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Ectopic *TAL-1/SCL* Expression in Phenotypically Normal or Leukemic Myeloid Precursors: Proliferative and Antiapoptotic Effects Coupled with a Differentiation Blockade

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The *TAL-1* gene specifies a basic helix-loop-helix domain (bHLH) transcription factor, which heterodimerizes with E2A gene family proteins. tal-1 protein is abnormally expressed in the majority of T-cell acute lymphoblastic leukemias (T-ALLs). tal-1 is expressed and plays a significant role in normal erythropoietic differentiation and maturation, while its expression in early myeloid differentiation is abruptly shut off at the level of late progenitors/early differentiated precursors (G. L. Condorelli, L. Vitelli, M. Valtieri, I. Marta, E. Montesoro, V. Lulli, R. Baer, and C. Peschle, Blood 86:164–175, 1995). We show that in late myeloid progenitors (the phenotypically normal murine 32D cell line) and early leukemic precursors (the human HL-60 promyelocytic leukemia cell line) ectopic tal-1 expression induces (i) a proliferative effect under suboptimal culture conditions (i.e., low growth factor and serum concentrations respectively), via an antiapoptotic effect in 32D cells or increased DNA synthesis in HL-60 cells, and (ii) a total or marked inhibitory effect on differentiation, respectively, on granulocyte colony-stimulating factor-induced granulopoiesis in 32D cells or retinoic acid- and vitamin D3-induced granulo- and monocytopoiesis in HL-60 cells. Furthermore, experiments with 32D temperature-sensitive p53 cells indicate that aberrant tal-1 expression at the permissive temperature does not exert a proliferative effect but causes p53-mediated apoptosis, i.e., the tal-1 proliferative effect depends on the integrity of the cell cycle checkpoints of the host cell, as observed for c-*myc* and other oncogenes. tal-1 mutant experiments indicate that ectopic tal-1 effects are mediated by both the DNA-binding and the heterodimerization domains, while the N-terminally truncated tal-1 variant (M3) expressed in T-ALL malignant cells mimics the effects of the wild-type protein. Altogether, our results (i) indicate proliferative and antidifferentiative effects of ectopic tal-1 expression, (ii) shed light on the underlying mechanisms (i.e., requirement for the integrity of the tal-1 bHLH domain and cell cycle checkpoints in the host cell, particularly p53), and (iii) provide new experimental models to further investigate these mechanisms.

The *TAL-1* gene (8) (also called *SCL* [5] or *TCL-5* [18]) specifies a basic helix-loop-helix domain (bHLH) transcription factor, which heterodimerizes with ubiquitous E2A family proteins (26).

The tal-1 protein, while absent in normal adult T lymphocytes (5), is constitutively expressed in >60% of T-cell acute lymphoblastic leukemias (T-ALLs), due to the (1;14)(p32;q11) translocation (5, 8, 18), specific interstitial deletions of the 5′ noncoding gene region (tal^d) (6), or unidentified alterations (4).

In the homologous-recombination mouse model, lack of tal-1 determines midgestation embryonic lethality due to the absence of primitive hematopoiesis (44, 48). Furthermore, in vivo and in vitro studies of tal-$1^{-/-}$ embryonic stem cells indicate a crucial role for tal-1 in the generation of all definitive lineages (40, 43). In early development tal-1 is also expressed in nervous system, smooth muscle, and endothelial cells (21, 29).

In adult cells, tal-1 is expressed in normal erythroblasts, megakaryocytes, and mast cells (36, 41), as well as in erythroleukemic, megakaryocytic, and mast cell lines (21, 56). In human hematopoiesis, tal-1 is expressed in hematopoietic progenitor cell (HPC) differentiation/maturation through the erythroid and megakaryocytic lineages (13, 36, 41, 53). Interestingly, tal-1 is also expressed in the early stages of HPC differentiation along the granulopoietic or monopoietic pathways; in both cases, however, the expression is abruptly shut off at the level of late HPC/early differentiated precursors (13). Functional studies with antisense oligodeoxynucleotides targeting tal-1 mRNA indicate a functional role for tal-1 in erythropoiesis, particularly at the advanced HPC differentiation stage, but not in granulo- and monocytopoiesis (13); the functional role in erythropoiesis is further indicated by studies on enforced tal-1 expression in the K562 erythroleukemic line (1, 20) and differentiating erythroid HPCs (54a).

The tal-1/E2A heterodimer recognizes a subset of E-box responsive elements (27, 28). However, tal-1/E2A target genes have not yet been identified. In erythroblasts, formation of the tal-1/E2A complex is inhibited by the HLH Id2 protein, which competes with tal-1 for E2A protein binding (13). Furthermore, tal-1 is associated in complex with Lmo2/rbtn-2 protein in erythroid (54; also, unpublished data) and T leukemic (58) cells.

While the function of tal-1 in normal hematopoiesis has been extensively investigated, the effects of aberrant tal-1 expression are under scrutiny (see Discussion). We have investigated the action of ectopic tal-1 on cell proliferation/differentiation and the underlying mechanisms. The selected cell models include phenotypically normal and leukemic myeloid precursors, frozen at the differentiation stage characterized by

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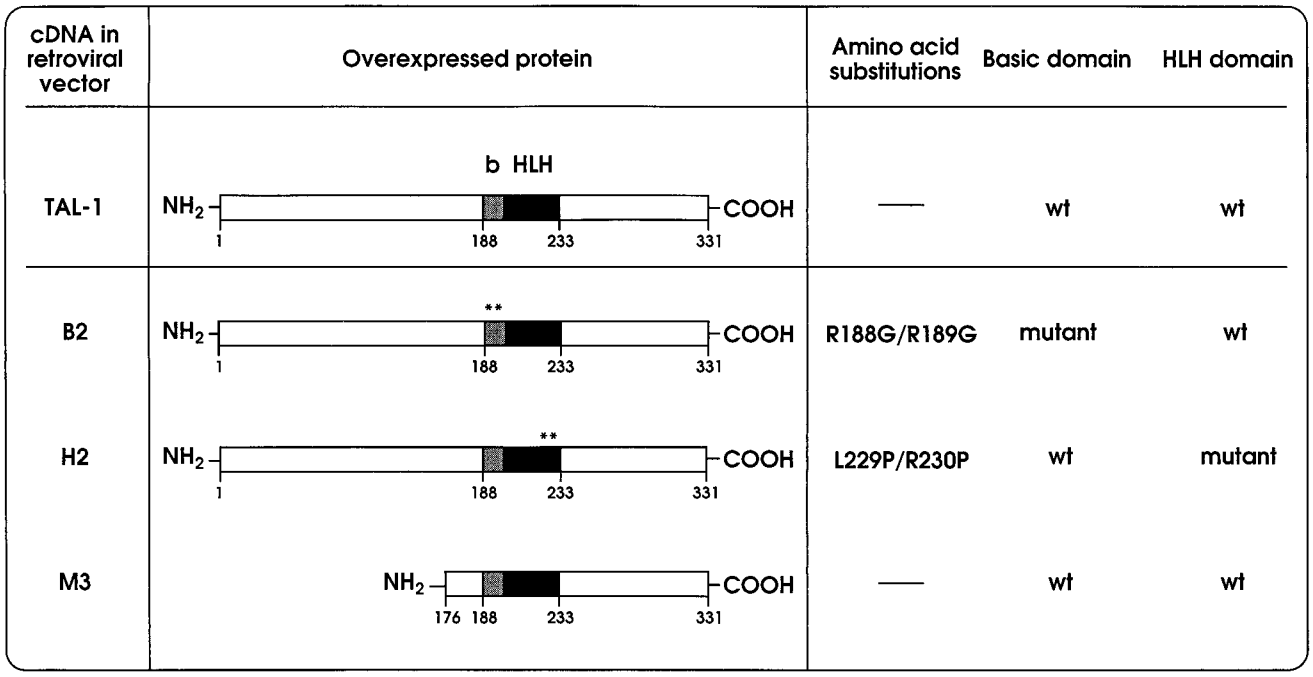


FIG. 1. Schematic diagram illustrating *TAL-1* wt and mutant cDNAs. Amino acid lengths and mutations (asterisks) are indicated.

tal-1 shutoff in normal myelopoiesis (i.e., 32D late myeloid progenitors and HL-60 leukemic promyelocytes).

MATERIALS AND METHODS

Cell lines, inducers, and growth factors. The HL-60 cell line was maintained in RPMI 1640 medium (GIBCO) supplemented with 10% fetal calf serum (FCS). All-*trans* retinoic acid (RA) and vitamin D3 (1α25-OH-VitD3) (VitD3) were obtained from Sigma (St. Louis, Mo.) and Roche Co. (Basel, Switzerland), respectively.

32D Cl3 cells were routinely cultured in Iscove modified Dulbecco medium (IMDM) supplemented with 15% FCS in the presence of 1 ng of recombinant murine interleukin 3 per ml (IL-3) (Preprotech, London, England) in a 5% partial CO_2 pressure atmosphere at 32°C and subcultured 1:20 twice a week in freshly prepared medium. Recombinant human granulocyte colony-stimulating factor (G-CSF) was purchased from R&D Systems (Minneapolis, Minn.).

32D Cl3 p53cG (Val^{135}) cells carrying the temperature-sensitive p53 Val-135 mutant gene were a kind gift of Silvia Soddu (Istituto Tumori Regina Elena, Rome, Italy) (7) and were grown as the original 32D Cl3 with the exception of experiments carried out at 39°C to inactivate the tsp53.

Gene transfer. The LXSN retroviral vector (35) kindly provided by D. A. Miller (University of Washington, Seattle) was used as a control vector and will be referred to as "neo control." Retroviral vectors containing wild-type (wt) tal-1 or tal-1 mutations were constructed. The full-length human tal-1 cDNA was inserted in LXSN. The tal-1 mutant M3 plasmid encodes the amino-truncated tal-1 species (residues 176 to 331) that is coexpressed with the full-length protein in T-ALL malignant cells (10). The tal-1 mutant B2 encodes a full-length tal-1 polypeptide with two amino acid substitutions in the basic region of the bHLH domain (R188G and R189G), which abolish the DNA consensus sequence binding (27). The tal-1 mutant H2 encodes a full-length tal-1 polypeptide with two proline substitutions (L229P and R230P) in the second helix of the bHLH domain, which abolish both E2A protein and DNA binding capacity (27).

The ecotropic GP+E-86 producer cell line and the amphotropic GP+envAM-12 packaging cell line used to generate the recombinant retroviruses were maintained in HXM medium, composed of Dulbecco modified Eagle medium (DMEM) (GIBCO), 10% FCS, and 15 μg of hypoxanthine, 250 μg of xanthine, and 25 μg of mycophenolic acid (all from Sigma) per ml. Amphotropic virus-producing cell lines were maintained in HXM medium supplemented with 0.4 mg of the neomycin analog Geneticin and 200 μg of hygromycin B (Sigma) per ml. Unless otherwise specified, all cell lines were maintained at 37°C in a 5% CO_2 atmosphere humidified incubator. Twenty-four hours prior to viral-supernatant harvesting, producer cells were cultured in DMEM supplemented with 10% FCS at 32°C in a 3% CO_2 atmosphere humidified incubator.

