T-cell Proto-oncogene Rhombotin-2 Is a Complex Transcription Regulator Containing Multiple Activation and Repression Domains*  

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The LIM domain protein rhombotin-2 (RBTN-2/TTG-2/LMO2) is involved in many processes, including leukemogenesis and erythropoiesis. It is thought that the principle role of RBTN-2 in these processes is to regulate transcription. To examine the potential for RBTN-2 to modulate transcription, we constructed RBTN-2/GAL4 DNA-binding domain fusion proteins and measured their ability to activate transcription of a reporter gene construct. From these studies we identified a transcription activation domain within the NH₂ terminus of RBTN-2. This activation domain was further localized within a proline-rich 19-amino acid region. A second activation domain of 11 amino acids was also identified. This domain was located within the COOH terminus of RBTN-2, and functioned in mammalian cells but not in yeast. Furthermore, the two LIM domains of RBTN-2 were shown to function as transcription repression domains. Each individual LIM domain acted as an independent transcription repression domain on a heterologous activation domain. However, in context of full-length RBTN-2, the LIM domains selectively repressed the NH₂-terminal activation domain, but had no effect on the COOH-terminal domain. Overall, these results demonstrate that the T-cell oncogene RBTN-2 is a complex transcription factor possessing multiple transcription regulatory modules, including two activation domains and two repression domains.

Chromosomal translocations, juxtaposing two different regions of the genome, are the recurrent events in human cancers, particularly leukemias (1, 2). Molecular analyses of the breakpoints in lymphocytic leukemias have revealed that genes specifying transcription factors are the most frequent target of the changes brought about by these translocations (2). Translocations cause two types of changes in the affected transcription factors. In one type, a chimeric fusion transcription factor is generated when the breakpoints occur in the coding regions of the affected genes. The resulting fusion transcription factor, possessing altered specificity, is produced in the cancer cells. The other type of change results in the increased, and usually ectopic, expression of a structurally intact transcription factor. This change most frequently occurs when translocation break-points occur in the regulatory region of a proto-oncogene, juxtaposing the proto-oncogene to either immunoglobulin or T-cell receptor genes in lymphocytic leukemias. Thus, either altered or ectopic expression of proto-oncogenes is thought to contribute toward the pathogenesis of human leukemias.

Translocations to the rhombotin-2 (RBTN-2/TTG-2/LMO2) locus represent the second type of change to a proto-oncogene. The RBTN-2 locus at chromosome 11p13 is the most frequent site of chromosomal translocation in childhood T-cell acute lymphoblastic leukemia (3–5). Translocations to this locus involve T-cell receptor genes, and almost invariably result in high expression of normal RBTN-2 (3, 4). While RBTN-2 is expressed in virtually all tissues during fetal and adult life, it is not expressed in T-lymphocytes (4, 6, 7). Thus, the ectopic expression of RBTN-2 in thymocytes contributes to leukemogenesis as demonstrated in transgenic mice with enforced expression in T-cells (8–10). A normal function for RBTN-2 in erythropoiesis has recently been found. Homozygous null mice lacking RBTN-2 die in utero around day 11 due to lack of red blood cells in the embryonic yolk sac (11). The mechanism of RBTN-2 protein in its leukemogenic and normal functions is implicated in transcriptional regulation because of its nuclear location in hematopoietic cells (7, 11), and recent demonstration that RBTN-2 forms complexes with other transcription factors: namely tal-1, GATA-1, and GATA-2 (12–14).

