The TAR RNA-binding Protein, TRBP, Stimulates the Expression of TAR-containing RNAs in Vitro and in Vivo Independently of Its Ability to Inhibit the dsRNA-dependent Kinase PKR*

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TRBP (HIV-1 transactivating response (TAR) RNA-binding protein) and PKR, the interferon-induced dsRNA-regulated protein kinase, contain two dsRNA binding domains. They both bind to HIV-1 TAR RNAs through different sites. Binding to dsRNA activates PKR that phosphorylates the eukaryotic initiation factor eIF-2α leading to protein synthesis inhibition. TRBP and PKR can heterodimerize, which inhibits the kinase function of PKR and has a positive effect on HIV-1 expression. In this study, an in vitro reticulocyte assay revealed the poor expression of TAR containing CAT RNAs compared with CAT RNAs. Addition of TRBP restored translation efficiency of TAR-CAT RNA and decreased the phosphorylation status of eIF-2α, confirming its role as a PKR inhibitor. Unexpectedly, eIF-2α was phosphorylated in the presence of TAR-CAT as well as CAT RNA devoid of the TAR structure. TRBP inhibited eIF-2α phosphorylation in both cases, suggesting that it restores the translation of TAR-CAT RNA independently and in addition to its ability to inhibit PKR. TRBP activity on gene expression was then analyzed in a PKR-free environment using PKR-deficient murine embryo fibroblasts. In a transient reporter gene assay, TRBP stimulated the expression of a TAR-containing luciferase 3.5-fold whereas the reporter gene with mutated TAR structures or devoid of TAR was stimulated 1.5- to 2.4-fold. Overall, the activity of TRBP2 was higher when the 5’-end of the mRNA was structured and was mediated independently by each dsRBD in TRBP. Increasing concentrations of TRBP showed no significant modification of the luciferase RNA levels, suggesting that TRBP stimulates translation of TAR-containing RNAs. Therefore, TRBP is an important cellular factor for efficient translation of dsRNA containing transcripts, both by inhibiting PKR and in a PKR-independent pathway.

TRBP1 (the HIV-1 transactivating response (TAR) RNA-binding protein) is a cellular protein, which was initially isolated from a HeLa cell expression library using TAR RNA as a probe (1). Two cDNAs have been isolated and have been named TRBP1 (originally TRBP) and TRBP2. They are identical but for an alternative first exon that arises from the use of a second internal promoter in the first intron of TRBP1. TRBP2 is the longer isoform and has 21 additional amino acids at its N terminus (2–4). TRBP1 and TRBP2 each contain two double-stranded RNA binding domains (dsRBD), although dsRBD1 binds RNA with low affinity whereas dsRBD2 binds TAR with high affinity because of the presence of a KR helix motif (5). TRP Bs, its dsRBD2, and a 24-amino acid TRBP peptide corresponding to the KR helix motif bind to two different sites of the highly ordered RNA structures of TAR. One site of high affinity is located between the bulge and the loop, and a second site of low affinity is located within the stem structure (1, 5–7). The dsRBDs have also been found in several other proteins (8, 9).

The interferon-induced, dsRNA-regulated protein kinase, PKR, a cellular serine/threonine kinase activated upon binding to dsRNA, induces inhibition of protein synthesis by phosphorylating the α subunit of eukaryotic initiation factor 2 (eIF-2α). Once phosphorylated, eIF-2α prevents translation initiation (10, 11). As a dsRNA-binding protein, PKR, also binds TAR RNA, which leads to its activation as a kinase (12). The PKR/TAR interaction can be displaced by the addition of a 60-amino acid synthetic peptide corresponding to residues of the first dsRBD present in PKR (13). The integrity of the stem structure of TAR is required for its interaction with PKR. TAR mutants whose structure is disrupted are no longer able to bind to and activate PKR (14).

