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Various Modes of Basic Helix-Loop-Helix Protein-Mediated Regulation of Murine Leukemia Virus Transcription in Lymphoid Cell Lines

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The transcriptionally regulatory regions of the lymphomagenic Akv and SL3-3 murine leukemia retroviruses (MLVs) contain two types of E-box consensus motifs, CAGATG. One type, $E_{A/S}$, is located in the upstream promoter region, and the other, E_{gre}, is located in a tandem repeat with enhancer properties. We have examined the requirements of the individual E-boxes in MLV transcriptional regulation. In lymphoid cell lines only, the E_{gre}-binding protein complexes included ALF1 or HEB and E2A basic helix-loop-helix proteins. Ectopic ALF1 and E2A proteins required intact E_{gre} motifs for mediating transcriptional activation. ALF1 transactivated transcription of Akv MLV through the two E_{gre} motifs equally, whereas E2A protein required the promoter-proximal E_{gre} motif. In T- and B-cell lines, the E_{gre} motifs were of major importance for Akv MLV transcriptional activity, while the $E_{A/S}$ motif had some effect. In contrast, neither E_{gre} nor $E_{A/S}$ motifs contributed pronouncedly to Akv MLV transcription in NIH 3T3 cells lacking DNA-binding ALF1 or HEB and E2A proteins. The Id1 protein was found to repress ALF1 activity in vitro and in vivo. Moreover, ectopic Id1 repressed E_{gre}-directed but not $E_{A/S}$-directed MLV transcription in lymphoid cell lines. In conclusion, E_{gre} motifs and interacting basic helix-loop-helix proteins are important determinants for MLV transcriptional activity in lymphocytic cell lines.

Endogenous retrovirus expression may result in induction of neoplastic diseases in mice (44). Within the first year of life, mice of the inbred AKR strain develop thymic lymphomas. SL3-3 murine leukemia virus (MLV), isolated from a lymphoid AKR tumor cell line, induces exclusively thymic lymphomas when injected in newborn NMRI mice, whereas the AKR endogenous MLV, Akv, induces a broader spectrum of diseases, including B and T lymphomas, myelogenous leukemia, and nondifferentiated leukemia (35). Lymphomagenesis is linked to the activity and cell type specificity of the MLV enhancer in the U3 part of the long terminal repeat (LTR); e.g., the enhancer of the T-lymphomagenic SL3-3 MLV directs greater transcriptional activity in T-cell lines than does the corresponding enhancer region of Akv MLV (6, 7, 23).

The MLV U3 regions represent an amalgamation of several *cis* transcriptional regulatory sequences. Recently, basic helix-loop-helix (bHLH) transcription factors and their cognate DNA recognition sequence, CANNTG (E-box), have been characterized as putative mediators of MLV transcription regulation (9, 21, 28, 31, 32). The bHLH transcription factors are involved in cell growth control and transformation, sex determination, pancreas gene expression, neurogenesis, muscle differentiation, and hematopoietic gene expression and differentiation (reviewed in reference 26). The cDNA cloning of the human bHLH protein SEF-2 or E2-2 was based on its in vitro capacity to bind E-boxes in the SL3-3 MLV enhancer (9), whereas murine ALF1 and ALF2 or E2A were cDNA cloned by affinity to the identical Akv MLV E-boxes (31). Human and rat analogs to ALF1 are termed HEB/HTF4 and REB, respectively (17, 20, 46). HEB has been reported to have affinity for E-boxes in the human immunodeficiency virus, immunoglobulin enhancers, and CD4 enhancer (10, 17, 38, 47). In transient-transfection assays, ALF1 can *trans* activate transcription from Akv, SL3-3, and the thymotropic Moloney MLV U3 regions, as well as the erythrotropic Friend spleen focus-forming virus, but not from the erythrotropic Friend MLV (32). Similarly, the bHLH transcription factor E47 can transactivate transcription of recombinant MLVs (21).

Class A bHLH proteins include E12, E47, and E2-5, all encoded by the E2A gene (13, 27); SEF-2 or E2-2 (9, 13); and ALF1, HEB, or HTF4 (17, 31, 46). Class A bHLH proteins regulate tissue-specific gene expression and differentiation by binding to DNA as either homodimers or heterodimers with other class A bHLH or with class B bHLH proteins, such as TAL1 or SCL and myogenic factors (15, 28). Class B bHLH proteins do not bind to DNA as homodimers but require heterodimer formation with class A bHLH proteins (28). Class A bHLH proteins can form heterodimers with helix-loop-helix proteins lacking the basic domain required for DNA binding, the Id proteins (4, 8, 37, 43). Id proteins are dominant negative for class A bHLH protein-mediated transcriptional activation, and Id mRNA expression is inversely correlated with class A bHLH protein activity (4, 36, 45). Moreover, a family of proteins coding for zinc finger domain proteins have also been identified as repressors of bHLH activity, e.g., ZEB and δEF1 (11, 39). Instead of mediating repression through protein-protein interactions, this protein family represses class A bHLH protein activity through competition for the E-box binding site (11).

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Class A bHLH proteins have been intensively studied with respect to transcriptional regulation in hematopoietic cells (13, 18, 29, 38). Ectopic expression of the class A bHLH dominant-negative Id1 protein in transgenic mice resulted in a decrease in B-cell formation (41). Targeted gene disruption in mice has recently shown the importance of class A bHLH proteins in hematopoietic development (2, 48). Mice with the E2A gene deleted contain no B cells, whereas other lineages, including T-cell, granulocyte, macrophage, and erythroid lineages, are relatively intact. This may suggest an incomplete functional redundancy in gene regulation among the very homologous class A bHLH proteins (2, 48).

In the present study, we found that MLV E-box-binding protein complexes in lymphoid cell lines included ALF1 or HEB and ALF2 or E2A bHLH subunits and that ALF1 and E2A proteins had stringent E-box requirements for transcriptional transactivation. Intact E-box motifs were found to have major importance for active MLV transcription in lymphoid cell lines, and ectopic Id1 reduced the MLV transcriptional rate. Our analysis showed that MLV enhancer E-boxes and corresponding bHLH transcription factors are important determinants for MLV transcription in Id-negative cells such as lymphocytes.

MATERIALS AND METHODS

Cell lines. NIH 3T3 fibroblasts, HeLa epitheloid carcinoma cells, and the Mpc11 immunoglobulin-secreting B-cell line were maintained in Dulbecco's modified Eagle's medium–10% newborn calf serum–2% penicillin-streptomycin. L691-6 and WEHI7.1 T-cell lines, the J558L immunoglobulin-secreting B-cell line, and K562 erythroleukemia cells were maintained in RPMI 1640 medium–10% newborn calf serum–2% penicillin-streptomycin. All cells were grown with 5.6% CO_2 at 37°C.

