

## A Growth Factor-inducible Nuclear Protein with a Novel Cysteine/Histidine Repetitive Sequence\*

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Growth factors rapidly induce transcription of a set of genes that encode regulatory proteins, many of which have been identified by cDNA cloning. Here we report the analysis of a cDNA corresponding to a gene induced in mouse 3T3 cells by growth factors and a variety of other extracellular signaling agents. The cDNA encodes a proline-, serine-, and glycine-rich nuclear protein designated Nup475 of 319 amino acids that contains two tandemly repeated cysteine- and histidine-containing sequences (CX<sub>8</sub>CX<sub>5</sub>CX<sub>3</sub>H) suggestive of a novel heavy metal-binding domain. Nup475 produced in *Escherichia coli* binds zinc. Its mRNA is present in a number of mouse tissues and cell lines, being especially abundant in intestine, thymus, and regenerating liver and in a macrophage cell line stimulated by  $\gamma$ -interferon. We hypothesize that Nup475 is a regulatory protein with a novel zinc finger structure.

Polypeptide growth factors and other extracellular ligands induce in target cells sequential changes in gene expression that appear to mediate long term cellular responses to the ligands. In mouse 3T3 fibroblastic cells, serum, platelet-derived growth factor, or fibroblast growth factor rapidly induce transcriptional activation of a set of "immediate early" genes that encode nuclear proteins (including known or probable transcription factors), putative cytokines, transmembrane proteins, and cytoskeletal proteins (reviewed in Refs. 1-3). Among the nuclear proteins are members of the Fos and Jun families, Myc, and three zinc finger proteins. Some of these proteins are thought to regulate the genomic response to growth factors by repressing immediate early genes and by activating genes expressed later.

Here we report the identification and initial characterization of another immediate early nuclear protein designated Nup475. Like many of the other immediate early proteins, Nup475 was identified by analyzing cDNA clones from a library prepared from BALB/c 3T3 cells stimulated with serum in the presence of an inhibitor of protein synthesis (4).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J05669.

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Nup475 has two tandemly repeated cysteine and histidine sequences of novel type suggestive of a heavy metal-binding domain and is shown to bind Zn<sup>2+</sup>. On the basis of these properties and its nuclear location, we suggest that Nup475 may be a nucleic acid-binding protein involved in regulating the response to growth factors.

### EXPERIMENTAL PROCEDURES

**Cell Culture and Transfection**—BALB/c 3T3 cells were maintained and stimulated with serum as described previously (4). Cos 1 cells (5) were maintained in minimal essential medium plus 10% fetal calf serum; for transfection, cells were treated with calcium phosphate-precipitated recombinant plasmid containing Nup475 cDNA (*EcoRI*-*Bgl*II fragment) cloned into the *EcoRI* site of pMT2 (6).

**Partial Hepatectomy**—Partial hepatectomy in which 70% of the liver was removed was carried out as described (7). At various times after hepatectomy, mouse livers were surgically removed and total RNA was isolated (8).

**DNA Sequencing**—Nup475 cDNA and genomic DNA isolates were cloned into the pGEM-2 plasmid (Promega Biotec), and sets of 5' and 3' deletions were prepared. The resulting subclones were sequenced as double-stranded plasmids by the dideoxynucleotide chain termination method (9) using deoxyadenosine 5'-[ $\alpha$ -<sup>35</sup>S]thiotriphosphate and Sequenase (United States Biochemical Corp.).

**Sequence Homology Search**—Homology search of the GenBank, EMBL, and NBRF databases was carried out with the use of the GenMenu program which incorporates the GCG sequence analysis software (10).

**Mapping the 5' End of Nup475 mRNA**—A <sup>32</sup>P-radiolabeled oligonucleotide complementary to nucleotides 17 to 45 of the Nup475 mRNA was hybridized to 25  $\mu$ g of total cellular RNA purified from BALB/c 3T3 cells 3 h after the addition of 20% serum and 10  $\mu$ g of cycloheximide per ml. Primer extension was carried out as previously described (11). The S1 nuclease assay was performed as described (12). The probe used was a <sup>32</sup>P-labeled DNA synthesized on a 3-kb mouse genomic clone of *nup475* (partial sequence shown in Fig. 5) using as primer an oligonucleotide complementary to nucleotides 17 to 45 of the mRNA.

**Southern Blot Analysis**—Genomic DNA was digested with *EcoRI* or *HindIII* (New England Biolabs), fractionated on 1% agarose gels, and then transferred to nitrocellulose (Optibind, Schleicher and Schuell) in 20  $\times$  SSC (SSC is 0.15 M NaCl, 15 mM sodium citrate, pH 7.0). DNA probes were prepared from the Nup475 cDNA by isolating the *NcoI*-*Bgl*II fragment from a low melt agarose gel. The fragments were labeled with [ $\alpha$ -<sup>32</sup>P]dCTP by nick translation (Boehringer Mannheim). Prehybridization and hybridization incubations were carried out in 5  $\times$  SSC at 65 °C for 14-24 h. After hybridization, the blots were washed twice in a buffer containing 2  $\times$  SSC at room temperature for 30 min. Then, the filters were washed in 1  $\times$  SSC at room temperature for 120 min and exposed to x-ray film at -70 °C.

**Production of Nup475 in *Escherichia coli* and Preparation of Antisera**—The coding region of the Nup475 cDNA (*NcoI*-*Bgl*II) was cloned into the pET8C plasmid and expressed in the *E. coli* T7 polymerase expression system (13). *E. coli* protein extracts were prepared by lysis in SDS sample buffer and electrophoresed in pre-

† The abbreviations used are: kb, kilobase(s); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PDGF, platelet-derived growth factor.

parative SDS-polyacrylamide gels (14). The expressed protein was visualized by staining with Coomassie Blue, the Nup475 band was excised, and the gel segment was pulverized in liquid nitrogen and used to immunize New Zealand White rabbits (15). To purify the antibody, purified bacterial Nup475 was covalently linked to Sepharose and used as an affinity column as described (45). Western blotting was carried out as described (16). Anti-peptide sera were raised against each of two synthetic peptides (corresponding to amino acids 84-99 and 304-319 of Nup475) coupled to bovine serum albumin.

