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ASB-2 Inhibits Growth and Promotes Commitment in Myeloid Leukemia Cells*

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In acute promyelocytic leukemia (APL) cells harboring the promyelocytic leukemia retinoic acid receptor α (PML-RAR α) chimeric protein, retinoic acid (RA)-induced differentiation is triggered through a PML-RAR α signaling resulting in activation of critical target genes. Induced differentiation of APL cells is always preceded by withdrawal from the cell cycle and commitment events leading to terminal differentiation. Here we have identified the human ankyrin repeat-containing protein with a suppressor of cytokine signaling box-2 (ASB-2) cDNA, as a novel RA-induced gene in APL cells. PML-RAR α strongly enhanced RA-induced ASB-2 mRNA expression. In myeloid leukemia cells, ASB-2 expression induced growth inhibition and chromatin condensation recapitulating early events critical to RA-induced differentiation of APL cells.

Hematopoiesis is organized as a hierarchy of events articulated by both a genetic commitment and cytokines. When exposed to specific cytokines, hematopoietic progenitor cells divide to generate an amplified progeny and give rise to the various lineages (1). Several hematopoietic cytokines have been shown to be positive regulators of hematopoiesis. However, negative feedback is likely to be important in regulating signal transduction elicited by a large variety of extracellular signals. To this extent, suppressors of cytokine signaling (SOCS)¹ proteins represent a family of feedback modulators of cytokine signal transduction that may play an important role in the regulation of normal hematopoiesis (2, 3). As in normal hema-

topoiesis, a hierarchy of events exists in leukemia hematopoiesis and it is assumed that leukemic cells descend from a very small pool of hematopoietic progenitors which are arrested at an immature stage of differentiation (4). It is expected that cytokine-responsive genes, which are critical to the maturation in normal hematopoietic progenitors, are repressed in those cells undergoing leukemic transformation, because of transcription factors and co-factors involved in chromosomal abnormalities (5, 6). Conversely, such genes should be induced in leukemia cells treated with inducers, such as retinoic acid (RA), which force *de novo* differentiation in leukemia cells. This should be the case in acute promyelocytic leukemia (APL) cells, which, as a result of a specific t(15;17) translocation, express the oncogenic PML-RAR α chimeric protein and can undergo complete remission in patients treated with all-*trans*-retinoic acid (ATRA) which induces differentiation of the leukemic cells *in vivo* (7, 8). Retinoic acid receptors (RARs) are RA-dependent transcription factors (9) involved in the control of hematopoietic cells differentiation (10, 11). PML-RAR α oncogenic effect is thought to involve transcriptional repression of RA target genes. In the presence of RA, PML-RAR α activity is switched from repression to activation, acting as a RA-dependent enhancer factor (12). Several observations suggest that growth arrest and differentiation of APL cells by ATRA is triggered directly through a PML-RAR α -dependent signaling pathway (12). In an early response to ATRA, PML-RAR α -expressing cells undergo two rounds of cell division before entering post-mitotic G₁ phase of the cell cycle. Although post-mitotic G₁ is a late event not crucially linked to the differentiation process, the G₁ to S phase transition of the first cell cycle is required for the differentiation process to occur (13). Indeed, limitation in cell proliferative activity together with chromatin condensation represent a hallmark in commitment, an irreversible step committing hematopoietic cells to terminal differentiation (14). Furthermore, it is well established that this process is enacted in RA-induced differentiation of myeloid leukemia cells (15) and especially in APL, where ATRA induces growth arrest as well as chromatin condensation, which is characteristic of the emergence of polymorphonuclear leukocytes (16).

To identify RA target genes, which are critical to the process of growth arrest and differentiation, we have used differential screening with the NB4 acute promyelocytic leukemia cell line, which harbors the t(15;17) translocation encoding PML-RAR α . These cells undergo granulocytic differentiation when treated with ATRA (17). We have identified and characterized the human ankyrin-repeat-containing protein with a SOCS box-2 (ASB-2) cDNA as induced by ATRA in NB4 cells. The ASB-2 mRNA was highly expressed in bone marrow cells. In ATRA-treated NB4 cells, increased ASB-2

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ251238.

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¹ The abbreviations used are: SOCS, suppressor of cytokine signaling; ATRA, all-*trans*-retinoic acid; ASB, ankyrin repeat-containing protein with a suppressor of cytokine signaling box; APL, acute promyelocytic leukemia; PML-RAR, promyelocytic leukemia retinoic acid receptor; RA, retinoic acid; RAR, retinoic acid receptor; contig, group of overlapping clones; NBT, nitro blue tetrazolium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PI, propidium iodide.