Chloramphenicol acetyltransferase (CAT) vector constructions. The pAKV6 CAT, pSL3-3CAT, and p1-99CAT constructs were described previously (34). To generate pE(c)CAT, the mutational primer 5231 (CCCCGGT*CATCTT*GGGA ACCTTG; E-box in italics and mutation underlined) and an oligonucleotide primer in excess corresponding to the R sequence at the *Kpn*I site were used in an asymmetric PCR with p1-99CAT as a template in a Hybaid OmniGene temperature cycler. The single-stranded PCR product was purified and used as a primer in an additional PCR with a primer corresponding to sequences including *Pst*I in the upstream U3 region. The fragment was digested with *Pst*I and *Kpn*I and cloned in the corresponding sites of pAKV6CAT. To generate pE (ab) CAT, a 139-nucleotide oligonucleotide with mutations in both Akv MLV enhancer E-boxes and a tag primer nonhomologous to Akv MLV sequences for recovery of the full-length PCR products (see reference 32) were used in a standard PCR with the *Kpn*I site corresponding primer. The PCR product was digested with *Kpn*I and partially digested with *Apa*I, and the longest *Apa*I-*Kpn*I fragment (343 bp) was cloned into the corresponding sites of p1-99CAT. pE(abc) was generated as described previously (32). Vector constructs with all combinations of wild-type and mutational E-boxes were constructed by swapping the largest fragment either from *Pst*I to a partially digested *Apa*I site or from *Kpn*I to a partially digested *Apa*I site between the constructs described above. For generation of E-box mutation constructs with one copy of the 99-bp repeat removed, an *Apa*I fragment was removed from pE(c) CAT, pE(ab)CAT, and pE (abc) CAT. The presence of specific mutations was verified by using an Applied Biosystem *Taq* sequencing kit on an Applied Biosystem 373A Sequencer.

Expression vectors and protein purification. pBNALF1A expressing ALF1 under control of the EF-1α promoter has been described elsewhere (32). The E12 and E2-5 expression vectors were kindly donated by R. Rivera, University of California, and T. Kadesch, University of Pennsylvania School of Medicine, respectively. Expression vectors pCMV4 and pCMV4-Id1 have been described previously and were kindly donated by R. Baer, University of Texas Southwestern Medical Center (16).

To generate the prokaryotic expression construct coding for ALF1 amino acids 562 to 706 ($ALF1_{562–706}$), PCR was done with the use of ALF1 primers with artificial restriction sites followed by *Bam*HI and *Eco*RI cloning of the generated fragment into pGEX2TK (Pharmacia). The prokaryotic Id expression vector was generated by cloning of the full-length coding region of Id1 into pGEX2TK by use of artificial restriction sites. Induction and purification procedures for the glutathione *S*-transferase (GST) fusion proteins were as described by the manufacturer (Pharmacia). The produced GST-$ALF1_{562–706}$ fusion protein was digested with thrombin and purified from the GST moiety.

Electrophoretic mobility shift assays and antibodies. Cellular nuclear extracts were prepared as described elsewhere (1). Double-stranded 34-nucleotide oligonucleotide probes labeled to the same specific activities were generated as described previously (32). Binding reactions of nuclear extracts with 2×10^4 cpm of probe were done at 25°C for 15 min in 25 μl of 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-NaOH (pH 7.9)–10 mM Tris-HCl (pH 7.9)–3 mM $MgCl_2$–80 mM NaCl–1 mM dithiothreitol–300 μg of bovine serum albumin per ml with 1 μg of poly(dI-dC)-poly(dI-dC). Prokaryotically expressed proteins were bound under the same conditions except for the addition of Tween 20 to 0.25% in the binding buffer and preincubation of the proteins at 37°C for 30 min before the addition of DNA to assist heterodimer formations. Following binding, the reaction products were electrophoresed through native gels (5% acrylamide, 0.1% bisacrylamide, 5% glycerol, 50 mM Tris, 380 mM glycine, 2 mM EDTA) and autoradiographed without drying at −80°C with intensifying screens. In competition analysis, a 50-fold excess of unlabeled oligonucleotides was added together with the probe to the binding reaction mixture. For supershift reactions, antiserum and control serum at the amounts indicated below were added to the binding reaction mixture, which was placed on ice prior to the addition of nuclear extract. Polyclonal ALF1 antibody has been described previously (33). The anti-HEB antiserum (αHEB) was generated by immunizing rabbits with HEB amino acids 530 to 554, a sequence region 100% identical to the ALF1 sequence (38). αHEB was kindly donated by D. R. Littman, University of California. The anti-E2A (αE2A)-specific antiserum was obtained from Santa Cruz Biotechnology.

Transfections and CAT assays. NIH 3T3 cells (5×10^5) were transfected as described previously (32). Each precipitate included 2 μg of CAT reporter plasmid, 2.0 μg of expression vector, and 5 μg of pCH110 as an internal standard expressing β-galactosidase from the simian virus 40 promoter. CAT assays were done for 0.5 to 4 h, and conversions were monitored by scintillation counting or by PhosphorImager analyses (Molecular Dynamics) of thin-layer chromatograms. Estimated values were normalized to the β-galactosidase activities to correct for variation in transfection efficiency. Transfections of Mpc11 and K562 cells were done as for NIH 3T3 cells except that the plates were seeded with 3×10^6 cells per 100-mm-diameter plate 24 h before transfection. DNA concentrations were 5 μg of CAT reporter plasmid, 5 μg of pCH110, and 6 μg of cytomegalovirus 4 expression vector.

L691-6 and J558L cells were transfected with DEAE-dextran as described previously, with modifications (40). Briefly, 5×10^6 cells were washed and suspended in 1 ml of TSD (140 mM NaCl, 5 mM KCl, 0.6 mM Na_2HPO_4, 25 mM Tris base [pH 7.4], 0.5 mM $MgCl_2$, 0.7 mM $CaCl_2$, 0.5 mg of DEAE-dextran [Pharmacia]) including 5 μg of CAT reporter plasmid and additionally, in cotransfection experiments, 5 μg of cytomegalovirus expression vector. After incubation for 20 min at 37°C, the cells were washed twice in complete medium and incubated for 48 h. Cellular extracts were prepared and CAT activity was measured as for the NIH 3T3 cells except that the extracts were preincubated at 60°C for 10 min and centrifuged before the CAT assay. CAT conversion was normalized to the total protein concentration, and all transfections were repeated three to nine times.