Additional evidence that RBTN-2 functions in transcriptional regulation is derived from the structural similarity between RBTN-2 and known transcription factors. The RBTN-2 gene encodes a 158-amino acid protein, approximately two-thirds of which is comprised of tandemly repeated cysteine-rich zinc-binding motifs called LIM domains (15, 16). The LIM domain was originally identified in the transcription factors Lin-11 (17), Isl-1 (18), and Mec-3 (19). However, unlike these LIM-homeodomain transcription factors, RBTN-2 has no demonstrable DNA binding activity (15, 16). Recently, the LIM domain has been shown to be a unique protein-protein interaction. In vitro studies have shown that the LIM domain can dimerize with other LIM domain (20, 21) or other motifs such as helix-loop-helix domain (12, 13). Thus, RBTN-2, lacking known transactivation domains and DNA-binding motifs, was thought to regulate transcription by binding other transcription factors and modulating their activity. However, recently, RBTN-2 was shown to possess transcription activity when fused to the DNA-binding domain of GAL4 (22). In this report, we confirm this transactivation activity and further show that RBTN-2 has complex transcriptional regulatory activities; it has two separate activation domains and two repression domains. Furthermore, we show that the LIM domains, in addition to their known function as protein-protein interaction

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domains, have transcription repression activity. These results support the notion that RBTN-2 may regulate the transcription of target genes in a complex manner that, when ectopically expressed in T-lymphocytes, may eventually lead to leukemias.

**MATERIALS AND METHODS**

**Plasmids—** All RBTN-2 clones and deletion mutants used in yeast were generated by polymerase chain reaction (PCR)\(^2\) and cloned into the NcoI site of the yeast fusion vector pAS2 (24). Every construct was sequenced fully to verify fidelity of the PCR. These constructs include full-length RBTN-2 (158 amino acids), and the following portions of RBTN-2: the \(\text{NH}_2\) terminus deletion (RdN, amino acids 27–158), the \(\text{NH}_2\) terminus region (RN, amino acids 1–26), the COOH terminus deletion (RdC, amino acids 1–147), the LIM domain region (R2L12, amino acids 27–150, containing both LIM domains), the first LIM domain (R2L1, amino acids 27–86), and the second LIM domain (R2L2, amino acids 92–150). The yeast vector pAS2 (a kind gift from Dr. Stephen Elledge, Baylor College of Medicine, Houston, TX) is a GAL4 DNA-binding protein (GBD) fusion protein expression vector. This vector contains a tryptophan (Tsp) selection marker and a yeast origin of replication. A positive control construct, pACTc/145, encodes the GAL4 activation domain fused with the COOH-terminal 69 amino acids of the retinoblastoma-binding protein-2 that has been shown to have transactivation activity (R2L7-2)\(^1\).

Among the RBTN-2 constructs used in mammalian cells, the \(\text{NH}_2\)-terminal region deletion mutants and the COOH terminus region mutant were generated using synthetic oligonucleotides (Center for Biotechnology, St. Jude Children’s Research Hospital, Memphis, TN) and cloned into the HindIII site of mammalian fusion vector pM3 (25). The \(\text{NH}_2\)-terminal region deletion mutants include: RN1–10 (amino acids 1–10), RN1–13 (amino acids 1–13), RN11–19 (amino acids 11–19), RN11–23 (amino acids 11–23), RN11–25 (amino acids 11–25), RN11–29 (amino acids 11–29), and RN14–23 (amino acids 14–23). The COOH terminus region mutant is RC (amino acids 148–158). All constructs were sequenced to verify the fidelity of the oligonucleotide synthesis, and the open reading frame of the cloned sequences. The pM3 vector is a mammalian version of the GBD fusion expressing vector under the control of the SV40 early promoter (25).

The other RBTN-2 constructs used in mammalian cells were generated by PCR. They include the \(\text{NH}_2\) terminus region (RN, amino acids 1–29), the \(\text{NH}_2\) terminus deletion mutant (RdN, amino acids 30–158), the COOH terminus deletion mutant (RdC, amino acids 1–145), the LIM domain region (R2L12, amino acids 30–147), the first LIM domain (R2L1, amino acids 30–86), and the second LIM domain (R2L2, amino acids 94–147). Of these PCR-generated mutants, RN, RdN, and RdC were cloned into the HindIII site of pM3 vector, while the LIM domain region mutants, R2L12, R2L1, and R2L2, were cloned into the EcoRI site of pM3 vector and the EcoRI site of the pM3/3CGln vector. The pM3/3CGln vector (a kind gift from Dr. Clare Sample, St. Jude Children’s Research Hospital, Memphis, TN), encodes the GBD fused with the glutamine-rich activation domain (3CGln) from Epstein-Barr virus transcription factor, EBNA3C (26). The LIM domain mutants cloned into this vector were positioned before and in-frame with the 3CGln transactivation domain. Authenticity of the above constructs was confirmed by sequencing.

