Trax (Translin-associated Factor X), a Primarily Cytoplasmic Protein, Inhibits the Binding of TB-RBP (Translin) to RNA*

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Trax (Translin-associated factor X) has been shown to interact with TB-RBP/Translin by its communoprecipitation and in yeast two-hybrid assays. Here we demonstrate that Trax is widely expressed, does not bind to DNA or RNA, but forms heterodimers with TB-RBP under reducing conditions. The heterodimer of TB-RBP and Trax inhibits TB-RBP binding to RNA, but enhances TB-RBP binding to specific single stranded DNA sequences. The in vitro interactions between TB-RBP and Trax are confirmed by similar interactions in the yeast two-hybrid system. Cell fractionation and confocal microscopy studies reveal that Trax is predominantly cytoplasmic. In contrast, TB-RBP is present in both the nuclei and cytoplasm of transfected cells and uses a highly conserved nuclear export signal to exit nuclei. In addition to a leucine zipper, two basic domains in TB-RBP are essential for RNA binding, but only one of these domains is needed for DNA binding. Trax restores DNA binding to TB-RBP containing an altered form of this domain. These data suggest that Trax-TB-RBP interactions modulate the DNA- and RNA-binding activity of TB-RBP.

The process of mammalian spermatogenesis is highly organized spatially and temporally. Highly controlled transcription and protein expression occur in each developmental stage. During the haploid interval, spermiogenesis, the spermatids become transcriptionally inactive, although there is a need for the synthesis of many proteins essential for the formation of spermatozoa (1). The sex chromosomes encode numerous genes essential for gametogenesis. Because the spermatids are haploid cells, they contain either the X or Y chromosome. Thus, intercellular transport of mRNA in the haploid cells is a critical process to ensure genetic equivalency.

The testis brain RNA-binding protein (TB-RBP)1 was identified and cloned on the basis of its ability to bind H and Y sequence elements in the 3'-untranslated repeats of mouse protamine 1 and 2 mRNAs (2). TB-RBP is the mouse orthologue of human Translin, a single-stranded DNA-binding protein that binds consensus sequence breakpoint junctions of chromosomal translocations in lymphoid malignancies (3). The TB-RBP/Translin consensus binding sequences are also found in TLS-CHOP reciprocal translocations, in therapy-related translocations in acute myeloid leukemias, and in BCR-ABL translocations in chronic myeloid leukemia (4–6). Sequence analysis in a meiotic recombination hot spot region of human chromosome 16 shows TB-RBP/Translin binding sequences near the breakpoint (7). TB-RBP/Translin has also been proposed to act as a single-stranded DNA-binding transcription factor, which activates early response gene expression in the brain (8).

TB-RBP/Translin also functions as a RNA-binding protein mediating intracellular and intercellular mRNA transport (9, 10). RNA binding of TB-RBP has been observed in brain and testis, and the binding is dependent upon Y and H sequence elements (2). Many testis- and brain-specific mRNAs have Y and H consensus sequences, and specific RNA-TB-RBP interactions have been demonstrated for testis mRNAs encoding protamine 1 and 2 and AKAP 82 and in brain for myelin basic protein mRNA, α-calmodulin kinase II mRNA, Tau mRNA, and BC1 RNA (11–13). Translationally suppressed mRNAs are bound to microtubules by TB-RBP in cellular extracts (14) and recombinant TB-RBP binds specific mRNAs in vitro to reconstituted microtubules (11). The presence of TB-RBP in the cytoplasmic bridges that connect germ cells in a syncytium, together with the association of TB-RBP with transported mRNAs and the cytoskeleton (10), suggest that TB-RBP acts as a transport molecule in the testis for mRNAs in intracellular (from nucleus to cytoplasm) and intercellular (between spermatids) mRNA transport. The shift of subcellular localization of TB-RBP in meiotic and post-meiotic mouse germ cells (10, 15), the dendritic translocation of BC1 RNA and TB-RBP in rat hippocampal neurons (13), and the role of TB-RBP for mRNA sorting in dendrites (16) support this hypothesis.

