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Specific *in vivo* association between the bHLH and LIM proteins implicated in human T cell leukemia

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The protein products of proto-oncogenes implicated in T cell acute lymphoblastic leukemia include two distinct families of presumptive transcription factors. *RBTN1* and *RBTN2* encode highly related proteins that possess cysteine-rich LIM motifs. *TAL1*, *TAL2* and *LYL1* encode a unique subgroup of basic helix–loop–helix (bHLH) proteins that share exceptional homology in their bHLH sequences. We have found that RBTN1 and RBTN2 have the ability to interact with each of the leukemogenic bHLH proteins (TAL1, TAL2 and LYL1). These interactions occur *in vivo* and appear to be mediated by sequences within the LIM and bHLH domains. The LIM–bHLH interactions are highly specific in that RBTN1 and RBTN2 will associate with TAL1, TAL2 and LYL1, but not with other bHLH proteins, including E12, E47, Id1, NHLH1, AP4, MAX, MYC and MyoD1. Moreover, RBTN1 and RBTN2 can interact with TAL1 polypeptides that exist in assembled bHLH heterodimers (e.g. TAL1–E47), suggesting that the RBTN proteins can influence the functional properties of TAL1. Finally, we have identified a subset of leukemia patients that harbor tumor-specific rearrangements of both their *RBTN2* and *TAL1* genes. Thus, the activated alleles of these genes may promote leukemia cooperatively, perhaps as a result of bHLH–LIM interactions between their protein products.

Key words: leukemia/oncogene/transcription factor

Introduction

Recent studies have identified at least nine different proto-oncogenes that are activated by chromosome translocation in T cell acute lymphoblastic leukemia (T-ALL) (reviewed by Rabbitts, 1991). Three of these genes (*TAL1*, *TAL2* and *LYL1*) encode proteins that possess the basic helix–loop–helix (bHLH) motif, a protein-dimerization and DNA-binding domain present in several known transcription factors. Although >60 different bHLH proteins have been identified, the bHLH domains of TAL1, TAL2 and LYL1 are more closely related to each other than to those of any other proteins (Baer, 1993).

Tumor-specific rearrangement of the *TAL1* gene (formerly called *TCL5*, *tal* or *SCL*) is the most common genetic lesion known to be associated with T-ALL. *TAL1* gene alterations occur in ~3% of T-ALL patients as a consequence of the (1;14)(p34;q11) chromosome translocation (Begley *et al.*, 1989; Finger *et al.*, 1989; Bernard *et al.*, 1990; Carroll *et al.*, 1990; Chen *et al.*, 1990). An additional 15–25% of patients harbor the 'tal^{d} rearrangement', a tumor-specific alteration of the *TAL1* gene that arises by local DNA recombination (Brown *et al.*, 1990; Bernard *et al.*, 1991; Aplan *et al.*, 1992a; Bash *et al.*, 1993). In contrast, activation of the *TAL2* or *LYL1* genes is far less common, affecting <2% of T-ALL patients (Mellentin *et al.*, 1989; Xia *et al.*, 1991). Although expression of *TAL1*, *TAL2* or *LYL1* has not been observed during normal T cell development, the rearranged alleles of these genes are readily transcribed in T-ALL cells. Hence, ectopic expression of these genes in T-lineage cells may be a contributing factor in T-ALL pathogenesis.

RBTN1/TTG1 and *RBTN2/TTG2* constitute a distinct family of proto-oncogenes that are also activated in T-ALL by tumor-specific chromosome translocations (Sanchez-Garcia and Rabbitts, 1993). The *RBTN1/TTG1* gene is altered as a result of the (11;14)(p15;q11) translocation, a rare defect observed in <1% of T-ALL patients (Boehm *et al.*, 1988; McGuire *et al.*, 1989). Activation of *RBTN2/TTG2* is mediated by a distinct chromosome abnormality, the (11;14)(p13;q11) translocation, that occurs in ~7% of T-ALL patients (Boehm *et al.*, 1991; Royer-Pokora *et al.*, 1991). *RBTN1* and *RBTN2* encode related polypeptides that possess two tandem LIM domains, a cysteine-rich sequence that coordinates zinc atoms (Hempe and Cousins, 1991; Crawford *et al.*, 1992; Sadler *et al.*, 1992; Hagemeier *et al.*, 1993; Michelsen *et al.*, 1993; Archer *et al.*, 1994; Kosa *et al.*, 1994). LIM motifs were originally identified in a subset of transcription factors (lin-11, Isl-1 and mec3) that bind DNA by virtue of an associated homeodomain (the 'LIM-HD' proteins) (Way and Chalfie, 1988; Freyd *et al.*, 1990; Karlsson *et al.*, 1990). However, the *RBTN1* and *RBTN2* gene products belong to a different class of 'LIM-only' proteins that do not have a homeodomain sequence. Since T cell tumors are induced in mice upon thymic expression of *RBTN1* or *RBTN2* transgenes (Fisch *et al.*, 1992; McGuire *et al.*, 1992), inappropriate expression of these proteins in T-lineage cells is likely to be leukemogenic.

We now report that the malignant cells of some T-ALL patients have tumor-specific alterations of both their *RBTN2* and *TAL1* genes. Moreover, the products of these genes form *in vivo* protein complexes that are assembled by interaction between their respective LIM and bHLH domains. The observed LIM–bHLH interactions are highly specific in that RBTN1 and RBTN2 uniquely associate with each of the TAL1-related bHLH proteins (TAL1, TAL2 or LYL1) but not with other bHLH proteins. These data suggest that a common leukemogenic pathway

may be elicited, in some cases synergistically, by the LIM and bHLH proteins implicated in T-ALL.

Results

T-ALL patients with tumor-specific rearrangements of both TAL1 and RBTN2

The *RBTN1* gene was originally identified upon analysis of an (11;14)(p15;q11) translocation in RPMI8402, a leukemic cell line derived from a T-ALL patient (Boehm *et al.*, 1988; McGuire *et al.*, 1989). Interestingly, these cells also harbor a *TAL1* allele that has been activated by tal^{d1} rearrangement (Brown *et al.*, 1990), suggesting the possibility that *TAL1* and *RBTN1* play complementary roles in the pathogenesis of T-ALL. To evaluate whether co-activation of the *TAL1* and *RBTN2* genes can also occur in T-ALL, we examined primary leukemic cells from T-ALL patients with the (11;14)(p13;q11) translocation. As illustrated in Figure 1A, Southern hybridization with a probe from the major breakpoint region of t(11;14)(p13;q11) revealed rearrangement of the *RBTN2* gene in leukemic cells of a representative patient (patient #1114) obtained at diagnosis (lanes 1 and 2) but not in normal cells obtained after remission (lane 3). The leukemic cells from this patient also showed a tumor-specific rearrangement of the *TAL1* gene that arose by tal^{d1} recombination (Figure 1A). When a similar analysis was applied to leukemic cells of seven other patients with the (11;14)(p13;q11) translocation, two were also found to harbor tal^{d1} rearrangements (Figure 1B). Therefore, tumor-specific co-activation of the *TAL1* and *RBTN2* genes is a recurrent feature of T-ALL.

