Gene Expression, Vol. 7, pp. 25-38, 1998
Printed in the USA.

1052-2166/98 $10.00 + .00

Induction of Basic Helix–Loop–Helix Protein-Containing Complexes During Erythroid Differentiation

JAMES A. LISTER[1] AND MARGARET H. BARON[2]

Department of Molecular and Cellular Biology, The Biological Laboratories, 16 Divinity Avenue, Harvard University, Cambridge, MA 02138

The involvement of basic helix-loop-helix (bHLH) transcription factors in erythroid differentiation and development has been established by forced expression of the proteins TAL1 and Id1 in cultured cell lines and by targeted disruption of the mouse *TAL1* gene. To better understand the mechanism by which bHLH proteins regulate erythropoiesis, we have investigated HLH protein–DNA interactions in mouse erythroleukemia (MEL) cells before and during chemically induced differentiation. Three bHLH (E-box) binding activities were found to be induced in nuclei from differentiating MEL cells. Using specific antisera, we have demonstrated that these complexes are dimers of TAL1 and ubiquitous E proteins. Similar complexes were detected in nuclear extracts from a human erythroid cell line, K562, and from mouse fetal liver. All three bHLH complexes were disrupted in vitro by Id1, a dominant-negative HLH protein that we and others have previously shown to antagonize MEL cell differentiation. During differentiation of an Id1-overexpressing MEL cell line, induction of a complex containing TAL1 and E2A was not only blocked but reduced below the levels seen in undifferentiating cells. These observations are consistent with the idea that TAL1 and Id1 have opposing effects on erythroid differentiation and that the level of TAL1/E2A heterodimer and/or another E protein-containing complex may influence the decision of a cell to terminally differentiate.

Erythroid differentiation Helix–loop–helix protein Transcription factors Globin switching Gene regulation

COMMITMENT and differentiation of hematopoietic stem cells along the erythroid lineage is controlled in part by tissue-restricted transcription factors [reviewed in (24,34,35)]. These include proteins of the basic helix-loop-helix (bHLH) family, which derives its name from a conserved structural motif consisting of a short basic region required for DNA binding, followed by two amphipathic α-helices separated by an intervening loop of variable length (31). The HLH domain mediates dimerization, and dimers of bHLH proteins recognize the consensus element CANNTG, which is found in the promoters and enhancers of many cell-specific genes and is known as the E-box (31). bHLH proteins can be subdivided into several groups (31,32). Among these, E proteins (class A bHLH proteins) are ubiquitously expressed and can form homodimers and heterodimers. Class B proteins such as MyoD (10) are expressed in a tissue-specific manner and homodimerize poorly but form heterodimers with the class A proteins. The dominant-negative HLH proteins, exemplified by Id1 (5), lack the basic region required for DNA binding. Therefore, complexes between Id and class A proteins or class B proteins cannot bind DNA and are inactive.

Received September 15, 1997; revision accepted November 12, 1997.

[1]Present address: University of Washington, Department of Biological Structure, Box 357420, Seattle, WA 98195.

[2]Address correspondence to Margaret H. Baron at her present address: The Mount Sinai School of Medicine, Box 1079, Research Building East, Rm 11-70B, 1425 Madison Avenue, New York, NY 10029. Tel: (212) 824-7420; Fax: (212) 996-1029; E-mail: mhbaron@msvax.mssm.edu

Hence, by functioning combinatorially, this family of proteins comprises a system of regulators with the potential for complex and sensitive control of gene expression.

Mouse erythroleukemia (MEL) (14) cells are thought to be developmentally arrested at the proerythroblast stage and can be chemically induced to undergo a limited program of terminal differentiation (28,29). MEL cells express a number of HLH genes in regulated patterns during their differentiation (6,15,26,39,43,45,48). Therefore, it has been suggested that erythroid cell differentiation can be promoted or antagonized through changes in the relative levels of different HLH protein complexes (1,26,43). For example, transcription of the *TAL1* gene, which encodes the major tissue-specific bHLH protein expressed in the erythroid lineage (4,15), increases during Me_2SO-induced differentiation of MEL cells (1,49). Overexpression of TAL1 enhances the spontaneous differentiation of MEL cells, whereas expression of antisense TAL1 RNA or a TAL1 mutant protein lacking the DNA binding basic region blocks chemically induced differentiation (1). Conversely, Id1 messenger RNA is rapidly downregulated in MEL cells during treatment with Me_2SO (6,26) and forced expression of Id1 blocks their differentiation (26,43). Although TAL1 and Id1 do not heterodimerize with each other (17,45), each may form heterodimers with E proteins such as those encoded by the *E2A* gene, E12 and E47/E2-5 (5,17,50). By analogy with MyoD and Id1 (21), TAL1 and Id1 may compete for interactions with E proteins in erythroid cells such that the relative levels of active (TAL1/E) and inactive (Id1/E) complexes influence the expression of E-box-containing target genes involved in the switch to the differentiated state. No target genes for TAL1 have yet been identified in erythroid cells.

A requirement for TAL1 in embryonic blood development in vivo has been established by targeted disruption of the mouse *TAL1* gene (38,40, 42). Analysis of these mutants indicates that, in addition to its role during terminal erythroid differentiation, TAL1 also functions very early in hematopoiesis. TAL1 does not homodimerize but forms heterodimers with class A bHLH proteins to bind DNA (17,18). Surprisingly, genetic ablation of the mouse *E2A* gene, which encodes potential dimerization partners of TAL1 in erythroid cells, has no apparent effect on erythropoiesis (52,53). This result suggests either that E2A proteins do not function in erythroid development and differentiation or that they are redundant with other E proteins in erythroid cells. Consistent with this notion of overlapping functions is the absence of any apparent defects in the erythroid lineage in mice carrying homozygous-targeted mutations in either the *HEB* or *E2-2* gene (54). The E proteins HEB (20) and E2-2 (16) could potentially substitute for E2A to form dimers with TAL1.

To elucidate the molecular mechanism(s) by which bHLH proteins regulate erythroid differentiation, we have examined the expression of E-box binding activities during Me_2SO-induced differentiation of MEL cells. We present evidence for three activities in MEL cell nuclear extracts that increase in level by 12–24 h of induction. Using antisera against TAL1 and E-type bHLH proteins, we show that these inducible DNA binding activities contain E2A and TAL1, HEB, or an as yet unidentified protein. All three complexes are disrupted in vitro by addition of exogenous Id1 and they are the only E-box binding activities so affected. Finally, in a cell line that constitutively overexpresses Id1, TAL1/E2A binding activity is reduced. Surprisingly, no heterodimers of TAL1 and HEB or E2-2 were detected in MEL cells either before or during differentiation. Together, these results suggest that the balance of heterodimers between E2A and TAL1 or other bHLH proteins and the negative regulator Id1 control terminal differentiation in the erythroid lineage.

MATERIALS AND METHODS

Cell Culture, Nuclear Extract Preparation, and Electrophoretic Mobility Shift Assay

MEL cells (subline DS19/sc9) and K562 cells (subline RA6) were maintained and induced as described previously (26). Stably transformed MEL lines C6 and B5 (26) were maintained in medium containing 0.2 mg/ml G418. Nuclear extracts were prepared from cultures (0.25–1.0 l) using the method of Dignam et al. (11) with the addition of protease inhibitors (2 μg/ml pepstatin A, 1 μg/ml leupeptin, and 5 μg/ml aprotinin) to buffers A, B, and C. Nuclear extracts were prepared from the livers (approx. 15) of 14.5-day embryos after homogenization of tissue in phosphate-buffered saline containing proteinase inhibitors.

Electrophoretic mobility shift assays (EMSA) were performed as described in Trepicchio et al. (46), except that binding reaction mixtures contained 2 μg poly(dI-dC) and 3.5–12 μg nuclear extract, and the binding reactions were analyzed on 4% or 5% nondenaturing polyacrylamide gels. For supershift experiments, antiserum (0.5–1 μl) was incubated with nuclear extract (6 μl) on ice for

20 min before addition of the other components of the binding reaction. Labeled oligonucleotide probe was added last. Reaction mixtures were incubated for 30 min at room temperature and then analyzed by nondenaturing acrylamide gel electrophoresis.

The sequences of the oligonucleotides used as probes and as cold competitors are shown in Table 1. Double-stranded oligonucleotides were radiolabeled at room temperature by end-filling reactions using Klenow polymerase for 30 min.