Using Translin as bait in a yeast two-hybrid assay, a protein of unknown function, Translin-associated factor X (Trax), was identified (7). TB-RBP/Translin and Trax are encoded by single-copy genes that are evolutionarily conserved. In addition to having highly conserved sequences in mammals, they are also found in frogs (Xenopus laevis), plants (Arabidopsis thaliana and Oryza sativa), insects (Drosophila), and yeast (Schizosaccharomyces pombe) (17). This extraordinary conservation from

1 The abbreviations used are: TB-RBP, testis brain RNA-binding protein; Trax, Translin-associated factor X; NLS, nuclear localization signal; NES, nuclear export signal; TBS, tris-buffered saline; DTT, dithiothreitol; RT, room temperature; GFP, green fluorescence protein; BFP, blue fluorescence protein; PBS, phosphate-buffered saline; kb, kilobase(s); ER, endoplasmic reticulum; hnRNP, heterogeneous nuclear ribonucleoprotein.

BFP, blue fluorescence protein; PBS, phosphate-buffered saline; kb, kilobase(s); ER, endoplasmic reticulum; hnRNP, heterogeneous nuclear ribonucleoprotein.
yeast to mammals suggests that these molecules play important biological functions. In the adult mouse, TB-RBP mRNA is widely expressed, with its highest levels in testis and brain (15). The subcellular localization of TB-RBP protein is both developmentally and subcellularly regulated during spermatogenesis. During meiosis, the TB-RBP protein primarily localizes in the nuclei of pachytene spermatocytes, whereas in late meiotic prophase and in all subsequent stages of germ cell differentiation, it is in the cytoplasm (10). TB-RBP/Translin also is predominantly a nuclear protein in malignant lymphoid cell lines but not in nonlymphoid cell lines (3). Treatment of nonlymphoid cells with DNA-damaging agents was reported to cause a shift of the TB-RBP/Translin from the cytoplasm into nuclei (18).

TB-RBP and Trax share a 28% identity at the protein level with a conservation of 38% in the C-terminal regions. Both have putative leucine zipper domains at the C terminus of TB-RBP and in the mid-region of Trax (3, 7). Interaction between TB-RBP and Trax has been demonstrated by coimmunoprecipitation (19) and in the yeast two-hybrid assay (7, 8). Yeast two-hybrid and in vitro binding studies indicate that TB-RBP dimers are the minimum unit needed for DNA or RNA binding (20). Unlike TB-RBP (20), when Trax is used as bait in the yeast two-hybrid system, it does not select itself, suggesting it does not homodimerize. TB-RBP/Translin contains two putative basic domains in the N-terminal region from amino acids 56 to 64 and from amino acids 86 to 97. Changes in the amino acid sequence in the 86–97 region abolish the DNA-binding activity of Translin (21).

Analysis of the Trax sequence has suggested it contains a putative nuclear localization signal (NLS) (7). The subcellular localization changes in meiotic and post-meiotic cells (10, 15) and in nonlymphoid cells after DNA damage (18) indicate a need to shuttle TB-RBP between the nucleus and cytoplasm. Comparison of TB-RBP sequences with known nuclear export signals (NES) suggests the presence of a putative leucine-rich Rev-like NES sequence in its C terminus. Although the Rev-like NES is one of the most commonly described NESs (22, 23), none of the Rev-like NES-containing proteins have been shown to interact with cellular mRNAs.

To date, little is known of the biological function(s) of Trax. Because heterodimeric partners often modulate the activity of proteins that can homodimerize such as c-Fos/c-Jun (25), we set out to determine whether TB-RBP/Trax heterodimers could alter the nucleic acid recognition properties of TB-RBP. Here we show that Trax alone does not bind to either DNA or RNA, but Trax does form heterodimers with TB-RBP. The heterodimer of TB-RBP and Trax is unable to bind to RNA, but binds to DNA. Thus, the heterodimerization modulates the nuclear export signal to exit the nucleus and requires two basic regions in its N terminus in addition to its leucine zipper to bind RNA.

**EXPERIMENTAL PROCEDURES**

**Expression of Trax Protein in Escherichia coli**—A cDNA encoding the complete open reading frame of human Trax was subcloned in-frame with a thioredoxin-6X His-S-peptide N-terminal tag in a pET32a vector (Novagen) and transformed into BL21(DE3) cells. The expressed fusion protein was purified by nickel-nitrotriacetic acid-agarose column chromatography, and the thioredoxin-6X His tag was removed by thermolin digestion to obtain Trax with an N-terminal S-peptide tag.

