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# The E2A-HLF Oncoprotein Activates *Groucho*-Related Genes and Suppresses *Runx1*

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**The *E2A-HLF* fusion gene, formed by the t(17;19)(q22;p13) chromosomal translocation in leukemic pro-B cells, encodes a chimeric transcription factor consisting of the transactivation domain of E2A linked to the bZIP DNA-binding and protein dimerization domain of hepatic leukemia factor (HLF). This oncoprotein blocks apoptosis induced by growth factor deprivation or irradiation, but the mechanism for this effect remains unclear. We therefore performed representational difference analysis (RDA) to identify downstream genetic targets of E2A-HLF, using a murine FL5.12 pro-B cell line that had been stably transfected with E2A-HLF cDNA under the control of a zinc-regulated metallothionein promoter. Two RDA clones, designated RDA1 and RDA3, were differentially upregulated in E2A-HLF-positive cells after zinc induction. The corresponding cDNAs encoded two WD40 repeat-containing proteins, Grg2 and Grg6. Both are related to the *Drosophila* protein Groucho, a transcriptional corepressor that lacks DNA-binding activity on its own but can act in concert with other proteins to regulate embryologic development of the fly. Expression of both Grg2 and Grg6 was upregulated 10- to 50-fold by E2A-HLF. Immunoblot analysis detected increased amounts of two additional Groucho-related proteins, Grg1 and Grg4, in cells expressing E2A-HLF. A mutant E2A-HLF protein with a disabled DNA-binding region also mediated pro-B cell survival and activated Groucho-related genes. Among the transcription factors known to interact with Groucho-related protein, only RUNX1 was appreciably downregulated by E2A-HLF. Our results identify a highly conserved family of transcriptional corepressors that are activated by E2A-HLF, and they suggest that downregulation of RUNX1 may contribute to E2A-HLF-mediated leukemogenesis.**

Transcription factor genes that regulate blood cell development are the most frequent targets of chromosomal translocations in the acute leukemias (31, 45). The chimeric or dysregulated proteins resulting from these rearrangements contribute to leukemogenesis, most likely by aberrantly controlling the expression of downstream genes critical for the growth, differentiation, or survival of hematopoietic progenitors. The *E2A* gene, a member of the basic helix-loop-helix (bHLH) family, encodes two alternatively spliced bHLH transcription factors, E12 and E47, which act synergistically to promote B-lymphocyte maturation (2, 3, 53, 54). *E2A* is targeted by two translocations in the human leukemias. The t(1;19) generates the E2A-PBX1 chimera, consisting of the AD1 and AD2 transactivation domains of E2A fused to the homeobox DNA-binding domain of PBX1 (26, 38), while the t(17;19) links the same two domains of E2A with the bZIP DNA-binding and protein dimerization domain of hepatic leukemia factor (HLF) (17, 21).

To elucidate the mechanism by which E2A-HLF subverts normal developmental pathways, we programmed t(17;19)-positive leukemia cells to express a dominant-negative suppressor of the oncoprotein (20). When E2A-HLF function was blocked, the transformed cells died by apoptosis, suggesting that the oncoprotein affects cell survival rather than cell growth. This interpretation was substantiated by additional experiments in which E2A-HLF reversed both apoptosis by interleukin-3 (IL-3) deprivation and p53 activation in murine pro-B lymphocytes (20). To determine the structural motifs that contribute to the antiapoptotic activity of E2A-HLF, we constructed a panel of E2A-HLF mutants and programmed their expression in IL-3-dependent murine pro-B cells, using a zinc-inducible vector (22). Neither the E12 nor the E47 product of the *E2A* gene nor the wild-type HLF protein was able to protect the cells from apoptosis induced by IL-3 deprivation. Surprisingly, different combinations of disabling mutations within the HLF bZIP domain had little effect on the antiapoptotic property of the chimeric protein, so long as the amino-terminal portion of E2A was left intact. In the context of an intact HLF bZIP domain, the AD1 but not the AD2 transactivation domain was required for antiapoptotic activity. However, in constructs with a defective bZIP domain, either transactivating region (AD1 or AD2) could promote cell survival after growth factor deprivation. Thus, the block of apoptosis imposed by E2A-HLF in pro-B lymphocytes depends critically on the transactivating regions of E2A. Our findings suggest dual mechanisms of E2A-HLF action, one in which the AD1 and bZIP domains act cooperatively to block apoptosis and another in which protein-protein interactions with the amino-

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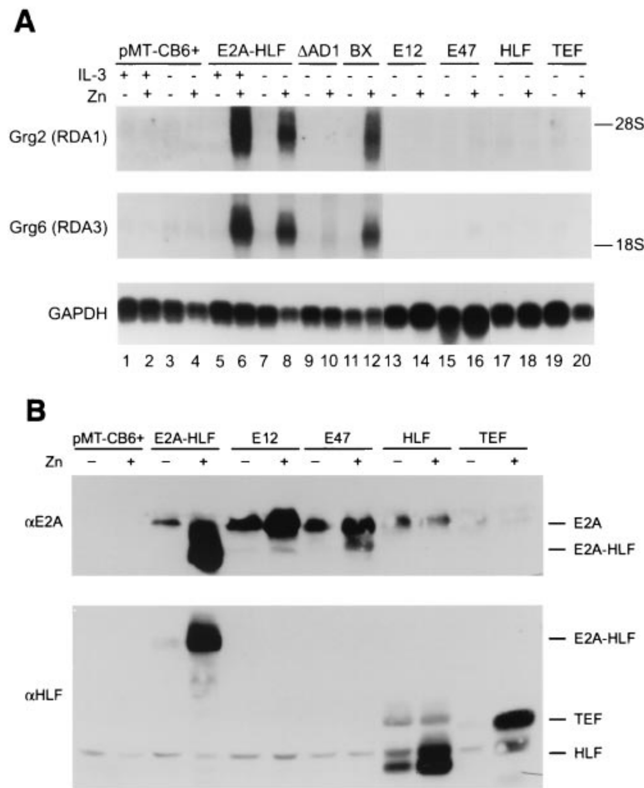


FIG. 1. Upregulation of expression of the Grg2 (RDA1) and Grg6 (RDA3) genes induced by E2A-HLF. (A) Northern blot analysis of poly(A)$^+$ RNA (1 μg per lane) prepared from FL5.12 cells stably transfected with the pMT empty vector (lanes 1 to 4) or with metallothionein promoter-regulated constructs containing cDNAs for E2A-HLF (lanes 5 to 8), the ΔAD1 E2A-HLF mutant lacking the AD1 transactivation domain of E2A (lanes 9 and 10), the BX E2A-HLF mutant with a disabled HLF DNA-binding domain (lanes 11 and 12), E2A (E12 [lanes 13 and 14] and E47 [lanes 15 and 16]), HLF (lanes 17 and 18), or TEF (lanes 19 and 20). Cells were cultured in IL-3-containing medium with (+) or without (−) 100 μM $ZnSO_4$ for 18 h to induce expression of the transfected cDNA and then continued in the same concentration of zinc and either maintained in IL-3 (+) or deprived of the cytokine (−) for an additional 12 h before RNA extraction. The blot was hybridized with the Grg2 (RDA1), Grg6 (RDA3), or GAPDH cDNA probe as indicated. The mobilities of the 18S and 28S rRNAs are shown. (B) Immunoblot analysis of FL5.12 cells transfected with E2A-HLF, E12, E47, HLF, or TEF protein. To verify that expression of the indicated genes was upregulated by zinc addition, lysates were prepared from FL5.12 cells and analyzed by immunoblotting with E2A rabbit antisera (αE2A) (top) and HLF(C) rabbit antisera (αHLF) (bottom). Cells were stably transfected with the pMT empty vector (pMT-CB6+) or with metallothionein promoter-regulated constructs containing cDNAs for E2A-HLF, E2A (E12 and E47), HLF, or TEF, with or without the addition of zinc as described for panel A.

terminal region of E2A act to block the expression of genes that normally control the apoptotic machinery of pro-B cells (22).