COS-1 Cell Transfection

COS-1 cells were transfected essentially as described previously (47). Cells were transfected with 10 μg of a plasmid consisting of the vector pMT23 (Genetics Institute, Cambridge, MA) containing the entire coding sequence of human E47 (31). Forty-eight hours after transfection, cells were harvested and pellets were resuspended in 150 μl of lysis buffer (47). For EMSA, 1 μl of this lysate diluted 1:40 with lysate from mock-transfected cells was added to binding reactions.

GST Fusion Proteins

The plasmid pGEX-2t-$Id_{PAH2}\Delta J$, encoding an Id1 helix-2 mutant (37) fused to glutathione *S*-transferase, was generated by replacing a AlwN1-Eag I fragment of pGEX-2t-IdΔJ (a gift from R. Benezra) spanning nucleotides 32–399 of the Id1 coding region (5) with the corresponding AlwN1-Eag I fragment from Id_{PAH2}er (26). The presence of the mutation was confirmed by DNA sequencing. Glutathione *S*-transferase fusion proteins were prepared according to a standard protocol (44). Protein concentration was determined by Bradford protein assay (Biorad) and integrity of the protein was verified by SDS-PAGE followed by Coomassie staining.

For EMSA experiments, nuclear extract (3.5 μg) was incubated with GST protein (0.5 μg) for 20 min at 37°C before addition of other binding reaction components.

TABLE 1
OLIGONUCLEOTIDES USED IN THIS STUDY

TAL1 preferred E-box oligonucleotide
5′-TGACCTGAA**CAGATG**GTCGGCT-3′
3′-TGGACTT**GTCTAC**CAGCCGAGT-5′
TAL1 mutant oligonucleotide
5′-TGACCTGAA**CCGATT**GTCGGCT-3′
3′-TCCACTT**GGCTAA**CAGCCGAGT-5′
μE5 oligonucleotide
5′-TGCAAGAA**CACCTG**CAAA**CACCTG**-3′
3′-GTTCTT**GTGGAC**GTTT**GTGGAC**TG-5′
MCK oligonucleotide
5′-GATCCCCCCAA**CACCTG**CTGCCTGA-3′
3′-GGGGGTT**GTGGAC**GACGGACTCTAG-5′
βmaj oligonucleotide
5′-TGATTGAG**CAAATG**CGTTCGC-3′
3′-TAACTC**GTTTAC**GCAAGCGGT-5′

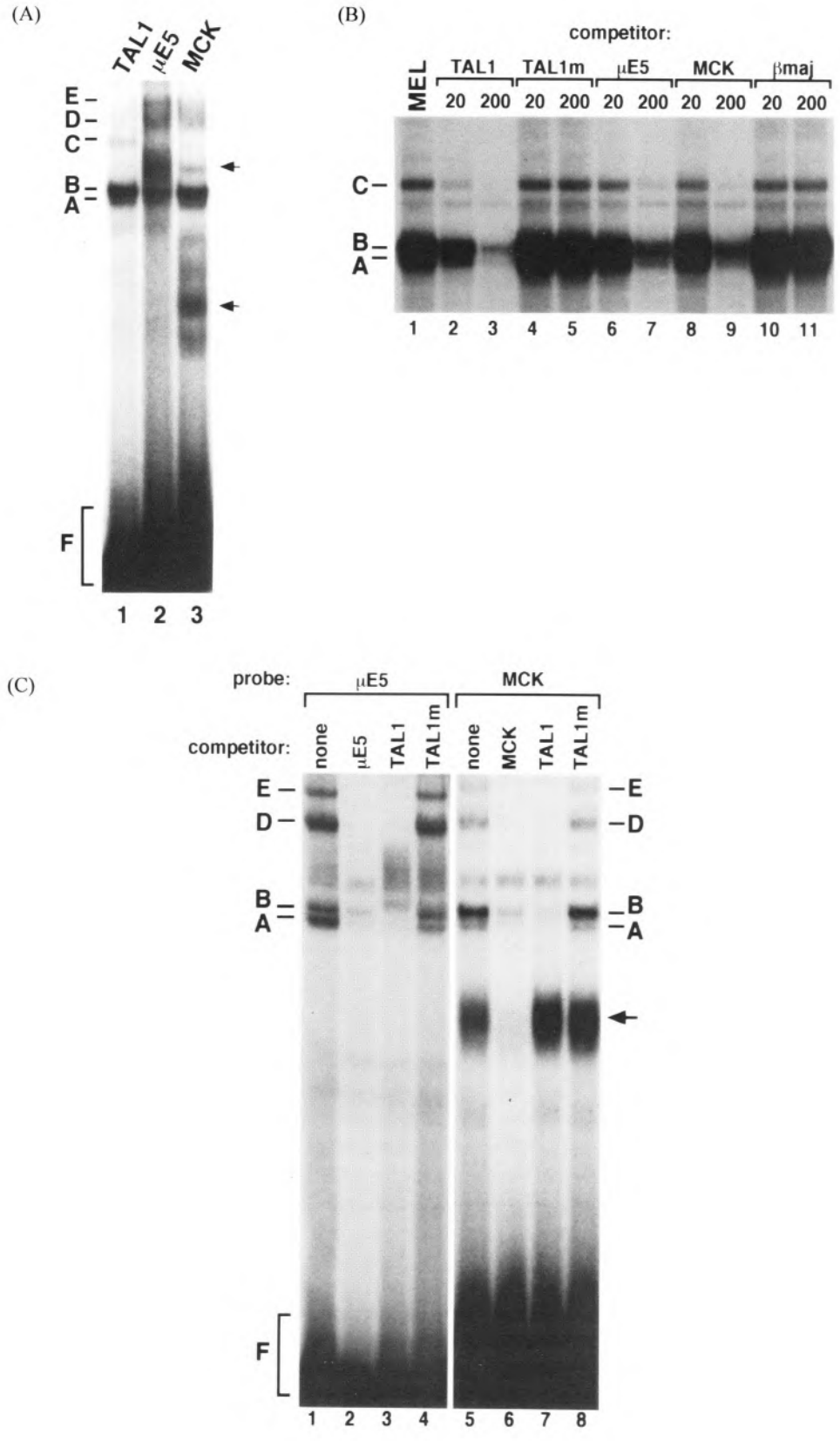
E-boxes (bHLH binding sites) are underlined and shown in boldface letters (E-box consensus: 5′-**CANNTG**-3′).

RESULTS

MEL Cell Nuclear Extracts Contain Several E-Box Binding Activities That Differ in Their Affinities for Distinct E-Box Sequences

MEL cells are known to transcribe several HLH genes (5,20,39,45,49), but translation of HLH proteins and their interactions in this system have been less well characterized. We and others have previously shown that overexpression of the dominant-negative HLH protein Id1 blocks MEL cell differentiation in response to Me_2SO (26,43), presumably by disrupting or preventing the formation of one or more DNA binding bHLH species. Therefore, to understand how HLH proteins are involved in promoting this differentiation switch, we asked which E-box DNA binding activities are present in MEL cells.

Nuclear extracts were prepared from undifferentiated MEL cells and used in EMSA experiments with a variety of different E-box DNA probes. Here we discuss results obtained only with three of these probes, the ones (TAL1, μE5, and MCK) for which some changes in protein binding complexes were detected during differentiation. The TAL1 preferred binding site (19) (hereafter referred to as the TAL1 E-box), μE5 (31), and MCK (2) E-boxes each contain the canonical CANNTG motif but differ in the central and flanking residues (see Table 1). The μE5 E-box sequence is found in the immunoglobulin heavy-chain enhancer, and the MCK E-box probe corresponds to the MCK-R site found in the murine muscle creatine kinase enhancer. As shown in Fig. 1A, each E-box probe is recognized by a distinct pattern of DNA binding activities in the nuclei of undifferentiated MEL cells. Based on their mobilities, we have denoted a subset of these activities A through E (Fig. 1). Complexes A and B (which appear as a single wide band in some of the figures but on lighter exposures and longer electrophoretic runs can be clearly seen as two distinct complexes; Fig. 1C) were present at similar


(A)
TAL1
μE5
MCK
E
D
C
B
A
F
1 2 3
(B)
competitor:
MEL
TAL1
TAL1m
μE5
MCK
βmaj
20 200 20 200 20 200 20 200 20 200
C
B
A
1 2 3 4 5 6 7 8 9 10 11
(C)
probe:
μE5
MCK
competitor:
none
μE5
TAL1
TAL1m
none
MCK
TAL1
TAL1m
E
D
B
A
F
1 2 3 4 5 6 7 8

levels for all three probes, whereas the slower migrating complex C was observed only with the TAL1 E-box probe (lane 1) and complexes D and E were observed only using the μE5 and MCK E-box probes (lanes 2 and 3). Several other complexes were detected for one or both of the μE5 and MCK probes (e.g., those indicated with arrows in Fig. 1A). This article will focus on complexes A–E.