Production of high-titer ecotropic recombinant retroviruses was carried out by calcium phosphate transfection (9) in the GP+E-86 producer cell line. Forty-eight hours after transfection, GP+E-86 supernatants were harvested, filtered, and used to transduce the amphotropic packaging cell line GP+envAM-12 in the presence of 8 μg of Polybrene per ml. Transduced GP+envAM-12 cells were selected in DMEM supplemented with 10% FCS and containing 0.8 mg of Geneticin per ml and then used to generate helper-free-virus-containing supernatants. The retrovirus titration was carried out on NIH 3T3 cells (15). The viral titers of *neo*-containing (LXSN) and *TAL-1* containing (LXSN-TAL-1) retroviral supernatants were 1.2×10^6 and 6×10^6 CFU/ml, respectively, as assessed by transfer of Geneticin resistance in NIH 3T3 cells. The viral titers of M3, B2, and H2 mutant retroviral supernatants were 3×10^5, 8×10^5, and 2×10^5 CFU/ml, respectively. Absence of helper virus generation in the producer cell lines was verified by the marker rescue assay (16).

The HL-60 cell line was transduced by the method described in reference 31. Briefly, 10^6 cells were incubated with 2-ml viral supernatants and Polybrene (8 μg/ml) and centrifuged at $670 \times g$ at 32°C. After a 90-min centrifugation, cells were incubated overnight at 32°C, washed once, resuspended in complete medium, and maintained at 37°C for 2 days. At day 3, they were split with complete medium supplemented with 0.4 mg of Geneticin per ml. Selection was carried out for 10 days; the cells were analyzed by immunofluorescence, and the presence of the transcript was confirmed at mRNA and protein levels by reverse transcriptase (RT)-PCR and Western blot analysis, respectively.

HL-60 tal-1 cell subcloning was performed in flat-bottom 96-microwell plates (0.5 cell/well). Single clones were grown in 0.1 ml of complete medium supplemented with 0.8 mg of Geneticin per ml. Cells from each clone were smeared onto glass slides by centrifugation and analyzed for tal-1 expression by anti-tal-1 immunofluorescence as specified below. Clones 22 and 24, displaying the highest positivity (95 to 99% and 80% strongly positive tal-1 cells, respectively), were maintained in complete medium with 0.4 mg of Geneticin per ml and utilized for functional studies.

Gene transfer into 32D Cl3 cells was performed by electroporation. Thus, 3×10^6 32D Cl3 cells were washed twice in phosphate-buffered saline (PBS), resuspended in 280 μl of ice-cold PBS, transferred in a gene pulser cuvette containing 20 μg of plasmid DNA in 20 μl of TE buffer (10 mmol of Tris-HCl [pH 7.5] and 1 mmol of EDTA per liter), electroporated with 0.25 kV and 975 μF, and then transferred in 5 ml of IMDM–15% FCS supplemented with 1 ng of recombinant murine IL-3 per ml. After 24 h 5 ml of fresh medium was added, and after 48 h the selection was applied (0.8 mg of Geneticin per ml). After 10 days the selection was terminated, and the transduced cells were maintained in the presence of 0.4 mg of Geneticin per ml.

For 32D tal-1 cell subcloning, cells were seeded (0.5 cell/well) in 100 μl of selective culture medium (Geneticin at 0.8 mg/ml) in 96-well U-bottom plates. Geneticin-resistant clones were analyzed for tal-1 expression by immunofluorescence as described below. Clones 2 and 14 displayed the highest positivity (85 and 83% strongly positive tal-1 cells, respectively) and were used for functional studies.

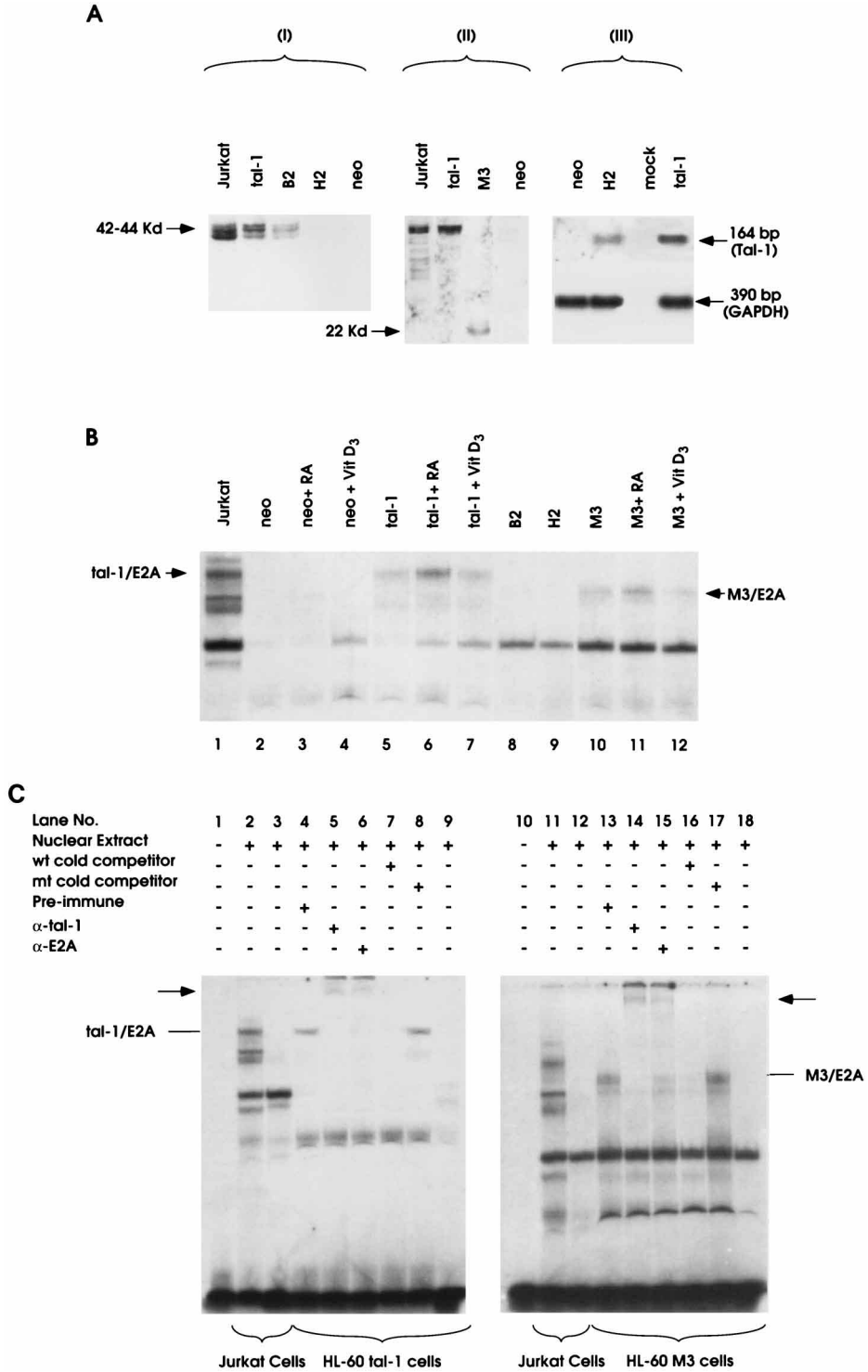
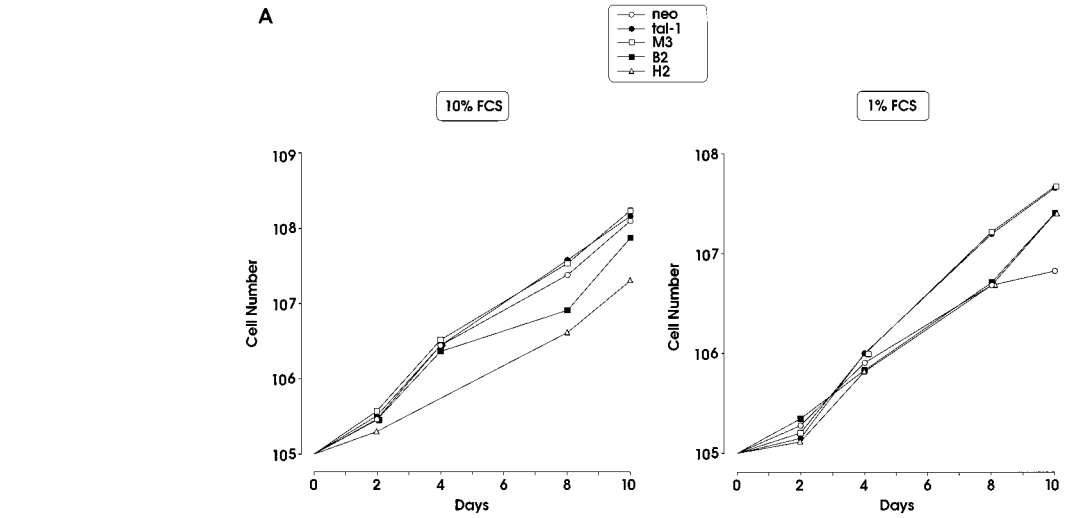


FIG. 2. [A(I)] Western blot analysis of tal-1 protein expression in HL-60 cells transduced with wt *TAL-1* or B2 and H2 mutants. Nuclear extracts from Jurkat and HL-60 neo cell lines (20 μg) were used as positive and negative controls, respectively. The filter was probed with the BTL73 MAb directed against a tal-1 carboxyl epitope. The BTL73 MAb recognizes the tal-1 wt, B2, and M3 polypeptides (41) but not the H2 polypeptide. [A(II)] Western blot analysis of tal-1 protein expression in HL-60 cells transduced with the M3 variant. Nuclear extracts from Jurkat and HL-60 tal-1 or HL-60 neo cells were used as positive or negative control, respectively. The filter was probed with the BTL73 MAb. A 22-kb band was detected in the HL-60 M3 nuclear extracts. [A(III)] Semiquantitative RT-PCR assay for *TAL-1* mRNA in HL-60 cells transduced with wt *TAL-1* (positive control) or the H2 mutant. HL-60 neo and mock reactions were used as negative controls. GAPDH was used for normalization (see Materials and Methods). (B) EMSA of DNA binding by the tal-1/E2A consensus E-box sequence on nuclear extracts of HL-60 cells transduced with wt *TAL-1* or the B2, H2, or M3 mutant (lanes 5 and 8 to 10). Jurkat (lane 1) and HL-60 neo (lane 2) nuclear extracts were used as positive and negative controls, respectively. In the case of HL-60 neo, HL-60 tal-1, or HL-60 M3, EMSA results for nuclear extracts from RA- and VitD3-treated cells are also shown (lanes 3, 4, 6, 7, 11, and 12). Bands corresponding to the tal-1/E2A and M3/E2A heterodimers are indicated. The H2 and B2 mutants produce a loss of binding activity for the tal-1/E2A canonical E-box sequence. (C) tal-1 and M3 DNA binding activities in HL-60 transduced cells. ^{32}P-labeled *TAL-1* consensus (lanes 1, 2, 4 to 11, and 13 to 17) or mutated (lane 3, 9, 12, and 18) E-box oligonucleotides (see Materials and Methods) were incubated with nuclear extracts from HL-60 tal-1 (lanes 4 through 9), HL-60 M3 (lanes 13 through 18), or Jurkat (lanes 2, 3, 11, and 12) cells. The HL-60 tal-1 and M3 extracts were preincubated with anti-tal-1 (α-tal-1) serum (no. 370) (lanes 5 and 14), anti-E2A serum (lanes 6 and 15), corresponding preimmune serum (lanes 4 and 13), or approximately 100-fold excesses of wt (lanes 7 and 16) and mutant (lanes 8 and 17) tal-1 E-box binding sequences. Protein-oligonucleotide complexes representing tal-1/E2A and M3/E2A heterodimers and the supershift band in lanes 5, 6, 14, and 15 (arrows) are indicated.