**Northern and Western Blotting**—Total RNA preparations were hybridized with 32P-labeled Trax cDNA under conditions previously described (15). For Western blotting, tissue extracts were prepared from sexually mature CD-1 male mice using the protocol of Wu et al. (2). Aliquots (30 μg) of protein were electrophoresed in 10% SDS-polyacrylamide gels, and the proteins were transferred onto nylon membranes.

The membranes were incubated overnight with TBS containing 5% nonfat dry milk at 4 °C and then incubated with a polyclonal antibody to Trax (1:2000) in TBS containing 0.25% nonfat dry milk for 1 h at RT. After washing, the membranes were incubated with protein A conjugated with horseradish peroxidase, and Trax was detected with the enhanced chemiluminescence protocol of Amersham Pharmacia Biotech.

**In Vitro Interactions between TB-RBP and Trax**—Recombinant mouse TB-RBP (200 ng) was incubated with recombinant human S-peptide-tagged Trax for 30 min at 4 °C in 200 μl of TBS-T (20 mM Tris, pH 7.6, 137 mM NaCl containing 1% Tween 20) or in 200 μl of 20 mM HEPES, pH 7.5, containing 1.5 mM MgCl2 with or without 5 mM DTT. To precipitate protein complexes, 50 μl of protein A-Sepharose beads (Sigma) was added to the mixture and incubation was continued for 30 min at 4 °C with gentle shaking. The mixture was centrifuged for 1 min at 1000 rpm at 4 °C, and the pellet was washed three times with TBS-T (1 ml) interspersed with centrifugation at 1000 rpm. The pellets were boiled in SDS loading buffer for 3 min, and proteins were resolved on a 10% SDS-polyacrylamide gel. The gel was stained with SYPRO Orange protein dye (BioRad), and the proteins were visualized by fluorescence according to the manufacturer’s protocol.

**RNA and DNA Mobility Shift Assays**—Electrophoretic mobility shift assays were performed with a DNA probe, Bcl-CL1, or an RNA probe, transcript c, as described previously by Wu et al. (20). For the RNA gel shifts, the RNA-protein complexes were routinely digested with T1 RNase (1 unit/assay) and incubated with heparin (5 mg/ml). Bcl-CL1 protein complexes with [γ-32P]ATP-labeled T4 polynucleotide transferase transcript c was separated from a pGEM 3ZI plasmid using SP6 RNA polymerase and [α-32P]CTP. Recombinant proteins were incubated with 40,000 cpm of DNA or RNA probe for 10 min at RT in 20 μl of binding buffer (20 mM HEPES, pH 7.6, 3 mM MgCl2, 40 mM KCl, 2 mM DTT, 5% glycerol), and DNA-protein and RNA-protein complexes were detected following electrophoresis in 4% polyacrylamide gels in TBE buffer. All electrophoretic mobility shift assays were performed after incubation of TB-RBP and Trax at 10 times the final concentration of the proteins in 20 mM HEPES (pH 7.5) containing 5 mM DTT and 1.5 mM MgCl2, for 30 min followed by 10-fold dilution of the mixture to reduce the DTT concentration to 0.5 mM.

**Site-directed Mutagenesis of Putative Domains of TB-RBP**—Site-directed mutagenesis of the two basic domains and a putative nuclear export signal of TB-RBP was carried out using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. The wild-type TB-RBP, TB-Nb mutant, and TB-Cb mutant cDNAs were subcloned into a pET28a vector for protein expression in E. coli.

**Wild Type TB-RBP and TB-RBP**

Wild type TB-RBP and TB-RBP mutant cDNAs were subcloned in-frame to the C terminus of GFP in the pEGFP C2 vector (CLON-TECH). We named the fusion proteins from these constructs EGFP-TB-RBP and EGFP-TB-RBP(NES), respectively. These constructs were subcloned into the pEBFP C1 or pDsRed1-N1 vectors, which produce fusion proteins with the blue fluorescence protein at the N terminus of Trax (EBFP-TRAX) or the red fluorescence protein at the C terminus of Trax (Trax-DsRed), respectively. The Trax cDNA was also cloned into the pEGFP C2 vector.