In this study, we used representational difference analysis (RDA) to identify genes that are differentially regulated in response to enforced expression of E2A-HLF in murine FL5.12 pro-B cells. Two such genes, designated *Grg2* and *Grg6*, are related to the *Drosophila* gene *Groucho*, which encodes a protein that acts as a transcriptional corepressor, interacting

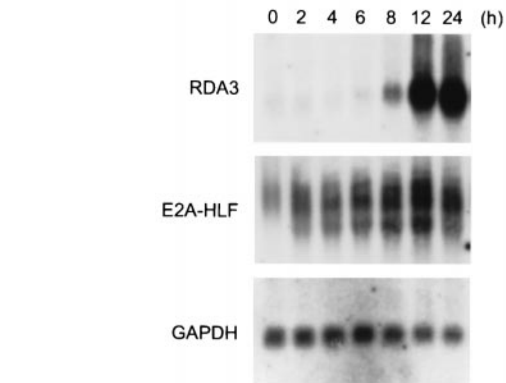


FIG. 2. Time course of RDA3 (*Grg6*) mRNA expression in response to E2A-HLF. Northern blot analysis was performed of total RNA (10 μg per lane) isolated from FL5.12 cells stably transfected with the metallothionein promoter-regulated E2A-HLF construct. Cells were cultured in IL-3-containing medium for the indicated times in hours after addition of 100 μM $ZnSO_4$. The blot was hybridized with the RDA3 (*Grg6*), *E2A-HLF*, or *Gapdh* cDNA probe as indicated.

with specific subsets of DNA-binding transcription factors. Immunoblot analysis identified two other Groucho-related proteins, Grg1 and Grg4, whose genes were coordinately upregulated with Grg2 and Grg6. Among the transcription factors known to interact with the Groucho family of corepressors, only RUNX1 was specifically downregulated by E2A-HLF. Thus, Groucho-related proteins and the RUNX1 transcription factor are likely candidates to participate as downstream effectors in E2A-HLF-mediated oncogenesis.

## MATERIALS AND METHODS

**Cell culture and cell survival assay.** IL-3-dependent FL5.12 murine pro-B cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and 10% WEHI-3B-conditioned medium (as a source of IL-3). The cell number was maintained below $10^6$/ml to avoid IL-3-independent growth. Cell lines expressing E2A-HLF or E2A-HLF mutants from the pMT-CB6+ eukaryotic expression vector (a gift from F. Rauscher III, Wistar Institute, Philadelphia, Pa.) have been described previously (22).

For cell survival assays, protein expression was induced by treating cells with 100 μM $ZnSO_4$ for 16 h prior to growth factor deprivation. IL-3 was removed by repeated centrifugation in fresh medium lacking the cytokine, and the cells were adjusted to $5 \times 10^5$ per ml on day 0 and cultured without IL-3. Viable cell counts were determined in triplicate by trypan blue dye exclusion.

**RDA.** RDA was performed according to the method of Hubank and Schatz (16) with additional modifications. Poly(A)$^+$ RNAs were isolated from FL5.12 cells harboring the E2A-HLF construct and grown in the presence or absence of 100 μM $ZnSO_4$ by use of the FastTrack 2.0 mRNA isolation system (Invitrogen). Double-stranded cDNAs were synthesized with the Superscript cDNA synthesis kit (GIBCO-BRL) according to the manufacturer's protocol. Double-stranded cDNA (2 μg) isolated from both cells grown in the presence of zinc and cells deprived of zinc was digested with *Dpn*II, ligated with adapter, and amplified by PCR (16, 28). RDA fragments were isolated after three cycles of subtractive hybridization and selective PCR amplification with different adapters. The third difference products were digested with *Dpn*II and cloned into the *Bam*HI site of the pBluescript phagemid (Stratagene). Double-stranded plasmid DNA was prepared and sequenced, and findings were compared with the GenBank database by use of the BLAST program.

**Screening of cDNA library.** The full-length cDNAs of RDA1 and RDA3 (designated Grg2 and Grg6, respectively) were isolated by screening a cDNA library that was constructed with mRNA isolated from E2A-HLF-expressing Baf-3 mouse pro-B cells. The pBK-CMV phagemid was purified from positive clones by in vivo excision from the lambda ZAP II vector, using ExAssist helper phage (Stratagene). The GenBank accession numbers for *Grg2* and *Grg6* are AF145958 and AF145957, respectively.

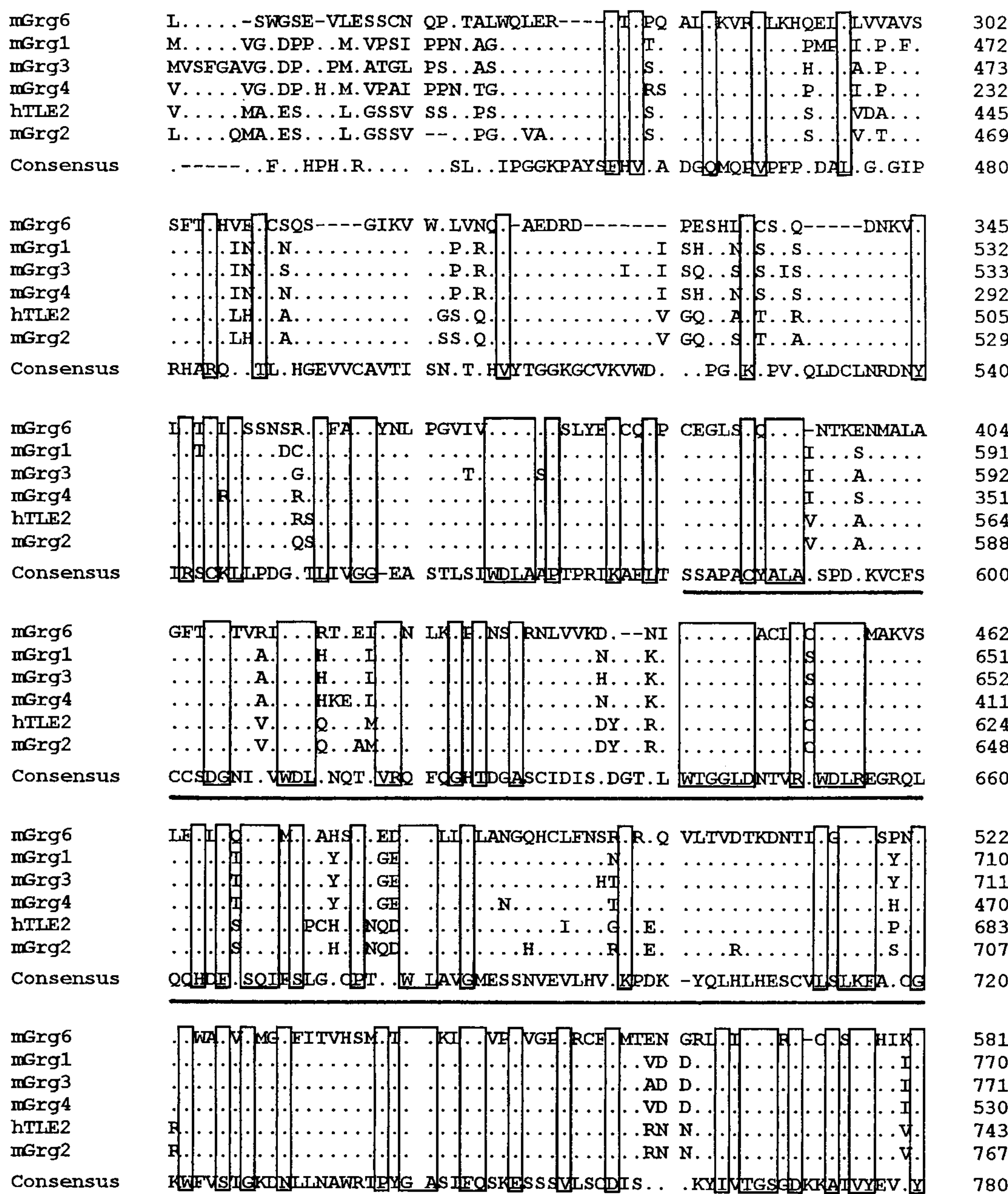

FIG. 3. Alignment of the Grg protein WD repeat domains. The C-terminal amino acid sequences of the newly identified Grg2 and Grg6 proteins are shown in comparison with the previously reported mouse (m) Grg1, -3, and -4 proteins and the human TLE2 sequences. Identical residues are boxed. Dashes indicate gaps in the alignment. The WD repeat domain is underlined.