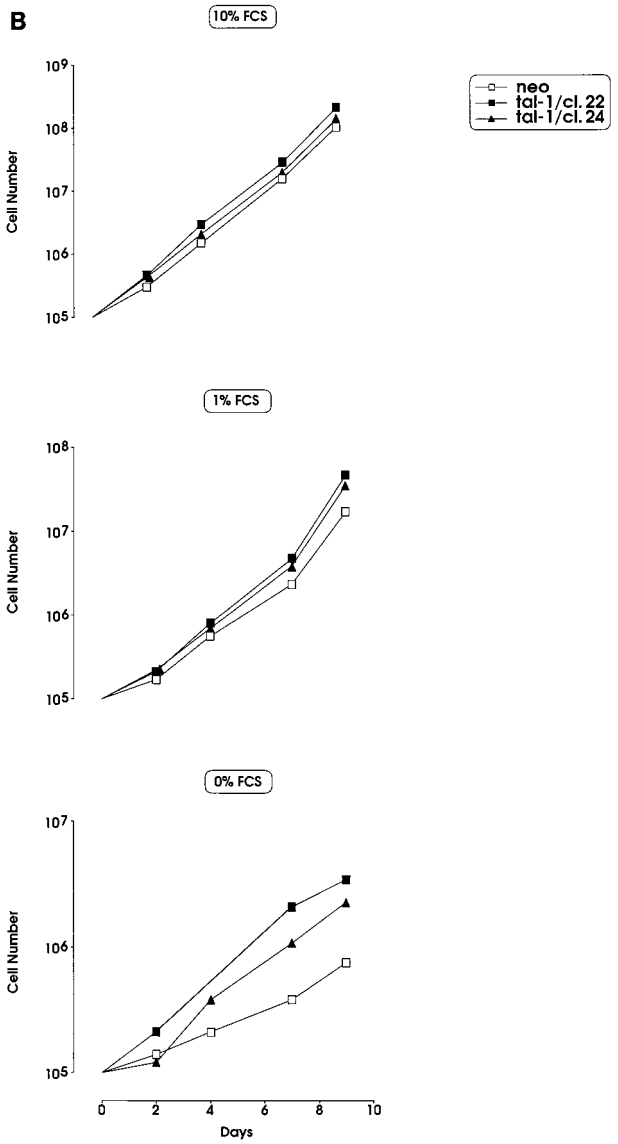


FIG. 3. (A) Growth curves of HL-60 cells transduced with neo, wt *TAL-1*, or the M3, B2, or H2 mutant. Cells were grown in 10 or 1% FCS medium. (B) Growth curves of HL-60 tal-1 subclones (cl.) 22 and 24 and HL-60 neo cells. Cells were grown in 10, 1%, or 0% FCS.

Transduced gene expression analysis. (i) RNA analysis by RT-PCR. Total RNA extracted by the guanidinium isothiocyanate-CsCl method (11) from the same number of cells in the presence of 12 μg of *Escherichia coli* rRNA was quantitated by dot hybridization with a human rRNA probe (13). After densitometric analysis, the same amount of RNA (~200 ng) was reverse transcribed (Moloney murine leukemia virus RT [Bethesda Research Laboratories, Gaithersburg, Md.]) with oligo(dT) as a primer. cDNAs were normalized by the glyceraldeyde-3-phosphate dehydrogenase (GAPDH) gene by using primers 5′-ACATCAAGAAGGTGGTGAAGCAGG-3′ (5′ primer) and 5′-CTCTTCCTCTTGTGCTCTTGCTGG-3′ (3′ primer) (probe, 880 to 914 bp) (2). Amplification of ~5 ng of RT RNA within the linear range was achieved by 20 PCR cycles.

To evaluate the expression of TAL-1 and mutant cDNAs, an aliquot of RT RNA (~20 ng) was amplified within the linear range by 40 PCR cycles, which allowed a linear cDNA dose response.

RT RNA from the K562 cell line was used as an internal positive control in each PCR.

Each sample was electrophoresed in a 2% agarose gel, transferred to a nitrocellulose filter, and hybridized with an internal oligomer as a probe. An aliquot of RNA (~20 ng) of each sample and a mock reaction product (negative control) were amplified to exclude the presence of contaminant DNA.

The following 5′ and 3′ primers and probe were used for wt tal-1 and mutants: 5′ primer, 5′-TATCTTCACCAACAGCCG; 3′ primer 5′-CTGCTTGGCCAAGAAGTT-3′; probe, 5′-CCGGACAAGAAGCTCAGCAAGAATGAGATC-3′.

The amplification procedure included denaturation at 95°C for 30 s, annealing at 56°C for GAPDH or at 54°C for tal-1, and extension at 72°C for 45 s for 40 PCR cycles, i.e., within the range of linear amplification.

(ii) Western blot analysis. Western blot analysis was performed with anti-tal-1 (no. 1080) rabbit serum (27) and the BTL73 monoclonal antibody (MAb) (41) by enhanced chemiluminescence, according to the manufacturer's protocol (Amersham, Little Chalfont, Buckinghamshire, United Kingdom).

(iii) Immunofluorescence analysis. The polyclonal rabbit anti-human tal-1 (no. 7742) was obtained by immunizing animals with a glutathione *S*-transferase–full-length tal-1 protein. The anti-p53 MAb (Ab-4) was purchased from Oncogene Science Inc. (Cambridge, Mass.).

The cells were smeared onto glass slides by cytospin centrifugation. For tal-1 analysis, both HL-60 cells and 32D cells were fixed for 5 min at room temperature with absolute methanol and for 2 min at −20°C with acetone. After a 10-min wash in PBS, the cells were incubated for 30 min at room temperature with a 1:100 dilution of anti-tal-1. After being washed in PBS, the cells were incubated for 30 min at room temperature with a 1:50 dilution of fluorescein isothiocyanate (FITC)-labeled sheep anti-mouse immunoglobulin (Dakopatts). For p53 analysis, wt 32D Cl3 or p53 cG (Val-135)-transduced cells were fixed for 5 min with absolute methanol at −10°C. The cells were then incubated with 10% goat serum in PBS for 20 min to suppress nonspecific immunoglobulin G binding. After an extensive wash, the cells were incubated for 40 min at room temperature with a 1:20 dilution of anti-p53 MAb (clone PAb 246; Calbiochem/Oncogene Research, Cambridge, Pa.). After a wash, a 1:75 dilution of FITC-labeled sheep anti-rabbit immunoglobulin was added to the cells for 40 min at room temperature. The slides were then washed in PBS, mounted in PBS-glycerol, and

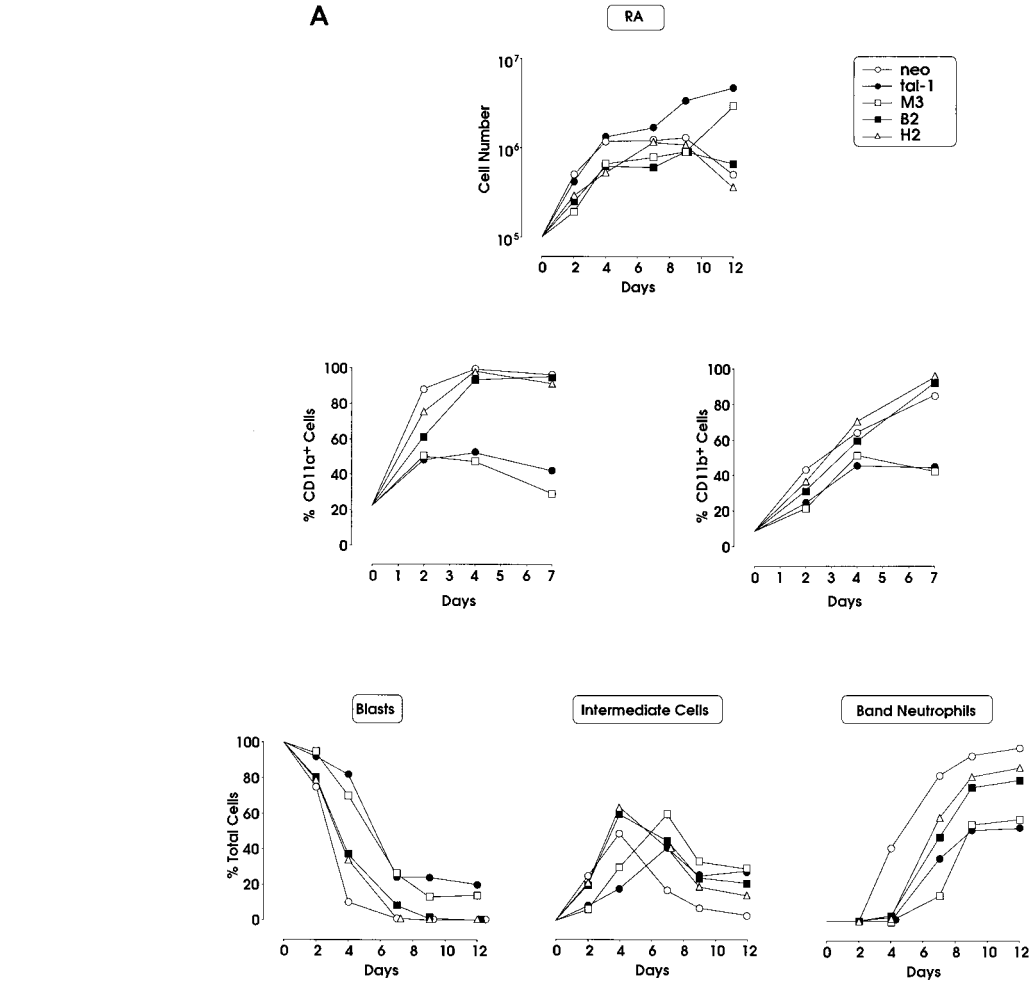


FIG. 4. (A) RA treatment of HL-60 cells expressing neo, wt tal-1, or the M3, B2, or H2 mutant. Cell number (upper panel), membrane antigen expression (percent positive cells) (middle panels), and morphology (lower panels) were evaluated. Cells were classified in three stages of differentiation/maturation: blasts, cells at an intermediate stage of differentiation, and band neutrophils (see Results). (B) Morphology of HL-60 cells expressing neo, wt tal-1, or the M3, B2, or H2 mutant grown for 7 days in the presence of RA. Control HL-60 neo cells grown without RA are shown at the upper left. At day 0, all groups had the same cell morphology. (C) RA treatment of the HL-60 tal-1 clone (cl.) 22. Cell number (upper panel), membrane antigen expression (middle panels), and cell morphology (lower panel) were evaluated. The expression of membrane antigens CD11a, CD11b, and CD54 was evaluated in terms of percentage of positive cells and mean fluorescence intensity.

observed under a fluorescence microscope (Zeiss Axiophot) equipped with a highly sensitive telecamera and a video-imaging system.