The three complexes formed on the TAL1 E-box probe in Fig. 1A were specific for the E-box motif, as shown in Fig. 1B. Addition of a 20- to 200-fold molar excess of unlabeled E-box oligonucleotide abolished complex formation with the labeled probe (lanes 2, 3, 6–9), whereas a mutated TAL1 E-box oligonucleotide, in which CCGATT was substituted for the CAGATG E-box core (see Table 1), had no effect (lanes 4, 5). Wild-type μE5 or MCK oligonucleotides competed for protein binding by the TAL1 probe less effectively than the TAL1 oligonucleotide itself (compare lanes 6 and 8 with lane 2, and lanes 7 and 9 with lane 3), indicating a preference of these proteins for the TAL1 E-box. An oligonucleotide spanning the region of the mouse β-major globin promoter from −268 to −250 (Table 1) (36) and containing a potential E-box (AGCAAATGCG) did not compete for binding (lanes 10, 11). We have been unable to detect specific protein binding to this sequence (data not shown).

Similar competition experiments indicated that complexes D and E are also specific for the E-box (Fig. 1C). Complexes A, B, D, and E, observed with the μE5 (lanes 1–4) and MCK (lanes 5–8) E-box probes, were competed by a 200-fold molar excess of unlabeled oligonucleotides corresponding to the μE5 (lane 2), MCK (lane 6), or TAL1 (lanes 3, 7) E-box, but not by the mutated TAL1 E-box (lanes 4, 8).

Induction of E-Box Binding Activities During Erythroid Differentiation

We next examined how the pattern of each DNA binding activity changes during MEL cell differentiation. Nuclear extracts prepared from uninduced MEL cells and from MEL cells induced to differentiate for 2 or 4 days were examined for E-box binding activity by EMSA. For each of the three probes tested, the pattern of protein-DNA complex formation changed with time of induction (Fig. 2A). The level of complex C, containing the TAL1 probe, increased by ~5-fold after 2 days (compare lanes 2 and 3) then decreased to the uninduced level by 4 days (lane 4). A similar pattern was observed for complex C when the MCK E-box was used as a probe (lanes 10–12). Complex D, formed by interaction of MEL cell proteins with the μE5 and MCK E-box probes, increased strongly by 4 days of induction (Fig. 2A, lanes 6–8, 10–12), as did complex E with the μE5 probe. Levels of complex A, containing the TAL1 or μE5 E-box probe, increased by 4 days (Fig. 2A, lanes 4 and 8). No significant changes were observed for complex B.

Because downregulation of Id1 mRNA occurs within hours after addition of Me_2SO to the medium (26), we evaluated binding to the three E-box probes using nuclear extracts prepared at 0, 12, 24, or 48 h (Fig. 2B). For the TAL1 probe, the levels of complex C increased gradually from 0 to 48 h (lanes 2–5), whereas complex D (clearly visible in this particular experiment with all three probes) showed a biphasic expression pattern, increasing markedly by 12 h (lanes 3, 8, 12) and then decreasing by 24 h (lanes 4, 9, 13). Complex E, not visible in this particular experiment, displayed kinetics similar to those of complex D at 12 and 24 h (data not shown).

FACING PAGE

FIG. 1. E-box binding activities in the nuclei of undifferentiated MEL cells. (A) Three E-boxes each give distinct patterns of complexes. Uninduced MEL cell nuclear extracts (5 μg) were incubated with 0.3 ng of each of the three E-box probes and analyzed by EMSA on a 4% nondenaturing acrylamide gel. Complexes A and B were observed with each of the three probes. Complex C was most prominent for the TAL1 probe, whereas complexes D and E were most abundant for the μE5 and MCK probes. Arrows indicate other complexes. F indicates free probe. (B) Specificity of complexes binding the TAL1 preferred E-box probe. The TAL1 E-box oligonucleotide probe (3 ng) was incubated with 10 μg of uninduced MEL cell nuclear extracts in the presence or absence of unlabeled competitor oligonucleotides. Lane 1, no competitior; even lanes 2–10, 6 ng (20-fold molar excess) competitor; odd lanes 3–11, 60 ng (200-fold molar excess) competitor. Competitor oligonucleotides were as follows: lanes 2–3, TAL1 E-box; lanes 4–5, TAL1 mutant E-box; lanes 6–7, μE5 E-box; lanes 8–9, MCK E-box; lanes 10–11, β-major globin promoter putative E-box. F indicates free probe. Binding reactions were analyzed by nondenaturing electrophoresis on a 5% acrylamide gel. Proteins in complexes A, B, and C specifically recognized the E-box motif, with a preference for the TAL1 E-box sequence. (C) Specificity of complexes containing the μE5 and MCK E-box probes. The indicated labeled oligonucleotide probes (0.12 ng) were incubated with 6 μg nuclear extracts from MEL cells treated for 4 days with Me_2SO and analyzed by EMSA on a 5% nondentauring acrylamide gel. A 250-fold molar excess (3 ng) of the indicated competitor oligonucleotides was included in reactions in lanes 2–4 and 6–8. Complexes A, B, D, and E are all specific for the E-box motif. The complex indicated by the arrow is not specific for the E-box sequence (figure and also data not shown).