The CAT reporter gene vector pG5EC contains the gene encoding chloramphenicol acetyltransferase (CAT), which is under the control of promoters containing the GAL4-binding sites (24). The CAT reporter gene constructs: E1b-CAT lacking GAL4-binding sites showed no activity with any of the constructs tested (Fig. 2A and B). In contrast, the activation domain was located in the 26-amino acid sequence of the \(\text{NH}_2\) terminus.

**RESULTS**

**RBTN-2 Has Intrinsic Transcription Activity in Yeast**—We\(^1\) and others (22) have found that RBTN-2, when fused to a DNA-binding domain, has transactivation activity in yeast. The full-length RBTN-2 coding sequence was cloned into the GBD fusion protein expression vector pAS2 and transformed into Saccharomyces cerevisiae strain Y190, which contains two reporter gene constructs: HIS3 and lacZ (24). As shown in Fig. 1, full-length RBTN-2, when fused to GBD, activated the reporter genes and the corresponding yeast transformants grew in the presence of histidine and turned blue in the filter-lift assay, indicating that RBTN-2 indeed has intrinsic transcription activation activity.

To identify the region(s) of RBTN-2 responsible for this transcription activity in yeast, we generated various deletion mutants of the RBTN-2 gene by the PCR (Fig. 1A). These deletion mutants were cloned into the pAS2 vector and transformed into the yeast strain Y190. As shown in Fig. 1 (panel A and B), deletion of the \(\text{NH}_2\)-terminal 26 amino acids (RdN) abolished the activation activity of RBTN-2; while deletion of the COOH terminus (RdC) had no effect on this activity. Neither LIM domain alone nor LIM domains together (R1L, R1L2 and R1L2) showed any transcription activity. This result further supported the transactivation activity of RBTN-2 and indicated that the activation domain was located in the 26-amino acid sequence of the \(\text{NH}_2\) terminus.

**Yeast Strain and Assays—** The yeast strain Y190 (24) was provided by Dr. Stephen Elledge. It contains two reporter genes, HIS3 and lacZ, under the control of promoters containing the GAL4-binding sites (24, 27). The yeast was grown on YPAD medium, or SD minimal medium supplemented as described (24).

The pAS2 constructs were transformed into Y190 as described (24). The activation of the reporter genes, HIS3 or lacZ, was judged by the ability of transformants: 1) to grow in the absence of histidine (50 mm 3-amino-1,2,4-triazole, a histidine synthesis inhibitor, was added to increase selection pressure) and 2) to turn blue on plates containing 5-bromo-4-chloro-3-indolyl-\(\beta\)-galactoside (40 ng/ml), or by filter-lift assay.

**CAT Assays—** Two micrograms each of the following three plasmids (6 \(\mu\)g total DNA), the CAT reporter construct pG5EC, GBD-RBTN-2 mutant fusions vectors (pM3), and pCMV-\(\beta\)-gal, were co-transfected into COS-1 cell lines, using 24 \(\mu\)l of LipofectAMINE\(^\text{TM}\) reagent based on the protocol provided by the supplier (Life Technologies, Inc.). After 72 h, the cells were harvested and the amount of the cell lysate used in each CAT assay was normalized on the basis of the \(\beta\)-galactosidase activity of each extract. The CAT assays were performed as described (28).

