J. Biochem. 123, 876-882 (1998)

Cloning and Characterization of Three Isoforms of OS-9 cDNA and Expression of the OS-9 Gene in Various Human Tumor Cell Lines[1]

Yukiko Kimura, Megumi Nakazawa, and Michiyuki Yamada[2]

Graduate School of Integrated Science, Yokohama City University, 22-2 Seto, Kanazawa-ku, Yokohama 236-0027

Received for publication, November 17, 1997

OS-9 gene is frequently coamplified with CDK4 gene in human sarcomas. We isolated and characterized three isoforms of OS-9 cDNA found in a myeloid leukemia HL-60 cDNA library. Isoform 1 consisted of 2,700 bp, from which a 667 amino acid sequence was deduced and found to be identical with that of OS-9 cDNA from osteosarcoma cells [Su *et al.* (1996) *Mol. Carcinogen.* 15, 270-275]. Isoform 2 cDNA lacked a 165 nucleotide sequence in the coding region. Isoform 3 cDNA had an additional 45 bp deletion in the coding region. Isoforms 2 and 3 encode 612 and 597 amino acid polypeptides, respectively. Comparison of their cDNA sequences with the genomic structure indicated that three isoforms are splice variants. Reverse transcription-polymerase chain reaction analysis showed predominant expression of isoform 2 mRNA in myeloid leukemia HL-60 cells, osteosarcoma OsA-CL cells and rhabdomyosarcoma Rh30 cells. Northern blotting revealed similar levels of expression of OS-9 gene in various tumor cell lines of sarcoma cells, carcinoma cells and myeloid leukemia cells, but 3-4 times higher expression in OsA-CL cells and Rh30 cells containing a homogeneously staining region of 12q13-15. OS-9 expression decreased in differentiation-induced HL-60 cells. Possible involvement of the OS-9 gene in cell growth is discussed.

Key words: alternative splicing, gene amplification, HL-60 cells, OS-9 cDNAs.

Amplification and overexpression of genes are involved in development and progression of human tumors. The q13-15 region of human chromosome 12 is frequently amplified in human sarcomas and gliomas (*1-3*). Amplified genes in this region include *MDM2*, which encodes the modulator for the tumor suppressor protein p53; *GLI*, the zinc finger protein; *GADD153/CHOP*, the transcription factor; *SAS*, a member of the transmembrane 4 superfamily; *CDK4*, cyclin-dependent kinase 4; and *A2MR*, the α_2 macroglobulin receptor (*4-8*). Recently, a novel cDNA, OS-9, was isolated by chromosome microdissection of this region and hybrid selection (*9*). The OS-9 gene is located near the CDK4 gene, which is most frequently amplified in sarcomas (*10*).

We are interested in the involvement of PTP in growth and differentiation of human myeloid leukemia HL-60 cells (*11-13*). An OS-9 cDNA clone cross-hybridized with a PTPcDNA probe has been isolated from an HL-60 cDNA library (*14*). Only one form of OS-9 cDNA has been reported (*15*). While cloning a full-length cDNA, we found various forms of OS9 cDNA clones. Therefore, we initiated isolation and characterization of these cDNAs. This paper describes cloning and characterization of three isoforms of OS-9 cDNA that are produced from mRNAs generated from a single transcript by alternative splicing, and their expression in myeloid leukemia cells and sarcoma cells. The amounts of the mRNA varied in types of tumor cells and states of differentiation, although isoform 2 predominated in the cell samples examined.

MATERIALS AND METHODS

Chemicals—[α-^{32}P]dCTP was purchased from the Institute of Isotopes of the Hungarian Academy of Science. RA was from Sigma. TPA was from Midland. Nitrocellulose filters BA 85 were from Schleicher and Schuell. Hybond-N nylon membranes were from Amersham Life Science. A digoxigenin (DIG) DNA labeling and detection kit was from Boehringer Mannheim. Oligotex™-dT30〈Super〉 was from Daiichi Pure Chemicals. Oligonucleotides were synthesized by Nippon Bio Service. A partial length PTP1B cDNA was cloned from an HL-60 cDNA library using TC-PTP cDNA as a probe.

Cell Culture—The human myeloid leukemia cell line HL-60, the human osteosarcoma cell line OsA-CL/SJSA-

[1] This work was supported by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan, the Japan Foundation for Applied Enzymology, the Takamura Foundation, and Grants in Support of the Promotion of Research at Yokohama City University. The nucleotide sequence data reported in this paper and the genomic sequence data will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with accession numbers AB002806 and AB002805.

[2] To whom correspondence should be addressed. Phone: +81-045-787-2214, Fax: +81-045-787-2370, E-mail: myamada@yokohama-cu.ac.jp

Abbreviations: G3PDH, glyceraldehyde 3-phosphate dehydrogenase; MPO, myeloperoxidase; PCR, polymerase chain reaction; PTP, protein tyrosine phosphatase; RA, all-*trans* retionoic acid; RACE, rapid amplification of cDNA ends; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

© 1998 by The Japanese Biochemical Society.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

1(obtained from ATCC), the human rhabdomyosarcoma cell line RH30, and other cell lines were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (General Scientific Laboratories) and kanamycin sulfate (50 μg/ml) at 37°C under 5% CO_2 in air. For induction of differentiation of HL-60 cells into granulocytes, the cells were seeded at 3×10^5 cells/ml and grown with 1 μM RA (*16*), and for differentiation into macrophages, the cells were seeded at 9×10^5 cells/ml and grown with 10 ng/ml TPA (*17*).

Cloning of OS-9 cDNAs—Approximately 1×10^6 plaques of an HL-60 cDNA library in λgt10 (*18*) were screened by hybridization on nitrocellulose filters for 40 h at 50°C with a ^{32}P-labeled PTP1B cDNA probe (*19*). The filters were washed in 6×SSC/0.1% SDS at 50°C twice and 2×SSC/0.1%SDS at 50°C twice. Clones positive for signal were obtained, and sequencing of these clones revealed that the one clone contained the reported 120 bp λOS-9 cDNA (*9*). The cDNA clone containing 2,300 bp was referred to as OS-9-1. For isolation of a full-length cDNA, we rescreened the HL-60 cDNA library. Hybridization was performed at 65°C with 10 ng/ml of a DIG-labeled *Eco*RI-*Pst*I fragment (800 bp) of OS-9-1 cDNA for 24-48 h. The filters were washed in 1×SSC/0.1% SDS at 65°C twice and 0.1×SSC/0.1% SDS at 65°C twice. Clones on the filters were detected by immunological assay with anti-DIG-alkaline phosphatase conjugate followed by colorimetric reaction coupled with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium salt solution (*20*). Twenty cDNA clones positive for signal were obtained. To detect cDNAs containing the longest 5′ region, cDNAs were subjected to PCR using λgt10 primers (forward 5′-GCA AGT TCA GCC TGG TTA AG-3′; reverse 5′-GCT TAT GAG TAT TTC TTC CAG GG-3′) and an internal OS-9 antisense primer (nt 1002-983 5′-GGT CCT CTG GCT CAT TAA GC-3′). The PCR products were subcloned into a pCR II vector (a TA cloning kit, Invitrogen) for sequencing. The cDNA containing the longest 5′ region was referred to as OS-9-2.