In vivo association between TAL1 and RBTN2 polypeptides

Protein dimerization is required for DNA binding and transcriptional regulation by bHLH proteins (reviewed by Kadesch, 1993). Although TAL1 polypeptides do not form homodimers *in vivo*, they readily assemble into heterodimers upon interaction with any of the known 'E proteins', a group of broadly expressed bHLH proteins that includes E47, E12, E2-2 and HEB−HTF4 (Hsu *et al.*, 1991, 1994a,b). Since RBTN1 and RBTN2 are also implicated in T-ALL, we sought to determine whether these polypeptides can influence the functional properties of TAL1. Initially, the two-hybrid system was used to evaluate *in vivo* association between TAL1 and RBTN2 polypeptides; in this assay, the transcription of GAL4-responsive reporter genes is activated by stable protein−protein interaction between the foreign moieties of hybrid GAL4 and VP16 polypeptides (Fields and Song, 1989; Dang *et al.*, 1991). Therefore, a mammalian expression vector was constructed that encodes the DNA-binding domain of the yeast GAL4 protein fused to the entire amino acid sequence of TAL1. The ability of this hybrid polypeptide (GAL4-TAL1) to induce RNA transcription was evaluated in the presence of various hybrid proteins that contain the herpesvirus VP16 trans-activation domain. The appropriate expression vectors were co-transfected into Jurkat T-ALL cells along with G5E1bLUC, a GAL4-responsive reporter plasmid that contains five GAL4-binding sites upstream of the firefly luciferase gene (Hsu *et al.*, 1994b). Transcription of the

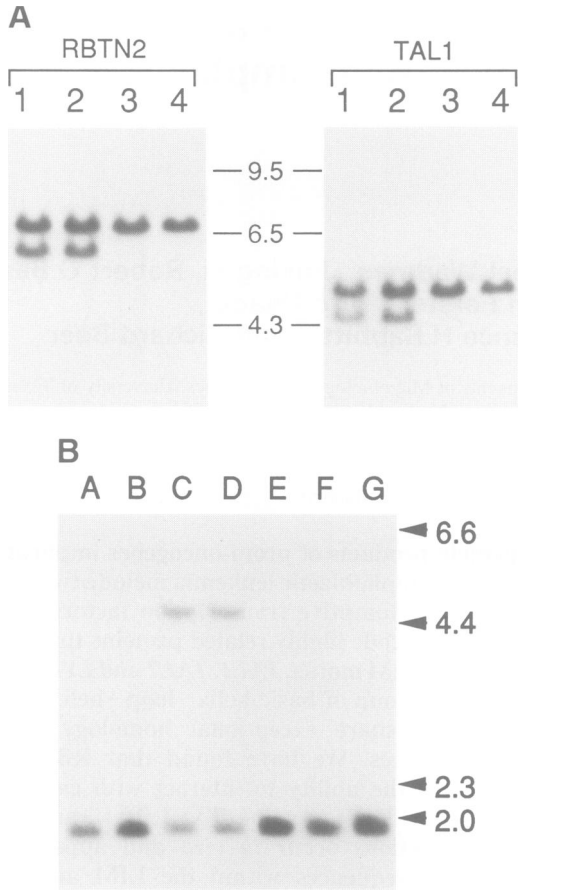

Fig. 1. Tumor-specific rearrangement of the RBTN2 and TAL1 genes in a patient with T-ALL. (**A**) A Southern filter of *Bam*HI-digested DNAs from patient #1114 was hybridized successively with probes from the human *RBTN2* and *TAL1* genes. Lanes 1, peripheral blood DNA obtained at diagnosis; lanes 2, bone marrow DNA obtained at diagnosis; lanes 3, remission DNA from the same patient; lanes 4, control DNA from an unrelated individual. The electrophoretic mobilities of marker DNA fragments are indicated in kb. The RBTN2 probe (W3XS-0.7) detects a rearranged *Bam*HI fragment of ~6.0 kb in the leukemic DNAs (lanes 1 and 2) that represents the der(14) junction of the (11;14)(p13;q11) chromosome translocation (Cheng *et al.*, 1990). The TAL1 probe (B2EE-2.0; Brown *et al.*, 1990) detects a rearranged fragment of ~4.5 kb in the leukemic DNAs (lanes 1 and 2); PCR amplification and sequence analysis confirmed that this fragment was generated by tal^{d1} recombination (data not shown). (**B**) Leukemic DNAs were prepared from peripheral blood or bone marrow cells obtained at diagnosis from seven T-ALL patients with the (11;14)(p13;q11) translocation. A Southern filter of *Eco*RI-digested leukemic DNAs was hybridized with the TAL1 probe (B2EE-2.0). In addition to the germline *Eco*RI fragment of 2.0 kb, this probe detects rearranged fragments of ~4.6 kb in the leukemic DNAs of patients C and D; PCR amplification and nucleotide sequence analysis confirmed that both rearrangements were generated by tal^{d1} recombination (data not shown).

reporter plasmid was evaluated by measuring the luciferase activity of lysates prepared from transfected cells. As illustrated in Figure 2A, expression of the GAL4-TAL1 hybrid polypeptide induced little luciferase activity in transfected Jurkat cells (lane 2). Likewise, significant luciferase activity was not observed in cells expressing VP16-RBTN2, a hybrid polypeptide that contains the VP16 transactivation domain fused to the entire amino acid sequence of RBTN2 (lane 4). However, co-expression of both hybrid polypeptides generated a large increase in luciferase activity (lane 3) to levels 20- or 200-fold higher,

