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ALY Is a Common Coactivator of RUNX1 and c-Myb on the Type B Leukemogenic Virus Enhancer[▽]

Jennifer A. Mertz,[1] Ryuji Kobayashi,[3] and Jaquelin P. Dudley[1,2]*

Section of Molecular Genetics and Microbiology[1] and Institute for Cellular and Molecular Biology,[2] University of Texas at Austin, Austin, Texas, and Department of Molecular Pathology, University of Texas M. D. Anderson Cancer Center, Houston, Texas[3]

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Type B leukemogenic virus (TBLV), a mouse mammary tumor virus (MMTV) variant, often induces T-cell leukemias and lymphomas by c-*myc* activation following viral DNA integration. Transfection assays using a c-*myc* reporter plasmid indicated that the TBLV long terminal repeat (LTR) enhancer is necessary for T-cell-specific increases in basal reporter activity. The sequence requirements for this effect were studied using mutations of the 62-bp enhancer region in an MMTV LTR reporter vector. Deletion of a nuclear factor A-binding site dramatically reduced reporter activity in Jurkat T cells. However, a 41-bp enhancer missing the RUNX1 site still retained minimal enhancer function. DNA affinity purification using a TBLV enhancer oligomer containing the RUNX1 binding site followed by mass spectrometry resulted in the identification of ALY. Subsequent experiments focused on the reconstitution of enhancer activity in epithelial cells. ALY overexpression synergized with RUNX1B on TBLV enhancer activity, and synergism required the RUNX1B-binding site. A predicted c-Myb binding site in the enhancer was confirmed after c-*myb* overexpression elevated TBLV LTR reporter activity, and overexpression of c-Myb and RUNX1B together showed additive effects on reporter gene levels. ALY also synergized with c-Myb, and coimmunoprecipitation experiments demonstrated an interaction between ALY and c-Myb. These experiments suggest a central role for ALY in T-cell enhancer function and oncogene activation.

Previous experiments have shown the importance of retroviral enhancers for disease induction. Substitutions of the duplicated enhancer element within the U3 region of a thymotropic Moloney murine leukemia virus (MuLV) long terminal repeat (LTR) with the comparable region from an erythroleukemia-inducing MuLV switched viral disease specificity (24, 32, 48). The type B leukemogenic virus (TBLV), a betaretrovirus, induces T-cell leukemias and lymphomas when injected into either neonatal or adult mice (3, 40). TBLV is highly related to mouse mammary tumor virus (MMTV), which induces primarily murine breast cancers (4, 5). Compared to the MMTV LTR, the TBLV LTR shows a loss of the MMTV negative regulatory elements (NREs) and triplication of the 18 and 44 bp flanking the NREs (5, 35). Molecular hybrids between the two viruses indicate that the loss of NRE function and the acquisition of a T-cell enhancer within the LTR are responsible for the altered disease specificity of TBLV (40).

Our experiments have shown that the triplicated region from the TBLV LTR acts as a T-cell-specific enhancer (35). The insertion of the TBLV LTR triplication upstream or downstream of the herpes simplex virus thymidine kinase promoter in either orientation increased the activity of reporter gene plasmids in transient transfections of $CD4^+$ or $CD4^+CD8^+$ T-cell lines but not fibroblasts. Substitution mutagenesis of the enhancer region revealed that mutations within the predicted RUNX1 (AML-1)-binding site dramatically reduced enhancer activity (35). Gel shift experiments with the TBLV enhancer region and antibody supershift and competition assays confirmed RUNX1 binding to the predicted site but also revealed the presence of several other binding activities that we referred to as nuclear factor A (NF-A) and NF-B (35). NF-A-binding activity was detectable in both B- and T-cell lines, whereas NF-B was more ubiquitously expressed (35). Mutagenesis experiments also indicated the presence of a functional glucocorticoid receptor (GR)-binding site downstream of the region bound by NF-A, NF-B, and RUNX1 (35). Efforts to detect enhancer activity in mouse mammary cells revealed that overexpression of a transcriptionally active form of RUNX1 (AML-1B) could activate TBLV LTR reporter expression approximately 30-fold, and this activation was dependent on an intact RUNX1-binding site (35). RUNX1, Ets1, lymphoid enhancer-binding factor 1 (LEF-1), GATA3, activating transcription factor/cyclic AMP response element-binding protein CATF/CREB, and c-Myb all have been shown to contribute to the T-cell specificity of viral and cellular enhancers (2, 33, 41, 49).

Three common integration sites, c-*myc*, *tblvi1*, and *Rorc*, have been identified in TBLV-induced T-cell lymphomas (13, 39, 44). Approximately 90% of TBLV-induced lymphomas overexpressed the c-*myc* gene, whereas more than 75% of these same tumors overexpressed one or more isoforms of the *Rorc* gene (13). To directly determine the effect of TBLV LTR insertion on c-*myc* gene expression, a reporter plasmid was constructed that contained the entire genomic c-*myc* locus, including 3 kb and 5 kb of upstream and downstream sequences, respectively (14). Insertion of the TBLV LTR upstream or downstream in either orientation elevated luciferase expression up to 150-fold compared to the parental plasmid in T cells (14). Interestingly, insertions of LTRs containing four-

* Corresponding author. Mailing address: Section of Molecular Genetics and Microbiology, University of Texas at Austin, One University Station, A5000, 24th Street and Speedway, ESB 226, Austin, TX 78712-0162. Phone: (512) 471-8415. Fax: (512) 471-7088. E-mail: jdudley@uts.cc.utexas.edu.

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repeat enhancers had a more modest effect on c-*myc* overexpression than those containing three-repeat enhancers, and characterization of several TBLV-induced T-cell lymphomas suggested that there was selection for the growth of tumor cells carrying TBLV proviruses with four-repeat enhancers (14). Such experiments indicate the importance of the enhancer for TBLV-induced disease. Moreover, c-*myc* activation by juxtaposition to novel cellular enhancers following chromosomal translocation is a common event in human T- and B-cell lymphomas (9, 20).

In this report, we have further dissected the anatomy of the T-cell-specific enhancer within the TBLV LTR. Transfection assays indicated that the enhancer was essential for effects on reporter activity from the c-*myc* expression plasmid, and the enhancer effect was observed only in T-cell lines. Enhancer deletions that removed the 5′ end of the enhancer or the 5′ end plus the RUNX1-binding site had minimal enhancer function, and this residual function could be partially attributed to c-Myb binding. Purification and analysis of enhancer-binding proteins identified ALY, which has previously been shown to promote LEF-1 and RUNX1 activity on several cellular enhancers in T cells (15, 26). Our experiments show that ALY similarly increases both RUNX1 and c-Myb activities on the TBLV enhancer, suggesting the general importance of this coactivator for c-*myc* expression in virally induced leukemias and lymphomas.

MATERIALS AND METHODS

Cell lines and transfections. Growth conditions for RLδ1 cells (murine $CD4^+CD8^+$ T cells) (37), Jurkat cells (human $CD4^+$ T cells) (46), A20 B cells (29), and HC11 mammary cells (6) have been described previously (35, 55). A20 cells (1.1×10^7) were used for electroporation in 4-mm cuvettes at 280 V and 975 μF in 0.4 ml of RPMI medium. A20 transfections were harvested at 24 h posttransfection, whereas all other cells were analyzed at 48 h posttransfection. Human embryonic kidney 293T cells (21) were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 50 μg/ml gentamicin sulfate, 50 μg/ml streptomycin, and 100 U/ml penicillin. Transfections of 293T cells were performed with SuperFect transfection reagent (QIAGEN) according to the manufacturer's directions. Alternatively, calcium phosphate transfections (18) were performed with 30 μg total DNA per 100-mm dish. Plasmid DNA for transfections was obtained by alkaline lysis and cesium chloride gradient purification as previously described (11). Each transfection contained the same amount of total DNA. Transfections were performed in triplicate, and each experiment was repeated at least three times with similar results.

