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The E2A and tal-1 helix–loop–helix proteins associate *in vivo* and are modulated by Id proteins during interleukin 6-induced myeloid differentiation

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ABSTRACT **The immunoglobulin enhancer-binding proteins, E12 and E47, encoded by the E2A gene belong to the basic helix–loop–helix (bHLH) family of regulatory proteins and act as transcriptional activators. In addition to their critical role in B-lymphocyte development, the E12 and E47 proteins have been implicated in the induction of myogenesis as heterodimeric partners of myogenic bHLH proteins, MyoD and myogenin. Here we demonstrate that the E2A proteins form heterodimers with the bHLH oncoprotein tal-1 in myeloid and erythroid cells and that these heterodimers specifically bind to the CANNTG DNA motif. Heterodimerization with tal-1 represses transactivation by E47 and could function to prevent the expression of immunoglobulin genes in cells other than B lymphocytes. DNA binding by E2A–tal-1 heterodimers in the M1 mouse myeloid cell line is abrogated upon terminal macrophage differentiation induced by the cytokine interleukin 6. The loss of E2A–tal-1 DNA binding is correlated with elevated expression of mRNA encoding the dominant negative HLH proteins, Id1 and particularly Id2. Moreover, recombinant Id proteins inhibit the E2A–tal-1-specific DNA binding activity from undifferentiated M1 cells. These results suggest that E2A–tal-1 heterodimers may play a role in preventing terminal differentiation in the myeloid lineage and provide a possible explanation for oncogenic transformation induced by ectopic tal-1 expression in acute T-cell lymphoblastic leukemias.**

The protein products of the E2A gene, E12 and E47, are members of the family of basic helix–loop–helix (bHLH) transcription factors that are involved in cell growth control and transformation, neurogenesis, sex determination, muscle differentiation, and pancreatic gene expression (1, 2). Their cognate DNA sequence motif CANNTG (E box) is present in the regulatory regions of a variety of genes and is critical for tissue-specific expression of these genes. The E12 and E47 proteins result from the alternative splicing of the E2A gene, share most of their amino acid sequence (1, 2), and form homo- and heterodimers that recognize the E-box motif present in the enhancers of the immunoglobulin genes (3). The enhancer-binding activity of E47 results in the transcriptional activation and induction of rearrangement of the immunoglobulin locus and plays a critical role in B-lymphocyte development (4). E12 and E47 also form heterodimers with the myogenic bHLH proteins MyoD and myogenin and are necessary for the induction of myogenesis (5, 6).

We have focused on the identification of HLH proteins that might play a role in hematopoietic differentiation similar to that of the myogenic bHLH proteins. Several bHLH genes that are restricted to the hematopoietic tissues are known. These genes, *tal-1*, *tal-2*, and *lyl-1*, were initially identified through their involvement in chromosomal translocations and are implicated in the development of human lymphoid malignancies (7–10). Approximately 30% of human acute T-cell lymphoblastic leukemias (T-ALLs) are associated with rearrangements that transcriptionally activate the *tal-1* gene (also called *SCL* or *TCL5*) (11). Normally, the tal-1 gene is expressed in bone marrow, fetal liver, mast cells, macrophages, and erythroid cells (7). The *tal-1* gene products, $p42^{tal\text{-}1}$ and $p22^{tal\text{-}1}$, referred to here as tal-1M1 and tal-1M3, respectively, were shown to heterodimerize with the E47 bHLH region *in vitro* (12). The goals of this study were to determine whether the E2A and tal-1 proteins are true heterodimeric partners *in vivo* and to investigate their physiological function in hematopoietic development. We show that E2A–tal-1 heterodimers are present in cells of the myeloid and erythroid lineages and in some T-ALL cell lines. We further show that these heterodimers specifically recognize the CANNTG DNA motif and can prevent transcriptional activation of a promoter containing E-box regulatory sequences. To assess the biological function of E2A–tal-1 heterodimers, we induced M1 myeloid cells to terminally differentiate into macrophages with the cytokine interleukin (IL) 6. When these cells terminally differentiate, DNA binding of E2A–tal-1 heterodimers is abrogated, an event correlated with and likely caused by the induction of expression of dominant negative Id proteins. These findings reveal complex interactions between HLH proteins with differing activation and DNA binding properties and suggest a role for the HLH proteins in the differentiation of hematopoietic cells.