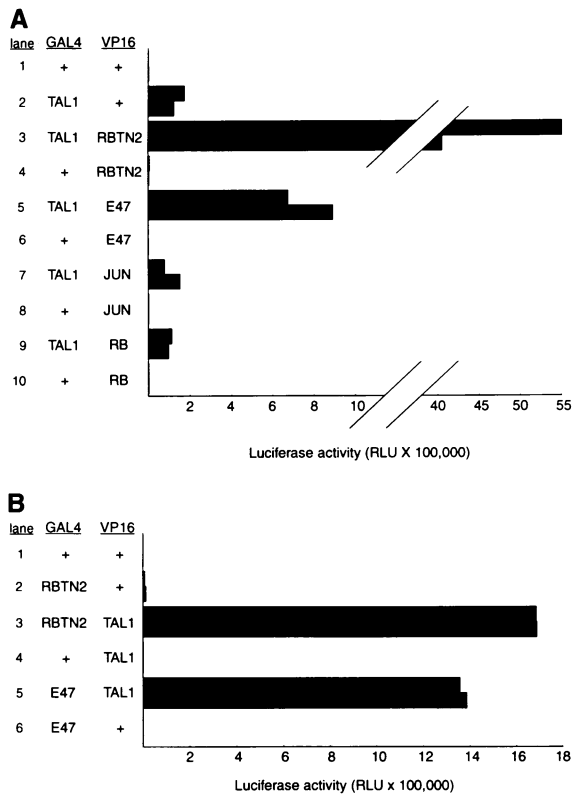

Fig. 2. *In vivo* interactions between TAL1 and RBTN2 revealed by two-hybrid analysis. Jurkat cells were transiently transfected with 15 μg of the G5E1bLUC reporter plasmid and 15 μg of each expression plasmid. Each cell culture was co-transfected with two expression plasmids: one encoded either the 'parental' GAL4 DNA-binding domain (denoted by '+' in the GAL4 column) or the indicated 'hybrid' GAL4 polypeptide, and the other encoded either the parental VP16 transactivation domain (denoted by '+' in the VP16 column) or the indicated hybrid VP16 polypeptide. Duplicate transfections were conducted for each combination of expression plasmids, and the normalized luciferase activities obtained in each transfection are illustrated. (**A**) Two-hybrid analysis of *in vivo* interactions with the GAL4-TAL1 fusion protein. (**B**) Two-hybrid analysis of *in vivo* interactions with the VP16-TAL1 fusion protein.

respectively, than those found with GAL4-TAL1 or VP16-RBTN2 alone. This suggests that TAL1 and RBTN2 have the potential to form heterologous protein complexes that are stable *in vivo*. Experiments with the reciprocal hybrids, GAL4-RBTN2 and VP16-TAL1, also provided evidence of an intracellular interaction between TAL1 and RBTN2. Thus, co-expression of these polypeptides generated 100-fold higher luciferase activity than either GAL4-RBTN2 or VP16-TAL1 alone (Figure 2B, lanes 2–4). The magnitude of the response obtained with two-hybrid analysis is striking; in both orientations of the two-hybrid assay the levels of luciferase activity induced by TAL1–RBTN2 association (lanes 3 of Figure 2A and B) are higher than or equal to those generated by TAL1–E47 association (lanes 5 of Figure 2A and B)—an established interaction mediated by bHLH heterodimerization (Hsu *et al.*, 1994b).

Co-immunoprecipitation experiments were carried out to confirm that TAL1 and RBTN2 associate *in vivo*. Therefore, COS1 cells, which do not express their endogenous *TAL1* or *RBTN2* genes (data not shown), were co-transfected with expression plasmids that encode either the wildtype TAL1 polypeptide or an epitope-tagged

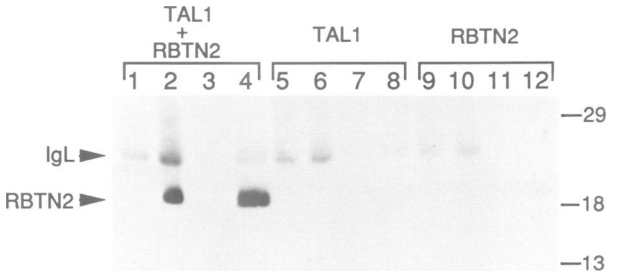

Fig. 3. Co-immunoprecipitation of TAL1 and RBTN2 polypeptides from COS1 cells. The cells were transfected with an expression vector encoding TAL1 (lanes 5–8), an expression vector encoding an epitope-tagged RBTN2 polypeptide (lanes 9–12), or both vectors together (lanes 1–4). After 2 days the cells were lysed under mild conditions, and aliquots of each lysate were immunoprecipitated with an antiserum specific for the TAL1 amino-terminus (lanes 4, 8 and 12), the corresponding pre-immune serum (lanes 3, 7 and 11), an antiserum specific for the TAL1 carboxy-terminus (lanes 2, 6 and 10), and the corresponding pre-immune serum (lanes 1, 5 and 9). The immunoprecipitates were fractionated by SDS–PAGE, and RBTN-tag polypeptides were detected by immunoblotting with the 12CA5 monoclonal antibody. The mobilities of molecular weight standards are indicated in kilodaltons.

RBTN2 polypeptide ('RBTN2-tag'); the latter includes a carboxy-terminal tag that is recognized by the 12CA5 monoclonal antibody (Field *et al.*, 1988). Two days after transfection the cells were lysed under mild conditions and aliquots of each lysate were immunoprecipitated with either a TAL1-specific rabbit antiserum or the corresponding pre-immune serum. To determine whether RBTN2-tag polypeptides were co-immunoprecipitated with TAL1, the precipitates were fractionated by SDS–PAGE and the presence of RBTN2-tag was evaluated by immunoblotting with the 12CA5 monoclonal antibody. As illustrated in Figure 3, the RBTN2-tag polypeptide was co-immunoprecipitated with TAL1-specific antisera (lanes 2 and 4) but not with the corresponding pre-immune sera (lanes 1 and 3). Co-immunoprecipitation of RBTN2-tag was observed with antisera raised against either the carboxy-terminal (lane 2) or amino-terminal (lane 4) sequences of TAL1. Moreover, RBTN2-tag co-immunoprecipitation was clearly dependent on the presence of TAL1 since it was not observed using lysates of COS1 cells transfected with the RBTN2-tag expression plasmid alone (lanes 9–12). Thus, the formation of *in vivo* complexes between TAL1 and RBTN2 can be demonstrated with two independent methods, the two-hybrid assay and co-immunoprecipitation analysis.