Coimmunoprecipitation experiments. The 293T cells were transfected by the calcium phosphate method and harvested after 48 h. Nuclear extracts were obtained as previously described (42). Equal amounts of extracts were used in each sample and incubated in a total volume of 200 μl immunoprecipitation buffer (25 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2 mM EDTA, and 0.5% NP-40) supplemented with protease inhibitors (Complete; Roche) containing 4 μg Myb-specific antibody overnight at 4°C with rotation. Protein A-Sepharose beads (Sigma) were added to each sample and incubated for 1 h at 4°C with rotation. The protein A-Sepharose beads were then washed three times with immunoprecipitation buffer before the addition of sodium dodecyl sulfate (SDS)-containing loading buffer and boiling for 5 min. Samples were then resolved on denaturing polyacrylamide gels and analyzed by Western blotting.

Plasmid constructs. The pd6 and pc-*mycRluc* vectors have been described previously (14, 35). The reporter vector pMTV-LUC (also called pC3H-LUC or pLC-LUC) contains the MMTV C3H LTR upstream of firefly luciferase (11, 47). The pTBLV-LUC vector has been described previously (35). Construction details for the other plasmids based on these vectors are available by request. The *myc*-tagged AML-1B (RUNX1B) expression plasmid (38) was provided by Shari Meyers (Louisiana State University Medical Center, Shreveport, LA). Linda Wolff (National Cancer Institute, Bethesda, MD) provided the c-*myb* expression vector (pcDNA3.1-FLMyb) (8), whereas the ALY expression plasmid (pCMV-T7-ALY) (15) was obtained from Rudolf Grosschedl (University of Munich, Germany).

Preparation of extracts and EMSAs. Whole-cell lysates for electrophoretic mobility shift assays (EMSAs) were prepared as previously described (35). EMSA probes were prepared by annealing the appropriate oligonucleotides and end labeling with Sequenase version 2.0 (Amersham Pharmacia Biotech, Piscataway, NJ) (35). The conditions for DNA-binding reactions have been described previously (35). To assess the specificity of DNA-protein complexes formed in EMSAs, unlabeled oligonucleotides were added in excess as indicated prior to the addition of the radiolabeled probe and then incubated on ice for 10 min prior to analysis. Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA).

Reporter gene analysis. Assays were performed using the Dual-Luciferase reporter assay system (Promega, Madison, WI) that independently measures *Renilla* and firefly luciferase activities or by analysis of the firefly luciferase activity after quantitation of green fluorescent protein (GFP)-positive cells by using a fluorescence-activated cell sorter.

Purification of ALY from Jurkat T cells. Whole-cell lysates were prepared from large-scale cultures of Jurkat T cells (4 to 6 liters) grown in spinner flasks. Lysates were prepared as previously described (35) using sonication, cell debris was removed, and the supernatant was separated using Sephacryl S-200 HR resin (Sigma, St. Louis, MO). The void volume was eluted, and then the collected fractions were analyzed for DNA-binding proteins by using EMSA and the TBLV enhancer probe (556WT26) (annealed sequences, 5′-GGG AAC AGG TGC GGT TCC CAA GGC TT-3′ and 5′-CCA AGC CTT GGG AAC CGC ACC TGT TC-3′). Samples containing NF-A- or NF-B-binding activity were independently pooled and heated (40°C or 45°C, respectively, for 10 min). After being heated, precipitated proteins were removed by centrifugation, and the soluble fraction was analyzed using a double-stranded DNA-cellulose column (6-cm by 2.5-cm bed volume; Sigma) equilibrated with N100 buffer (0.1 M NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 20 mM HEPES [pH 7.9], 0.05% Brij-35, 20% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.2 μg/ml pepstatin A). After the column was washed, bound proteins were eluted by E250 buffer (with a substitution of 250 mM ammonium sulfate for 0.1 M NaCl in N100 buffer). Protein-containing fractions were analyzed for NF-A- and NF-B-binding activities by EMSA. Fractions containing NF-A or NF-B activity were independently pooled. Each pool was then affinity purified using streptavidin MagneSphere paramagnetic particles (Promega, Madison, WI). A cloned concatemer containing 10 copies of the 556WT oligomer was excised and end labeled with biotinylated dCTP prior to binding to streptavidin beads. Bound proteins were washed extensively with EMSA-binding buffer containing 1 μg/ml or 2.5 μg/ml of poly(dA-dT) (GE Healthcare Biosciences, Piscataway, NJ) as a nonspecific competitor in the first two or second two washes, respectively. Proteins were eluted with EMSA-binding buffer containing 2 M NaCl and dialyzed against EMSA-binding buffer before analysis on 10% polyacrylamide gels containing SDS. Gels were stained in 50% (vol/vol) methanol, 0.05% (vol/vol) Coomassie brilliant blue G-250 (Sigma), 10% (vol/vol) acetic acid, and 40% (vol/vol) water, followed by multiple changes, until a minimal background was achieved. The protein bands were excised from the gel and subjected to in-gel trypsin digestion. The extracted peptide fragments were then analyzed by tandem mass spectrometry as previously described (17).

Western blot analysis. Western blot analyses were performed as previously described (36) using Western Lightning enhanced chemiluminescence reagent (PerkinElmer). The antibodies used were T7 tag-specific mouse monoclonal antibody (Novagen), c-Myc-specific mouse monoclonal antibody (Santa Cruz Biotechnology, Inc.), actin-specific mouse monoclonal antibody (Calbiochem), and Myb-specific mouse monoclonal antibody (clone 1-1; Upstate).

RESULTS

TBLV enhancer requirement for upregulation of c-*myc* expression. Our previously published experiments used a c-*myc* reporter plasmid to measure the effect of the TBLV enhancer on the c-*myc* promoters (14). The pc-*mycRluc* vector contains the *Renilla* luciferase gene downstream of the *myc* start codon as well as 3 kb of 5′ flanking sequence and 5 kb of 3′ flanking sequence. Substitution of *myc* coding exons 1 and 2 with the luciferase gene prevented any potential apoptotic effects of c-*myc* overexpression. Our results established that insertions of the TBLV LTR at multiple positions upstream and down-

