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Masking of the Translin/Trax complex by endogenous RNA

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Abstract Translin and Trax are components of an RNA binding complex initially detected in extracts of brain and testes. Although other tissues appear to contain much lower or negligible levels of the Translin/Trax gel-shift complex, we found, unexpectedly, that several of these peripheral tissues express Translin and Trax proteins at levels comparable to those present in brain. In this study, we demonstrate that the paradoxically low levels of the Translin/Trax complex in kidney and other peripheral tissues are due to masking of these sites by endogenous RNA. Thus, these findings indicate that the Translin/Trax complex is involved in RNA processing in a broader range of tissues than previously recognized.

Key words: RNA binding protein; Dendritic RNA; RNA localization; Cerebellum

1. Introduction

Translin and Trax have been identified as protein components of an RNA binding complex that was found to be highly enriched in brain and testes [1–6]. Although the functional role of this complex has not yet been clarified, it has been implicated in regulating cytoplasmic mRNA localization. In spermatocytes, its association with intercellular bridges has prompted the suggestion that it is involved in intercellular transport of mRNAs between spermatocytes [7]. In the brain, immunohistochemical studies have detected prominent Translin staining in neuronal dendrites [8,9], consistent with recent studies indicating that it plays a role in localization of dendritic RNAs [10].

Although recent studies have focused attention on the possible role of the Translin/Trax complex in mRNA targeting, it is important to keep in mind that Translin homomeric complexes or heteromeric Translin/Trax complexes also bind with high affinity to single-stranded DNA oligonucleotides in a sequence-specific fashion [4,5,11,12]. Accordingly, Translin and Trax have also been implicated in DNA repair or chromosomal translocations [11–15]. Thus, it is possible that the Translin/Trax complex is multi-functional and may bind to either RNA or single-stranded DNA species in vivo.

Since gel-shift studies indicated that the Translin/Trax complex is highly enriched in brain and testes compared to a wide range of other tissues [1,2,4,5], we had assumed that Translin and Trax proteins would display a similar, restricted pattern of expression. However, recent studies examining the tissue distribution of Trax mRNAs have questioned this assumption, since several tissues with low or negligible levels of the Translin/Trax gel-shift complex display Trax mRNA levels comparable to those found in brain [16,17]. In the present study, we have examined the tissue distributions of Translin and Trax proteins to see if they parallel that of the Translin/Trax gel-shift complex. Furthermore, since we detected a marked discrepancy in some tissues between the level of the Translin/Trax gel-shift complex and its component proteins, we have investigated the basis for this mismatch.

2. Materials and methods

2.1. Antibody generation and immunoblot analysis

The generation of rabbit polyclonal antibodies against the C-terminal peptides of either Translin or Trax has been described previously [8]. In this study, we also used N-terminal Trax antibodies prepared in guinea pigs and antibodies generated against full-length Translin fused with glutathione *S*-transferase (GST–Translin). To generate the guinea pig Trax antisera an N-terminal peptide of rat Trax (KMNGKEGPGGFRKRKHDN) was conjugated to thyroglobulin. In brief, the peptide (1 mg/ml) was incubated with thyroglobulin (20 mg/ml) in the presence of 0.1% glutaraldehyde for 1 h at room temperature. The coupling reaction was stopped by the addition of glycine (200 mM) and then dialyzed against phosphate-buffered saline overnight at 4°C. Immunoblot analysis of crude cerebellar homogenates (5 μg of protein) confirmed that the N-terminal Trax antiserum detects a single protein band that migrates at the expected molecular weight for Trax. Furthermore, preincubation of the antiserum with the N-terminal antigen peptide (10 μg/μl) abolished detection of this band.

To generate antibodies directed against the coding sequence of Translin, a full-length rat Translin cDNA insert was subcloned into the pGEX-2T (Pharmacia) glutathione *S*-transferase expression vector. Fusion protein purification, immunization, test bleeds, and production bleeds were performed as described previously [18].

For immunoblot analysis of Translin and Trax in tissue homogenates (5 μg protein), crude antisera were used at dilutions of 1:2000 (Translin) or 1:7000 (Trax). Horseradish peroxidase-conjugated anti-rabbit (Translin) or anti-guinea pig (Trax) antibodies were used at a 1:5000 dilution.