**Northern blot analysis.** Two micrograms of mRNA or 10 μg of total RNA was subjected to denaturing agarose electrophoresis in 1 M formaldehyde and transferred to a nylon membrane (Stratagene). The blots were then hybridized with cDNA probes labeled with [$^{32}$P]dCTP. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control in quantifying the levels of mRNA expression. After hybridization, the blots were analyzed with a PhosphorImager (Molecular Dynamics).

**Protein expression.** Expression plasmids for human *TLE1* and *TLE2* and mouse *Grg6* were generated by subcloning the full-length coding sequences of these cDNA inserts into the pMT-CB6+ eukaryotic expression vector, which provides inducible gene expression under control of the sheep metallothionein promoter. This vector also contains the neomycin resistance gene (*neo*) driven by the simian virus 40 (SV40) promoter. A Kozak sequence was placed in front of ATG in these cDNA inserts.

**Antibodies and Western blot analysis.** Rabbit polyclonal antibodies were raised against the N terminus (3 to 68 amino acids; Bio-80) and middle portion (186 to 272 amino acids; Bio-81) of Grg6. A rabbit polyclonal antibody against HLF was made as previously described (23). The pan-transducin-like enhancer-of-split (TLE) monoclonal antibody C597.4A and rabbit polyclonal antibodies against amino acids unique to each human TLE protein (TLE1, TLE2, TLE3, and TLE4) were prepared as indicated previously (18, 50). A rabbit polyclonal antibody against Runx1 (AML1/RHD; Ab-2) was obtained from Calbiochem, and a goat polyclonal antibody against actin (C-11) was obtained from Santa Cruz Biotechnology.

Cells were lysed in NP-40 lysis buffer (150 mM NaCl, 1.0% NP-40, 50 mM Tris [pH 8.0]), and total cellular proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrotransfer onto nitrocellulose membranes, the blots were stained either with specific TLE anti-

bodies (1:2,000 dilution) and anti-Grg6 (1:400 dilution) followed by horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) or with a pan-TLE antibody (1:400 dilution) followed by an anti-rat IgG. The bands were visualized by enhanced chemiluminescence according to the manufacturer's instructions (Amersham).

**RNase protection analysis.** An RNase protection assay (RPA) was performed with the In Vitro Transcription/Ribonuclease Protection Assay Kit (PharMingen) according to the manufacturer's instructions. In brief, total RNA (20 μg) isolated from FL5.12 cells was hybridized with [$^{32}$P]UTP-labeled antisense RNA probes (~$10^7$ cpm per reaction mixture) at 56°C for 16 h, then heated at 90°C for 3 min, and digested with RNase. The templates from *Grg6* and *Runx1* for RPA were prepared by inserting a 199-bp PCR fragment of *Grg6* cDNA (bp 152 to 350) and a 310-bp *Runx1* fragment (bp 822 to 1131) into the pcDNA3 vector in antisense orientations relative to the T7 polymerase transcription origin. The 95-bp mouse *Gapdh* cDNA fragment (PharMingen) was used as an internal control.

**RT-PCR.** Total RNA was extracted from cells using the Trizol reagent (LTI, Inc.), by following the manufacturer's directions. Reverse transcription was performed on 2 μg of total RNA. RNA was resuspended in a final volume of 11 μl with 50 ng of random hexamer (Pharmacia Biotech), incubated at 80°C for 5 min, and cooled on ice. Reverse transcription was performed using 200 U of SuperScriptII reverse transcriptase (GIBCO-BRL) in the manufacturer's buffer in the presence of 0.5 mM deoxynucleoside triphosphates (dNTP)–1.0 mM dithiothreitol in a final volume of 20 μl, which was incubated at 42°C for 1 h. After the reaction, each sample was added to 1 μl of RNase H (GIBCO-BRL) and incubated at 37°C for 20 min. For the negative control, the reaction was also performed on each sample without reverse transcriptase (RT). cDNA samples were stored at −20°C. PCRs were performed using a DNA Thermal cycler (Perkin-Elmer). PCR mixtures contained 1 μl of cDNA, 1× reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 10 pmol of each primer, and 2.5 U of *Taq* polymerase (Perkin-Elmer) in a total volume of 50 μl. The thermocycling parameters and the sequences of the specific primers for PCR are as follows: for TLE1, 30 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min with sense primer 5′-AATCG CCAAGAGATTGAATA-3′ and antisense primer 5′-GTGCCTCTTAGACTG TCGG-3′; for TLE2, 40 cycles at 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min with sense primer 5′-AGCAGAGAGCAGATGAGAAG-3′ and antisense primer 5′-GAGAGGTCTCCGTTGAGAGT-3′; for TLE3, 30 cycles at 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min with sense primer 5′-TACGACAGTG ATGGAGACAA-3′ and antisense primer 5′-CATTATACCTATCGGGTCCA-3′; for TLE4, 30 cycles at 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min with sense primer 5′-GTTTGTAAGCACTGGAAAGG-3′ and antisense primer 5′-AAAAAGGATGACAGAGCAAA-3′; for AML-1, 40 cycles at 94°C for 30 s, 54°C for 30 s, and 72°C for 1 min with sense primer 5′-AGACATCGGCAGA AACTAGA-3′ and antisense primer 5′-CCAGGTATTGGTAGGACTGA-3′; for c-ABL, 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min with sense primer 5′-GTATCATCTGACTTTGAGCC-3′ and antisense primer 5′-GTAC AGGAGTGTTTCTCCA-3′. Amplification of c-ABL mRNA was performed to assess the quality of RNA extraction. These primer sets amplify a 342-bp product of TLE1, a 501-bp product of TLE2, a 501-bp product of TLE3, a 501-bp product of TLE4, a 253-bp product of AML-1, and a 288-bp product of c-ABL. Ten microliters of the PCR products was electrophoresed in a 2.0% agarose gel and transferred onto a nylon membrane (NEN Life Science) with 0.4 N NaOH. Membranes were hybridized with an internal probe end labeled with $^{32}$P using polynucleotide kinase, and autoradiography was performed. The sequences of the internal probes were as follows: for TLE1, 5′-ACCCTCCCTGACCTGTCT CG-3′; for TLE2, 5′-CTCATTGTGGCCCTTATGTA-3′; for TLE3, 5′-GAGA AATTGACTGCTCGAGT-3′; for TLE4, 5′-TAACTAAAGGTGAAAAGCTC C-3′; for AML-1, 5′-AACCCTCAGCCTCAGCGTCA-3′; and for c-ABL, 5′-T AACTAAAGGTGAAAAGCTCC-3′.

**Nucleotide sequence accession numbers.** The GenBank accession numbers for *Grg2* and *Grg6* are AF145958 and AF145957, respectively.