TABLE I. **Primers used in 5′-RACE and PCR analysis of cDNA clones.**

Primers	Positions relative to OS-9 cDNA	Primer sequences
5′-RACE		
1st primer	nt 427-409	5′-TGG ATG TGG CGT CCA TAA CC-3′
2nd primer	nt 230-211	5′-GAC AAT CAC CAC GTC CGA AG-3′
5′-C-primer		5′-GGC CCG ACG TCG CAT GAA TTC GCC CCC CCC CCC C-3′
5′-Ap I primer		5′-GGC CCG ACG TCG CAT G-3′
Primer pairs		
1	nt 422-441	5′-CAT CCA GCA ATA CCA CAT GG-3′
1	nt 1227-1208	5′-GGA CTT CTG CAG CAT CAT CC-3′
2	nt 805-824	5′-ACA TGG CCT ACG TTC AGA GG-3′
2	nt 1536-1515	5′-TGT TGA GAG TGG ATG TGA GAG C-3′
3	nt 1568-1587	5′-TCC AGA GCT GGT GAA GAA GC-3′
3	nt 2043-2024	5′-AGA AGT CAA ATT CGT CCA GG-3′

Rapid Amplification of the cDNA 5′End (5′RACE)—For further sequencing of the 5′ end region, 5′ RACE cDNAs were obtained essentially as described (*21*). Total cellular RNAs of HL-60 cells and OsA-CL cells were prepared by the acid guanidium thiocyanate method (*22*) using TRIZOL reagent (Gibco BRL). Poly(A)$^+$ RNA was obtained from the total RNA using oligotex-dT30〈Super〉. Primers used for 5′RACE are included in Table I. Nucleotides are numbered in accord with the isoform 1 cDNA sequence. First strand cDNA was synthesized from 2 μg of poly(A)$^+$ RNA using a 1st antisense primer (nt 427-409) and 200 units of Moloney murine leukemia virus reverse transcriptase (Superscript II, Gibco BRL) in a total volume of 20 μl, then polyguanylated using 15 units of terminal deoxynucleotidyl transferase (Gibco BRL) by incubation with 0.1 mM dGTP at 37°C for 15 min. The reaction mixture for 2nd strand cDNA synthesis and amplification consisted of a tenth portion of the polyguanylated cDNA, a 5′-C primer, a 2nd primer (nt 230-211), and a 5′-Apa I primer. The 1st one-cycle reaction consisted of denaturing for 2 min at 95°C, annealing for 2 min at 50°C and extension for 40 min at 72°C, and the 2nd 35-cycle reaction consisted of denaturing for 40 s at 94°C, annealing for 2 min at 55°C and extension for 3 min at 72°C. The PCR products were subcloned into a pCR II vector and sequenced.

Nucleotide Sequencing—Inserts subcloned were sequenced with an Li-COR dna sequencer model 4000L, using a cycle sequencing kit containing a laser dye (IRD41)-labeled primer (Epicenter Technology). For PCR products, at least three plasmids were sequenced. The complete sequence was determined from these consensus sequences with Gene Works software (Intelligenetics). Homology searches were conducted using the BLAST and FASTA programs.

PCR Analysis of cDNA Clones—PCR analyses of OS-9 cDNA clones were performed using primer pairs 1, 2, and 3 listed in Table I. The PCR products were separated by electrophoresis in 1.2% agarose gel and eluted with Geneclean (Bio 101) for subcloning into a pCR II vector.

Northern Blotting—Total cellular RNAs obtained as above were separated by electrophoresis in denaturing 1.0% agarose gel containing formaldehyde (*19*), transferred to a Hybond-N nylon membrane, and UV-cross linked. An *Eco*RI fragment (2,289 bp) of OS-9-1 cDNA was labeled with [α-^{32}P]dCTP by the random primer DNA labeling method (*23*) and used as a probe. Hybridization with the probe was performed at 42°C in 50% formamide, 6×SSPE, 0.1% SDS, 0.1 mg/ml of sonicated-heat denatured salmon sperm DNA, 5×Denhardt's solution, and 5% dextran sulfate for 24-48 h. The filters were washed in 2×SSC/0.1% SDS at room temperature and in 0.1×SSC/0.1% SDS at 65°C, then were exposed to X-ray films at −80°C.

RT-PCR Analysis—First strand cDNA was synthesized from 2 μg of poly(A)$^+$ RNA obtained as above using an oligo-$dT_{12\text{-}18}$ primer and 200 units of reverse transcriptase (Superscript II) in a total volume of 20 μl. A tenth portion of the first strand cDNA was amplified using a primer pair 2 or 3 listed in Table I. A similar portion of the same reverse transcription product was amplified with a G3PDH primer pair (Clontech) as a control. The PCR products were separated by electrophoresis in 2.5% agarose gel and eluted for subcloning and sequencing.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

RESULTS

Cloning and Characterization of OS-9 cDNAs—A cDNA clone, OS-9-1, cross-hybridized with a human PTP 1B cDNA probe was obtained from an HL-60 cDNA library. Sequencing indicated that the cDNA contained a large portion of OS-9 cDNA sequence (Fig. 1A). For cloning a full-length OS-9 cDNA, the HL-60 cDNA library was screened by plaque hybridization using a 5′ *Eco*RI-*Pst*I fragment (800 bp) of OS-9-1 cDNA as a probe and 20 OS-9 cDNA clones were obtained. The cDNA clone containing the longest 5′ region was selected by PCR using the OS-9 primer (nt 1002-983) and the λ primers and was referred to as OS-9-2. OS-9-2 was 367 bp longer in the 5′ region than OS-9-1 (Fig. 1A). In addition, we prepared 5′ RACE cDNAs from HL-60 and OsA-CL poly(A)$^+$ RNAs using the antisense primer (nt 427-409) and the nested primer (nt 230-211). These cDNAs extended further to the 5′ ends. The complete nucleotide sequence of cDNA was determined by alignments of the sequences of OS-9-1, OS-9-2, and 5′ RACE cDNAs and named OS-9 (Fig. 1B). The sequence of 2,700 bp was identical with the reported seqence of OS-9 cDNA (*15*), except that the 16 bp sequence at the 5′ terminus differed obviously. The sequence presented here contained a 41 bp 5′ untranslated region, a 2,001 bp coding region encoding 667 amino acid residues, and a 658 bp 3′ untranslated region. The 5′ untranslated region has an inframe stop codon TAA at nt 24. The deduced amino acid sequence has an acidic stretch (amino acid 414-429) consisting of glutamic acid and aspartic acid (ED-rich sequence) and a potential nuclear targeting sequence TKKGK in the middle portion of the protein, as noted originally by Su *et al.* (*15*).