**Yeast Two-hybrid Assays**—The complete cDNAs of TB-RBP, its mutant alleles, and Trax were subcloned into the EcoRI/SalI sites of pBD-GAL4am and pAD-GAL4 (Stratagene, La Jolla, CA). Pairs of binding domain and activation domain plasmid constructs were co-transformed into the yeast strain YRG-2. Transformants were selected on SD medium lacking leucine and tryptophan. Protein-protein interactions were detected by growth on SD medium lacking leucine, tryptophan, and histidine, and by the 5-bromo-4-chloro-3-indolyl β-galactopyranoside (X-gal) filter lift assay on SD medium lacking leucine and tryptophan. Strength of interaction was determined by the addition of 3-aminotriazole (Sigma Chemical Co., St. Louis, MO) to the medium at concentrations of 50–100 μM. The transformation and filter lift assay procedures were performed following the manufacturer’s instructions (Stratagene).

**Cell Culture, Transfections, and Confocal Fluorescence Microscopy**—NIH 3T3 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and streptomycin. Cells were transiently transfected with the plasmid constructs described above using FuGENE 6 reagent (Roche Molecular Biochemicals) according to the manufacturer’s protocol. Cells were grown on two-well chamber slides (Lab-Tek) and fixed 18 h post-transfection using 4% paraformaldehyde in PBS, washed three times in PBS and mounted using Vectashield mounting medium (Vector Scientific).
For amounts of undegraded mRNAs on the filter (Fig. 1).

Expression of Trax in Various Mouse Tissues. Total RNAs (10 μg) from brain, heart, kidney, liver, lung, spleen, and testis (lanes 1–7, respectively) were hybridized to the complete open reading frame of a human Trax cDNA. B, rehybridization of the blot from A with a mouse β-actin cDNA. C, Western blot analysis for Trax protein expression in various mouse tissues. Post-mitochondrial extracts (30 μg) from brain, heart, kidney, lung, liver, spleen, and testis (lanes 1–7, respectively) were separated by 10% SDS-polyacrylamide gel electrophoresis, transferred to nylon membranes, and probed with an antibody to Trax. Recombinant histidine-tagged human Trax (10 ng) is included as a control (lane 8). D, Western blot analysis (30 μg) of Trax expression in cytoplasmic and nuclear extracts from: testis cytoplasm, lane 1; testis nuclei, lane 2; NIH 3T3 cytoplasm, lane 3; and NIH 3T3 nuclei, lane 4. For C and D, equal protein loading in each lane was confirmed by Coomassie staining.

RESULTS

Expression of Trax in Mouse Tissues—To determine the extent of Trax expression in various mouse tissues, a Northern blot hybridization was performed with the complete open reading frame of Trax as probe (Fig. 1A). In the mouse, Trax is present as a single transcript of about 2.5 kb. As previously reported for TB-RBP (15), brain and testis contain the highest levels of Trax mRNA, whereas heart, kidney, liver, lung, and spleen contain lower amounts of Trax mRNA. These differences are not due to RNA degradation or unequal loading, because hybridization of the blot to a β-actin cDNA reveals similar amounts of undegraded mRNAs on the filter (Fig. 1A).

To determine the protein levels of Trax in mouse tissues, a Western blot was performed using a polyclonal rabbit anti-Trax antibody to detect a protein band of 34 kDa that comigrates to Trax. As seen for the mRNAs, the highest levels of Trax expression are seen in testis and brain. In general, Trax protein levels reflect the Trax mRNA levels, and the level of Trax protein in the mouse tissues examined was similar to the pattern reported for TB-RBP (2, 15). Quantitation of Western blots using recombinant Trax and recombinant TB-RBP as standards revealed TB-RBP to be present at about a 2- to 3-fold molar excess compared with Trax in these tissue extracts (data not shown).

To define the subcellular distribution of Trax, Western blot analyses of cytoplasmic and nuclear extracts from mouse testis and from NIH 3T3 fibroblasts were performed (Fig. 1D). In extracts from testis and transfected cells, Trax is predominantly found in the cytoplasm (Fig. 1D, lanes 1 and 3), although lower levels are seen in the nuclear fractions (Fig. 1D, lanes 2 and 4). Similar results are seen with confocal microscopy (see below).