Electrophoretic mobility shift assay (EMSA). The protein concentrations of all extracts were determined by the Bradford assay (Bio-Rad). Each reaction mixture contained, in 20 μl, 10 to 25 μg of nuclear extract, 10 mmol of HEPES (pH 7.9) per liter, 50 mmol of KCl per liter, 2 mmol of $MgCl_2$ per liter, 4% Ficoll, 1 mmol of EDTA per liter, 1 mmol of dithiothreitol per liter, 0.5 μg of poly(dI-dC), and 1 to 3 pmol of a ^{32}P-labeled, double-stranded oligonucleotide probe containing the preferred sequence for DNA binding by *TAL1/E2A* heterodimers (top strand) (ACCTGAACAGATGGTCGGCT) or corresponding mutant oligonucleotides that bear two nucleotide substitutions in the E-box core (top strand) (ACCTGAACCGATTGTCGGCT) (27). After a 15-min incubation at room temperature, the assay mixture was loaded onto a 15-cm 4% polyacrylamide gel containing 0.25× Tris-borate-EDTA electrophoresis buffer and electrophoresed at 180 V for 2 to 3 h at 4°C. In some binding reactions, the nuclear extract was preincubated for 10 min at room temperature with 1 μl of tal-1 rabbit antiserum no. 370, the E2A antiserum, or the same amount of normal rabbit immunoglobulin. Where indicated, an approximately 100-fold excess of unla-

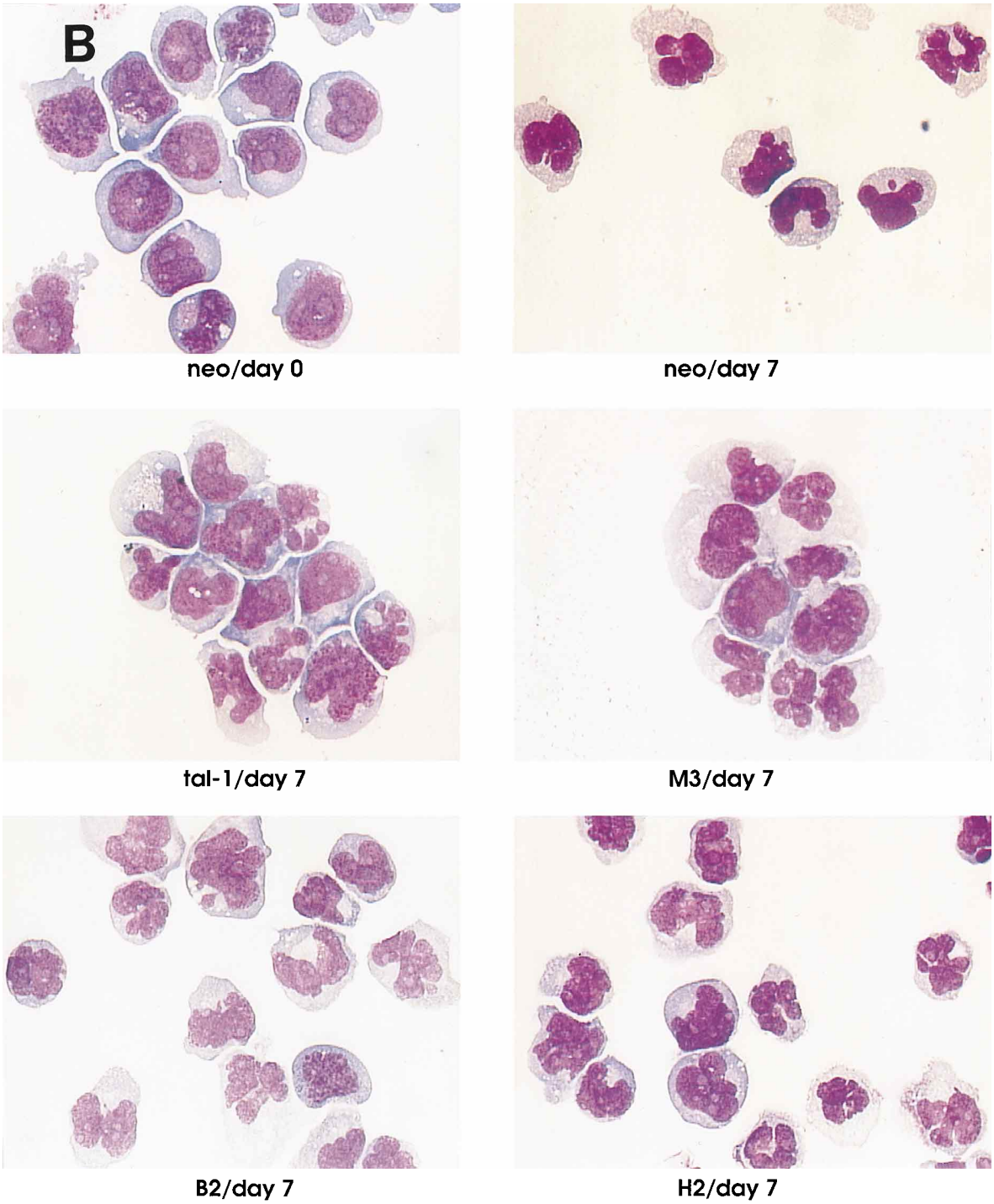


FIG. 4—*Continued.*

beled wt or mutant competitor oligonucleotides was included in the binding reaction.

Culture conditions and cell analysis. HL-60 cells were diluted at 10^5/ml in RPMI 1640 medium containing 10% FCS and resuspended at the initial cell number when their number exceeded 1.5×10^6 to 2×10^6/ml.

(i) Differentiation experiments with HL-60 cells. Cells were resuspended at 10^5/ml in RPMI 1640 medium containing 10% FCS in the presence of either 10^{-6} M RA or 100 ng of VitD3 per ml. Cells were counted daily; membrane antigen expression was determined with fluorochrome-conjugated anti-CD-11a, -CD-11b, -CD-14, and -CD-54 MAbs (Becton Dickinson, Mountain View, Calif.) as previously reported (22); cell morphology was analyzed in cytosmears stained with May-Grünwald Giemsa stain.

(ii) 32D Cl3 proliferation. Cells were diluted at 5×10^4/ml in IMDM containing 15% FCS supplemented with 0.01 or 1 ng of IL-3 per ml. Cells were counted daily and resuspended at the initial cell number when their number exceeded 5×10^5/ml.

(iii) Differentiation experiments with 32D Cl3. Cells were washed twice in PBS and then resuspended at 2×10^5 or 4×10^5/ml in IMDM–15% FCS in the presence of 30 U of recombinant human G-CSF. Cells were counted daily and diluted not to exceed 10^6/ml, and morphology was analyzed in cytosmears stained with May-Grünwald Giemsa stain.

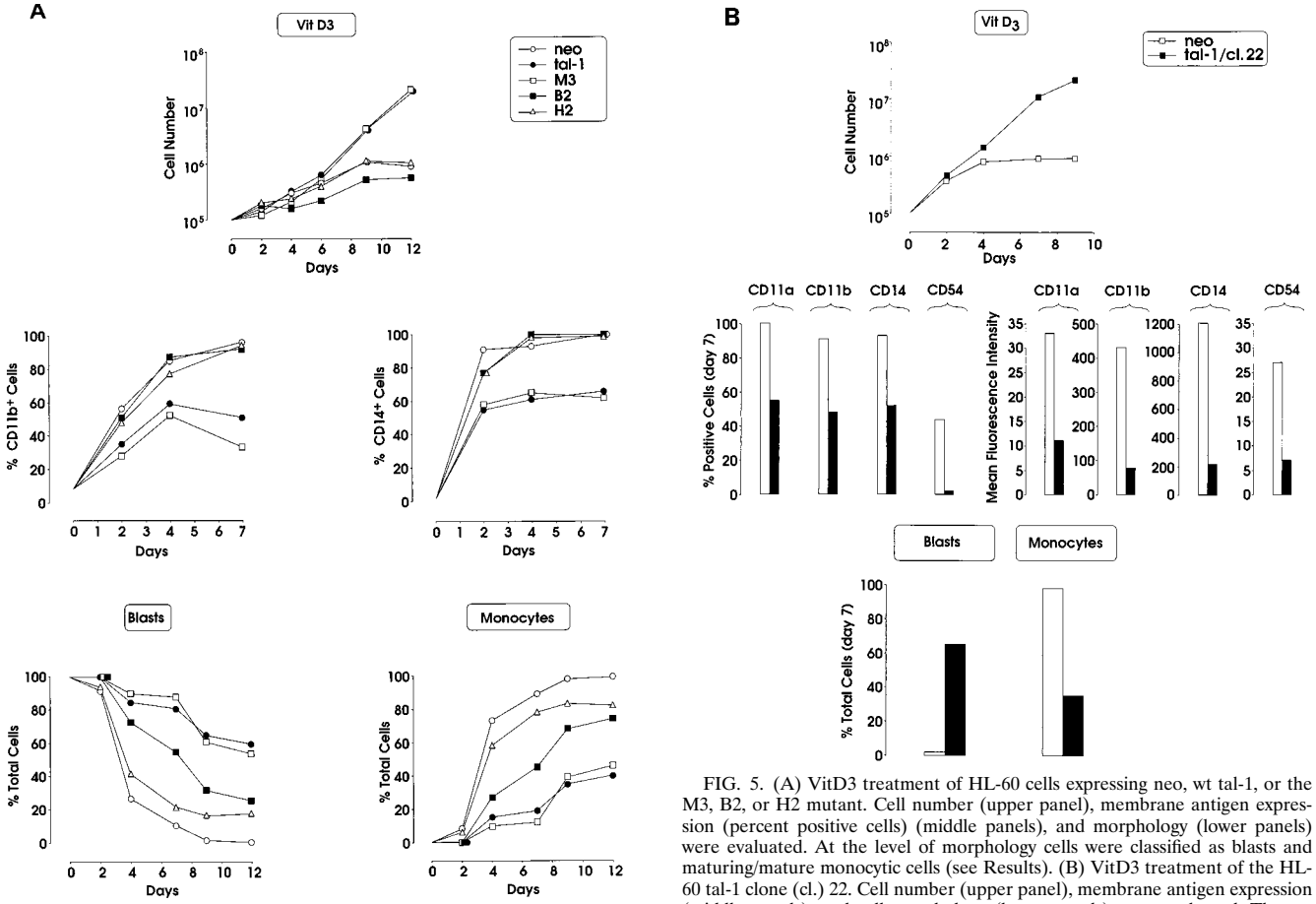


FIG. 5. (A) VitD3 treatment of HL-60 cells expressing neo, wt tal-1, or the M3, B2, or H2 mutant. Cell number (upper panel), membrane antigen expression (percent positive cells) (middle panels), and morphology (lower panels) were evaluated. At the level of morphology cells were classified as blasts and maturing/mature monocytic cells (see Results). (B) VitD3 treatment of the HL-60 tal-1 clone (cl.) 22. Cell number (upper panel), membrane antigen expression (middle panels), and cell morphology (lower panels) were evaluated. The expression of membrane antigens CD11a, CD11b, CD14, and CD54 was evaluated in terms of percentage of positive cells and mean fluorescence intensity.

