The TAL1 gene (also abbreviated SCL or TCL5) is found transcriptionally activated in 25% of T-cell acute lymphoblastic leukemia (T-ALL) by tumor-specific rearrangements such as chromosomal translocations or local DNA recombinations (1–3). Under normal conditions, TAL1 gene expression is restricted to some cells of the endothelial and hematopoietic lineages (24, 25). As well as TAL1, other transcription factors such as GLI5, E2A, myc, and Ets that have class A bHLH protein DNA binding activity. Opposite, TAL1/E2A is a significantly better transcription activator than the E2A homodimer, due to a higher stability of the complex (26–29).

In this study, we report that the bHLH MLV transactivator protein ALF1 heterodimerizes with TAL1 in vitro and in vivo and that such heterodimers bind a MLV enhancer E-box sequence. Ectopic expression of ALF1 in NIH3T3 fibroblasts activates MLV transcription to a level that is modulated by coexpression with TAL1. Similar transcriptional modulations by ectopic TAL1 were observed in hematopoietic cell lines, which have class A bHLH protein DNA binding activity. Oppositely, transcriptional repression by TAL1 of ALF1-mediated
transactivation was observed using artificial E-box reporter constructs. Our studies suggest that TAL1 is a transcriptional modulator under the appropriate cellular conditions and that MLVs provide a model system for studies of the transcriptional regulatory functions of TAL1 in vivo.

MATERIALS AND METHODS

Cell Lines—NIH3T3 fibroblasts and Mpc11 immunoglobulin-secret ing B-cells were maintained in Dulbecco's modified Eagle's medium, 10% calf newborn serum, 2% penicillin/streptomycin. L691-6 T-lymphoma cells and K562 erythroleukemia cells were maintained in RPMI 1640 medium, 10% calf newborn serum, 2% penicillin/streptomycin. Eukaryotic Vectors—pAKV6CAT and pSL3-3CAT, including the chloramphenicol acetyltransferase (CAT) gene under control of complete MLV long terminal repeats and pEabcCAT lacking E-boxes on the background of pAVK6CAT sequences, have been described (32). Friend and Moloney MLV CAT constructs were kindly donated by D. Kabat (34) and N. A. Speck (35), respectively. The minimal E-box CAT construct has been described (36). pNALF1A expressing ALF1 under the control of the EF-1α promoter has been described (32). Expression vector pCMV4-TAL1 coding for full-length human p42AL1, pCMV4-TAL162 coding for a TAL1 version with point mutations in the DNA-binding basic region, and pCMV4-I-d1 were kindly donated by R. Baer (University of Texas Southwestern Medical Center) (30).

For expression of ALF1 and TAL1 proteins in Saccharomyces cerevisiae, the vectors described by P. Chevray were used (37). TAL1 and TAL1Δ65 were digested with EcoRI and XbaI and inserted in the EcoRI and SpeI sites of pC86. An ALF1 deletion protein, ALF1Δ66, lacking amino acids 46–499 of ALF1 cDNA, was inserted in pC86 and pPC97. Constructs in pPC97 and pPC86 were cotransfected into the yeast strain CBY-1.4.1-a and were plated on appropriate selective plates. Assays for monitoring the expression level of β-galactosidase were done under standard conditions.

Prokaryotic Expression Vectors and Protein Purification—For expression of ALF1 amino acids 562–706, ALF1(562–706), in bacteria ALF1 cDNA was polymerase chain reaction-amplified with primers including synthetic BamHI and EcoRI restriction sites followed by cloning into the corresponding sites of pGEX2TK (Pharmacia, Sweden). TAL1 and TAL1Δ62 bacterial expression vectors were generated by cloning of EcoRI fragments including the complete coding region into the EcoRI site of pGEX2TK. Induction and purification procedures for recombinant glutathione S-transferase (GST) fusion proteins were as described by the manufacturer (Pharmacia). ALF1-(562–706) was purified from the GST moiety by thrombin cleavage as described by the manufacturer (Pharmacia), whereas the TAL1 proteins were obtained as GST fusions.