TB-RBP Forms a Heterodimer with Trax in Vitro under Reducing Conditions—Because Trax coprecipitates with TB-RBP with an affinity-purified antibody to mouse recombinant TB-RBP (19) and the proteins interact in yeast two-hybrid assays (7), we set out to determine the conditions needed for Trax-TB-RBP interactions. An S-peptide-tagged recombinant Trax protein was mixed with recombinant TB-RBP and incubated in a series of buffers. Proteins precipitated with S-protein-agarose beads were then separated on SDS-polyacrylamide gels and stained using a SYPRO-Orange dye (Fig. 2). Trax and TB-RBP do not stably interact in buffers such as TBS or HEPES at neutral pH (Fig. 2, lanes 1 and 2). The presence of divalent cations such as Ca2+ or Mg2+ in PBS, TBS, or HEPES buffer also does not facilitate interaction. However, the addition of 5 mM DTT in HEPES buffer induces the proteins to interact at roughly a ratio of 1:1 suggesting a heterodimer, although a larger oligomer may also be formed (Fig. 2, lane 3).

The heterodimer is maintained when the DTT level is diluted to 0.5 mM, but prolonged dialysis of the protein mixture against HEPES buffer in the absence of DTT dissociates the heterodimer (data not shown).

Trax Inhibits TB-RBP-RNA Interactions but Enhances DNA Binding—To determine whether the TB-RBP-Trax interaction alters the nucleic acid binding of TB-RBP, gel shift assays were performed. RNA electrophoretic mobility shift assays were performed with transcript c and DNA electrophoretic mobility shifts assays with the single-stranded DNA, Bcl-CL1, two probes previously used to characterize Translin/TB-RBP binding to nucleic acids (20). Although Trax itself does not bind to RNA (Fig. 6, lane 2), the addition of increasing amounts of Trax under conditions where heterodimers are formed leads to about a 75% decrease in RNA binding at approximately a 1:1 ratio of Trax and TB-RBP (Fig. 3A, lane 5). The free RNA in these samples is degraded by the T1 RNase incubation routinely performed for the RNA gel shifts. (Fig. 3, lanes 2–5). In the absence of T1 RNase digestion, the RNA probe remains undegraded (Fig. 6).

The addition of increasing amounts of Trax to TB-RBP enhances binding to one of the DNA target sequences for TB-RBP, Bcl-CL1 (Fig. 3B). Severalfold increases in DNA binding are seen at a Trax:TB-RBP ratio of 2:1 (Fig. 3B, lane 4). A higher level of Trax (4:1) does not elicit any further increase in DNA binding (Fig. 3B, lane 5). As seen for the RNA binding assay, Trax alone does not bind DNA (Fig. 7, lane 2).

The TB-RBP-Trax Heterodimer Is the Cause of the Changes in RNA and DNA Binding—To demonstrate that it is the heterodimer of Trax and TB-RBP that is causing changes in TB-RBP binding to RNA and DNA, heterodimers were prepared using thioredoxin-tagged Trax and recombinant TB-RBP. The heterodimer migrates more slowly than either a TB-RBP homodimer or a Trax-TB-RBP heterodimer, because of the additional amino acids at its N terminus. Gel shift assays carried out using this protein nucleic acid complex reveal that it is the heterodimer of Trax and TB-RBP that binds poorly to RNA (Fig. 4, lanes 4 and 5) and the heterodimer that binds well to specific single-stranded DNA probes such as Bcl-CL1 (Fig. 4, lanes 9 and 10).

Mutations in Either of Two Basic Domains, Nb and Cb, of
determine their effect on RNA binding. Using radiolabeled transcript c, recombinant Trax, and recombinant TB-RBP as controls, electrophoretic mobility shift assays were performed to evaluate nucleic acid binding changes caused by altering these two basic domains of TB-RBP. As previously seen, the addition of Trax to TB-RBP reduces TB-RBP RNA binding (Fig. 6, compare lane 3 to lane 4). A mutation of the N-basic domain of TB-RBP (TB-Nb) drastically reduces the RNA binding in the absence (Fig. 6, lane 5) or presence of Trax (Fig. 6, lane 6). The protein-RNA complex formed by the TB-Nb mutant also migrates more rapidly than the wild type TB-RBP-RNA complex. A mutation of the C-basic domain of TB-RBP (TB-Cb) leads to the complete abolition of RNA binding (Fig. 6, lane 7). The addition of Trax to the TB-Cb protein does not show any effect on the reduction in RNA binding of TB-Cb (Fig. 6, lane 8). We conclude that both the N-basic and C-basic domains of TB-RBP are essential for RNA binding and the addition of Trax does not substantially alter its RNA interactions with these mutated proteins.