(iv) Apoptosis analysis. In HL-60 and 32D cells apoptosis was evaluated by double staining with FITC-labeled annexin V and propidium iodide (PI) (R&D Systems) (25, 37). Briefly, 5×10^4 HL-60 or 32D cells were washed twice in cold PBS and resuspended in 250 μl of binding buffer (HEPES-buffered saline solution supplemented with 0.25 mM $CaCl_2$). Five microliters of FITC-conjugated annexin V and 5 μl of PI reagent were added to the cells, and the mixtures were gently vortexed and incubated for 15 min at room temperature in the dark. Within 1 h cells were analyzed at 488 mm in a FACsort cytometer.

All experiments on cell proliferation, differentiation, and apoptosis were reproduced at least three times; representative data are presented in Results.

RESULTS

tal-1 promotes proliferation and inhibits RA- and VitD3-induced differentiation of HL-60 cells via the basic and HLH domains. An LXSN-based *TAL-1* retroviral vector and a high-titer producer AM12 cell line were constructed due to the difficulty of transfecting HL-60 cells by electroporation.

To characterize the molecular domain(s) responsible for the tal-1 effects, mutants of tal-1 in LXSN-based vectors and packaging cell lines were also produced (Fig. 1). The B2 mutant is a full-length tal-1 molecule with a double point mutation (R188G/R189G) in the basic domain, which abolishes the binding of the tal-1/E2A heterodimer to the DNA consensus sequence in vitro (27). However, the B2 mutant retains the ability to interact with E12 or E47 protein and thus represents a dominant-negative mutant that disrupts E-protein functions such as homodimerization and DNA binding (27). The H2 mutant bears a double point mutation in the second helix of the cDNA; it does not dimerize with E2A protein in vitro and thus represents an inactive (recessive-negative) tal-1 mutant (27). M3 is a tal-1 variant, isolated from a T-ALL patient and often coexpressed in the malignant cells with wt tal-1; it specifies a 22-kDa protein and includes the bHLH–C-terminus part of the tal-1 molecule (10).

After retroviral transfer in HL-60 cells and Geneticin selection, wt and mutant tal-1 proteins were expressed at significant, comparable levels, as evaluated by RT-PCR, Western blotting (Fig. 2A), immunofluorescence (not shown), and EMSA (Fig. 2B), under standard culture conditions. The gel shift assay of the protein-oligonucleotide complexes representing tal-1/E2A heterodimers also included self-competition by a cold E box and lack of competition by an E-box mutant (see Materials and Methods) or a supershift with anti-tal-1 and anti-E2A sera (Fig. 2C). The expression after differentiative stimuli (Fig. 2B) is discussed below. Furthermore, starting from the bulk HL-60 tal-1 cells, we have isolated by limiting dilution two subclones (clones 22 and 24) exhibiting a particularly elevated tal-1 expression (see Materials and Methods).

(i) Effect on cell proliferation. Bulk culture results are shown in Fig. 3A. (i) In 1% FCS medium HL-60 tal-1 cells showed a more elevated proliferative rate than HL-60 neo control cells; interestingly, the proliferative effect of the M3 variant was similar to that of the full-length tal-1 polypeptide, while B2 and H2 exerted a less marked stimulus and a modest stimulus, respectively. In all groups, apoptotic cells were barely detected (<1% annexin V-positive cells); the wt or mutant tal-1 effects

were mediated by a corresponding increase of the frequency of S-phase cells (results not shown). (ii) Under optimized culture condition, i.e., in 10% FCS medium, the proliferative rates did not significantly differ among groups (similar results were obtained in two other experiments). Interestingly, tal-1 clone 22 and 24 cells exhibited a growth advantage over neo cells at 10% FCS and even more at 1 or 0% FCS (Fig. 3B).

(ii) Effect on RA-induced differentiation. In HL-60 neo cells, RA (10^{-6} M) induced a wave of differentiation/maturation of the blasts to intermediate differentiated cells (with decreased size and condensed chromatin but devoid of the granules present in corresponding normal cells) reaching, at day 7 to 9, a level of up to 95% mature band neutrophilic cells; this was coupled with both a rise of $CD11a^+$/-$11b^+$ cell frequency and growth arrest (Fig. 4). Starting from day 7 to 9, the cells progressively undergo apoptosis (38; also, data not shown).

In HL-60 tal-1 bulk (Fig. 4A and B) and clone 22 (Fig. 4C) or clone 24 (not shown) cultures, RA-induced differentiation was sharply inhibited, i.e., the band neutrophil frequency at day 9 was only 50% for HL-60 tal-1 bulk cells and 25% for subclone 22, while the rise in $CD11a^+/CD11b^+$ cell frequency was similarly inhibited: cycling cells, present through day 12 and onward, were mainly represented by blasts (not shown). RA induced increased tal-1 expression compared with uninduced cells, as shown by gel shift assay and Western blotting (Fig. 2B and results not shown), which is seemingly mediated by enhanced transcription from the long terminal repeat promoter (12).

The M3 variant closely mimicked the effects of the full-length tal-1 polypeptide. Conversely, the B2 and H2 mutants exerted a modest inhibitory effect on cell differentiation, essentially restricted to the terminal stages, while they did not affect cell proliferation (Fig. 4A and B).

These results indicate that (i) ectopic tal-1 partially blocks RA-induced granulopoietic differentiation while maintaining blast cell growth and (ii) both the basic and the HLH domains of tal-1 are functionally required to mediate the RA effect on HL-60 differentiation and maturation.

(iii) Effect on VitD3-induced differentiation. To test whether the differentiation-blocking effect of tal-1 on HL-60 cells is restricted to the granulopoietic lineage, we explored the influence of tal-1 on monocytic differentiation (Fig. 5) (51). HL-60 neo and tal-1 cells were treated with 100 ng of VitD3, and the effects on cell proliferation and monocytic differentiation/maturation were monitored through day 12. neo cells showed (i) a block of proliferation and progressive rise in the number of monocytes, associated with an inverse decrease in the number of undifferentiated cells, as well as a gradual increase in the number of $CD11b^+/CD14^+$ cells; (ii) slower differentiation and growth arrest than those observed after RA treatment; and (iii) terminal maturation coupled with cell death, as observed for granulocytes after RA addition (see above). Conversely, tal-1 cells showed exponential growth and only modest differentiation/maturation, as evaluated in terms of morphology and $CD11b^+/CD14^+$ cell frequency. This pattern was observed for tal-1 bulk (Fig. 5A) and clone 22 (Fig. 5B) and 24 (not shown) cultures. The M3 variant mimicked the antidifferentiative and proliferative effects of tal-1. The B2 and H2 mutants exerted modest inhibitory effects on monocytic differentiation, while they did not significantly affect cell proliferation.

Altogether, these results indicate that (i) ectopic tal-1 expression partially blocks both the differentiative response and the growth arrest response to VitD3 and (ii) both the basic and the HLH domains are involved in the monocytic differentiation blockade.

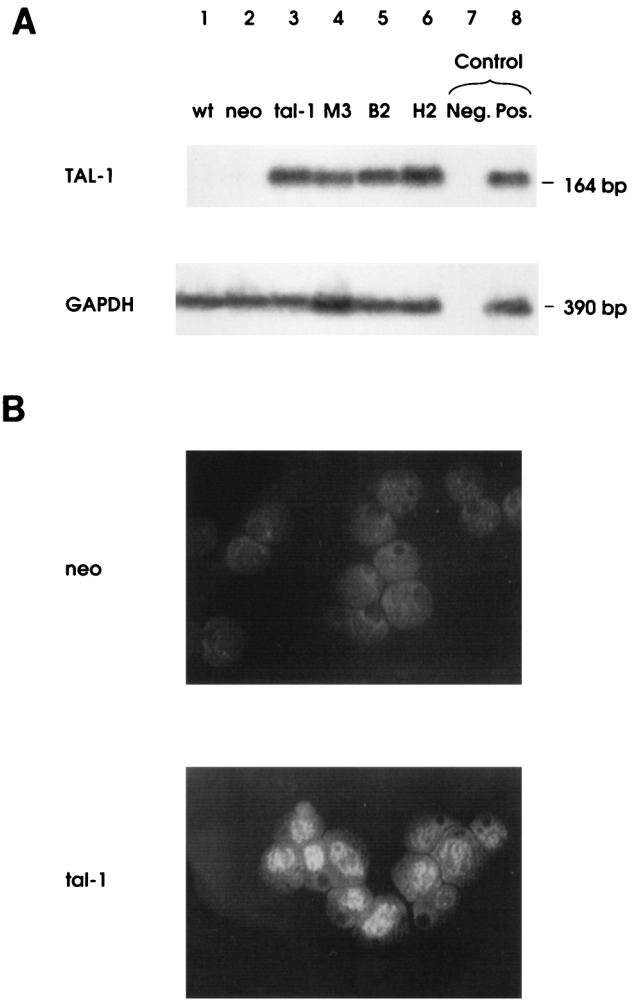


FIG. 6. (A) Semiquantitative RT-PCR assay of *TAL-1* mRNA in 32D cells, transduced with neo, wt *TAL-1*, or the M3, B2, or H2 mutant (lanes 2 to 6). Positive and negative (Pos. and Neg.) controls (lanes 7 and 8) are detailed in Materials and Methods. Untransduced 32D wt cells were also used as negative control (lane 1). GAPDH was used for normalization. (B) Immunofluorescence of 32D neo or 32D tal-1 cells with an anti-tal-1 serum (no. 7742).

tal-1 enhances IL-3-dependent proliferation and inhibits G-CSF-induced differentiation in 32D cells: role of basic and HLH domains. In the 32D cell line, derived from murine HPC long-term culture, late myeloid HPCs proliferate in the presence of IL-3 and undergo granulopoietic differentiation when treated with G-CSF (55). The 32D cells, while containing a structural rearrangement of the env locus (30), closely mimic the physiological proliferative/differentiative program of myeloid HPCs in response to G-CSF; therefore, it seemed of particular interest to evaluate the effects of ectopic wt and mutant tal-1 expression in this cell model. Thus, 32D cells electroporated with neo, wt *TAL-1*, or the M3, H2, or B2 mutant were Geneticin selected; the transfected wt and mutant genes were expressed at significant, comparable levels, as evaluated by semiquantitative RT-PCR (Fig. 6A), Western blot (not shown), and immunofluorescence (Fig. 6B and results not shown) analyses.