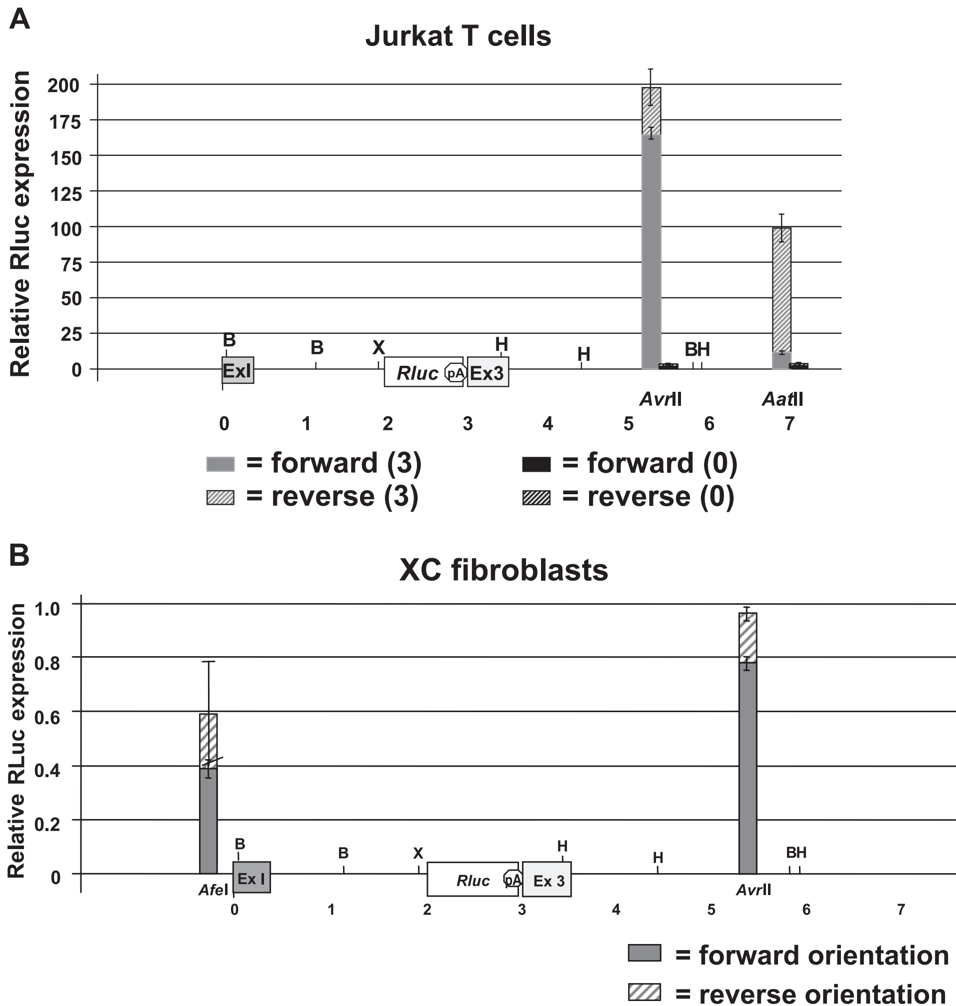


FIG. 1. Transcriptional activity of c-*myc* reporter plasmids containing wild-type or mutant TBLV LTRs. (A) Comparison of TBLV LTR insertions with different numbers of enhancer elements in transient transfections of Jurkat T cells. Relative luciferase (Rluc) activity was determined after normalization for DNA uptake. Luciferase activity was determined relative to the activity of the c-*myc* reporter plasmid in the absence of LTR insertion (assigned a value of 1). The means for triplicate assays (± standard deviations) are shown. Each experiment (performed in triplicate) was repeated at least three times with similar results. Note the difference in scale between panels A and B. The positions of LTR insertions are shown relative to the first exon (Ex 1) of c-*myc*, and the numbers below the *x* axis indicate the distance in kilobases. Assay results are shown using plasmids containing LTRs either in the same transcriptional orientation as the c-*myc* gene (forward) or in the transcriptional orientation opposite that of the c-*myc* gene (reverse). The numbers in parentheses indicate the number of enhancer repeats in the LTR (either three or zero). B, BamHI; X, XbaI; H, HindIII. (B) Activity of the c-*myc* reporter plasmid after transient transfections in XC fibroblast cells. Values were determined as described for panel A.

stream of the *myc* coding region, which mimicked the position and orientation of integrated TBLV proviruses observed in virally induced T-cell lymphomas (44), increased reporter gene activity up to 150-fold in T-cell lines (14).

To determine whether the activity of the TBLV LTR was dependent on the enhancer elements within the U3 region, we inserted TBLV LTRs lacking the enhancer elements in either orientation at two positions downstream of the c-*myc* gene. Reporter constructs were then transiently transfected into Jurkat T cells prior to luciferase assays (Fig. 1A). LTRs containing enhancer elements increased reporter expression up to 100- and 200-fold when introduced into the AatII and AvrII sites, respectively. However, differences in reporter gene activity were not observed when reporter plasmids lacking TBLV LTRs and plasmids with LTRs lacking the enhancer were compared. Moreover, the enhancer activity of the TBLV LTR was cell type specific since transfection of plasmids containing the TBLV LTR with a three-repeat enhancer upstream or downstream of the c-*myc* coding region in XC rat fibroblasts had either no effect or an inhibitory effect on reporter gene activity (Fig. 1B). Therefore, the enhancer was responsible for the cell-type-specific and stimulatory activity of the TBLV LTR on the adjacent c-*myc* promoters.

Mutational analysis of the TBLV enhancer. Because of the importance of the enhancer for c-*myc* activation, we explored the nature of enhancer sequence requirements. The pTBLV-LUC reporter vector containing the TBLV enhancer has been shown to exhibit the same cell type specificity as the pc-*mycRluc* vector containing the TBLV LTR (35). The 62-bp triplicated enhancer element within the TBLV LTR is com-

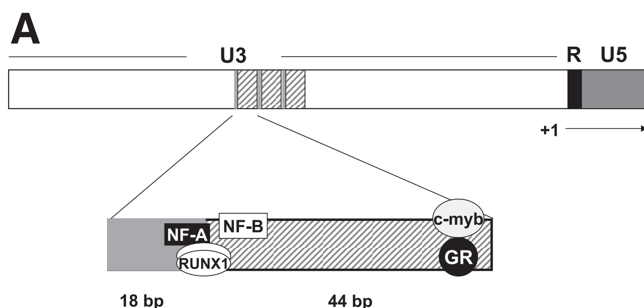
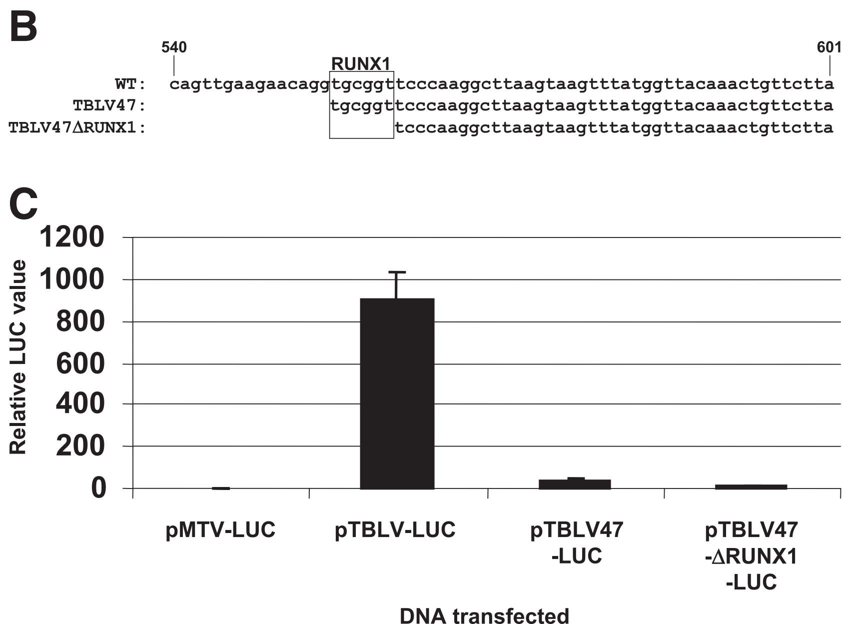


FIG. 2. Activity of 5′ deletion enhancer mutants in transient transfection assays of Jurkat T cells. (A) Diagram of TBLV enhancer with binding sites for NF-A, NF-B, RUNX1, c-Myb, and GR. Transcription begins at the first base of the R region (+1). (B) Sequences of wild-type (WT) and 5′ deletion TBLV enhancers. The RUNX-binding site is boxed. Numbering is given relative to the first base of the TBLV LTR (5). (C) Results of a reporter gene assay using 5′ deletion enhancer mutants. Luciferase (LUC) activity is given in light units/100 μg of protein normalized for DNA uptake, as measured by cotransfection with the pRL-TK plasmid. LUC activity is reported relative to that of pMTV-LUC, which has been assigned a value of 1; standard deviations from the means for triplicate assays are shown.



posed of a fusion of 18 bp 5′ to the MMTV NREs and 44 bp 3′ to the MMTV NREs (Fig. 2A) (5). A consensus RUNX1-binding site spans the junction of the two fragments and is flanked by or overlaps with binding sites for two unknown factors, NF-A and NF-B, on the 5′ and 3′ sides, respectively (35). To determine the contribution of each of these sequences to enhancer activity, two constructs were prepared. One sequence lacked the first 15 bp of the enhancer element (TBLV47), whereas the second sequence lacked the same 15 bp and the 6-bp RUNX1-binding site (TBLV47ΔRUNX1) (Fig. 2B). Each of these sequences and the wild-type sequence were triplicated and inserted into the MMTV LTR lacking the NREs upstream of the firefly luciferase reporter gene (pd6) (35) to mimic the enhancer configuration in the TBLV LTR.