MATERIALS AND METHODS

Cell Culture. The primitive myeloid NFS-60 cell line and KD83 plasmacytoma cells were grown in RPMI medium 1640 supplemented with mouse recombinant (r) IL-6. Bone marrow-derived clone K cells were grown in RPMI medium supplemented with rIL-7. MC9 mast cells were grown in RPMI medium supplemented with mouse rIL-4. PT18 mast cells were grown in RPMI medium supplemented with mouse recombinant granulocyte/macrophage colony-stimulating factor. HT2 mouse T cells were grown in RPMI medium supplemented with mouse rIL-2. M1 cells were grown as described (13). M1 cells were treated with mouse rIL-6 at 500 units/ml, and cells were harvested as specified (13).

Electrophoretic Mobility Shift Assay (EMSA). Nuclear extracts were prepared from cultured cells (14), and assayed for the ability to form specific complexes with an E-box oligonucleotide probe as described (3). The nucleotide sequences for the E-box probes used in the EMSA were as follows: κE2, TCGACTCCCAGGCAGGTGGCCCAGATTAC; MCK, TCGAGAGGCAGCAGGTGTTGGGG; β-globin locus control

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Abbreviations: bHLH, basic helix–loop–helix; IL, interleukin; T-ALL, acute T-cell lymphoblastic leukemia; 2-ME, 2-mercaptoethanol; EMSA, electrophoretic mobility shift assay; MCK, muscle creatine kinase; FBS, fetal bovine serum; r, recombinant; CAT, chloramphenicol acetyltransferase.

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region, TCGACAGGGCAGATGGCAAAC; AP-1, TCGAGCTATGACTCATCCG.

Plasmids and Transfections. The E47 expression vector has been described (4). Chloramphenicol acetyltransferase (CAT) reporter plasmids were generated by inserting multimerized E-box oligonucleotides into a *Sal* I site of pΔ56fosCAT plasmid (15). The oligonucleotide sequences for the sense strand were as follows: κE2, TCGACTCCCAGGCAGGTGGCCCAGATTAC; MCK, TCGAGAGGCAGCAGGTGTTGGGG; β-globin locus control region, TCGACAGGGCAGATGGCAAAC. Multimers of five oligonucleotides were used in each case. The tal-1M1 and tal-1M3 expression plasmids were as described (16). The 293 human embryonic kidney cells were transfected using the calcium phosphate method with 5 μg of reporter plasmid plus 5 μg of activator and/or vector plasmid (17), harvested, and assayed for enzymatic CAT activity as described (18).

RNA PCR Assays. Randomly primed cDNA was synthesized from 5 μg of total RNA and PCR analysis was performed as described (4). PCR with actin-specific primers was used as a control (data not shown). PCR primer oligonucleotides for E47 were as described (4). PCR primers were designed to amplify sequences spanning exon junctions in all cases. The other oligonucleotide sequences were as follows: β actinL, TATGGAATCCTGTGGCATCCATGAAAC; β actinR, TAAAACGGAGCTCAGTAACAGTCCG; tal-1L, TTGGGAA/GCCGGATGCCTTCC; tal-1R, TCCCGCCTGTTGGTGAAG; E12L, GTTG/CCGT/CTCTCGCACC/TTGCTGCTCC; E12R, TCCGCTCTCGCACCTGCTGCTCCAG; Id1L, CACTCTGTTCTCGAGCCTCCTC; Id1R, GGCTGGATCCATCTGGTCCCT; Id2L, ACGGGATCCCTTCCACCAATGG; Id2R, AAGATCTTGTAGAATTCTTCTTGTGC.