Complex formation between the endogenous TAL1 and RBTN2 polypeptides

The experiments described above showed that TAL1 and RBTN2 interact *in vivo* upon over-expression in mammalian cells. We then sought to determine whether TAL1–RBTN2 complexes are also formed from the endogenous polypeptides of hematopoietic cells. The *RBTN2* gene is expressed in murine (MEL) and human (HEL) erythroleukemia cell lines but not in Jurkat T-ALL cells (Valge-Archer *et al.*, 1994). Thus, each of these lines was cultured for 2 h with [^{35}S]methionine and lysed in a mild detergent (NP-40 buffer). The radiolabeled cell lysates were then immunoprecipitated under low stringency conditions with anti-TAL1(n), an antiserum raised

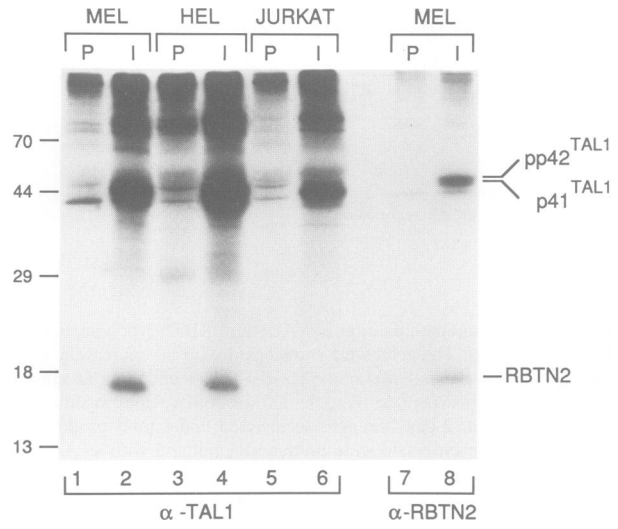

Fig. 4. Co-precipitation of endogenous TAL1 and RBTN2 polypeptides. Hematopoietic cell lines that do (MEL and HEL) or do not (Jurkat) express RBTN2 were labeled with [^{35}S]methionine and lysed in a mild detergent. Equivalent aliquots of each lysate were immunoprecipitated under low-stringency conditions with a TAL1-specific immune serum (lanes 2, 4 and 6) or the corresponding pre-immune serum (lanes 1, 3 and 5). Aliquots of the MEL cell lysate were also immunoprecipitated under high-stringency conditions with a RBTN2-specific immune serum (lane 8) or the corresponding pre-immune serum (lane 7). The mobility of the RBTN2 polypeptide is denoted on the right of the autoradiogram, along with those of the hyperphosphorylated (pp42^{TAL1}) and underphosphorylated (pp41^{TAL1}) TAL1 polypeptides. Molecular weight standards are indicated (in kilodaltons) on the left.

against the amino-terminal 121 residues of TAL1 (Hsu *et al.*, 1994a). We had previously shown that HEL and Jurkat cells produce both full-length (residues 1–331) and truncated (residues 176–331) TAL1 polypeptides (Cheng *et al.*, 1993). As expected, immunoprecipitation with anti-TAL1(n) (lanes 2, 4 and 6), but not the corresponding pre-immune serum (lanes 1, 3 and 5), revealed abundant quantities of the full-length TAL1 species (p41^{TAL1} and pp42^{TAL1}) in MEL, HEL and Jurkat cells. Significantly, however, the immunoprecipitates from MEL and HEL also contain a protein of ~18 kDa that is conspicuously absent from Jurkat cells. To ascertain whether this species co-migrates with RBTN2, radiolabeled lysates of MEL were immunoprecipitated with a RBTN2-specific antiserum under high-stringency conditions (RIPA buffer). As shown in Figure 4, the RBTN2 polypeptide recognized by this antiserum (lane 8) migrates with the 18 kDa species that was co-precipitated with TAL1 (lanes 2 and 4). Furthermore, the anti-RBTN2 immunoprecipitate also contains a doublet that resembles the hyperphosphorylated (pp42^{TAL1}) and underphosphorylated (p41^{TAL1}) forms of TAL1 (lane 8). Therefore, endogenous TAL1 and RBTN2 polypeptides form an *in vivo* complex that is stable even under relatively stringent conditions.

The TAL1–RBTN2 interaction is dependent on the bHLH domain of TAL1

To identify the TAL1 sequences responsible for TAL1–RBTN2 association, a series of expression plasmids was prepared which encode the GAL4 DNA-binding domain fused to various segments of TAL1 (Figure 5A). Each GAL4-TAL1 polypeptide was then evaluated by two-hybrid analysis for its ability to interact *in vivo* with the VP16-RBTN2 polypeptide. At least two distinct TAL1 polypeptides are expressed in T-ALL cells, a full-length gene product (amino acid residues 1–331) and a truncated species (residues 176–331), both of which harbor the intact bHLH domain (Cheng *et al.*, 1993). As illustrated in Figure 5B, VP16-RBTN2 associates equally well with GAL4-TAL1 polypeptides that include either the entire TAL1 sequence (lane 3) or the truncated sequence (lane 7). A substantial interaction was also observed using a hybrid polypeptide in which the TAL1 moiety was comprised almost entirely of bHLH sequences (lane 13). In contrast, the TAL1–RBTN2 association was not mediated by polypeptides in which the bHLH domain was either absent (lanes 11) or disrupted (lanes 5 and 9). Together, these results indicate that an intact bHLH domain is required for *in vivo* interaction between TAL1 and RBTN2.

Since most of the RBTN2 polypeptide is comprised of LIM sequences, these data suggest that the association between TAL1 and RBTN2 is mediated by their respective bHLH and LIM domains. In this regard it is noteworthy that direct binding of bHLH sequences with other known protein motifs has also been reported. In particular, the bHLH domain of MyoD1, a muscle-specific transcription factor, was shown to interact with both the leucine zipper of c-Jun and the binding pocket of Rb (Bengal *et al.*, 1992; Gu *et al.*, 1993). However, two-hybrid analysis suggests that the bHLH domain of TAL1 does not share these properties. As shown in Figure 2A, co-expression of GAL4-TAL1 with VP16 hybrid polypeptides containing either the c-Jun leucine zipper (lane 7) or the Rb pocket (lane 9) did not increase luciferase activity to levels higher than those obtained with GAL4-TAL1 alone (lane 2).

The specificity of TAL1 association with LIM-only proteins

To evaluate the specificity of TAL1–RBTN2 association, we also tested the ability of TAL1 to interact with a panel of LIM-only polypeptides, including some that have not been implicated in leukemogenesis. The cysteine-rich intestinal protein (CRIP), the cysteine-rich protein (CRP) and the zyxin polypeptide possess one, two or three LIM domains, respectively (Birkenmeier and Gordon, 1986; Liebhaber *et al.*, 1990; Sadler *et al.*, 1992). Expression vectors encoding GAL4-CRIP, GAL4-CRP and GAL4-zyxin fusion polypeptides were constructed for use in the two-hybrid system. In Figure 6, the ability of these polypeptides to induce reporter gene transcription in the presence of VP16-TAL1 is compared with that of GAL4-RBTN2. As expected, co-expression of VP16-TAL1 and GAL4-RBTN2 elicits a large increase in luciferase activity (lane 3). In contrast, co-expression of VP16-TAL1 with each of the other GAL4 fusion proteins failed to generate a detectable increase in luciferase activity (lanes 5, 7 and 9). Thus, TAL1 specifically associates with some (e.g. RBTN2) but not all (CRIP, CRP and zyxin) LIM-only proteins.