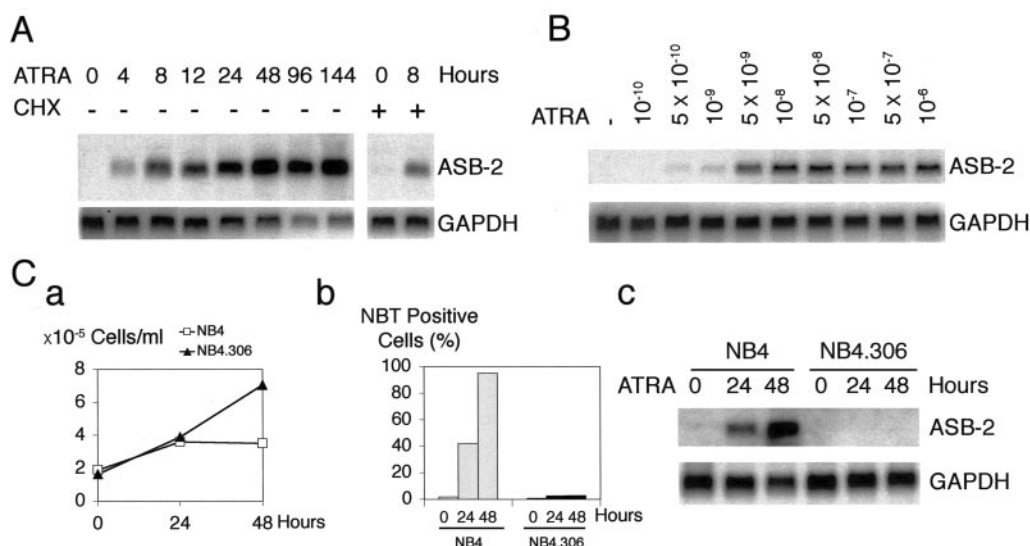


FIG. 1. Induction of ASB-2 mRNA in RA-treated cells. *A*, time response to ATRA in NB4 cells. Cells were treated for different times with 5×10^{-7} M ATRA. To inhibit protein synthesis, NB4 cells were pretreated for 30 min with $10 \mu\text{g/ml}$ cycloheximide (CHX) and then incubated with or without ATRA for 8 h. *B*, dose response to ATRA in NB4 cells. Cells were exposed to 0 – 10^{-6} M of ATRA for 24 h. *C*, NB4 and NB4.306 cells were cultured with 10^{-6} M ATRA and harvested after 0, 24, and 48 h. Viable cells (*a*) and percentage of NBT-positive cells (*b*) were counted each day, and ASB-2 mRNA expression was analyzed (*c*). Northern blot analysis were performed using $8 \mu\text{g}$ (*A*, *B*) or $3 \mu\text{g}$ (*C*, *c*) of total RNA. In *A*, *B*, and *C* (*c*), upper and lower panels are autoradiograms of ASB-2 transcript and hybridization with GAPDH probe as assessment of RNA quantities in each lane.

mRNA expression correlated with the onset of myeloid differentiation. We demonstrate that, in ATRA-treated U937 myeloid cells, an increase in ASB-2 mRNA expression was mediated through an exogenously expressed PML-RAR α . Expression of an inducible exogenous ASB-2 in PLB-985 myeloid leukemia cells resulted in growth inhibition and chromatin condensation, which recapitulate early steps in induced differentiation of APL cells.

EXPERIMENTAL PROCEDURES

Leukemia Cells, Culture Conditions, Induction, and Assessment of Differentiation—Myeloblastic PLB-985 (18), promyelocytic NB4 (17), and RA-resistant subclones of NB4 cells (19) were kindly provided by W. H. Miller, M. Lanotte, and C. Nervi, respectively. U937-PR9 and U937-MT cells were generously provided by P. G. Pelicci (20). Cells were grown and used as described (21, 22). PLB-985/MT-ASB-2 and PLB-985/MT cell lines were used for induction of ASB-2 expression or as control respectively. For this, cells were cultured either with or without ZnSO $_4$. U937-MT and U937-PR9 cells were generated by stable transfection of the zinc-inducible MTPR plasmid containing no cDNA and the PML-RAR α cDNA, respectively (20). These cells were preincubated with $100 \mu\text{M}$ ZnSO $_4$ for 12 h and then treated with ATRA. To inhibit *de novo* protein synthesis, NB4 and U937-PR9 cells were pretreated for 30 min with $10 \mu\text{g/ml}$ cycloheximide (Sigma) and then incubated with or without ATRA. Cell viability was estimated using standard trypan blue dye exclusion assay. Differentiation was assessed by: (i) the percentage of cells with nitro blue tetrazolium (NBT) (Sigma) and (ii) cell morphology under light microscopy on May-Grünwald-Giemsa-stained cytospin slides. PML-RAR α -expressing primary APL cells obtained from patients at Saint-Louis Hospital (Paris, France) were kindly provided by C. Chomienne. Cells were purified and cultivated with 10^{-7} M ATRA as described (23).

Sequencing and Computer Analysis—Plasmid DNA were purified through Nucleobond columns (Macherey-Nagel), and double-stranded DNA templates were sequenced using pBluescript and internal primers. Sequence analyses and alignments were carried out with the GCG programs (Wisconsin Package, version 10.2-UNIX, Genetic Computer Group, Inc.). Sequence homologies were identified using the FastA and BLAST programs by searching GenBank[®] (release 124.0) and EMBL (release 67.0) data banks and in the case of translated sequences by searching SWISS-PROT (release 39.0), PIR/NBRF (release 68.0), and SPTREMBL (release 17.0) data banks.

ASB-2 cDNA Cloning—Differential screening was applied to identify RA-induced genes in NB4 cells. Subtracted probes were prepared as described (24). Briefly, untreated and RA-treated (24 h) NB4 single-

stranded cDNA probes were obtained through hybridization to an excess of nonhematopoietic HT-29 and NB4 poly(A)⁺ RNA, respectively. Remaining single-stranded cDNAs were then separated by hydroxyapatite chromatography and used as specific probes. Two cDNA fragments (813 and 870) isolated from the RA-treated NB4 cDNA library contained 1007 bp (position 45–1051) and 1351 bp corresponding to the 3' end of ASB-2 cDNA, respectively. A remaining 5' cDNA sequence (44 bp) was amplified by rapid amplification of cDNA ends PCR from RA-treated NB4 RNA (with 5×10^{-7} M for 48 h) using the Marathon cDNA amplification kit (CLONTECH) with the primer: 5'-GCTTCAG-GCAGGAACGAGGAT-3'. Using the same kit, a contiguous cDNA was generated by reverse transcription-PCR amplification of ASB-2 from RA-treated NB4 cells.

In Vivo Expression—The ASB-2 coding sequence was subcloned into the pMTCB6⁺-derived expression vector (25) under the control of the zinc-inducible sheep metallothionein promoter resulting in the pMT-ASB-2 vector. Exponentially growing PLB-985 cells ($5 \times 10^6/0.5$ ml of Opti-MEM (Invitrogen)) were electroporated (Gene Pulser, Bio-Rad) at 300 V, 960 microfarads in the presence of $20 \mu\text{g}$ of the pMT-ASB-2 vector or the empty vector. Transfected PLB-985 cells were cultured for 48 h, selected with $0.4 \mu\text{g/ml}$ G418 (Invitrogen), and cultured under limiting dilution conditions.

Northern Blot Analysis—Total RNA extraction and hybridization were as described (21). Human RNA master blot and human immune system multiple tissue Northern blot II were obtained from CLONTECH. The ASB-2 probe corresponded to the 3' end of the 1351-bp ASB-2 cDNA. Radioactivity was quantified using a Storm 860 Phosphor-Imager (Amersham Biosciences, Inc.).

