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Isolation and characterization of a novel PDGF-induced human gene

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Abstract

Using a differential display RT–PCR strategy to identify novel growth-factor-induced transcripts, we cloned and characterized the human homolog of yeast *NOP5*/*NOP58*, whose gene product has been implicated in the execution of early pre-rRNA processing steps. Human *NOP5* cDNA was isolated from an M426 fibroblast cDNA library. Determination of the cDNA nucleotide sequence revealed an open reading frame of 1587 nucleotides encoding a predicted gene product of 529 amino acids and mass of 59 554 Da. The yeast and human *NOP5* gene products were found to share 63% homology and 46% identity. *NOP5* mRNA was induced within 2 h of platelet-derived growth factor (PDGF) treatment of human M426 fibroblasts. Pretreatment with cycloheximide enhanced, while actinomycin blocked induction of the *NOP5* transcript. In vitro translational analysis of the cDNA revealed a 60 kDa species, consistent with the predicted molecular weight of the gene product. Ubiquitous, but differential *NOP5* mRNA expression was revealed after Northern blot analysis of total RNA from several human tissues. Moreover, *NOP5* mRNA expression was also demonstrated in cell lines of fibroblast, epithelial, and myeloid origin. A highly charged carboxy terminal domain and consensus phosphorylation sites were identified. The presence of potential regulatory elements, together with growth factor induction and widespread expression is consistent with the hypothesis that the *NOP5* gene product may play a role in fundamental cellular growth processes.

Keywords: NOP5/NOP58; PDGF; Ribosome biogenesis

1. Introduction

Receptor tyrosine kinase signal transduction to the nuclear compartment is a fundamental process through which cells respond to environmental stimuli (Hunter, 1997). The attendant transcriptional activation that results often mediates a host of cellular functions, including growth, differentiation, metabolic responses and other unique processes characteristic of a given cell type (Lau and Nathans, 1991). Among the many metabolic events influenced by growth factors, cytokines and oncogenes, are the induction of ribosome biogenesis and the subsequent increase in protein synthesis that is prerequisite to cellular proliferation (Peterson and Schreiber, 1998; Schmidt, 1999; Willis, 1999).

Within the eukaryotic nuclear envelope, the nucleolus contains over 80 ribosomal proteins that assemble with rRNA during the process of ribosome biogenesis (Tollervey, 1996). Early events in ribosome assembly include the transcription and processing of pre-rRNA to rRNA by endo- and exonucleases (Weinstein and Steitz, 1999). In yeast, for example, the 35S pre-RNA is transcribed by RNA polymerase I and processed to 18S, 5.8S, and 25S rRNAs (Venema et al., 1995). A key processing event in the splicing of the 35S precursor rRNA is the removal of the promoter proximal 5′-externally transcribed spacer (5′-ETS) at sites designated A_0 and A_1 (Hughes and Ares, 1991). Cleavage of the 35S rRNA at site A_2 results in formation of the 3′ end of the 18S rRNA. Many small ribonucleoproteins (snoRNPs) have been shown to be involved in the A_0, A_1, and A_2 splicing events (Venema et al., 1995). The resultant 18S ribosomal RNA is incorporated into the small 40S ribosomal subunit.

The *NOP5*/*NOP58* gene product has been shown to be required for both efficient yeast growth and the processing of pre-35S rRNA to 18S rRNA (Wu et al., 1998; Gautier et al., 1997). Genetic depletion studies have demonstrated that loss of *NOP5* lengthened yeast

Abbreviations: ORF, open reading frame; PDGF, platelet-derived growth factor.

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doubling time about 5-fold. Processing of 35S pre-rRNA at the A_0 and A_2 cleavage sites was dramatically impaired (Wu et al., 1998). Additionally, the encoded Nop5p was shown to be associated with the snoRNAs (u3, snR13, U14, U18) as well as the snoRNP Nop1p (Gautier et al., 1997; Wu et al., 1998). Here, we describe the cloning and expression of a human homolog of the yeast *NOP5*. Consistent with its role in yeast growth processes, the human *NOP5* transcript was found to be induced by the potent mitogen, PDGF, in human fibroblasts. Like its yeast counterpart, the deduced human amino acid sequence was found to contain a highly charged motif, within its carboxy terminal domain and consensus phosphorylation sites, potentially important for biologic function. The extensive expression of the human *NOP5* transcript in multiple human tissues and cell lines suggests a fundamental function consistent with the lethality of its deletion in yeast.