RESULTS

Several distinct protein complexes interact with the MLV E-boxes. Previous studies have shown that sequences within the U3 part of Akv and SL3-3 MLV LTRs are determinants for transcriptional activity (6, 12, 25). Recently, consensus E-box motifs (CANNTG) were identified in SL3-3 MLV and Akv MLV U3 regions (9, 31, 32). The U3 region of Akv MLV contains three E-box consensus motifs (Fig. 1). E_{gre}(A) and E_{gre}(B) in the tandem repeat are identical with the sequence AACAGATGGT; the E_{gre} sequence overlaps the glucocorticoid response element. $E_{A/S}$(C) (CCCAGATGAC) is located between the tandem enhancer repeat and the promoter region. The SL3-3 MLV U3 region contains a triplicated region carrying the E_{gre} E-box and an $E_{A/S}$ in the upstream promoter (Fig. 1).

To examine the binding of cellular proteins to the sequence regions corresponding to the E-boxes, we did electrophoretic mobility shift assays using oligonucleotide probes with the Akv MLV and SL3-3 MLV E-box sequence E_{gre} or $E_{A/S}$ (Fig. 2). We have analyzed nuclear extracts from T-cell lines (L691-6 and WEHI7.1), a B-cell line (J558L), and a fibroblast cell line (NIH 3T3) for their ability to form complexes with affinity for the E-box sequences. All major bands were specific for the E-box sequences, i.e., they were not formed with oligonucleotides including a mutated E-box sequence such as AANNTG (mutated nucleotide underlined) (data not shown). When the E_{gre} oligonucleotide probe was used, nuclear extracts from T- and B-cell lines formed E-box-specific complexes termed nu-

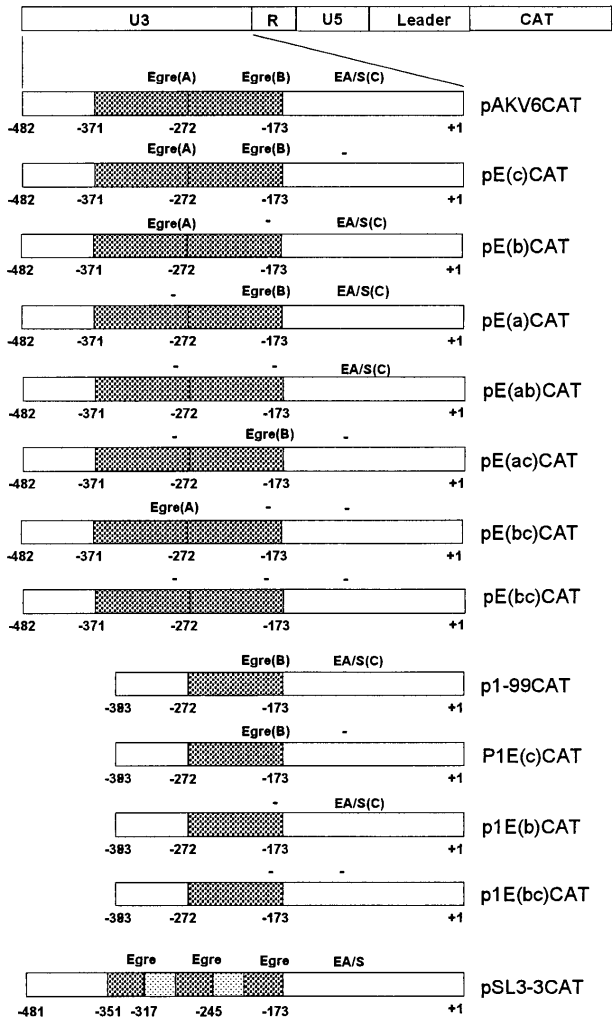

FIG. 1. Structure of MLV LTR CAT gene constructs. In the pAKV6CAT construct, the CAT reporter gene was set under transcriptional control of the Akv MLV LTR and flanking sequences. The pSL3-3CAT construct was generated by replacing the Akv MLV U3 region with the SL3-3 MLV U3 region. p1-99CAT was derived from pAKV6CAT by removing one copy of the enhancer repeat. By PCR, the mutation AAGATG was introduced into each E-box (CAGATG), and all possible combinations of E-box mutations were created in the background of pAKV6CAT or p1-99CAT. Introduced E-box mutations (−) and enhancer repeat structures (shaded boxes) are indicated. Numbers of nucleotides are given below the boxes, with +1 corresponding to the start point of transcription.

clear factor complexes (NFC_{gre}s) (Fig. 2A). The intensities of the bands varied for the different cell types; $NFC_{gre}4$ was always the most prominent band, whereas $NFC_{gre}2$ and $NFC_{gre}3$ were barely detectable in WEHI7.1 cells. The E-box specificity of the bands was verified by electrophoretic mobility shift assay competition analysis with mutant E-box probes (data not shown). E-box-specific complexes termed nuclear factor complexes ($NFC_{A/S}$s) were formed with the $E_{A/S}$ probe (Fig. 2B and data not shown).

In lymphocytic cell lines, E-box-interacting protein complexes include ALF1 or HEB and ALF2 or E2A subunits. To examine if the identified E-box-specific protein complexes formed with the oligonucleotide probes E_{gre} and $E_{A/S}$ included class A bHLH components, we did electrophoretic mobility shift assays, using polyclonal antisera raised against either ALF1 or the murine E2A protein ALF2. Binding of nuclear extracts from T-cell lines (L691-6 or WEHI7.1) and a B-cell line (J558L) to the E_{gre} oligonucleotide after the addition of ALF1 antiserum showed a decrease in formation of $NFC_{gre}1$ complexes (Fig. 2A). With the ALF2-E2A antiserum, we observed the same pattern but more pronounced. In this case, $NFC_{gre}1$ was completely undetectable in the presence of antiserum. In NIH 3T3 cells, none of the E-box complexes reacted with the antisera, in agreement with previous observations that NIH 3T3 fibroblasts are depleted of DNA-binding class A bHLH proteins (32, 38). Interestingly, the two antibodies supershifted none of the complexes formed with the $E_{A/S}$ probe (Fig. 2B). Thus, ALF1 and ALF2 or E2A are not involved in complex formation at the E-box sequence $E_{A/S}$, localized between the enhancer and the promoter regions in both Akv and SL3-3 MLVs.