RBTN2 interacts specifically with the TAL1 family of leukemic bHLH proteins

The TAL1, TAL2 and LYL1 proteins display an exceptional degree of amino acid sequence identity within their bHLH domains. Thus, TAL2 and LYL1 are likely to

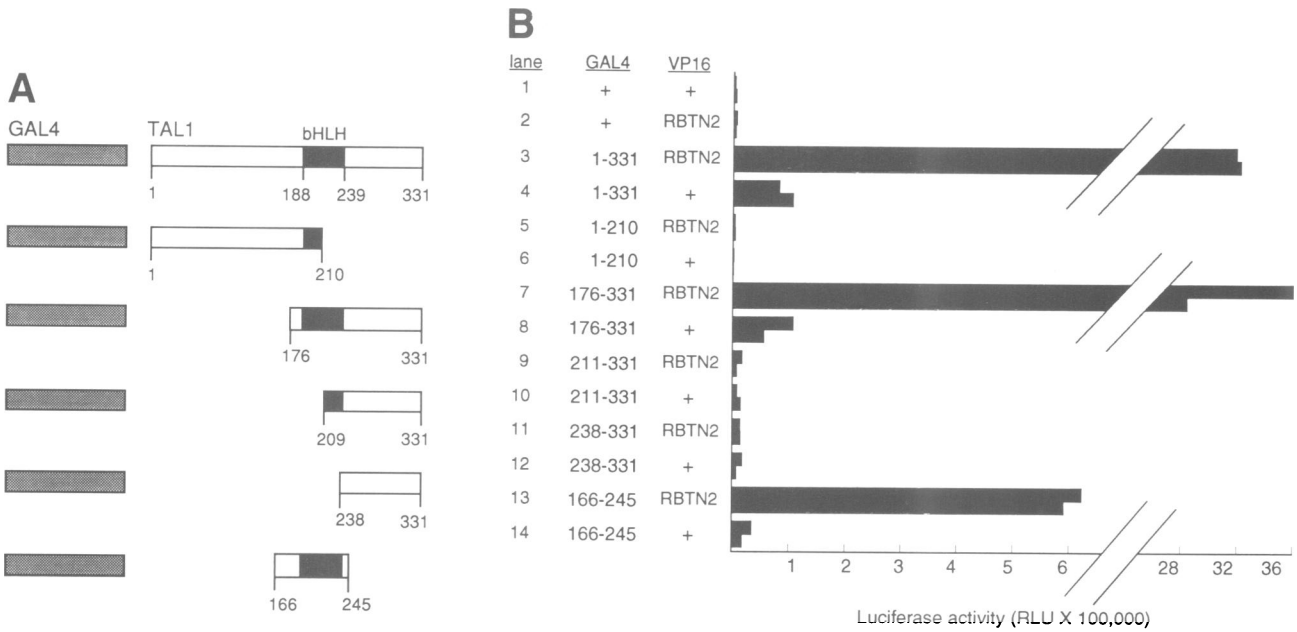

Fig. 5. TAL1 sequences responsible for *in vivo* association with RBTN2. (**A**) Schematic diagram illustrating the GAL4-TAL1 hybrid polypeptides. The hybrids are comprised of the DNA-binding domain of GAL4 (shaded bars) and segments of the TAL1 polypeptide (open bars). The numbers indicate amino acid residues of TAL1. The bHLH domain of TAL1 (residues 188–239) is denoted by a black box. (**B**) Two-hybrid analysis of *in vivo* interactions between VP16-RBTN2 and the various GAL4-TAL1 polypeptides. The two-hybrid assays were conducted in Jurkat cells as described in the legend of Figure 2. The TAL1 amino acid residues present in each GAL4-TAL1 polypeptide are indicated in the 'GAL4-TAL1' column; the parental GAL4 DNA-binding domain is denoted by a '+'.

share certain functional properties with TAL1, including, perhaps, the ability to associate with RBTN2. Therefore, two-hybrid analysis was used to compare *in vivo* interactions between the VP16-RBTN2 polypeptide and a panel of GAL4 hybrid polypeptides containing different bHLH sequences. As illustrated in Figure 7, large increases in luciferase activity were observed upon co-expression of VP16-RBTN2 with GAL4-TAL1 (lane 3), GAL4-TAL2 (lane 5) and GAL4-LYL1 (lane 7). However, RBTN2 sequences did not associate with the bHLH domains of E12, E47 or Id1 (lanes 9–14). Additional two-hybrid experiments indicate that RBTN2 also fails to interact with the bHLH domains of other proteins, including AP4, NHLH1, MAX, MYC and MyoD1 (data not shown). Thus, RBTN2 appears to associate specifically with the TAL1-related bHLH proteins.

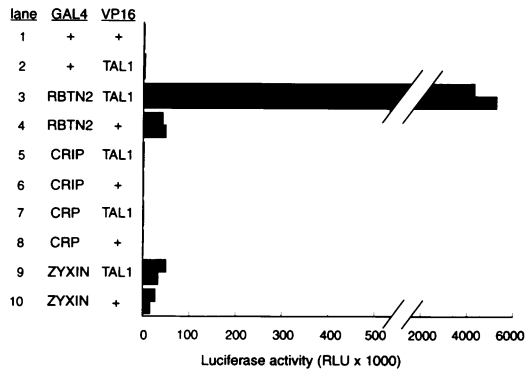

Fig. 6. Two-hybrid analysis of *in vivo* interactions between TAL1 and a panel of LIM-only proteins. Comparison of VP16-TAL1 interactions with the GAL4-RBTN2, GAL4-CRIP, GAL4-CRP and GAL4-zyxin polypeptides. The two-hybrid assays were conducted in Jurkat cells as described in the legend of Figure 2.

RBTN1 also interacts specifically with TAL1, TAL2 and LYL1

The *RBTN1* and *TAL1* genes are both activated by tumor-specific chromosome defects in RPMI8402, a leukemic cell line derived from a T-ALL patient. This observation, along with the structural and functional similarities of RBTN1 and RBTN2, suggests that RBTN1 may also associate *in vivo* with bHLH proteins of the TAL1 subgroup. In order to test RBTN1-mediated interactions, an expression vector was constructed that encodes VP16-RBTN1, a hybrid polypeptide containing the entire sequence of RBTN1. As shown in Figure 8, two-hybrid analysis of RBTN1 reveals an interaction profile indistinguishable from that of RBTN2. Thus, RBTN1 can associate specifically with the TAL1-related polypeptides (lanes 2–8) but not with other bHLH proteins such as E12, E47, Id1, NHLH1 (lanes 9–16), AP4, MAX, MYC and MyoD1 (data not shown). Together, these data indicate that each of the LIM proteins implicated in T-ALL has the potential to associate specifically with TAL1, TAL2 and LYL1.