2.2. Gel-shift assays

Gel-shift assays were performed on crude tissue homogenates prepared as follows. Fresh or frozen tissues (cerebellum, kidney, lung, or heart) were homogenized in a solution containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 20% glycerol, 1.5 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 μg/ml leupeptin, 5 μg/ml aprotinin, and 20 mM NaF, incubated on ice for 15 min, and then centrifuged at 14 000 rpm in a Microfuge for 15 min. The concentration of protein in the supernatant was determined using the BCA protein assay (Pierce).

Gel-shift assays were performed with a single-stranded DNA oligonucleotide probe, referred to as GS1, using procedures described pre-

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viously [8]. Briefly, the GS1 oligonucleotide (5′-CTAGGAGCGG-GGGCGCTCATG-3′) was end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase (NEB) and purified with a Sephadex G-50 column. 5 μg of extract protein was incubated in 12 mM HEPES (pH 7.9), 4 mM Tris–HCl (pH 7.9), 50 mM KCl, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 12% glycerol, and 2 μg of poly(dI-dC) with 40 000 cpm of end-labeled GS1 probe in 30 μl for 15 min at room temperature. After this incubation, the reaction was loaded on a 4% polyacrylamide gel. After electrophoresis to separate free and bound probe, the gel was dried and exposed to Kodak XAR-5 film.

For 'supershift' experiments, 1 μl of the GST–Translin or rabbit Trax antisera were added 15 min after the addition of the probe. The binding reaction was then extended for an additional 15 min. For competition experiments, the tissue was preincubated with a 10× or 50× excess of cold GS1 oligonucleotide for 15 min before the addition of the end-labeled GS1 probe. For the RNase experiments, 10 μg of RNase A (Sigma) was added immediately prior to the addition of the radiolabeled GS1 probe.

In some experiments, equal amounts (5 μg protein) of cerebellar and kidney extracts were combined in the gel-shift reaction. When indicated, the kidney extract was boiled for 5 min, then cooled to room temperature before being added to the cerebellar extracts. Proteinase K (Life Technologies) (0.06 μg) or DNase I (Sigma) (0.1 μg) was incubated with the indicated tissue for 5 min at 37°C, then boiled for 5 min to inactivate the enzymes prior to use in the gel-shift assay.

2.3. Immunoprecipitation

Kidney extracts prepared identically to those used for gel-shift assays were first pre-cleared by incubating them with protein A-Sepharose beads (Pierce) for 1 h at 4°C. Trax antibody (5 μl/ml) that had been preincubated with protein A-Sepharose beads was added to the pre-cleared supernatant. After an additional 1 h incubation at 4°C, the beads were pelleted by centrifugation, washed extensively, and processed for immunoblot analysis with Translin or Trax antibodies.

3. Results

In previous studies, we and others have reported that the Translin/Trax gel-shift complex is highly enriched in brain and testes with low or negligible levels of binding activity in a variety of other tissues. Paradoxically, we found in an immunoblot survey of Translin and Trax protein expression that the pattern of expression of Translin and Trax proteins does not parallel that predicted by previous gel-shift studies. For example, similar levels of Translin and Trax protein are detected by immunoblotting in extracts of kidney, lung or cerebellum (Fig. 1A), even though the first two contain much lower levels of the Translin/Trax gel-shift complex labeled with the GS1 probe (Fig. 1B). In contrast, heart extracts have very low levels of both Translin and Trax proteins, as well as the Translin/Trax gel-shift complex.

To help resolve this discrepancy between the level of expression of Translin and Trax proteins and the corresponding gel-shift complex in several peripheral tissues, we focused our investigation on kidney. First, we considered the possibility that Translin and Trax do not form a complex in this tissue. However, we found that these proteins co-precipitate from kidney extracts, as observed previously in studies conducted on brain extracts (Fig. 2) [8].