Antibodies and Western Blot Analysis—An NH $_2$ -terminal peptide (SAPSRSTAPPESSPA) was synthesized according to the ASB-2 deduced amino acid sequence and coupled to keyhole limpet hemocyanin through a cysteine residue added to the carboxyl-terminal amino acid of the peptide (Eurogentec). The rabbit serum (2PNA) was collected following the initial injection and three booster immunizations (Eurogentec). Immunoblot analyses were carried as described (22) except that the antiserum was diluted 1:2000.

Staining of Cellular DNA, Cell Cycle Analysis—Cells were cytocentrifuged onto microscope slides, fixed in 1% formaldehyde in ice-cold phosphate-buffered saline, and kept overnight at 4 °C in 70% ethanol. Cells were then stained with $5 \mu\text{g/ml}$ propidium iodide (PI; Molecular Probes) in phosphate-buffered saline containing $100 \mu\text{g/ml}$ DNase-free RNase A (Sigma) for 60 min and mounted under a coverslip in a solution of 9 parts of glycerol and 1 part of the PI staining solution.

Detection of Apoptotic Cells—Both *in situ* detection of DNA strand breaks by terminal dUTP nick-end labeling assay using the APO-BRDU kit (Phoenix Flow Systems) (26), and immunocytochemical detection of

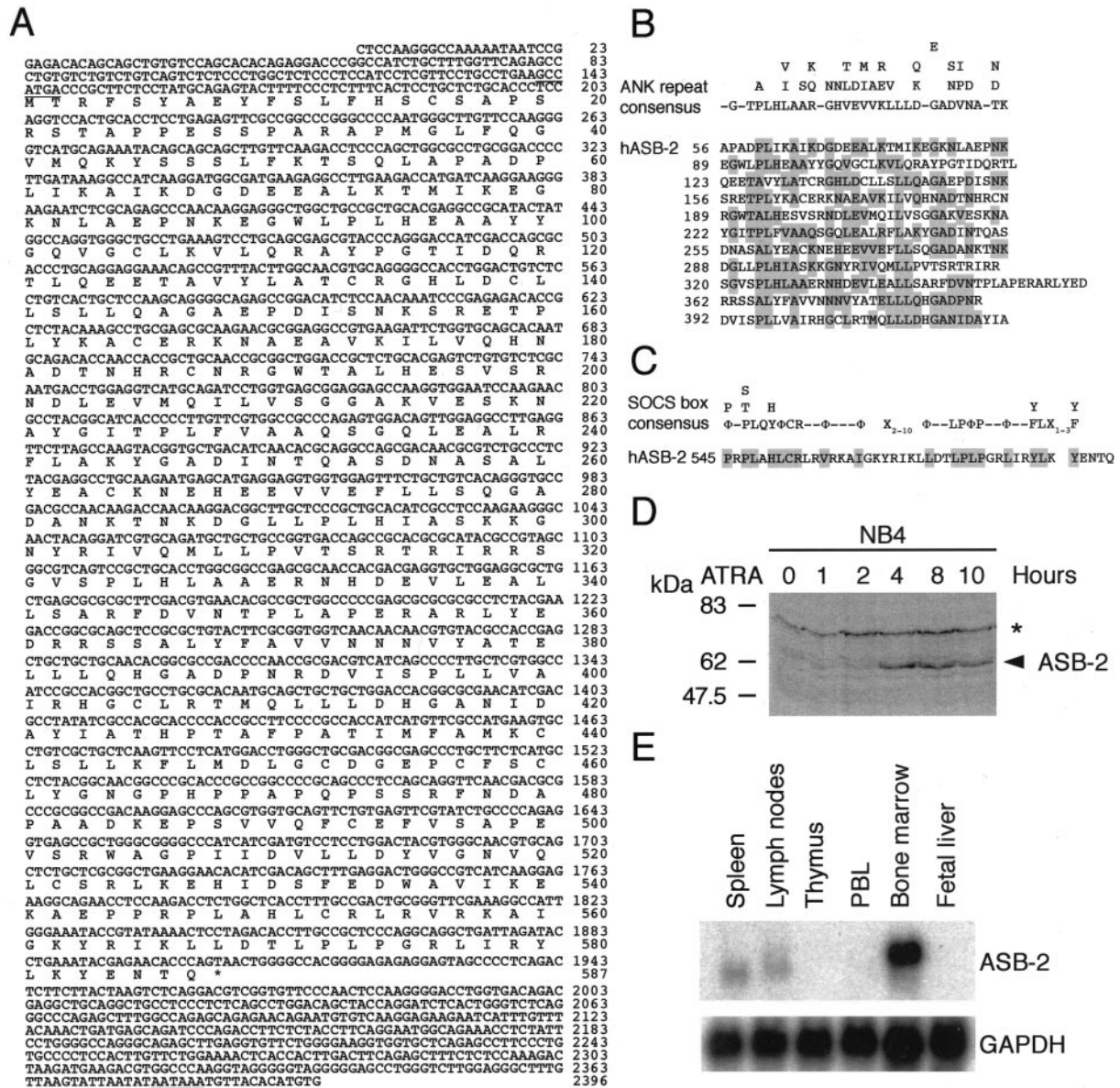


FIG. 2. ASB-2 cDNA, protein, and expression in hematopoietic tissues. A, cDNA and deduced amino acid sequences of human ASB-2. Nucleotide residues are numbered in the 5' to 3' orientation, and amino acids in the reading frame are designated by the one-letter code. Kozak and poly(A) addition signal sequences are underlined. Asterisk indicates the stop codon. These sequence data are available from EMBL/GenBank/DBJ under accession no. AJ251238. B, alignments of the 11 ankyrin repeats of ASB-2. C, alignments of the SOCS box with the consensus sequence. Shaded regions represent residues identical to the consensus sequences for ankyrin repeats (33) and SOCS box (31). Φ represents hydrophobic residues. D, ASB-2 protein is induced upon ATRA treatment in NB4 cells. NB4 cells were treated for different times using 10^{-6} M ATRA. Protein extracts from 10^5 cells were separated by SDS-PAGE and immunoblotted for ASB-2. Asterisk indicates a nonspecific band. E, expression of ASB-2 mRNA in hematopoietic tissues. PBL, peripheral blood lymphocytes. Top and bottom panels are autoradiograms of ASB-2 transcript and hybridization with GAPDH probe as assessment of RNA quantities in each lane, respectively.

poly(ADP-ribose) polymerase cleavage product (27) were used for identification of apoptotic cells. Apoptotic cells were labeled with green fluorescing fluorescein isothiocyanate, and cellular DNA was counterstained with fluorescein red PI (28).