Characterization of Three Isoforms of OS-9 cDNA—Restriction analyses and sequencing of OS-9-2 cDNA indicated that its sequence lacked the sequence of 165 bp from nt 1642 through nt 1806 without change of the reading frame (Fig. 1B). To examine the heterogeneity among the 20 cDNA clones further, we performed PCR analyses using the primer pairs 1, 2, and 3 (Table I). The PCR products were separated electrophoretically, subcloned and sequenced. Figure 2 shows the electrophoretic mobilities of the PCR products from 7 representative clones. PCR using primer pair 1 delimiting nt 422-1227 gave the same band from all the 7 clones (lanes 1-7). PCR using primer pair 2 delimiting nt 805-1536 gave a 732 bp band from clones 3/OS-9-2, 7-2, 9-1, 13-1, and 18 (lanes 8, 9, 11, 13, and 14), while clones 8-3/OS-9-3 and 12-2 gave a 687 bp band migrating slightly ahead (lanes 10 and 12). Subcloning and sequencing of this band showed a 45 bp deletion from nt 1407 to 1451 in the OS-9 sequence (the boxed sequence 1 in Fig. 1B). This coincided exactly with a stretch coding 15 amino acids in the reading frame. PCR using primer pair 3

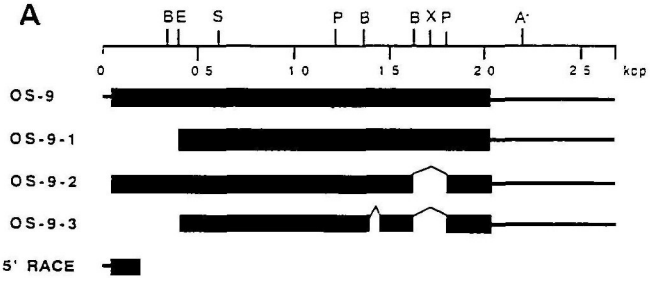

Fig. 1. **Nucleotide sequence and deduced amino acid sequence of OS-9 cDNA isoforms.** (A) Restriction map of OS-9 cDNA isoform 1. E, *Eco*RI; B, *Bam*HI; P, *Pst*I; X, *Xho*I; S, *Sma*I; A, *Apa*I. The closed rectangles represent reading frames, and thick lines show untranslated regions. The thin lines connecting rectangles indicate the absence of sequences between the rectangles. (B) Nucleotide and amino acid sequences. The reading frame begins at the putative start codon ATG (nt 42) and stops at the first inframe stop codon (nt 2043) marked with an asterisk. The potential nuclear targeting signal TKKGK is marked with a bold underline, an ED-rich domain with a shaded box, and the polyadenylation signal AATAAA (nt 2672) with a dotted underline. Isoform 2 lacks the boxed sequence marked with asterisk 2. This and another boxed sequence marked with asterisk 1 are lost in isoform 3. The 5′ end of the clone OS-9-1 is nt 403, that of the clone OS-9-2 nt 36, and that of the clone OS-9-3 nt 403. The 5′-RACE product spans from nt 1 to nt 211.

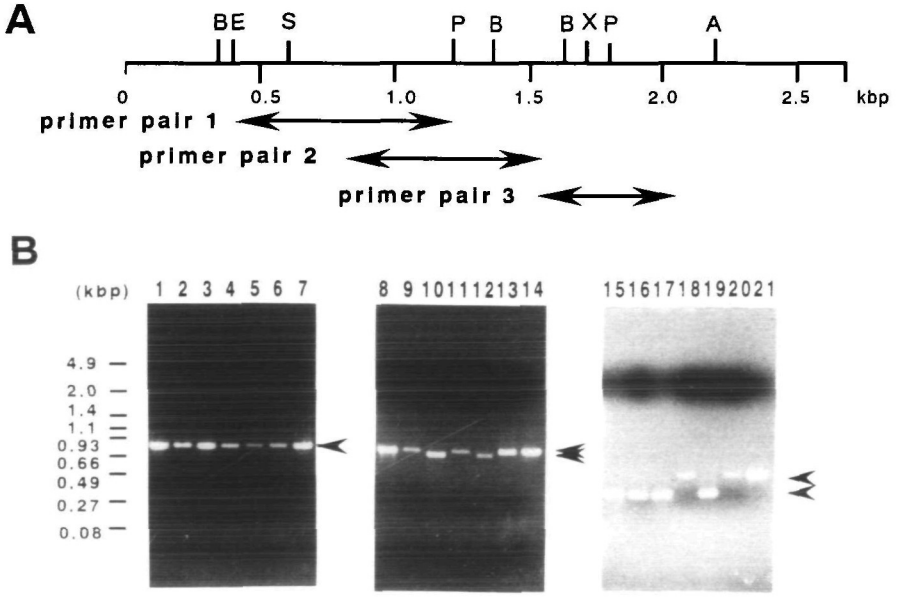

Fig. 2. **PCR analysis of OS-9 cDNAs.** A: The top line represents a restriction map of OS-9 cDNA isoform 1. Bars with arrowheads represent portions of the coding region amplified in PCR with the indicated primer pair. Three primer pairs are listed in Table I. B: Seven representative OS-9 cDNA clones in λgt10 were amplified by PCR with primer pair 1 (lanes 1-7), 2 (lanes 8-14), or 3 (lanes 15-21). PCR products were separated by electrophoresis in 1.2% agarose gel and stained with ethidium bromide. Lanes 1, 8, and 15, clone 3/OS-9-2; lanes 2, 9, and 16, clone 7-2; lanes 3, 10, and 17, clone 8-3/OS-9-3; lanes 4, 11, and 18, clone 9-1; lanes 5, 12, and 19, clone 12-2; lanes 6, 13, and 20, clone 13-1; lanes 7, 14, and 21, clone 18. The positions of standard size markers are shown on the left. Arrowheads indicate the positions of the PCR products.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