RBTN1 and RBTN2 interact with heterodimeric bHLH complexes involving TAL1 and E47

Although TAL1 polypeptides do not self-associate to form bHLH homodimers, they readily interact with any of the known E proteins (E47, E12, E2-2 and HEB) to form bHLH heterodimers (Hsu *et al.*, 1991, 1994a,b). Therefore, to understand the influence of RBTN polypeptides on TAL1 function, we sought to discern whether these polypeptides will associate with assembled TAL1 heterodimers (e.g. E47−TAL1) or whether TAL1 interactions with the RBTN proteins and E proteins are mutually exclusive. A variation on the two-hybrid assay was employed to determine if E47, TAL1 and RBTN1 sequences will associate

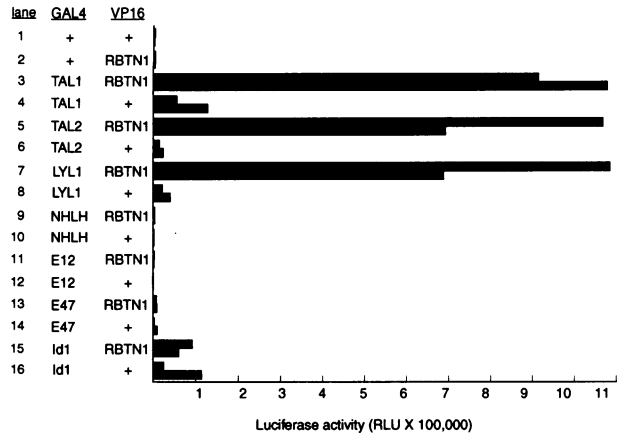

Fig. 7. Analysis of RBTN2 interaction with a panel of bHLH proteins. Two-hybrid analysis of *in vivo* interaction between the VP16-RBTN2 polypeptide and GAL4 fusion proteins containing the bHLH domains of TAL1, TAL2, LYL1, E12, E47 or Id1. The two-hybrid assays were conducted in Jurkat cells as described in the legend of Figure 2.

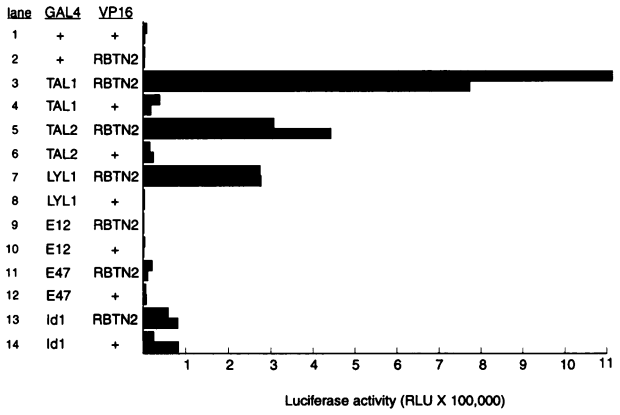

Fig. 8. RBTN1 associates specifically with TAL1, TAL2 and LYL1. Two-hybrid analysis of *in vivo* interaction between the VP16-RBTN1 polypeptides and GAL4 fusion proteins containing the bHLH domains of TAL1, TAL2, LYL1, NHLH1, E12, E47 or Id1. The two-hybrid assays were conducted in Jurkat cells as described in the legend of Figure 2.

simultaneously *in vivo* (Figure 9). As expected, co-expression of VP16-RBTN1 with a GAL4 fusion polypeptide containing the bHLH domain of E47 failed to generate significant luciferase activity (Figure 9A, lane 3). Likewise, induction of luciferase activity did not occur when wildtype TAL1 polypeptides were co-expressed with either the GAL4-E47 (lane 4) or VP16-RBTN1 (lane 5) hybrid polypeptides. However, co-expression of TAL1 with both hybrid polypeptides generates a huge increase in luciferase activity, indicating that TAL1 can serve as a bridge to allow *in vivo* formation of a stable multi-component complex involving E47, TAL1 and RBTN1 (lane 6). These results were confirmed in two-hybrid experiments using the reciprocal combination of fusion polypeptides (i.e. GAL4-RBTN1 and VP16-E47) (data not shown). A similar experiment, illustrated in Figure 9B, shows that TAL1 also promotes complex formation between the GAL4-E47 and VP16-RBTN2 polypeptides (lane 6). Thus, both RBTN polypeptides have the ability to associate *in vivo* with assembled bHLH heterodimers comprised of TAL1 and E47.

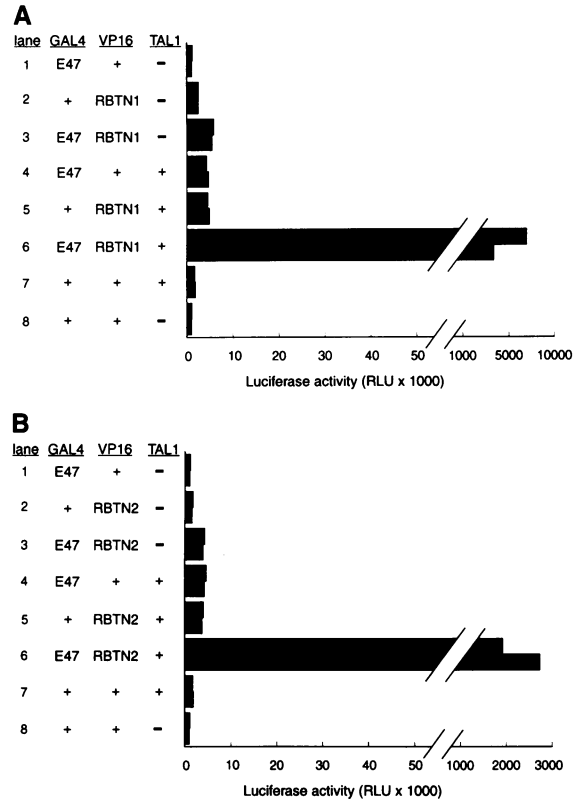

Fig. 9. RBTN2 polypeptides associate with assembled bHLH heterodimers that contain TAL1. (**A**) Two-hybrid analysis of the *in vivo* association between the GAL4-E47 and VP16-RBTN1 fusion proteins in the presence or absence of wildtype TAL1 polypeptides. The wildtype TAL1 protein was provided by co-transfection of the cells with 5 μg of the TAL1/pCMV4 expression vector (lanes 4–7); the other samples were co-transfected with 5 μg of the parental pCMV4 vector (lanes 1, 2, 3 and 8). (**B**) Two-hybrid analysis of the *in vivo* association between the GAL4-E47 and VP16-RBTN2 fusion proteins in the presence or absence of wildtype TAL1 polypeptides.