TRBP has been reported to behave as an inhibitor of PKR. By cotransfection analysis, TRBP was found to enhance the translational efficiency of DHFR mRNAs and to restore vaccinia protein synthesis in E3L-vaccinia mutant-infected cells (15). Yeast two-hybrid assays as well as Far-Western techniques showed heterodimerization between PKR and TRBP and conveyed the idea that PKR and TRBP need dsRNA as a bridge for their mutual interaction (16). The ability of TRBP to inhibit PKR activity was confirmed by overexpression of TRBP cloned...
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into the nef gene of the HIV-1 genome. This allowed the virus to escape the PKR-mediated inhibition of virus replication. In addition, this work demonstrated that PKR and TRBP can heterodimerize in the absence of dsRNA (17). Furthermore, recent data have shown that TRBP reverses the PKR-induced HIV-1 LTR inhibition of expression (18, 19). The binding sites between TRBP and PKR have been mapped in each dsRB of TRBP and are independent of the KR helix motif that mediates major RNA binding activity in TRBP (19). TRBP function has been mainly studied in the context of viral infection. These activities include its binding to HIV-1 TAR RNA and PKR inhibition and activation of HIV LTR expression. Recent data indicate a direct correlation between a targeted disruption of the tarbp2 gene and the regulation of its translation and plays a role in spermatogenesis (20, 21). Mice that carry a targeted disruption of the tarbp2 gene have a growth defect, are sterile, and severely oligospermic. Xlrpbpa, the Xenopus homologue of TRBP, is associated with ribosomes and hnRNPs (22). These data point to a stimulatory role of TRBP on translation by a direct activation through its RNA binding properties and/or through its inhibitory effect on PKR.

Here, we analyzed the effects of TRBP on the in vitro translation of CAT RNAs with or without the HIV-1 TAR and/or through its inhibitory effect on PKR. PRPB, the murine homologue of TRBP, binds the 3'-UTR of Prm1 protamine RNA, regulates its translation and plays a physiological role in spermatogenesis (20, 21). Mice that carry a targeted disruption of the tarbp2 gene have a growth defect, are sterile, and severely oligospermic. Xlrpbpa, the Xenopus homologue of TRBP, is associated with ribosomes and hnRNPs (22). These data point to a stimulatory role of TRBP on translation by a direct activation through its RNA binding properties and/or through its inhibitory effect on PKR.

**MATERIALS AND METHODS**

**Antibodies**—Rabbit polyclonal antibodies directed against a synthetic 13-residue phosphorylated rat eIF2-α peptide were obtained from Research Genetics (Huntsville, AL). Mouse polyclonal antibodies directed against eIF2-α were a gift of C. Proud (University of Dundee). Anti-mouse and anti-rabbit polyclonal antibodies conjugated to horse-radish peroxidase were obtained from Amersham Biosciences.

**Cell Cultures**—PKR-deficient murine embryo fibroblasts (MEFs) were provided by B. R. G. Williams (23). They were cultured in Glutamax-1 Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 5 μg of penicillin-streptomycin per ml and containing 10% fetal calf serum.

**Plasmids**—The different TAR constructs (wild type and mutants) that have been previously described (1). These pUC18-T7-TAR-CAT plasmids contain the CMV promoter (700 bp), T7 RNA polymerase binding site (20 bp), TAR (80 bp), and the chloramphenicol acetyltransferase (CAT) gene (HindIII-BamHI) inserted of 1600 bp. The plasmid T7-CAT was constructed by removing the CMV, T7, and TAR sequences (NdeI-HindIII) and inserting the T7-promoter as an oligonucleotide (5'-TACCATGCTAATAGTATAGC-3' and 5'-GCGAGTCTTACGCGTGCTAGCTA-3') in NdeI-HindIII sites of the same plasmid. The plasmid pG2-TAR-Luc and its derivatives were constructed as follows: an 800-bp region containing the CMV and the T7 promoters followed by TAR were excised from the pUC18-T7-TAR-CAT plasmids by HindIII digestion and inserted into the pG2 basic vector at the HindIII site upstream of the luciferase coding sequence. The coding sequence was cut by HindIII and inserted in pG2 basic vector. All constructs were confirmed by sequence analysis. The pMAL-TRBP2 plasmid in which TRBP2 was fused in-frame with the maltose binding protein (MBP) has been described previously (7). TRBP2 cDNA (3) was inserted in the pcDNA1/AMP vector at the BamHI site to make pcDNA1-TRBP2. The pcDNA1 expressing TR-A, TR-B, and TR-C have been described previously (19). The pcDNA3-TR-AB was constructed by amplification of the AB part from pBS-TRBP2 by PCR and insertion into pcDNA3 BamHI-XbaI sites.

