Vol. 213, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 4, 1995 Pages 325-333

A MAMMALIAN DELAYED-EARLY RESPONSE GENE ENCODES HNP36, A NOVEL, CONSERVED NUCLEOLAR PROTEIN+

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Received June 26, 1995

Summary. Murine fibroblasts respond to mitogens by sequential gene expression in which immediate-early, or primary response, gene transcription factors direct expression of secondary transcripts encoded by delayed-early response (DER) genes. DER gene products include growth progression factors, but the products of several novel cDNAs are unknown. Murine and human cDNAs derived from one novel DER gene (DER12) were characterized to identify its product and to probe its role in the growth response. Both sequences encoded a hydrophobic 36 kD protein (HNP36) that was related to the yeast protein, FUN26. Anti-murine HNP36 antibodies were prepared and shown to immunoprecipitate both mammalian *in vitro* translation products. Immunocytochemical staining indicated localization of HNP36 to the nucleolus where its concentration increased after mitogen stimulation. Although HNP36 protein function is unknown, its identification as a nucleolar gene transcriptionally activated by growth factors implicates it as participating in the proliferative response.

After binding to surface receptors on quiescent cells, mitogens activate a signal transducing cascade (1,2) that reactivates a dormant genetic program essential for cell division. In quiescent murine BALB/c fibroblasts, stimulation by serum, platelet derived growth factor (PDGF), or fibroblast growth factor (FGF) directs sequential gene expression that begins with rapid transcriptional activation of primary, or immediate-early response (IER) genes (3-5). The IER gene products include established or putative transcription factors that act in the interval between IER gene transcription and DNA replication to induce transcription of secondary, or delayed-early response (DER) genes (4). Several DER cDNAs were recently cloned and among the gene products were cell cycle progression and replication-related proteins [Cyclin D1, DNA polymerase δ, and nonhistone high mobility group proteins HMG-I(Y) and IC], a cytokine (Macrophage inhibitory factor, MIF), and a water channel (CHIP28/Aquaporin-1) (6). However, not all of the

+The murine and human nucleotide sequences reported here have been deposited into the EMBL database under accession numbers X86682 and X86681, respectively.

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0006-291X/95 $12.00

DER cDNAs were homologous to sequences in the databases, and those that remained unidentified (DER genes 9, 11, 12, and 13) may encode proteins with novel growth related functions. In pursuit of this notion, the partial DER12 cDNA was used to isolate full length murine and human cDNAs which appeared to encode highly conserved and nearly identical hydrophobic 36 kD proteins. The predicted proteins were homologous to a yeast protein of unknown function (FUN26), but all lacked recognizable motifs that suggested its biological function. Regardless, immunocytochemistry localized the gene product to the nucleolus of fibroblasts where mitogen action increased the amount of detectable protein. Hence, this conserved DER gene encodes a 36 kD hydrophobic nucleolar protein (designated as HNP36) whose expression is correlated with mitogen action. These findings identify a genetic component of the observations that activation of nucleolar functions in ribosomal DNA transcription and ribosome biogenesis are early proliferative responses of BALB/c fibroblasts.

Experimental Procedures

Cell Culture. Culture of murine BALB/c 3T3 fibroblasts (clone A31) and stimulation with fetal bovine serum (20%) or purified growth factors was performed as described (6). All other cell lines used were maintained in standard culture media as specifically indicated by the providers.

Isolation of cDNAs. The original DER12 cDNA (900bp), renamed as the HNP36 cDNA, was radiolabeled with [α-^{32}P]dCTP (New England Nuclear, 111 TBq/mmol) by random priming (Pharmacia) and hybridized (6X SSC, 50mM Tris at pH 6.8, 5X Denhardt's solution, 10mM EDTA, 100μg/ml denatured sperm DNA, 0.2% SDS, 68^{o}C) to a cDNA library prepared from BALB/c fibroblast mRNAs (6). One cDNA (clone 62) was nearly the size of the 2.1 kb mRNA and the DNA sequence of both strands of this insert was determined by dideoxynucleotide methods (7).