\(^2\)The abbreviations used are: PCR, polymerase chain reaction; GBD, GAL4 DNA-binding domain; CAT, chloramphenicol acetyltransferase.\n
\(^1\)Stephen Elledge, Baylor College of Medicine, Houston, TX.) is a GAL4...
Transcription activation in mammalian cells. These findings were confirmed when the NH₂-terminal 29-amino acid region fused to GBD also showed 6-fold higher transcription activity (Fig. 2A, lane 3) as compared to the control cells (lane 1). However, in contrast to yeast, we found that deletion of 29 amino acids from the NH₂ terminus region or RBTN-2 did not abolish the transcription activation in mammalian cells. These findings were

examined the transcription activity of this 19-amino acid region in detail. There are only 4 proline residues in full-length RBTN-2, and all these 4 prolines are present in this 19-amino acid stretch (Fig. 3A). When we deleted the sequence from 24 to 29 amino acids, which contains two consecutive prolines, the transcription activation was reduced by about 50% (Fig. 3B, lane 4), suggesting the presence of other transactivation domain(s). Overall, these results suggested a more complex transcription regulatory behavior of RBTN-2 in mammalian cells than that seen in yeast.

The NH₂-terminal Activation Domain Is Located within a Proline-rich Region—To further localize the NH₂ terminus activation domain, a series of deletion mutants were made either by PCR or synthesis of oligonucleotides and then cloned into the pM3 vector (Fig. 3A). These constructs were transfected into COS cells along with the CAT reporter gene constructs. After 72 h, cells were lysed and CAT activity measured. The amount of cell lysates used in CAT assays was normalized based on transfection efficiency measured using an internal control plasmid expressing β-galactosidase activity. Panel A shows a representative CAT assay using the pG5EC reporter plasmid which contains five tandemly repeated GAL4 DNA-binding sites in the promoter region. Lane 1, pM3 empty vector; lane 2, vector expressing full-length RBTN-2 (pM3/RBTN2); lane 3, vector expressing only the first 29 amino acids of RBTN-2 (pM3/RN1–29); lane 4, vector expressing a RBTN-2 mutant lacking the first 29 amino acids (pM3/RdN). Panel B shows that the GAL4 DNA-binding domain is required for transcriptional activity of RBTN-2 constructs. In these experiments, RBTN-2 constructs were co-transfected with either pG5EC (lanes 1 and 2) or pEB1-CAT (lanes 3–6). pEB1-CAT is identical to pGSEC except that it lacks the 5 GAL4 DNA-binding sites upstream of the adenovirus E1b promoter. Lanes 1 and 3, pM3 empty vector; lanes 2 and 4, vector expressing the NH₂-terminal activation domain of RBTN-2 (pM3/RN1–29); lane 5, vector expressing the COOH-terminal activation domain of RBTN-2 (pM3/RC); lane 6, vector expressing full-length RBTN-2 (pM3/RBTN2).

FIG. 1. Transcriptional activity of RBTN-2/Gal4 DNA-binding domain fusion proteins in yeast. A, schematic representation of the regions of RBTN-2 cloned in the yeast expression vector pAS2, and summary of the transcription activity of each fusion protein when transformed into yeast Y190. The topmost figure depicts full-length RBTN-2, while those below indicate the portions of RBTN-2 tested for transcription activity in yeast. Transcription activity of each construct was determined by the ability of transformants: (i) to produce histidine and grow in the presence of histidine synthesis inhibitor 3-amino-1,2,4-triazole; and (ii) to generate blue color (lacZ activity) in filter lift assays. The two LIM domains of RBTN-2 are indicated by shaded boxes. B, transcription activity of RBTN-2 fusion proteins as determined by lacZ activity. Fusion constructs depicted in Panel A were transformed in yeast Y190 and assayed for lacZ activity by filter lift assay. Positive β-galactosidase activity is indicated by blue coloring (dark) of the colonies. A positive control (R2L12/RBP2) is also shown in the figure. This control is a yeast co-transformant expressing the GBD fusion of both NH₂ terminus region of RBTN-2 (pAS2/R2L12) and the GAL4 activation domain fusion of the COOH-terminal end of retinoblastoma-binding protein-2, a RBTN-2 interactive partner (see Footnote 1).