**Northern Blot Analysis**—Northern blot analysis was carried out as described (17).

**In Vitro Translation**—*In vitro* translation of cDNA transcripts and immunoprecipitation were carried out as described (40).

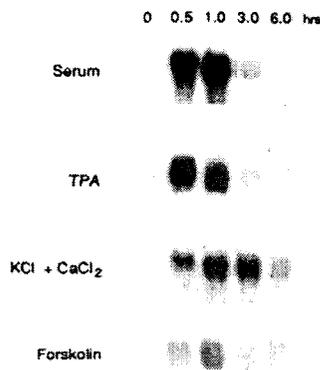
**Comparison of Nup475 mRNA with the Cloned cDNA**—Total RNA from regenerating mouse liver was annealed to a  $^{32}$ P-labeled antisense transcript corresponding to a 338-nucleotide fragment of Nup475 cDNA derived from nucleotides 355 to 17 (Fig. 2). The RNA duplex was treated with RNase and fractionated by electrophoresis, as described (41). Amplification of cDNA prepared from total cellular RNA was carried out as described (42). The primers corresponded to nucleotides 2 to 22 (5' primer) and nucleotides 1804 to 1783 (3' primer) of the Nup475 cDNA (Fig. 2).

**Immunofluorescence Staining of Cultured Cells**—Serum-stimulated BALB/c 3T3 cells or Cos cells grown on 12-mm coverslips were processed for immunostaining, as described (18), using affinity-purified anti-Nup475 antibody and rhodamine-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories).

**Zinc Binding**—Cell lysates and protein fractions were fractionated by discontinuous SDS-PAGE (14), and the proteins were electrophoretically transferred to nitrocellulose (Bio-Rad TransBlot). After transfer, the filters were washed in metal-binding buffer (100 mM Tris-HCl, pH 6.8, 50 mM NaCl) for 1 h, and the proteins were denatured in 6 M guanidine HCl for 30 min at 4 °C. The proteins were then renatured by serial incubations in buffer containing decreasing amounts of guanidine HCl. The filters were finally incubated in metal-binding buffer with  $^{65}$ ZnCl<sub>2</sub> and washed as described (19).

## RESULTS

**Analysis of Nup475 cDNA**—Nup475 cDNA was initially identified by differential screening of a cDNA library prepared from poly(A<sup>+</sup>) RNA of BALB/c mouse 3T3 cells stimulated with serum in the presence of cycloheximide (clone 475 of Ref. 4). As is typical of immediate early genes, *nup475* is transcriptionally activated within minutes after stimulation of quiescent 3T3 cells with serum or PDGF, its mRNA increases transiently thereafter, and it is superinduced in the presence of an inhibitor of protein synthesis ((4) and Fig. 1).



**FIG. 1. Northern blot analysis showing response of Nup475 mRNA in BALB/c 3T3 cells to signaling agents.** Total RNA prepared from cells at various times after stimulation with 20% fetal calf serum (15  $\mu$ g of RNA), 10  $\mu$ M forskolin (10  $\mu$ g), 50 mM 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (10  $\mu$ g), or 60 mM KCl plus 10 mM CaCl<sub>2</sub> (10  $\mu$ g) was fractionated by electrophoresis and blotted onto nitrocellulose, and the filter was probed with Nup475 cDNA. The numbers above each lane indicate the time in hours after stimulation. Only the 1.8-kb region of each autoradiogram is shown.

Nup475 mRNA also increases following treatment of 3T3 cells with a phorbol ester, forskolin, and depolarizing concentrations (20) of K<sup>+</sup> and Ca<sup>2+</sup> (Fig. 1).

Nup475 mRNA is about 1.8 kb in length, as estimated by gel electrophoresis. The nucleotide sequence of a nearly full length Nup475 cDNA is shown in Fig. 2. It contains a long open reading frame beginning at nucleotide 60 (the first ATG codon) that is 959 nucleotides in length and encodes a protein of 319 amino acids with a predicted molecular mass of 33,592 daltons. Following the termination triplet TGA at position 1,017, there are 785 nucleotides and a poly(A) tail. A typical poly(A) addition signal is present at nucleotides 1,781 to 1,786. Several ATTTA or related sequences are present in the 3' untranslated region, which may contribute to the observed instability of Nup475 mRNA (4, 21).

The 5' end of the mRNA was identified by reverse transcription of RNA prepared from superinduced 3T3 cells using an oligonucleotide primer (nucleotides 45-17) and by S1 nuclease analysis using a genomic DNA probe made with the same primer (Fig. 3). Both procedures revealed that the 5' end of the mRNA maps predominantly to a site 16 nucleotides upstream of the cDNA clone; this site is 29 nucleotides downstream from the start of a TATA-like sequence (ATAAA) present in a *nup475* genomic clone (Fig. 2).

**Predicted Protein Sequence**—The protein encoded by the long open reading frame of the Nup475 cDNA is rich in glycine, serine, and proline residues, including 3 PPPPG sequences. Another notable feature of the amino acid sequence is the presence of a partial repeat of 26 amino acids from residue 95 to 120 and 133 to 158 (*underlined* in Fig. 2). Of the 26 amino acid residues in each repeat, 17 are identical or closely related (Fig. 4), including identically spaced cysteine and histidine residues of the form CX<sub>n</sub>CX<sub>n</sub>CX<sub>n</sub>H, suggestive of a heavy metal-binding finger structure of novel type.