These constructs were transiently transfected into Jurkat T cells and then assayed for reporter gene activity (Fig. 2C). The pTBLV47-LUC construct had approximately 4% of the luciferase expression of the wild-type pTBLV-LUC plasmid. The larger deletion in pTBLV47ΔRUNX1-LUC further reduced expression to 1.2% of that of pTBLV-LUC, which remained higher than the activity of the MMTV LTR or the MMTV LTR missing the NRE (Fig. 2C and data not shown). Although sequences at the 5′ end of the enhancer appear to be most important for enhancer activity, sequences downstream of the RUNX1 site also contribute to function. In addition, these experiments corroborate our previous substitution mutation analysis, indicating that sequences between nucleotides 548 and 573 are critical for TBLV enhancer function in T cells (35).

To perform finer mapping of sequences surrounding the RUNX1-binding site, we performed oligonucleotide competition analysis using EMSAs and the radiolabeled 556WT26 probe (Fig. 3A). An oligomer containing a 3-bp mutation of the RUNX1 site (34) competed for NF-A binding and less strongly for NF-B binding but, as expected, failed to compete for RUNX1 binding (Fig. 3B, compare lanes 2 and 6). NF-A binding can be resolved into at least three distinct complexes in EMSAs. Since initial substitution mutations in the TBLV enhancer were large (6 to 8 bp), smaller substitution mutations were subsequently tested by competition for transcription factor binding. Although the M1 oligomer competed for NF-A, NF-B, and RUNX binding, the other oligonucleotides had more-selective effects on NF-A binding. One mutant oligonucleotide, M2, competed for RUNX1, NF-B, and the higher-mobility NF-A complexes (Fig. 3B, lanes 11 and 12) but had little effect on the lowest-mobility NF-A complex. The M3, M4, and M5 oligomers did not interfere with NF-A binding at the lower concentrations and were not as effective as M1 and M2 in competing for RUNX1 binding. The latter results suggested

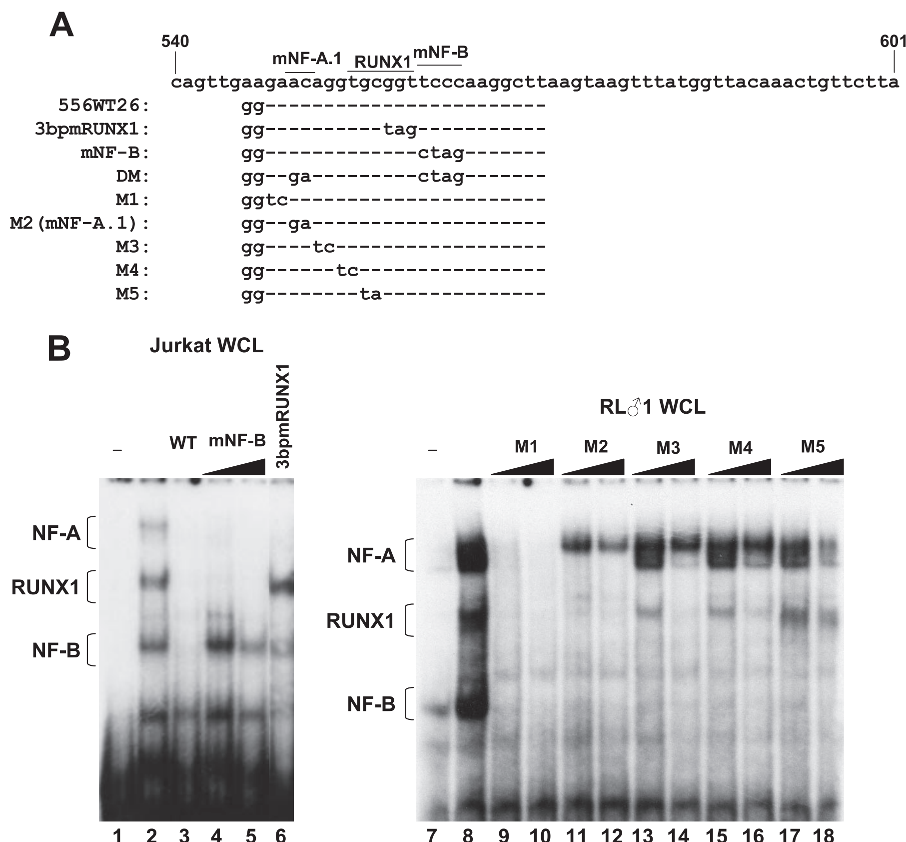


FIG. 3. Gel shift assays define critical binding sites within the TBLV enhancer. (A) Diagram of substitution mutations within the TBLV enhancer. (B) Results of competition assays with oligonucleotides from the wild-type (WT) and mutant enhancers. Whole-cell lysates (WCL) from human Jurkat cells (5 μg) (lanes 1 to 6) or mouse RL♂1 T cells (10 μg) (lanes 7 to 18) were incubated with the indicated wild-type end-labeled oligonucleotide (1 pmol) in the absence or presence of a 50-fold or a 200-fold molar excess of the indicated oligonucleotide. Complexes were resolved on nondenaturing 4% polyacrylamide gels. The positions of known complexes are indicated. m, mutant.

that some NF-A complexes are dependent on RUNX1 binding to the TBLV enhancer or overlap the RUNX1-binding site.

To determine the effect on enhancer activity, the 2-bp M2 mutation was substituted into a reporter construct containing a single enhancer element (pmNF-A.1-LUC) and compared to a reporter construct containing a single wild-type enhancer element after transient transfections into Jurkat T cells (pTBLV-1R-LUC) (Fig. 4). This mutation decreased transcriptional activity from the TBLV promoter approximately five- to sixfold (Fig. 4A). Therefore, the upper NF-A complex (NF-A.1) makes a major contribution to the T-cell-specific enhancer activity.

Based on our previously published results (35), the NF-B-binding site was predicted to overlap or juxtapose the RUNX1-binding site. An oligonucleotide containing a 4-bp substitution mutation 3′ of the RUNX1-binding site (43) was determined to specifically abolish only NF-B binding, but not NF-A or RUNX1 binding, to the TBLV enhancer (Fig. 3B, lanes 4 and 5). This mutation was transferred into the pTBLV-1R-LUC vector, resulting in the plasmid pmNF-B-LUC. Transient transfections of these plasmids into Jurkat T cells revealed that mutation of the NF-B-binding site reduced reporter gene levels approximately twofold (Fig. 4A). Similarly, a 3-bp mutation that affected only RUNX1 binding (p3bp-mRUNX-LUC) decreased reporter activity ~2-fold.

To determine the effect of both NF-A.1 and NF-B complex binding on TBLV enhancer activity, a double mutant vector (pDM-LUC) that contained both the 2-bp mutation of NF-A.1 and the 4-bp mutation from NF-B was constructed. Transient transfections of Jurkat cells revealed that the combined mutations lowered reporter levels less than twofold compared to the NF-A.1 mutation alone. Similar results were also obtained with RL♂1 T cells (Fig. 4B). These results suggested that the NF-A.1 complex is the major contributor to TBLV enhancer activity in T cells.