## RESULTS

**Identification of genes that are upregulated by E2A-HLF.** Overexpression of E2A-HLF protects pro-B cells from death induced by growth factor deprivation or irradiation (20). We have previously shown that IL-3-dependent murine pro-B cells (FL5.12 or BAF-3 cell lines) can survive in the absence of the cytokine when E2A-HLF expression is controlled by a metallothionein-regulated vector (20, 22). To identify downstream genes targeted by the E2A-HLF oncoprotein, we performed RDA on the mRNAs of FL5.12 pro-B cells shortly after removal of IL-3 from the culture medium. The so-called "tester" E2A-HLF$^+$ mRNAs were extracted from cells that had been incubated in the presence of both IL-3 and 100 μM $ZnSO_4$ for 18 h to induce E2A-HLF expression and maintained in the same concentration of zinc but deprived of IL-3 for an additional 12 h. The control or "driver" mRNAs were extracted

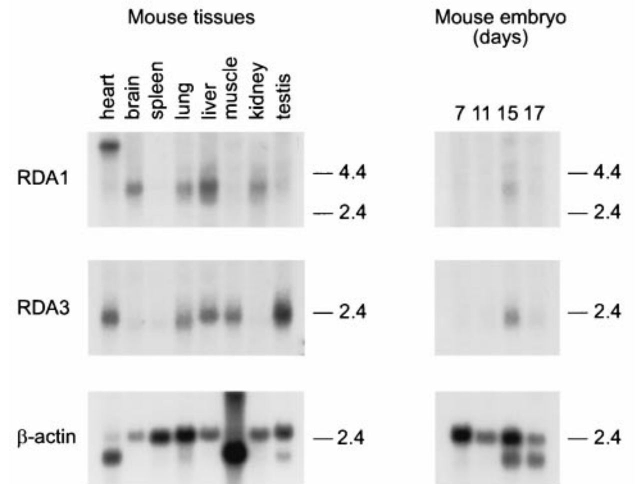


FIG. 4. Expression of the RDA1 (*Grg2*) and RDA3 (*Grg6*) genes in adult mouse tissues and embryos. Northern blot analysis was performed of poly(A)$^+$ RNA (2 μg per lane) from adult mouse tissues, including the heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis (left panel) and from mouse embryos from days 7, 11, 15, and 17 postconception (right panel). The blot was hybridized with the RDA1 (*Grg2*), RDA3 (*Grg6*), or human β-actin cDNA probe as indicated. Mobilities of size standards (in kilobases) are shown.

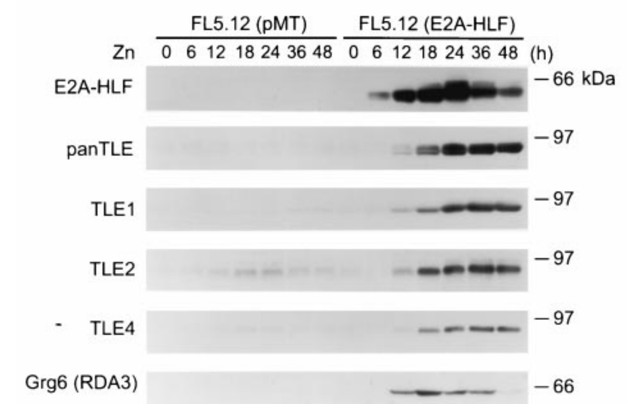


FIG. 5. Immunoblot analysis of Grg proteins induced by E2A-HLF. Extracts were prepared from cells stably transfected with either the pMT-CB6+ vector (left) or the metallothionein promoter-regulated E2A-HLF construct (right) after the cells were cultured in IL-3-containing medium for the indicated times in hours after the addition of 100 μM $ZnSO_4$. The blots were immunostained with the HLF(C) rabbit antiserum, the pan-TLE rat monoclonal antibody C597.4A, TLE1-, TLE2-, or TLE4-specific rabbit antisera, or the Grg6 (RDA3)-specific rabbit antiserum. Relative mobilities of molecular size markers (in kilodaltons) are shown.

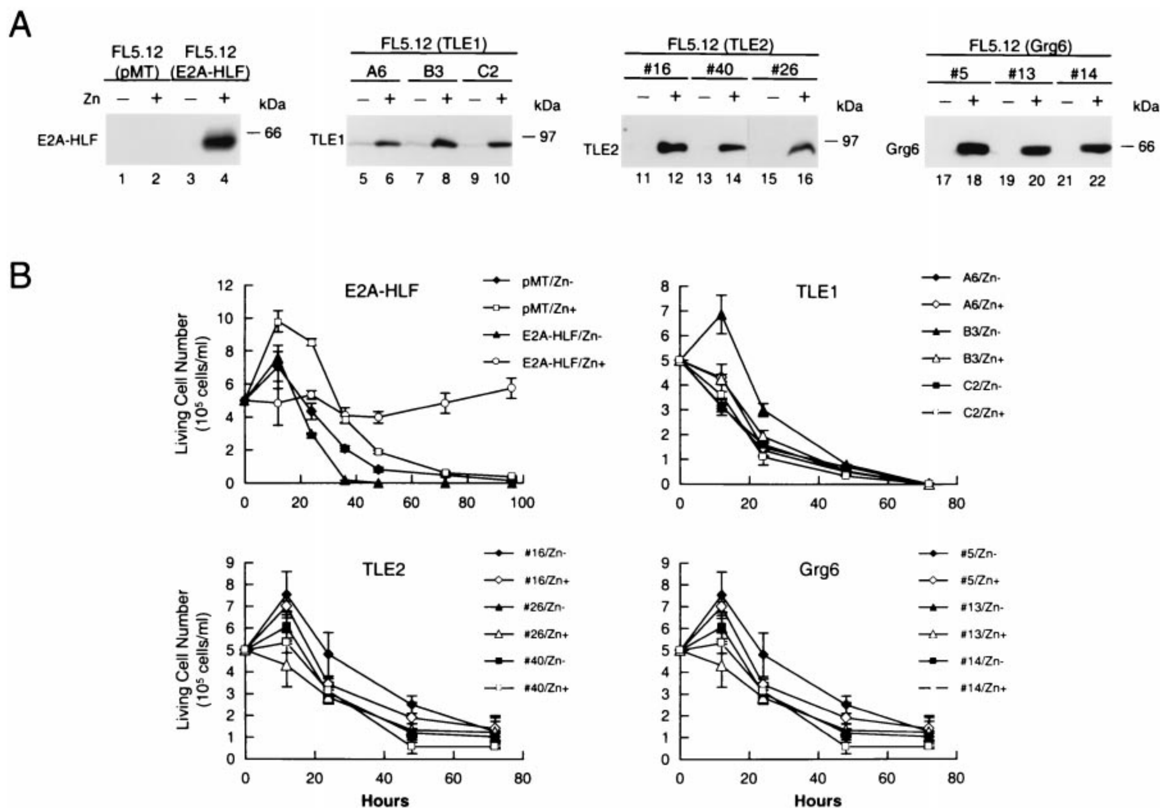


FIG. 6. Effects of E2A-HLF, TLE1, TLE2, and Grg6 on the survival of FL5.12 cells deprived of IL-3. (A) Immunoblot analysis of three independently derived clones of G418-resistant FL5.12 cells stably transfected with the indicated cDNAs under the control of the zinc-regulated metallothionein promoter, as well as controls transfected with the empty vector pMT-CB6+ or the vector containing E2A-HLF. Blots were stained with antisera recognizing E2A-HLF, TLE1, TLE2, and Grg6 (see the legend to Fig. 5). (B) Survival of FL5.12 cells expressing E2A-HLF, TLE1, TLE2, or Grg6 in the absence of IL-3. Cells growing exponentially in IL-3-supplemented medium for 16 h in the presence or absence of zinc (100 μM) were adjusted to $5 \times 10^5$ cells per ml on day 0, and viable cell counts were determined at the indicated times after the removal of IL-3.

from the same cell clone after identical treatment, except that E2A-HLF expression was not induced by the addition of zinc to the medium.

After three rounds of sequential hybridization and PCR amplification according to the RDA protocol of Hubank and Schatz (16), we isolated three clones (RDA1, RDA3, and RDA9) that were differentially expressed in E2A-HLF-positive cells compared with controls. RDA1 contains a 239-bp cDNA fragment that hybridized with an mRNA of approximately 3 kb (Fig. 1). This transcript was expressed at high levels in FL5.12 cells induced to express E2A-HLF, whether or not IL-3 was present in the culture medium (Fig. 1A, lanes 6 and 8). The same cells did not express detectable levels of the RDA1 mRNA in the absence of zinc (Fig. 1A, lanes 5 and 7). The 300-bp RDA3 cDNA fragment hybridized with an mRNA of approximately 2 kb in E2A-HLF-transfected FL5.12 cells (Fig. 1). RDA3 mRNA was also undetectable in control FL5.12 cells but was strikingly upregulated after addition of zinc in the presence or absence of IL-3 (Fig. 1A, lanes 6 and 8). A 454-bp RDA9 cDNA fragment also hybridized with a 2-kb transcript (data not shown). Subsequent analysis of the complete sequence of the cDNA clones identified by these probes showed that both RDA3 and RDA9 are represented in the 3′ and middle portions of the same mRNA.