2. Materials and methods

2.1. Materials

Cycloheximide and actinomycin D were purchased from US Biological (Swampscott, MA) and Sigma (St. Louis, MO) respectively.

2.2. Library construction and screening

Murine *NOP5* was isolated as described in (Nelson et al., 2000). Briefly, 2 μg of total cellular RNA isolated from PDGF BB-treated (100 ng/ml) and untreated NIH 3T3 fibroblasts was reverse transcribed using random primers. Differential display RT–PCR was performed as described by Liang and Pardee (1992). Amplicons were separated on 6% polyacrylamide gels, fragment isolated, and TA cloned into PCRII (Invitrogen; Carlsbad, CA). After bacterial transformation, cDNA was prepared from individual colonies, and rescued inserts were tested by Northern hybridization and sequenced by the dideoxy chain termination method using T7 polymerase (US Biochemical).

A cDNA library was constructed using oligo (dT) primed human M426 fibroblast cDNA packaged into λpCEV27 (Miki et al., 1991). For library screening, the bacterial strain Y1088 was infected with phage (2×10^4 plaques per 150-mm plate) and plated on agar plates. After a 16 h incubation at 37°C, the plates were overlayed with Nytran filters (Schleicher and Schuell). Filters were then hybridized with ^{32}P-labeled mouse *NOP5* cDNA using standard protocols (Miki et al., 1991; Beeler et al., 1997). Plaques giving positive signals were isolated and subjected to secondary and tertiary screenings to insure plaque purification. The cDNA inserts from plaque purified clones were sequenced by the dideoxy chain termination method (Sanger et al., 1977) using T7 polymerase (US Biochemical).

2.3. Northern blot analysis

M426 fibroblasts were maintained in DMEM/10% fetal calf serum throughout this study unless otherwise indicated (Patel et al., 1996). For Northern blot analysis, total RNA was prepared after PDGF BB treatment (100 ng/ml) in the presence of TriZol (Gibco/BRL, Gaithersburg, MD), extracted with chloroform, and recovered by precipitation with isopropanol. In some instances, cells were pretreated with cycloheximide (100 μg/ml) or actinomycin D (100 μg/ml) for 60 min before growth factor addition. In other experiments, M426 fibroblasts, M413 fibroblasts, HA153B fibroblasts, B5/589 and BC1 breast epithelial cells, HEL-92-1, K562, U937 and TF-1 cells were grown in 100-mm tissues culture dishes, washed with sterile PBS, lysed in the presence of RNAzol (TelTest), extracted with chloroform, and precipitated as described.

Typically, 20 μg of total RNA was resolved by electrophoresis on 1.0% formaldehyde agarose gels and transferred to Nytran nylon membranes. After UV-crosslinking of the RNA to the membrane, filters were prehybridized for 4 h at 42°C in Hybrisol-I (Oncor, Gaithersburg, MD) [50% formamide, 10% dextran sulfate, 1% sodium dodecyl sulfate (SDS), $6 \times$ SSC and blocking agents] and then hybridized for 20 h in the same solution containing a ^{32}P-labeled probe corresponding to a 1.7 *Bam*HI fragment of the human *NOP5* cDNA. Filters were washed twice (30 min each) at room temperature in $2 \times$ SSC–0.1% SDS and twice at 50°C in $0.1 \times$ SSC–0.1% SDS and exposed to Kodak XAR film.

2.4. In vitro transcription and translation

A plasmid designated pCEV27-human *NOP5* was analyzed for in vitro transcription/translation using the TNT Coupled Reticulocyte Lysate System (Promega). The purified plasmid (1 μg) was added to a rabbit reticulocyte lysate in the presence of SP6 RNA polymerase and 40 μCi of [^{35}S]methionine (10 mCi/ml, specific activity 1078 Ci/mmol, translation grade Dupont, NEN) according to the manufacturer's specifications (Promega). Five μl of sample were mixed with 20 μl of $2 \times$ SDS sample buffer and resolved by SDS–polyacrylamide gel electrophoresis (PAGE) under reducing conditions. Gels were dried and exposed to X-ray film at −70°C for 4 h.