B

```
AGGGCGGAAACAGATTCTCTGCATAAGAAGGGGAACGAAAGATGGCGGCGGAAACGCTGCTGTCCAGTTTGTTAGGACTG      80
                                          M  A  A  E  T  L  L  S  S  L  L  G  L       13
CTGCTTCTGGGACTCCTGTTACCCGCAAGTCTGACCGGCGGTGTCGGGAGCCTGAACCTGGAGGAGCTGAGTGAGATGCG     160
L  L  L  G  L  L  L  P  A  S  L  T  G  G  V  G  S  L  N  L  E  E  L  S  E  M  R       40
TTATGGGATCGAGATCCTGCCGTTGCCTGTCATGGGAGGGCAGAGCCAATCTTCGGACGTGGTGATTGTCTCCTCTAAGT     240
 Y  G  I  E  I  L  P  L  P  V  M  G  G  Q  S  Q  S  S  D  V  V  I  V  S  S  K  Y       67
ACAAACAGCGCTATGAGTGTCGCCTGCCAGCTGGAGCTATTCACTTCCAGCGTGAAAGGGAGGAGGAAACACCTGCTTAC     320
  K  Q  R  Y  E  C  R  L  P  A  G  A  I  H  F  Q  R  E  R  E  E  E  T  P  A  Y       93
CAAGGGCCTGGGATCCCTGAGTTGTTGAGCCCAATGAGAGATGCTCCCTGCTTGCTGAAGACAAAGGACTGGTGGACATA     400
Q  G  P  G  I  P  E  L  L  S  P  M  R  D  A  P  C  L  L  K  T  K  D  W  W  T  Y       120
TGAATTCTGTTATGGACGCCACATCCAGCAATACCACATGGAAGATTCAGAGATCAAAGGTGAAGTCCTCTATCTCGGCT     480
  E  F  C  Y  G  R  H  I  Q  Q  Y  H  M  E  D  S  E  I  K  G  E  V  L  Y  L  G  Y      147
ACTACCAATCAGCCTTCGACTGGGATGATGAAACAGCCAAGGCCTCCAAGCAGCATCGTCTTAAACGCTACCACAGCCAG     560
  Y  Q  S  A  F  D  W  D  D  E  T  A  K  A  S  K  Q  H  R  L  K  R  Y  H  S  Q      173
ACCTATGGCAATGGGTCCAAGTGCGACCTTAATGGGAGGCCCCGGGAGGCCGAGGTTCGGTTCCTCTGTGACGAGGGTGC     640
T  Y  G  N  G  S  K  C  D  L  N  G  R  P  R  E  A  E  V  R  F  L  C  D  E  G  A      200
AGGTATCTCTGGGGACTACATCGATCGCGTGGACGAGCCCTTGTCCTGCTCTTATGTGCTGACCATTCGCACTCCTCGGC     720
 G  I  S  G  D  Y  I  D  R  V  D  E  P  L  S  C  S  Y  V  L  T  I  R  T  P  R  L      227
TCTGCCCCCACCCTCTCCTCCGGCCCCCACCCAGTGCTGCACCACAGGCCATCCTCTGTCACCCTTCCCTACAGCCTGAG     800
  C  P  H  P  L  L  R  P  P  P  S  A  A  P  Q  A  I  L  C  H  P  S  L  Q  P  E      253
GAGTACATGGCCTACGTTCAGAGGCAAGCCGACTCAAAGCAGTATGGAGATAAAATCATAGAGGAGCTGCAAGATCTAGG     880
E  Y  M  A  Y  V  Q  R  Q  A  D  S  K  Q  Y  G  D  K  I  I  E  E  L  Q  D  L  G      280
CCCCCAAGTGTGGAGTGAGACCAAGTCTGGGGTGGCACCCCAAAAGATGGCAGGTGCGAGCCCGACCAAGGATGACAGTA     960
 P  Q  V  W  S  E  T  K  S  G  V  A  P  Q  K  M  A  G  A  S  P  T  K  D  D  S  K      307
AGGACTCAGATTTCTGGAAGATGCTTAATGAGCCAGAGGACCAGGCCCCAGGAGGGGAGGAGGTGCCGGCTGAGGAGCAG    1040
  D  S  D  F  W  K  M  L  N  E  P  E  D  Q  A  P  G  G  E  E  V  P  A  E  E  Q      333
GACCCAAGCCCTGAGGCAGCAGATTCAGCTTCTGGTGCTCCCAATGATTTTCAGAACAACGTGCAGGTCAAAGTCATTCG    1120
D  P  S  P  E  A  A  D  S  A  S  G  A  P  N  D  F  Q  N  N  V  Q  V  K  V  I  R      360
AAGCCCTGCGGATTTGATTCGATTCATAGAGGAGCTGAAAGGTGGAACAAAAAAGGGGAAGCCAAATATAGGCCAAGAGC    1200
 S  P  A  D  L  I  R  F  I  E  E  L  K  G  G  T  K  K  G  K  P  N  I  G  Q  E  Q      387
AGCCTGTGGATGATGCTGCAGAAGTCCCTCAGAGGGAACCAGAGAAGGAAAGGGGTGATCCAGAACGGCAGAGAGAGATG    1280
  P  V  D  D  A  A  E  V  P  Q  R  E  P  E  K  E  R  G  D  P  E  R  Q  R  E  M      413
GAAGAAGAGGAGGATGAGGATGAGGATGAGGATGAAGATGAGGATGAACGGCAGTTACTGGGAGAATTTGAGAAGGAACT    1360
E  E  E  E  D  E  D  E  D  E  D  E  D  E  D  E  R  Q  L  I  G  I  F  I  Y  I  :      440
GGAAGGGATCCTGCTTCCGTCAGACCGAGACCGGCTCCGTTCGGAGGTGAAGGCTGGCATGGAGCGGGAACTTGAGAACA    1440
 E  G  I  L  L  P  S  D  R  D  R  L  R  S  E*1 V  K  A  G  M  E  R  E  L  E  N  I      467
TCATCCAGGAGACAGAGAAAGAGCTGGACCCAGATGGGCTGAAGAAGGAGTCAGAGCGGGATCGGGCAATGCTGGCTCTC    1520
  I  Q  E  T  E  K  E  L  D  P  D  G  L  K  K  E  S  E  R  D  R  A  M  L  A  L      493
ACATCCACTCTCAACAAACTCATCAAAAGACTGGAGGAAAAACAGAGTCCAGAGCTGGTGAAGAAGCACAAGAAAAAGAG    1600
T  S  T  L  N  K  L  I  K  R  L  E  E  K  Q  S  P  E  L  V  K  K  H  K  K  K  R      520
GGTTGTCCCCAAAAAGCCTCCCCCATCACCCCAACCTACAGAGGAGGATCCTGAGCACAGAGTCCGGGTCCGGGTCACCA    1680
 V  V  P  K  K  P  P  P  S  P  Q  P  T*2 E  E  D  P  E  H  R  V  R  V  R  V  T  K      547
AGCTCCGTCTCGGAGGCCCTAATCAGGATCTGACTGTCCTCGAGATGAAACGGGAAAACCCACAGCTGAAACAAATCGAG    1760
  L  R  L  G  G  P  N  Q  D  L  T  V  L  E  M  K  R  E  N  P  Q  L  K  Q  I  E      573
GGGCTGGTGAAGGAGCTGCTGGAGAGGGAGGGACTCACAGCTGCAGGGAAAATTGAGATCAAAATTGTCCGCCCATGGGC    1840
G  L  V  K  E  L  L  E  R  E  G  L  T  A  A  G  K  I  E  I  K  I  V  R  P  W  A      600
TGAAGGGACTGAAGAGGGTGCACGTTGGCTGACTGATGAGGACACGAGAAACCTCAAGGAGATCTTCTTCAATATCTTGG    1920
  E  G  T  E  E  G  A  R  W  L  T  D  E  D  T  R  N  L  K  E  I  F  F  N  I  L  V      627
TGCCGGGAGCTGAAGAGGCCCAGAAGGAACGCCAGCGGCAGAAAGAGCTGGAGAGCAATTACCGCCGGGTGTGGGGCTCT    2000
  P  G  A  E  E  A  Q  K  E  R  Q  R  Q  K  E  L  E  S  N  Y  R  R  V  W  G  S      653
CCAGGTGGGGAGGGCACAGGGGACCTGGACGAATTTGACTTCTGAGACCAACACTACACTTGACCCTTCACGGAATCCAG    2080
P  G  G  E  G  T  G  D  L  D  E  F  D  F  *                                          667
ACTCTTCCTGGACTGGCTTGCCTCCTCCCCACCTCCCCACCCTGGAACCCCTGAGGGCCAAACAGCAGAGTGGAGCTGAG    2160
CTGTGGACCTCTCGGGCAACTCTGTGGGTGTGGGGGCCCTGGGTGAATGCTGCTGCCCCTGCTGGCAGCCACCTTGAGAC    2240
CTCACCGGGCCTGTGATATTTGCTCTCCTGAACTCTCACTCAATCCTCTTCCTCTCCTCTGTGGCTTTCCTGTTATTGTC    2320
CCCTAATGATAGGATATTCCCTGCTGCCTACCTGGAGATTCAGTAGGATCTTTTGAGTGGAGGTGGGTAGAGAGAGCAAG    2400
GAGGGCAGGACACTTAGCAGGCACTGAGCAAGCAGGCCCCCACCTGCCCTTAGTGATGTTTGGAGTCGTTTTACCCTCTT    2480
CTATTGAATTGCCTTGGGATTTCCTTCTCCCTTTCCCTGCCCACCCTGTCCCCTACAATTTGTGCTTCTGAGTTGAGGAG    2560
CCTTCACCTCTGTTGCTGAGGAAATGGTAGAATGCTGCCTATCACCTCCAGCACAATCCCAGCGAAAAAGGTGTGAAGCA    2640
CCCACCATGTTCTTGAACAATCAGGTTTCTAAATAAACAACTGGACCATCAAAAAAAAAA                          2700
```