There was no accumulation of RDA1 or RDA3 mRNAs in cells transfected with cDNAs encoding the intact E2A protein E12 or E47, wild-type HLF, or the HLF-related TEF, a bZIP transcription factor (Fig. 1A, lanes 13 to 20), indicating that the genes detected by RDA were specifically upregulated by E2A-HLF. To exclude the possibility that expression of these RDA fragments was induced nonspecifically by zinc, we analyzed mRNAs from cells transfected with the empty pMT-CB6+ expression vector. As shown in Fig. 1A, neither RDA1 nor RDA3 mRNAs were detectable in these cells, whether or not zinc was added to the medium (lanes 1 to 4).

In a recent study, we found that at least one of the transactivation domains of E2A is required for the antiapoptotic activity of E2A-HLF, regardless of the functional status of the HLF bZIP domain (22). To relate this observation to the ability of E2A-HLF to upregulate RDA1 and RDA3, we analyzed the mRNA levels of both genes, using FL5.12 cell lines that had been stably transfected with cDNAs encoding two mutant E2A-HLF proteins. As shown in Fig. 1A, the ΔAD1 mutant, which lacks the entire AD1 transactivation domain but possesses an intact HLF DNA-binding domain, failed to induce expression of either RDA1 or RDA3 (lanes 9 and 10). By contrast, a mutant defective in DNA-binding activity [E2A-HLF (BX)], as a result of substitutions for six critical amino acids in the HLF basic region (22), stimulated RDA1 and RDA3 expression as efficiently as the wild-type protein (Fig.

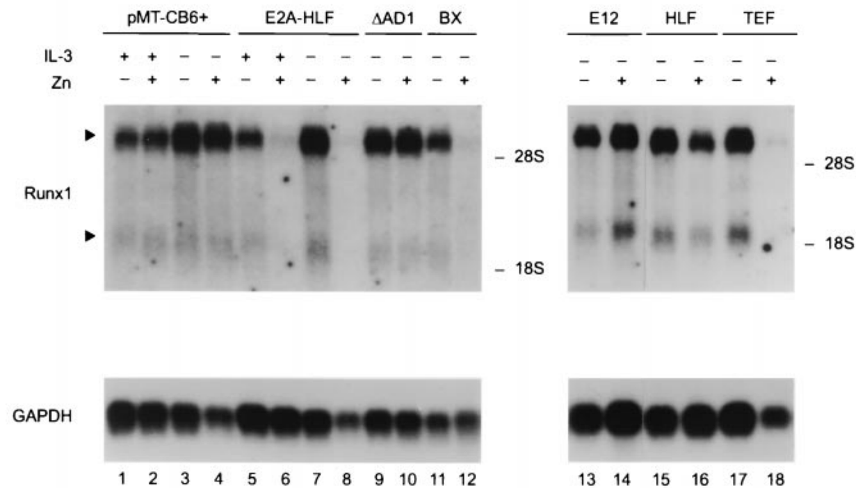


FIG. 7. Downregulation of expression of the *RUNX1* gene induced by E2A-HLF. Northern blot analysis was performed on poly(A)$^+$ RNA (1 μg per lane) prepared from FL5.12 cells expressing the indicated cDNAs under the control of a zinc-regulated metallothionein promoter. Cells were cultured in the presence or absence of zinc (100 μM) and IL-3 as described in the legend to Fig. 1. The blot was hybridized with the *RUNX1* or *Gapdh* cDNA probe as indicated. Mobilities of the 18S and 28S rRNAs are shown.

1A, lanes 11 and 12). Thus, activation of these candidate genes requires an intact AD1 domain but not sequence-specific DNA binding through the bZIP domain of HLF, mirroring the ability of these mutants to prolong cell survival in the absence of cytokine.

The timing of RDA3 mRNA accumulation relative to the induction of E2A-HLF expression is shown in Fig. 2. Low baseline levels of E2A-HLF maintained by the metallothionein vector increased markedly within 2 h after zinc addition, while expression of RDA3 mRNA was first detected 8 h after incubation of the cells with zinc, reaching maximal levels within 12 h. A similar kinetic profile was observed for RDA1 mRNA (data not shown).

**Both RDA1 and RDA3 are homologous to the *Drosophila* gene *Groucho*.** DNA sequence analysis revealed that RDA1 is closely related to the human gene *TLE2*, one of several mammalian genes related to the *Drosophila* gene *Groucho* (27, 29, 36, 50). Although murine homologues have been identified for most of the *TLE* genes, none has been found for *TLE2* (29). To clone the full-length coding sequence of RDA1, we isolated multiple cDNA clones from a library prepared from a Baf-3 murine pro-B cell line programmed to express E2A-HLF. The longest cDNA consisted of 2,749 bp, including one long open reading frame that encoded a 767-amino-acid protein (GenBank accession number AF145958) which did not correspond to any of the previously identified murine Grg proteins (29). Because of the high degree of amino acid sequence identity (87%) between the murine protein and human TLE2, we concluded that RDA1 represents the murine homologue, Grg2 (Fig. 3). The predicted Grg2 protein contains a 23-amino-acid insertion near its amino terminus that is not shared with TLE2, but otherwise these proteins are very highly conserved (90% amino acid identity).

Sequence analysis of two overlapping cDNA clones hybridizing to RDA3 and RDA9 revealed a span of 2,015 bp, including a single open reading frame that encodes a 581-amino-acid protein with a predicted molecular mass of 65 kDa (GenBank accession number AF145957). The main region of homology with previously identified Grg proteins was within the C-terminal domain containing the so-called WD40 repeats (29, 37). Short regions showing identity with the highly conserved amino acids of other Grg family members were also noted. Alignment of this protein, which we have designated Grg6, with the murine Grg1, -2, -3, and -4 proteins is shown in Fig. 3.

The tissue distribution of Grg2 and Grg6 mRNAs was determined in adult mice as well as in embryos. The 3.5-kb Grg2 transcript was detected predominantly in the heart, brain, and liver, with much lower levels found in the lung, muscle, kidney, testis, and spleen (Fig. 4). A larger Grg2 mRNA of approximately 6 kb was identified in the heart. The 2.2-kb Grg6 transcript was expressed predominantly in the heart, lung, liver, muscle, and testis and at very low levels in the brain, spleen, and kidney (Fig. 4). Like other *Groucho*-related genes (33), neither Grg2 nor Grg6 was expressed in embryos until 11 days postconception, with both mRNAs detected by day 15 (Fig. 4).