***In Vitro* Transcription and Translation.** To generate Id1 and Id2 proteins by *in vitro* translation, the DNA templates were prepared by inserting Id1 and Id2 cDNA obtained by PCR using the specific primers described above into the pBCSK plasmid (Stratagene) and used in coupled transcription/translation rabbit reticulocyte lysate *in vitro* system, TNT (Promega), as described by the manufacturer.

RESULTS

To investigate whether the E2A and tal-1 HLH proteins heterodimerize *in vivo*, nuclear extracts from a panel of murine cell lines were analyzed by the EMSA by using an E-box DNA probe (Fig. 1). To identify complexes containing E2A proteins, nuclear extracts were incubated with E2A-specific serum prior to the addition of the DNA probe (3). Nuclear extracts prepared from 293 cells transfected with an E47 cDNA eukaryotic expression vector (4) were used as a positive control for the E2A homodimer complex, which is supershifted in the presence of the antibodies (Fig. 1*A*, lanes 1 and 2). A distinct pattern of complexes that specifically bind to the E-box DNA probe was observed in the nuclear extracts from myeloid and mast cells (Fig. 1). Notably, one set of the complexes migrating faster than E47 homodimers disappeared upon preincubation with the E2A antiserum (Fig. 1*A*) but not with preimmune serum (data not shown), indicating that these particular complexes contain E2A proteins or proteins closely related to E2A.

Pretreatment of the extracts with tal-1-specific serum (12) was used in the binding reactions to identify complexes containing tal-1 proteins. The antiserum interferes with tal-1-specific DNA binding (12). The antibodies specific for tal-1 proteins eliminated the same DNA–protein complex bands that were specifically recognized by anti-E2A serum in myeloid and mast cell nuclear extracts (Fig. 1*B*). Similarly, E2A–tal-1 DNA binding complexes were detected in MEL erythroid cells and P815 T-ALL cells but not in B cells or thymocytes (data not shown). Thus E2A and tal-1 proteins form heterodimers *in vivo* in myeloid, erythroid, and some T-ALL cells.

To understand the functional significance of E2A–tal-1 heterodimers, we examined whether tal-1 proteins modulate transcriptional activation by E2A proteins *in vivo*. A reporter construct containing multimerized E boxes from the immunoglobulin κ gene enhancer controlling the bacterial gene encoding CAT has very low level of CAT expression when transfected alone into 293 cells (Fig. 2). Cotransfection of the reporter construct and an E47 cDNA expression vector results in a high level of transcription of the reporter CAT gene (Fig. 2*A*). tal-1M1 and tal-1M3 expression plasmids separately or together failed to induce CAT expression. However, when the tal-1M1 or the tal-1M3 expression plasmids were transfected with the E47 expression vector, CAT activity was significantly reduced compared to the E47 expression vector alone. This reduction of E47-induced