Our initial search of the EMBL/GenBank/NBRF protein and nucleic acid databases (versions 21, 62, 23, 35, respectively) did not reveal significant homology between Nup475 and known proteins. However, just prior to submission of this manuscript, we discovered a similarity of parts of the Nup475 cDNA sequence to that for a recently described cDNA (designated TIS11) derived from an mRNA induced by phorbol ester (43). The nucleotide sequence of Nup475 cDNA between positions 300 and 1731 (Fig. 2) is present in TIS11 cDNA except that Nup475 has an additional C at position 607 in its coding sequence (beyond which the reading frames of Nup475 and TIS11 differ). Upstream of the region of identity, the two cDNAs show no sequence similarity, and downstream of the identity region Nup475 cDNA has 62 additional nucleotides prior to the poly(A) tail, including an AATAAA polyadenylation site. As a result of the above differences, Nup475 cDNA encodes a protein of 319 amino acids whereas TIS11 encodes a protein of 183 amino acids, with identical sequences corresponding to amino acids 81 to 183 of Nup475. That the predicted sequence of the C-terminal segment of Nup475 is correct is supported by the ability of antiserum raised against a peptide corresponding to the C-terminal 15 amino acids of Nup475 to precipitate the Nup475 *in vitro* translation product (see Fig. 7).

Several lines of evidence indicate that the Nup475 cDNA clone actually corresponds to a cellular RNA. First, as shown in Fig. 3, the 5' end of Nup475 mRNA has been mapped on the cloned gene, and overlapping genomic and cDNA sequences have been identified (Fig. 2). Second, when the  $^{32}$ P-antisense transcript of a 5' Nup475 cDNA fragment of 338 nucleotides (nucleotides 355 to 17 of Fig. 2) was annealed with RNA from regenerating liver (in which Nup475 mRNA

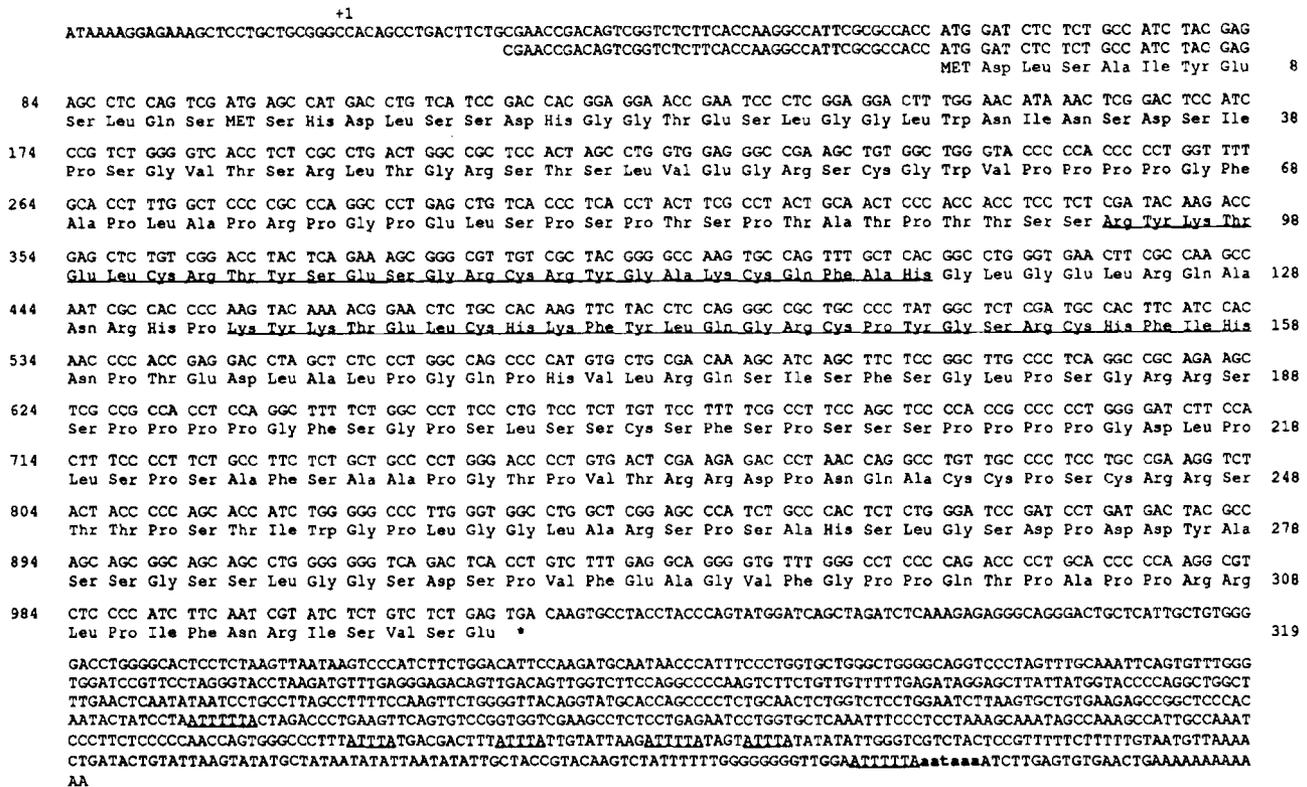


FIG. 2. The cDNA sequence and predicted amino acid sequence of Nup475. Numbers appearing in the left margin refer to the first nucleotide listed on that line. Numbers appearing in the right margin refer to the last amino acid on that line. The principal start of the mRNA is indicated by +1. Nucleotides in the top line were determined by sequencing a genomic clone. The underlined amino acids represent a conserved repeat of 26 amino acids. The underlined nucleotides in the 3' untranslated region represent sequences associated with unstable mRNAs (20). Lowercase letters indicate a poly(A) addition signal.

is induced, see below), treated with RNase, and fractionated by gel electrophoresis, the cellular RNA protected the anti-sense transcript from RNase digestion (data not shown), indicating that Nup475 mRNA contains a continuous sequence corresponding to nucleotides 17 to 355 of the cDNA. Finally, when oligonucleotide primers corresponding to the 5' and 3' ends of Nup475 cDNA (nucleotides 2-22 and 1804-1783, respectively) were used to amplify cDNA prepared from total RNA of serum-stimulated 3T3 cells and the resulting amplification product was analyzed, the product was of the predicted size for a nearly full length Nup475 cDNA and gave identical BamHI and BclI restriction patterns as the cloned cDNA (data not shown). We conclude that the Nup475 cDNA whose sequence is shown in Fig. 2 is derived from a single cellular mRNA species.