To isolate cDNAs providing additional sequence at the 5' terminus, an oligonucleotide (5'-GAGCGTGAAGAGCAGTAGAGGCAG-3') derived from 5' terminus of clone 62 was radiolabeled with [γ-^{32}P]ATP (New England Nuclear, 111TBq/mmol) and polynucleotide kinase (New England Biolabs), and used to screen a cDNA library prepared by priming of cDNA synthesis with random hexamer oligonucleotides (In Vitrogen) Hybridization (42^{o}C) was performed, a solitary isolate (clone 72) was obtained, and its DNA sequence was determined.

To isolate the human HNP36 cDNA, clone 62 was radiolabeled by random priming and used to probe a heart cDNA library (Stratagene). Reduced stringency hybridization (56^{o}C) yielded a solitary candidate full-length isolate (clone 8b), and the DNA sequence was determined. All sequence analysis was performed with MacDNAsis 3.0 software (Hitachi) using default parameters of the algorithms.

Northern Hybridizations. Total RNA was prepared by lysis of cells in guanidinium buffer followed by sedimentation through CsCl (8). RNA (10μg/lane) was fractionated in formaldehyde/agarose gels, transferred to nitrocellulose, and hybridized overnight to radiolabeled cDNA probes (5X SSPE at pH 7.4, 50% formamide, 4X Denhardt's solution, 0.1% sodium pyrophosphate, 0.1% SDS, 100μg/ml denatured sperm DNA, 42^{o}C) prior to autoradiography.

Antibody Preparation. Oligonucleotide primers containing BamHI restriction sites were used to amplify by polymerase chain reaction (PCR) a 162 bp segment (nucleotides 478 to 640) of the murine HNP36 cDNA that encoded 54 amino acids from the "loop" of the protein. The PCR product (35 cycles, 52^{o}C annealing) was digested with BamHI, and cloned into plasmid pGEX 2T (Amrad Corp, Ltd) that encodes Shistosomal glutathione-S-transferase, GST (9). The integrity of the DNA sequence was confirmed and the HNP36-GST fusion protein was purified by glutathione-agarose (Sigma) chromatography of bacterial lysates (9). Specific antisera were generated in rabbits (HRP, Inc) and enriched for anti-HNP36 antibodies.

Anti-HNP36 serum or preimmune serum (3 ml each) was diluted four-fold in Tris-buffered saline (TBS, 50mM Tris, 0.15M NaCl, pH 8.0) and the IgG was bound to Protein A-Sepharose (Sigma). After washing with TBS, the IgG was eluted with 0.1M sodium citrate, pH 3.0, into 1M

Tris, pH 8.0. Peak fractions (280 nm) were pooled, and both preimmune and anti-HNP36 antibodies were dialyzed vs. TBS. GST affinity columns were generated by coupling GST purified from bacterial lysates to activated Sepharose 4B (Pharmacia) according to the manufacturer's recommendations. Anti-HNP36 IgG was passed in quadruplicate over the column, the flow through was collected, concentrated, and stored in TBS at 4° C. The resulting anti-HNP36 IgG was devoid of Western blot reactivity vs. GST and specifically bound to the HNP36 epitopes of the fusion protein. Preimmune IgG did not bind to GST or HNP36 epitopes.

***In Vitro* Translations.** Murine (clone 62) and human (clone 8b) HNP36 cDNAs in pBluescript II were linearized and used for *in vitro* RNA synthesis (Ribomax, Promega) according to protocol. *In vitro* RNA was translated using reticulocyte (Flexilysate, Promega) and wheat germ (Promega) systems according to protocol with identical results. After electrophoresis on 10% SDS/polyacrylamide gels and fixation in Fluoro-hance (RPI, Inc), the gels were dried and autoradiography was performed. Positive control RNA (Brome Mosaic virus, Promega) reliably produced the appropriate product, and negative control reactions (no added RNA) yielded no significant endogenous translation products. To determine if the HNP36 protein was modified post-translationally, pancreatic microsomes (1.8μL, Promega) were included in the HNP36 and control translations. Results indicating appropriate signal peptide processing and glycosylation of the positive controls were consistently obtained.