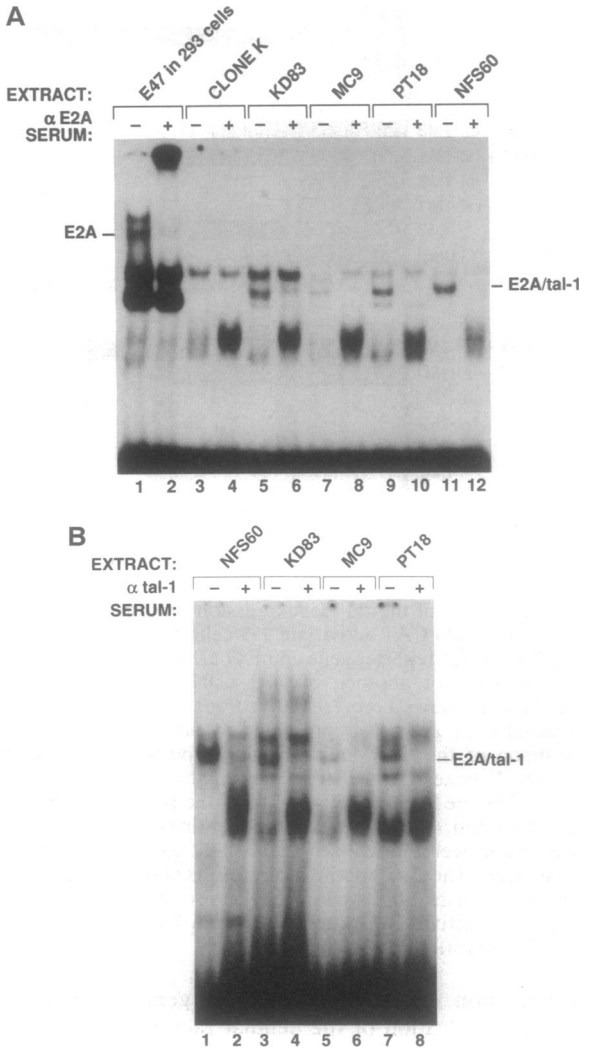

FIG. 1. EMSA of nuclear extracts from mouse hematopoietic cells. The indicated nuclear extracts were assayed for the ability to form specific complexes with the MCK E box oligonucleotide probe. (*A*) E2A-specific antiserum was used in the DNA binding reactions (indicated by +) to identify DNA–protein complexes containing E12 or E47 subunits. Nuclear extracts prepared from 293 cells transfected with an E47 cDNA eukaryotic expression vector (4) were used as a positive control. Position of the E2A homodimer complex is indicated. (*B*) tal-1-specific antiserum was used in the DNA binding reactions to identify complexes containing tal-1 proteins (lanes +). The mobility of the E2A–tal-1 heterodimer is indicated.