**In Vitro Transcription and Translation**—In vitro transcription, the pUC18-T7-CAT or pUC18-T7-TAR-CAT plasmids were linearized with BamHI. Transcription was performed at 37 °C for 2 h in a total volume of 25 μl containing 5 μg of linearized DNA and 10 units of T7 RNA polymerase according to the manufacturer’s protocol (Promega). The DNA template was removed by DNase treatment for 20 min at 37 °C (1 unit of DNase/μg of DNA), and the transcribed RNA was purified by phenol extraction and resuspended in diethyl pyrocarbonate-treated distilled water. The integrity of all RNAs was assessed by electrophoresis on a 1% agarose gel and visualized by fluorography.

The concentration of the RNA preparations was estimated by spectrophotometry using known amounts of DNA as standards (Smart Ladder, Eurogentech). 25 ng of the in vitro transcribed RNAs were translated at 30 °C for 45 min as previously described (24), unless otherwise indicated. 5-μl aliquots were analyzed by SDS, 12.5% PAGE.

**Fusion Protein Production and Purification**—Plasmid DNA of the empty pMAL-c vector (New England Biolabs) and pMAL-TRBP constructs were transformed in Escherichia coli DH5α. An overnight culture from a single colony was diluted 1:100 (v/v) and grown to an OD600 of 0.4. After 4 h of induction in 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG), the bacteria were harvested and resuspended in cold sonication buffer A (20 mM Tris-HCl, pH 7.6, 200 mM NaCl, 1 mM EDTA, and 100 μg/ml lysozyme containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 100 μg/ml leupeptin, 10 μg/ml pepstatin). The suspension was sonicated and clarified by centrifugation at 10,000 × g for 30 min at 4 °C. The fusion proteins were purified to near homogeneity by amyllose column chromatography as described by the manufacturer (New England Biolabs). Protein concentration was determined using the BioRad dye reagent with bovine serum albumin as a standard. For each protein preparation, increasing concentrations (50–500 nM) of MBP or MBP-TRBP proteins were added in a preliminary in vitro translation assay, to determine the best concentrations to use.

**Immunoblotting**—The reticulocyte lysates were electrophoresed with an equal amount of lysis buffer (20 mM Tris-HCl, pH 7.6, 50 mM KCl, 400 mM NaCl, 1 mM EDTA, 1% Triton X-100, 5 mM 2-mercaptoethanol, 0.005% aprotinin, 0.2 mM phenylmethylsulfonyl fluoride, 20% glycerol) supplemented with phosphatase inhibitors (5 mM sodium fluoride, 10 mM paraoxonase/phyosphatase, 1 mM sodium orthovandate, and 10 mM β-glycerophosphate). After 5 min of incubation on ice, the samples were diluted twice with 2× protein electrophoresis buffer, and the proteins were separated by SDS, 12.5% PAGE. The proteins were transferred to immobilon polyvinyldene difluoride membranes (Millipore), and the membranes were processed for immunoblotting and for incubation with the primary and secondary antibodies as described previously (17).

**Reporter Assay by Microtransfection**—The PKR-deficient MEFs (23) were seeded at 20,000 cells per well in 96-well plates. They were transfected, by a calcium phosphate precipitation-glycerol shock technique as previously described (18, 25), with the same amounts of plasmids by addition of the empty vector pcDNA1/AMP.