To immunoprecipitate the native protein, radiolabeled HNP36 translation products were incubated (2 hr, 4°C) with preimmune and specific antisera. Protein A-sepharose (Sigma) was added (1 hr, 4°C), the immunoprecipitates were pelleted (2500xg, 3 min), and washed three times. The proteins were solubilized in SDS loading buffer, electrophoresed, and detected as described.

Immunocytochemistry. BALB/c fibroblasts, F9 embryonal carcinoma cells, and rat intestinal epithelial, RIE, cells were grown in chamber slides (Nunc), washed with PBS, and fixed (3.8% paraformaldehyde, 5 min). The cells were permeabilized (0.1% Triton X-100 in PBS, 5 min), endogenous peroxidase was blocked (0.3% H_2O_2/methanol, 20 min), and the cells were incubated with either preimmune IgG or anti-HNP36 antibodies (1:250, 24 hr, 4°C or 1:50, 1hr, 20°C). Following washing, the cells were developed with peroxidase-IgG (Vectastain, Vector Labs) and SG substrate (Vector Labs). No staining was obtained when either primary or secondary antibody was excluded. During preadsorbtion studies, anti-HNP36 antibodies were incubated with excess HNP36-GST fusion protein (100 fold, 1 hr, 4°C) prior to addition to the cells. Similar treatment of the preimmune antibodies had no effect on background staining. Silver staining of nucleolar organizing regions, Ag(NOR) staining, was performed as described (10).

Results and Discussion

***HNP36* Gene Expression in BALB/c Fibroblasts.** Total RNA was isolated from the cells at intervals after stimulation and an approximately 2.1 kb mRNA was identified by Northern hybridization (Fig 1). Serum, PDGF, and FGF induced the HNP36 mRNA similarly whereby the transcript was nearly undetectable in quiescent cells and most abundant 7.5 h after serum stimulation. Like all DER genes, nuclear run-on assays had established that activated transcription accounts for the increase of HNP36 transcripts (6), and transcriptional activation was prevented by application of protein synthesis inhibitors during the proliferative response. The HNP36 transcript returned to quiescent levels 12 h after stimulation, demonstrating the transient expression typical of growth related DER gene products (Cyclin D1, DNA polymerase δ, High Mobility Group proteins). These observations suggested that characterizing the cognate protein encoded by this novel cDNA might provide new information about the cellular response to growth stimulation.

HNP36 cDNAs. A candidate full length murine sequence (clone 62, 2006 bp) was obtained whose open reading frame extended 1153 bp and originated at the 5' terminus. Consequently, it was assumed that the extreme 5' portion of the cDNA was absent and additional 5' sequence was obtained by screening another fibroblast cDNA library prepared by random