Next, we assessed whether kidney extracts might contain a factor that inhibits the binding activity of the Translin/Trax complex. To this end, we examined the effect of kidney extracts on the prominent Translin/Trax gel-shift complex found in cerebellar extracts. We found that mixing kidney and cerebellar extracts produced a marked reduction in the intensity of the cerebellar Translin/Trax gel-shift band (Fig. 3A). Immunoblot analysis of these gel-shift samples confirmed that mixing the extracts does not cause degradation of either

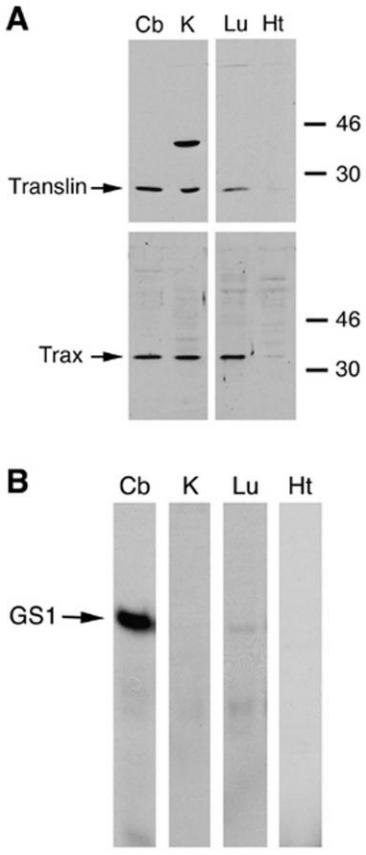

Fig. 1. Tissue distribution of Translin and Trax proteins and the Translin/Trax gel-shift complex. (A) Immunoblot analysis of Translin (top panels) and Trax (bottom panels) in cerebellum (Cb), kidney (K), lung (Lu) and heart (Ht) indicates that the first three tissues have comparable levels of these proteins, with much lower levels present in heart. Arrows at the left indicate the location of either the Translin or Trax bands. (B) Gel-shift assays of the Translin/Trax complex in these tissues with the GS1 probe detect a robust gel-shift band in cerebellar extracts with negligible binding activity in the other three tissues. The arrow labeled GS1 indicates the location of the Translin/Trax gel-shift complex.

Translin or Trax (data not shown), which suggests that kidney extracts contain a factor that blocks detection of the Translin/Trax gel-shift complex. To help determine whether this inhibitory factor might be a protein, we examined the effect of boiling the kidney extract prior to adding it to the cerebellar extract. However, we found that boiling did not diminish the inhibition (Fig. 3A). In fact, we found that boiling enhanced the inhibitory effect of kidney extracts, as boiled kidney extracts were approximately 5-fold more potent in suppressing the cerebellar Translin/Trax gel-shift band than control kidney extracts (data not shown).

Although the resistance of the inhibitory factor to boiling argues against it being a protein, it does not rule out the possibility that it is a heat stable protein or peptide. Accordingly, we examined the effect of preincubating kidney extracts with proteinase K which was then inactivated by boiling prior to mixing these treated kidney extracts with cerebellar extracts. However, the inhibitory factor present in kidney extracts was also unaffected by this protease treatment. To check that the absence of a gel-shift band was not merely due to incomplete inactivation of the proteinase K, we confirmed that addition of the heat-treated proteinase K, in the

absence of kidney extracts, did not diminish the intensity of the cerebellar gel-shift band. However, as expected, inclusion of proteinase K that had not been heat-inactivated in the gel-shift assay abolished the gel-shift band (Fig. 3A).

Since RNA and single-stranded DNA can bind to the Translin/Trax gel-shift complex with high affinity, we examined the possibility that endogenous RNA or single-stranded DNA species might be bound to the complex, masking detection of the gel-shift complex. As predicted by this hypothesis, we found that treatment of kidney extracts with RNase abolished their ability to suppress detection of the gel-shift complex in cerebellar extracts (Fig. 3B).

To check whether single-stranded DNA might also be involved in masking detection of the complex, we performed comparable experiments with DNase treatment of kidney extracts (Fig. 3B). However, since inclusion of DNase in the gel-shift assay would be expected to degrade the GS1 probe, we first heat-inactivated the DNase-treated kidney extracts by boiling prior to mixing it with the cerebellar extracts and the probe. Following this procedure, we found that DNase treatment did not mimic the ability of RNase to neutralize the inhibitory activity present in kidney extracts. To confirm that heat treatment did inactivate the DNase as expected, we checked that adding heat-treated DNase directly to the gel-shift assay of cerebellar extracts did not inhibit the intensity of the gel-shift band. Lastly, as a positive control to check that the DNase was able to degrade DNA, we confirmed that addition of DNase to the gel-shift reaction mixture abolished detection of the gel-shift complex by degrading the probe.