Electrophoretic Mobility Shift Assays—The 34-base pair oligonucleotide E-box probes were 32P-labeled to equivalent specific activities using the method described in Ref. 32. Prokaryotic proteins were preincubated at 37 °C for 30 min in binding buffer before the addition of DNA to assist heterodimer formations. Binding reactions including 2 × 105 cpm of probe were done at 25 °C for 15 min in 25 μl of 10 mM HEPES-NaOH (pH 7.9), 10 mM Tris-HCl (pH 7.9), 3 mM MgCl2, 80 mM NaCl, 1 mM dithiothreitol, 300 μM bovine serum albumin with 1 μg of poly(dI-dC)-poly(dI-dC), TWEEN 20 to 0.25%. Following binding, reactions were electrophoresed as described (33). In competition analysis, a 50-fold excess of unlabeled oligonucleotide was added with the probe in the binding reaction.

Transfections and CAT Assays—NIH3T3 cells were transfected by calcium phosphate-mediated precipitation as described (32). Each precipitate included 2 μg of CAT reporter plasmid, 2 μg of either pALF1A expression vector or 2 μg of the parental pBSNEN expression vector, 2 μg of TAL1 expression vector or 2 μg of the parental CMV4 expression vector, and 3 μg of pCH110, an internal standard coding for β-galactosidase (TAL1 competition analysis and β-gal association analysis both included 8 μg of total CMV4 expression vectors). CAT assays were done for 0.5–2 h, conversions were monitored by PhosphorImager analyses (Molecular Dynamics) of thin layer chromatograms, and CAT conversions were normalized to the level of β-galactosidase. The presented CAT values are the means of at least three independent transfection experiments. Transfections of Mpc11 and K562 cells were as described, for NIH3T3 cells except that cells were seeded to 3 × 105 cells/100-mm diameter plate 24 h before transfection. DNA concentrations used were 3 μg of pCH110, 5 μg of CAT reporter plasmid, and, in cotransfections, 6 μg of TAL1 expression vectors or 6 μg of the parental expression vector. L691-6 cells were transfected by a modification of the DEAE method (33). The transfection mix included 5 μg of CAT reporter plasmid.

RESULTS

TAL1 and ALF1 Interact in Vitro and Bind to a Subset of the E-boxes—The heterodimer between TAL1 and class A bHLH proteins was by DNA-binding site selection methods found to have the preferred DNA-binding sequence, AACAGATGGT, with the imperfect palindromic E-box core sequence underlined (18). The binding site includes half-sites for class A bHLH partner (AACAG) and for class B bHLH protein TAL1 (ATGGT).

Until now candidate genes regulated by the TAL1/class A bHLH heterodimer have not been identified in either T-cell acute lymphoblastic leukemia or hematopoietic lineages normally expressing TAL1. The MLV enhancer in the U3 region of the long terminal repeat (LTR) includes, for most MLVs, an E-box motif, Egre, which shares high identity with the preferred TAL1/class A bHLH heterodimer binding site (Fig. 1) (38), suggesting that MLVs might form a target for transcriptional regulation of TAL1. Previous examinations have shown that the class A bHLH protein ALF1 has the characteristics expected for a hematopoietic E-box-dependent MLV transactivator protein (20, 32, 33, 39).

To determine if ALF1 and TAL1 could form stable heterodimers in vitro, we performed electrophoretic mobility shift assays using labeled double-stranded oligonucleotides including the Egre motif, with ALF1(562–704), GST, GST-TAL1, and GST-TAL162; the last is a version of TAL1 with no affinity to DNA. The proteins were expressed in Escherichia coli (see “Materials and Methods.” TAL1 or TAL162 GST fusion proteins were not able to form DNA-binding homodimers (Fig. 2A, lanes 4 and 6). ALF1(562–706) alone formed two complexes.
with the $E_{grev}$ oligonucleotide (Fig. 2A, lane 2). Mixing ALF1-(562–706) and TAL1-GST generated a doublet of putative ALF1/TAL1 heterooligomeric complexes, with retarded mobilities compared with the ALF1 homooligomeric complexes (Fig. 2A, lane 5). As expected, mixing ALF1-(562–706) and GST-TAL1β2 did not result in generation of heterooligomeric bands but in a decreased ALF1-(562–706) DNA binding. Thus, the mutation in TAL1β2 gives the protein properties as an Id protein (Fig. 2A, lane 7). It is notable that the two TAL1/ALF1 bands represent heterodimeric and higher oligomeric complexes, since changing the stoichiometry between ALF1 and TAL1 as well as the total concentration altered the band composition in the binding reactions (Fig. 2B).