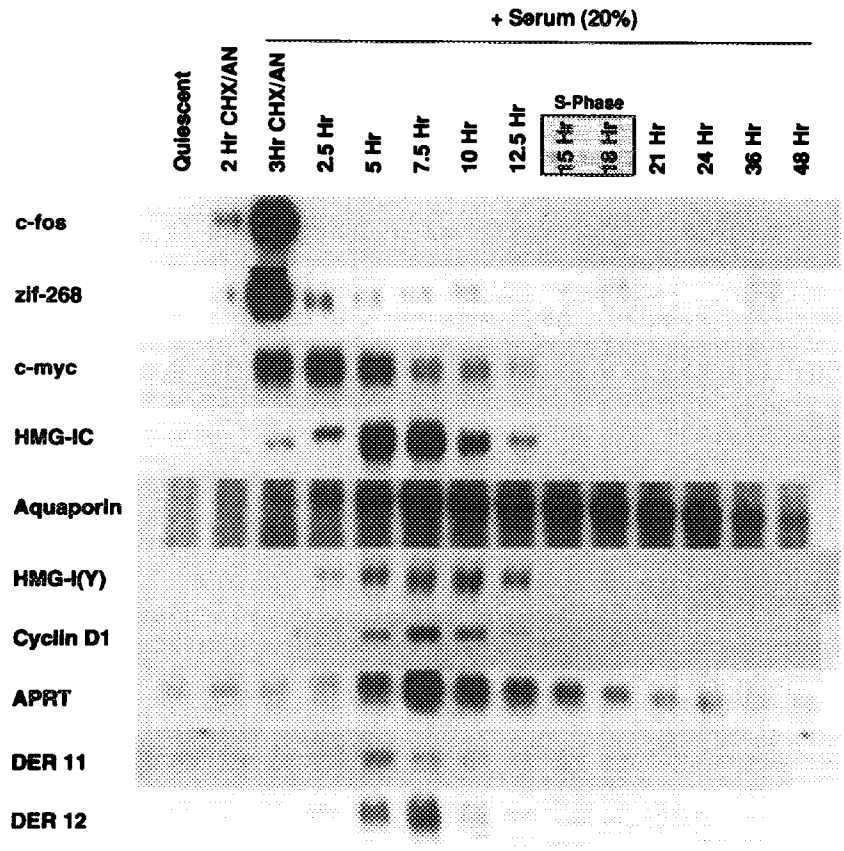

Figure 1. Time course of mRNA levels of the DER12/HNP36 gene and representative serum inducible genes of BALB/c fibroblasts. Quiescent cells were stimulated with culture medium containing 20% serum as described in Experimental Procedures, and total RNA was isolated at the indicated times. Each lane represents 10μg of total fibroblast RNA. Autoradiographic exposure times of the Northern hybridizations vary and the period corresponding to DNA synthesis is indicated. CHX/AN indicates the concurrent addition of protein synthesis inhibitors cycloheximide (10μg/ml) and anisomycin (10μM) during serum stimulation of the cells. Immediate-early genes are represented by *c-fos, zif-268, and c-myc*. All others are delayed-early genes: HMG; *high mobility group*, Aquaporin; *aquaporin-1*, APRT; *adeninephosphorylribosyl tranferase*.

oligonucleotide priming. A single isolate (clone 72, 278 bp) contained a 252 bp overlap of clone 62 and provided 26 bp of upstream sequence. The length of the composite cDNA sequence (2032 bp, Fig 2) was in accord with the mRNA and translation was presumed to initiate from the first AUG codon (199 bp).

Southern hybridization of the murine cDNA to selected genomic DNAs identified homologous sequences in all mammalian DNA examined (not shown). This indicated that homologous cDNAs could be analyzed to corroborate the murine reading frame predictions. From a human heart cDNA library a candidate full length cDNA (clone 8b) was isolated (2281 bp) and contained an open reading frame whose 3' terminus (1365 bp) matched exactly that of the murine cDNA. The human clone had 85% nucleotide identity with the corresponding murine reading frame

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FUN26     181 MMLVVISSMG TAMTQNGIMA IANVFGSEYS QGVMVGQAVA GVLPSL-VLF   229
MDER12      1 MASVWFINSP CAVLQGSLFG QLGTMPSTYS TLFLSGQGLA GIFAALAMLM    50
HDER12      1 MASVCFINSF SAVLQGSLFG QLGTMPSTYS TLFLSGQGLA GIFAALAMLL    50

FUN26     230 ALAFIENSSV STTGGILLYF FTT---TLVV TICVVMFSVS KISRK-VNEN   275
MDER12     51 SLA----SGV DAQTSALGYF ITPCVGILLS IVCYLSLPHL KFARYYLTEK    96
HDER12     51 SMA----SGV DAETSALGYF ITPYVGILMS IVCYLSLPHL KFARYYLANK    96

FUN26     276 WNVEDGHITD VLLGSLRSNE EEIRIVGRID QMEDEDHRRT NGTRDDNDEG   325
MDER12     97 LSQAPTQELE TKAELLQADE KNGVPISPQQ ASPTLDLDPE KEPE--PEEP   144
HDER12     97 SSQAQAQELE TKAELLQSDE -NGIPSSPQK VALTLDLDLE KEPESEPDEP   145

FUN26     326 EELQLKVPFE VLFAKLKYLV LSIFTTFVVT L-VFPVFASA TYVTGLPLSN   374
MDER12    145 QKPGKPSVF- VVFRKIWLTA LCLVLVFTVT LSVFPAITAM VTTSSNSPGK   193
HDER12    146 QKPGKPSVF- TVFQKIWLTA LCLVLVFTVT LSVFPAITAM VT-SSTSPGK   193