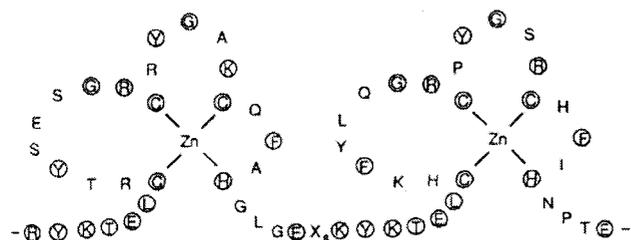
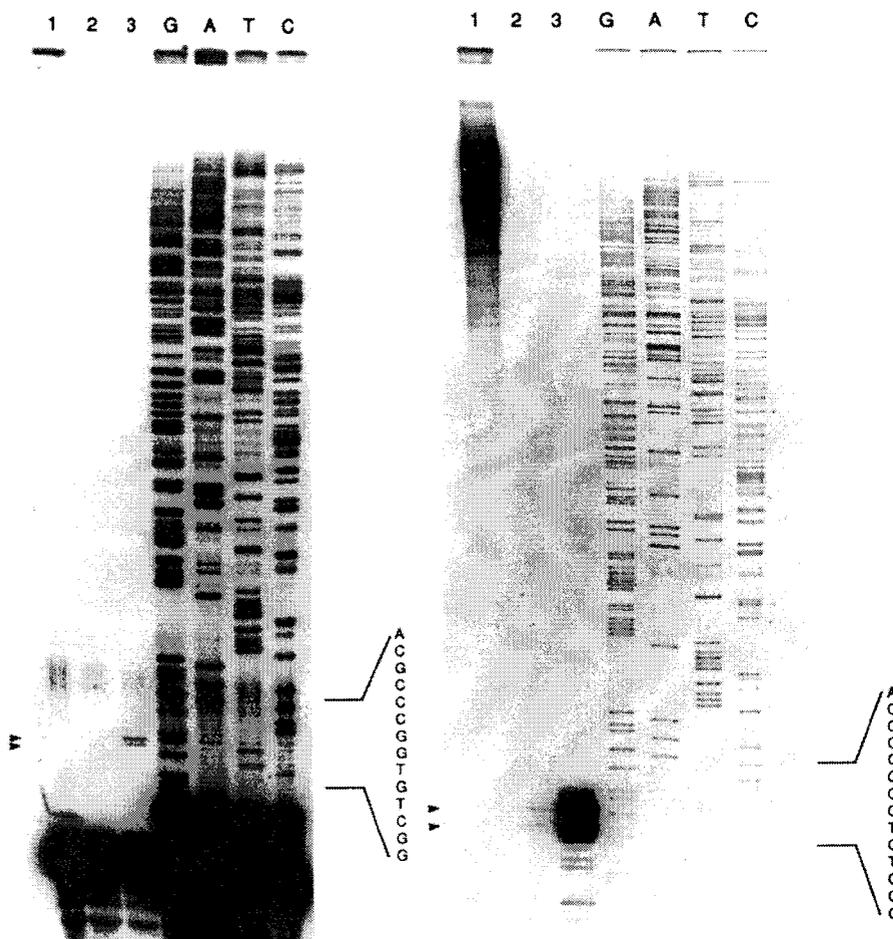
**Nucleotide Sequence of the Upstream Genomic Region—**Murine genomic clones of Nup475 were isolated using the cDNA as a probe, and the 5'-most clone was identified. The nucleotide sequence just upstream of the transcription start site is shown in Fig. 5. The sequence ATAAAA at -29 relative to the start site presumably functions as a TATA element. Also present are two core Sp1 sequences at -34 and -362 (22) and two binding sites for another immediate early protein, Zif268, at -45 and -77 (23). There is also a core glucocorticoid-like response element (24) present at nucleotide position -705. The sequence GATTTTC which has been shown to bind transcription factor H4Tf-1 (25) is present at nucleotide -831, and there is a potential serum response element (CCATAAAAGG) (26) at position -31, overlapping the TATA-like sequence. There are two potential AP-2 sites (27), one at -43 which overlaps a Zif268 site and another at

-363 which overlaps an Sp1 site. The functional significance of these various elements is not presently known.

**Southern Blot Analysis of Genomic DNA—**Southern blot analysis of mouse genomic digests under stringent conditions (see "Experimental Procedures") revealed single intensely hybridizing EcoRI and HindIII fragments (8.8 kb and 7.1 kb, respectively), data not shown. Human and rat DNA also showed simple EcoRI and HindIII digest patterns. These results are consistent with the presence of a single copy of nup475 in the mouse, rat, and human genomes. However, other faintly hybridizing fragments were observed in all digests, which could represent predominantly intronic or gene-flanking regions, or related genes.

**Presence of Nup475 mRNA in Mouse Cell Lines and Tissues—**To determine how widely expressed the nup475 gene is in mouse cell lines and tissues, total RNA samples from various proliferating lines, adult mouse tissues, and mouse liver after partial hepatectomy were analyzed by Northern blot analysis for the presence of Nup475 RNA (Fig. 6). The RNA was detected in many murine cell lines including a macrophage line (RAW, Ref. 28) and B cell lines (29-31). In the macrophage cell line stimulated with spleen extract (RAW S of Fig. 7) or  $\gamma$ -interferon (data not shown), the amount of Nup475 mRNA was severalfold greater than that in unstimulated cells. Of the tissues examined, thymus, regenerating liver, and small intestine had a high level of mRNA, while quiescent liver, kidney, and spleen had lower levels (Fig. 6). The RNA was also present in 8- and 12-day placenta but was barely detectable in 16-day placenta. None was detectable in whole brain RNA. We conclude that nup475 is expressed in many tissues and cell lines and its mRNA level increases in response to diverse signals.