Competition analyses assured the DNA binding specificity of TAL1/ALF1-(562–706) heterooligomers to the $E_{grev}$ motif, using an unlabeled $E_{grev}$ oligonucleotide, which efficiently competed DNA binding, whereas oligonucleotides $E_{mut}$ and $E_{AAS}$ having an E-box mutation and another type of E-box, respectively, competed DNA binding only slightly (Fig. 2C).

TAL1/ALF1 Heterodimers Bind One Specific Type of E-box in Vitro—Several other types of E-boxes can be identified in the U3 section of MLV LTRs besides the $E_{grev}$ motif. Fig. 1, A and B, show the distribution of E-boxes in the MLV enhancers and sequences of MLV E-boxes used in our analysis. To determine to which specific types of the E-boxes ALF1 homodimers and ALF1/TAL1 heterodimers bind, we performed electrophoretic mobility shift assay analysis using E-box-containing oligonucleotides, differing only in the E-box sequence. ALF1-(562–706) homooligomeric complexes were found binding to the $E_{grev}$, $E_{LVa}$, $E_{Mpr}$, $E_{M5}$, and $E_{cont}$ but not to the $E_{AAS}$, $E_{mut}$, and $E_{MP}$ (Fig. 3A). In contrast, the TAL1/ALF1-(562–706) oligomeric complex is formed only with the $E_{grev}$ probe (Fig. 3B). Our results agree with observations showing that in class A bHLH protein/TAL1 heterodimers, the class A bHLH protein and TAL1 subunits have the DNA-binding half-sites, AACAG and ATGGT, respectively (18). None of the MLV E-boxes besides the $E_{grev}$ carry the TAL1 half-site (Fig. 1A). In conclusion, the constraints for ALF1 homooligomeric E-box binding are less than for ALF1/TAL1 heterooligomeric E-box binding, and MLVs include an ALF1/TAL1 heterodimeric DNA response element, the $E_{grev}$, in the enhancer region.

TAL1 and ALF1 Heterodimerize in Vivo—ALF1/TAL1 heterodimerization was further studied using the yeast two-hybrid system. A truncated ALF1 version, ALF1Δ6, that lacks amino acids 46–499 but retains the bHLH domain was fused to the GAL4 DNA-binding domain, GAL4DB, or to the GAL4 transactivation domain, GAL4TA. Full-length TAL1 and TAL1β2 were fused to GAL4TA. Previous examinations have shown that TAL1 is not able to form stable homodimers in vivo (40). We cotransfected constructs coding for GAL4DB and for GAL4TA fusion proteins into yeast cells (Fig. 4). GAL4DB-ALF1 coexpressed with GAL4TA-TAL1 caused a large increase in the $\beta$-galactosidase activity compared with the basal level (Fig. 4). We obtained an equivalent $\beta$-galactosidase activity level with GAL4DB-ALF1 and GAL4TA-TAL1β2, which showed that the introduced mutations in the basic region af-
TAL1 Modulates ALF1-mediated Transcriptional Activity—

Given that ALF1 and TAL1 dimerize in vivo and interact with the E-box, identified in several MLV enhancers, we wanted to examine the transcriptional regulatory capacity of TAL1. NIH3T3 fibroblasts contain no activity of DNA-binding class A bHLH proteins (32, 33, 39). In NIH3T3 cells, ectopically expressed ALF1 increases the transcriptional activity of the LTR enhancers of Akv MLV (pAKV6CAT, SL3-3 MLV (pSL3-3CAT), Moloney MLV (pMoCAT), or Friend spleen focus-forming virus (pFrSFFVCAT) (Fig. 1 and Table I), all including the E-box motif (32). Coexpression of ALF1 and TAL1 resulted in a transcriptional decrease in activity (Table I). Coexpression of TAL1β2 with ALF1 reduced the activated transcription level from the reporters to the basal level (Table I). Thus, we achieved a complete out-titrated of ALF1 homodimers when TAL1 protein was expressed in the amount used in our experiments. The modulation of transcription obtained by TAL1 expression depends on the existence of heterodimeric complexes including TAL1. Additionally, the positive modulatory effect of TAL1 required a functional TAL1 DNA-binding basic region.