2.5. Nucleotide sequence Accession No.

The GenBank Accession No. for the human *NOP5* cDNA sequence is AF263608.

3. Results

3.1. Isolation of a novel human PDGF-induced cDNA

Serum growth factors maintain tissue homeostasis by mediating proliferation and differentiation of responsive cells. Characterization of changes in transcript expression that occur in response to a given growth factor is an essential step in understanding growth factor function. We used differential display RT–PCR analysis to identify novel amplicons induced or repressed in the presence of PDGF. Using this technique, we identified a novel PDGF-induced gene in mouse NIH3T3 cells (Nelson et al., 2000). Isolated amplicons induced by PDGF were first screened by Northern blot analysis. After DNA sequence analysis to determine uniqueness, we used the murine cDNA to screen 500 000 plaques from a human M426-λCEV27 cDNA library (Miki et al., 1990, 1991). After secondary and tertiary screenings, six positive clones were identified. Digestion of the phagemids with *Not* I enabled the rescue of the clones as pCEV27 plasmids. Further analysis of these plasmids revealed *Sal* I inserts 1.9–2.2 kb with similar restriction endonuclease digestion patterns.

Isolated cDNAs were subjected to DNA sequence analysis. We identified an open reading frame that encompassed 1587 bp, beginning with an initiation codon at position 193 and ending with a termination codon at position 1780 (Fig. 1). 5′ and 3′ UTRs of 192 and 280 nucleotides flanked the open reading frame, with a potential polyadenylation signal (AATAAA) at position 1986. The predicted sequence of 529 amino acids encoded a polypeptide with a calculated molecular mass of 59 554 Da (p*I* of 9.56). The predicted translation of the longest open reading frame of the human cDNA is shown in Fig. 1.

We further analyzed databases using the National Center for Biotechnology Information (NCBI) Blast network service to search for similarity between our encoded gene product and other previously identified proteins (Altschul et al., 1990). Utilization of the Blast network service revealed that the human gene product was highly related to *Drosophila* and yeast *NOP5*, and it was thus designated human *NOP5* (*hNOP5*). *Drosophila NOP5* showed 75% homology (62% identity) when compared with our human *NOP5* cDNA sequence. The yeast *NOP5* coding region showed 63% homology (46% identity) to human *NOP5*. Searches of NCBI databases (Swiss Prot 25.0, Pir 36.0 and Gen-Pept-Gen Bank 76.0) indicated that in addition to the yeast and *Drosophila NOP5* gene product, the human *NOP5* also showed similarity to *Mus musculus* SIK protein and *Pisum sativum* SAR DNA-binding protein-1.

The predicted amino acid sequence of human Nop5p was analyzed using the Prosite and CBS PhosphoBase 2.0 programs that identify conserved protein sequence motifs. Computer analysis revealed that the predicted sequence contained a number of potential protein kinase phosphorylation sites, including those for protein kinase C (S-219, S-331, T-335, S-351, T-409, T-416, S-440, T-455, S-483), protein kinase A (T-335, T-397, S-407), p34cdc2 (T-34, S-514), and p70 S6K (T-335). Three consensus tyrosine kinase phosphorylation sites were also predicted (Y-204, Y-217, Y-338).

3.2. Human NOP5 is a novel delayed early response gene

The relatively rapid induction of *hNOP5* led us to investigate whether it might be characterized as an early response gene. Studies of the kinetics of *hNOP5* transcript expression showed that after stimulation of M426 fibroblasts with PDGF BB, the hNOP5 message was first detected within approx. 2 h (Fig. 2A). Human NOP5 expression continued to increase approx. 3–4 fold over basal expression levels after as long as 4 h and reached sustained expression for as long as 8 h (Fig. 2A). Pretreatment of M426 fibroblasts with actinomycin inhibited *hNOP5* induction (Fig. 2B), demonstrating that an increase in *NOP5* transcript stability did not explain the observed induction.