**Mutation in the C-basic Domain of TB-RBP Abolishes DNA Binding but Trax Restores DNA Binding**—DNA gel shift assays were carried out using an α[32P]-labeled Bcl-CL1 probe with control recombinant TB-RBP and Trax and with the two TB-RBP proteins with altered basic domains, TB-Nb and TB-Cb (Fig. 7). As previously demonstrated, DNA binding of TB-RBP is enhanced by heterodimerization of TB-RBP with Trax (Fig. 7, compare lane 3 to lane 4). The mutation in the N-basic domain of TB-RBP does not appear to affect binding to Bcl-CL1, although a more rapidly migrating DNA-protein complex is seen (Fig. 7, lane 5). Interestingly, when Trax is added to TB-Nb under conditions in which heterodimers are formed, no enhanced DNA binding is seen (Fig. 7, lane 6). The mutation in the second basic domain of TB-RBP, TB-Cb, abolishes DNA binding completely (Fig. 7, lane 7). Surprisingly, the addition of Trax, forming a Trax-TB-Cb heterodimer, restores DNA binding of the TB-Cb mutant (Fig. 7, lane 8). These data suggest that Trax can induce changes in mutant TB-RBP conformation, which influence its binding to DNA.

**TB-RBP and Trax Interact in Vivo**—To verify that TB-RBP, TB-Nb, TB-Cb, TB- NES, and Trax interact in vivo, a yeast two-hybrid assay was employed (Fig. 8). As previously reported, TB-RBP readily forms homodimers in yeast (20) (Fig. 8A) and heterodimers with Trax (Fig. 8B) (7). Trax, however, does not homodimerize (Fig. 8C). The TB-Nb, TB-Cb, and TB- NES mutants can also homodimerize, dimerize with wild-type TB-RBP, and heterodimerize with Trax in yeast (Fig. 8, D–L), suggesting that the changes in nucleic acid binding we detect by the gel mobility shift assays are specific and mediated by changes produced in the heterodimer as a result of mutations in TB-RBP not due to nonspecific interactions.

**Trax Is Predominantly a Cytoplasmic Protein**—To determine the subcellular locations of TB-RBP and Trax, transfections were performed in NIH 3T3 mouse fibroblasts using a fusion protein of TB-RBP and green fluorescence protein and Trax with a blue or red fluorescence protein. Confocal fluorescence microscopy reveals that Trax is predominantly cytoplasmic with a high concentration localized around the nucleus (Fig. 9C). TB-RBP is also mostly in the cytoplasm, although a reproducible low level is seen in nuclei, but not in nucleoli (Fig. 9, B and D). Cotransfections of TB-RBP with Trax also show a predominantly cytoplasmic localization for the two proteins. Identical results are obtained whether the fluorescence protein tag is on the N or C terminus of Trax (Fig. 9, D–F). The staining of the GFP-Trax-transfected cells with the fluorescent Golgi/ER marker BODIPY 558/568 suggests that much of the Trax is Golgi/ER-associated (Fig. 9G). This is supported by
studies where the disruption of the Golgi with brefeldin A leads to a more diffused cytoplasmic localization of Trax (Fig. 9H).

TB-RBP Has a Functional Leucine-rich Nuclear Export Signal—By sequence comparison of TB-RBP with other nuclear-cytoplasmic shuttling proteins such as HIV Rev and c-Abl (22, 23), we noted a putative nuclear export signal N-terminal to the leucine zipper of TB-RBP (Fig. 5). This sequence shows high sequence homology to other leucine-rich NES sequences present in a number of shuttling proteins (Fig. 10A) and is highly conserved from Drosophila to humans (Fig. 10B). To test the functionality of this sequence, site-directed mutagenesis was used to disrupt the putative nuclear export signal (from LASELSRLSVN to LASEQSRLSVN) (Fig. 10A). The mutated TB-RBP, TB-RBPNES, was then fused to GFP and transfected into NIH 3T3 cells. In contrast to wild type TB-RBP, which is mostly seen in the cytoplasm (Fig. 9B and D), the GFP-TB-RBPNES mutant protein localizes mostly in the nucleus with little staining in the nucleoli (Fig. 9I).