**FIG. 3. Mapping the 5' end of Nup475 mRNA.** The autoradiogram on the left shows the results of reverse transcription in which an antisense  $^{32}\text{P}$ -labeled oligonucleotide primer (nucleotides 45 to 17 of the cDNA, Fig. 2) was annealed to total 3T3 cell RNA and extended with reverse transcriptase, and the products were fractionated by electrophoresis in a sequencing gel. Lane 1, primer alone; lane 2, RNA from quiescent cells; lane 3, RNA from cells stimulated for 3 h with serum in the presence of 10  $\mu\text{g}/\text{ml}$  cycloheximide; lanes 4-7, genomic DNA sequence: reaction products with the oligonucleotide primer indicated above. Arrows indicate the primer extension products on the RNA template. The autoradiogram on the right shows the results of S1 mapping of 3T3 RNA on a  $^{32}\text{P}$ -labeled DNA probe prepared from cloned Nup475 genomic DNA using the oligonucleotide primer indicated above (nucleotides 45 to 17). After annealing of probe with total 3T3 cell RNA, the S1-resistant DNA was fractionated by electrophoresis in a sequencing gel. Lane 1, DNA probe; lane 2, RNA from quiescent cells; lane 3, RNA from cells stimulated for 3 h with serum in the presence of 10  $\mu\text{g}/\text{ml}$  cycloheximide; lanes 4-7, genomic DNA sequence: reaction products with the oligonucleotide primer indicated above. Arrows indicate the S1-resistant probe.



**FIG. 4. Repetitive amino acid sequence of Nup475 depicted as hypothetical zinc fingers.** The circled amino acids are either identical or similar in the repetitive sequence.

**Production of Nup475 in *E. coli* and Preparation of Antisera**—To produce Nup475 proteins, *E. coli* strain BL21 (DE3) was transformed with a recombinant pET8C-Nup475 plasmid, as detailed under "Experimental Procedures." Induced plasmid-containing strains produced a protein of approximately 37 kDa not present in vector-transformed bacteria (Fig. 7). The 37-kDa protein band was excised and used for immunization of rabbits. In addition, antisera against two synthetic peptides (residues 304 to 319 and 84 to 99, respectively) were also prepared. The sera reacted with the *E. coli*-produced 37-kDa protein (data not shown) and with a 37-kDa protein synthesized in reticulocyte lysates programmed with RNA transcripts of Nup475 cDNA (Fig. 7). These results establish the identity of the *E. coli* protein and the activity of the antisera.

**Subcellular Localization of Nup475**—To determine the cel-

lular localization of Nup475, BALB/C 3T3 cells were stimulated with serum, and Nup475 was detected by immunofluorescence with affinity-purified antibody raised against the *E. coli* 37-kDa protein (Fig. 8). Quiescent cells showed a faint signal in their nuclei, which increased markedly by 2 h after serum stimulation and returned to baseline by 4 h. Partially purified Nup475 blocked the immunofluorescence seen in stimulated cells. Similar experiments were carried out in cos cells transfected with a Nup475/pMT plasmid, which replicates in cos cells. Nup475 antigen was localized to nuclei of transfected cells (Fig. 8); similar results were obtained by analyzing cytoplasmic and nuclear fractions for Nup475 antigen (data not shown). We conclude that Nup475 is predominantly a nuclear protein.

**Zinc Binding by Nup475**—As indicated above, the predicted amino acid sequence of Nup475 suggests that the protein may have two heavy metal-binding fingers of novel type. To determine whether the protein binds metal ions, an extract of *E. coli* containing Nup475 was fractionated by SDS-PAGE, the separated proteins were transferred to nitrocellulose, renatured, and then incubated with  $^{65}\text{Zn}^{2+}$ , as described under "Experimental Procedures." As shown in Fig. 9, although many *E. coli* proteins bind zinc, in the pET8C-Nup475 strain there is an additional zinc-binding protein that corresponds in mobility to the predominant protein that reacts with anti-Nup475 serum. The intensity of zinc labeling of Nup475 is similar to that seen with an *E. coli* extract containing Zif268 (Fig. 9), a DNA-binding zinc finger protein of the TF III A type (23).

FIG. 5. Nucleotide sequence of the *nup475* 5' flanking region. Numbers on the left side refer to the first nucleotide listed on the line with the mRNA start site designated as +1. The TATA-like element is labeled, and other potential protein binding sites are underlined and labeled appropriately (Zif268, Sp1, GRE (glucocorticoid-like response element), H4TF1, AP-2). The last 24 nucleotides correspond to the first 8 codons in the cDNA.