**Preparation of Total RNA and RT-PCR**—PKR-deficient MEFs (23) cultured in 10-cm Petri dishes (seeded at 2 × 105 cells per dish) were transfected by a calcium phosphate precipitation-glycerol shock technique with 10 μg of plasmid pG2 CMV-T7-TAR-Luc in the absence or in the presence of increasing amounts of pcDNA1-TRBP2. Total RNA was extracted 24 h post-transfection with TriZol isolation reagent (Invitrogen) and treated by DNaseI (G4000 units of Superscript II (Invitrogen). Incubation was performed at 37 °C for 1 min, followed by a 5-min incubation at 58 °C. RNA was extracted on ice with Trizol isolation reagent (Invitrogen). In order to retain the integrity of each RNA sample, 150 ng of each RNA was transcribed in vitro with 3′-UTR SV40 antisense (5′-GGAGGAGTAAGGTGTTGGAGTAC-3′) and GAPDH antisense-specific primer (5′-CCAAATGTTGCTACGGATGACC-3′) in a 25-μl reaction containing 30 units of RNasin (Amerham Biosciences), 1 μl dNTP, 10 μM dithiothreitol and 10 μM β-mercaptoethanol. The reaction mixture was incubated at 42 °C for 1 h and 5 μl of the resulting reaction containing each strand cDNA template were used for PCR amplification. Conditions for luciferase and GAPDH amplifications were 95 °C for 5 min, 20 cycles of 95 °C for 1 min, 58 °C for 1 min, and 72 °C for 2 min, followed by a 5-min incubation at 72 °C. PCRs amplifications were performed in a 100-μl reaction volume containing 2 μl of each cDNA, 0.25 μl Taq Polymerase (Invtrogen), 1.5 μM MgCl2, 0.2 mM dNTP, and 1× Taq buffer (Invitrogen). The products were fractionated on a 1.5% agarose gel. The lucif-
FIG. 1. The presence or absence of different TAR structures in the 5'-UTR, determine the efficiency of translation of the CAT transcripts. A, secondary structure and thermodynamic stability of the different 5'-UTR RNAs used in the in vitro translation assay. The 5'-UTR of the transcripts including the wild type TAR sequence (TAR) and different TAR mutants previously described (1) were folded up to the AUG start codon using the Mfold software version 3.1 (bioinfo.math.rpi.edu/mfold/rna/form1.cgi). The most stable folded structures are represented, and their calculated free energy is \( \Delta G = -54.7 \text{ (TAR-CAT)} \) and \( -55.2 \text{ (L135)}, -50.1 \text{ (BL234)}, -40.7 \text{ (S3-11)}, -34.2 \text{ (S3-17)} \) and \(-16.9 \text{ (CAT)} \) kcal/mol. The dots in the BL234, B123, and the L135 structures indicate that the structure that follows is identical to TAR CAT. The base changes in the TAR mutants L135, B123, and BL234 are shown in bold letters. B, ethidium bromide staining of in vitro transcribed TAR- and
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FIG. 2. Purification of MBP and MBP-TRBP after expression in E. coli. A. Coomassie Blue-stained gel of total proteins prepared either without induction (lane 1), or after IPTG induction of E. coli cultures transformed with the control plasmid pMAL (lane 2) or with the recombinant plasmid pMAL-TRBP (lane 3). B. Coomassie Blue-stained gel of purified MBP proteins. Lane 2, MBP; lane 3, MBP-TRBP. Apparent molecular weights are indicated on the right.

Fig. 3. TRBP increases the translation efficiency of TAR-CAT RNA. The CAT (upper panel) or the TAR-CAT (lower panel) RNAs were translated in the rabbit reticulocyte lysate in the absence of additional protein (lane 1) or in the presence of 50 nM (lanes 2 and 6), 250 nM (lanes 3 and 7), 1000 nM (lanes 4 and 8), and 5000 nM (lanes 5 and 9) of MBP (lanes 2–5) or MBP-TRBP (lanes 6–9).

Fig. 4. Translation kinetics of CAT and TAR-CAT RNAs in the presence of TRBP. The yield of CAT protein translated either from the CAT (upper panel) or the TAR-CAT (lower panel) transcript was monitored after 0 (lanes 1 and 5), 5 (lanes 2 and 6), 15 (lanes 3 and 7), and 30 (lanes 4 and 8) min of incubation in the presence of 250 nM of MBP (lanes 1–4) or MBP-TRBP (lanes 5–8).