Fluorescence Measurement—Cellular red and green fluorescence intensities were measured by laser scanning cytometer (CompuCyte) as described (29, 30). DNA content frequency histograms were deconvoluted to obtain the percentage of cells in different phases of the cell cycle. The percentage of cells with condensed chromatin, *i.e.* with increased intensity of the DNA-associated maximal pixel PI fluorescence and decreased nuclear area, was obtained by gating analysis of the bivariate red integrated fluorescence versus maximal red fluorescence pixel distributions (29). The frequency of apoptotic cells was estimated from the bivariate distributions of PI versus fluorescein isothiocyanate integrated fluorescence intensities (28). At least 3000 cells were measured in each sample. The experiment was repeated three times.

RESULTS

ASB-2 mRNA Is Up-regulated during RA-induced Granulocytic Differentiation of NB4 Cells—Using subtractive hybridization, we have identified two novel cDNA clones (813 and 870) showing homology with the murine ASB-2 cDNA originally deduced from the analysis of contigs derived from expressed sequence tags (31). Increased ASB-2 mRNA expression occurred 4 h after exposure of NB4 cells to 5×10^{-7} M ATRA with a maximum expression at 96 h (Fig. 1A). Sequential treatment of NB4 cells with cycloheximide and ATRA indicated that ATRA-induced ASB-2 mRNA up-regulation occurred even in the absence of *de novo* protein synthesis (Fig. 1A). Dose-response studies with ATRA-treated NB4 cells showed that 24 h of treatment with 10^{-8} M ATRA induced peak levels of the

ASB-2 mRNA (Fig. 1B), which correlated with differentiation as assessed by the NBT assay (data not shown). As expected, ATRA did not affect growth and differentiation (Fig. 1C, a and b, respectively) of NB4.306 cells that have lost the capacity to encode an intact ATRA-binding PML-RAR α (19). In these cells, no induction of ASB-2 mRNA was observed (Fig. 1C, c). In contrast, ASB-2 mRNA was strongly up-regulated in NB4 cells induced to growth arrest and differentiation by ATRA (Fig. 1C). These data indicate that an increased ASB-2 mRNA expression correlated with the capacity of ATRA-treated leukemia cells to undergo growth arrest and granulocytic differentiation.

ASB-2 Is a Member of the SOCS Family Highly Expressed in Human Bone Marrow—We have cloned a 2396-bp cDNA (GenBankTM accession no. AJ251238) (Fig. 2A) showing: (i) complete homology to a 1635-bp unpublished human sequence (GenBankTM accession no. AF159164) representing a partial human ASB-2 cDNA (positions 267–1907 in Fig. 2A) and (ii) 87% homology to a 1758-bp murine sequence (32) (GenBankTM accession no. AF155353) representing the complete coding sequence of the mouse ASB-2 cDNA (positions 144–1904 in Fig. 2A). Alignments of sequences from the 2396-bp ASB-2 cDNA, the partial human ASB-2, and the human chromosome 14 (GenBankTM accession no. AL079302) revealed some differences. Sequences of the 2396-bp ASB-2 cDNA and chromosome 14 were identical except an insertion of a G at position 1234, which was also found in partial ASB-2. This together with five 1-bp substitutions and two 3-bp insertions found in the 2396-bp ASB-2 cDNA when compared with the partial sequence indicates that the complete ASB-2 cDNA sequence reported here corresponds to the human ASB-2 mRNA. The complete open reading frame of ASB-2 consisted of 1764 bp translating into a 587-amino acid protein sharing 91% identity and 94% similarity with its mouse counterpart (data not shown). This protein harbors 11 ankyrin repeats and a SOCS box as defined by Michaely *et al.* (33) and Starr *et al.* (34), respectively. Ankyrin repeats contained 33 amino acids except repeats 2, 8, 9, and 10, which contained 34, 32, 42, and 30 amino acids, respectively (Fig. 2B). The ~50-amino acid SOCS box motif is composed of two well conserved blocks of sequence, which are separated by 2–10 nonconserved residues (Fig. 2C). The carboxyl-terminal conserved region is an leucine/proline-rich sequence of unknown function, and the amino-terminal conserved region is a 15-amino acid BC box known to interact with the elongin BC complex (35). In ATRA-treated NB4 cells, anti-ASB-2 polyclonal antibodies detected a specific 64-kDa protein after 4 h of treatment (Fig. 2D).

In the human immune system, ASB-2 mRNA was mainly expressed in bone marrow and to a lower extent in spleen and lymph nodes (Fig. 2E). Using the Human RNA Master BlotTM, ASB-2 mRNA expression was also detected in skeletal muscle, heart, fetal heart, small intestine, appendix, bladder, aorta, stomach, uterus, prostate, and colon (data not shown). Our results indicated that ASB-2 is a member of the SOCS family relevant to normal hematopoiesis.

Expression of an Exogenous PML-RAR α in U937 Leukemia Cells Enhances ATRA-induced ASB-2 Expression—To explore the role of PML-RAR α in the induction of ASB-2 mRNA by ATRA, we used U937-MT and U937-PR9 cells. ATRA-treatment of U937-PR9 cells expressing PML-RAR α resulted in a 7-fold increased expression of the ASB-2 mRNA (Fig. 3A, a), which occurred even in the absence of *de novo* protein synthesis (Fig. 3A, b). A 2-fold-increase was observed in U937-MT cells following ATRA treatment (Fig. 3A, a), as expected because U937 cells express an active RAR (36). ASB-2 mRNA was induced *in vitro* by ATRA in PML-RAR α -expressing leukemia promyelocytes freshly obtained from peripheral blood of newly