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-997 TAGTCTGACA TTSAACGCCT GATCCCCCTT CCTCCACCTC CAAATGCCAA GATTACAACC ATATGCCACT GTCTTGTCTT
-917 TTGPTTTGTT TTCAAGACA GGGTTTCTCA GCCGAGCATG GTGACACAGC CCTTTGATCC CAGCACTCCG GAGGCAGAGG
-837 CAGCGCGATT TCTGAGTTCA AGGCCAGCCT GGTCTACAAA GTGAGTTCCA GGACAGCCAG AGCTATACAG AAAAAACCTG
      2H4TF-1
-757 TCTCGAAAAA CAAAACAAAC AAACAACAAA AAAAGACAGG GTTTCTGTGT GTAGTCTTGG TGTCTCTGGA ACTCACTCTG
      2GRE
-677 TAGACCAGGC TAGCCCTGAA CTCATAGAAA TCCGTCTGCT TCTGCCTTCC AAGTGTCTGAA ATTAAGTGT TGGGCCATCA
-597 GTGATCTGGC ATCTTTTAC TTCTAAGAAA AAGGCTCTCA CCAAGTTACT CAAGTGTCCC TAGAACCACG TGTATATGGG
-517 ASCCAGGCCT TGAACACTCA GCTCCTGCCC TCAGCCTCCC TGAGGGGGCC GAATCACAGT CCTAAACCAG CAGATCCTAC
-437 TTGTGAATGT CTCTCACTGT CTCCTTGTIT TTCTCTGGC TTCTGGCCAT CTCTGTCTCT TCCAACCTGT CTCTCCCCCG
      2AP2
      sp1
-357 CCCCCTCTGT CTCACACGCA CAGCCCTCA GTCTCTGCC TTGTCAATTG TCCTAGAGC CCTGCCCCA CTCTGCCCC
-277 AGTTCTCTTC CCSAGTGCCT TCCACCTCTG AAAAACACTA GGAGCCGCCG CTACCATCAC CTCCAGTTGG TCGTACACAA
-197 CCTCTCTAC TACCCCCGA GAGGGGGCCG AGCTCCGGGA GGGCCAAGTT CAGGCACATT TTGCATGCTA ACCAGCAGAT
-117 AGGCGGAAGG GGCTAGTAC CAGGCCGCC SAGCTAAGCC GCGGGGGCC GTCCCGGAAG CTCTAGTGGC CAGCCCCCA
      2AP2
      zif/268
-37 GCGCGCCCA TAAAAGGAGA AAGTCTCTGC TCGGGGCCAC AGCCTGACTT CTGCGAACCG ACAGTGGTCT TCTTACCACAA
      TATA
      sp1 2SRE +1
+44 GGCCATTTCG GCCACC ATG GAT CTC TCT GCC ATC TAC GAG
    
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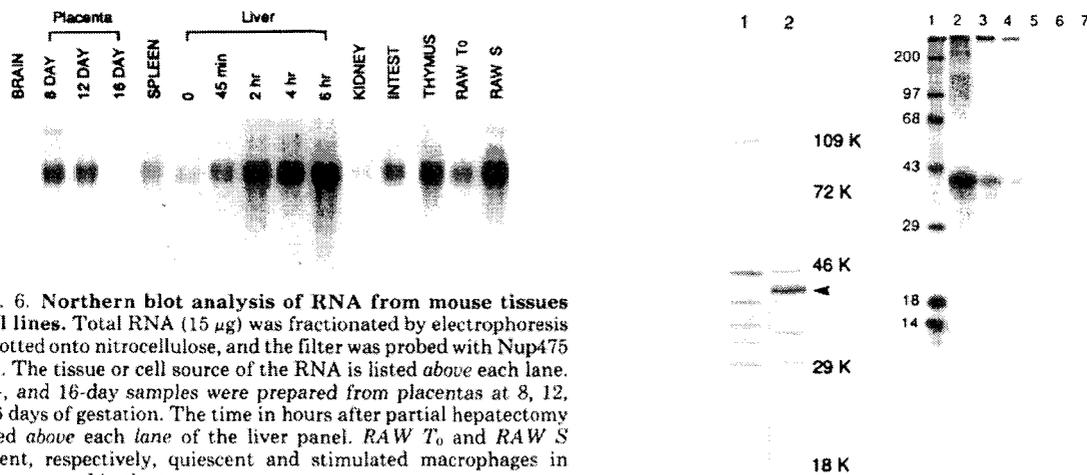


FIG. 6. Northern blot analysis of RNA from mouse tissues or cell lines. Total RNA (15 µg) was fractionated by electrophoresis and blotted onto nitrocellulose, and the filter was probed with *Nup475* cDNA. The tissue or cell source of the RNA is listed above each lane. 8-, 12-, and 16-day samples were prepared from placentas at 8, 12, and 16 days of gestation. The time in hours after partial hepatectomy is listed above each lane of the liver panel. RAW T<sub>0</sub> and RAW S represent, respectively, quiescent and stimulated macrophages in culture, as noted in the text.

DISCUSSION

Of the genes so far defined that are rapidly activated in mouse 3T3 cells by serum growth factors, several encode regulatory proteins that modulate gene expression, including cellular counterparts of viral oncoproteins; others encode potential cytokines, transmembrane proteins, or cytoskeletal proteins (1-3). A number of activated genes, identified by cDNA cloning, remain to be analyzed. In this report, we describe the analysis of one of these previously isolated cDNAs (4) and its encoded protein, designated *Nup475*.

The *nup475* gene is a typical growth factor-induced, immediate early gene, i.e. it is transcriptionally activated in 3T3 cells within minutes of addition of serum or PDGF and is superinduced by growth factor in the presence of an inhibitor of protein synthesis (4). The level of *Nup475* mRNA rises rapidly after growth stimulation and rapidly declines. The decline is due to two processes: shut-off of transcription and lability of the RNA (4). Such lability may be related to AUUUA sequences present in the 3' untranslated region of the mRNA, as suggested for cytokine mRNAs (38) and other immediate early RNAs (3).