To examine the importance of intact E-boxes in mediation of the transcriptional activity of the ALF1/TAL1 heterodimeric complex, we used two different constructs: (i) pE(abc)CAT, which is pAKV6CAT with the enhancer E-box motifs and the upstream promoter E\textsubscript{0.2} motif mutated to prevent binding of TAL1/ALF1 heterodimers and ALF1 homodimers, and (ii) pFrCAT, a Friend MLV LTR-based construct, which lacks E-box motifs in the LTR enhancer (Fig. 1). Expression of pE(abc)CAT and pFrCAT were affected neither by expression of ALF1 alone nor by TAL1 and ALF1 in combination (Table I), showing that TAL1/ALF1 heterodimers require intact LTR E-box motifs for mediating the transcriptional modulation.

Transcriptional Modulation by TAL1 Has E-box Environmental Constraints—We wanted to examine if the TAL1 modulation of ALF1 transcriptional regulatory capacity was reflected only in the environment of intact enhancers or could be observed on artificial E-box constructs as well. For this purpose, we used a CAT construct, p1-37CAT, with two E-box motifs placed immediately upstream of the TATA-box on the background of the adenovirus E1b promoter (Fig. 1C). In NIH3T3 cells, the basal CAT activity from p1-37CAT was very low and was significantly activated by ALF1 overexpression (Table I). Coexpression of ALF1 and TAL1, using the same amounts of expression vectors as described above, decreased CAT activity from p1-37CAT to the background level (Table I). The same decrease in activity was observed by coexpression of ALF1 and TAL1β2 (Table I). Thus, on the background of p1-37CAT, TAL1 functions as a repressor of ALF1-mediated transactivation. This result opposed the observations made with intact MLV enhancers. To examine the difference between the MLV enhancer CAT constructs and p1-37CAT further, we performed an experiment where we competed ALF1 transactivation of pSL3-3CAT and p1-37CAT with increasing amounts of TAL1 (Fig. 5). For pSL3-3CAT, about 2000 ng of TAL1 expression vector was required to reduce the ALF1-induced activation to 50%. However, for p1-37CAT 10-fold less TAL1 expression vector was required to obtain the 50% reduction. Thus, the positive effect of TAL1 was exhibited primarily when the target E-box was within a natural enhancer environment, where TAL1 complexes may be assisted by accessory factors. The orientation of the E-box relative to the promoter and the distance to the promoter may influence the effect, as well. However, an artificial construct with inverted orientation of the E-boxes responded as p1-37CAT to TAL1 (data not shown).

Ectopic Expression of TAL1 in Hematopoietic Cell Lines Modulates Transcription—Previous examinations of nuclear extracts from lymphoid cell lines such as L691-6 T-cells have shown the presence of oligomeric class A bHLH protein complexes, including ALF1 and E2A, with affinity to the E-box motif, and introduction of mutations in the E-boxes on the background of MLV enhancers was found to decrease transcrip-

![Fig. 4. TAL1 and ALF1 interact in vivo. Yeast cells were cotransfected with various plasmids coding for parental GAL4DB, GAL4DB-ALF1, parental GAL4TA, GAL4TA-ALF1, GAL4TA-TAL1, or GAL4TA-TAL1β2 as indicated. The β-galactosidase activity corresponding to each cotransfection was measured by an o-nitrophenyl-β-D-galactopyranoside assay and given in relative amount.](image)