As these studies indicated that endogenous RNA present in kidney was able to mask the Translin/Trax complex present in cerebellum, we reasoned that a similar mechanism accounted for our inability to detect the Translin/Trax gel-shift complex

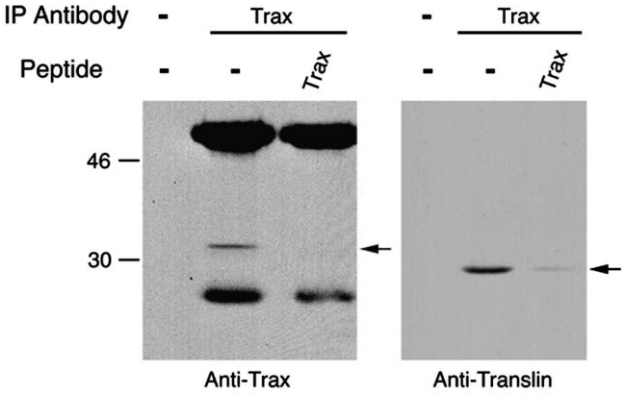

Fig. 2. Co-precipitation of Translin and Trax from kidney extracts. The immunoprecipitate obtained from kidney extracts with Trax antibodies was analyzed by immunoblotting with Trax (left panel) and Translin (right panel) antibodies. Arrows to the right of each panel indicate the expected location of either Trax (left panel) or Translin (right panel). Neither Trax nor Translin is detected in the immunoprecipitate when Trax antibody is omitted from the immunoprecipitation step. Furthermore, preincubation of the Trax antibody with the antigen peptide prior to the immunoprecipitation step blocks immunoprecipitation of both proteins. As the same guinea pig Trax antiserum was used for immunoprecipitation and immunoblotting, the prominent bands present in the two right lanes of the left panel reflect detection of the Trax IgG by the anti-guinea pig secondary antibody. Accordingly, these bands are not present in the Translin blot as it was processed with an anti-rabbit secondary antibody.

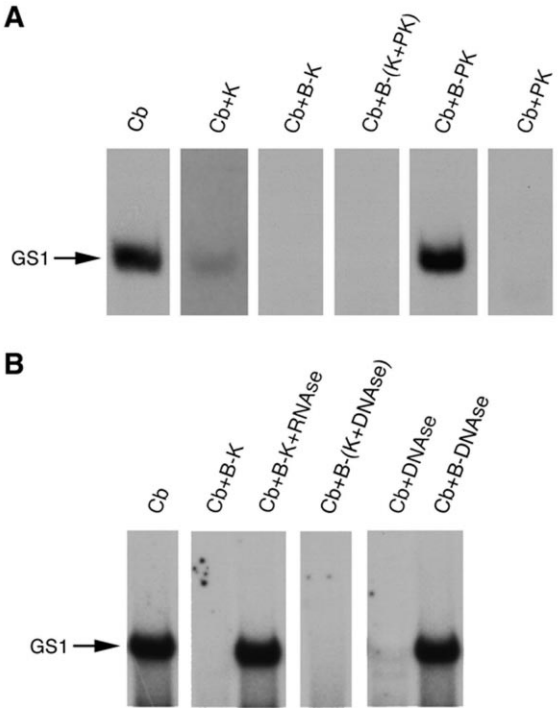

Fig. 3. Characterization of a factor present in kidney extracts that suppresses detection of the Translin/Trax gel-shift complex in cerebellar extracts. (A) As shown above, cerebellar extracts (Cb) display a robust GS1 gel-shift complex indicated by the arrow in the left margin. Mixing of equal amounts of cerebellum and kidney extracts (Cb+K) markedly suppresses the intensity of the gel-shift band. Boiling of the kidney extract prior to mixing it with cerebellar extracts (Cb+B-K) enhances the suppression of the GS1 band. Treatment of the kidney extract with proteinase K (PK) and then boiling this mixture to inactivate the proteinase K prior to mixing it with cerebellar extracts (Cb+B-(K+PK)) does not reverse the suppression. The remaining two lanes confirm that boiling of the proteinase K inactivates it, since it does not reduce the intensity of the GS1 band present in cerebellar extracts (Cb+B-PK). As expected, in the absence of boiling, proteinase K abolishes the GS1 complex (Cb+PK). (B) The first two lanes show the Translin/Trax gel-shift complex present in cerebellar extracts (Cb) and the ability of boiled kidney extract to suppress detection of this complex (Cb+B-K). The next two lanes illustrate that treatment of the boiled kidney extract with RNase reverses this inhibitory effect (Cb+B-K+RNase), while DNase treatment does not (Cb+B-(K+DNase)). In order to prevent degradation of the GS1 DNA oligonucleotide probe used in this gel-shift assay, the DNase-treated kidney extracts were boiled prior to mixing them with the cerebellar extracts. This precaution is necessary since, as expected, addition of DNase to the cerebellar extracts abolishes the GS1 gel-shift band (Cb+DNase). As shown in the last lane to the right, we confirmed that the boiling treatment used was sufficient to inactivate the DNase, as addition of boiled DNase to the cerebellar extracts does not reduce the intensity of the GS1 gel-shift band (Cb+B-DNase).