DISCUSSION

Trax is Widely Expressed—As previously shown for TB-RBP (2, 15), Trax is widely expressed in mouse tissues with high levels of mRNA and protein in brain and testis (Fig. 1). By Northern blotting, we detect one transcript of about 2.5 kb, in agreement with the 2.7-kb mRNA reported by Aoki et al. in humans (7). In general, this pattern of expression of Trax in mouse tissues is similar to the expression pattern observed for TB-RBP (2, 15), suggesting a functional relationship between these two similar proteins.

Trax and TB-RBP Form Heterodimers—To begin to define the functional relationship between Trax and TB-RBP, we have examined the interactions of recombinant TB-RBP with recombinant Trax. Trax and TB-RBP interact poorly under nonre-
TB-RBP nuclear export signals in various species. BODIPY and EGFP-Trax; Heterodimerization of Trax and TB-RBP appears to be sufficient for TB-RBP binding to either single-stranded DNA or RNA (20). Heterodimerization of Trax and TB-RBP protein coimmunoprecipitates with TB-RBP with an affinity-purified monospecific anti-TB-RBP antibody (19). A modest change in the electrophoretic mobility of the DNA-protein complex is seen when Trax interacts with the TB-Nb protein and Trax dramatically alters the binding of TB-Cb to DNA (Fig. 7). Enhanced DNA binding observed upon heterodimer formation of wild type TB-RBP or the TB-Cb mutant with Trax may be due to improved interaction of the N-basic domain with DNA. Thus, modulation of TB-RBP nucleic acid binding by Trax may be through conformational changes in TB-RBP that are induced by Trax. These changes in protein-protein interactions are likely due to heterodimer formation, because similar interactions are seen in vivo using the in vitro.
yeast two-hybrid assay. All of the mutant alleles interact as homodimers or with Trax as a partner for a heterodimer. These interactions indicate that, in vivo, the introduced mutations do not significantly alter the interacting capabilities of the fusion proteins (29). Although interaction strength was not quantified, we detected differences among the TB-RBP alleles. Wild type TB-RBP and TB-RBP<sub>NES</sub> interact strongly as homodimers and heterodimers, whereas TB-Nb interacts noticeably weaker. These results suggest that the effects on nucleic acid binding in the gel mobility shift assays are predominantly due to the mutations in TB-RBP, not gross alteration of protein–protein interactions.

Subcellular Locations of TB-RBP and Trax—Using confocal microscopy of N-terminal GFP-tagged TB-RBP in transfected NIH 3T3 cells, we detect the majority of TB-RBP in the cytoplasm with low levels in nuclei. No TB-RBP is seen in nucleoli, suggesting that TB-RBP is not directly involved with ribosomal RNA transscripton or transport. It has been reported that treatment of HeLa cells with mitomycin C or etoposide induces endogenous TB-RBP to move into nuclei (18). However, treatment of the transfected NIH 3T3 cells with doxorubicin, a compound that causes DNA double-strand breaks (at concentrations to 30 μM) does not alter the location of the TB-RBP fusion protein in NIH 3T3 cells (data not shown).

Trax has been proposed to have a bipartite nuclear localization signal in its N terminus that could facilitate the movement of TB-RBP/Translin into the nucleus (7). Western blot analyses of cytoplasmic and nuclear protein fractions from mouse testis and NIH 3T3 cells indicate that Trax is predominantly a cytoplasmic protein (Fig. 1D), although a low level of Trax is found in the nuclear fractions (Fig. 1D, lanes 2 and 4). Although we cannot exclude the possibility that Trax transiently enters and rapidly exits nuclei as part of a transport function, most of the “steady-state” levels of Trax protein in our Western blot assays appear to be cytoplasmic. Comparison of the Trax protein sequences of <i>S. pombe</i>, <i>Drosophila melanogaster</i>, <i>A. thaliana</i>, mouse, rat, and human indicate that, although Trax is a very conserved protein, the putative NLS sequence is situated in a region of the protein with lower sequence conservation.