FUN26     375 -AQYIPLI-- FTLWNLGDLY GRVIAD---W PMFRDQKFTP RKTFIYSLLR   418
MDER12    194 WGLFFNPICC FLLFNVMDWL GRSLTSYFLW PDEDSQQLLP --LLVC--LR   239
HDER12    194 WSQFFNPICC FLLFNIMDWL GRSLTSYFLW PDEDSR-LLP --LLVC--LR   238

FUN26     419 VAAIPLFLMF TAITSSSSGD EEHNGSVIV- -DLCYMLLQF LFGVTNGHVI   466
MDER12    240 FLFVPLFML- ------CHVP QHARLPIIFR QDAYFITFML LFAVSNGYLV   282
HDER12    239 FLFVPLFML- ------CHVP QRSRLPILFP QDAYFITFML LFAVSNGYLV   281

FUN26     467 SMSFMKVPEQ LDNDDEKEAA GGFTNIFVST GLALGSIISY VFVFIIDFIIR   517
MDER12    283 SLTMCLAPRQ V-LPHEREVA GALMTFFLAL GLSCGASLSF LF----KALL*   327
HDER12    282 SLTMCLAPRQ V-LPHEREVA GALMTFFLAL GLSCGASLSF LF----KALL*   326
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Figure 2. Homology comparison of human and murine HNP36 proteins and *S. cerevisiae* FUN 26 protein. The complete HNP36 proteins are represented, but only the homologous carboxyl terminus of the 58.3 kD FUN26 protein is shown. Sequences were aligned using the Higgins-Sharp algorithm of the MacDNAsis 3.0 software (Hitachi). The segment of the murine "loop" sequence used as immunogen for antibody production is underlined.

and homology was preserved in the 5' untranslated region, as well as in segments of the 3' untranslated region (not shown). Of the two possible AUG start codons within the human reading frame, only the second (386 bp) was conserved in the murine sequence as the presumptive initiation codon (199 bp). Both had identical flanking nucleotides (ACC**AUG**GCC) that correlated with the defined mammalian consensus for favorable translational initiation (11,12). Hence, the mammalian HNP36 cDNAs differed in length but contained coincident potential reading frames.

The proteins predicted by the murine and human HNP36 cDNAs contained 327 and 326 amino acids respectively, with 90% identity and predicted masses of 36 kD (Fig 2). When the databases were analyzed (Blastp and Tblastn algorithms, NCBI) homology to *S. cerevisiae* protein, FUN26, was discovered (Fig 2). *Fun26* was identified during sequence determination of yeast chromosome I (13) and the reading frame predicts a 58.3 kD protein. The carboxyl terminal 331 amino acids of FUN 26 were 50% similar to the entire HNP36 proteins due to conservative alterations of hydrophobic amino acids and further analysis (Kyte-Doolittle algorithm) indicated a

unique distribution (Fig 3). In all three proteins, the hydrophobic residues were clustered in domains resembling putative transmembrane sequences (14,15) that flanked a hydrophilic "loop" (amino acids 82-159 in murine HNP36) enriched in proline and charged amino acids, particularly aspartic and glutamic acids. The pattern of hydrophobic domains did not resemble the characteristic patterns of numerous mammalian transporters or channel-forming proteins (16-18). Both mammalian HNP36 protein sequences had consensus sites for possible phosphorylation by casein kinase II at serine 54 and threonine 107, but these were not present in the FUN26 protein. These conserved features suggested that the HNP36 proteins were structurally/functionally related to a novel yeast protein, but no additional protein motifs were identified to suggest a possible function. FUN26 protein may participate in growth of yeast because deletion of this locus (along with the adjacent *fun25* and *CCR4* genes) caused slow growth on rich medium (13).