As ALF1 and ALF2 or E2A are very homologous in primary structure, the polyclonal sera for ALF1 and ALF2/E2A were found to cross-react to some degree (data not shown). Therefore, we repeated our examinations with sera more specific for each component. αHEB reacts with a domain specific for ALF1 or HEB, and αE2A reacts specifically with the ALF2 or E2A proteins (38). αHEB decreased the mobility of $NFC_{gre}1$ from the T-cell lines (Fig. 2C). Nuclear extract from the B-cell line J558L includes an αHEB-retarded complex comigrating with the αHEB-retarded complexes from the T-cell lines (Fig. 2C). Hence, $NFC_{gre}1$ includes ALF1 or HEB subunits. Incubation with αE2A antiserum always completely removed the $NFC_{gre}1$ (Fig. 2D). Thus, in all tested T- and B-cell lines, $NFC_{gre}1$ includes E2A subunits. In conclusion, class A bHLH protein complexes of B- and T-cell lines include both ALF1 and E2A subunits that bind to the E_{gre} motif but not to the very homologous $E_{A/S}$.

Since nuclear extracts from lymphoid cell lines contained DNA-binding ALF1 and ALF2 or E2A bHLH proteins, we did transient-transfection assays to examine the transcriptional regulatory capacity of various combinations of ALF1 and ALF2 or E2A proteins. We cotransfected into NIH 3T3 cells the pAKV6CAT construct, having the CAT gene under transcriptional control of the Akv MLV LTR with expression vectors for the bHLH proteins. Both ALF1 and E2A proteins activated pAKV6CAT transcription approximately fourfold (Fig. 3). Cotransfection of ALF1 and E2A proteins showed no synergistically transactivated transcription from the Akv MLV enhancer to the same level as the putative heterodimeric complex (Fig. 3). We should draw attention to the fact that homodimeric ALF1 or HEB and E2A proteins have different preferred DNA-binding sites (5, 42). In addition, ALF1 and E2A proteins require different compositions of enhancer E-boxes for mediating transcriptional activity (see below and Fig. 4).

We find E-box-binding complexes without ALF1 and ALF2 or E2A proteins. Similar observations have been made in other studies, i.e., ubiquitous protein complexes lacking class A bHLH protein interact with the CD4 enhancer E-box (38). We do not relate these E-box-binding protein complexes, without ALF1 and ALF2 or E2A proteins, to any DNA-binding protein family, but they could make up transcription factor complexes. Notably, the transcriptional activity mediated by protein complexes formed on the $E_{A/S}$ could not be repressed by ectopic expression of Id1 (see below and Table 2).

MLV E-boxes are weak transcriptional elements in NIH 3T3 and HeLa cells. To examine the contribution from individual E-box sequences to the MLV enhancer function, by PCR we introduced identical point mutations into each of the three Akv MLV E-boxes and created constructs with all possible combinations of wild-type and mutated E-box motifs. The in-

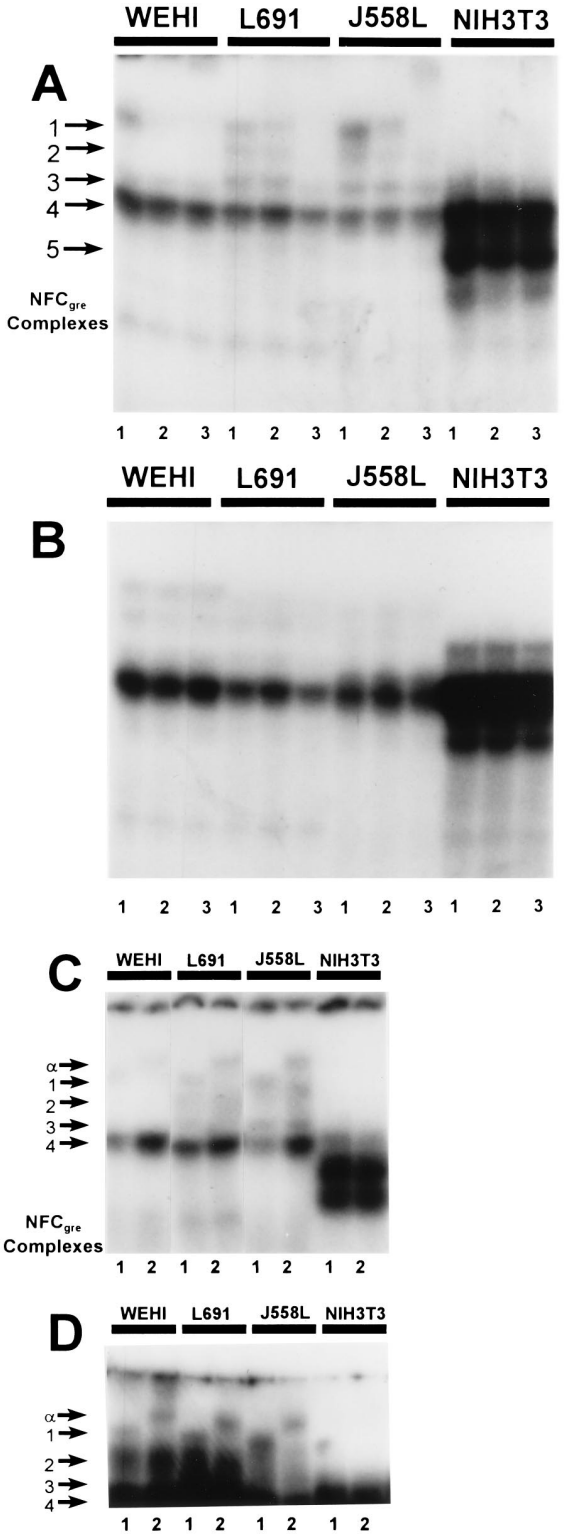

FIG. 2. E_{gre}-binding protein complexes include ALF1 and E2A subunits. (A and B) Electrophoretic mobility shift assay reaction mixtures included nuclear extracts, rabbit antiserum against ALF1 (αALF1) or E2A proteins (αALF2), and the E_{gre} probe (A) or the $E_{A/S}$ probe (B). Lanes 1, 1 μl of a 10-fold dilution of preimmune serum; lanes 2, 1 μl of a 10-fold dilution of αALF1; and lanes 3, 1 μl of a 10-fold dilution of αALF2. The positions of the E_{gre}-specific complexes NFC_{gre} are indicated. (C and D) Electrophoretic mobility shift assays with ALF1-HEB-specific antibody αHEB and ALF2-E2A-specific antibody αE2A, respectively. Reaction mixtures included nuclear extracts, the E_{gre} oligonucleotide, and either unspecific serum (lanes 1) or specific antiserum (lanes 2).