Fig. 1B

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

delimiting nt 1568-2043 gave a 476 bp band from clones 9-1, 13-1, and 18 (lanes 18, 20, and 21), while clones 3, 7-2, 8-3, and 12-2 gave a faster 311 bp band (lanes 15, 16, 17, and 19). Sequencing of the faster band showed a 165 bp deletion from nt 1642 to 1806 in the OS-9 sequence (the boxed sequence 2 in Fig. 1B). This resulted in loss of a 55 amino acid sequence and converted a glutamic acid residue into a glycine residue in the splice junction. The three isoforms are referred to as OS-9-1, OS-9-2, and OS-9-3, respectively, in the order of isolation and characterization of the cDNAs. These data taken together suggested at least three isoforms of OS-9 mRNA. The origins of clones of 9-1, 13-1, and 18 were ascribed to isoform 1, which has the longest open reading frame; those of clones of 3 and 7-2 to isoform 2, which lacks the 165 bp sequence; and those of clones of 8-3 and 12-2 to isoform 3, which lacks the 45 and 165 bp sequences. The OS-9 gene is composed of 15 exons, and all exon-intron junction sequences of the gene are known (*14*). Comparison of these isoform sequences with the genomic organization of the gene revealed that the three isoforms were splice variants (Fig. 3). Isoform 2 cDNA lacked the entire exon 13 and encodes a 612 amino acid polypeptide. Isoform 3 cDNA lacked the 45 bp 3′ end of exon 11 and the entire exon 13 and encodes a 597 amino acid polypeptide. These alternative splicings conformed to the GT-AG rule.

Expression of OS-9 Gene in Various Cells—Expression of the OS-9 gene in various tumor cell lines of carcinoma cells, sarcoma cells, and myeloid leukemia cells was analyzed by Northern blotting using a total RNA (Fig. 4A). The OS-9 mRNA of approximately 3.0 kb was found in all tumor cells examined. Its content was much higher in osteosarcoma OsA-CL and rhabdomyosarcoma RH30 cells bearing a homogeneously staining region of 12q13-15. However, the OS-9 mRNA appeared to be nearly equal in amount in the other nonbearing cells, including myeloid leukemia HL-60, osteosarcoma NY, fibrosarcoma HT1080, vulva epidermoid carcinoma A431, colon adenocarcinoma WiDr, hepatoma HLE, gastric adenocarcinoma STKM-1, and cervical carcinoma HeLa. The G3PDH mRNAs were shown as a control of the loading and transfer of the RNA preparations.

Subsequently, we examined the changes in the expression of OS-9 gene during differentiation of HL-60 cells in the same way. When the cells were induced to differentiate into granulocytes by treatment with RA, the OS-9 mRNA content increased during the first two days of the treatment (Fig. 4B), remained at a similar level on the third day, and decreased to the initial level by the fifth day. The OS-9 mRNA content in untreated cells increased significantly on

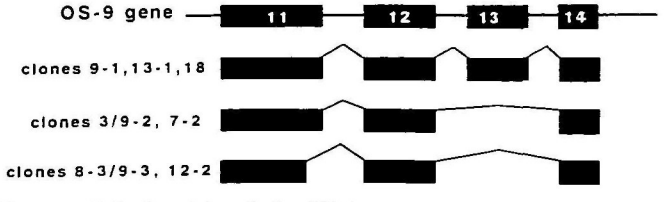

Fig. 3. **Relationship of the OS-9 gene and the three isoform mRNAs.** Black boxes of the gene show exons; solid lines show introns. Exon numbers are shown in the boxes. Three patterns of alternative splicing of mRNA are shown parallel to the gene organization. Black boxes that run parallel with the gene show portions of the mRNA; thin lines connecting the boxes show splicing of the mRNAs.