The gene transfer effects on 32D cell proliferation were evaluated in the presence of saturating- or low-level IL-3 (1 or 0.01 ng/ml, respectively) (Fig. 7A to C). In bulk culture, tal-1

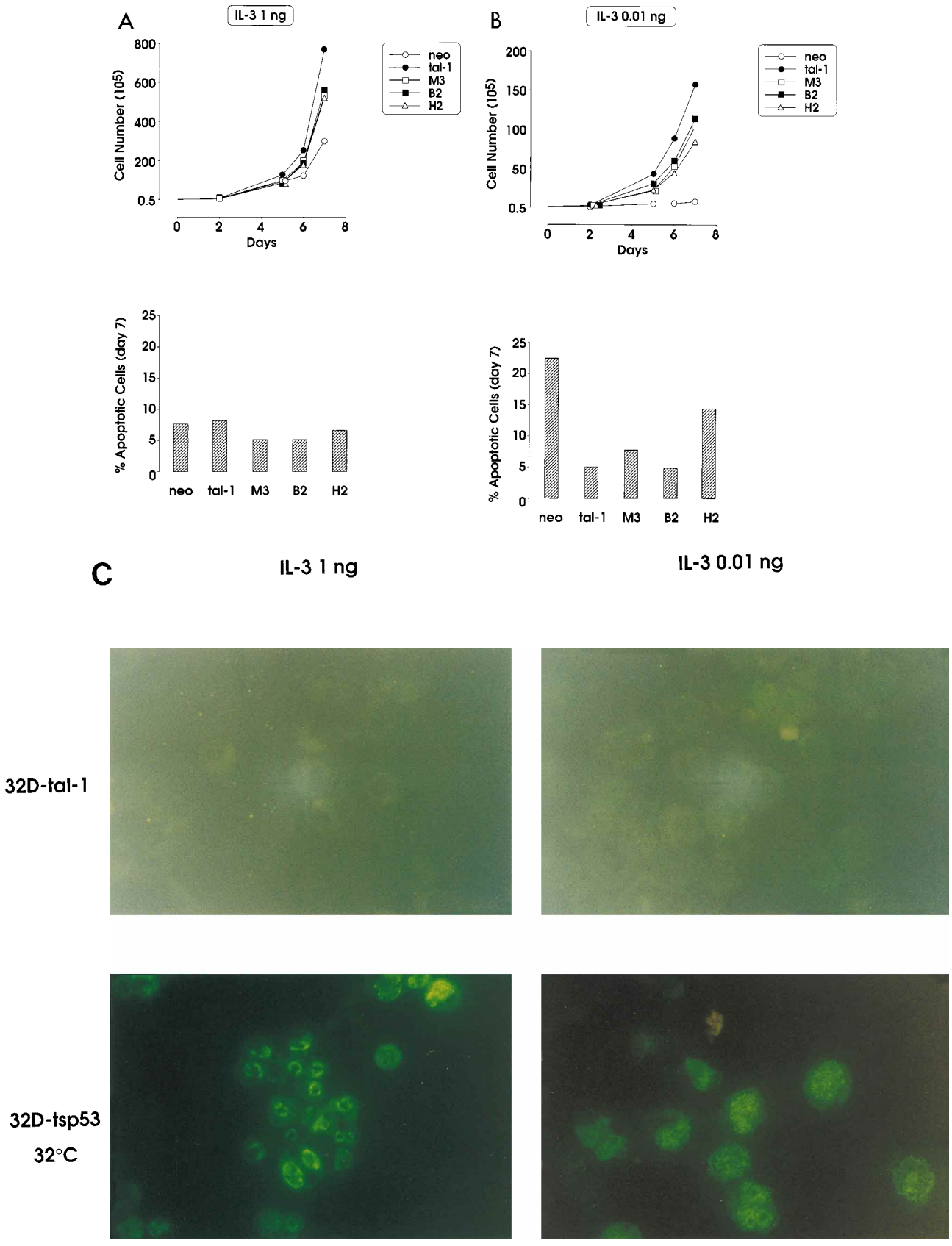

A
IL-3 1 ng
Cell Number (10⁵)
800
600
400
200
0.5
0
2
4
6
8
Days
neo
tal-1
M3
B2
H2
B
IL-3 0.01 ng
Cell Number (10⁵)
200
150
100
50
0.5
0
2
4
6
8
Days
neo
tal-1
M3
B2
H2
% Apoptotic Cells (day 7)
25
20
15
10
5
0
neo
tal-1
M3
B2
H2
% Apoptotic Cells (day 7)
25
20
15
10
5
0
neo
tal-1
M3
B2
H2
C
IL-3 1 ng
IL-3 0.01 ng
32D-tal-1
32D-tsp53
32°C

FIG. 7.

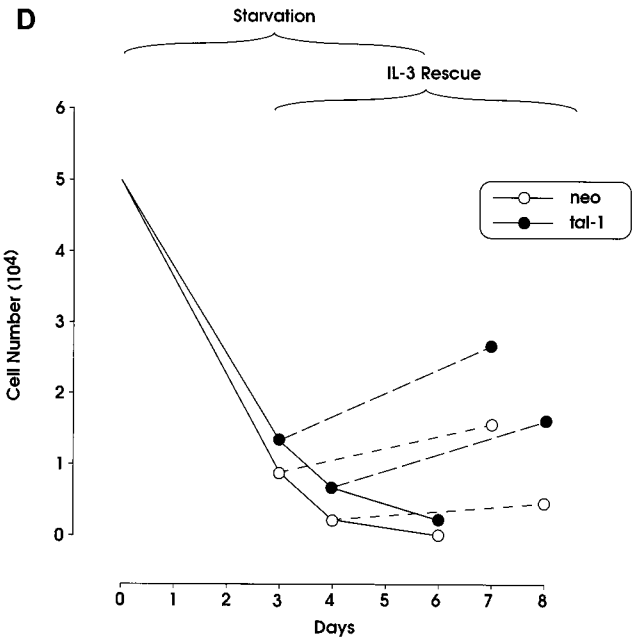


FIG. 7. Cell growth and apoptosis of 32D cells expressing neo, wt tal-1, or the M3, B2, or H2 mutant, grown in the presence of 1 (A) or 0.01 (B) ng of IL-3 per ml. The number of viable cells was determined at different days of culture (upper panels); the percentage of apoptotic cells (i.e., annexin V-positive cells) was evaluated at day 7 of culture (lower panels). (C) p53 immunofluorescence labeling in 32D tal-1 and 32D tsp53 cells. Cells were grown for 4 days at 32°C in the presence of either a low (0.01-ng/ml) or a high (1-ng/ml) IL-3 concentration, fixed, and stained by indirect immunofluorescence with the PAb246 anti-p53 MAb. (D) Cell growth rescue of 32D neo and tal-1 cells. After IL-3 starvation, at day 0, cells were rescued with 1 ng of IL-3 per ml from either day 3 or day 4. The number of viable cells was determined by the Trypan blue exclusion test.

significantly enhanced cell growth, particularly upon a suboptimal IL-3 stimulus, while the effects of the mutants (particularly B2) were less pronounced (Fig. 7A and B). The tal-1 proliferative effect was also observed for the tal-1 clones 2 and 14 (results not shown). In 32D neo cells the low IL-3 stimulus was associated with a high frequency of apoptotic cells, which was sharply lower in 32D tal-1 cells and in M3 and B2 clones; the H2 mutant displayed a less pronounced protective activity against apoptosis (Fig. 7B and results not shown). Conversely, the high IL-3 concentration induced a lower frequency of apoptotic cells, which did not differ significantly among groups (Fig. 7A and results not shown). Altogether, these data indicate that ectopic tal-1 expression in 32D cells induces a proliferative stimulus; at a low IL-3 level this action is coupled with an antiapoptotic effect, thus maximizing the difference between the tal-1 and neo cell growth curves. These effects are partially induced by the M3, B2, and H2 mutants; as an exception, H2 does not exert a significant antiapoptotic effect at a low IL-3 level.

It is noteworthy that 32D tal-1 cell proliferation in the presence of an elevated or low IL-3 level (1 or 0.01 ng/ml) is not coupled with detectable p53 expression, as evaluated by immunofluorescence (Fig. 7C; the positive control is represented by 32D tsp53 cells grown at permissive temperature) and Western blotting (49; also, data not shown).

Along these lines, experiments involving starvation followed by IL-3 rescue showed that the proliferative recovery was more pronounced for 32D tal-1 cells than for neo control cells (Fig. 7D).

Addition of G-CSF caused gradual 32D granulopoietic differentiation/maturation with ~80% terminal granulocytes at day 7 in the neo group (Fig. 8). Strikingly, ectopic tal-1 expression caused a virtually complete differentiation blockade (the frequency of tal-1$^+$ cells as determined by immunofluorescence was substantially unchanged from day 0 through day 7). This inhibitory action was also observed in M3 cells, except for initial blast differentiation, but was less pronounced in B2 and H2 cells. Accordingly, neo cells showed little proliferation, compared with tal-1, M3, and B2 cells, while H2 showed an intermediate growth pattern. Here again, it is apparent that both basic and HLH domains cooperate to mediate the differentiation blockade induced by ectopic tal-1 expression. It is noteworthy that in all these experiments cell overcrowding was avoided, i.e., cell density was $\leq 10^6$ cells/ml.

Effect of tal-1 on apoptosis in 32D tsp53 cells. In HL-60 cells both p53 alleles are inactive (59). In 32D cells the wt p53 is barely expressed if at all (7, 49) (Fig. 7C), as in normal human myeloid precursors (unpublished results).

Overexpression of p53 has been extensively used to study the effect of transforming oncogenes (45). To test this key aspect, we used a 32D cell line carrying the tsp53 Val-135 mutant gene (7). The overexpressed p53 Val-135 protein is stable and active at 32°C, while it is rapidly degraded and hence largely inactive at 39°C (33, 49).

It has been shown that the p53 apoptotic effect on the M1 murine leukemic cell line is blocked by a high, but not low, IL-6 concentration (60). Therefore, a series of experiments were performed at a low IL-3 level (Fig. 9A). At 32°C 32D tsp53 tal-1 cells showed a slower proliferation than neo control cells; in contrast, at 39°C the growth of 32D tsp53 tal-1 cells was more rapid, compared with controls. Control immunofluorescence labeling with anti-p53 Ab showed that 32D tsp53 neo or tal-1 cells, grown at a low IL-3 concentration, displayed a nuclear localization of p53 at 32°C and an almost complete cytoplasmic localization at 39°C (Fig. 9B and results not shown; see also Fig. 7C).

In order to understand whether these phenomena are mediated by modulation of apoptosis, cells were stained with annexin V and PI for fluorescence-activated cell sorter analysis (Fig. 9A). The percentage of apoptotic cells was then evaluated in 32D tsp53 neo and tal-1 cells. At 32°C the frequency of apoptotic cells was more elevated in tal-1 than neo cells, thus suggesting that the slower proliferative rate of tal-1 cells may be mediated by increased apoptosis; at 39°C the frequencies of apoptotic cells were similar for tal-1 and neo cells, thus unmasking the proliferative stimulus exerted by ectopic tal-1 expression.