Purification of factors that contribute to TBLV enhancer function. To determine the identity of factors that bind to TBLV enhancer sequences adjacent to the RUNX1 site, we attempted conventional and affinity purification using extracts from Jurkat T cells. Jurkat cells were used for protein purification since they appeared to have the highest levels of NF-A and NF-B protein complexes and could be grown in large-scale spinner cultures. Whole-cell lysates were initially fractionated on a Sephacryl S-200 HR resin column. Fractions were then analyzed by EMSA using a 556WT oligonucleotide probe, which could detect NF-A, NF-B, and RUNX1 DNA-binding activities (35). Fractions containing NF-A and NF-B were pooled independently prior to heating and double-stranded DNA-cellulose chromatography as described in Materials and Methods. Fractions containing NF-A- and NF-B-binding activ-

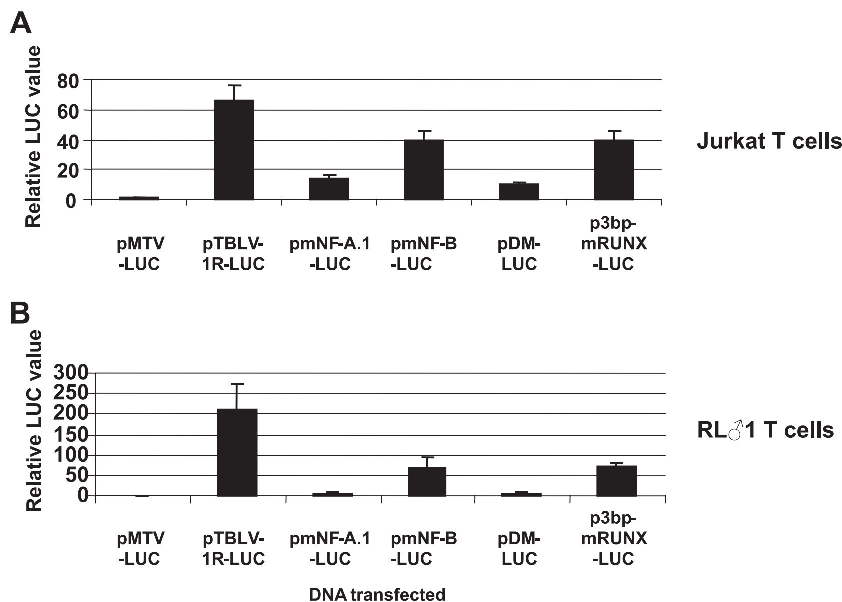


FIG. 4. Effect of TBLV enhancer mutations in T cells. (A) Jurkat cells were transfected with various reporter constructs to compare their activities relative to those of wild-type MMTV and TBLV reporter constructs. The mean value for triplicate transfection assays with the pMTV-LUC vector was assigned a relative value of 1 and compared to the means for triplicate transfection assays with TBLV LTR or mutant LTR reporter constructs. Standard deviations for triplicates are also shown. Each of the values from TBLV mutant LTR transfections was compared to values from transfections using pTBLV-1R-LUC containing one copy of the wild-type enhancer sequence; each of the mutant values was statistically different from that of pTBLV-1R-LUC by the Student *t* test ($P < 0.05$). (B) RL♂1 cells were transiently transfected with various reporter constructs to compare their activities relative to those of wild-type MMTV and TBLV reporter constructs. Luciferase (LUC) levels are expressed as described for panel A. Each of the values from TBLV mutant LTR transfections was compared to values from transfections using pTBLV-1R-LUC containing the wild-type enhancer sequence; each of the mutant values was statistically different from that of pTBLV-1R-LUC by the Student *t* test ($P < 0.05$).

ities were again identified by EMSA and affinity purified with concatemerized 556WT probe conjugated to magnetic beads. The eluted material from the pooled NF-A fraction was subjected to SDS-denaturing gel electrophoresis, and there appeared to be multiple protein bands by Coomassie staining (data not shown). Major protein bands were then excised from the gel and subjected to tandem mass spectrometry. The proteins identified from the NF-A pool analysis included nucleolin, hnRNP A1/A2, HMG1 and -2, and ALY. Purifications from the NF-B pools were not successful.

The purified protein of most interest was ALY (also known as BEF, REF, or THOC4), a transcriptional coactivator that interacts with RUNX proteins and LEF-1 on the T-cell receptor α (TCRα) enhancer (15, 28, 50). Since ALY has previously been shown to facilitate RUNX function (15) and since RUNX1 has been shown to be important for TBLV enhancer activity (35), we focused our efforts on the role of ALY in TBLV enhancer-mediated transcription. To determine whether ALY was capable of increasing transcriptional activation by RUNX1, T7-tagged ALY and c-*myc*-tagged RUNX1 expression vectors were transiently transfected in non-T cells (epithelial) to examine their effect on pTBLV-LUC reporter gene levels (Fig. 5A). When this experiment was performed with HC11 mouse mammary cells, RUNX1 overexpression alone activated reporter gene levels 2.5- to 7-fold when suboptimal levels of RUNX1 vector were transfected into the cells. Expression of ALY alone with pTBLV-LUC had no effect on reporter activity, but transfection of ALY and RUNX1 together elevated expression approximately 10- to 18-fold, suggesting a synergistic effect. Western blot analyses with T7 and c-*myc* antibodies confirmed ALY and RUNX1 overexpression, respectively, in this experiment (Fig. 5B). The ALY effect was abolished in the presence of the TBLV LTR mutation at position 556, which affects RUNX1 binding, but not in the presence of the mutation at position 572, which has no effect on RUNX1 activity (Fig. 5C) (35). These results suggest that ALY acts as a coactivator for RUNX1 on the TBLV enhancer.

Identification of a c-Myb-binding site in the TBLV enhancer. Software analysis indicated that there was a predicted GR-binding site downstream of the RUNX1-binding site in the TBLV enhancer. Mutation of this binding site prevented glucocorticoid induction of TBLV LTR-directed luciferase expression in rat XC fibroblasts, consistent with binding of GR (35). Jurkat T cells, which show dramatic activity of the TBLV enhancer, have little functional GR, and enhancer activity is not dependent on the presence of glucocorticoids (35). Nevertheless, mutation of the TBLV enhancer at position 586 (Fig. 6A) greatly reduced the activity in Jurkat cells (35).

A number of leukemia viruses that induced T-cell lymphomas contain a c-Myb-binding site (23) within their enhancers (10, 22, 31, 41, 53), and TRANSFAC software predicted a c-Myb-binding site in the TBLV enhancer region spanning the mutation at position 586 when the stringency for detection was reduced (Fig. 6A). Therefore, a c-*myb* plasmid was overexpressed transiently with pTBLV-LUC in 293T embryonic kidney cells, which have very low levels of endogenous c-Myb. An approximately 35-fold elevation of TBLV enhancer activity