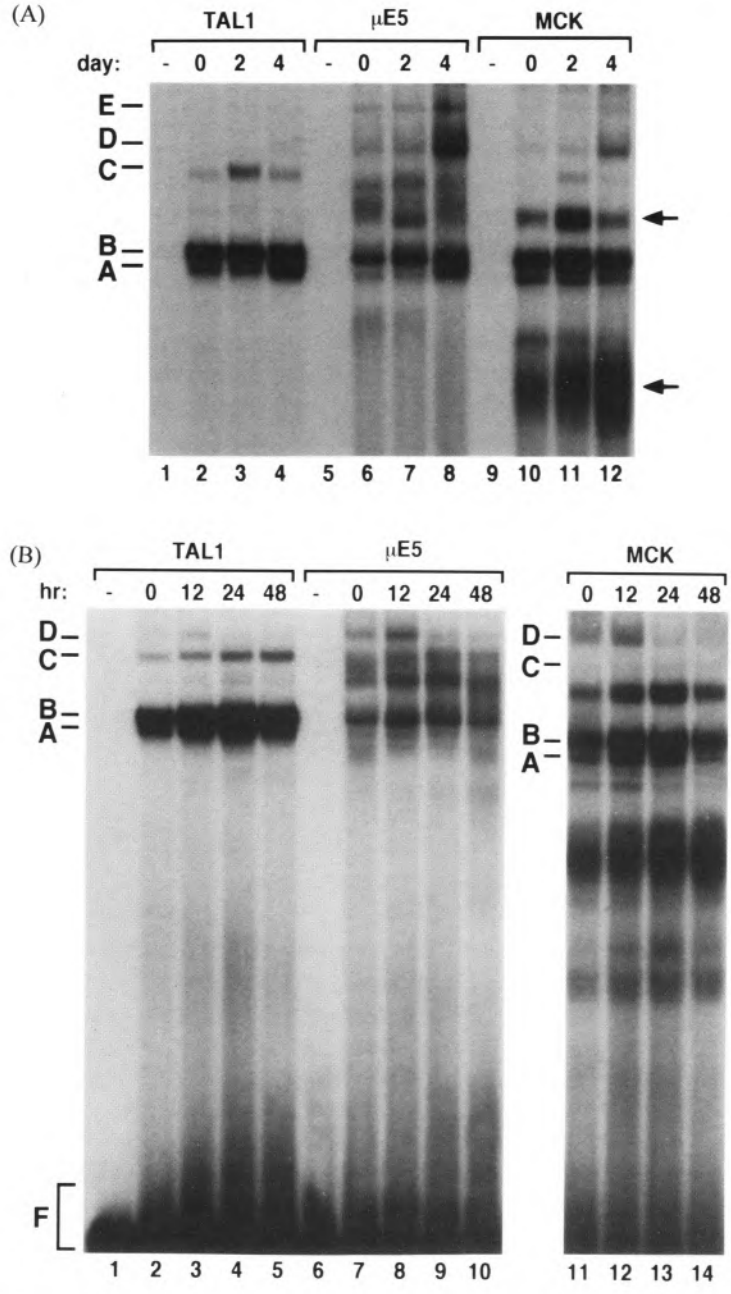


FIG. 2. Changes in E-box binding activities during Me_2SO-induced MEL cell differentiation. (A) Four-day time course. Labeled oligonucleotide probes (lanes 1–4, 0.12 ng TAL1; lanes 5–8, 0.06 ng μE5; lanes 9–12, 0.06 ng MCK) were incubated without protein (lanes 1, 5, 9) or with 5 μg nuclear extracts from uninduced MEL cells (lanes 2, 6, 10) or MEL cells treated with Me_2SO for 2 days (lanes 3, 7, 11) or 4 days (lanes 4, 8, 12), and analyzed by nondenaturing electrophoresis on a 5% acrylamide gel. Complexes A and B were observed for all three probes. Complex C was most prominent for the TAL1 probe. Complexes indicated by the arrows are not specific for the E-box sequence. (B) Two-day time course. Labeled oligonucleotide probes (lanes 1–5, 0.24 ng TAL1; lanes 6–10, 0.24 ng μE5; lanes 11–14, 0.2 ng MCK) were incubated without protein (lanes 1, 6) or with 6 μg nuclear extract from uninduced MEL cells (lanes 2, 7, 11) or MEL cells treated with Me_2SO for 12 h (lanes 3, 8, 12), 24 h (lanes 4, 9, 13), or 48 h (lanes 5, 10, 14). The level of complex C (TAL1 probe) increased during the period from 12 to 48 h after addition of Me_2SO but dropped by 4 days (A). Complex D showed a biphasic expression pattern with a low-level increase within 12 h (μE5 and MCK probes) and more sharply by 4 days of Me_2SO treatment.

Protein Composition of Inducible E-Box Binding Activities

The TAL1 protein exists in two forms (331 and 156 amino acids) resulting from translational initiation at distinct AUG codons (8). The smaller protein $pp22^{TAL1}$ corresponds to residues 176–331 of the larger protein $pp42^{TAL1}$. Both protein species have been found in MEL cells (33) and heterodimers of each protein with E2A have distinct mobilities in EMSA experiments (17). To examine the protein composition of TAL1 complexes, we used an antiserum raised against the carboxy-terminal 94 amino acids common to both polypeptides (19) and another that is specific for residues 1–121 found only in the longer translation product. Nuclear extracts from MEL cells grown for 48 h in the presence of Me_2SO were preincubated with immune or control serum prior to addition of the TAL1 E-box probe and DNA binding was assayed by EMSA. Figure 3A shows that the inducible complex C was specifically ablated by preincubation of nuclear extract with either TAL1 antiserum (lanes 2, 4), whereas control sera had no effect (lanes 3, 5). No changes were observed for the faster-migrating complexes A and B. We conclude that most or all of the TAL1 protein in DNA binding complexes in MEL cells is the longer translation product, $pp42^{TAL1}$.

We performed a similar analysis using antisera against the E proteins E2A (31), HEB (20), and E2-2 (16). The E2A antiserum (17) (Fig. 3B, lane 3), but not the anti-HEB (41) or anti-E2-2 (2) sera (lanes 5, 7), blocked formation of the inducible complex C but had no effect on complexes A or B. Extracts from a mouse B cell line were used as positive controls for the HEB and E2-2 antisera (not shown). A complex containing $pp42^{TAL1}$ and E2A was also detected in nuclear extracts from the human erythroid cell line K562 (data not shown). These experiments did not distinguish between the two proteins encoded by the *E2A* gene (E12 and E47/E2-5) (16,31).

We next assayed for the presence of TAL1 and E proteins in inducible complexes D and E. Nuclear extracts from MEL cells treated for 4 days with Me_2SO were preincubated with antisera against the E proteins and TAL1 before addition of the E-box probe. In experiments with the μE5 and MCK E-box probes, antiserum against E2A supershifts and/or ablates both complexes D and E (Fig. 4, lane 2). Complex D is not affected by any other antiserum; thus, it appears to contain E2A but not TAL1 or either of the other two E proteins, HEB or E2-2. Complex E was supershifted not only by anti-E2A serum but also by antiserum against HEB (lane 4), suggesting that this complex is a heterodimer of E2A and HEB proteins. As with the TAL1/E2A complex, we detected both "E2A/X" and E2A/HEB in K562 nuclear extracts (see below and data not shown).

The observation that complex D reacted with antisera against E2A proteins but not with any of the other antisera and its preferential binding to the μE5 E-box suggest that this activity may be a homodimer of E2A proteins. To address this question, we prepared lysates from COS-1 cells transfected with a human E47 construct and compared the mobility of human E47 homodimers produced in these cells with that of complex D from K562 cells. As shown in Fig. 5, two closely migrating complexes containing the μE5 E-box probe were observed in EMSA with COS-1 lysates from transfected (lane 3) but not mock-transfected cells (lane 2). The slower migrating of the two bands migrated with the same mobility as complex D from K562 cells (lane 1). It is therefore most likely that complex D is a homodimer of E2A proteins.

TAL1/E2A and E2A/X Are Present in Mouse Fetal Liver

To examine the potential relevance of the E-box binding activities observed in transformed erythroid cell lines to erythroid development in vivo, nuclear extracts were prepared from mouse fetal livers (14.5 days postcoitum) and analyzed by EMSA. At this stage of development, the liver is the primary site of hematopoiesis [for review, see (12)], and TAL1 protein is highly expressed in this tissue (23). In nuclear extract from mouse fetal liver, we detected a profile of TAL1 E-box binding activities similar to that found in MEL cells (Fig. 6A, lanes 1 and 2). Antibody supershift experiments established that in normal erythroid cells of the mouse, as in MEL cells, the majority of TAL1 protein in DNA binding complexes is found as heterodimers between the longer TAL1 translation product and E2A proteins (complex C, Fig. 6B). Similar analysis using μE5 or MCK E-box probes identified a complex that comigrated with MEL complex D and was disrupted only by the E2A antiserum (data not shown). Therefore, two complexes observed in induced MEL cells at high abundance are both found in nuclear extracts from normal erythroid cells.