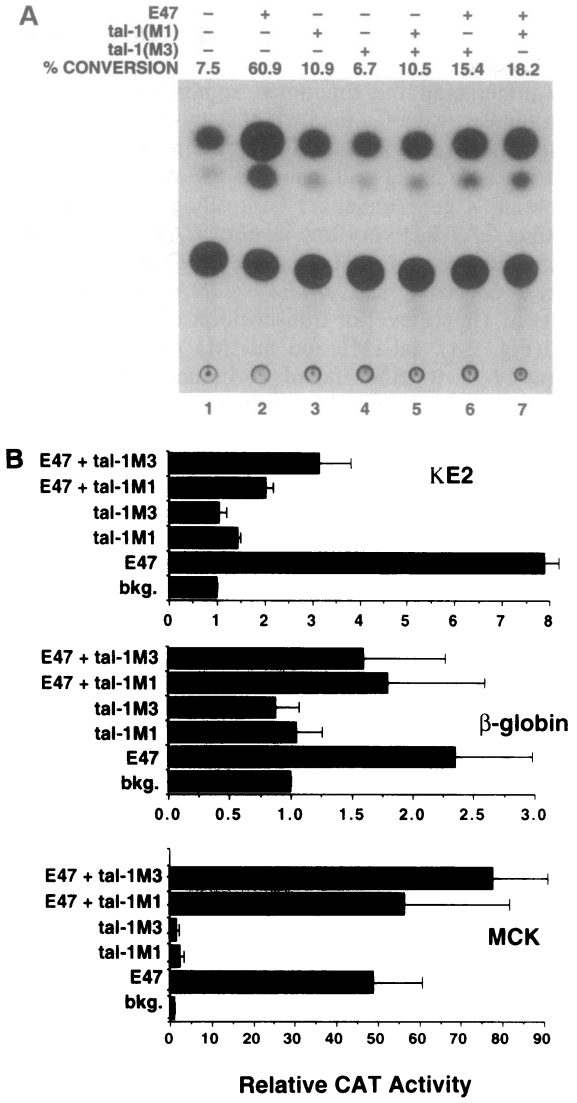

FIG. 2. Effect of the tal-1 coexpression on transcriptional activation by E47. (*A*) CAT activity in 293 cells cotransfected with the (κE2)Δ56fos-CAT reporter gene and E47 cDNA expression plasmid in the presence or absence of tal-1 cDNA expression vectors. Autoradiogram shows a representative transfection experiment, and normalized CAT activity is presented as percent acetylation. (*B*) Comparison of the CAT activity using reporter gene constructs containing E boxes derived from the immunoglobulin κ gene enhancer (κE2), the hypersensitive site 2 of the β-globin locus control region (β-globin), or the muscle creatine kinase enhancer (MCK). CAT activities were normalized against that obtained with reporter plasmid alone. The data are the mean results and SD obtained from two to five transfection experiments performed in duplicate. The CAT enzyme activity was quantitated on a PhosphorImager (Molecular Dynamics). bkg., Background.

transactivation of the CAT reporter gene did not result merely from titration of the cellular factors needed for the expression of the cytomegalovirus promoter utilized in the E47 and tal-1 expression vectors, because cotransfection of the vector alone without the cDNA inserts did not result in down regulation of CAT expression (data not shown). Transcriptional repression by tal-1 was not limited exclusively to the E-box sequence derived from the immunoglobulin κ gene enhancer; similar results were obtained using a reporter gene containing E-box sequences from the hypersensitive site 2 of the β-globin locus control region (19), taking into account the higher background of this reporter construct (Fig. 2*B*). In contrast, cotransfection of tal-1M1 or tal-1M3 expression plasmids did not repress E47 transactivation when the E box from the muscle creatine kinase (MCK) enhancer was used in the reporter gene construct. The immunoglobulin κ gene E box and MCK gene-derived E boxes contain the identical six core nucleotides but they differ in the flanking sequences, which may mediate different regulatory properties, although E2A–tal-1 heterodimers bind equally well to these three E boxes as determined by EMSA (Figs. 1 and 3, and data not shown).

Because HLH proteins have been associated with various differentiation processes, we examined E2A–tal-1 heterodimer DNA binding using the M1 early myeloid leukemic cell line as a model. Addition of the cytokine IL-6 to cultures of M1 cells rapidly inhibits their proliferation and induces phagocytic activity and morphologic changes characteristic of differentiated mature macrophages (13). Nuclear extracts derived from undifferentiated M1 cells contain E-box DNA binding complexes that were recognized specifically by both E2A- and tal-1-specific sera (Fig. 3*A*). Nuclear extracts were prepared from mock-induced M1 cells and from M1 cells induced with mouse rIL-6 harvested at specified times and analyzed by EMSA with the MCK E-box probe. DNA binding of the E2A–tal-1 heterodimers became undetectable at 24 h after addition of IL-6 to the cells, at a time when both morphologic and molecular changes become apparent in terminally differentiating M1 cells (13). In contrast, in the mock-induced M1 cells, the complexes persisted for up to 72 h, though the cells begin to die at this point from overcrowding (Fig. 3*B*).

Several experiments show that the abrogation of the DNA binding is specific for the E-box DNA sequence. Mixing of nuclear extracts from M1 cells induced with IL-6 for 48 h with extracts from the uninduced M1 cells inhibited specific DNA binding of E2A–tal-1 heterodimer from uninduced nuclear extract (Fig. 3*C*, lane 3). Similarly, addition of the nuclear extracts from IL-6-induced M1 cells to nuclear extracts derived from 293 cells transfected with the E47 cDNA resulted in the disappearance of the E47 homodimer DNA-binding complex (Fig. 3*C*, lane 4). However, addition of the nuclear extracts from IL-6-induced M1 cells produced no effect on the DNA binding activities specific for the AP-1 or NF-κB sequences (Fig. 3*C*, lane 7, and data not shown). Thus, the mixing experiments suggest that abrogation of E-box DNA binding is likely to result from the induction of a specific dominant inhibitory activity in M1 cells upon treatment with IL-6.