FIG. 2. RBTN-2 functions as a transcription factor in mammalian cells. Full-length, or portions of RBTN-2, were cloned into the mammalian GBD fusion protein expression vector pM3 and co-transfected into COS cells with CAT reporter gene constructs. After 72 h, cells were lysed and CAT activity measured. The amount of cell lysates used in CAT assays was normalized based on transfection efficiency measured using an internal control plasmid expressing β-galactosidase activity. Panel A shows a representative CAT assay using the pG5EC reporter plasmid which contains five tandemly repeated GAL4 DNA-binding sites in the promoter region. Lane 1, pM3 empty vector; lane 2, vector expressing full-length RBTN-2 (pM3/RBTN2); lane 3, vector expressing only the first 29 amino acids of RBTN-2 (pM3/RN1–29); lane 4, vector expressing a RBTN-2 mutant lacking the first 29 amino acids (pM3/RdN). Panel B shows that the GAL4 DNA-binding domain is required for transcriptional activity of RBTN-2 constructs. In these experiments, RBTN-2 constructs were co-transfected with either pG5EC (lanes 1 and 2) or pEB1-CAT (lanes 3–6). pEB1-CAT is identical to pGSEC except that it lacks the 5 GAL4 DNA-binding sites upstream of the adenovirus E1b promoter. Lanes 1 and 3, pM3 empty vector; lanes 2 and 4, vector expressing the NH₂-terminal activation domain of RBTN-2 (pM3/RN1–29); lane 5, vector expressing the COOH-terminal activation domain of RBTN-2 (pM3/RC); lane 6, vector expressing full-length RBTN-2 (pM3/RBTN2).
The NH₂-terminal activation domain resides within a proline-rich region. Various portions of the NH₂-terminal region were cloned into the mammalian GAL4 fusion protein expression vector pM3 and analyzed for transcription activity as described in the legend to Fig. 2. A, schematic representation of the NH₂-terminal region and its deletion constructs. Each construct is named based on the position of the first and the last amino acid residues. Each bar corresponds to the length of the amino acid sequence and the position of the construct relative to the NH₂-terminal region. B, the relative CAT activity of the NH₂-terminal deletion constructs. Quantification of CAT activity was performed using a PhosphorImager (Molecular Dynamics) and the associated software ImageQuant. The relative CAT activity of each construct is represented as a percentage of the CAT activity of the complete NH₂ terminus (RN1–29). C, equivalent amounts of GAL4 fusion proteins are expressed in transfection assays. Extracts from the transfected cells were immunoprecipitated with a GAL4 DNA-binding domain antibody, and fusion proteins were detected by Western blot using the same antibody. Lane 1, empty pM3 vector expressing only the GAL4 DNA-binding domain (GBD); lanes 2–8, pM3 vector containing the various NH₂-terminal deletion mutants of RBTN-2 as indicated in Panel A. Molecular mass in kilodaltons (kDa) is shown to the right of the blot. The intense band at 51 kDa corresponds to the GAL4 DNA-binding region antibody used in the initial immunoprecipitation.

The COOH Terminus of RBTN-2 Has Transactivation Activity in Mammalian Cells—In contrast to yeast, deletion of the NH₂ terminus did not abolish the activity of RBTN-2 in mammalian cells (Fig. 2A, lane 4). These results show that additional activation domain(s) exist in regions other than the NH₂ terminus of RBTN-2. Since deletion of the COOH terminus greatly reduced the overall activity (Fig. 4, lane 4), we therefore tested the transactivation activity of this region by cloning the COOH terminus of RBTN-2 into pM3 vector. COS cells transfected with the pM3 construct containing the COOH terminus region (11 amino acids) and the CAT reporter gene construct pG5EC showed 4 times more CAT activity than the control cells (Fig. 4, compare lanes 1 and 3). These results show that the COOH terminus region of RBTN-2 possesses transactivation activity in mammalian cells. Surprisingly, the existence of the COOH terminus seems to be critical to the overall activation activity of RBTN-2 in mammalian cells, since its deletion (Fig. 4, lane 4) greatly reduced the overall transcription activation, even though the truncated RBTN-2 molecule contained the NH₂-terminal activation domain.