Discussion

The LIM and bHLH proteins implicated in T-ALL

Recent studies have uncovered several proto-oncogenes that are activated in the leukemic cells of patients with T-ALL (Rabbitts, 1991). These include two unique families of genes: *RBTN1* and *RBTN2*, which encode homologous LIM-only proteins, and the *TAL1*-related genes (*TAL1*, *TAL2* and *LYL1*), which encode a distinct subgroup of bHLH proteins (Baer, 1993; Sanchez-Garcia *et al.*, 1993). We now report that some T-ALL patients have tumor-specific rearrangements of both their *RBTN2* and *TAL1* genes. The recurrent co-activation of these genes suggests that they play complementary roles in T-ALL pathogenesis. This view is supported by the fact that their protein products readily assemble into complexes that are stable *in vivo*. As discussed below, the intracellular interaction between RBTN2 and TAL1 polypeptides appears to be mediated by residues within their respective LIM and bHLH domains. Moreover, both RBTN1 and RBTN2 associate avidly with each of the TAL1-related bHLH proteins (TAL1, TAL2 and LYL1) but not with a broad spectrum of other bHLH proteins including E12, E47, Id1, NHLH1, AP4, MAX, MYC and MyoD1. The striking specificity of these interactions implies a functional relationship between the LIM and bHLH proteins implicated in T-ALL.

TAL1 polypeptides also form heterodimeric complexes with the E proteins. The interaction between TAL1 and the E proteins is mediated by dimerization of their respective bHLH domains. The resultant bHLH heterodimers (e.g. E47−TAL1) bind DNA by specific recognition of the E-box sequence (CANNTG), a common regulatory element of eukaryotic transcription enhancers. We now show that the LIM proteins implicated in T-ALL (RBTN1 and RBTN2) can associate with TAL1 in the context of E47−TAL1 heterodimers. Thus, RBTN polypeptides may modulate the expression of target genes that are otherwise regulated by bHLH heterodimers containing TAL1. We have not as yet observed a consistent effect of RBTN2 on the transcriptional activity of TAL1 (data not shown); however, these preliminary studies were conducted with artificial reporter genes bearing multimerized binding sites for E47−TAL1 heterodimers (Hsu *et al.*, 1994c). Thus, natural target genes may be required to discern the functional consequences of the TAL1−RBTN2 interaction—particularly if TAL1 heterodimers (e.g. E47−TAL1) and RBTN2 polypeptides are components of larger multiprotein complexes that assemble on natural transcriptional promoter (or enhancer) sequences. In any case, future studies should reveal whether RBTN2 and TAL1, as well as the other LIM and bHLH proteins implicated in T-ALL, promote leukemogenesis by regulating a common subset of target genes.

The molecular nature of the LIM−bHLH interaction

The TAL1−RBTN2 association described here represents the first example of a protein−protein interaction mediated by the LIM and bHLH motifs. The present data do not establish whether this association is facilitated by intermediary factors or whether it involves direct physical contact between LIM and bHLH sequences. The ability of RBTN proteins to associate with assembled E47−TAL1 heterodimers places constraints on the bHLH sequences responsible for TAL1−RBTN interaction. The bHLH motif consists of two amphipathic α-helices separated by an intervening loop; in most cases the HLH segment is preceded by a short cluster of basic amino acids that facilitate DNA recognition (recently reviewed by Kadesch, 1993). It was originally proposed that bHLH polypeptides dimerize by hydrophobic interactions between the amphipathic helices of their respective bHLH moieties (Murre *et al.*, 1989). Structural studies have provided support for this view by showing that bHLH homodimers fold into a parallel, left-handed, four-helix bundle stabilized by van der Waals forces within its highly conserved hydrophobic core (Ferré-D'Amaré *et al.*, 1993, 1994; Ellenberger *et al.*, 1994). Since RBTN proteins will associate with E47−TAL1 heterodimers, the TAL1 sequences that mediate RBTN interaction are likely to reside on the exposed surface of the four-helix bundle, either as hydrophilic amino acids in the amphipathic helices or as available residues in the loop or basic regions. Significantly, these segments of the bHLH domain feature amino acids that are found uniquely in the TAL1-related proteins, and thus could account for the specificity of RBTN interaction with this subgroup of bHLH polypeptides.

The *RBTN1* and *RBTN2* genes encode LIM-only proteins of 156 and 158 amino acids, respectively. These polypeptides contain two tandem LIM domains, each consisting of 50–60 amino acids. Since the RBTN polypeptides are mostly comprised of LIM sequences, it is likely that their association with bHLH proteins is mediated by one or both LIM domains. Moreover, the amino acid homology shared by RBTN1 and RBTN2 occurs within the LIM motifs, further implicating these sequences as mediators of intracellular interaction with TAL1, TAL2 and LYL1. The RBTN−TAL1 association described here suggests that the LIM domain functions as an interface for *in vivo* protein−protein interactions as previously proposed by Rabbitts and Boehm (1990).

LIM−bHLH interactions in normal development

The specificity of interaction between the RBTN polypeptides and the leukemic bHLH proteins implies that LIM−bHLH complexes also function during normal development. Previous studies have shown that *TAL1* is normally expressed in hematopoietic cells of the erythrocytic, megakaryocytic and mastocytic lineages, as well as in restricted populations of neural and endothelial cells (Green *et al.*, 1992; Hwang *et al.*, 1993; Mouthon *et al.*, 1993). Moreover, the growth and differentiation of erythroid cells are influenced by experimental changes in TAL1 expression (Green *et al.*, 1991; Aplan *et al.*, 1992b). Significantly, the *RBTN2* gene is co-expressed with *TAL1* in cells of erythroid origin (Valge-Archer *et al.*, 1994). Indeed, mice with null mutations of the *Rbtn2* gene die at E10.5 due to a complete absence of erythroid precursors, indicating that Rbtn2 is required for normal erythroid development (Warren *et al.*, 1994). Thus, the regulatory functions of RBTN2 and TAL1 during erythroid development may be mediated, at least in part, by interaction between their respective LIM and bHLH domains.

It has become increasingly clear that the transcriptional regulatory activities of LIM-HD proteins are influenced by their LIM sequences. For example, the LIM moieties of Isl-1 have an inhibitory influence on the DNA-binding activity of its associated homeodomain, perhaps reflecting an intramolecular interaction between the LIM and homeodomain sequences (Sanchez-Garcia *et al.*, 1993). Moreover, transcription of the insulin gene is activated synergistically by lmx-1, a B-cell specific LIM-HD protein and shPAN-1, the hamster homolog of E47; although the mechanism by which these proteins cooperatively activate transcription is not understood, the effect is clearly dependent on the LIM domains of lmx-1 (German *et al.*, 1992).