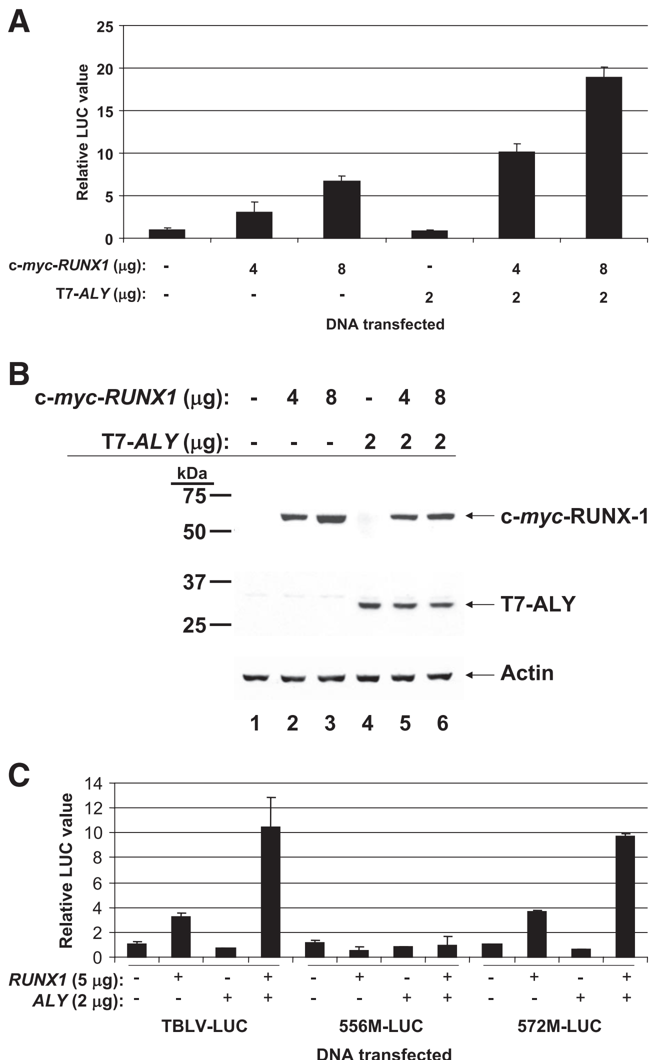


FIG. 5. ALY and RUNX1 synergize to enhance expression from the TBLV promoter. (A) Effect of ALY and RUNX1 on the TBLV promoter in HC11 mammary cells. T7-tagged *ALY* and c-*myc*-tagged *RUNX1* expression vectors were cotransfected with pTBLV-LUC into HC11 cells and, after 48 h, harvested and analyzed for luciferase (LUC) activity as described for Fig. 2. The mean for triplicate transfections with the pTBLV-LUC vector alone was assigned a relative value of 1 and compared to the means and standard deviations for assays in the presence of *RUNX1* and *ALY* expression vectors as indicated. (B) Western blot for detection of ALY and RUNX1 expression. Whole-cell lysates were prepared from transfected cells and analyzed for RUNX1 and ALY protein expression with c-Myc-specific antibody (top panel) and T7 tag-specific monoclonal antibody (middle panel), respectively. The lower panel shows blots after incubation with actin-specific antibody as a loading control. (C) ALY-mediated increases in RUNX1 activity on the TBLV enhancer are dependent on the RUNX1-binding site. *RUNX1* and *ALY* expression vectors were cotransfected with pTBLV-LUC, p556M-LUC, or p572M-LUC into HC11 cells. After 48 h, cells were harvested and analyzed for luciferase activity as described for Fig. 2. Means for triplicate transfections with either pTBLV-LUC or pTBLV-LUC carrying the 556 or 572 mutation were assigned relative values of 1. Means for triplicate cotransfections with RUNX1 or ALY expression plasmids (± standard deviations) were compared to the mean of transfection values of the same reporter vector without the RUNX1 or ALY vector.

was observed, and this activity was dependent on the amount of cotransfected c-*myb* construct (Fig. 6B and data not shown). A TBLV LTR reporter construct carrying the mutation at position 586 was less responsive to c-*myb* overexpression, indicating that the predicted mutation affected c-Myb binding to the enhancer. Consistent with the site-specific nature of this response, overexpression of the c-*myb* plasmid with a TBLV-LUC mutant affecting the RUNX1-binding site (556M-LUC) in 293T cells had no effect (Fig. 6B). Overexpression of c-Myb was verified by Western blot analysis (Fig. 6C).

To determine whether c-Myb and RUNX1B synergize to activate the TBLV enhancer, HC11 cells were cotransfected with various amounts of the two transcription factors and pTBLV-LUC. Using suboptimal levels of both c-*myb* and *RUNX1B* vectors, c-Myb and RUNX1 overexpression showed approximately 2-fold and 15-fold enhancement, respectively (Fig. 7A), consistent with higher endogenous c-Myb levels (data not shown). Cotransfection of both c-*myb* and *RUNX1B* expression vectors elevated reporter activity ~30-fold. Thus, in agreement with results of c-Myb overexpression on the mutation at position 556, which affects RUNX1 binding (Fig. 6B), the effect of these transcription factors on the TBLV enhancer appeared to be additive rather than synergistic.

Since ALY increases the activity of RUNX1 on the TBLV enhancer, the ability of ALY to facilitate c-Myb activity on the TBLV enhancer was tested. Because c-Myb levels are lower in 293T kidney epithelial cells than in HC11 cells (data not shown), 293T cells were transiently cotransfected with wild-type TBLV-LUC and a c-Myb expression vector in the absence or presence of an ALY expression vector (Fig. 7B). When c-Myb was transfected alone, an approximately 40-fold elevation of reporter activity was observed over the basal level, and when ALY was cotransfected, a further 7.5-fold enhancement was observed. The 586M-LUC vector has a mutation at the 5′ end of the c-Myb binding site but retains some residual responsiveness to c-Myb overexpression (~6.5-fold), and coexpression of ALY enhances this activity. Similar results were obtained with HC11 cells (data not shown). Therefore, the coactivator ALY increases the activity of several transcription factors that bind to the T-cell-specific TBLV enhancer.

Since the transfection experiments suggest an interaction between ALY and c-Myb, coimmunoprecipitation experiments were performed. Expression constructs for full-length c-Myb or T7-tagged ALY were transfected into 293T cells, and extracts were incubated with c-Myb-specific monoclonal antibody prior to analysis of protein complexes by Western blotting. Interactions between endogenous c-Myb and T7-tagged ALY were detectable (Fig. 7C, lane 2) and were greatly enhanced by exogenous c-Myb expression (Fig. 7C, lane 4). These data suggest that ALY and c-Myb interact directly or indirectly to increase expression through the TBLV enhancer.

DISCUSSION

A common mechanism of TBLV-induced T-cell lymphomas appears to be insertional activation of the c-*myc* gene (14, 44), and ca. 90% of TBLV-induced tumors overexpress this protooncogene (13, 44). Our previous experiments indicate that insertion of the TBLV LTR upstream or downstream of the c-*myc* promoters in either orientation leads to increased ex-