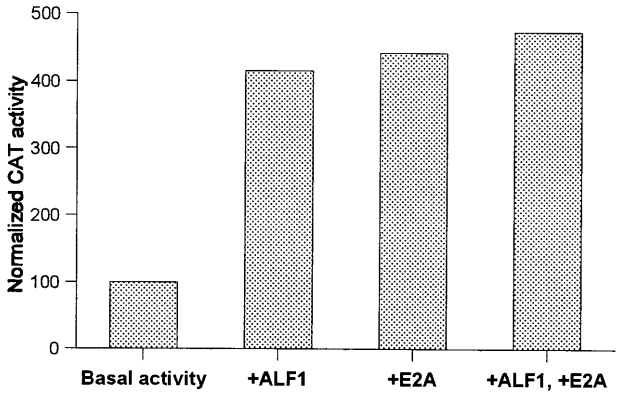

FIG. 3. ALF1 and E2A proteins do not activate Akv MLV transcription synergistically. The pAKV6CAT construct (2 μg) was cotransfected into NIH 3T3 cells (5×10^5) with a total of 2 μg of expression vector, either parental empty expression vectors (basal), vector expressing ALF1 (encoding expression vectors pBNALF1A and pBNALF1B) or E2A (protein-encoding expression vectors E47 and E2-5), or ALF1 and E2A expression vectors in combinations, as indicated. A 2-μg amount of expression vector yields half-saturation of the synthesis, as measured by either ALF1 or E2A expression. Five micrograms of pCH110 was included as an internal standard expressing β-galactosidase from the simian virus 40 promoter. The β-galactosidase-normalized CAT activities were estimated and visualized by setting the value of the basal expression to 100. The standard deviation from the mean values was ≤25%.

troduced mutation changed the E-box core sequence CAG ATG to AAGATG, a mutation found to eliminate the binding of class A bHLH proteins in vitro and transactivation in vivo (32). The panel of constructs generated is shown schematically in Fig. 1. According to the nomenclature of Speck et al. (40), the mutated E-boxes are shown in parentheses [e.g., pE (ab) CAT includes mutations in E_{gre}(A) and E_{gre}(B)]. Studies of Akv MLV transcriptional regulation in NIH 3T3 fibroblast cells have shown that removal of one copy of the 99-bp enhancer repeat reduced the transcriptional activity (25). To examine the function of E-boxes in enhancers with only one copy of the repeat, we included such constructs in our analysis (Fig. 1).

We transfected the series of CAT gene constructs into NIH 3T3 fibroblasts and evaluated CAT activities (Table 1). Muta-

TABLE 1. E-boxes contribute to the MLV transcriptional activity in lymphoid cell lines

Construct	Normalized CAT activity in the following cell line[a]:						
	NIH 3T3	HeLa	WEHI7.1	L691-6	J558L	Mpc11	K562
pAKV6CAT	1.0[b]	1.0[b]	1.0[b]	1.0[b]	1.0[b]	1.0[b]	1.0[b]
pE(c)CAT	1.0	0.8	0.5	0.4	0.6	0.7	0.6
pE(b)CAT	0.8	0.8	0.3	0.3	0.6	0.3	0.5
pE(a)CAT	1.2	1.1	0.3	0.3	0.9	0.6	0.6
pE(ab)CAT	1.1	0.8	0.2	0.1	0.4	0.3	0.5
pE(ac)CAT	1.0	0.9	0.2	0.1	0.6	0.6	0.6
pE(bc)CAT	1.1	0.8	0.3	0.2	0.3	0.3	0.5
pE(abc)CAT	0.8	0.8	0.1	0.1	0.2	0.2	0.4
p1-99CAT	1.0[b]	ND[c]	ND	1.0[b]	1.0[b]	1.0[b]	1.0[b]
p1E(c)CAT	0.8	ND	ND	0.2	0.3	0.6	0.7
p1E(b)CAT	0.8	ND	ND	0.2	0.1	0.3	0.7
p1E(bc)CAT	0.7	ND	ND	0.1	0.1	0.2	0.5

[a] The standard deviation from the mean value was ≤20% except for K562 and NIH 3T3 cells, for which the standard deviation was ≤25%.
[b] Value set to 1.0.
[c] ND, not done.

tions in either E_{gre}(A) or E_{gre}(B) or the double mutation resulted in no significant change in transcriptional activity. Mutation of the promoter-proximal E-box $E_{A/S}$(C) did not affect the level of transcription. Constructs with only one copy of the 99-bp repeat (Fig. 1) all showed a small reduction in transcriptional activity by introduction of E-box mutations (Table 1). In conclusion, under our assay conditions, the Akv MLV E-boxes seemed to contribute only weakly to the high Akv MLV transcriptional activity in NIH 3T3 cells. We notice that NIH 3T3 cells lack DNA-binding bHLH proteins ALF1 and ALF2 (Fig. 2).

We repeated the analysis with HeLa cells also lacking DNA-binding class A bHLH proteins. As observed in NIH 3T3 cells, the mutations of Akv MLV E-boxes resulted in only a reduced transcription relative to the levels of the parental construct (Table 1). Thus, in HeLa cells, as in NIH 3T3 cells, the MLV E-boxes contributed only slightly to MLV transcription.

E-boxes contribute to Akv MLV transcriptional activity in T- and B-cell lines. In previous studies, E-boxes were found to contribute to the transcriptional activity of lymphoid cell-expressed genes, such as the immunoglobulin and CD4 genes (22, 38). To examine if intact E-boxes contributed to the Akv MLV transcriptional activity in lymphoid cells relevant for MLV oncogenesis, we tested the panel of Akv MLV mutant constructs in the L691-6 and WEHI7.1 T-cell lines. The pE(c) CAT construct had a twofold-lower transcriptional activity than the parental construct (Table 1). Mutation of either E_{gre}(A) or E_{gre}(B) decreased the transcriptional activity approximately fourfold, whereas the pE(ab)CAT construct had a 5- to 10-fold-decreased activity (Table 1). Thus, for Akv MLV, E-boxes are essential for transcriptional activity in T-cell lines. Removal of one copy of the repeat did not affect Akv MLV transcriptional activity pronouncedly in L691-6 cells (24). Introduction of either single or double E-box mutations reduced the transcriptional activity equivalently to the decrease caused by the same mutations in the parental construct (Table 1). Thus, in a T-cell line, the absence of one copy of the Akv MLV enhancer repeat did not enhance the importance of the remaining E-box motifs. The greater importance of the E_{gre}(A) and E_{gre}(B) motifs in T-cell lines than in fibroblasts and HeLa cells correlates with the appearance of the class A bHLH complex $NFC_{gre}1$ (Fig. 2).