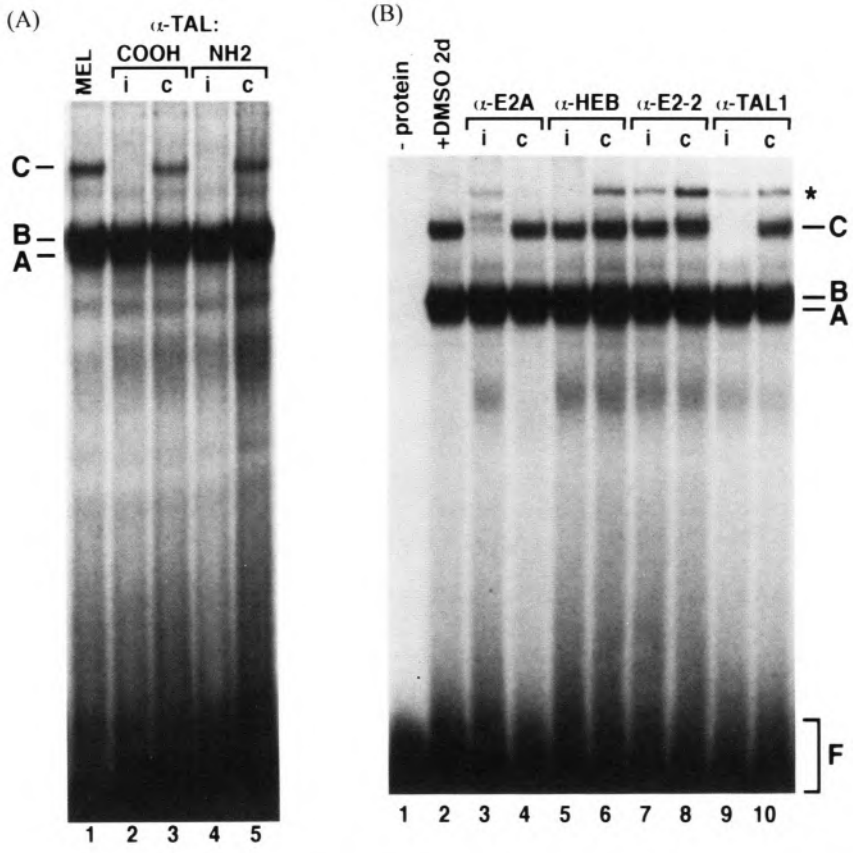


FIG. 3. Supershift and/or disruption of TAL1 E-box binding complexes with antisera to HLH proteins. (A) Inducible complex C contains full-length TAL1 protein. Nuclear extracts (4 μg) from MEL cells induced for 2 days with Me_2SO were incubated with antisera against the C-terminus (lane 2) or N-terminus (lane 4) of the TAL1 protein, or control sera (lanes 3, 5) prior to the addition of 0.6 ng TAL1 E-box probe and other binding reaction components. Samples were analyzed by electrophoresis on a 5% nondenaturing acrylamide gel. Complex C was ablated by the C-terminal antiserum, which recognizes both TAL1 polypeptides, and the N-terminal antiserum, which recognizes only the full-length protein. No other complexes were affected. Pre-immune serum (lane 3) and normal rabbit serum (lane 5) were used as controls. (B) Complex C is a heterodimer of $pp42^{TAL1}$ and E2A. Nuclear extracts (5 μg) from MEL cells treated for 2 days with Me_2SO were incubated with 1 ml of the indicated immune (i) or control (preimmune, c) antisera on ice for 20 min before addition of 0.12 ng TAL1 E-box oligonucleotide probe and other binding reaction components. After 30-min incubation at room temperature, reactions were analyzed by nondenaturing electrophoresis on a 5% acrylamide gel. The sample analyzed in lane 1 contained TAL1 E-box probe but no protein and the sample analyzed in lane 2 contained TAL1 E-box probe and 5 μg protein but no antiserum. *Nonspecific binding activity contributed by serum (data not shown). F, free probe. The inducible complex C was disrupted only by E2A (lane 3) and TAL1 (lane 9) antisera. Complexes A and B were not affected by any of the antisera.

The TAL1/E2A and E2A/X Complexes Are Disrupted by Id1

To extend our analysis to bHLH proteins for which antisera are not available, we used a protein complex disruption assay. Glutathione *S*-transferase fusion proteins containing wild-type or mutant Id1 were incubated with MEL cell nuclear extract before addition of other binding reaction components. GST-Id1 contains the wild-type Id1 protein, whereas GST-$Id1_{PAH2}$ contains a proline-to-valine substitution in amphipathic α-helix 2 (37). This mutant is unable to dimerize with E proteins in vitro (26,37) or to inhibit MyoD activity (37) or MEL cell differentiation (26) in vivo.

As shown in Fig. 7, preincubation of MEL nuclear extract with GST-Id1, but not with the mutant protein GST-$Id1_{PAH2}$, eliminated nearly all of the TAL1/E2A DNA binding activity (compare lanes 2 and 3), and completely eliminated the E2A/X (lanes 5, 8) and E2A/HEB (lane 5) complexes. The two faster migrating complexes A and

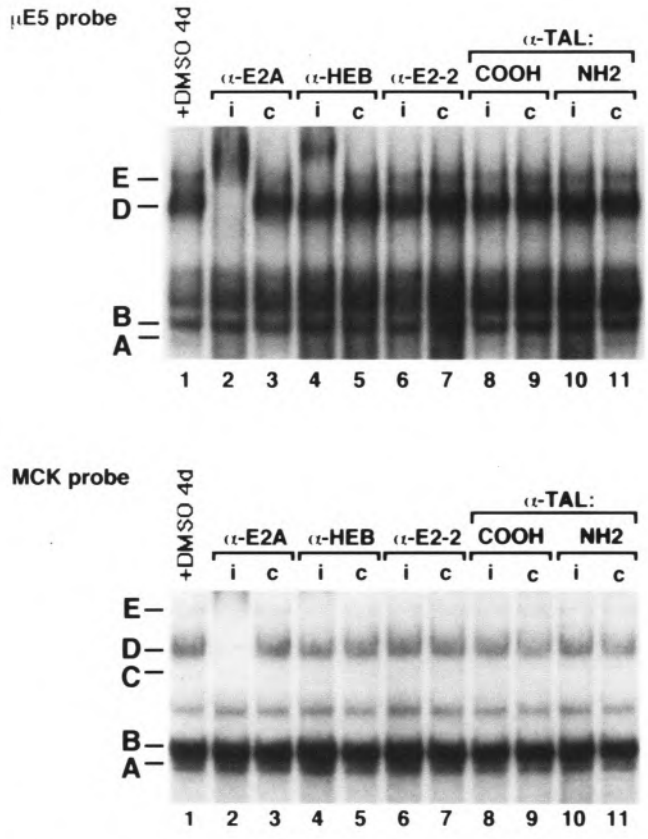


FIG. 4. Supershift/disruption of μE5 and MCK E-box complexes: inducible complexes D and E contain E2A. Nuclear extract (top panel, 4.5 μg; bottom panel, 5 μg) from MEL cells treated for 4 days with Me_2SO was incubated with 0.5 μl of the indicated immune (i) or control (c) sera prior to the addition of 0.6 ng μE5 (top) or 0.24 ng MCK (bottom) probe and EMSA analysis on a 4% (top) or 5% (bottom) nondenaturing acrylamide gel. Complex D was affected only by the anti-E2A serum, whereas complex E was affected by anti-E2A and anti-HEB sera. Normal rabbit serum was used as a control for the sample in lane 11; all other control samples contained preimmune serum.

B were unaffected by the addition of either protein to the binding reaction mixture, suggesting that they are formed by bHLH proteins that are resistant to Id1, such as those of the bHLH-leucine zipper class [e.g., USF or Myc-like proteins (7,27)].

Induced But Not Basal TAL1/E2A DNA Binding Levels Are Reduced in an Id1-Overexpressing Cell Line

We (26) and others (43) had previously shown that MEL cell lines constitutively overexpressing Id1 are blocked in their ability to terminally differentiate. To determine whether the levels of any of the inducible complexes are affected in these cell lines, nuclear extracts were prepared and examined by EMSA. The line B5 constitutively overexpresses Id1 at high levels. As a control, the C6 line, which was transfected with but does not express the Id1 construct (26), was used. As shown in Fig. 8 (lanes 2 and 6), after 48 h of Me_2SO treatment, an increase in TAL1/E2A binding was observed for the control line C6 (consistent with the results shown in Fig. 2A for parental MEL cells), whereas the Id1-overexpressing line B5 showed a decrease in TAL1/E2A binding below the levels seen in uninduced cells (lanes 4, 8). The decrease in TAL1/E2A binding activity observed for the Id1-overexpressing line B5 correlates with its block in differentiation; however, we have not detected consistent differences in E-box binding for other Id1-overexpressing cell lines (data not shown). No significant change in expression level was observed for complexes A, B, D, or E (Fig. 8; data not shown). Therefore, only complex C (TAL1/E2A) showed any change in these cells.

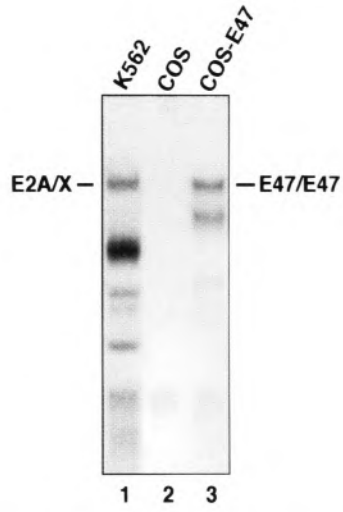


FIG. 5. Complex D comigrates with E47 homodimer. Radiolabeled μE5 probe (6 ng) was incubated with 6 μg of nuclear extract from K562 cells (lane 1) or 1 μl of lysate from mock-transfected COS-1 cells (lane 2) or COS-1 cells transfected with an expression construct for human E47 (lane 3). Samples were analyzed by EMSA on a 4% nondenaturing acrylamide gel. E47-transfected COS-1 cells contained two binding activities the slower of which comigrated with K562 complex D.