The Id1 and Id2 HLH proteins both lack the DNA binding basic region, can selectively dimerize with and inhibit DNA binding of a number of bHLH proteins including E12 and E47 (20), and so represent possible candidates for the activity that inhibits E2A–tal-1 DNA binding. We found a dramatic increase in Id2 mRNA levels in M1 cells induced with IL-6 compared to uninduced cells, and the levels of Id1 mRNA were slightly elevated in M1 cells treated with IL-6 (Fig. 4*A*). In contrast, the steady-state levels of E12 and E47 mRNA, the two alternatively spliced products of the E2A gene, remained unchanged in M1 cells upon treatment with IL-6 (Fig. 4*B*). Transcription of the *tal-1* gene in M1 cells induced with IL-6 was diminished after 24 h compared to uninduced cells (Fig. 4*C*). However, the observed small decrease in the tal-1 mRNA level does not reflect the amount of decrease of specific DNA binding by the E2A–tal-1 complex. In addition, the mixing experiments demonstrate the presence of a dominant inhibitory factor. We conclude that the elevation in the expression of two dominant negative HLH genes, Id1 and particularly Id2, may mediate the observed inhibitory effect on DNA binding activity of the E2A–tal-1 heterodimers in M1 leukemic cells upon terminal differentiation induced by IL-6.

To test directly whether Id proteins are capable of inhibiting specific DNA binding by the E2A–tal-1 heterodimers,

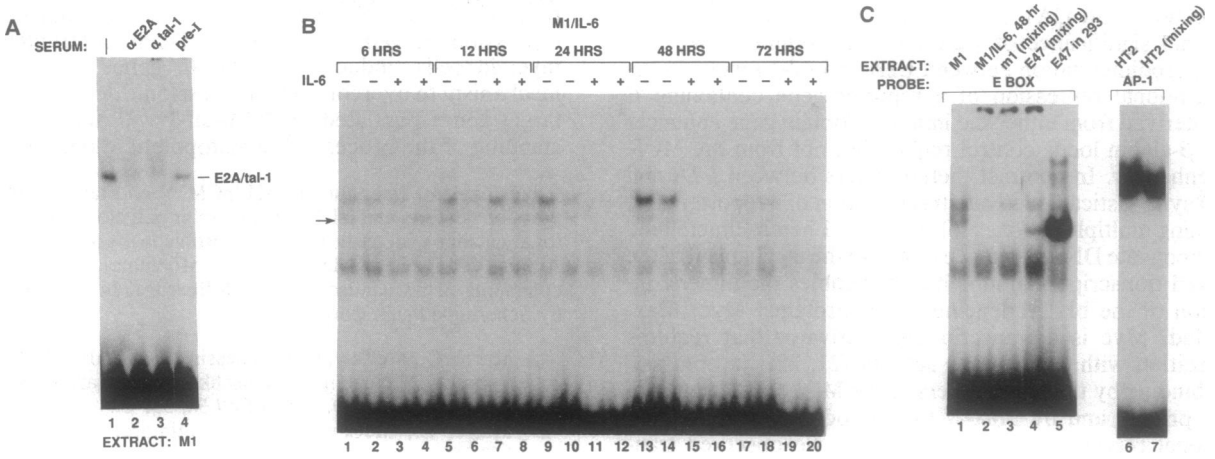

FIG. 3. DNA binding activity of E2A–tal-1 heterodimers is abrogated in the mouse myeloid leukemic cell line M1 upon terminal differentiation induced by IL-6. (*A*) E2A–tal-1 heterodimers were identified in the nuclear extracts from M1 myeloid leukemic cells by EMSA in the presence of the E2A- and tal-1-specific antisera as indicated. The E-box probe is from the hypersensitive site 2 of the β-globin locus control region. (*B*) Inhibition of the specific DNA binding of E2A–tal-1 correlates with the time course of M1 cell terminal differentiation induced by IL-6. Nuclear extracts from mock-induced or IL-6-induced M1 cells for specified times were analyzed by EMSA with the MCK E box probe. Protein concentrations were measured for each sample and equal amounts of protein were used in the DNA binding reactions. The arrow indicates the E2A–tal-1 heterodimer. The identity of the slower migrating band that also became undetectable at the same time as the E2A–tal-1 heterodimer is not known. (*C*) Inhibition of DNA binding is specific for the E box sequence. The indicated nuclear extracts were mixed in a 1:1 ratio with extracts derived from M1 cells treated with IL-6 for 48 h and analyzed by EMSA with the DNA probes as indicated at the top. AP-1 DNA probe-specific complexes derived from T-cell line HT2. One-half the amount of nuclear extract was used in the mixing reactions compared with the positive controls, M1, E47 in 293, and HT2 alone.