We also tested the importance of Akv MLV E-boxes for transcriptional activity in two B-cell lines. In the B-cell lines, the pE(c)CAT construct had a lower transcriptional activity than the wild-type construct, as observed in T-cell lines (Table 1). In both cell lines, the pE(b)CAT construct had a decreased transcriptional activity compared with that of the pE(a)CAT construct, indicating that the promoter-proximal E_{gre}(B) was more important for the enhancer function than the distal E_{gre}(A) motif. In agreement, the E(b) mutation and the double mutation E (ab) had equivalent activities, and pE(bc)CAT had lower activity than pE(ac)CAT (Table 1). These data suggested that in B-cell lines the E_{gre}(B) motif was more important than E_{gre}(A) in control of Akv MLV transcription. The construct with the triple mutation had a fivefold-reduced transcriptional activity compared with that of the parental construct, showing the major importance for Akv MLV E-boxes in mediating transcription in B-cell lines (Table 1). Removal of one copy of the enhancer repeat decrease the transcriptional activity of Akv MLV threefold in the B-cell lines (data not shown). Introduction of a mutation in E_{gre}(B) decreased transcriptional activity pronouncedly in the background of p1-99CAT (Table 1). Moreover, the mutations E(c) and E(bc) decreased the transcriptional activity (Table 1). Thus, removal of one copy of the 99-bp repeat increased the importance for intact E-boxes in B-cell lines. In conclusion, in T- and B-cell lines, E-boxes and in particular enhancer E_{gre} E-boxes have major importance for proper Akv MLV transcription. In T-cell lines, E_{gre}(A) and E_{gre}(B) might be of equivalent importance, whereas E_{gre}(B) might be more important than E_{gre}(A) in B-cell lines.

In the K562 erythroleukemia cell line, class A bHLH proteins exist in heterodimeric complexes with the class B bHLH oncoprotein TAL1 (14, 30). In K562 cells, a twofold reduction in transcription resulting from the E_{gre} mutations E(a), E(b), and E(ab), as well as the $E_{A/S}$ mutation E(c), was observed (Table 1). Removal of one copy of the Akv MLV enhancer repeat decreased the transcriptional activity in K562 cells twofold compared with that of the parental-type enhancer (data not shown). By further introduction of mutations in the E-boxes, we observed a decrease in activity smaller than the decrease observed for E-box mutations in the parental-type construct (Table 1).

In conclusion, in T-cell lines and an erythroleukemia cell line, E-box-binding protein complexes transactivate equivalently through both enhancer E_{gre} E-boxes, and in B-cell lines, protein complexes transactivate preferentially through the most promoter-proximal enhancer E-box, E_{gre}(B).

ALF1 and E2A transactivations have different enhancer E-box constraints. Since bHLH proteins were found to interact solely with the motifs E_{gre}(A) and E_{gre}(B) and since distinct E-boxes contribute differently to Akv MLV transcriptional activity in lymphocytes, we wanted to examine the activator capacity of class A bHLH proteins. We cotransfected the different Akv MLV-based CAT constructs into NIH 3T3 cells with the ALF1 expression vector and evaluated CAT activities for the examination of the putative activator function of ALF1. As previously observed, ALF1 transactivates the wild-type pAKV6CAT construct (Fig. 3 and 4) (32). The pE(c)CAT construct was transactivated by ALF1, whereas the pE(ab) CAT and pE(abc) CAT constructs did not respond positively to ALF1 overexpression (Fig. 4A). Thus, the enhancer E-boxes are necessary for ALF1 function, which is in accordance with the observations that enhancer-localized E_{gre} motifs are binding sites for ALF1 (Fig. 2) (32). The constructs pE(a)CAT and pE(b)CAT responded identically to transactivation by ALF1 (Fig. 4A). Thus, the existence of a single enhancer E-box is sufficient for mediating a positive ALF1 transcriptional signal, similar to the observations for T-cell lines with DNA-binding ALF1 subunits. Furthermore, we examined CAT constructs for transcriptional transactivation mediated by the *E2A* gene-encoded protein, E2-5. Like ALF1, E2-5 required intact E_{gre} motifs for mediating transcriptional activation (Fig. 4B). Interestingly, the pE(a)CAT construct was transactivated, while the pE(b)CAT construct was not significantly activated (Fig. 4B). Thus, the ALF1 and E2A proteins have different E-box constraints for mediating transcriptional activation. The importance of the E_{gre}(B) motif in E2A-mediated transcriptional activation parallels the importance of the intact motif for mediating transcriptional activity in B-cell lines, a cell type in which E2A subunit-including complexes bind to the motif (Fig. 2). In constructs in which one copy of the enhancer repeat is removed, E2A protein E2-5 and ALF1 transactivated transcription equally (Fig. 4B).

The different E-box requirements for transcriptional activation mediated by E2A protein E2-5 and ALF1 suggest that these two very homologous proteins somehow may contact the transcriptional machinery formed on the MLV promoter-enhancer differently.

Id1 out-titrates bHLH protein-mediated MLV transactivation. To examine the effect of Id1 on MLV E-box DNA-bind-

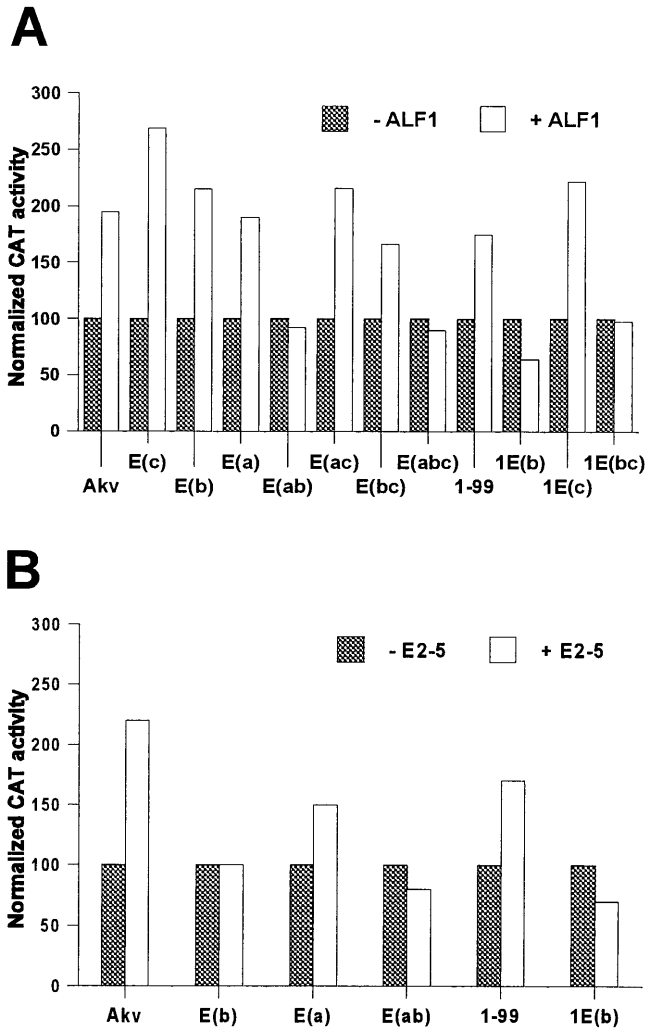

FIG. 4. ALF1 and E2A proteins have different constraints for E-box-mediated transcriptional activation. The CAT reporter constructs (2 μg) depicted in Fig. 1 were cotransfected in NIH 3T3 cells (5×10^5) with empty expression vectors and vectors expressing either ALF1 (A) or E2-5 (B). In all cases, the total amount of expression vector was 2 μg per transfection. Five micrograms of pCH110 was included as an internal standard expressing β-galactosidase from the simian virus 40 promoter. CAT activities normalized for β-galactosidase activities were estimated and visualized by setting the value for reporter constructs without bHLH overexpression to 100. The standard deviation from the mean values was ≤20%.