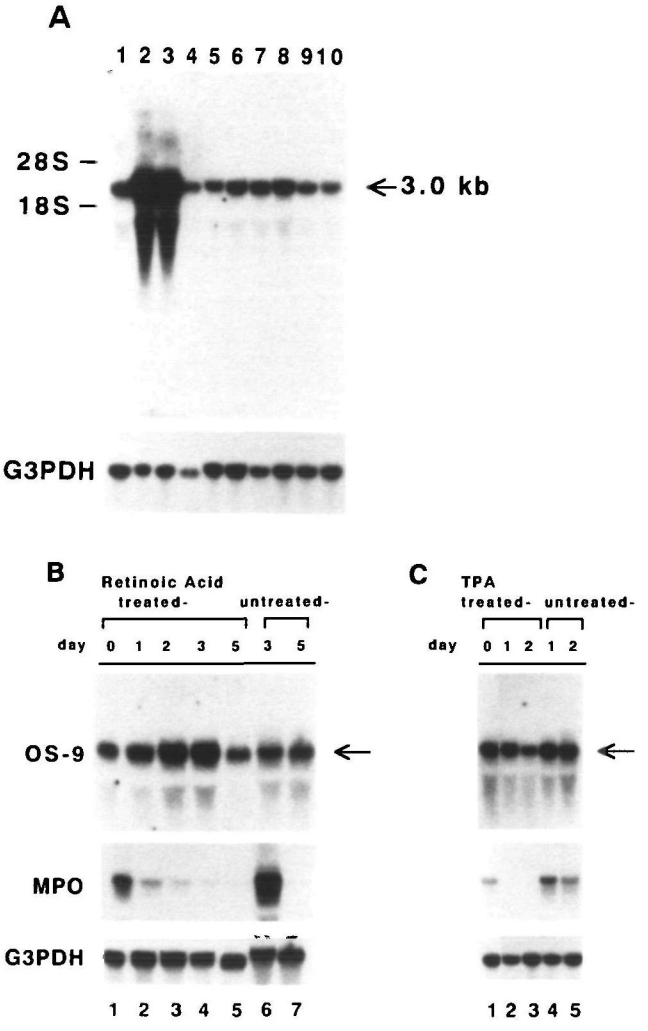

Fig. 4. **Northern blot analysis of OS-9 mRNA.** Total RNA preparations (30 μg) isolated from various tumor cell lines (A) and HL-60 cells treated with or without RA (B) and with or without TPA (C) for the indicated number of days were analyzed by Northern blot hybridization with a ^{32}P-labeled *Eco*RI 3′ fragment of OS-9 cDNA as described under "MATERIALS AND METHODS." The same blots were hybridized successively with OS-9 cDNA, G3PDH cDNA, and MPO cDNA probes. Arrows indicate the bands of OS-9 mRNA. G3PDH and MPO mRNAs were used as a control of RNA loading and a differentiation marker of HL-60 cells, respectively. A: OS-9 gene expression in various tumor cells lines. Lane 1, myeloid leukemia HL-60; lane 2, osteosarcoma OsA-CL; lane 3, rhabdomyosarcoma RH30; lane 4, osteosarcoma NY; lane 5, fibrosarcoma HT1080; lane 6, vulva epidermoid carcinoma A431; lane 7, colon adenocarcinoma WiDr; lane 8, hepatoma HLE; lane 9, gastric adenocarcinoma STKM-1; lane 10, cervical carcinoma HeLa. The positions of 18 and 28S RNA bands detected by ethidium bromide staining are shown on the left. B: Changes in the amounts of OS-9 mRNA in HL-60 cells induced to differentiate into granulocytes with RA. Lane 1, cells before treatment; lanes 2, 3, 4, and 5, cells treated with RA for 1, 2, 3, and 5 days, respectively; lanes 6 and 7, cells cultured without RA for 3 and 5 days, respectively. C: Changes in the amount of OS-9 mRNA in HL-60 cells induced to differentiate into macrophages with TPA. Lane 1, cells before treatment; lanes 2 and 3, cells treated with TPA for 1 and 2 days, respectively; lanes 4 and 5, cells cultured without TPA for 1 and 2 days, respectively.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

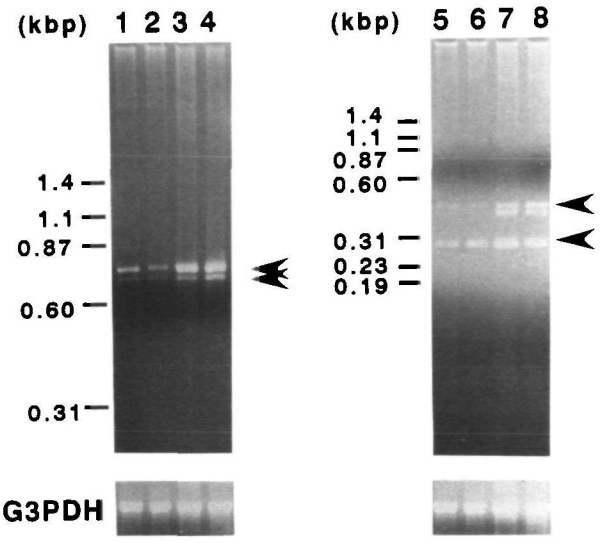

Fig. 5. **Expression of three isoforms of OS-9 mRNA.** First strand cDNA was synthesized from 2 μg of poly(A)$^+$ RNA from HL-60, RA-treated HL-60, OsA-CL, and RH30 cells using an oligo(dT) primer and amplified by PCR using primer pairs 2 (lanes 1-4) and 3 (lanes 5-8) used in Fig. 2. RT-PCR products were separated by electrophoresis in 2.5% agarose gel and stained with ethidium bromide. Lanes 1 and 5, HL-60; lanes 2 and 6, RA-treated HL-60; lanes 3 and 7, OsA-CL; lanes 4 and 8, RH30. The positions of standard size markers are shown on the left. Arrowheads indicate the bands of the RT-PCR products. The band marked G3PDH is the RT-PCR product obtained from the same 1st strand cDNA using the G3PDH primer pair.

the third day, but to a smaller extent than in the treated cells. The MPO mRNA content used as a differentiation-linked marker (*16*) decreased rapidly in the treated cells, whereas it increased markedly in the untreated cells. The G3PDH mRNA content did not change appreciably under the culture conditions. Differentiation of cells into macrophages by TPA caused a considerable decrease in the OS-9 mRNA content at the second day of the treatment (Fig. 4C). This was preceded by a rapid decrease in the MPO mRNA content as observed previously (*17*). The OS-9 mRNA contents in human erythroid leukemia K562 cells and human monocytic leukemia SKM-1 and THP-1 cells were comparable to that in HL-60 cells. The TPA treatment of SKM-1 and THP-1 cells also decreased their mRNA contents (data not shown).