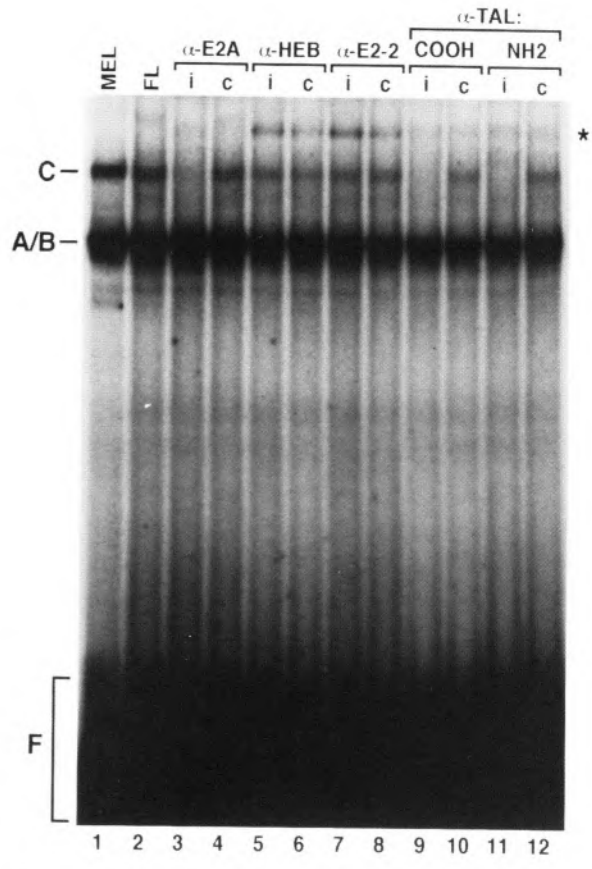


FIG. 6. Complex C, the TAL1/E2A heterodimer, is present in nuclear extract from mouse fetal liver. Nuclear extracts (4 μg) from MEL cells treated for 2 days with Me_2SO (lane 1) or prepared from mouse fetal livers (lanes 2–12) were incubated with the indicated immune (i) or control (c) sera prior to addition of 0.6 ng TAL1 E-box probe and other binding reaction components, followed by electrophoresis on a 5% nondenaturing acrylamide gel. Formation of complex C was blocked by antisera against E2A and TAL1 but not by control serum. None of the complexes were affected by HEB or E2-2 antiserum. *Nonspecific binding activity contributed by the serum. F, free probe.

The observation that overexpression of Id1 reduces the induced but not the basal level of TAL1/E2A binding suggests that there is some difference in complex C detected in undifferentiated versus induced cells. To examine this possibility, we examined these complexes in supershift experiments using anti-TAL and other anti-bHLH sera, at a number of time points following induction. However, no differences were observed, suggesting that if complex C changes upon induction, the change is not one that can be detected by available antisera.

DISCUSSION

Our previous work established a functional relationship between downregulation of Id1 and terminal differentiation of erythroid cells. In those studies, we showed that overexpression of Id1 blocked Me_2SO-induced differentiation of mouse erythroleukemia cells, and that this inhibition required a functional Id1 helix–loop–helix dimerization domain (26). By analogy with the effect of Id1 on differentiation of myoblasts in culture (21), we reasoned that Id1 in proliferating erythroid cells may prevent the formation of particular dimeric bHLH complexes involved in activating the differentiation program. In this report, we have begun to identify the components of such complexes by determining biochemically which E-box binding activities are present in actively dividing (undifferentiated) and terminally differentiating erythroid cells.

We have identified three inducible E-box binding activities in MEL cell nuclear extracts. One (complex C) preferentially recognizes a TAL1 preferred binding site out of a sampling of E-boxes and is a heterodimer between E2A and $pp42^{TAL1}$ proteins. The other two inducible E-box binding activities also contain E2A and interact preferentially with a different E-box core sequence. Complex E is a heterodimer between HEB and E2A; the binding partner of E2A in complex D is unknown. The levels of all three complexes change during differentiation. Induction of complexes D and E is biphasic, peaking first at 12 h and again

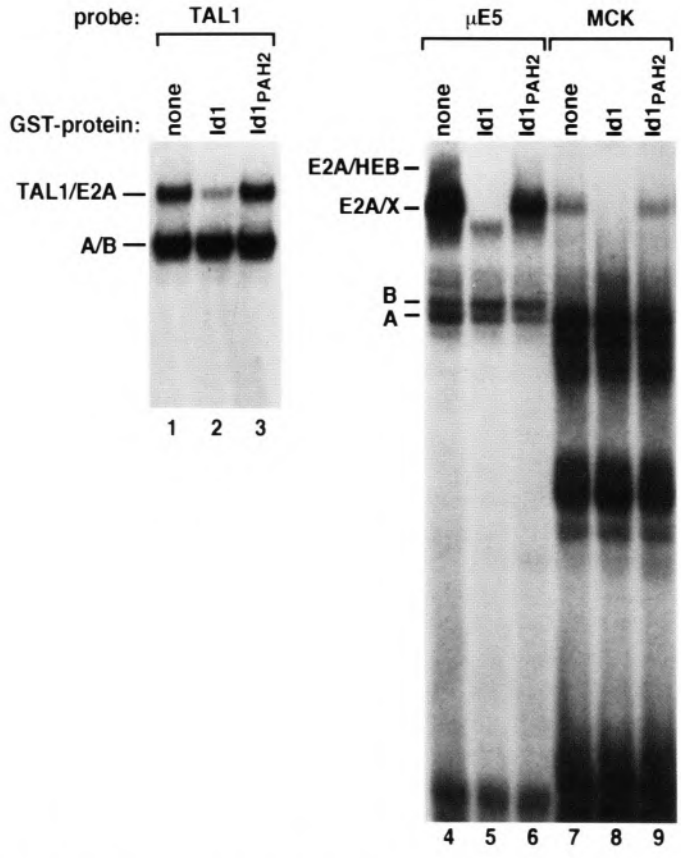


FIG. 7. Disruption of the inducible MEL cell complexes by Id1. Nuclear extract (3.5 μg) from MEL cells treated for 2 days (lanes 1–3) or 4 days (lanes 4–9) with Me_2SO was incubated with PBS/10 mM EDTA (lanes 1, 4, 7) or with 0.5 μg GST-Id1 (lanes 2, 5, 8) or GST-$Id1_{PAH2}$ (nondimerizing mutant) (lanes 3, 6, 9) for 20 min at 37°C before addition of the indicated E-box probe (0.6 ng) and other binding reaction components. Formation of the TAL1/E2A, E2A/X, and E2A/HEB complexes was specifically disrupted by GST-Id1 but not GST-$Id1_{PAH2}$ protein. Complexes A and B were unaffected by addition of either fusion protein.

at 96 h (4 days). TAL1/E2A levels increase steadily for 48 h before declining by 4 days. It has been reported that the DNA binding activity of TAL1 and E2A synthesized in vitro (19,50) or expressed in cultured hematopoietic progenitor cells (9) can be antagonized by addition of Id proteins. We have shown that all three of the inducible complexes in MEL cells are disrupted by Id1. Me_2SO triggers a rapid downregulation of Id1 mRNA in MEL cells (5,26); the subsequent decrease in the level of Id1 protein may therefore account for the observed increases in TAL1/E2A, E2A/HEB, and E2A/X during MEL cell differentiation. Although induction of TAL1/E2A is blocked in one Id1-overexpressing MEL cell line, we have not been able to detect consistent differences in the levels of E2A-containing complexes in all of our Id1-overexpressing lines. Therefore, other bHLH complexes important for erythroid differentiation may not have been detected by the E-box probes used in this study. Others have reported that overexpression of Id1 inhibits the increase in an MCK E-box binding activity at 4 days of Me_2SO induction (43). This activity may correspond to the E2A/X complex, which we also find is most abundant after 4 days of Me_2SO treatment.

The full-length TAL1 protein, but not its amino terminal-truncated form, was detected in E-box binding complexes. Both proteins are present in MEL cells (33) and have DNA binding activity as heterodimers with E2A proteins in vitro and in vivo (17). The amino-terminus of $pp42^{TAL1}$ has been reported to contain a weak transcriptional activation domain (51). This region is absent in the truncated form of TAL1. Whether or not the two proteins have distinct functions in erythroid cells is not known (40). Although no gene has yet been demonstrated to be a bona fide target for

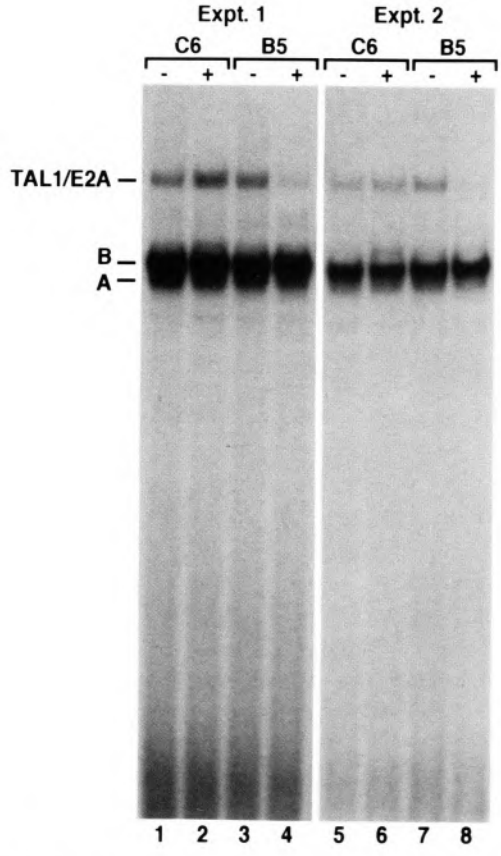


FIG. 8. Reduction of TAL1/E2A levels in an Id1-overexpressing cell line treated with Me_2SO. Labeled TAL1 E-box probe (0.12 ng) was incubated with 12 μg of nuclear extract from uninduced (odd-numbered lanes) or 2-day Me_2SO-induced (even-numbered lanes) cells. Samples in lanes 3, 4, 7, and 8 contained extract from the Id-overexpressing line B5. Samples in lanes 1, 2, 5, and 6 contained extract from the control line C6. EMSA analysis was carried out on a 5% nondenaturing acrylamide gel. In the two experiments shown here, reduced levels of TAL1/E2A complex were observed for the Id1-overexpressing cell line B5.