LIM Domains of RBTN-2 Act as Transcriptional Repressors—We observed an interesting phenomenon in that the overall activation activity of RBTN-2 did not reflect the sum of the individual activation domains of the NH₂ terminus and the COOH terminus in mammalian cells. In fact, the activity of the NH₂ terminus domain alone is stronger than that of the full-length RBTN-2 (Fig. 2A, lanes 2 and 3), suggesting that the activity of this domain is somehow repressed in the context of the whole molecule. Besides the two activation domains at the two termini, the two LIM domains constitute virtually the rest of the RBTN-2 protein. Therefore, we examined the possibility that the LIM domains act as transcription repressor. We positioned the LIM domain region of RBTN-2 between the GBD and a heterologous activation domain. We used the glutamic acid-rich region (3CGln) of the Epstein-Barr virus transactivation factor EBNA3C which has been shown to possess transactivation activity (26). The transactivation activity of the 3CGln (pM3/3CGln) is shown in lane 2 of Fig. 5. This activity was completely repressed when 3CGln was fused to the LIM domain region of RBTN-2 (pM3/L12–3CGln, lane 5), indicating that the LIM domain region of RBTN-2 can repress the transcription activity of a heterologous activation domain. We further examined if this repression effect required both LIM domains. Each LIM domain was cloned independently between the GBD region and 3CGln domain. As shown in lanes 3 and 4 of Fig. 5, the activation activity of 3CGln was abolished in the presence of either LIM domain. As expected, each individual LIM domain (Fig. 5, lanes 6 and 7) had no activation activity. The absence of CAT activity was not due to unequal amounts of the fusion proteins expressed in the transfected cells. Western blot analysis showed that roughly equal amounts of the GAL4 fusion proteins were present (data not shown). Overall, these results indicate a single LIM domain can act as a transcription repression domain. In addition, LIM domain repression of a heterologous activation domain suggests that the LIM domain may function in other proteins as a transcriptional repressor.

**DISCUSSION**

Most oncoproteins activated by chromosomal translocations in lymphocytic leukemias are transcription factors (2). Accumulated evidence suggests that RBTN-2 is involved in transcription regulation: (i) immunofluorescence studies show that RBTN-2 is predominantly nuclear in cells of hematopoietic...
The LIM domain acts as transferable transcription repression domain. The LIM domains of RBTN-2 were positioned between the GBD of pM3 and a heterologous activation domain (3CGln) from the Epstein-Barr viral transcription factor EBNA3C (26). Transcription activity of each construct was determined as described in the legend to Fig. 2. Lane 1, pM3 empty vector; lane 2, construct expressing the transcription activation domain (3CGln) of EBNA3C (pM3/3CGln); lane 3, construct expressing 3CGln fused with the first LIM domain of RBTN-2 (pM3/L1–3CGln); lane 4, construct expressing 3CGln fused with the second LIM domain of RBTN-2 (pM3/L2–3CGln); lane 5, construct expressing 3CGln fused with both RBTN-2 LIM domains together (pM3/L1–2–3CGln); lane 6, construct expressing the first LIM domain of RBTN-2 (pM3/LIM1); lane 7, construct expressing the second LIM domain of RBTN-2 (pM3/LIM2).

Lineages (7, 11); (ii) as an LIM-only protein, RBTN-2 interacts with the transcription factors Tal-1 (12, 13), GATA-1 and GATA-2 (14), and retinoblastoma-binding protein-2; (iii) RBTN-2 fused to GBD activates transcription (22). In this study, we have confirmed that RBTN-2 functions as a transcription activator in both yeast and mammalian systems. In addition, we have localized the NH2 terminus activation domain to a 19-amino acid region and identified a second activation domain at the COOH terminus of RBTN-2. The COOH-terminal activation domain, unlike the NH2 terminus activation domain, functions in mammalian cells but not in yeast. Furthermore, we have demonstrated that the LIM domains, currently recognized as protein-protein interaction domains, also function as repression domains. The presence of LIM domains result in the overall low transcription activity of full-length RBTN-2 compared to those of the individual activation domains at both termini.