RESULTS

Alteration of the HIV-1 TAR Structure Affects the Efficiency of Translation of TAR-CAT Transcripts—We have first analyzed whether the structure of the TAR element could affect the translation of a downstream CAT reporter RNA. Different CAT RNA transcripts either with or without a TAR structure at their 5′-end were used for in vitro transcription. These TAR RNA structures were previously described (1) and are either wild type TAR (TAR) or TAR mutated in the loop (TAR L135), in the bulge (TAR B123), between the bulge and loop (TAR B123L), or deleted from nucleotides 3 to 11 (S TAR). TAR RNA structures were previously described (1) and are either wild type TAR (TAR) or TAR mutated in the loop (TAR L135), in the bulge (TAR B123), between the bulge and loop (TAR B123L), or deleted from nucleotides 3 to 11 (S TAR). TAR and TAR mutants L135, B123, and the synthesized products were analyzed by SDS-PAGE (Fig. 1C). The in vitro synthesis of the CAT protein markedly decreased when translation was performed with TAR CAT transcript, and with the TAR B123 CAT and TAR L135 CAT RNAs (Fig. 1C, lanes 2, 3, and 5, respectively) compared with its synthesis from the CAT RNA (lane 1). In contrast, translation of TAR BL234 CAT (lane 4) was more efficient than the translation of the other TAR CAT constructs and translation products from TAR SΔ3–11 CAT and TAR SΔ3–17 CAT RNAs (lanes 6 and 7) had equal intensity as that from CAT RNA. Overall, the translation efficiency of each RNA is in good agreement with its calculated free energy (Fig. 1). Therefore, the in vitro translation data indicate that the presence of the TAR dsRNA structure at the 5′-end of mRNAs severely affects their translation efficiency. In contrast, attenuation or disruption of this structure leading to a decreased thermodynamic stability increases translation of the corresponding RNAs.

TRBP Stimulates the Translation of TAR CAT Transcript—TRBP was initially isolated through its ability to bind to TAR RNA (1). We have analyzed the effect of TRBP on the translation of CAT or TAR-CAT RNAs in reticulocyte lysates. MBP, MBP-TRBP fusion protein (5) were produced and purified in E. coli (Fig. 2). These proteins were added in translation assays performed with CAT and TAR-CAT used as RNA representatives of efficiently or poorly translated mRNAs, respectively.

As previously, translation of CAT was much more efficient than translation of TAR-CAT RNA (Fig. 3, lane 1). Increasing concentrations of the MBP-TRBP protein (50–5000 nM) progressively enhanced the efficiency of translation of the TAR-CAT RNA (Fig. 3, lanes 6–8, bottom) with the best efficiency

