. We have shown elsewhere that NuMA has the ability to polymerize into lattice-like structures *in vivo* (8), and we have also shown that NuMA can self-assemble *in vitro* into multiarm oligomers, which may be the structural unit for a nuclear scaffold (9). Obviously, to prove such a link further evidence has to be provided to show that GAS41 is involved in transcription and to define the nature and stoichiometry of the GAS41-NuMA complex.

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GENES: STRUCTURE AND REGULATION:

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