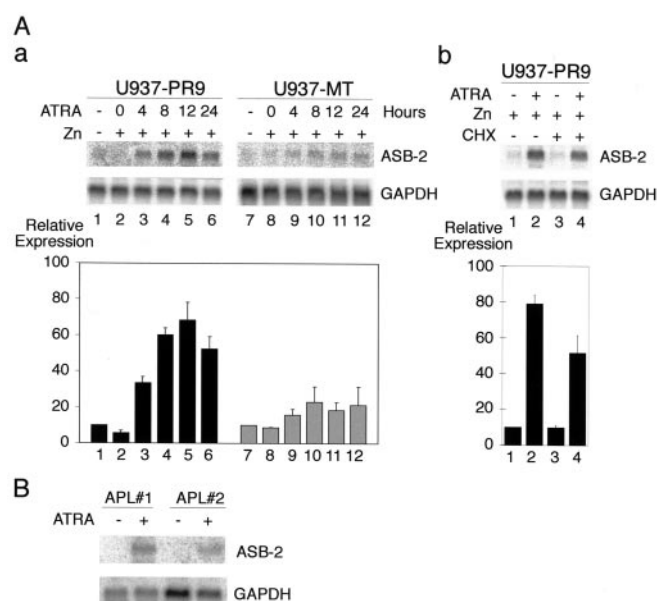


FIG. 3. Marked increase in ASB-2 mRNA by expression of an exogenous PML-RAR α in ATRA-treated U937 and primary APL cells. *A, a*, autoradiogram (*upper panel*) and relative expression (*lower panel*) of ASB-2 mRNA in U937-PR9 and U937-MT cells untreated (–) or treated (+) with 100 μ M ZnSO₄ (Zn) for 12 h and then treated with 5 \times 10^{–7} M ATRA for 0, 4, 8, 12, and 24 h. *b*, *de novo* protein synthesis is not required for ASB-2 mRNA expression. Autoradiogram (*upper*) and relative expression (*lower*) of U937-PR9 cells pre-treated for 12 h with 100 μ M ZnSO₄ (Zn), and then untreated or treated with either 5 \times 10^{–7} M ATRA, both cycloheximide (CHX) and 5 \times 10^{–7} M ATRA, or cycloheximide alone for 8 h. In *a* and *b* (*lower panels*), signals were quantified using densitometric analysis of PhosphorImager data. ASB-2 mRNA quantities were adjusted on the basis of GAPDH expression. Mean values and standard errors from two independent experiments are shown. *B*, ASB-2 mRNA is induced in ATRA-treated primary APL cells. Cells were purified from 2 untreated patients and cultivated in the absence (–) or presence (+) of 10^{–7} M ATRA for 5 (APL#2) or 6 (APL#1) days. Northern blots were performed using 8 μ g (*A*) or 2 μ g (*B*) of total RNA. Hybridization with a GAPDH probe controlled for RNA quantities in each lane.

diagnosed patients with APL (Fig. 3B). This induction correlated with ATRA-induced differentiation of these cells as assessed morphologically and by NBT assay (data not shown). Altogether, these results suggest that up-regulation of ASB-2 by ATRA is mediated by PML-RAR α .

Expression of an Exogenous ASB-2 Induces Growth Arrest and Chromatin Condensation in Myeloid Leukemia Cells—To investigate whether induction of ASB-2 expression was involved in myeloid leukemia cell growth, an ASB-2 expression vector (MT-ASB-2) under the control of a zinc-inducible metallothionein promoter was stably transfected into PLB-985 cells. The MT vector was used as an empty control. When ZnSO₄ was added to the media, ASB-2 mRNA (Fig. 4A, a) and protein (Fig. 4A, b) expression increased dramatically in PLB-985/MT-ASB-2 cells. In the presence of ZnSO₄, a ~40% growth inhibition was observed in PLB-985/MT-ASB-2 cells whereas no significant change was detected in PLB-985/MT cells (Fig. 4B, a and b).

Laser scanning cytometer was used for measurements of cellular fluorescence intensity as assessment of DNA contents. Two representative samples of PLB-985/MT-ASB-2 cells, either untreated or treated with ZnSO₄ for 60 h, are shown in Fig. 4B (c and d, respectively). Based on the intensity of the DNA-associated red integrated fluorescence PI, we identified cells in G₁ versus S and G₂/M as shown on DNA content frequency histograms. Mitotic cells with condensed chromatin were distinguished from G₂ cells by an increased intensity of maximal