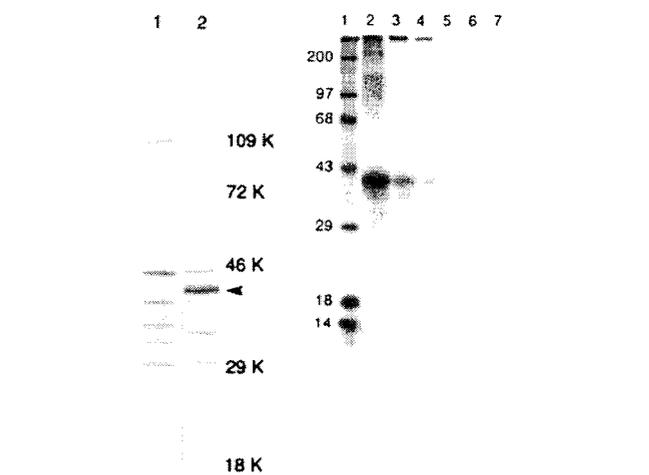
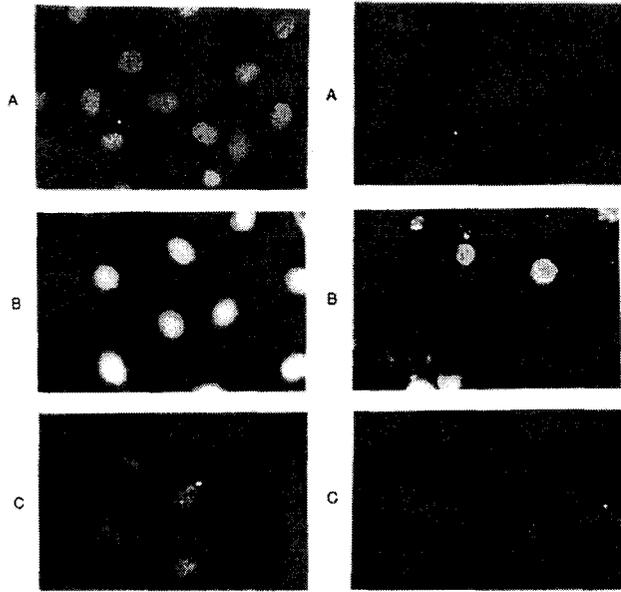


FIG. 7. Left, expression of *Nup475* in *E. coli* strain BL21(DE3). Extracts of *E. coli* harboring vector (pET8c) or *Nup475* recombinant plasmid (pET8c-475) were fractionated by electrophoresis in 12.5% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. Lane 1, pET8c-transformed *E. coli*; lane 2, pET8c *Nup475*-transformed *E. coli*. Arrowhead indicates the *Nup475* protein band. Right, [<sup>35</sup>S]methionine-labeled *in vitro* translation products of transcripts of *Nup475* after immunoprecipitation. Lane 1, protein markers (kDa); lane 2, antiserum prepared against *Nup475* from *E. coli*; lane 3, antiserum prepared against peptide corresponding to amino acids 84-99 of *Nup475*; lane 4, antiserum against peptide corresponding to amino acids 304-319 of *Nup475*; lanes 5-7, the respective preimmune sera.

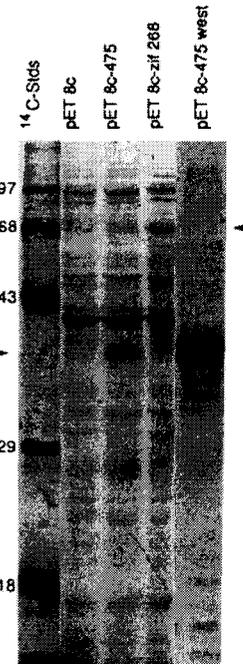
The protein encoded by *Nup475* cDNA is rich in glycine, serine, and proline, including three copies of the sequence PPPPG. A notable feature is the presence of two tandem copies of a 26-amino acid sequence in which 17 of the 26 amino acids are identical or closely related. Each sequence repeat contains 3 spatially conserved cysteine residues and 1 histidine residue of the form CX<sub>2</sub>CX<sub>3</sub>CX<sub>4</sub>H, suggesting a novel



**FIG. 8. Immunofluorescence of Nup475 in BALB/c 3T3 cells (left) and transfected cos cells (right).** BALB/c 3T3 cells were grown to confluence and maintained for 2 days in medium containing 0.5% serum (panel A), stimulated for 2 h with medium containing 20% fetal calf serum (B and C), and then reacted sequentially with affinity-purified Nup475 rabbit antibody and rhodamine-conjugated anti-rabbit IgG. In C, Nup475 was added prior to reaction with anti-Nup475 antibody. Cos cells were transfected with pMT2 vector (A) or with the pMT2/Nup475 construct (B and C) and then assayed for Nup475 antigen. In C, Nup475 was added prior to reaction with anti-Nup475 antibody.

type of heavy metal-binding finger structure (32–34). Although Nup475 binds zinc ions, we do not yet know if zinc is bound to the putative fingers or to other parts of the molecule. Compared to the classes of known zinc finger proteins, exemplified by TFIIIA and Xfin (35, 36), steroid hormone receptors (37), GAL 4 and related fungal proteins (38), and one of the retroviral gag proteins (33, 39), the suspected fingers of Nup475 have a different order and spacing of cysteine and histidine residues. Further work will be required to determine whether Nup475 represents a new class of zinc finger proteins and whether its nuclear localization is an indication that it is a nucleic acid-binding protein involved in regulating transcription. If Nup475 is a transcription factor, its proline-rich sequence could be part of an effector domain (44).

Little is known about the regulation of *nup475* expression. It is widely, but not universally, expressed in mouse tissues and cell lines, and its expression is markedly stimulated in appropriate cells by PDGF, forskolin, depolarizing ionic conditions,  $\gamma$ -interferon, or 12-*O*-tetradecanoylphorbol-13-acetate, and in regenerating liver following partial hepatectomy. Thus Nup475 appears to be part of a rather general response to extracellular signaling agents. The promoter region of *nup475* contains a number of potential binding sites for known transcription regulators, including Sp1 sites (22), a core glucocorticoid-like response element (24), two Zif268 binding sites (23), a possible serum response element (26), and two potential AP-2 binding sites (27). A number of immediate early genes have Zif268 sites (23), and it has been suggested that binding to these sites may play a role in down-regulating gene transcription after initial growth factor-induced activation. Further experiments should clarify the role