***In Vitro* Generated Proteins .** To document that the reading frame predictions were correct, the respective cDNAs were used for generation of *in vitro* RNA and translation in lysates. Both cDNAs produced proteins of identical electrophoretical mobilities (36 kD, Fig 4), matching the predicted size of the proteins. Translations were also used to characterize the antisera and to determine whether addition of pancreatic microsomes identified a signal sequence or glycosylation of the HNP36 proteins. Though the control reactions proceeded normally, the electrophoretical mobility of the murine HNP36 protein was unaffected by the microsomes indicating absence of post-translational modification. The utility of the antisera was evaluated by immunoprecipitation of the native translation products (Fig 4). The specific, but not the preimmune, antisera precipitated the murine and human proteins. Therefore, the identical mobilities of the translation products and

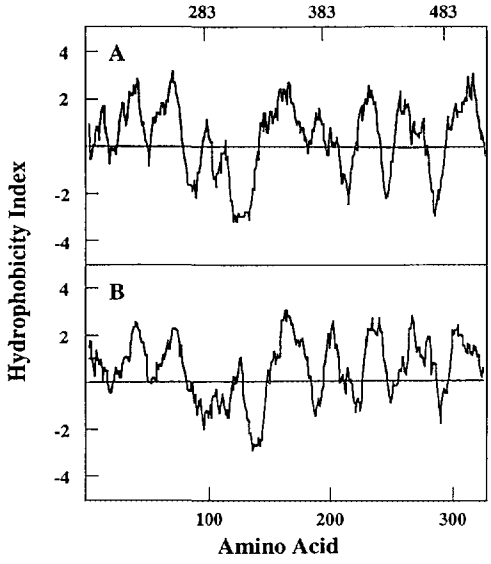

Figure 3. Comparison of the hydrophobic character of the HNP36 and FUN26 proteins. The hydrophobicity plots of the entire murine and human HNP36 and the homologous region of the *S. cerevesiae* FUN26 protein sequences were analyzed by the algorithm of Kyte and Doolittle using MacDNAsis software (Hitachi). A, FUN26. B, Murine HNP36. The plots of the respective HNP36 protein sequences are superimposable. The "loop" structure of the HNP36 proteins ranges from residues 82-159 and is conserved in the FUN26 protein.

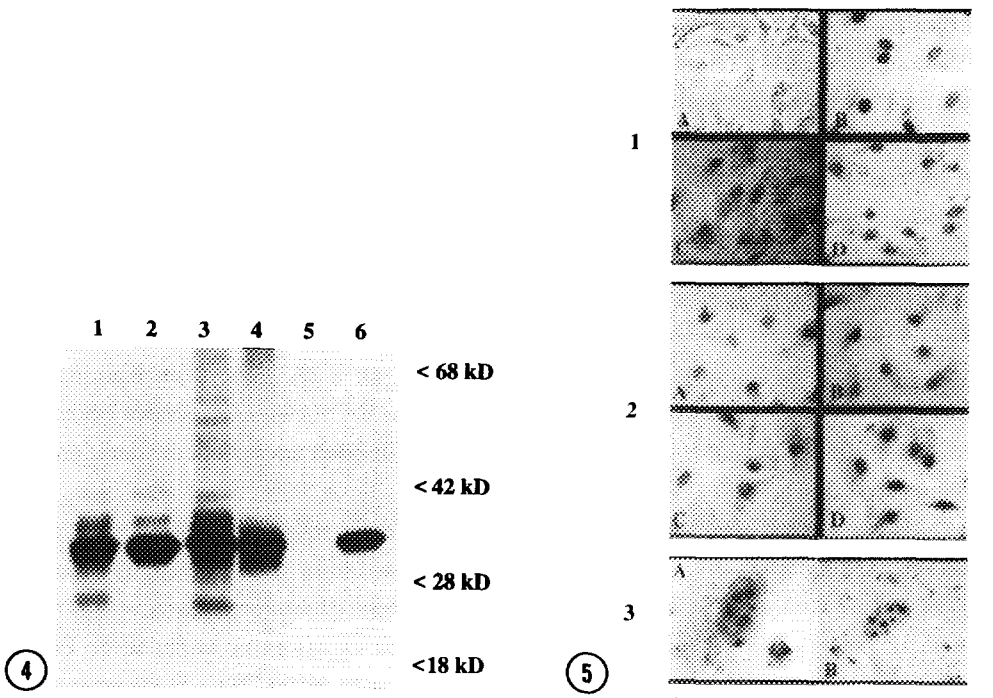

Figure 4. Immunodetection of the *in vitro* translated and endogenous fibroblast HNP36 proteins by specific antisera. Radiolabeled human and murine HNP36 proteins were generated *in vitro* from RNA templates and analyzed by SDS polyacrylamide gel electrophoresis as described in Experimental Procedures. Murine and human HNP36 translation products (lanes 1 and 3, respectively) and after translation with added pancreatic microsomes (lanes 2 and 4, respectively). *In vitro* generated murine HNP36 was immunoprecipitated with preimmune antiserum (lane 5) and with anti-HNP36 antiserum (lane 6).