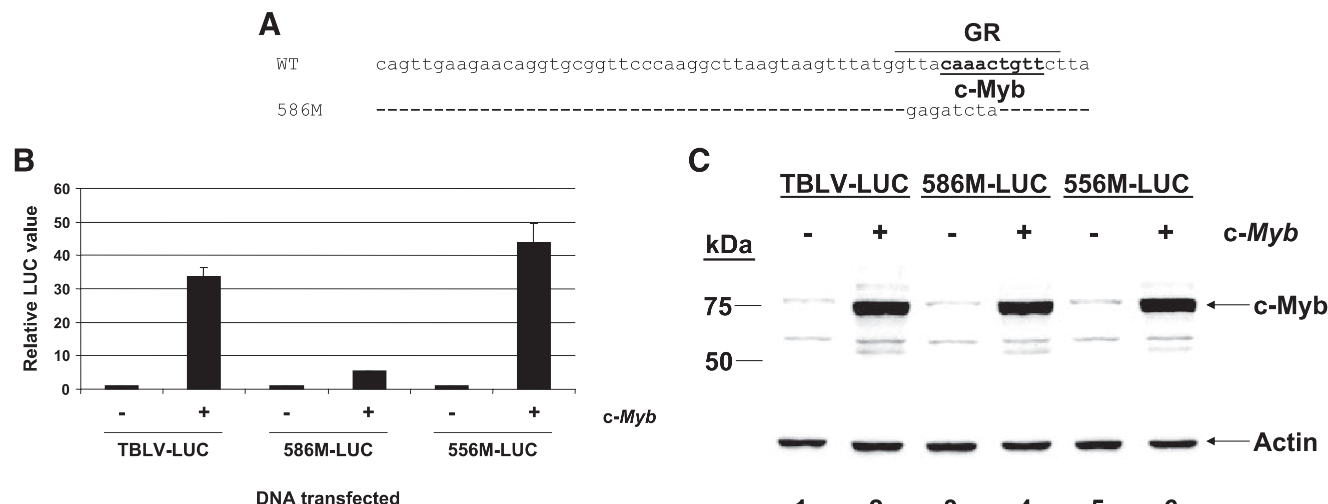


FIG. 6. c-Myb overexpression enhances TBLV promoter activity in non-T cells. (A) Diagram of wild-type (WT) and mutant GR- and c-Myb-binding sites in the TBLV enhancer. The 586 mutant sequences are shown below the wild-type TBLV enhancer sequence. Identical sequences are indicated by dashes. The c-Myb-binding site is indicated in bold and underlined, and the GR-binding site is overlined. (B) Comparisons of c-Myb activity in wild-type and mutant TBLV LTR constructs. 293T cells were transiently transfected with wild-type or mutant TBLV-LUC reporter constructs and a full-length c-Myb expression construct. After 48 h, cells were harvested, and lysates were prepared and analyzed for luciferase (LUC) activity as described for Fig. 2. The means of reporter values for pTBLV-LUC, p586M-LUC, and p556-LUC were assigned relative values of 1 and compared to the means (± standard deviations) for assays with cotransfected c-*myb* vector. (C) Western blotting confirms c-Myb overexpression. Whole-cell lysates were prepared from transfected cells and analyzed by Western blotting for the expression of c-Myb by incubation with Myb-specific monoclonal antibody (top panel) and for equal protein loading by incubation with actin-specific monoclonal antibody (bottom panel).

pression. In this report, we show that this effect was observed only in T-cell lines. Increased reporter expression from the c-*myc* promoters was dependent on the presence of the TBLV enhancer (Fig. 1), suggesting the importance of the enhancer for TBLV-induced disease.

Mutational analysis of the TBLV enhancer containing three copies of the 62-bp element revealed that deletion of the 15 bp upstream of the RUNX1-binding site had 25-fold lower activity than the wild-type enhancer (Fig. 2). Further deletion, including the RUNX1 site, reduced activity another fourfold. However, the residual enhancer had some activity compared to the NRE^- MMTV LTR (pd6) in Jurkat T cells, and such constructs had altered spacing with respect to the promoter.

Competition assays indicated that multiple NF-A complexes bind upstream and overlap with the RUNX1 site in the TBLV enhancer. The largest of the complexes appears to bind 5′ to the two smaller complexes (Fig. 3B), whereas NF-B bound 3′ to the RUNX1 site. A mutation that did not change spacing and affected only the larger NF-A complex reduced enhancer activity approximately fivefold when a single copy of the 62-bp element was present, whereas mutation of the NF-B site had less than a twofold effect. These data suggest that the largest of the NF-A complexes has the greatest effect on T-cell enhancer function.

Attempts to purify NF-A and NF-B have been unsuccessful to date. However, affinity purification methods revealed the presence of ALY, which has been shown to interact with RUNX1 and LEF-1 on the TCRα and major histocompatibility complex class I enhancers (15, 26). Previous data indicated that NF-A is preferentially expressed in lymphoid cells, whereas NF-B activity is ubiquitously expressed (35). Based on the tissue distribution of NF-A, its association with a RUNX1-binding site, and our affinity purification of ALY by using TBLV enhancer sequences, we considered the possibility that NF-A is LEF-1. However, oligonucleotides containing a consensus LEF-1-binding site did not compete for NF-A binding in EMSAs. In addition, preliminary data suggest that small interfering RNAs that reduce LEF-1 expression have no effect on TBLV enhancer activity (data not shown). Results from overexpression experiments and EMSAs with Ets-specific competitors suggested that NF-A is not Ets1 (35).

Interestingly, like the MuLV enhancers, the TBLV 62-bp element contains a consensus GR-binding site, and this site mediates glucocorticoid-stimulated transcription in XC fibroblasts (35). The mutation at position 586 in the TBLV enhancer spans both GR- and c-Myb-binding sites (Fig. 6A). c-Myb binds the consensus sequence NNCNTAACGGTTTT based on a binding site selection assay for yeast (7), whereas GR binds to the consensus sequence AGAACANNNTG TTCT (19). Our previous results have shown that the +586 site is more important for enhancer function in Jurkat $CD4^+$ T cells than in $CD4^+CD8^+$ T cells (35). Experiments using hormone-stripped serum indicated that GR is not the factor that binds to the +586 site in Jurkat cells (35).

The contribution of c-Myb to enhancer function was confirmed by overexpression in HC11 mouse mammary epithelial or 293T human kidney epithelial cells, resulting in activation of the TBLV LTR (Fig. 6). The additivity of c-Myb and RUNX1 overexpression on reporter gene expression suggests that these two binding regions within the TBLV enhancer function relatively independently. RUNX1 and c-Myb have been shown to synergistically activate the myeloperoxidase promoter region, which contains binding sites for both proteins (12); however, binding of RUNX1 and c-Myb to the myeloperoxidase pro-