in kidney extracts. As predicted, we found that addition of RNase to kidney extracts revealed the presence of a robust gel-shift complex (Fig. 4). To insure that this effect of RNase was due to its enzymatic activity, we confirmed that pretreatment of the extracts with RNasin blocked the effect of RNase.

As these findings strongly supported the view that the inhibitory factor present in kidney extracts is composed of RNA, we wondered whether Translin/Trax complexes present in brain extracts were also partially masked by binding to endogenous RNA species. However, RNase had little effect on the intensity of the gel-shift band in cerebellum indicating

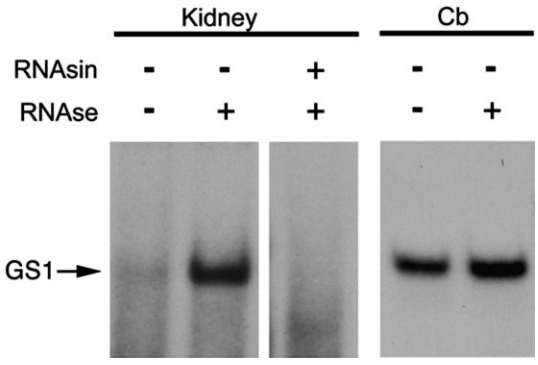

Fig. 4. RNase treatment unmasks the GS1 gel-shift complex in kidney. Treatment of kidney extracts with RNase reveals the presence of a GS1 gel-shift complex (indicated by arrow in the left margin) that co-migrates with that detected in cerebellar extracts. This effect of RNase is blocked by pre-treatment with RNasin. As shown in the panel at the right, RNase does not produce a comparable effect in cerebellar extracts.

that this is not the case (Fig. 4). Conversely, homogenization of cerebellum in the presence of RNase inhibitor did not produce a consistent decrease in the intensity of the gel-shift band (data not shown).

To check our assumption that the gel-shift band detected in kidney extracts after RNase treatment corresponds to the Translin/Trax complex, we performed 'supershift' studies with antibodies to these proteins. In previous studies conducted on cerebellar extracts, we found that antibodies to Translin or Trax were able to slow the migration of the complex (data not shown) [8]. We confirmed that the gel-shift complex induced by RNase treatment behaved in the same way (Fig. 5A). Furthermore, we found that the sensitivity of the kidney complex to inhibition by unlabeled GS1 oligonucleotide matched that displayed by the Translin/Trax complex present in cerebellar extracts (Fig. 5B) [4].