In light of our results, it is conceivable that the LIM domains of other proteins also participate in functional interactions with bHLH polypeptides. For example, the synergistic activation of insulin gene transcription by lmx1 and shPAN-1 may involve association between their respective LIM and bHLH motifs (German *et al.*, 1992). Considering the broad spectrum of cellular activities that are governed by transcription factors of the LIM and bHLH families, LIM−bHLH interactions may potentially influence various aspects of cell growth and differentiation. These interactions would allow coordinate control of transcription by LIM and bHLH proteins, and they would greatly expand the regulatory functions of both families of transcription factors.

Materials and methods

Southern analysis of patient DNA

Patient #1114 was diagnosed with T-ALL at the Children's Medical Center, Dallas. The cytogenetic analysis and tissue specimens of patient #1114 were described previously (Yoffe *et al.*, 1989). The other leukemic specimens were provided by the Pediatric Oncology Group (POG) Cell Bank; these represent peripheral blood or bone marrow aspirates obtained prior to treatment from seven T-ALL patients with the (11;14)(p13;q11) translocation. Complete immunophenotyping and karyotyping of POG samples were performed by Drs Michael P.Link (Stanford University) and Andrew J.Carroll (University of Alabama), respectively. DNAs were extracted from patients' specimens and analyzed by Southern hybridization as described previously (Chen *et al.*, 1990).

Two-hybrid analysis

Expression plasmids encoding VP16-TAL1, GAL4-TAL2, VP16-TAL2, GAL4-E47 and VP16-E47 have been described (Hsu *et al.*, 1994b; Xia *et al.*, 1994). Plasmids encoding GAL4-Jun, VP16-Jun, GAL4-E12, VP16-E12, GAL4-MyoD1, VP16-MyoD1, GAL4-Id1, VP16-Id1, GAL4-MYC, VP16-MYC, GAL4-MAX and VP16-MAX were kindly provided by Dr Chi Dang (Dang *et al.*, 1991; Finkel *et al.*, 1993). Plasmids encoding GAL4-TAL1 fusion proteins with the complete TAL1 sequence (amino acid residues 1–311) or with partial TAL1 sequences (see Figure 5A) were constructed by inserting the appropriate segments of TAL1 cDNA into the pSG424, pM1 or pM2 expression vectors (Sadowski and Ptashne, 1989; Sadowski *et al.*, 1992). Plasmids encoding GAL4-RBTN1, VP16-RBTN1, GAL4-RBTN2, VP16-RBTN2 and GAL4-CRIP were constructed by inserting PCR-generated segments of cDNA into the pNLVP16 or pM1 expression vectors (Dang *et al.*, 1991; Sadowski *et al.*, 1992). An expression plasmid encoding GAL4-CRP was prepared by transferring a 1.8 kb *Eco*RI–*Pst*I fragment (containing the entire human CRP cDNA sequence) from the pGEM4-hCRP plasmid (Liebhaber *et al.*, 1990) into the pM1 vector. The GAL4-zyxin expression plasmid was constructed by transferring a 2.0 kb *Bam*HI fragment from the pBScZyx-5 plasmid (Sadler *et al.*, 1992) into pM2; the GAL4-zyxin fusion protein contains the carboxy-terminal 400 amino acids of zyxin (residues 143–542), including the three LIM domains. An expression plasmid encoding the hybrid VP16-Rb polypeptide was constructed in two steps: first, the cDNA insert of the pGEX2T-RB(379–792) plasmid (Kaelin *et al.*, 1991) was excised with *Bam*HI and ligated into the *Bam*HI site of the p34H vector (Tsang *et al.*, 1991); second, the cDNA insert of the resultant p34H-Rb(379–792) plasmid was excised with *Sma*I and ligated to pNLVP16 plasmid DNA that had been linearized with *Nde*I and end-filled with Klenow DNA polymerase. The final plasmid, VP16-Rb(379–792), encodes a hybrid polypeptide containing the transactivation domain of VP16 fused to the binding pocket of Rb (residues 379–792). The G5E1bLUC reporter plasmid, and the methods for two-hybrid analysis in Jurkat cells have been described (Hsu *et al.*, 1994b). In Figure 9, the indicated samples (lanes 4–7) were also co-transfected with 5 μg of TAL1/pCMV4, a mammalian expression vector that encodes full-length wildtype TAL1 polypeptides (Cheng *et al.*, 1993).

Co-immunoprecipitation

The TAL1 expression plasmid (TAL1/pCMV4) has been described (Cheng *et al.*, 1993). The RBTN2-tag/pCMV5 plasmid was produced by inserting a PCR-amplified fragment of mouse RBTN2 cDNA (Boehm *et al.*, 1991) into the pCMV5 expression vector (Andersson *et al.*, 1989); the resultant plasmid encodes RBTN2-tag, a full-length RBTN2 polypeptide with a carboxy-terminal tag of nine amino acids (YPYDVPDYA) that is recognized by the 12CA5 monoclonal antibody (Field *et al.*, 1988). Methods for DNA transfection of COS1 cells have been described (Cheng *et al.*, 1993). Cell lysates in 1 ml of low stringency NP40 buffer were prepared from 10^7 cells (Hsu *et al.*, 1994b), and 10 μl of immune or pre-immune rabbit serum were added to each lysate. Antisera specific for the amino-terminal 121 residues (#1080) or the carboxy-terminal 19 residues (#460) of TAL1 have been described (Hsu *et al.*, 1994a,b). After gentle rocking at 4°C for 1 h, 50 μl of staphylococcal protein A–Sepharose beads (Pharmacia) were added to each lysate and the mixture was rocked at 4°C for an additional hour. The beads were then pelleted by a brief centrifugation and washed four times in low stringency buffer. Finally, the beads were resuspended in 2× loading buffer, boiled for 10 min and pelleted by centrifugation. The supernatant was then fractionated by electrophoresis on a 15% SDS–polyacrylamide gel. The protein was then electroblotted from the fractionated gel onto Hybond-ECL nitrocellulose for Western analysis by enhanced chemiluminescence (Amersham) with 12CA5 monoclonal antibodies. For co-immunoprecipitation of endogenous TAL1 and RBTN2 polypeptides, suspension cultures of leukemia cell lines were labeled with [^{35}S]methionine (Cheng *et al.*, 1993) and the radiolabeled cells were lysed in low-stringency NP-40 buffer (Hsu *et al.*, 1994b). Immunoprecipitation of cell lysates was conducted in either low-stringency (NP-40 buffer) or high-stringency (RIPA) conditions (Hsu *et al.*, 1994b).

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