### Table 1

| Constructs       | Basal\(a\) | +TAL1 | +TAL1β2 | +ALF1 | +TAL1/+ALF1 | +TAL1β2/+ALF1 |
|------------------|------------|-------|---------|--------|-------------|--------------|
| pSL3-3CAT        | 1.0 ± 0.2  | 1.0 ± 0.2 | ND     | 4.6 ± 0.9 | 3.0 ± 0.6 | 1.3 ± 0.3     |
| pMoCAT           | 1.0 ± 0.2  | 0.7 ± 0.1 | ND     | 4.2 ± 0.8 | 2.1 ± 0.4 | 1.1 ± 0.2     |
| pFrSFFVCAT       | 1.0 ± 0.2  | 1.3 ± 0.3 | ND     | 4.0 ± 0.8 | 2.6 ± 0.5 | ND            |
| pAKV6CAT         | 1.0 ± 0.2  | 0.7 ± 0.2 | 0.8 ± 0.2 | 1.7 ± 0.4 | 1.3 ± 0.3 | 0.8 ± 0.2     |
| pE(abc)CAT       | 1.0 ± 0.2  | 0.7 ± 0.2 | ND     | 0.9 ± 0.2 | 0.9 ± 0.2 | ND            |
| pFrCAT           | 1.0 ± 0.2  | ND     | ND     | 0.8 ± 0.2 | 0.9 ± 0.2 | ND            |
| p1-37CAT         | 1 ± 0.2    | 1 ± 0.2 | ND     | 50 ± 10  | 1 ± 0.2   | 1 ± 0.2       |

\(a\) All values represent a minimum of three transfection experiments in NIH3T3 fibroblast cells, and the standard deviation is given as ±n.

\(b\) Transfections were including the parental expression constructs and values were normalized to 1.

ND, not done.
Id1 Does Not Repress Transcription Mediated by TAL1/ALF1 Heterodimers—Id1 proteins repress class A bHLH-mediated transcriptional activity through the formation of heterodimers, which cannot bind to DNA (26–29, 33). Since TAL1/ALF1 heterodimers and homodimeric ALF1 in vivo displayed different stability (see Fig. 4), we wanted to examine the capacity of Id protein to dissociate these two types of complexes. In NIH3T3 cells ALF1 activation of SL3-3 MLV expression had no effects on the transcriptional level, whereas TAL1 does not affect E2A-mediated transcriptional activity in lymphoid cells (19, 33). To examine the effect of TAL1 ectopic expression on class A bHLH-mediated transcriptional activity in lymphoid cells, we transfected pSL3-3CAT with TAL1 expression constructs into the T-cell lineage L691-6 and the B-cell lineage Mpc11. With TAL1 coexpression in Mpc11 cells CAT activity was decreased, whereas in L691-6 the reduction was insignificant. Ectopic expression of TAL1 with Id1 expression construct as indicated. The total amounts of CMV4 expression constructs were adjusted to 8 \text{ng} by the addition of parental expression vector. The normalized CAT activity obtained with empty expression vector was given a value of 1.0. This experiment was repeated three times, and the standard deviation was \leq 15\% of the mean values.

**DISCUSSION**

We show that the class A bHLH protein ALF1 and the class B bHLH protein TAL1 interact both \textit{in vitro} and \textit{in vivo}. The TAL1/ALF1 heterodimerization is favored compared with ALF1 homodimerization (Fig. 2A and Fig. 4), which might be a shared property with other bHLH proteins (30, 31). TAL1 heterodimer formation with other class A bHLH protein products has been examined (18, 30, 40, 42, 43), and heterodimers are identified in myeloid, erythroid, and Jurkat T-ALL cells. The preferred binding sequence of TAL1 heterodimers with class A bHLH proteins was determined to be the E-box, AACAGATGGT (18). An equivalent E-box, E_{\text{pre}} is present in the enhancers of several MLVs and was previously found to bind ALF1 homodimers (22, 32).

By electrophoretic mobility shift assays, we found using an array of oligonucleotides, including different MLV E-box motifs, that TAL1/ALF1 heterodimers bind exclusively to the E_{\text{pre}} site, whereas ALF1 homodimers have affinity to an assortment of E-boxes (Fig. 3). Thus, heterodimerization results in an increased specificity of DNA binding. An off-rate constant determination assay showed that ALF1 homodimers and TAL1/ALF1 heterodimers dissociate from the E_{\text{pre}} site with the same kinetics (data not shown). Since there is a large preference for heterodimerization \textit{in vivo}, the stoichiometry of TAL1 and ALF1 in the cell will be reflected directly in the stoichiometry of TAL1/ALF1 complexes binding to DNA.