ing bHLH proteins in vitro, we did electrophoretic mobility shift assays. An oligonucleotide including the E_{gre} motif was used as a DNA probe (Fig. 5A, lane 1). Purified $ALF1_{562–706}$ resulted in the generation of a doublet of retarded bands constituting DNA-binding ALF1 oligomers (Fig. 5A, lanes 2 and 3). Addition of GST-Id1 to $ALF1_{562–706}$ resulted in a nearly 100% reduction of ALF1 DNA binding (Fig. 5A, lane 5). Thus, Id1 functions in vitro as a repressor of ALF1 DNA-binding activity, as observed for other class A bHLH proteins.

We examined the repressive effect of Id1 in vivo on MLV transcription in NIH 3T3 cells, using pAKV6CAT or pSL3-3CAT as a reporter construct. Ectopic expression of Id1 did not influence the transcription of pAKV6CAT (Fig. 5B). However, the ALF1-mediated transcriptional activation was significantly reduced when we coexpressed Id1 with ALF1 (Fig. 5B). The repressive effect of Id1 on ALF1 transactivation was ob-

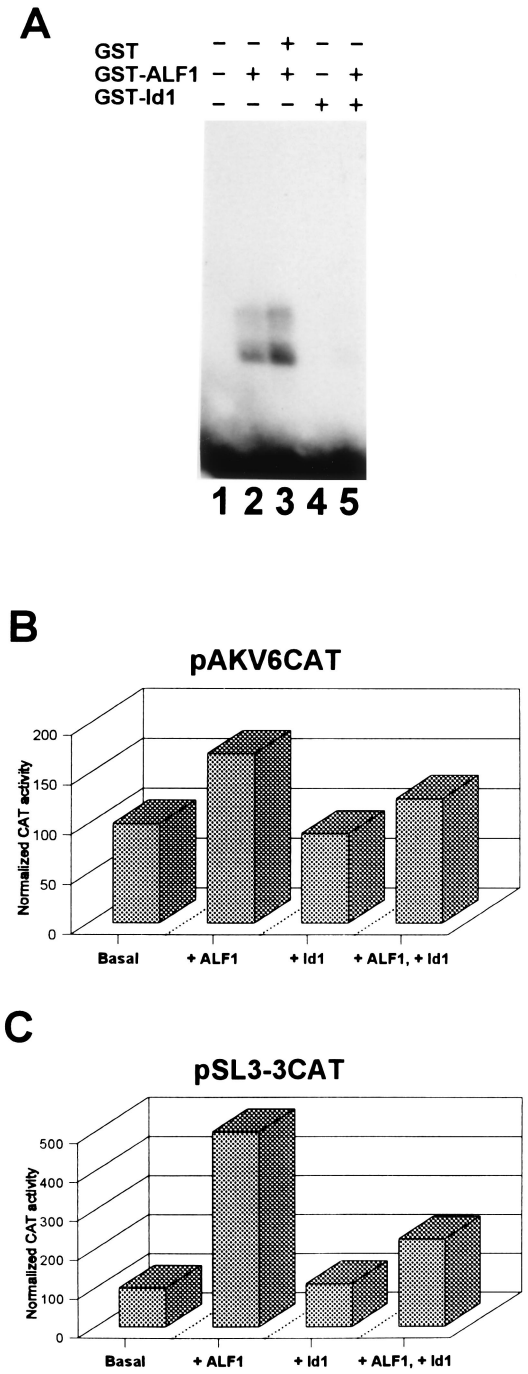

FIG. 5. Id1 represses ALF1-HEB activity in vitro and in vivo. (A) Id1 inhibits ALF1 DNA binding in vitro. An electrophoretic mobility shift assay was done with ^{32}P-labeled double-stranded E_{gre} oligonucleotide and the purified proteins GST-$ALF1_{562–706}$, GST, and GST-Id1 as indicated. (B and C) Id1 inhibits ALF1 activity in vivo. In NIH 3T3 cells (5×10^5), pAKV6CAT (2 μg) (B) or pSL3-3CAT (2 μg) (C) was cotransfected with empty parental expression vectors and expression vector Id1 or ALF1, as indicated. In all cases, a total of 2 μg of expression vector was used per transfection. Five micrograms of pCH110 was included as an internal standard expressing β-galactosidase from the simian virus 40 promoter. β-Galactosidase-normalized CAT activities were estimated by using the parental expression vectors arbitrarily set to 100. The standard deviations from the mean values were ≤15% in panel B and ≤20% in panel C.

TABLE 2. E_{gre}-directed MLV transcription is repressed by Id1 in lymphoid cell lines

Construct	Normalized CAT activity[a]							
	L691-6[b]		Mpc11[b]		Mpc11[c]		J558L[b]	
	Without Id1	With Id1	Without Id1	With Id1	Without Id1	With Id1	Without Id1	With Id1
pAKV6CAT	1.0[d]	0.5	1.0[d]	1.0	1.0[d]	0.6	1.0[d]	1.1
pE(ab)CAT	1.0[d]	1.0	ND[e]	ND	ND	ND	1.0[d]	1.1
pSL3-3CAT	1.0[d]	0.5	1.0[d]	0.4	1.0[d]	0.2	1.0[d]	0.5

[a] The standard deviation from the mean value was ≤20%.
[b] Five micrograms of CAT reporter construct was included in each transfection.
[c] One microgram of CAT reporter construct was included in each transfection.
[d] Value set to 1.0.
[e] ND, not done.

served with the pSL3-3CAT construct as well (Fig. 5C). Thus, Id1 may function as a repressor of ALF1-mediated MLV transcription in vivo.