**Upregulation of a family of Groucho-related proteins in response to E2A-HLF expression.** Identification by RDA of two *Grg* genes that are upregulated by E2A-HLF suggested that increased amounts of other Grg family members might be detectable after enforced expression of the oncoprotein. As shown in Fig. 5, E2A-HLF expression was apparent within 6 h after addition of zinc to FL5.12 cells bearing the fusion construct and reached maximal levels by 24 h. To analyze the pattern of Grg protein expression, we first used a monoclonal antibody (C597.4A) (50) that recognizes all the human TLE proteins and then used antibodies specific for each of these proteins. The Grg proteins recognized by the pan-TLE antibody were expressed at low levels in murine FL5.12 cells before induction of E2A-HLF expression, with increased expression apparent 12 h after zinc addition (Fig. 5). Maximal expression of TLE proteins occurred after 24 h and was maintained through 48 h. The Grg1, Grg2, and Grg4 proteins

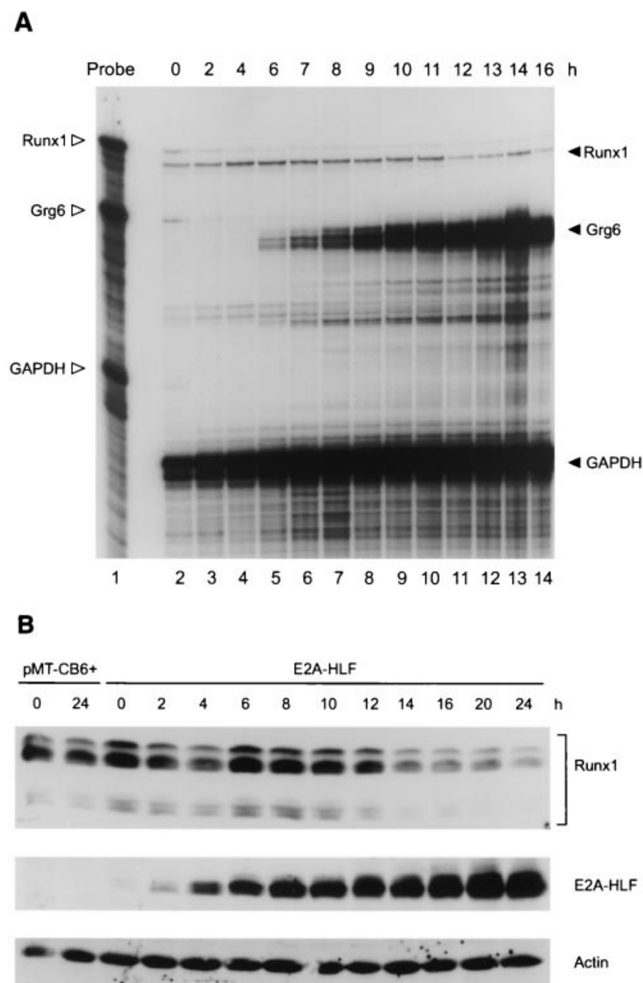


FIG. 8. Time course of *RUNX1* and *Grg6* mRNA expression in E2A-HLF-transfected FL5.12 cells. (A) Total RNA was isolated at different time points after addition of zinc (100 μM) and hybridized with *RUNX1*, *Grg6*, and *Gapdh* antisense mRNA probes. GAPDH was used as an internal control. The protected RNA fragments (solid arrowheads) were separated by an 8% sequencing gel. Open arrowheads, RNA probes. (B) Immunoblot analysis of Runx1 in E2A-HLF-transfected FL5.12 cells. Extracts were prepared from cells stably transfected with either the pMT-CB6+ vector or the metallothionein promoter-regulated E2A-HLF construct after the cells were cultured in IL-3-containing medium for the indicated times in hours after the addition of 100 μM $ZnSO_4$. The blot was immunoblotted with Runx1 rabbit antisera, HLF(C) rabbit antisera, and actin goat antisera (C11), respectively.

showed similar expression patterns (Fig. 5), while Grg3 was not detected at any time (data not shown).

*Grg6* encodes a predicted 65-kDa protein that was not recognized in FL5.12 cells by antibodies to the human TLE proteins. However, it was detectable by Western blot analysis with a polyclonal antibody to a recombinant polypeptide from the N-terminal region of the protein (see Materials and Methods). Grg6 expression was clearly upregulated by enforced expression of E2A-HLF, reaching maximal levels 18 h after zinc addition (Fig. 5). In contrast to results for the Grg1, Grg2, and Grg3 proteins, Grg6 expression was markedly reduced 48 h after zinc addition, despite continued expression of E2A-HLF.

**Lack of antiapoptotic activity by Grg proteins.** Since E2A-HLF protects IL-3-dependent murine pro-B cells from apoptosis due to IL-3 deprivation (20, 22), we asked whether any of the the Groucho-related proteins TLE1, TLE2, and Grg6 could replace this function in the absence of the oncoprotein. We therefore transfected FL5.12 cells with a zinc-regulated vector encoding either the murine Grg6 protein or human TLE1 or TLE2. Independent clones isolated by G418 selection expressed each of the proteins in the presence of 100 μM $ZnSO_4$ at levels approximately 10-fold higher than the background levels in medium lacking the metal (Fig. 6A). As shown in Fig. 6B, FL5.12 cells expressing E2A-HLF survived with little loss of viability, while those expressing TLE1, TLE2, or Grg6 rapidly underwent apoptosis after removal of IL-3 from the growth medium. These observations indicate that enforced expression of the individual Groucho-related proteins cannot replace the antiapoptotic function of E2A-HLF.

**Analysis of RUNX1 (PEBP2αb) and other transcription factors known to interact with Grg proteins.** The Groucho protein and its mammalian homologues lack DNA-binding domains and act as transcriptional corepressors, that is, they interact with DNA-binding transcription factors to downregulate gene expression (11, 41). Thus, we reasoned that E2A-HLF might block programmed cell death by regulating both a subset of the Grg proteins and one or more of the transcription factors with which these proteins interact. This would account for the failure of individual Grg proteins to block apoptosis in cells deprived of IL-3. In *Drosophila melanogaster*, Groucho interacts with discrete families of transcription factors, including Hairy, Runt, Engrailed, Dorsal, and Tcf, and acts as a corepressor with widespread roles in development (1, 5, 9, 25, 51).

In mammalian cells, Groucho-related proteins repress gene expression by interacting with several transcription factors, including RUNX1, LEF-1, Tcf and a family of Hes (Hairy-enhancer-of-split) proteins (15, 19, 30). Thus, we examined E2A-HLF-positive FL5.12 cells for evidence of such transcription factors. The only detectable candidate was RUNX1, which was markedly downregulated by E2A-HLF. As shown in Fig. 7, *RUNX1* mRNA levels in FL5.12 pro-B cells transfected with the pMT-CB6+ vector were not altered in response to IL-3 deprivation, whether or not zinc was added to the medium (Fig. 7, lanes 1 to 4). However, *RUNX1* mRNA levels were strikingly decreased in E2A-HLF-transfected cells grown for 30 h in the presence of zinc (Fig. 7, lanes 6 and 8), compared with the same cells grown in the absence of the metal. They were unaffected by the presence or absence of IL-3 (Fig. 7, lanes 5 and 7). We also tested the regulation of RUNX1 expression in FL5.12 cells transfected with two E2A-HLF mutants (ΔAD1 and BX). The AD1 domain of the E2A portion was required for this inhibitory effect, as deletion of this region completely abolished the downregulation of RUNX1 by E2A-HLF. By contrast, the E2A-HLF(BX) mutant was as potent as wild-type E2A-HLF in mediating RUNX1 downregulation, indicating that sequence-specific DNA binding by E2A-HLF is not required for this effect of the protein, as was previously observed for its antiapoptotic activity and its ability to upregulate the expression of *Groucho*-related genes. Enforced expression of E12 or wild-type HLF had little discernible effect on *RUNX1* mRNA levels, whereas TEF, a transcription factor

that recognizes the same DNA-binding motif as HLF, markedly downregulated *RUNX1* mRNA levels.

To determine the kinetics of the downregulation of *Runx1* in relation to the increase in the expression of *Groucho*-related genes induced by E2A-HLF, we used the RPA to examine their mRNA levels in E2A-HLF-expressing cells at serial times after zinc induction. Expression of Grg6 was induced 6 h after the addition of zinc (Fig. 8), with kinetics similar to those of Grg2 (data not shown) and the E2A-HLF protein (Fig. 5). By contrast, the decrease in the level of *RUNX1* mRNAs did not occur for an additional 6 h (Fig. 8), indicating that its change was temporally delayed compared with the increase in Groucho expression.

No change in expression of other genes that encode proteins interacting with Groucho-related proteins was detected in E2A-HLF-expressing cells. Most of them, such as those encoding Hes-1, Lef-1, and Sox4, were not detectable in FL5.12 cell RNA by RPA, while their expression was detected in mouse bone marrow cells, as shown in Fig. 9. The expression of other genes, such as *Tcf-1* and *En-1*, was detected in FL5.12 cells but was unchanged by E2A-HLF expression (data not shown).