The existence of a TAL1/ALF1 heterodimer binding site in MLV enhancers suggests that MLVs constitute a group of genes transcriptionally regulated by TAL1. Heterodimerization with TAL1 decreased the transactivation by E47 through immunoglobulin gene enhancer E-boxes and a b-globin locus control region E-box, whereas TAL1 does not affect E2A-mediated transcriptional activity from a muscle creatine kinase.
enhancer E-box (31). Equivalently, a reporter construct with six TAL1/E2A-binding E-box motifs arranged in tandem responded weakly positively to the presence of TAL1/E2A heterodimers (30). Using several intact MLV enhancers, we found in our analysis that coexpression of TAL1 modulated MLV transcriptional activation by ALF1 (Table I). The transcriptional activity observed with coexpression of ALF1 and TAL1 was not due to residual ALF1 homodimers, because expression of a dominant negative TAL1 mutant protein reduced the transcription to the basal level. Thus, the TAL1/ALF1 heterodimer has transcriptional activator capacity on the background of intact enhancer sequences.

Using an artificial E-box reporter construct, TAL1 was found to function as a repressor of ALF1-mediated transcriptional activity. Titration analysis showed that significantly more TAL1 was required to reduce ALF1-mediated transcription of the SL3-3 MLV enhancer, compared with an artificial E-box reporter construct p1-37CAT (Fig. 5). The reduced transcription from the SL3-3 MLV enhancer at higher TAL1 concentrations might be due to a general transcriptional squelching. It deserves notice that the CAT activity from ALF1-activated SL3-3CAT was about 5-fold higher than ALF1-activated p1-37CAT (data not shown). In conclusion, in our analysis only intact enhancers form an environment for transcriptional activation by TAL1/ALF1 heterodimers. This might be a consequence of a synergistic function with additional transcription factors binding to the enhancer sequences. For example, it has been reported that TAL1 interact with Lin-11, ISL1, Mec-3 (LIM) domain proteins (44).

That intact E-boxes are required for the TAL1/ALF1 heterodimer-mediated transcriptional modulations was shown by using either the Akv MLV-derived construct E(abc)CAT lacking intact E-boxes in the LTR U3 region or the Friend MLV CAT construct including no efficient ALF1 homodimer or TAL1/ALF1 heterodimer binding sites in the LTR U3 region. Neither ALF1 homodimers nor TAL1/ALF1 heterodimers transactivated these two constructs (Table I).

Id proteins are expressed in multiple lineages that in vitro can be induced to differentiate terminally, and the level of Id is often decreased during terminal differentiation. A notable exception is myeloid differentiation, where the Id levels are only transiently depressed (45). All members of the Id family can act as transcriptional squelching. It deserves notice that the CAT activity from ALF1-activated SL3-3CAT was about 5-fold higher than ALF1-activated p1-37CAT (data not shown). In conclusion, in our analysis only intact enhancers form an environment for transcriptional activation by TAL1/ALF1 heterodimers. This might be a consequence of a synergistic function with additional transcription factors binding to the enhancer sequences. For example, it has been reported that TAL1 interact with Lin-11, ISL1, Mec-3 (LIM) domain proteins (44).

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Id proteins are expressed in multiple lineages that in vitro can be induced to differentiate terminally, and the level of Id is often decreased during terminal differentiation. A notable exception is myeloid differentiation, where the Id levels are only transiently depressed (45). All members of the Id family can probably function as inhibitors of cellular differentiation (45–47). According to this, the transcriptional activation mediated by ectopically expressed ALF1 was strongly repressed by the dominant negative Id1 protein (Fig. 6). Therefore, in the context of Id expression, ALF1 is a poor transcriptional activator. In contrast, transcription mediated by TAL1/ALF1 heterodimers was resistant to Id inhibition (Fig. 6). Consequently, in the presence of Id protein the appearance of TAL1/ALF1 heterodimers was resistant to Id inhibition (Fig. 6). Consequently, in the presence of Id protein the appearance of TAL1/ALF1 heterodimers was resistant to Id inhibition (Fig. 6).

In approximately 25% of all analyzed T-ALLs, the TAL1 locus is genetically rearranged in the 5’ noncoding region, resulting in TAL1 overexpression (4–6). Thus, in these T-ALL cells, expression of genes, which are transcriptionally regulated through non-Ebox E-boxes, might be repressed by the TAL1 activation, whereas Ebox E-box-directed gene expression may be enhanced. This may partly explain the oncogenic effect of TAL1.

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