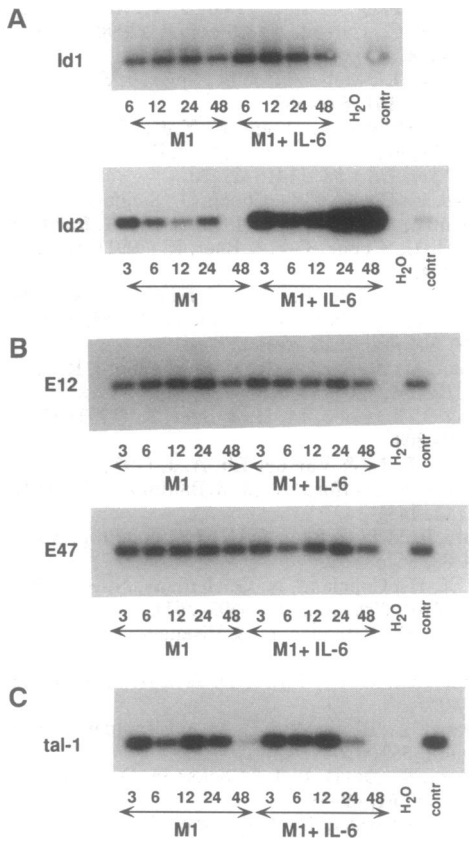

FIG. 4. Induction of the mRNA for the dominant negative HLH proteins, Id1 and Id2, in M1 cells upon stimulation with IL-6. RNA was prepared from unstimulated M1 cells and from the cells treated with IL-6 for the number of hours indicated below the lanes and subjected to reverse transcription–PCR analysis as described in the text. Lane C, RNA isolated from day 14 mouse embryo used as control; lane H contains PCR products when no cDNA template was added. (*A*) PCR with Id1- and Id2-specific primers. (*B*) PCR with E12- and E47-specific primers. (*C*) PCR with tal-1-specific primers.

we synthesized both Id1 and Id2 proteins *in vitro* using rabbit reticulocyte lysates. Recombinant Id1 or Id2 inhibited the DNA binding activity of the E2A–tal-1 heterodimer when added to the nuclear extracts derived from undifferentiated M1 cells (Fig. 5). Thus these experiments suggest that abrogation of the DNA binding by E2A–tal-1 heterodimers in M1 cells upon terminal macrophage differentiation induced by IL-6 is likely caused by the elevated expression of dominant negative Id proteins.

DISCUSSION

In this study we demonstrate *in vivo* heterodimerization between the E2A and tal-1 HLH proteins and address the question of the function of these heterodimers. By using EMSAs and antibodies specific for E2A or tal-1 proteins, we show the existence of E2A–tal-1 heterodimers in cells of the myeloid and erythroid lineages and some T-ALLs. Transient

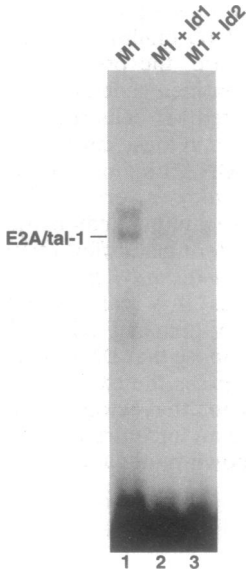

FIG. 5. Inhibition of E2A–tal-1 DNA binding by Id1 and Id2. Nuclear extracts from uninduced M1 cells were mixed with Id1 or Id2 proteins synthesized *in vitro*. The probe used is the E box derived from the MCK enhancer.