**Runx1 expression levels are unaffected by enforced expression of Groucho-related proteins.** The Runx1 protein contains a WRPY motif at its carboxyl terminus, and Groucho-related proteins are known to interact through this motif to convert Runx1 from a transcriptional activator into a repressor (1, 19, 30). Furthermore, the *Runx1* promoter contains its own core DNA-binding motif, and published evidence suggests that *Runx1* may in part regulate its own expression (13). Thus, we tested whether overexpression of Groucho-related proteins alone in FL5.12 cells was sufficient to downregulate RUNX1 gene expression through an autoregulatory mechanism. The expression levels of Runx1 were tested by RPA in FL5.12 cells expressing zinc-inducible Grg proteins (see Fig. 6) and were unchanged in TLE1-, TLE2- and Grg6-transfected FL5.12 cells after addition of the metal (Fig. 10, lanes 6 to 11). Similarly, equal levels of the 310-bp protected band of *Runx1* were detected in control pMT vector-transfected cells in the presence or absence of zinc (Fig. 10, lanes 2 and 3). By contrast, the *Runx1* mRNA level in E2A-HLF-transfected cells was significantly decreased by the oncoprotein after addition of zinc (Fig. 10, lanes 4 and 5). As expected, the 199-bp protected *Grg6* band was detected only in E2A-HLF-positive and Grg6-positive cells, not in TLE1- and TLE2-transfected cells. These results suggested that expression of TLE1, TLE2, and Grg6 alone could not substitute for E2A-HLF in mediating the repression of Runx1.

**Expression of *Groucho*-related genes in early B lineage leukemias.** To assess the expression of the human homologues of *Groucho* in early B-lineage leukemia cells, we performed RT-PCR analysis of leukemic cell lines with and without the t(17;19) translocation. As shown in Fig. 11, all t(17;19)-positive cell lines, including UOC-B1, YCUB-2, and HAL-01, expressed mRNAs encoding TLE1, TLE2, and TLE4 (lanes 1 to 3). Moreover, TLE3 was expressed in HAL-01 cells but not in the other two t(17;19)-positive cell lines. Examples of RNAs from five other leukemia cell lines lacking t(17;19) are also shown in Fig. 11, including the Nalm6, REH, and 697 early B-lineage, Jurkat T-cell, and U937 monocytic cell lines. Like t(17;19)-positive cell lines, all these t(17;19)-negative cell lines except U937 expressed TLE1, TLE2, and TLE4, but only Jurkat and REH cells expressed TLE3. By contrast, expression level of RUNX1 was very low in all of the cell lines and could not be detected under the same PCR conditions used for the analysis of the TLE genes (data not shown). Thus, our analysis suggests that *Groucho*-related genes are expressed in human leukemias including those with the t(17;19) translocation and that the expression level of Runx1 is very low.

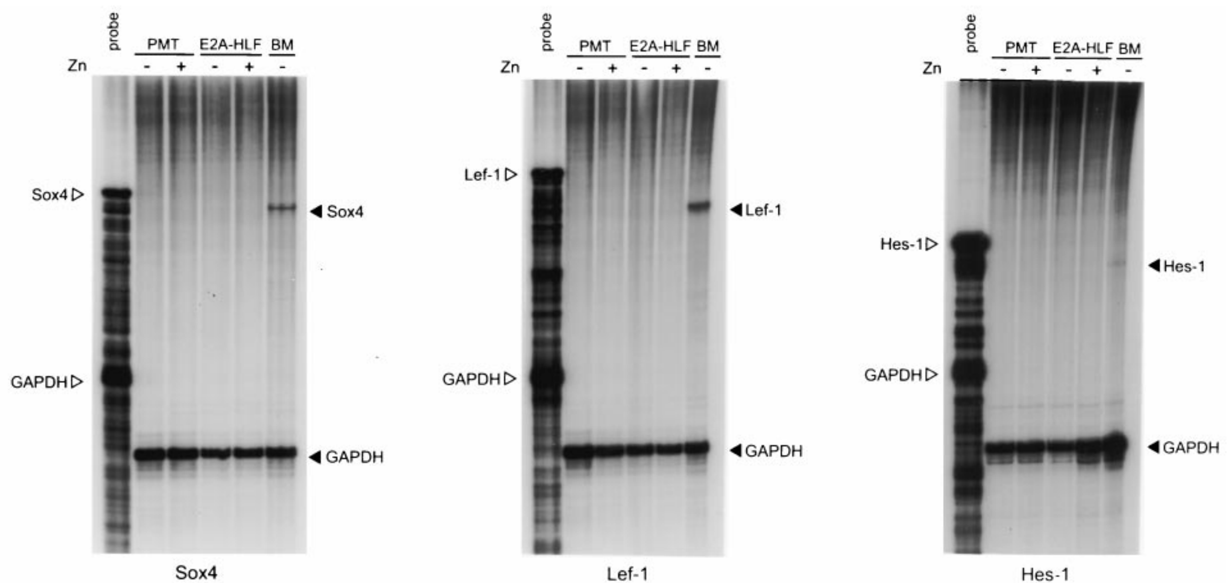


FIG. 9. RPA of *Hes-1*, *Lef-1*, and *Sox4* mRNAs. Total RNAs were isolated from FL5.12 cells stably transfected with plasmids expressing E2A-HLF or the pMT vector, with or without a 24-h incubation with zinc (100 μM), and from mouse bone marrow (BM) cells and were hybridized with *Hes-1*, *Sox4*, *Lef-1*, and *Gapdh* antisense RNA probes. GAPDH was used as an internal control. The protected RNA fragments (solid arrowheads) were separated by an 8% sequencing gel. Open arrowheads, RNA probes.

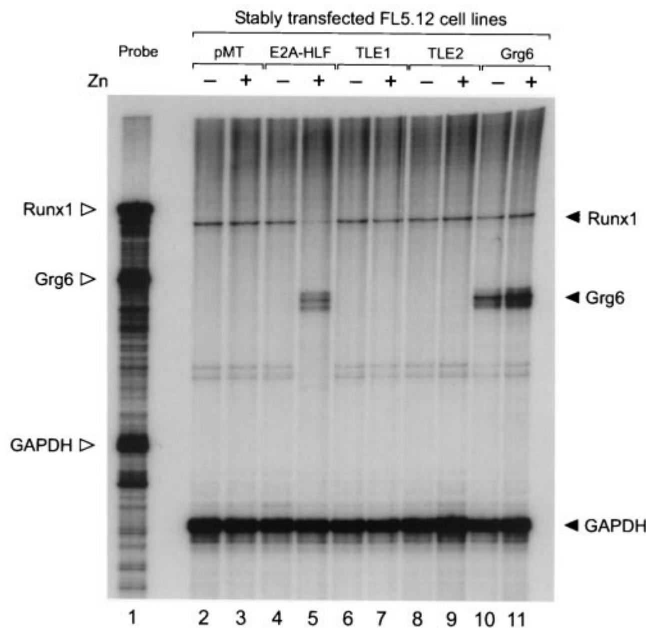


FIG. 10. Expression of RUNX1 and Grg6 in FL5.12 cells stably transfected with plasmids expressing E2A-HLF, TLE1, TLE2, and RDA3. Total RNA was isolated from cells with or without a 24-h incubation with zinc (100 μM) and was hybridized with *RUNX1*, *Grg6*, and *Gapdh* antisense RNA probes. Solid arrowheads, protected RNA fragments; open arrowheads, RNA probes.