We find that Trax fusion constructs either tagged with blue fluorescence protein at their C terminus or with red fluorescence protein at their N terminus localize predominantly to the cytoplasm of 3T3 cells (Fig. 9, C, E, and G). Although we find colocalization of GFP-TB-RBP and BFP Trax in the cytoplasm of doubly transfected cells (Fig. 9, D–F), we only detect substantial amounts of TB-RBP in the nucleoplasm of the cells. The Trax fusion protein localizes predominantly around the nuclei in the transfected cells, reminiscent of a Golgi/ER distribution. Staining the fixed cells with BODIPY 558/568 reveals a colocalization of Trax with the Golgi/ER (Fig. 9G). Moreover, Trax delocalizes from the Golgi into the cytoplasm following disruption of the Golgi with brefeldin A (Fig. 9H).

Although the retention of the Trax fusion protein in Golgi may be preventing Trax from utilizing its putative NLS for nuclear entry, following the disruption of the Golgi, we do not see any substantial increase of Trax in the nucleus. This may be due to rapid transport into and out of the nuclei, levels of nuclear Trax too low to be detected by these methodologies, or the presence of cytoplasmic retention sequences in Trax that override the NLS sequences (30). Subcellular fractionation of Translin and Trax in cerebellar extracts also suggests the two proteins are enriched in the cytoplasm (31).

Other cytoplasmic proteins such as the Fanconi anemia complementation group A gene product, FAA, also contain a putative bipartite NLS sequence (30). A hybrid protein containing the NLS of SV40 large T antigen and FAA also localizes in the cytoplasm of transfected human 293 cells, showing a specific cytoplasmic retention. The subcellular distribution of the <i>Drosophila</i> Cubitus interruptus protein, which mediates Hedgehog signaling, appears to be regulated by opposing bipartite NLS and cytoplasmic targeting/retention signals (32). Trax may also contain similar cytoplasmic retention sequences that override its putative NLS. From our confocal studies, Trax appears to predominantly localize to the Golgi/ER, although we cannot exclude low levels of Trax in the nuclei (Fig. 9G). Studies using Trax as bait in yeast two-hybrid assays have primarily detected Trax interactions with cytoplasmic and Golgi resident proteins.

The selective localization and movement of proteins between the nucleus and cytoplasm is often influenced by protein phosphorylation. A GFP fusion of MAPKAP kinase 2, which contains a bipartite NLS, exits the nuclei of transfected cells under stress following phosphorylation (33). Phosphorylation of the adenomatous polyposis coli protein is required for its nuclear import (34). We have found that recombinant Trax can be phosphorylated in testis germ cell nuclear extracts (unpublished data). Whether a phosphorylated (or nonphosphorylated) form of Trax localizes transiently in the nucleus, thereby helping TB-RBP to enter the nucleus as proposed by Aoki et al. (7), remains to be determined.

**TB-RBP Contains a Nuclear Export Signal**—Previous studies have indicated that TB-RBP exits nuclei of male germ cells toward the end of meiosis (10), is associated with various mRNAs in germ cell cytoplasmic extracts (11), and moves through the intercellular bridges in haploid spermatids (10). Movement of proteins from the nucleus to the cytoplasm utilizes specific nuclear export signal sequences. The HIV I Rev-like NES sequence binds directly to a nuclear export receptor CRM1/Exportin, which is involved in the export of various proteins such as MAPKK, PKI-alpha, FMRF, and p53 in a RanGTP-dependent manner (22). Various cellular RNA-binding proteins utilize other shuttling signals such as the M9 signal in hnRNP A1, KNS signal in hnRNP K, and HNS signal in HuR (23). Comparing TB-RBP with known NES sequences, we have found a highly conserved putative leucine-rich NES sequence at its C terminus (Fig. 10). We believe this is a functional sequence, because when we alter the NES of TB-RBP by site-directed mutagenesis, the GFP-TB-RBP fusion protein accumulates in the nuclei (Fig. 9F). This suggests that TB-RBP utilizes a Rev-like NES to move from the nucleus to the cytoplasm. We propose that TB-RBP has a role in mRNA binding and export in male germ cells and neuronal cells where it associates with a specific subset of mRNAs. In the cytoplasm, Trax interacts with TB-RBP producing a heterodimer with reduced affinity for RNA leading to the release of mRNAs in the cytoplasm.

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Trax Inhibits TB-RBP Binding to RNA
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