Differential Synthesis of OS-9 mRNA Isoforms—To see whether or not OS-9 mRNA isoforms are generated differentially in HL-60 cells, RA-treated HL-60 cells, OsA-CL cells, and RH30 cells, we performed RT-PCR using primer pairs 2 and 3 (Fig. 2A and Table I). The products separated by agarose gel electrophoresis were eluted for subcloning and sequencing. Figure 5 lanes 1-4 show that the primer pair 2 gave a major band (732 bp) and a minor band (687 bp) directed by poly(A)$^+$ RNA preparations from all cell types, although the preparations from HL-60 cells yielded lesser amounts than those from OsA-CL and RH30 cells. The 732 bp product could be derived from either isoform 1 or 2. The 687 bp product was definitely derived from isoform 3, suggesting that it constituted minor portions of total OS-9 mRNA in these cells. The primer pair 3 gave a major band (311 bp) and two minor bands (476 and 456 bp) with all poly(A)$^+$ RNA preparations (Fig. 5 lanes 5-8). The major band could be derived either from isoform 2 or 3. The slower minor band (476 bp) undoubtedly represented isoform 1. The faster minor band (456 bp) was probably an artificial product in RT-PCR, since it was devoid of one of the primer sequences (nt 1568-1587). The G3PDH primer pair gave a 983 bp band, showing integrity of all poly(A)$^+$ RNA preparations used. These data suggested that isoform 2 comprised major portions of total OS-9 mRNA in all cell types examined.

DISCUSSION

Screening of an HL-60 cDNA library with PTP 1B cDNA led us to an unexpected finding of OS-9 cDNA clones. The nucleotide sequences as well as the deduced amino acid sequences suggest that there are at least three isoforms of OS-9 mRNA. The sequence of isoform 1 cDNA coincided with the reported OS-9 cDNA sequence (*15*), except in the 5′-terminal hexadecanucleotide sequence. The isoform 1 cDNA encodes a 667 amino acid polypeptide containing an ED-rich sequence and a potential nuclear targeting sequence TKKGK (*24*). Isoform 2 is devoid of the 165 bp sequence (nt 1642-1806) of isoform 1. This means deletion of a 55 amino acid sequence without affecting the flanking amino acid sequences. Isoform 3 has an additional deletion of the 45 bp sequence (nt 1407-1451) in the coding sequence, resulting in deletion of a 15 amino acid sequence. Isoform 2 appeared to be the major form of transcript, irrespective of cell types and states of differentiation. The ED-rich region (amino acid 398-433) in the three OS-9 isoforms showed high homology to the corresponding sequence (amino acid 138-174) in human nucleolin (*25*). Nucleolin is a multifunctional nucleolar protein in exponentially growing cells and its ED-rich region is thought to be involved in the induction of chromatin decondensation by binding to histone H1 (*26-28*). The presence of both the ED-rich region and the potential nuclear targeting signal sequence in all the isoforms suggests that OS-9 proteins may be transported into the nucleus and function like nucleolin.

Studies on the organization of the OS-9 gene have shown that the gene covers approximately 30.1 kbp and consists of 15 exons (*14*). All the splice junction sequences of the 15 exons have been determined (*14*). Examination of the sequences of the cDNAs and the genomic DNA indicated that the three isoforms of OS-9 mRNA were produced from a single transcript of the gene by alternative splicing.

Furthermore, no typical TATA box but two repeats of an ATTGG box and an Sp1 binding site are found in the 5′ upstream sequence of the OS-9 gene (*14*). These sequence features are common in the 5′ upstream sequences of a number of housekeeping genes and cell cycle-regulated genes. Indeed, expression of the OS-9 gene was seen in all kinds of tumor cell lines examined in this work and also in various normal tissues (*15*). This suggests that OS-9 proteins may play a role in cell viability. This is also consistent with the observations that the decreased expressions of OS-9 gene in myeloid leukemia HL-60 cells, SKM-1 cells, and THP1 cells treated either with RA or TPA were associated with the differentiation of these myeloid leukemia cells. Accompanying these differentiations, apoptosis of these cells is also induced under the conditions. It

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

should be noted that OS-9 mRNA isoforms were overexpressed in OsA-CL cells and Rh 30 cells. OS-9 gene is located about 46 kbp proximal to *CDK4* on the band q13 of chromosome 12 (*29*). Because of this proximity, the two genes are coamplified in similar, higher frequencies in human sarcomas (*10*). CDK4 protein alone in excess has been shown to inactivate retinoblastoma protein and may lead to tumor development (*8*). Future characterization of OS-9 proteins is necessary for understanding their roles in growth and development of myeloid cells and development and progression of tumors.

A 98 bp sequence (nt 1657-1754) of OS-9-1 cDNA was 50% identical with a sequence (nt 652-743) of the PTP1B cDNA (*30*). This nucleotide sequence similarity accounts for hybridization of the PTP 1B cDNA probe with the OS-9-1 cDNA clone. However, no amino acid sequence homology was detected between the corresponding regions of OS-9 protein and PTP1B. Thus, the biological significance of this nucleotide sequence similarity remains unclear.

We are grateful to Dr. Kiyoshi Kurahashi (Professor Emeritus, Osaka University) for stimulating discussions and encouragement and to Dr. Tatuo Senshu (Tokyo Metropolitan Institute of Gerontology) for a critical reading of the manuscript. We thank Dr. Yoshifumi Nishimura, Naoto Tsuchiya, and Daisuke Kamei (this university) for helpful discussions, Dr. Kaoru Miyazaki (Kihara Institute for Biological Research) for kindly providing tumor cell lines, Dr. Peter J. Houghton (St. Jude Children's Hospital) for a gift of RH30 cells, and Dr. Edmond E. Fischer (University of Washington) for a gift of TC-PTPcDNA.