Besides rapid induction, another characteristic of early response genes is transcriptional activation independent of de novo protein synthesis. To determine whether induction of *hNOP5* required protein synthesis, M426 fibroblasts were pretreated with 100 mM cycloheximide for 30 min, followed by PDGF BB treatment for the indicated time period. As shown in Fig. 2C, *NOP5* induction after both cycloheximide and PDGF BB treatment was not inhibited. Consistent with previous reports, induction of the immediate early gene *JE* was not inhibited and superinduction was observed (data not shown). In contrast to the greatly enhanced *JE* transcription in the presence of both cycloheximide and PDGF BB, only a modest increase (approx. 2-fold) in *hNOP5* message levels was observed when compared with PDGF treatment alone (Fig. 2A). Thus, human *NOP5* exhibited the transcriptional activation characteristics of a delayed early response gene.

3.3. In vitro translation of the human NOP5 gene product

To examine the integrity and relative molecular weight of the encoded gene product, we transcribed/translated each plasmid using an in vitro rabbit reticulocyte lysate system. As shown in Fig. 3, the in vitro translation product of the human pCEV27-*NOP5* plasmid revealed a major 60 kDa protein consistent with the predicted size of human Nop5p, as well as the known size of the yeast gene product. The control pCEV27 plasmid did not translate a 60 kDa protein (Fig. 3). Further investigation of the identity of the p40