**FIG. 9. Binding of  $^{65}\text{Zn}^{2+}$  by Nup475.** Extracts of *E. coli* harboring vector (pET8c) or Nup475 recombinant plasmid (pET8c-475) or Zif268 recombinant plasmid (pET8c-Zif268) (23) were fractionated by electrophoresis in 12.5% SDS-polyacrylamide gel, and the proteins were transferred to nitrocellulose. The nitrocellulose sheet was then incubated with  $^{65}\text{ZnCl}_2$ , and zinc-binding proteins were visualized by autoradiography. The same sheet was then reacted sequentially with affinity-purified anti-Nup475 antibody, alkaline phosphatase-conjugated goat anti-rabbit IgG, and phosphatase substrate to visualize Nup475 antigen (pET8c-475 west). On the left are  $^{14}\text{C}$ -protein markers (numbers in kDa). Arrowheads indicate the positions of Zif268 (upper) and Nup475 (lower).

of the various protein-binding sites in regulating the *nup475* gene.

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#### REFERENCES

- Rollins, B. J., and Stiles, C. D. (1989) *Adv. Cancer Res.* **53**, 1–32
- Bravo, R. (1989) in *Growth Factors, Differentiation Factors, and Cytokines* (Habenich, A., ed) Springer Verlag New York Inc., New York
- Lau, L. F., and Nathans, D. (1990) in *Molecular Aspects of Cellular Regulation* (Cohen, P., and Foulker, J. G., eds) Vol. 6, Elsevier Science Publishers B.V., Amsterdam
- Lau, L. F., and Nathans, D. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 1182–1186
- Gluzman, Y. (1981) *Cell* **23**, 175–182
- Jang, S. K., Davies, M. V., Kaufman, R. J., and Wimmer, E. (1989) *J. Virol.* **63**, 1651–1660
- Higgins, G. M., and Anderson, R. M. (1931) *Arch. Pathol.* **12**, 186–191
- Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) *Biochemistry* **18**, 5294–5299
- Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 5463–5467
- Devereux, J., Haerberli, P., and Smithies, O. (1984) *Nucleic Acids Res.* **12**, 387–395
- Ryder, K., Lau, L. F., and Nathans, D. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 1487–1491
- Sharp, P. A., Berk, A. J., and Berget, S. M. (1980) *Methods Enzymol.* **65**, 750–768

13. Studier, F. W., and Moffatt, B. A. (1986) *J. Mol. Biol.* **189**, 113-130
14. Laemmli, U. K. (1970) *Nature* **227**, 680-685
15. Harlowe, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
16. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 4350-4354
17. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
18. Dang, C. V., and Lee, W. M. F. (1989) *J. Biol. Chem.* **264**, 18019-18023
19. Schiff, L. A., Nibert, M. L., and Fields, B. N. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 4195-4199
20. Greenberg, M., Ziff, E., and Greene, L. A. (1986) *Science* **234**, 80-83
21. Shaw, G., and Kamen, R. (1986) *Cell* **46**, 659-667
22. Kadonaga, J. T., Jones, K. A., and Tjian, R. (1986) *Trends Biochem. Sci.* **11**, 20-23
23. Christy, B., and Nathans, D. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 8737-8741
24. Jantzen, K., Fritton, H. P., Igo-Kemenes, T., Espel, E., Janich, S., Cato, A. C. B., Mugele, K., and Beato, M. (1987) *Nucleic Acids Res.* **15**, 4535-4552
25. Dailey, L., Hanley, S. M., Roeder, R. G., and Heintz, N. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 7241-7245
26. Triesmann, R. (1985) *Cell* **42**, 889-902
27. Imagawa, M., Chin, R., and Karin, M. (1987) *Cell* **51**, 251-260
28. Raschke, W. C., Baird, S., Ralph, P., and Nakoinz, I. (1978) *Cell* **15**, 261-267
29. Yancopoulos, G. D., Desiderio, S. V., Paskind, M., Kearney, J. F., Baltimore, D., and Alt, F. W. (1984) *Nature* **311**, 727-733
30. Alt, F. W., Rosenberg, N., Enea, V., Siden, E., and Baltimore, D. (1982) *Mol. Cell. Biol.* **2**, 386-400
31. Kappler, J., White, J., Wegmann, D., Mustain, E., and Marrack, P. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79**, 3604-3607
32. Miller, J., McLachlin, A. D., and Klug, A. (1985) *EMBO J.* **4**, 1609-1614
33. Berg, J. (1986) *Science* **232**, 485-487
34. Klug, A. and Rhodes, D. (1987) *Trends Biochem. Sci.* **12**, 464-469
35. Brown, R. S., Sander, C., and Argos, P. (1985) *FEBS Lett.* **186**, 271-274
36. Lee, M. S., Gippert, G. P., Soman, K. V., Case, D. A., and Wright, P. E. (1989) *Science* **245**, 635-637
37. Evans, R. M. (1988) *Science* **240**, 889-895
38. Pfeifer, K., Kim, K.-S., Kogan, S., and Guarente, L. (1989) *Cell* **56**, 291-301
39. Summers, M. F., South, T. L., Kim, B., and Hare, D. R. (1990) *Biochemistry* **29**, 329-340
40. Nakabeppu, Y., Ryder, K., and Nathans, D. (1988) *Cell* **55**, 907-915
41. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Strubel, K. (1988) in *Current Protocols in Molecular Biology*, John Wiley & Sons, New York
42. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1988) *Science* **239**, 487-491
43. Varnum, B. C., Lim, R. W., Sukhatme, V. P., and Herschman, H. R. (1989) *Oncogene* **4**, 119-120
44. Mermod, N., O'Neill, E. A., Kelly, T. J., and Tjian, R. (1989) *Cell* **58**, 741-753
45. Pharmacia LKB Biotechnology Inc. (1983) *Technical Bulletin*, Vol. 2, Pharmacia LKB Biotechnology Inc., Piscataway, NJ