transfections and CAT assays were used to investigate the functional significance of E2A–tal-1 heterodimers. We find that heterodimerization between E2A and tal-1 results in the transcriptional repression of a reporter gene containing E boxes derived from either the immunoglobulin gene enhancer or the β-globin locus control region, but not from the MCK gene enhancer. In contrast, heterodimers between E2A and MyoD synergistically activate transcription of a reporter gene containing multiple E boxes (6). E2A–tal-1 heterodimers bind to their cognate DNA sequence motif, so the mechanism of the observed transcriptional inhibition resembles the dimeric interaction of the bHLH/leucine-zipper proteins, Myc, Max, and Mad. Myc is a transcriptional activator that requires dimerization with Max for its activity (21, 22). In contrast, DNA binding by the homodimers of the Max bHLH/leucine-zipper protein and by Max–Mad heterodimers causes transcriptional repression (21, 23). Because we observed transcriptional repression of a reporter gene driven by E boxes derived from the immunoglobulin κ gene enhancer, one of the possible functions for E2A–tal-1 heterodimers in hematopoietic lineage commitment could be to prevent the expression of the immunoglobulin genes in cells other than B lymphocytes. In addition, the possibility of positive transcriptional activation exerted by the E2A–tal-1 protein complexes on other DNA binding sites cannot be excluded.

To elucidate the functional significance of E2A–tal-1 heterodimers in hematopoietic development, we examined the E-box DNA binding activity of E2A–tal-1 heterodimers in the mouse myeloid leukemic cell line M1. We show that DNA binding of E2A–tal-1 heterodimers is abrogated in M1 cells upon the induction of terminal macrophage differentiation with IL-6 and that this effect is most likely explained by the elevated expression of the dominant negative HLH proteins, Id1 and particularly Id2. Interestingly, we observe increases in Id1 and Id2 mRNA levels in M1 myeloid cells when these cells are induced to terminally differentiate. In contrast, previous studies in other cell types have shown high expression of the Id1 and Id2 genes in undifferentiated tissues with a decline of Id gene expression in mature cells such as T and B lymphocytes, erythroid cells, and muscle cells (20, 24, 25). However, a biphasic pattern with a transient decrease of Id1 mRNA followed by an increase in expression was reported for the promyeloid cell line 32DC13 induced to terminally differentiate with granulocyte colony-stimulating factor (26). Thus, elevation of Id expression during the later stages of terminal differentiation could be a characteristic of cells of the myeloid lineage.

Experiments presented here have explored the relationship between *tal-1* gene expression and the developmental state of hematopoietic cells. We find that DNA binding activity of E2A–tal-1 heterodimers and, therefore, the possible transcriptional repression of as yet unidentified gene(s) is correlated with proliferating undifferentiated M1 cells. Induction of terminal macrophage differentiation in these cells by IL-6 results in abrogation of E2A–tal-1 DNA binding. Previous analysis of purified human megakaryocytes, erythroblasts, basophilic granulocytes, and their progenitors demonstrated that tal-1 is down-modulated during terminal differentiation in these cells (27). Further, although the *tal-1* gene is not normally expressed in T lymphocytes, it is rearranged and transcriptionally activated in ≈30% of human T-ALLs (11). Based on data presented here, we propose that DNA binding by E2A–tal-1 heterodimers might repress the expression of genes needed for cell differentiation and that ectopic expression of the *tal-1* gene could promote an uncontrolled proliferation of immature T cells by down-modulation of the transcriptional activation by E2A proteins and/or by the repression of genes needed for T-cell differentiation. In myeloid cells, induction of the Id genes may well be a mechanism to overcome this repression. Identification of the target genes regulated by E2A–tal-1 will aid in the understanding of the process of hematopoietic differentiation.

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