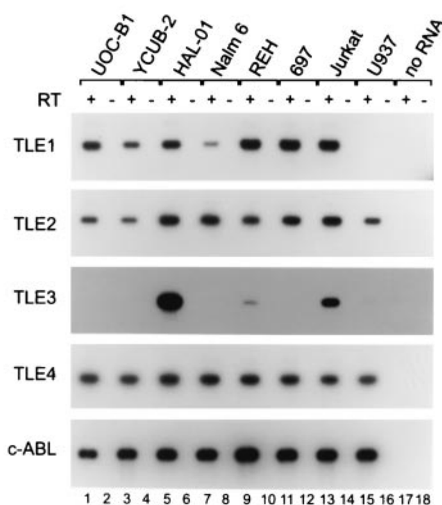


FIG. 11. RT-PCR analysis. Each RNA was transcribed with (+) (odd lanes) or without (−) (even lanes) RT. PCR products were transferred to a nylon membrane and analyzed by hybridization with end-labeled internal oligonucleotide probes specific for each gene. RNAs were evaluated for the UOC-B1 (lanes 1 and 2), YCUB-2 (lanes 3 and 4), and HAL-01 (lanes 5 and 6) t(17;19)-positive human leukemic cell lines and for the t(17;19)-negative Nalm6 (lanes 7 and 8), REH (lanes 9 and 10), and 697 (lanes 11 and 12) B-lineage leukemic cell lines, the t(17;19)-negative Jurkat T-lineage leukemic cell line (lanes 13 and 14), and the t(17;19)-negative U937 myeloid cell line (lanes 15 and 16). In addition, a control PCR lacking RNA template was performed (lanes 17 and 18).

## DISCUSSION

Many chimeric and dysregulated transcription factors have been identified in association with chromosomal translocations in the human acute leukemias (31). In recent years, major efforts have been made to link these genes and their mutated counterparts in multistep pathways that regulate programs of gene expression during development. Recent studies revealed that E2A-HLF protects IL-3-dependent pro-B cells from apoptosis induced by growth factor deprivation or irradiation, suggesting that this chimeric transcription factor may cause leukemia by interfering with apoptotic responses in normal cells. In this study, we used RDA to identify E2A-HLF-regulated genes, including two murine homologues of the gene encoding the Groucho developmental regulator in *Drosophila*, designated *Grg2* and *Grg6*. Analysis of other members of the Grg family revealed that Grg1 and Grg4 were also activated by E2A-HLF. Among transcription factors that have been shown to interact with Groucho-related proteins, we found that RUNX1, a frequent target of chromosomal abnormalities and inactivating point mutations in human leukemia (8, 40, 49), was suppressed by E2A-HLF.

The *Drosophila* Groucho protein is a widely expressed nuclear protein that has critical roles in many development processes (11, 41, 42). Groucho lacks a DNA-binding domain but contains a conserved C-terminal WD repeat domain that is present in a large number of proteins performing various cellular functions, including transcriptional regulation and signal transduction, and is believed to mediate protein-protein interactions (37, 48). In *Drosophila*, Groucho acts as a transcriptional corepressor to modulate gene expression through its associations with numerous transcription factors including Hairy, Runt, engrailed, Tcf, Dorsal, and Hkb (1, 5, 9, 14, 25, 51). These sequence-specific transcription factors recruit Groucho to the target gene promoters through different interaction domains. Hairy and Runt proteins interact with Groucho through their C-terminal WRPW or WRPY motifs (1, 12), whereas Dorsal binds Groucho through its Rel homology domain (52) and Tcf interacts with Groucho through an HMG domain that also mediates its interaction with CBP (46). Once targeted to the promoter region in a heterocomplex with one of these transcription factors, Groucho can actively repress both basal and activated transcription (11, 25). The mechanism underlying Groucho-mediated repression appears to be linked to the direct recruitment of histone deacetylase (HDAC) with its associated chromatin-modifying activities. More recently, it has been shown that the HDAC Rpd3 activity is required for Groucho-mediated transcriptional repression during *Drosophila* development (6). In addition, NK-3, a homeodomain transcription factor, has recently been shown to recruit both the Groucho corepressor and an HDAC complex with HIPK2 to repress gene expression (7). Many mammalian Groucho homologues have been identified in humans (TLE genes) and mice (Grg) (27, 29, 32, 36, 50). These mammalian Groucho family members are highly conserved and have similar functions in transcriptional repression (11, 15, 37). In mammalian cells, Groucho proteins have been shown to be involved in both Notch and Wnt signaling pathways through interaction with Hes and Tcf transcription factors, respectively (5, 10, 30, 35, 43, 44).

Activation of several Grg family members by E2A-HLF suggests the possibility that they may contribute to an E2A-HLF-mediated antiapoptotic pathway of leukemogenesis. In FL5.12 cells transfected with *Grg* genes, however, we did not see protection from cell death after growth factor deprivation, indicating that increased expression levels of Groucho proteins alone were insufficient to block apoptosis. Since Groucho acts as a corepressor in transcriptional regulation, we considered the possibility that specific DNA-binding transcription factors might be required to prevent apoptosis. In *Drosophila*, a number of transcription factors are known to interact with Groucho and control cell fate determination. Recently, it has been shown that Re1/NF-κB, a mammalian homologue of Dorsal, is involved in suppressing p53-independent apoptosis induced by Ras (34) and that inhibition of NF-κB activity induces apoptosis (4). Jehn et al. have reported that Notch-1 protects T-cell receptor (TCR)-induced apoptosis (24), suggesting that Hes, the downstream genes activated by Notch signaling, may be involved in cell survival. Among these Groucho-interacting transcription factors (including RUNX1, NF-κB, Hes-1, Tcf-1, Lef-1, and En-1), however, only the expression levels of RUNX1 were affected in E2A-HLF-expressing cells.

RUNX1 is affected by several of the most frequent chromosomal abnormalities associated with human leukemia (39, 40, 47). Since this transcription factor controls many genes that are important in hematopoiesis (40), downregulation of RUNX1 may play an important role in E2A-HLF-mediated leukemogenesis. TLE1 has been demonstrated to inhibit RUNX1-induced transactivation by interacting with this protein (19, 30), effectively converting RUNX1 from an activator to a repressor of gene expression. For example, in conjunction with TLE1, RUNX1 negatively regulates the activity of TCRα and TCRβ enhancers (30). Binding of TLE1 to RUNX1 was also found to suppress activities of the macrophage colony-stimulating factor and neutrophil elastase promoters (19). This binding is necessary for RUNX1-mediated repression, because RUNX1-induced transactivation was recovered when the interaction was abolished by deletion of the C terminus of RUNX1. Thus, downregulation of RUNX1 by E2A-HLF may block Groucho-mediated repression through AML pathways and provides another mechanism through which inhibition of RUNX1 contributes to leukemia in humans (8).

Upregulation of Grg proteins and downregulation of RUNX1 in response to E2A-HLF emphasize the fact that this chimeric oncoprotein is able to regulate gene expression both positively and negatively, either directly or through intermediate pathways of transcriptional control. Recently, we have shown that E2A-HLF-mediated prevention of apoptosis in IL-3-dependent pro-B cells depends critically on protein-protein interactions mediated by the intact transactivation domains of E2A (23). Data presented here support this mechanism, showing that E2A-HLF activation of *Groucho*-related genes depends on the AD1 transactivation domain of E2A but not on the DNA-binding activity of HLF. We have detected a significant increase in *Grg6* promoter activity in E2A-HLF-transfected cells (data not shown). Identification of E2A-HLF-responsive elements in the promoter regions of *Grg* genes will be required to implicate a direct effect of E2A-HLF in the activation of *Grg* genes.

Chimeric and dysregulated transcription factors, created by chromosomal translocation in the acute leukemias, act in concert with other mutations in multistep pathways leading to leukemogenesis. Identification of a family of *Groucho*-related genes and RUNX1 as potential downstream targets of the E2A-HLF oncoprotein provide direct evidence for its ability to profoundly alter the transcriptional status of early B-lineage cells. Although the relevance of Grg proteins to leukemogenesis has not been conclusively established, *RUNX1* is the gene most commonly disrupted by chromosomal abnormalities (8) and inactivated by point mutation in human leukemia (49). The functional analysis of downstream genes in E2A-HLF transcriptional programs may therefore provide useful insights into E2A-HLF-mediated survival advantages as well as leukemogenesis. In addition, identification of the transcription factor(s) involved in regulation of Grg and/or RUNX1 expression should be valuable for elucidating the mechanism by which the Groucho corepressors are activated by E2A-HLF.

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