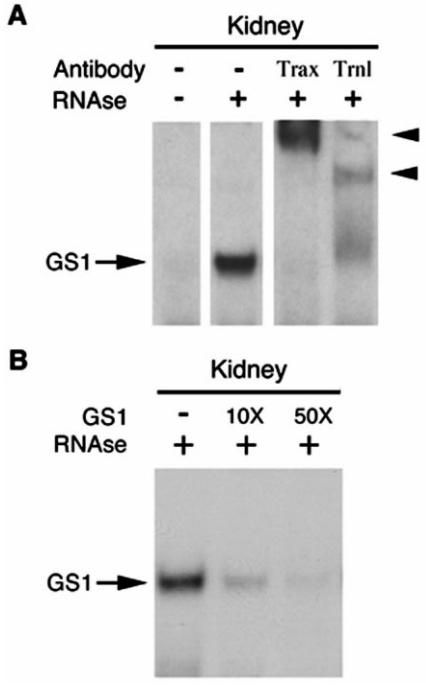

Fig. 5. Characterization of the GS1 gel-shift complex in kidney extracts. (A) Addition of antibodies to either Trax or Translin slows the migration of the GS1 complex. Arrowheads in the right margin indicate the location of the 'supershifted' gel-shift complexes. Incubation with the corresponding pre-immune antisera does not produce this effect (data not shown). (B) Competition studies with unlabeled GS1 oligonucleotide demonstrate that the Translin/Trax complex in kidney displays similar affinity to that found previously for the Translin/Trax complex in cerebellum. 10× and 50× indicate that the concentration of unlabeled GS1 oligonucleotide was 10-fold and 50-fold higher than that of the radiolabeled GS1 probe. Unlabeled oligonucleotide was incubated with kidney extracts for 15 min prior to adding the GS1 probe.

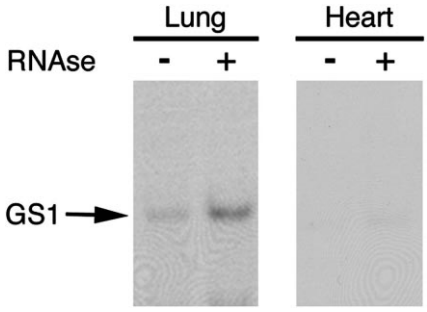

Fig. 6. Effect of RNase treatment on the GS1 gel-shift complex in lung and heart. As found for kidney extracts, RNase increases the intensity of the GS1 gel-shift band in lung (indicated by arrow). However, RNase treatment of heart extracts does not reveal the presence of a cryptic GS1 complex in this tissue.

As RNase treatment is able to unmask cryptic Translin/Trax complexes in kidney extracts, we also checked whether it has a similar effect in lung, another tissue in which there is a discrepancy between the level of the Translin/Trax gel-shift band and its component proteins (Fig. 1). As found in kidney, RNase treatment also increased the intensity of the Translin/Trax gel-shift band detected in lung extracts (Fig. 6). However, as expected, RNase treatment did not unmask the presence of Translin/Trax binding complexes in heart, which has negligible levels of both Translin and Trax proteins (Figs. 1 and 6).

4. Discussion

The goal of this study was to explain the marked discrepancy in the intensity of the Translin/Trax gel-shift bands detected in brain and kidney, even though these tissues contain comparable amounts of Translin and Trax proteins. We have found that endogenous RNA masks the complex in kidney, preventing it from being detected by exogenous radiolabeled probes. In contrast, Translin/Trax complexes present in cerebellar extracts are not masked by endogenous RNAs. Conceivably, this reflects greater susceptibility of brain RNAs to endogenous RNase activity. Alternatively, it is possible that kidney is enriched in RNA species that have high affinity for the Translin/Trax complex. We favor the former alternative since we have found that RNAs isolated from cerebellum or kidney under denaturing conditions have comparable potencies in inhibiting binding of radiolabeled GS1 probe to the Translin/Trax complex (P.M.F., unpublished observations).

In vitro studies have demonstrated that the Translin/Trax complex can bind with high affinity to either RNA or single-stranded DNA species. Thus, it is possible that one of these nucleic acid species, or perhaps both, are targeted by the Translin/Trax complex in vivo. Our demonstration that the

Translin/Trax complex present in several tissues is masked by binding to endogenous RNA supports the view that the complex binds to RNA in vivo. However, it is conceivable that 'masking' of the complex by endogenous RNA occurs during homogenization of the tissue. Nevertheless, we favor the former alternative, since we were unable to detect binding of radiolabeled GS1 probe to the Translin/Trax complex, even when the GS1 probe was included in the homogenization buffer (P.M.F., unpublished observations).

Although some of the proteins implicated in dendritic RNA processing appear to be expressed selectively in neurons, such as Nova-1 [19,20] and the neuronal Hu proteins [21], many others are widely expressed RNA binding proteins that have been adapted to this purpose in neurons, such as Staufen [22–24]. The broad tissue distribution of Translin and Trax proteins indicates that these proteins fall into the latter class. To date most studies have focused on the role of this complex in RNA processing in brain and testes. However, our results support the view that its role in other tissues warrants study as well. Expanding the scope of these studies may provide important clues to understanding the regulation and function of this complex in RNA processing in general and also shed light on its role in specialized situations such as dendritic RNA processing.

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