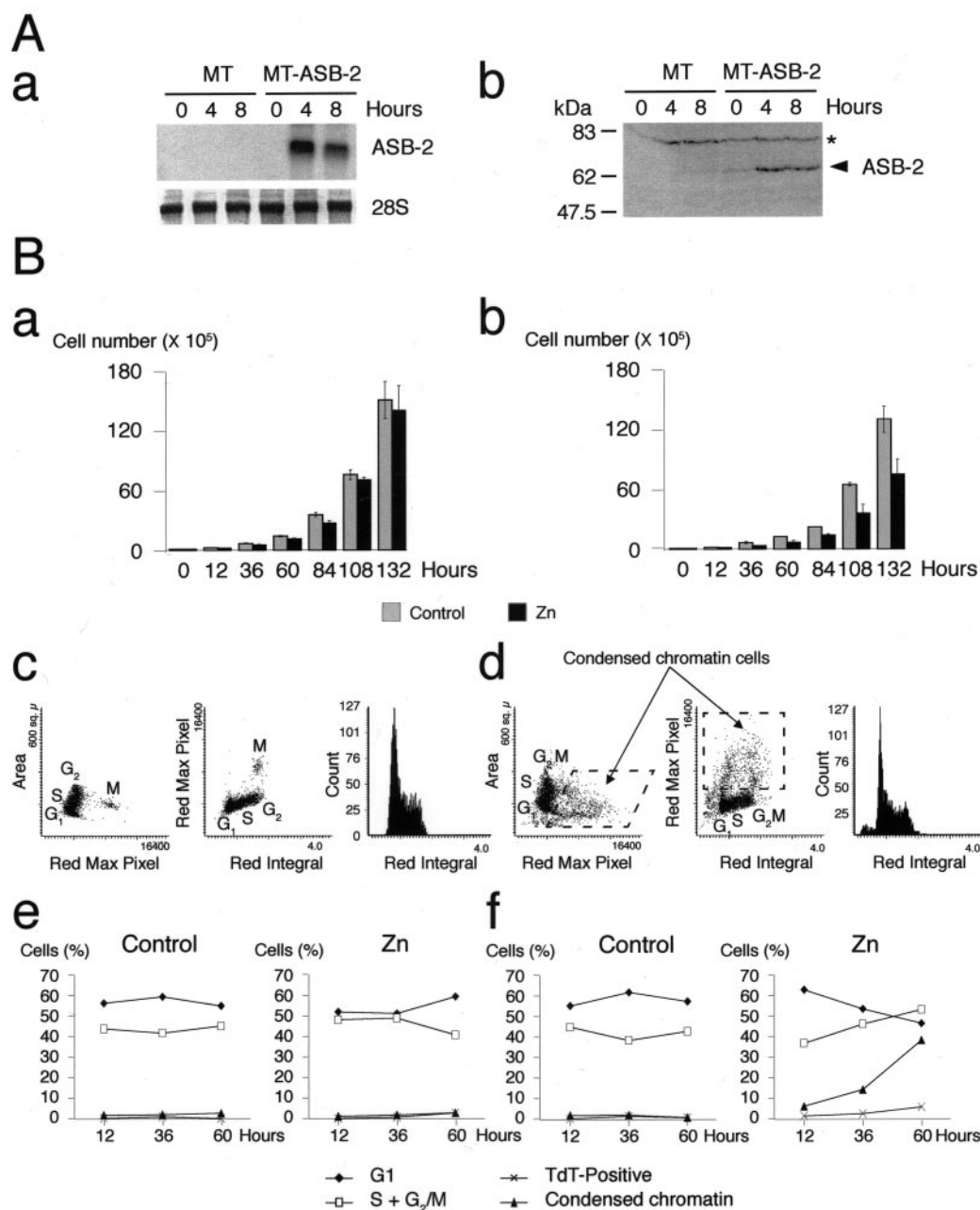


FIG. 4. Growth inhibition and chromatin condensation in myeloid cells induced to express an exogenous ASB-2 protein. A, ASB-2 mRNA (a) and protein (b) were induced in PLB-985/MT-ASB-2 cells treated with 100 μ M ZnSO₄ for the indicated times. a, autoradiogram of ASB-2 mRNA expression (upper panel) and methylene blue-stained 28 S rRNA after transfer on membrane as assessment of RNA quantities in each lane (lower panel). Northern blot analysis was performed using 5 μ g of total RNA. b, protein extracts (50 μ g) were separated by SDS-PAGE and immunoblotted for ASB-2. Asterisk indicates a nonspecific band. B, ASB-2 expression induced growth inhibition and chromatin condensation. PLB-985/MT (a) and PLB-985/MT-ASB-2 (b) cells that were either untreated (control) or treated with 50 μ M ZnSO₄ (Zn) for the indicated times were maintained at 5×10^5 cells/ml. Viable cells were counted each day. In a and b, mean values and standard errors from two independent experiments are shown. Representative experiments showing distribution of cells through the cell cycle and chromatin condensation were analyzed in PLB-985/MT-ASB-2 cells, prior to the treatment (c) and after 60 h of ZnSO₄ treatment (d). Distinction of these subpopulations were made based on the differences in intensity of the integrated PI fluorescence (DNA content), intensity of maximal pixel of red fluorescence, and fluorescence area (number of pixels within the integrated area). The latter two parameters reflect the status of nuclear chromatin condensation (29). Changes in percentage of cells in G₁, S+G₂/M, terminal deoxynucleotidyltransferase (TdT)-positive, or with condensed chromatin were analyzed in PLB-985/MT (e) and PLB-985/MT-ASB-2 (f) cell populations following 12, 36, and 60 h of ZnSO₄ treatment.

pixel of red fluorescence (*Red Max Pixel*) and by a decreased fluorescence area (29, 30). A large number of PLB-985/MT-ASB-2 cells treated with ZnSO₄ exhibited an increased intensity of maximal pixel of red fluorescence. After their relocation by laser scanning cytometer and examination by microscopy, they appeared to be interphase cells with smaller nuclei and condensed chromatin (Fig. 4B, d). The pattern of chromatin condensation in these cells did not resemble apoptosis-associ-

ated changes but rather was consistent with changes occurring in ATRA-induced HL-60 cells (29). We then analyzed the effect of ZnSO₄ on the DNA contents of PLB-985/MT-ASB-2 versus PLB-985/MT cells as well as the frequency of interphase cells with condensed chromatin. No change in proportions of untreated cells at different phases of the cell cycle or with condensed chromatin were detected (Fig. 4B, e and f). However, in the presence of ZnSO₄, few changes were observed with PLB-

985/MT cells (Fig. 4B, c), whereas the proportion of PLB-985/MT-ASB-2 cells with condensed chromatin was markedly increased (up to 35% at 60 h) (Fig. 4B, f). This was associated with an increased percentage of cells with a DNA content equivalent of that of S and G₂/M cells (Fig. 4B, f). A large proportion of PLB-985/MT-ASB-2 cells with condensed chromatin had DNA content equivalent of that of S and G₂/M cells (Fig. 4C, d). In separate measurements, we assessed the percentage of apoptotic cells with DNA strand breaks (terminal dUTP nick-end labeling-positive) (Fig. 4B, e and f) and cleaved poly-(ADP-ribose) polymerase (data not shown). The proportion of apoptotic cells remained below 5% whether PLB-985/MT-ASB-2 and PLB-985/MT were untreated or treated with ZnSO₄. Thus, in light of the evidence that proliferation of ASB-2 transfected cells was suppressed by ZnSO₄ whereas apoptosis was minimal, the observed increase in proportion of S and G₂/M cells including those cells with condensed chromatin indicates that, although commitment events were induced, cells were arrested in their progression through S and G₂/M. However, expression of differentiation markers such as CD11b, ICAM-1, MCP-1, interleukin-8, and defensin 1 was not induced in ZnSO₄-treated PLB-985/MT-ASB-2 cells (data not shown). Altogether, our results strongly suggest that ASB-2 expression is part of the mechanisms controlling growth arrest and chromatin condensation known to be associated with early phases of ATRA-induced differentiation of myeloid leukemia cells.