Studies on the eukaryotic transcription factors indicate that, as opposed to DNA-binding domains, the transcription activation domains of these proteins are less well defined (29). However, based on primary amino acid sequences, several classes of activation domains have been loosely defined, e.g. acidic, proline-rich, glutamine-rich, serine/threonine-rich, and recently identified isoleucine-rich domains (30–32). But some activation domains do not fit into any of these classes (33). In agreement with Sanchez-Garcia et al. (22), we have found that the NH2 terminus region of RBTN-2 serves as an activation domain in yeast, as well as in mammalian cells. In addition, we have localized the activation domain to a 19-amino acid stretch which displays the full transcription activity of the NH2 terminus region. It has been suggested that an 8-amino acid region (residues 15–23), with homology to the acidic activation domain of VP16 (34), may account for the transcription activity of RBTN-2 (22). However, we found that the “VP16 homologous sequence” in a deletion construct (RN14–23, Fig. 3) gave only ~40% of the total activation activity of the NH2 terminus domain. Thus, our data suggest that the VP16 homologous sequence contributes significantly but does not account for the total transcription activity of the NH2 terminus activation domain of RBTN-2. This notion is further supported by our observation that an 8-amino acid stretch (RN11–19, Fig. 3) gave ~60% of the total activation activity even though it lacked 4 of the 8 amino acids constituting the VP16 homologous region.

We found that the aromatic amino acid proline seems to play an important role in the transcription activity of the NH2 terminus activation domain. This conclusion is based on the following observations: (i) there are only 4 proline residues in RBTN-2, but all of them are present within this 19-amino acid activation domain; (ii) deletion of two consecutive prolines, Pro-24,25, from this region (RN1–23 and RN11–23, Fig. 3) resulted in an approximately 50% decrease in the transcription activity; (iii) the level of transcription activation exhibited by the NH2 terminus activation domain of RBTN-2 is similar to that seen in other proline-rich domains of transcription factors, such as AP-2 (3–6-fold activity over the background) (35, 36). These transcription factors usually act in a TATA-proximal position and have considerably lower activity compared to the transcription factors containing acidic-rich domain (35, 36). However, further studies involving site-directed mutagenesis are required to determine whether or not proline residues play a critical role in the NH2-terminus activation domain of RBTN-2.

We also discovered a second activation domain in the COOH terminus region of RBTN-2 that was active in mammalian cells, but was silent in yeast. This activation domain, comprised of 11 amino acids, does not easily fit into any class of the activation domains, but it has four isoleucine residues. Thus, the COOH-terminal region may represent either a novel class or an isoleucine-rich activation domain (32). It is noteworthy that the COOH-terminal activation domain is required for the transactivation activity of the entire full-length RBTN-2 protein. Removal of the COOH terminus alone results in silencing of the NH2 terminus activation domain in mammalian cells (Fig. 4, lane 4).

The presence of two activation domains in RBTN-2 is not too surprising since it is not unusual for eukaryotic transcription factors to have multiple activation domains (37). For example, the HLH family member E2A contains at least two separate activation domains; one functions efficiently in a variety of mammalian cell lines, whereas the second functions preferentially in pancreatic beta cell lines (38). In some eukaryotic transcription factors, the situation is even more complex. C-Fos has three activation domains in its COOH terminus which show weak activity independently but act synergistically in combination (39, 40). Recently, two additional activation domains and an inhibitory region has been identified in NH2 terminus of c-Fos (39). Multiple, regulatory domains in the eukaryotic transcription activation factors, such as RBTN-2, may allow these proteins to respond to different signals and/or endow them with multiple functions.