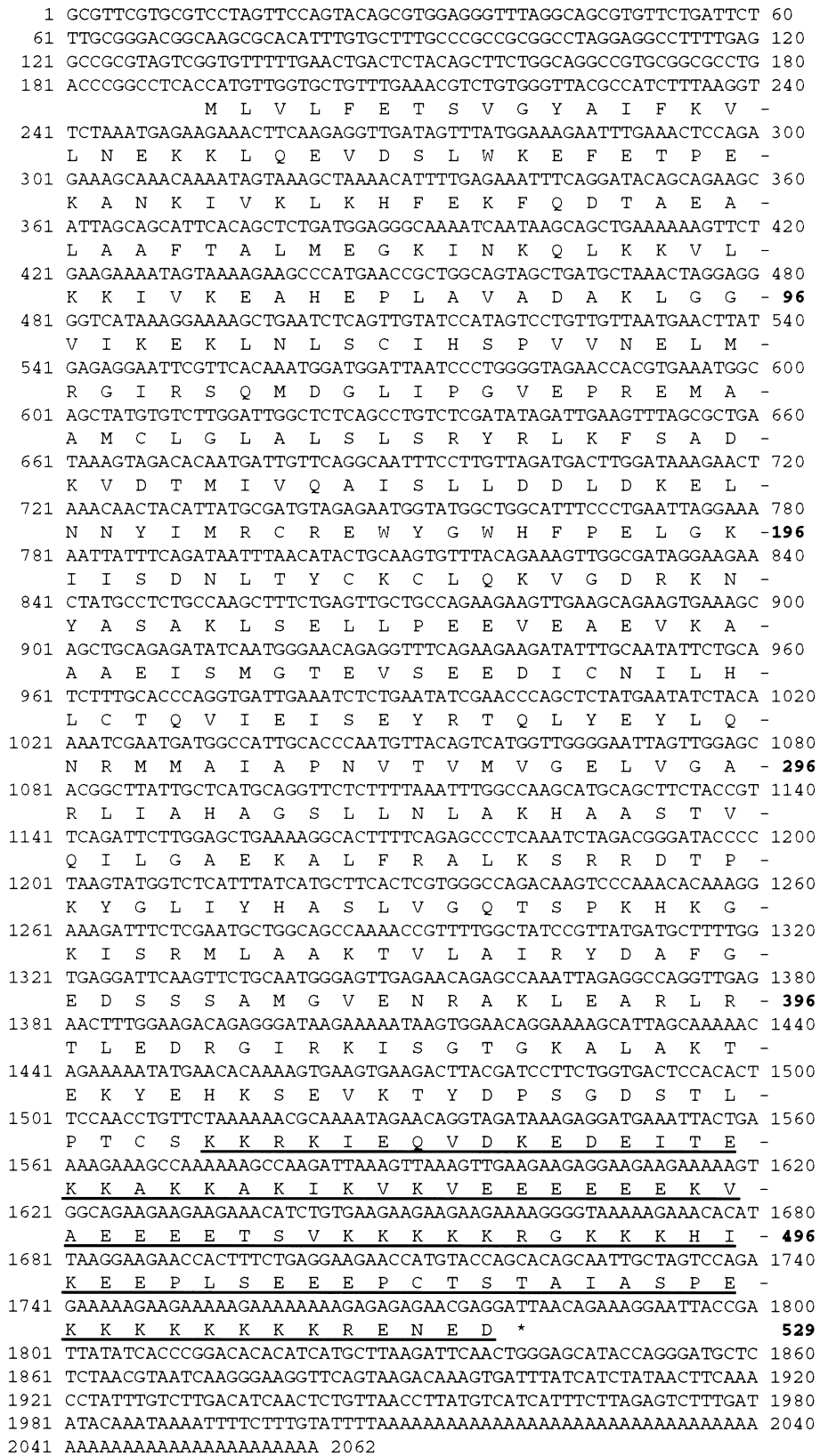

Fig. 1. The nucleotide and deduced amino acid sequence of human *NOP5*. The *hNOP5* nt sequence is printed for the 5′→3′ strand. Amino acids are numbered in bold, and single-letter designations are used. The highly charged carboxy terminal domain is underlined.

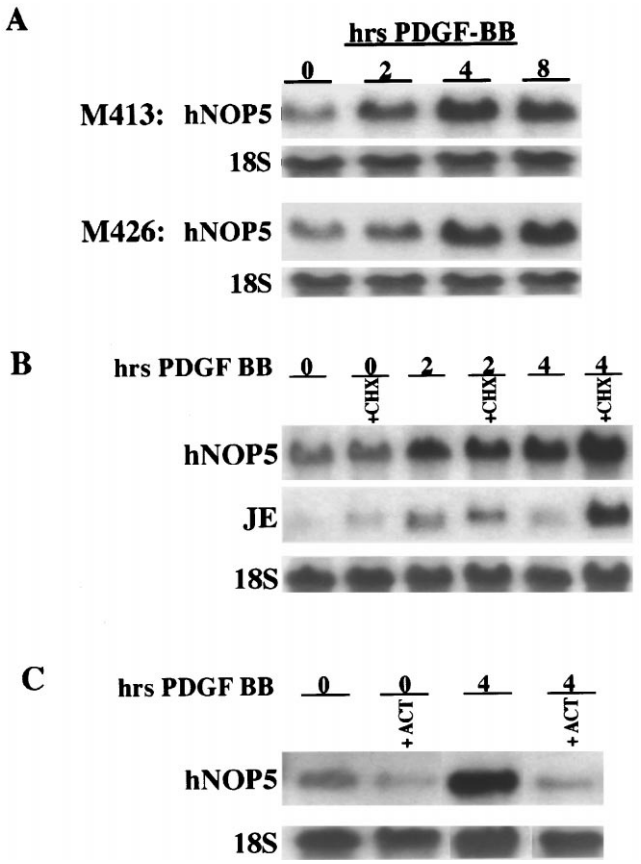

Fig. 2. Platelet-derived growth factor induction of *hNOP5* transcript. M426 and M413 fibroblasts (A) were treated with PDGF for 0, 2, 4 or 8 h. Total RNA was prepared and Northern blot analysis performed as described in Materials and methods. In some instances, M426 fibroblasts were pretreated with cycloheximide (B) or actinomycin (C). Filters were hybridized with ^{32}P-labeled cDNA probes to human NOP5 or 18S ribosomal RNA and subjected to autoradiography. *hNOP5* transcript levels were quantitated with NIH Image 1.6 over the time course of induction.