TAL1, good matches for the TAL1 preferred E-box are found in several promoters (18). Recent work from two laboratories has implicated a conserved E-box in hypersensitive site 2 (HS2) of the β-globin locus control region in regulating LCR enhancer activity (13,25). In one study (13), mutation of the E-box at position 8701 diminished overall enhancer activity. In vitro, TAL1 but not E2A was detected in complexes formed by MEL cell nuclear proteins with this E-box sequence. Elnitski and coworkers (13) also reported inducible binding of the bHLH/ZIP protein USF to the HS2 8701 E-box. Complex A (this work) displays properties consistent with those expected of USF, including specificity for the E-box, mobility relative to the TAL1/E2A complex, and insensitivity to Id1.

We speculate that complex D (E2A/X) may be an E2A homodimer based on its preference for binding to the μE5 E-box and its mobility relative to that of human E47 expressed in COS-1 cells, but we cannot unequivocally rule out the possibility that it contains another, unidentified protein. The TAL1-related protein LYL1 is also expressed in MEL cells [our unpublished results; (20)], and can form heterodimers with E2A proteins. However, because LYL1 displays an almost identical E-box preference as TAL1 (30) and complex D shows a clear preference for the μE5 and MCK E-boxes over the TAL1 E-box, we think it is unlikely that the partner of E2A in complex D is LYL1. The presence of complexes containing LYL1 or the truncated form of TAL1 might have been obscured by other, more abundant complexes in MEL cell nuclear extracts.

E2A proteins were present in all three inducible complexes in MEL cells. Curiously, although the *TAL1* gene is required for erythropoiesis (40,42), the products of the E2A gene are dispensable (3,53). These findings suggest that erythroid cells contain other proteins that can compensate for E2A activity. However, although TAL1/E2A complexes were easily detected in MEL cell nuclei, we did not detect TAL1/HEB or TAL1/E2-2 complexes in nuclei from any erythroid cells, including nontransformed mouse fetal liver erythrocytes. E2A homodimers are known to regulate the expression of immunoglobulins (3,22,53) and are necessary for development of the B cell lineage (3,53), whereas dimers of E2A and HEB have been previously shown to bind an E-box in the CD4 enhancer in T cells (41). Whether or not either of these complexes regulate transcription of genes in erythroid cells, either directly or indirectly (e.g., by influencing the levels of other HLH dimers), is unknown.

In conclusion, we have shown that the DNA binding activity of members of the helix–loop–helix protein family is regulated during MEL cell differentiation, consistent with the suggested role of these proteins in erythropoiesis. Complexes containing an E2A protein dimerized with TAL1, HEB, or another as yet unidentified partner may be of particular importance for terminal erythroid differentiation.

ACKNOWLEDGMENTS

We thank Richard Baer, Tom Kadesch, Glenn Begley, Robert Benezra, Steve Hauschka, Dan Littman, and Andrew Lassar for providing antisera and some cell lines and plasmids used in these experiments, and Maria Belaoussoff for help with mouse embryo dissections. We are grateful to members of our laboratory and to Andrew Lassar

for helpful discussions. This work was supported by grants (to M.H.B) from the National Institutes of Health (RO1 GM42413) and the Lucille P. Markey Charitable Trust (Grant 87-24). J.A.L. was supported during part of this work by an N.I.H. predoctoral training grant (GM 07620). M.H.B. was a Lucille P. Markey Scholar in Biomedical Science.

REFERENCES

1. Aplan, P. D.; Nakahara, K.; Orkin, S. H.; Kirsch, I. R. The SCL gene product: A positive regulator of erythroid differentiation. EMBO J. 11:4073–4081; 1992.
2. Apone, S.; Hauschka, S. D. Muscle gene E-box control elements. Evidence for quantitatively different transcriptional activities and the binding of distinct regulatory factors. J. Biol. Chem. 270: 21420–21427; 1995.
3. Bain, G.; Maandag, E. C. R.; Izon, D. J.; Amsen, D.; Druisbeek, A. M.; Weintraub, B. C.; Krop, I.; Schlissel, M. S.; Feeney, A. J.; Roon, M. v.; Valk, M. v. d.; Riele, H. P. J. t.; Berns, A.; Murre, C. E2A proteins are required for proper B cell development and initiation of immunoglobulin gene rearrangements. Cell 79:885–892; 1994.
4. Begley, C. G.; Aplan, P. D.; Denning, S. M.; Haynes, B. F.; Waldmann, T. A.; Kirsch, I. R. The gene SCL is expressed during early hematopoiesis and encodes a differentiation-related DNA-binding motif. Proc. Natl. Acad. Sci. USA 86:10128–10132; 1989.
5. Benezra, R.; Davis, R. L.; Lockshon, D.; Turner, D. L.; Weintraub, H. The protein Id: A negative regulator of helix-loop-helix DNA binding proteins. Cell 61:49–59; 1990.
6. Benezra, R.; Davis, R. L.; Lassar, A.; Tapscott, S.; Thayer, M.; Lockshon, D.; Weintraub, H. Id: A negative regulator of helix-loop-helix DNA binding proteins. Ann. NY Acad. Sci. 599:1–11; 1990.
7. Bresnick, E. H.; Felsenfeld, G. Evidence that the transcription factor USF is a component of the human β-globin locus control region heteromeric protein complex. J. Biol. Chem. 268:18824–18834; 1993.
8. Cheng, J.-T.; Hsu, H.-L.; Hwang, L.-Y.; Baer, R. Products of the TAL1 oncogene: Basic helix-loop-helix proteins phosphorylated at serine residues. Oncogene 8:677–683; 1993.
9. Condorelli, G.; Vitelli, L.; Valtieri, M.; Marta, I.; Montesoro, E.; Lulli, V.; Baer, R.; Peschle, C. Coordinate expression and developmental role of Id2 protein and TAL1/E2A heterodimer in erythoid progenitor differentiation. Blood 86:164–175; 1995.
10. Davis, R. L.; Weintraub, H.; Lassar, A. B. Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell 51:987–1000; 1987.
11. Dignam, J. D.; Lebovitz, R. M.; Roeder, R. G. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. 11:1475–1489; 1983.
12. Dzierzak, E.; Medvinsky, A. Mouse embryonic hematopoiesis. Trends Genet. 11:359–366; 1995.
13. Elnitski, L.; Miller, W.; Hardison, R. Conserved E boxes function as part of the enhancer in hypersensitive site 2 of the β-globin locus control region. J. Biol. Chem. 272:369–378; 1996.
14. Friend, C.; Scher, W.; Holland, J. G.; Sato, T. Hemoglobin synthesis in murine virus-induced leukemic cells in vitro: Stimulation of erythroid differentiation by dimethyl sulfoxide. Proc. Natl. Acad. Sci. USA 68:378–382; 1971.
15. Green, A. R.; Salvaris, E.; Begley, C. G. Erythroid expression of the helix-loop-helix gene, SCL. Oncogene 6:475–479; 1991.
16. Henthorn, P.; Kiledjian, M.; Kadesch, T. Two distinct transcription factors that bind the immunoglobulin enhancer μE5/kE2 motif. Science 247: 467–470; 1990.
17. Hsu, H.-L.; Wadman, I.; Baer, R. Formation of in vivo complexes between the TAL1 and E2A polypeptides of leukemic T cells. Proc. Natl. Acad. Sci. USA 91:3181–3185; 1994.
18. Hsu, H.-L.; Cheng, J.-T.; Chen, Q.; Baer, R. Enhancer-binding activity of the tal-1 oncoprotein in association with the E47/E12 helix-loop-helix proteins. Mol. Cell. Biol. 11:3037–3042; 1991.
19. Hsu, H.-L.; Huang, L.; Tsan, J. T.; Funk, W.; Wright, W. E.; Hu, J.-S.; Kingston, R. E.; Baer, R. Preferred sequences for DNA recognition by the TAL1 helix-loop-helix proteins. Mol. Cell. Biol. 14:1256–1265; 1994.
20. Hu, J.-S.; Olson, E. N.; Kingston, R. E. HEB, a helix-loop-helix protein related to E2A and ITF2 that can modulate the DNA-binding ability of myogenic regulatory factors. Mol. Cell. Biol. 12:1031–1042; 1992.
21. Jen, Y.; Weintraub, H.; Benezra, R. Overexpression of Id protein inhibits the muscle differentiation program: In vivo association of Id with E2A proteins. Genes Dev. 6:1466–1479; 1992.
22. Kadesch, T. Helix-loop-helix proteins in the regulation of immunoglobin gene transcription. Immunol. Today 13:31–36; 1992.
23. Kallianpur, A. R.; Jordan, J. E.; Brandt, S. J. The SCL/TAL-1 gene is expressed in progenitors of both the hematopoietic and vascular systems during embryogenesis. Blood 83:1200–1208; 1994.
24. Kehrl, J. H. Hematopoietic lineage commitment: Role of transcription factors. Stem Cells 13:223–241; 1995.
25. Lam, L. T.; Bresnick, E. H. A novel DNA-binding protein, HS2NF5, interacts with a functionally important sequence of the human beta-globin locus control region. J. Biol. Chem. 271:32421–32429; 1996.
26. Lister, J.; Forrester, W. C.; Baron, M. H. Inhibi-