Figure 5. Immunocytochemical localization in BALB/c fibroblasts of HNP36 protein using specific antibodies. Asynchronous cycling fibroblasts were fixed in paraformaldehyde and imaged as described in Experimental Procedures. Part 1, 220X. Cells were photographed after (A) phase contrast microscopy; (B) staining with preimmune IgG; (C) staining with anti-HNP36 IgG; and (D) silver staining of nucleolar organizing regions of nucleoli by the Ag(NOR) technique. Part 2, (220X). Fibroblasts were stained with anti-HNP36 IgG in (A) the quiescent state, and after serum stimulation for (B) 4h, (C) 12h, and (D) 24 h. Part 3, (550X). Nucleolar staining of fibroblasts with (A) anti-HNP36 IgG, and (B) Ag(NOR) technique.

cross reactivity of anti-murine HNP36 antibodies with the human protein provided experimental support for the sequence-derived predictions of the HNP36 proteins.

Immunocytochemical Detection of HNP36 Protein. Localization of the protein was examined by immunocytochemical staining of cycling fibroblasts. When stained with preimmune antibodies, background staining of the nucleus was noted, but focal regions appearing as punctate lucencies remained unstained (Fig 5,1B). These unstained regions corresponded to the phase contrast images of nucleoli (Fig 5,1A). In contrast, the anti-HNP36 antibodies specifically stained the nuclear structures (Fig 5, 1C) that were not recognized by the preimmune antibodies. To ensure that staining by the anti-HNP36 antibodies was specific, cells were stained after antibodies were preadsorbed by excess HNP36-GST immunogen. Preadsorbtion eliminated staining of the focal nuclear areas and the results were identical to those obtained with preimmune antibodies. To

confirm nucleolar staining, parallel slides were stained by a modified silver technique, Ag(NOR) staining, documented to stain nucleolar organizing regions (19,20). Ag(NOR) staining readily identified nucleoli (Fig 5, 1D) and appeared indistinguishable from the anti-HNP36 antibody staining. Examination at higher magnification suggested that the nucleolar staining was not uniform and, in contrast to the silver staining (Fig 5, 3B), was most intense in the periphery of the nucleolus (Fig 5, 3A). Supporting the conserved features of the protein, identical results were obtained by staining of murine F9 and rat intestinal epithelial (RIE) cells (not shown).

The immunocytochemical approach was used to investigate whether the cytological detection of the protein was altered by the proliferative state. When the fibroblasts were rendered quiescent, nucleolar staining was markedly reduced (Fig 5, 2A). However, staining clearly increased within 4 hr of serum stimulation (Fig 5, 2B) as the nucleolar structures became more readily identifiable. This accentuated nucleolar staining persisted throughout the duration (24 hr) of stimulation (Fig 5, 2C and D).

These studies report that the HNP36 delayed-early response gene encodes a highly conserved nucleolar protein, the first nucleolar gene noted to be transcriptionally activated by growth stimuli. Although the biological function has not been determined, this novel hydrophobic protein is likely to participate in the nucleolar response to mitogenic stimulation. Enhanced ribosome biogenesis is known to occur early in the response to serum (21), and the gene encoding ribosomal protein L32 was also shown to be transcriptionally activated during the cultured fibroblast response to serum (22). Presumably, transcription of nucleolar genes during cell growth supports enhanced ribosome production and may explain the observations of nucleolar activation during proliferation associated with activated lymphocytes, liver regeneration, and tumor growth (21,23,24).

Acknowledgments: The technical assistance of Ms. Laura Bliss and Mrs. Sarada Siripurapu are gratefully acknowledged. We wish to thank Dr. Daniel Nathans for advice and suggestions. This work was supported in part by the Lucille Markey Foundation and the University Research Council of Vanderbilt University.

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