In another set of experiments, we tested the ability of tal-1 to rescue IL-3-starved 32D tsp53 cells (Fig. 9C). At 32°C, 32D tsp53 tal-1 cells died early during starvation, while 32D tsp53 neo or 32D wt cells showed a slower death rate. At 39°C, tal-1 cells exhibited a proliferative rescue, which was absent in neo cells.

Altogether, these studies indicate that in 32D cells expressing p53 at permissive temperature ectopic tal-1 does not induce a proliferative effect but causes p53-mediated apoptosis.

DISCUSSION

The effects of ectopic tal-1 expression on cell proliferation, differentiation, and apoptosis are under scrutiny. In this regard, studies with M1 and TF1 leukemic cell lines suggest that tal-1 overexpression interferes with monocytic differentiation (24, 50, 51). However, these studies bear significant limitations. (i) Both cell lines express endogenous tal-1 (51; also, unpublished data); (ii) in M1 cells, enforced expression of exogenous

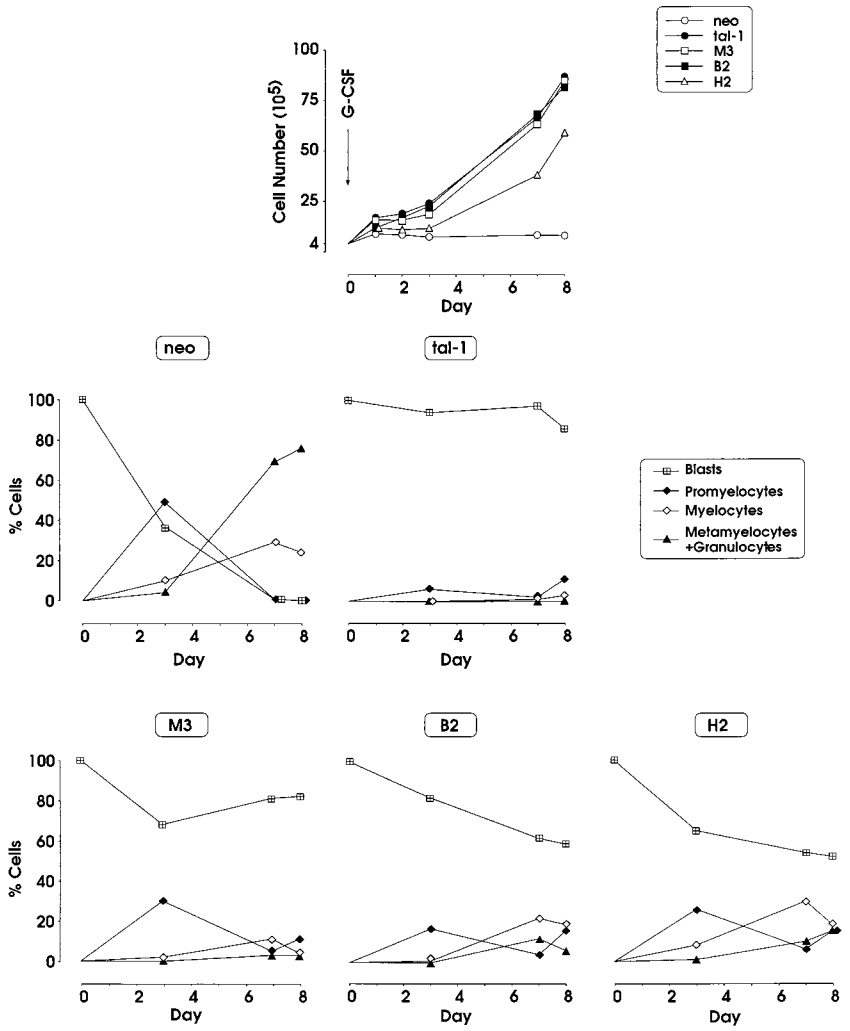


FIG. 8. (A) G-CSF-induced differentiation of 32D cells expressing neo, wt tal-1, or the M3, B2, or H2 mutant. Cell number (upper panel) and morphology (middle and lower panels) were determined. (B) Morphology analysis of 32D cells expressing neo, wt tal-1, or the M3, B2, or H2 mutant. 32D neo cells grown in the presence of IL-3 are shown at the upper left. At day 0, all groups had the same cell morphology.

tal-1 inhibited monocytic differentiation induced by the leukemia-inhibitory factor but not by IL-6; and (iii) in TF-1 cells with enforced expression of exogenous tal-1, cell differentiation was monitored on the basis of the expression of CD45 and FcγRII, which are not strictly specific for the monocytic lineage (in our experience, the TF-1 cell line cannot be induced into canonic monocytic differentiation [unpublished results]). On the other hand, *TAL-1* transduction in 5-fluorouracil-resistant hematopoietic precursors did not affect their differentiation along nonerythroid lineages after transplantation in recipient mice (17; also, see below). Circumstantial evidence on the effect of ectopic tal-1 on apoptosis has been provided by a subclone of the Jurkat T-ALL cell line which exhibits loss of ectopic tal-1 expression coupled with loss of viability at a low serum concentration (32).

The present results provide unequivocal evidence for the proliferative/antidifferentiative effects of ectopic tal-1 expression, shed light on the underlying mechanisms, and establish appropriate models for further investigation of these mechanisms.

We show that enforced ectopic tal-1 expression in leukemic (HL-60) and phenotypically normal (32D) myeloid precursors (i) stimulates cell proliferation, particularly under suboptimal culture conditions, and (ii) blocks granulopoietic and monocytic cell differentiation. It is noteworthy that in the murine 32D HPC line tal-1 induces both a marked proliferative effect at a low IL-3 level and a total differentiation blockade upon G-CSF addition; these results strongly suggest that ectopic tal-1 expression in normal myeloid HPCs is potentially oncogenic. Interestingly, aberrant tal-1 expression in acute myeloid leukemia patients is associated with a poor prognosis (47). More important, parallel studies by our group (14) indicate that in transgenic mice tal-1 exerts a leukemogenic effect upon ectopic expression in T cells.

As mentioned above, transduction of *TAL-1* in post-5-fluorouracil exposure primitive hematopoietic cells does not affect their differentiation pattern after transplantation into irradiated recipients (17). This study can be reconciled with our observations, showing an inhibitory effect of enforced tal-1 expression on granulocytic differentiation of both 32D cells and HL-60 cells. Indeed, tal-1 expression was enforced in sharply different target cells, i.e., in vivo-growing stem cells

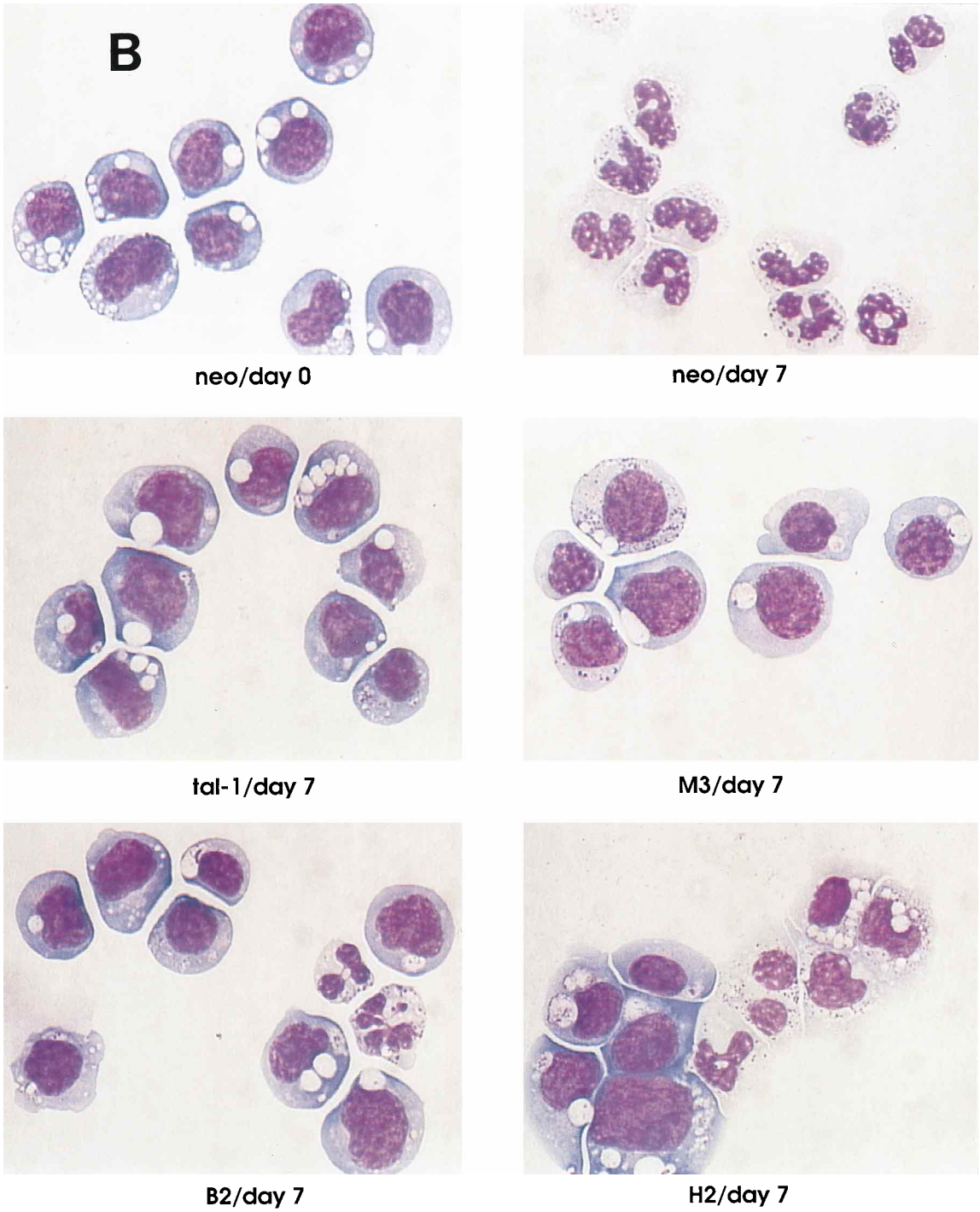


FIG. 8—*Continued.*

(CFU-S) (17) versus in vitro-growing late granulocytic progenitors (32D) or early leukemic precursors (HL-60) (this study). Also, *TAL-1* expression in myeloid precursors and mature cells, while not assayed at the protein level, was evaluated at the mRNA level by semiquantitative RT-PCR in myeloid colonies (17). Thus, it was not established whether tal-1 mRNA/protein was expressed in both early and intermediate-late precursors of the granulocytic and/or monocytic lineage. As previously mentioned, *TAL-1* expression in normal hematopoiesis is shut off at the level of early myeloid precursors (13). Furthermore, Porcher et al. (40) showed that constitutive tal-1 expression in tal-1$^{-/-}$ ES cells rescued monocytic differentiation, while granulopoietic rescue was not documented. Here again, the apparent divergence between these findings and our study can be reconciled in view of (i) differences between transduced cells, i.e., totipotent ES cells (40) and HL-60 leukemic precursors (this study), and (ii) different levels of tal-1 expression in transduced cells (see discussion in reference 40).