tion of an erythroid differentiation switch by the helix-loop-helix protein Id1. J. Biol. Chem. 270: 17939-17946.; 1995.
27. Lüscher, B.; Eisenman, R. N. New light on Myc and Myb. Part I. Myc. Genes Dev. 4:2025-2035; 1990.
28. Marks, P.; Rifkind, R. Erythroleukemic differentiation. Annu. Rev. Biochem. 47:419-448; 1978.
29. Marks, P. A.; Richon, V. M.; Kiyokawa, H.; Rifkind, R. A. Inducing differentiation of transformed cells with hybrid polar compounds: A cell cycle-dependent process. Proc. Natl. Acad. Sci. USA 91: 10251-10254; 1994.
30. Miyamoto, A.; Cui, X.; Naumovski, L.; Cleary, M. L. Helix-loop-helix proteins LYL1 and E2a form heterodimeric complexes with distinctive DNA-binding properties in hematolymphoid cells. Mol. Cell. Biol. 16:2394-2401; 1996.
31. Murre, C.; McCaw, P. S.; Baltimore, D. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD and myc proteins. Cell 56:777-783; 1989.
32. Murre, C.; McCaw, P. S.; Vaessin, H.; Caudy, M.; Jan, L. Y.; Jan, Y. N.; Cabrera, C. V.; Buskin, J. N.; Hauschka, S. D.; Lassar, A. B.; Weintraub, H.; Baltimore, D. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell 58:537-544; 1989.
33. Murrell, A. M.; Bockamp, E.-O.; Göttgens, B.; Chan, Y. S.; Cross, M. A.; Heyworth, C. M.; Green, A. R. Discordant regulation of SCL/TAL-1 mRNA and protein during erythroid differentiation. Oncogene 11:131-129; 1995.
34. Ness, S. A.; Engel, J. D. Vintage reds and whites: Combinatorial transcription factor utilization in hematopoietic differentiation. Curr. Opin. Genet. Dev. 4:718-724; 1994.
35. Orkin, S. H. Transcription factors and hematopoietic development. J. Biol. Chem. 270:4955-4958; 1995.
36. Orkin, S.; Goff, S. The duplicated human alpha-globin genes: Their relative expression as measured by RNA analysis. Cell 24:345-351; 1981.
37. Pesce, S.; Benezra, R. The loop region of the helix-loop-helix protein Id1 is critical for its dominant negative activity. Mol. Cell. Biol. 13:7874-7880; 1993.
38. Porcher, C.; Swat, W.; Rockwell, K.; Fujiwara, Y.; Alt, F.; Orkin, S. H. The T cell leukemia oncoprotein SCL/tal-1 is essential for development of all hematopoietic lineages. Cell 86:47-57; 1996.
39. Quertermous, E. E.; Hidai, H.; Blanar, M. A.; Quertermous, T. Cloning and characterization of a basic helix-loop-helix protein expressed in early mesoderm and the developing somites. Proc. Natl. Acad. Sci. USA 91:7066-7070; 1994.
40. Robb, L.; Lyons, I.; Li, R.; Hartley, L.; Köntgen, F.; Harvey, R. P.; Metcalf, D.; Begley, C. G. Absence of yolk sac hematopoiesis from mice with a targeted disruption of the scl gene. Proc. Natl. Acad. Sci. USA 92:7075-7079; 1995.
41. Sawada, S.; Littman, D. R. A heterodimer of HEB and an E12-related protein interacts with the CD4 enhancer and regulates its activity in T-cell lines. Mol. Cell. Biol. 13:5620-5628; 1993.
42. Shivdasani, R. A.; Mayer, E. L.; Orkin, S. H. Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL. Nature 373:432-437; 1995.
43. Shoji, W.; Yamamoto, R.; Obinata, M. The helix-loop-helix protein Id inhibits differentiation of murine erythroleukemia cells. J. Biol. Chem. 269: 5078-5084; 1994.
44. Smith, D. B.; Johnson, K. S. Single-step purification of polypeptides expresed in *Escherichia coli* as fusions with glutathione S-transferase. Gene 67:31-40; 1988.
45. Sun, X.-H.; Copeland, N. G.; Jenkins, N. A.; Baltimore, D. Id proteins, Id1 and Id2, selectively inhibit DNA binding by one class of helix-loop-helix proteins. Mol. Cell. Biol. 11:5603-5611; 1991.
46. Trepicchio, W. L.; Dyer, M. A.; Baron, M. H. Developmental regulation of the human embryonic β-globin gene is mediated by synergistic interactions among multiple tissue- and stage-specific elements. Mol. Cell. Biol. 13:7457-7468.; 1993.
47. Tsai, S.-F.; Martin, D. I. K.; Zon, L. I.; D'Andrea, A. D.; Wong, G. G.; Orkin, S. H. Cloning of cDNA for the major DNA-binding protein of the erythroid lineage through expression in mammalian cells. Nature 339:446-451; 1989.
48. Visvader, J.; Begley, C. G. Helix-loop-helix genes translocated in lymphoid leukemia. Trends Biochem. Sci. 16:330-333; 1991.
49. Visvader, J.; Begley, C. G.; Adams, J. M. Differential expression of the LYL, SCL, and E2A helix-loop-helix genes within the hematopoietic system. Oncogene 6:187-194; 1991.
50. Voronova, A. F.; Lee, F. The E2A and tal-1 helix-loop-helix proteins associate in vivo and are modulated by Id proteins during interleukin 6-induced myeloid differentiation. Proc. Natl. Acad. Sci. USA 91:5952-5956; 1994.
51. Wadman, I.; Li, J.; Bash, R. O.; Forster, A.; Osada, H.; Rabbitts, T. H.; Baer, R. Specific in vivo association between the bHLH and LIM proteins implicated in human T cell leukemia. EMBO J. 13:4831-4839; 1994.
52. Zhuang, Y.; Kim, C. G.; Bartelmez, S.; Cheng, P.; Groudine, M.; Weintraub, H. Helix-loop-helix transcription factors E12 and E47 are not essential for skeletal or cardiac myogenesis, erythropoiesis, chondrogenesis, or neurogenesis. Proc. Natl. Acad. Sci. USA 89:12132-12136; 1992.
53. Zhuang, Y.; Soriano, P.; Weintraub, H. The helix-loop-helix gene E2A is required for B cell formation. Cell 79:875-884; 1994.
54. Zhuang, Y.; Cheng, P.; Weintraub, H. B-Lymphocyte development is regulated by the combined dosage of three basic helix-loop-helix genes, E2A, E2-2, and HEB. Mol. Cell. Biol. 16:2898-2905; 1996.