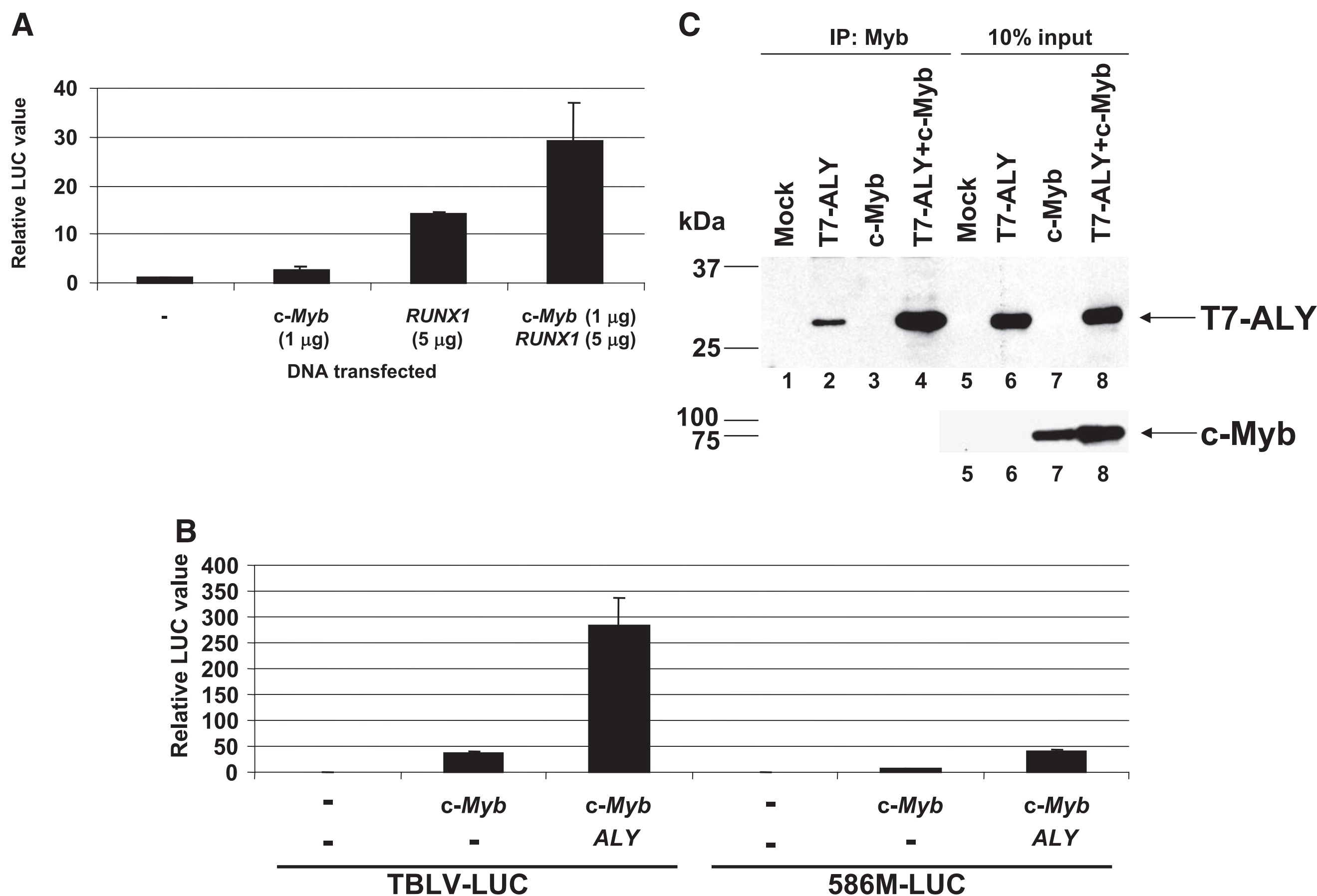


FIG. 7. Effects of RUNX1 and ALY on c-Myb activity in epithelial cells. (A) RUNX1 and c-Myb have additive effects. The indicated amounts of DNA were transfected into HC11 mammary cells. After 48 h, cytoplasmic extracts were analyzed for luciferase (LUC) activity. Activity is expressed as described in the legend to Fig. 2. The mean for triplicate assays with pTBLV-LUC alone was assigned a relative value of 1 and compared to the means for triplicate assays that had cotransfected c-*myb* or *RUNX1* expression vector. Standard deviations also are shown. (B) ALY and c-Myb synergize to enhance expression from the TBLV promoter. Cultured 293T cells were transiently transfected with wild-type TBLV-LUC or 586M-LUC together with expression vectors for c-Myb (0.5 μg) alone or c-Myb (0.5 μg) and ALY (0.5 μg). A small amount (0.5 μg) of a GFP expression vector (pEGFPN3) was included in each transfection. After 48 h, cells were harvested, and a portion of each sample was analyzed by using a fluorescence-activated cell sorter. The remaining cells were used for preparation of cell lysates and analysis of luciferase activity as described for Fig. 2, except that normalization for DNA uptake was assessed by the percentage of GFP-positive cells. The means for triplicate assays with either pTBLV-LUC or p586-LUC were assigned relative values of 1 and compared to the means (± standard deviations) for transfections with cotransfected c-*myb* or *ALY* expression vector. (C) ALY and c-Myb coimmunoprecipitate. Transfection of 293T cells was performed using the calcium phosphate method with 10 μg of the indicated expression plasmid; cells were harvested after 48 h. Nuclear extracts (200 μg) were incubated with 4 μg monoclonal anti-Myb antibody overnight prior to immunoprecipitation (IP). Lanes 5 to 8 contain 20 μg nuclear extract of the indicated sample (10% input) and were incubated with T7-specific (top panel) or Myb-specific (bottom panel) antibody to verify expression.

moter is not cooperative. The T-cell-specific genes, those encoding TCRγ, TCRδ (Dδ2), and CD4, are transcriptionally activated by c-Myb binding (1, 16, 27). The TCRγ promoter contains a RUNX1-binding site adjacent to a c-Myb site that is necessary, but not sufficient, for activation, and functional cooperation between RUNX1 and c-Myb has been demonstrated (25). Together with previous findings, our data suggest that c-Myb, rather than GR, binding to the +586 region contributes to the T-cell specificity of the TBLV enhancer.

RUNX1-binding activity is critical for the activity of retroviral enhancers that function in T cells (48, 52, 53). SL3-3 rapidly induces T-cell lymphomas, and there are four RUNX1-binding sites in the typical enhancer (53). Moloney MuLV induces a wider range of tumors and has a total of two RUNX1-binding sites, whereas TBLV, which induces only T-cell lymphomas, has a total of three RUNX1 sites in the prototypical enhancer element (35, 41). Previous data indicate that proviruses carrying mutations in the RUNX1 site of SL3-3 enhancers could induce T-cell lymphomas, and insertions near the c-*myc* gene retain the RUNX1 mutation (2). Other experiments argue that similar MuLV mutants in the RUNX1-binding sites have decreased enhancer activity and leukemogenicity (41, 53). These data may be reconciled by considering the results of Zaiman et al. (53), who demonstrated that RUNX1 requires binding of additional transcription factors to constitute the T-cell enhancer. Like SL3-3, the TBLV enhancer has a c-Myb-binding site downstream of the RUNX1 site (Fig. 2A). Mutation of the c-Myb-binding site in the SL3-3 or the TBLV enhancers dramatically reduced enhancer activity in the CD4$^+$ Jurkat line (35, 41), whereas the c-Myb-binding mutation in

the TBLV enhancer had little effect in a $CD4^+CD8^+$ cell line (35). Together with previous data (35), the current results argue that sequences immediately upstream of the RUNX1-binding site, including the NF-A-binding site, are critical for the function of the TBLV enhancer in several T-cell types.

ALY is a coactivator that cannot bind DNA alone (15). Instead, other transcription factors tether ALY to target DNA and activate transcription (15). Expression of ALY in HC11 mammary epithelial cells had no effect on TBLV promoter activity (Fig. 5A). However, when RUNX1 and ALY were coexpressed in HC11 cells, ALY enhanced RUNX1-mediated activation of the TBLV promoter, and this effect was dependent on the presence of a functional RUNX1-binding site (Fig. 5C). ALY also potentiated enhancer function dependent on c-Myb binding (Fig. 7). The interaction between ALY and c-Myb was demonstrated by coimmunoprecipitation experiments with both endogenous and exogenously expressed c-Myb in 293T cells. Attempts to demonstrate an interaction between RUNX1 and ALY were unsuccessful using tagged expression vectors and coimmunoprecipitation with 293T cell nuclear extracts or supershift experiments, perhaps due to weak interactions. However, the interaction between RUNX1 and ALY has been demonstrated previously by glutathione *S*-transferase pull-down assays (15), and our DNA affinity purification experiments also showed that ALY copurified with a concatemerized 26-bp oligonucleotide spanning the RUNX1-binding site in the TBLV enhancer. Further, the effects of ALY on RUNX1 and c-Myb appear to be independent since mutation of either transcription factor-binding site largely abolishes the coactivator activity. Together, these results indicate that the observed ALY effect on the TBLV enhancer is dependent on DNA-binding factors rather than the effects of ALY on RNA export (54).

To our knowledge, our experiments represent the first reports of ALY function with c-Myb as well as ALY function on a viral enhancer/promoter. Because ALY and other proteins, such as hnRNP A1/A2 and HMG1/2, were purified by affinity chromatography using the TBLV enhancer, it is tempting to speculate that ALY acts as a scaffolding protein in the assembly of enhanceosomes as well as the recruitment of RNA processing and export factors (30, 51). Although current data suggest that the transcription/export complex is recruited cotranscriptionally in mammalian cells through the splicing machinery (45), our results suggest that at least a portion of these proteins are recruited by the enhanceosome. Further, ALY may represent a common component of enhanceosome assembly necessary for c-*myc* overexpression in leukemias and lymphomas, and this coactivator might be an attractive target for the treatment of such diseases.

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