DISCUSSION

ASB-2 Is a Member of the SOCS Protein Family—ASB proteins are evolutionarily conserved because translation of the M60-7 gene of the *Caenorhabditis elegans* cosmid M60 indicated that it encodes a full-length ASB (37) with *bona fide* ankyrin repeats and SOCS box organization. These proteins belong to the SOCS protein family identified as containing a SOCS box consensus (31). They differ in their NH₂-terminal part, sharing sequences involved in protein-protein interactions such as a Src homology 2 domain (SOCS proteins), a SPRY domain (SSB proteins), WD-40 repeats (WSB proteins), or ankyrin repeats (ASB proteins). Several cytokines induce transcriptional activation of one or more SOCS genes, suggesting that SOCS proteins play central roles in determining the intensity and duration of cellular responses to many cytokines (3). Whether ASB-2 is involved in a specific cytokine signaling pathway remains to be determined.

PML-RAR α Enhances RA-induced Expression of ASB-2—We found that the drastic increase in ASB-2 mRNA expression induced by RA in NB4 cells was mediated through a PML-RAR α signaling and correlated with growth arrest and differentiation of these cells. Little is known about the signaling pathway through which pharmacological concentrations of RA can induce growth arrest and differentiation in APL cells. PML-RAR α behaves as a constitutive transcriptional repressor of RA target genes in the presence of physiological concentrations of RA (38–41), and a central role of RAR α pathway deregulation was found in the pathogenesis of APL (42). Therefore, PML-RAR α may repress expression of ASB-2 and thus affect signaling at physiological concentrations of RA. The fact that high RA concentrations can switch PML-RAR α from repressor to activator (12) may account for a capacity of PML-RAR α to transcriptionally activate the ASB-2 gene in the presence of RA. The causal role of PML-RAR α in the pathogenesis of APL has been demonstrated in several transgenic mouse models (43). Furthermore, expression of the PML-RAR α gene in murine bone marrow cells greatly blocked myeloid maturation and led to the immortalization of myeloid progenitors. The ASB-2 transcripts were strongly detected in normal bone marrow and up-regulated upon RA-induced differentiation of NB4

cells. Because target cells for transformation in APL are likely to be myeloid progenitors (44), genes involved in myeloid differentiation might be repressed in APL cells and induced when these cells are exposed to RA. Our results suggest that ASB-2 may be one of these genes.

ASB-2 Expression Recapitulates Early Differentiation Events in Myeloid Leukemia Cells—In APL cells, RA induces growth arrest and terminal differentiation possibly via the induction of genes implicated in cell cycle arrest and the acquisition of a differentiated phenotype. However, although normal differentiation in most cell types is associated with an arrest in G₁, a large proportion of the cells expressing an exogenous ASB-2 were actually arrested in the S and G₂/M phases. This was quite evident from the presence of cells with condensed chromatin that had a S+G₂/M DNA content and from the increase in overall proportion of S+G₂/M cells after treatment with ZnSO₄. During normal differentiation, the induction process appears to be sequential, first by arresting cells in G₁ (by causing an accumulation of pRb in hypophosphorylated state) (45) and, subsequently, by inducing their differentiation phenotype. However, it should be noted that, in differentiating NB4 cells, hypophosphorylated pRb was found both in G₁ and S+G₂/M (46). In the case of ASB-2-expressing cells, the signaling appears to by-pass the cell cycle regulatory system and directly trigger commitment to differentiation. This observation is in agreement with the fact that, in PML-RAR α -expressing cells exposed to the differentiative stimulus of RA, G₁ arrest occurs as a late phase not causally linked to commitment and differentiation (13). It should also be noticed that, in uridine-induced HL-60 cells, an arrest in the G₁ phase is not required for differentiation as this process is preceded by accumulation of cells in the G₂/M phase of the cell cycle (47).

The fact that ASB-2 expression induced a limitation in cell proliferation together with chromatin condensation strongly suggests that it promotes commitment events found at early stages in RA-induced differentiation of leukemia cells. Further studies on the mechanisms of action of ASB-2 and the identification of its partners will contribute greatly to the understanding of the signaling pathway interrupted by PML-RAR α at the early stages of leukemia transformation and reactivated during RA-induced commitment to differentiation in APL cells.

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Addendum—After this manuscript was submitted, Kohroki *et al.* reported the cloning of ASB-2 as induced by ATRA in HL-60 leukemia cells (Kohroki, J., Fujita, S., Itoh, N., Yamada, Y., Imai, H., Yumoto, N., Nakanishi, T., and Tanaka, K. (2001) *FEBS Lett.* **505**, 223–228).

REFERENCES

- Reddy, G. P., Tiarks, C. Y., Pang, L., Wu, J., Hsieh, C. C., and Quesenberry, P. J. (1997) *Blood* **90**, 2293–2299
- Begley, C. G., and Nicola, N. A. (1999) *Blood* **93**, 1443–1447
- Starr, R., and Hilton, D. J. (1999) *Bioessays* **21**, 47–52
- Lowenberg, B., and Touw, I. P. (1993) *Blood* **81**, 281–292
- Ida, K., Kitabayashi, I., Taki, T., Taniwaki, M., Noro, K., Yamamoto, M., Ohki, M., and Hayashi, Y. (1997) *Blood* **90**, 4699–4704
- Look, A. T. (1997) *Science* **278**, 1059–1064
- Huang, M. E., Ye, Y. C., Chen, S. R., Chai, J. R., Lu, J. X., Zhou, L., Gu, L. J., and Wang, Z. Y. (1988) *Blood* **72**, 567–572
- Castaigne, S., Chomienne, C., Daniel, M. T., Ballerini, P., Berger, R., Fenaux, P., and Degos, L. (1990) *Blood* **76**, 1704–1709
- Chambon, P. (1996) *FASEB J.* **10**, 940–954
- Tsai, S., Bartelmez, S., Sitnicka, E., and Collins, S. (1994) *Genes Dev.* **8**, 2831–2841
- Kastner, P., Lawrence, H. J., Waltzinger, C., Ghyselinck, N. B., Chambon, P., and Chan, S. (2001) *Blood* **97**, 1314–1320
- Lin, R. J., Egan, D. A., and Evans, R. M. (1999) *Trends Genet.* **15**, 179–184
- Casini, T., and Pelicci, P. G. (1999) *Oncogene* **18**, 3235–3243
- Cole, R. D. (1987) *Int. J. Pept. Protein Res.* **30**, 433–449
- Tsiftoglou, A. S., Wong, W., Hyman, R., Minden, M., and Robinson, S. H. (1985) *Cancer Res.* **45**, 2334–2339
- Miyauchi, J., Ohyashiki, K., Inatomi, Y., and Toyama, K. (1997) *Blood* **90**, 803–813
- Lanotte, M., Martin-Thouvenin, V., Najman, S., Balerini, P., Valensi, F., and