Id1 represses MLV transcription in lymphoid cell lines. The bHLH protein complexes from lymphoid cell nuclear extracts that were found to interact with the E_{gre} motifs are targets for dissociation by Id proteins (Fig. 2). It deserves notice that covalent linking between the protein members in class A bHLH homodimers has been identified (3) and that Id proteins dissociate TAL1-E2A heterodimers inefficiently (16, 30). In the L691-6 T-cell line, ectopic expression of Id1 resulted in a 50% reduction in the transcriptional activity from pAKV6 CAT (Table 2). Use of the pE (ab) CAT construct showed that repression by Id1 required intact E_{gre} motifs (Table 2). That ectopic Id1 in a T-cell line can function as a transcriptional regulator by mediating MLV transcriptional repression verifies that class A bHLH proteins are important Akv MLV transcription factors in T-cell lines. Use of the pSL3-3CAT construct as a reporter showed that Id1 also has the capacity to regulate SL3-3 MLV's transcriptional level (Table 2). Thus, the existence of identical E-boxes in Akv and SL3-3 MLV enhancers resulted in the same response to Id1 protein in a T-cell line.

In B-cell lines (Mpc11 and J558L), ectopic expression of Id1 again resulted in a roughly 50% reduction of transcription from the pSL3-3CAT construct (Table 2). pAKV6CAT was not repressed by ectopic expression of Id1 in the B-cell lines under the experimental conditions used for the T-cell line, i.e., use of 5 μg of CAT reporter construct (Table 2). However, by using a smaller amount of Akv MLV reporter construct (1 μg), a repression by Id1 was detectable, confirming that Akv MLV was under transcriptional regulation by class A bHLH proteins in B-cell lines (Table 2). Thus, although Akv MLV and SL3-3 MLV carry almost the same set of E-box sequences, they did not respond exactly alike to ectopic Id1 in the B-cell lines tested, indicating that sequences outside the E-boxes are important determinants for cell-type-directed function of the E-boxes in Akv MLV and SL3-3 MLV.

DISCUSSION

For study of transcriptional regulation, MLVs represent an excellent model system, with their regulatory sequences localized in a well-defined DNA segment in the LTR and with regulation dependent exclusively on cellular transcription factors. By electrophoretic mobility shift assays, we found that the two types of promoter-enhancer-localized E-boxes, E_{gre} and $E_{A/S}$, in Akv MLV and SL3-3 MLV bind distinct protein complexes. Supershift analysis using specific antibodies directed against ALF1 or HEB or E2A showed that the E_{gre} E-boxes bound ALF1 or HEB and E2A proteins in nuclear extracts from lymphoid cell lines, whereas the $E_{A/S}$ E-box complex did not include ALF1 or HEB or E2A. The E_{gre} E-box complex was found to include both ALF1 or HEB and E2A, suggesting the existence of a heterodimer. Previous reports have described such a heterodimeric E-box binding complex (38).

Nuclear extracts from NIH 3T3 and HeLa cells included no E-box-binding capacity of ALF1 or E2A proteins. In transfection analysis, we showed that ALF1, HEB or E2A homodimeric protein complexes transactivated transcription from the Akv MLV enhancer to the same level as when the proteins were coexpressed (Fig. 3). Thus, there is no synergistic effect of ALF1 or HEB and E2A proteins. However, a putative heterodimerization may result in stabilization compared with the relatively labile homodimer complex, e.g., resulting in greater resistance to dissociation, as observed for E2A-TAL1 heterodimers (16, 30), or a changed DNA specificity, which we were not able to observe using the MLV E-boxes. We should draw attention to the fact that homodimeric ALF1/HEB and E2A proteins have different preferred DNA-binding sites (5, 42).

Some of the E-box complexes detected in this work did not contain ALF1 or ALF2 or E2A proteins. Complexes formed on E_{gre} and $E_{A/S}$ without class A bHLH components might be transcriptional repressors, as proposed for E-boxes in immunoglobulin enhancers, in which the transcriptional repressor ZEB regulates transcriptional regulation through E-box sequences (11).

By introduction of mutations in the Akv MLV E-boxes, we examined their putative function as targets for activators and repressors. In fibroblasts and HeLa cells, mutation of the E-boxes only moderately affected the transcriptional activity. This is in accordance with the absence of functional class A bHLH E-box DNA-binding activity in fibroblasts (Fig. 2). Analysis of Akv MLV reporter constructs with mutations in the two E_{gre} E-boxes (A and B) and the $E_{A/S}$ showed that the expression was reduced to 10 to 20% of that of the parental Akv MLV reporter, suggesting a very central role in the enhancer function of E-box-binding proteins either alone or in a larger complex with other proteins involved in the transcriptional regulation. This shows that the E-boxes in the enhancer region are essential for proper Akv MLV transcription in lymphoid cell lines and suggests an important role for the observed E-box-binding ALF1 or HEB and E2A proteins in Akv MLV transcriptional regulation (Fig. 2 and Table 1).

In T-cell lines, mutation of each of the E_{gre} E-boxes [E_{gre}(A) and E_{gre}(B)] reduced the transcriptional activity equally, and the double mutation decreased the activity even further in L691-6 cells (Table 1). Interestingly, in B-cell lines, the promoter-proximal enhancer E-box, E_{gre}(B), was most important for Akv expression, indicating a different composition of the E-box binding complex in T- and B-cell lines.

Ectopic expression of ALF1 in NIH 3T3 cells showed that ALF1 transactivated equivalently through the two enhancer E_{gre} E-boxes, similar to the situation of E-box regulation in T-cell lines, whereas overexpression of the E2A protein, E2-5, transactivated with preference through the more promoter proximal of the enhancer E-boxes, E_{gre}(B), equivalent to the E-box requirement in B-cell lines (Fig. 4). The different E-box requirements in E2A protein E2-5- and ALF1-mediated transcriptional activation suggest that these two very homologous proteins contact the transcriptional machinery formed on the MLV promoter-enhancer differently.

The only known function of the family of Id proteins is to repress the function of class A bHLH proteins. We have measured the effect of Id overexpression to see if repression may be detected in MLV transcription. Id1 was previously described to be a repressor of DNA binding and of transcriptional activation by E2A proteins (4, 19, 43). We found that Id1 repressed ALF1 DNA binding in vitro and that ectopic expression of Id1 resulted in a decrease in ALF1-mediated transactivation of Akv MLV and SL3-3 MLV transcription in vivo. Thus, Id1 has the characteristics expected for a repressor of ALF1-mediated transcriptional activation. According to this, we found that in a T-cell line ectopic Id1 repressed transcription of Akv MLV and SL3-3 MLV. Repression by Id1 required intact E_{gre} motifs (Table 2). This is in agreement with our observations showing that the E_{gre} motifs, and not the $E_{A/S}$ motif, bind class A bHLH protein-including protein complexes (Fig. 2). We found different sensitivities to Id repression of Akv and SL3-3 MLV expression in B-cell lines. This may reflect various concentrations of endogenous bHLH proteins in the different cell types.

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