The proliferative and antidifferentiative effects of tal-1 are linked to the bHLH domain: both the HLH heterodimerization and the basic DNA-binding domains are functionally relevant. Thus, in HL-60 and 32D cells the H2 and B2 mutants, bearing a defective HLH or basic domain, respectively, partially exert the proliferative and antidifferentiative effects observed for wt tal-1 (however, in RA- or VitD3-treated HL-60 cells, B2 and H2 mutants induce a moderate block of cell differentiation but no growth stimulus, compared to a more pronounced differentiation blockade and a significant proliferative action exerted by wt tal-1; further studies will be required to elucidate this complex situation). Also, the truncated M3 variant, bearing a normal bHLH domain, closely mimics the tal-1 action in HL-60 cells, while exerting biological effects comparable to those observed for H2 and B2 mutants in the 32D cell line; this difference may relate to differential protein-protein interactions in these two cell systems.

tal-1 heterodimerizes with E2A protein to exert its effect, putatively via binding to the E-box sequence in target gene promoters (3). In normal erythropoiesis, the HLH protein Id2 blocks differentiation by competition with tal-1 for E2A binding (14). Similarly, *TAL-1* gene transduction impedes muscle differentiation by sequestering E protein and preventing heterodimerization with the bHLH MyoD transcription factor; interestingly, this effect is abolished when HLH- but not basic-domain *TAL-1* mutants are transduced (19). In our studies on ectopic tal-1 expression, the HLH heterodimerization domain is relevant in mediating both proliferative and antidifferentiative effects; this suggests that the tal-1–E2A protein interaction underlies these phenomena. It is noteworthy that E protein per se has the ability to induce G_1 arrest when overexpressed in fibroblasts (39); hypothetically, the cell cycling stimulus induced by ectopic tal-1 may be partially accounted for by E-protein sequestration.

The mechanism(s) underlying the requirement for the tal-1 basic domain is a matter of speculation. The possibility exists that DNA binding of the tal-1/E2A heterodimer to target E-box sequences requires both tal-1 and E2A protein DNA-binding basic domains; alternatively or additionally, the tal-1 basic and HLH domains may cooperate to interact with another nuclear factor(s) involved in transcriptional control mechanisms, e.g., Lmo2/rbtn-2 (see the introduction).

It is noteworthy that the tal-1 antiapoptotic effect in 32D cells is not mediated by the basic domain. Conversely, the HLH domain is involved in this effect; this is in line with results for a Jurkat subclone, with a mutant tal-1 exhibiting loss of heterodimerizing capacity (32).

The phenomena described here in phenotypically normal or leukemic myeloid precursors may be of widespread significance, in light of the fact that ectopic tal-1 expression in SAOS-2 osteosarcoma and T98G glioblastoma cell lines similarly causes a proliferative effect under low serum culture conditions, mediated by decreased apoptosis or increased entrance into S phase, respectively (results not shown).

Overexpression of p53 has been extensively used to study the effect of transcription factor oncogenes. If p53 is overexpressed, forced expression of an oncogene promotes cell death, while in the presence of inactive p53 the putative oncogene enhances cell proliferation or becomes frankly oncogenic (45); this pattern has been observed for the *E2F* (42, 46) and *myc* (23, 34, 57) oncogenes, the latter being a bHLH transcription factor like tal-1.

To test this paradigm for ectopic tal-1 expression, we used a 32D cell line transduced with the tsp53 mutant gene (7). The overexpressed p53 protein is stable and active at 32°C (permissive temperature), while it is rapidly degraded and largely inactive at 39°C (nonpermissive temperature) (33, 49; also, this study). Aberrant tal-1 expression either stimulates proliferation or favors apoptosis, depending on p53 expression. Thus, we can state the following. (i) Under suboptimal culture conditions (i.e., low IL-3 concentration), enforced tal-1 expression favors cell proliferation and inhibits apoptosis in wt 32D cells, which do not express detectable p53 levels (7; also, this study). (ii) Under the same culture conditions, tal-1 increases apoptosis and impairs cell growth upon p53 overexpression in 32D tsp53 cells grown at permissive temperature; at nonpermissive temperature p53 function is largely lost (7, 49; also, this study), cell cycling is favored by tal-1, while apoptosis patterns in tal-1 and control cells are similar. (iii) IL-3 rescue experiments confirm the above results. (iv) The leukemogenic effect of tal-1 expression in T cells is dramatically potentiated in $p53^{-/+}$ transgenic mice (14).

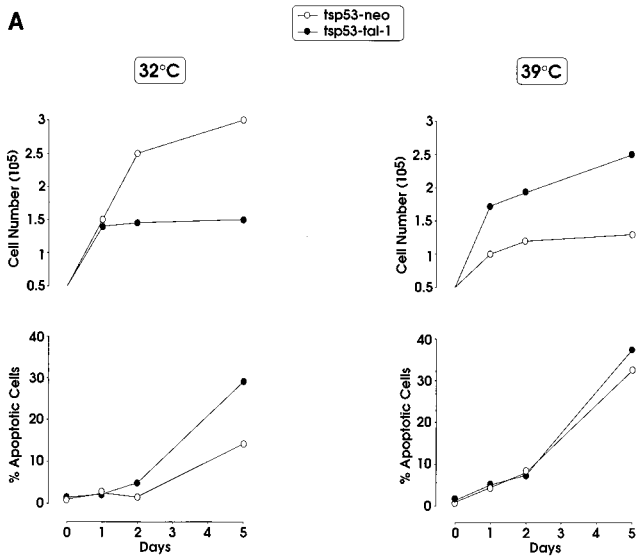


FIG. 9. (A) Cell growth and apoptosis of 32D tsp53 cells expressing neo or tal-1 grown in the presence of a low IL-3 concentration (0.01 ng/ml). Cells were grown at either 32°C or 39°C. Numbers of viable cells were determined by the Trypan blue exclusion test. Percentages of apoptotic cells (i.e., annexin-V-positive cells) are plotted below. (B) p53 localization in 32D tsp53 tal-1 cells at 32 and 39°C. Cells were grown for 4 days in the presence of a low (0.01-ng/ml) IL-3 concentration, fixed, and stained by indirect immunofluorescence with the PAb246 anti-p53 MAb. (C) Cell growth rescue of 32D wt, tsp53 neo, and tsp53 tal-1 cells, grown at either 32°C or 39°C. After IL-3 starvation at day 0, cells were rescued with 1 ng of IL-3 per ml starting from either day 3 or day 5. The number of viable cells was determined by the trypan blue exclusion test.

Altogether, the results indicate that aberrant tal-1 expression induces proliferative/antidifferentiative effects in diverse cell systems; this provides strong though indirect support for the oncogenic function of tal-1 ectopic expression. Furthermore, our studies shed light on mechanisms underlying the potential oncogenic effects of aberrant tal-1 expression: the tal-1 effects depend on the integrity of the bHLH domain of tal-1 and cell cycle checkpoints in the host cells, particularly p53. Finally, these studies provide novel experimental models (i.e., a leukemic cell line [HL-60] and a phenotypically normal late HPC line [32D]) to further investigate mechanisms underlying the effects of ectopic tal-1 expression.

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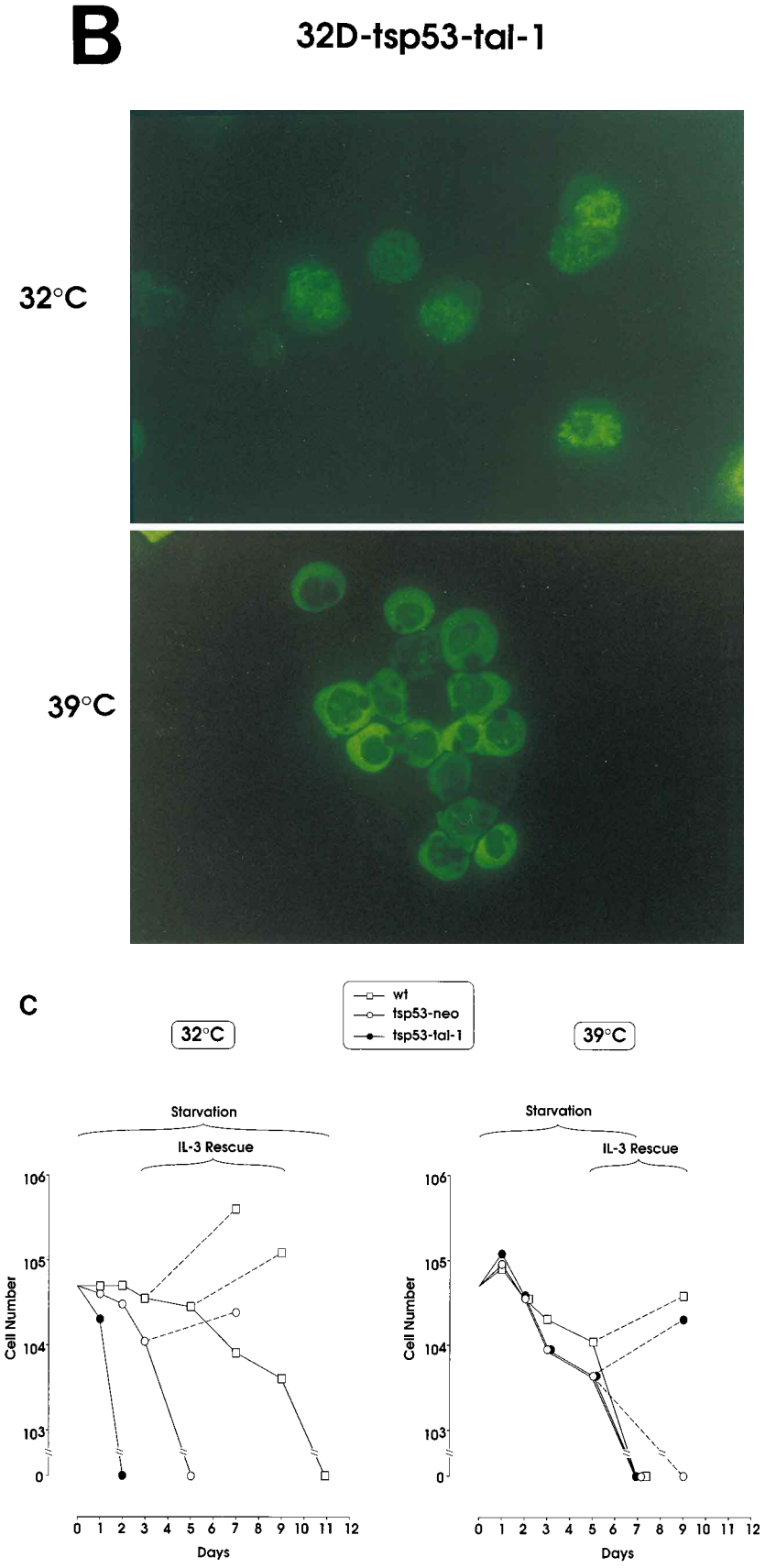

FIG. 9—*Continued.*

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