REFERENCES

1. Elkahloun, A.G., Bittner, M., Hoskins, K., Gemmill, R., and Meltzer, P.S. (1996) Molecular cytogenetic characterization and physical mapping of 12q13-15 amplification in human cancer. *Genes, Chromosome & Cancer* **17**, 205-214
2. Berner, J.-M., Forus, A., Elkahloun, A.G., Meltzer, P.S., Fodstad, O., and Myklebost, O. (1996) Separate amplification regions encompassing CDK 4 and MDM2 in human sarcomas. *Genes, Chromosome & Cancer* **17**, 254-259
3. Reifenberger, G., Ichimura, K., Reifenberger, J., Elkahloun, A.G., Meltzer, P.S., and Collins, V.P. (1996) Refined mapping of 12q13-15 amplicons in human malignant glioma suggested CDK4/SAS and MDM2 as independent amplification targets. *Cancer Res.* **56**, 5141-5145
4. Oliner, J.D., Kinzler, K.W., Meltzer, P.S., George, D.L., and Vogelstein, B. (1992) Amplification of a gene encoding a p53-associated protein human sarcomas. *Nature* **358**, 80-83
5. Roberts, W.M., Douglass, E.C., Peiper, S.C., Houghton, P.J., and Look, A.T. (1989) Amplification of the gli gene in childhood sarcomas. *Cancer Res.* **49**, 5407-5413
6. Crozat, A., Aman, P., Mandahl, N., and Ron, D. (1993) Fusion of CHOP to a novel RNA-binding protein in human myxoid liposarcoma. *Nature* **363**, 640-644
7. Jankowski S.A., Mitchel D.S., Smith S.H., Trent, J.M., and Meltzer, P.S. (1994) SAS, a gene amplified in human sarcomas, encodes a new member of the transmembrane 4 superfamily of protein. *Oncogene* **9**, 1205-1211
8. Khatib, Z.A., Matsusime, H., Valentine, M., Shapiro, D.N., Sherr, C.J., and Look, A.T. (1993) Coamplification of the CDK4 gene with MDM2 and GLI in human sarcomas. *Cancer Res.* **53**, 5535-5541
9. Su, Y.A., Trent, J.M., Guan, X.-Y., and Meltzer, P.S. (1994) Direct isolation of genes encoded within a homogeneously staining region by chromosome microdissection. *Proc. Natl. Acad. Sci. USA* **91**, 9121-9125
10. Elkahloun, A.G., Meltzer, P.S., Guan, X.-Y., McNinch, J.S., Trent, J.M., and Jong, P.J. (1996) Isolation of a cosmid sublibrary for a region of chromosome 12 frequently amplified in human cancers using a complex chromosome microdissection probe. *Genomics* **31**, 343-347
11. Frank, D.A. and Sartorelli, A.C. (1986) Regulation of protein phosphotyrosine content by changes in tyrosine kinase and protein phosphotyrosine phosphatase activities during induced granulocytic and monocytic differentiation of HL-60 leukemia cells. *Biochem. Biophys. Res. Commun.* **140**, 440-447
12. Frank, D.A. and Sartorelli, A.C. (1988) Biochemical characterization of tyrosine kinase and phosphotyrosine phosphatase activities of HL-60 leukemia cells. *Cancer Res.* **48**, 4299-4306
13. Zhao, Z., Shen, S.-H., and Fischer, E.H. (1994) Phorbol ester-induced expression, phosphorylation, and translocation of protein tyrosine phosphatase 1C in HL-60 cells. *Proc. Natl. Acad. Sci. USA* **91**, 5007-5011
14. Kimura, Y., Nakazawa, M., Tsuchiya, N., Asakawa, S., Shimizu, N., and Yamada, M. (1997) Genomic organization of the OS-9 gene amplified in human sarcomas. *J. Biochem.* **122**, 1190-1195
15. Su, Y.A., Hutter, C.M., Trent, J.M., and Meltzer, P.S. (1996) Complete sequence analysis of a gene (OS-9) ubiquitously expressed in human tissues and amplified in sarcoma. *Mol. Carcinogen.* **15**, 270-275
16. Yamada, M. and Kurahashi, K. (1984) Regulation of myeloperoxidase gene expression during differentiation of human myeloid leukemia HL-60 cells. *J. Biol. Chem.* **259**, 3021-3025
17. Kasugai, I. and Yamada, M. (1986) Regulation of myeloperoxidase gene expression by the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate in human leukemia HL-60 cells. *J. Biochem.* **100**, 381-388
18. Hashinaka, K., Nishio, C., Hur, S.-J., Sakiyama, F., Tsunasawa, S., and Yamada, M. (1988) Multiple species of myeloperoxidase messenger RNAs produced by alternative splicing and differential polyadenylation. *Biochemistry* **27**, 5906-5914
19. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
20. Mcgadey, J.A. (1970) A tetrazolium method for non-specific alkaline phosphatase. *Histochemie* **23**, 180-184
21. Frohman, M.A., Dush, M.K., and Martin, G.R. (1988) Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. USA* **85**, 8998-9002
22. Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159
23. Feinberg, A.P. and Vogelstein, B. (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**, 6-13
24. Dingwall, C. and Laskey, R.A. (1986) Protein import into the cell nucleus. *Annu. Rev. Cell. Biol.* **2**, 367-390
25. Srivastava, M., Fleming, P.J., Pollard, H.B., and Burns, A.L. (1989) Cloning and sequencing of the human nucleolin cDNA. *FEBS Lett.* **250**, 99-105
26. Lapeyre, B., Bourbon, H., and Amalric, F. (1987) Nucleolin, the major nucleolar protein of growing eukaryotic cells: An usual protein structure revealed by the nucleotide sequence. *Proc. Natl. Acad. Sci. USA* **84**, 1472-1476
27. Erard, M.S., Belenguer, P., Caizergues-Ferrer, M., Pantaloni, A., and Amalric, F. (1988) A major nucleolar protein, nucleolin, induces chromatin decondensation by binding to histone H1. *Eur. J. Biochem.* **175**, 525-530
28. Tuteja, N., Huang, N.W., Skopac, D., Tuteja, R., Hrvatic, S., Zhang, J., Pongor, S., Joseph, G., Faucher, C., Amalric, F., and Falaschi, A. (1995) Human DNA helicase IV is nucleolin, an RNA helicase modulated by phosphorylation. *Gene* **160**, 143-148
29. Elkahloun, A.G., Krizman, D.B., Wang, Z., Hofmann, T A., Roe, R., and Meltzer, P.S. (1997) Transcript mapping in a 46-kb sequenced region at the core of 13q13.3 amplification in human cancers. *Genomics* **42**, 295-301
30. Brown-Shimer, S.B., Johnson, K.A., Lawrence, J.B., Johnson, C., Bruskin, A., Green, N.P., and Hill, D.E. (1990) Molecular cloning and chromosome mapping of the human gene encoding protein phosphotyrosylphosphatase 1B. *Proc. Natl. Acad. Sci. USA* **87**, 5148-5152

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012