The two activation domains of RBTN-2 behave quite differently in that the NH2-terminal one functions in both yeast and mammalian cells, whereas, the COOH-terminal one has activity only in mammalian cells. One possibility for this different behavior of activation domains is that it reflects the basic differences in the general transcriptional machinery between mammalian cells and yeast. Indeed, recent studies have shown that some activation domains, e.g. the class of glutamine-rich domains such as those of SP1, AP2, Oct-1, Oct-2 (29, 31, 41), do not stimulate transcription in yeast S. cerevisiae, even when human TATA-binding protein or human-yeast hybrid TATA-binding protein is supplied (31). Thus, the COOH-terminal activation domain of RBTN-2 may target transcription factor(s) in mammalian cells that are absent in yeast.
The distinct behaviors of the two separate activation domains of RBTN2 imply that each domain may have different function. This view is consistent with the observations that RBTN2 is a multifunctional protein. RBTN2 is required for erythropoiesis (11), and is involved in T-cell leukemogenesis (8, 10). In addition, RBTN2 is implicated in the acute-phase response (7). In this context, it is noteworthy that RBTN2 binds multiple protein partners, especially transcription factors through its LIM domains (12–14). It is likely that each function requires different partner(s) that may interact with the distinct LIM domains of RBTN2. Thus, the functions of RBTN2 may depend upon the relative availability of the protein partners, that in turn may be influenced by the developmental stages, different tissue distribution, or cellular environment. In this context, it will be interesting to determine the relative activity of the two activation domains in different tissues.

We found that the overall transcription activity of RBTN2 was lower than that of each individual activation domain, suggesting the presence of a repressor domain in RBTN2. The most obvious candidates for the repressor effect are the LIM domains since, other than the NH2 and COOH terminus activation domains, they virtually constitute the remaining portion of RBTN2. Indeed, our results show that RBTN2 LIM domains, either individually or in combination, suppress the transcription induced by a heterologous activation domain (the EBNA3C protein of the Epstein-Barr virus, Fig. 5). Since each LIM domain can inhibit transcription activation independently, we consider that RBTN2 has two repressor domains. A similar “mix and match” of protein domain approach has led various investigators to identify repressor domains in different transcription factors such as WT1, eve, en, c-Evb-A, and v-Evba (37, 42). These repressor domains are termed “portable” or “transferable” because they function in the context of heterologous activation and DNA-binding domains. Thus, RBTN2 possesses two portable repressor domains. These repressor domains function in a position independent manner, i.e. repression was seen when LIM domains were positioned either amino-terminal (Fig. 5, lanes 3–5) or carboxyl-terminal (Fig. 4, lane 4) to activation domains. Despite this observation, the COOH terminus activation domain is not repressed by LIM domains (Fig. 2A, lane 4). Therefore, it is important to note that in context of the whole RBTN2 protein, the LIM domains selectively repress the NH2 terminus activation domain (Fig. 4, lane 4). The mechanism of this selective repression is not known.

The selective repression of the NH2-terminal domain provides a mechanism for differential activity of RBTN2. It is possible that repression of the NH2-terminal domain is abrogated through binding LIM interacting proteins. Based on this hypothesis, LIM interacting proteins would be present in yeast, but not in COS cells, since the NH2-terminal domain is active in yeast despite the presence of the LIM domains (Fig. 1). It is likely that the NH2-terminal domain is also active in mammalian cells. The unmasking of the NH2-terminal domain activity in selective tissues, and/or during fetal development would be dependent on expression of LIM interacting proteins in these tissues. Tissue-specific unmasking of activation domains is known to occur, e.g. an activation domain of E2A functions only in pancreatic beta cells (38).

In summary, our study establishes that the primary cellular role of T-cell oncogene, RBTN2-2, is to serve as a transcription regulator. The mapping of two distinct activation domains and two repression domains suggest a complex behavior of RBTN-2 on transcription regulation. In this context, RBTN2-2 is similar to other complex, multifunctional transcription regulatory proteins such as p53 (43), c-Fos (39), SRF (44), and IRF (23), that possess multiple negative and positive regulatory domains. The presence of multiple transcription regulatory modules suggests that the overall activity of RBTN-2 depends on the interplay among these activation and repression domains, as well as on the differential binding of LIM domain to different target proteins during fetal development and in different tissues.

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