- Berger, R. (1991) *Blood* **77**, 1080–1086
18. Tucker, K. A., Lilly, M. B., Heck, L., Jr., and Rado, T. A. (1987) *Blood* **70**, 372–378
 19. Dermime, S., Grignani, F., Clerici, M., Nervi, C., Sozzi, G., Talamo, G. P., Marchesi, E., Formelli, F., Parmiani, G., Pelicci, P. G., and Gambacorti-Passerini, C. (1993) *Blood* **82**, 1573–1577
 20. Grignani, F., Ferrucci, P. F., Testa, U., Talamo, G., Fagioli, M., Alcalay, M., Mencarelli, A., Peschle, C., Nicoletti, L., and Pelicci, P. G. (1993) *Cell* **74**, 423–431
 21. Lutz, P. G., Houzel-Charavel, A., Moog-Lutz, C., and Cayre, Y. E. (2001) *Blood* **97**, 2449–2456
 22. Moog-Lutz, C., Peterson, E. J., Lutz, P. G., Eliason, S., Cave-Riant, F., Singer, A., Di Gioia, Y., Dmowski, S., Kamens, J., Cayre, Y. E., and Koretzky, G. (2001) *J. Biol. Chem.* **276**, 22375–22381
 23. Chomienne, C., Ballerini, P., Balitrand, N., Daniel, M. T., Fenaux, P., Castaigne, S., and Degos, L. (1990) *Blood* **76**, 1710–1717
 24. Tomasetto, C., Regnier, C., Moog-Lutz, C., Mattei, M. G., Chenard, M. P., Lidereau, R., Basset, P., and Rio, M. C. (1995) *Genomics* **28**, 367–376
 25. Cook, D. M., Hinkes, M. T., Bernfield, M., and Rauscher, F. J., III (1996) *Oncogene* **13**, 1789–1799
 26. Gorczyca, W., Bruno, S., Darzynkiewicz, R., Gong, J., and Darzynkiewicz, Z. (1992) *Int. J. Oncol.* **1**, 639–648
 27. Li, X., and Darzynkiewicz, Z. (2000) *Exp. Cell Res.* **255**, 125–132
 28. Darzynkiewicz, Z., Juan, G., Li, X., Gorczyca, W., Murakami, T., and Traganos, F. (1997) *Cytometry* **27**, 1–20
 29. Bedner, E., Burfeind, P., Gorczyca, W., Melamed, M. R., and Darzynkiewicz, Z. (1997) *Cytometry* **29**, 191–196
 30. Darzynkiewicz, Z., Bedner, E., Li, X., Gorczyca, W., and Melamed, M. R. (1999) *Exp. Cell Res.* **249**, 1–12
 31. Hilton, D. J., Richardson, R. T., Alexander, W. S., Viney, E. M., Willson, T. A., Sprigg, N. S., Starr, R., Nicholson, S. E., Metcalf, D., and Nicola, N. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 114–119
 32. Kile, B. T., Viney, E. M., Willson, T. A., Brodnicki, T. C., Cancilla, M. R., Herlihy, A. S., Croker, B. A., Baca, M., Nicola, N. A., Hilton, D. J., and Alexander, W. S. (2000) *Gene (Amst.)* **258**, 31–41
 33. Michaely, P., Bennett, V. (1992) *Trends Cell Biol.* **2**, 127–129
 34. Starr, R., Willson, T. A., Viney, E. M., Murray, L. J., Rayner, J. R., Jenkins, B. J., Gonda, T. J., Alexander, W. S., Metcalf, D., Nicola, N. A., and Hilton, D. J. (1997) *Nature* **387**, 917–921
 35. Kamura, T., Sato, S., Haque, D., Liu, L., Kaelin, W. G., Jr., Conaway, R. C., and Conaway, J. W. (1998) *Genes Dev.* **12**, 3872–3881
 36. Botling, J., Castro, D. S., Oberg, F., Nilsson, K., and Perlmann, T. (1997) *J. Biol. Chem.* **272**, 9443–9449
 37. Wilson, R., Ainscough, R., Anderson, K., Baynes, C., Berks, M., Bonfield, J., Burton, J., Connell, M., Copsey, T., Cooper, J., et al. (1994) *Nature* **368**, 32–38
 38. Grignani, F., De Matteis, S., Nervi, C., Tomassoni, L., Gelmetti, V., Cioce, M., Fanelli, M., Ruthardt, M., Ferrara, F. F., Zamir, I., Seiser, C., Lazar, M. A., Minucci, S., and Pelicci, P. G. (1998) *Nature* **391**, 815–818
 39. Guidez, F., Ivins, S., Zhu, J., Soderstrom, M., Waxman, S., and Zelent, A. (1998) *Blood* **91**, 2634–2642
 40. He, L. Z., Guidez, F., Tribioli, C., Peruzzi, D., Ruthardt, M., Zelent, A., and Pandolfi, P. P. (1998) *Nat. Genet.* **18**, 126–135
 41. Lin, R. J., Nagy, L., Inoue, S., Shao, W., Miller, W. H., Jr., and Evans, R. M. (1998) *Nature* **391**, 811–814
 42. Du, C., Redner, R. L., Cooke, M. P., and Lavau, C. (1999) *Blood* **94**, 793–802
 43. He, L. Z., Merghoub, T., and Pandolfi, P. P. (1999) *Oncogene* **18**, 5278–5292
 44. Turhan, A. G., Lemoine, F. M., Debert, C., Bonnet, M. L., Baillou, C., Picard, F., Macintyre, E. A., and Varet, B. (1995) *Blood* **85**, 2154–2161
 45. Juan, G., Li, X., and Darzynkiewicz, Z. (1998) *Exp. Cell Res.* **244**, 83–92
 46. Brooks, S. C., III, Sturgill, R., Choi, J., and Yen, A. (1997) *Exp. Cell Res.* **234**, 259–269
 47. Sokoloski, J. A., Lee, C. W., Handschumacher, R. E., Nigam, A., and Sartorelli, A. C. (1991) *Leuk. Res.* **15**, 1051–1058

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