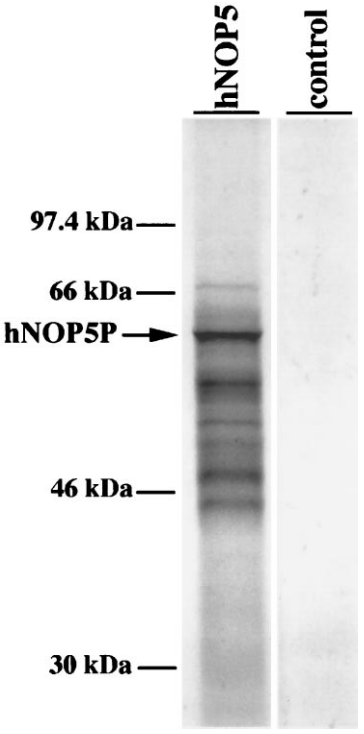

Fig. 3. In vitro translation of human *NOP5* cDNA. A plasmid containing human NOP5 was isolated from a human M426 fibroblast cDNA library and subjected to in vitro transcription/translation using the TNT Coupled Reticulocyte Lysate System as described in Materials and methods. Rabbit reticulocyte lysates were incubated in the presence of pCEV27-*NOP5* or pCEV27 and analyzed by SDS–PAGE (10% acrylamide). After drying, the gel was exposed to X-ray film for 24 h. The relative mobility of each molecular mass standard is indicated.

and minor p30 species suggested internal initiation of the *hNOP5* cDNA consistent with internal Met residues.

3.4. Analysis of human NOP5 mRNA expression in human tissues and cell lines

To gain clues regarding human *NOP5* biologic function, we examined the tissue distribution of *hNOP5* transcript expression. Northern blot analysis was performed on RNAs isolated from a variety of different human tissues. Under stringent hybridization conditions, the *hNOP5* cDNA probe revealed a single transcript of approx. 2.2 kb in various tissues consistent with its size in M426 fibroblasts (Fig. 4). *NOP5* was expressed at moderate amounts in heart, brain, skeletal muscle and kidney, and at lower levels in spleen. Highest levels of mRNA expression were observed in lung, liver and testes.

To further investigate the cellular pattern of human

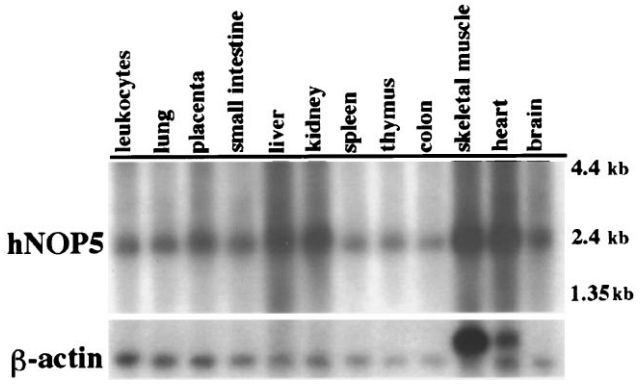

Fig. 4. Tissue distribution of human NOP5 mRNA. A multiple tissue Northern blot (Clontech, USA; 5 μg of poly(A)$^+$ RNA) was hybridized with ^{32}P-labeled human NOP5 or human actin cDNA and subjected to autoradiography as described in the Materials and methods. The positions of molecular weight markers from a 0.24–9.5 kb RNA ladder are indicated.

NOP5 mRNA expression, we performed Northern blot analysis on total RNA isolated from cell lines representing a variety of human tissues. As shown in Fig. 5, the human *NOP5* cDNA probe revealed a single transcript of approx. 2.2 kb in the cell lines tested. *hNOP5* mRNA

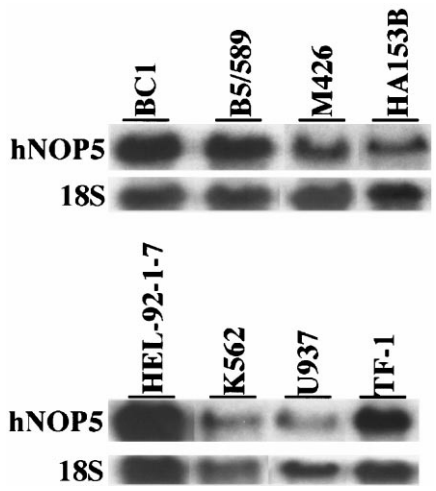

Fig. 5. Analysis of the human NOP5 transcript in human cell lines of diverse tissue origin. Total RNAs were extracted from M426 fibroblasts, brain-derived fibroblasts (HA153B), breast epithelial cells (B5/589, BC1), erythroid cells (HEL-92-1),erythroid progenitors (K562), myeloid progenitors (U937) and T cells (TF-1), and then subjected to Northern blot analysis. The filter was hybridized (Beeler et al., 1997) with ^{32}P-labeled cDNA probes to human *NOP5* or 18S ribosomal RNA and subjected to autoradiography as described in the Materials and methods.

was expressed in M426 fibroblasts, brain-derived fibroblasts (HA153B), breast epithelial cells (B5/589, BC1), erythroid cells (HEL-92-1),erythroid progenitors (K562), myeloid progenitors (U937) and T cells (TF-1). We conclude that *hNOP5* is differentially expressed in tissues of various origins. The widespread detection of the *hNOP5* transcript was suggestive of a role in fundamental cellular events.

3.5. Identification of a highly charged domain in the Nop5p gene product

Analysis of the predicted human Nop5p protein sequence against the NCBI databases found a highly charged carboxy terminal domain conserved between human Nop5p and Nop5p isolated from evolutionarily diverse species such as yeast and *Drosophila*. The highly charged human carboxy terminal domain (K-441 to D-529) contained numerous acidic (24.7% E) and basic (32.6% K) residues. A 10 amino acid pocket containing two prolines, two serines, and two threonines divided this domain at residues 506–516. The major difference observed between human and yeast *NOP5* was observed in the carboxy terminal region in which the yeast Nop5p contains numerous KKD/E repeats.

4. Discussion

In this study, we report the isolation and characterization of a cDNA encoding the human homolog of the yeast *NOP5* gene. The human *NOP5* cDNA encodes a deduced protein sharing 75% and 63% homology to *Drosophila* and yeast *NOP5*, respectively. Human *NOP5* is induced by the mitogen PDGF, and displays characteristics of an early response gene, including synthesis of transcript within 2 h of growth factor induction and transcription independent of de novo protein synthesis. Human *NOP5* RNA is widely and differentially expressed in human tissues and cell lines, including those derived from fibroblasts, epithelial, and myeloid cell lineages. The human *NOP5* gene product was identified as an approx. 60 kDa translation product and contains multiple consensus kinase phosphorylation sites as well as a highly charged carboxy terminal domain that has been reported for several nucleolar proteins.

In yeast, *NOP5* has been shown to be required for the biogenesis of the 40S ribosomal subunit and functional activity appears to be influenced by carboxy terminal KKX repeats (Wu et al., 1998). For example, removal of the basic KKD/E motif from Nop5p results in substantially reduced growth at 37°C, but has no effect on growth at 25°C, suggesting a role in molecular stabilization (Wu et al., 1998). Carboxy terminal KKX repeats are also characteristic of several nucleolar proteins implicated in rRNA synthesis, including Cbf5p (Jiang et al., 1993) and Dbp3p (Weaver et al., 1997). Human *NOP5*, while highly charged at its carboxyl terminus, has only two KKX motifs. Future studies will resolve whether human*NOP5* will prove to have similar functions.

Growth factor receptors transduce external stimuli into nuclear events, ultimately resulting in the activation or repression of a specific complement of genes (Herschman, 1991). In yeast, adverse growth conditions result in arrested transcription of genes encoding ribosomal proteins (Planta, 1997). Recently, Wu et al. (1998) have elegantly demonstrated that yeast deficient in Nop5p undergo growth arrest due to defects in early pre-rRNA processing events necessary for ribosome assembly. Similarly, in mammalian cells, PDGF (Rosenkranz and Kazlauskas, 1999) clearly increases transcriptional/translational activity to promote effective cell division, requiring increased levels of ribosomal proteins. It is tempting to speculate that human *NOP5* regulation may provide a context through which cells interpret external proliferative signals, and that altering *hNOP5* activity may present a unique approach for arresting aberrant cell growth associated with many disease processes.

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