2 M. Zuker, personal communication.

TAR-mutant-CAT RNAs. Each of the RNA expressing CAT either alone or preceded by one of the TAR sequence shown above was transcribed in vitro by the T7 RNA polymerase after linearization of each corresponding template plasmid (pUC) with BamHI. Each RNA migrates as a 0.9-kb band in a 1% agarose gel. An inverted image of the fluorogram is presented. C. Translation of the TAR and mutant-CAT transcripts. The CAT or TAR-CAT RNAs were translated in vitro in rabbit reticulocyte lysates. Lane 1, CAT; lane 2, TAR CAT; lane 3, TAR B123 CAT; lane 4, TAR BL234 CAT; lane 5, TAR L135 CAT; lane 6, TAR ΔS3–11 CAT; lane 7, TAR ΔS3–17 CAT. In the absence of RNA, no synthesized product was detected (data not shown). Apparent molecular weight is indicated on the right.
and performed in the presence of 250 nM MBP (results, the efficiency of translation of TAR-CAT RNA was markedly after 15 min and with the highest amount of product translation was identical to CAT translation, increasing lanes 5 compare mRNAs of species and tissues, including rabbit reticulocytes. In the presence of dsRNA, PKR is activated and phosphorylates its substrate eIF-2α. A concentration as low as 0.02 μg/ml of poly(rI)-poly(rC) is sufficient to activate the endogenous PKR from rabbit reticulocytes and to induce eIF-2α phosphorylation (29) and Fig. 5B, lane 12. We therefore expected to observe the phosphorylated form of eIF-2α in the presence of TAR-CAT RNA but not in the presence of CAT RNA. Kinetic experiments were performed to explore the phosphorylation state of eIF-2α in a translation assay. After the indicated incubation times, the lysates were analyzed for eIF-2α phosphorylation by immunoblot using antibodies directed against an eIF-2α peptide phosphorylated at serine 51 and for total amounts of eIF-2α by using a mouse monoclonal antibody directed against the full eIF-2 complex. Incubation of the lysates in the presence of TAR-CAT RNA (Fig. 5A, middle panel, lanes 9–14) resulted in eIF-2α phosphorylation, as expected from the dsRNA structure of TAR-CAT RNA that activates PKR. Surprisingly, eIF-2α was also phosphorylated in the presence of CAT RNA with the same kinetics (Fig. 5A, middle, lanes 2–7). However, as already shown in Fig. 1C, 3 and 4, translation of CAT RNA was much more efficient than translation of TAR-CAT RNA (Fig. 5A, upper panel). We can therefore conclude that both RNAs activate PKR and that the phosphorylation of eIF-2α is not sufficient to explain the translation difference between CAT and TAR-CAT RNAs.
Stimulation of TAR-CAT Translation by TRBP Cannot Be Solely Attributed to PKR Inactivation—We next explored whether the ability of TRBP to increase the translation efficiency of TAR-CAT RNA could be attributed to its ability to inhibit PKR activity and eIF-2α phosphorylation. Because TRBP has been shown to block PKR autophosphorylation (17), we hypothesized that TRBP would prevent PKR phosphorylation, which in turn will result in the non-phosphorylation of its substrate eIF-2α. Therefore, the ultimate effect of increased TRBP would be a decrease in eIF-2α phosphorylation and an increase in translational initiation. The CAT and TAR-CAT RNAs were incubated for 30 min either in the absence or in the presence of two different concentrations of MBP or MBP-TRBP (Fig. 5B). As in Fig. 5A (middle panel, lanes 6 and 13), at 30 min after the onset of incubation, incubation of the lysates in the presence of CAT or TAR-CAT RNAs (Fig. 5B, lanes 2 and 7) resulted in eIF-2α phosphorylation. In addition, incubation of both types of lysates containing CAT RNA (lanes 2–6) or TAR-CAT RNA (lanes 7–11) resulted in a strong inhibition of eIF-2α phosphorylation with MBP-TRBP (250 nm, lanes 5 and 10, and at 500 nm, lanes 6 and 11), whereas no inhibition was seen when extracts were incubated in the presence of MBP (lanes 3, 4, 8, and 9). The difference in eIF-2α phosphorylation was only due to the addition of TRBP as the levels of endogenous eIF-2α were similar in all samples (Fig. 5B, lower panel).

Therefore, the ability of TRBP to behave as a strong inhibitor for PKR, is confirmed in the presence of rabbit endogenous PKR. Because TRBP inhibits eIF-2α phosphorylation in the presence of both RNAs but only increases TAR CAT translation, we concluded that the ability of TRBP to restore the translation of the TAR-CAT RNAs is at least in part, independent from its ability to inhibit PKR phosphorylation.

TRBP Stimulates Expression of TAR-containing Transcripts in PKR-deficient MEFs—TRBP has been reported to act as an inhibitor of PKR in several assays (17–19) suggesting a major function in cellular response to infection. Another function of TRBP in normal cells, is to bind dsRNA segments of mRNAs to form ribonucleoproteins with highly ordered structures (7, 20).

**Fig. 6.** TRBP stimulates the luciferase expression of TAR-containing transcripts in PKR-deficient MEFs. A, TRBP2 stimulates expression of TAR-containing plasmids to a higher extent than 5′-unstructured RNAs. 100 ng of plasmids pGL2 expressing TAR-luciferase (TAR), TAR-BL234-luciferase (BL234), TAR-S3–17-luciferase (S3–17), or luciferase alone (ΔTAR) under the control of the CMV-T7 promoter were transfected in PKR-deficient MEFs in the absence or in the presence of increasing concentrations of pcDNA1-TRBP2 (10–300 ng). B, each dsRBD in TRBP2 stimulates the expression of TAR-containing plasmids. 100 ng of plasmid pGL2 expressing TAR-luciferase under the control of the CMV promoter were transfected in PKR-deficient MEFs in the absence or in the presence of 10–300 ng of pcDNA1-TRBP2, pcDNA3-TR-AB, TR-A, TR-B, or TR-C (19). Each DNA sample was adjusted to the same final DNA content with the empty pcDNA1/Amp vector. The luciferase activity per microgram of protein (Luc Index) was calculated as the means of the transfection in four different wells. In Fig. 6, the data shown are expressed as fold stimulation of luciferase index and are the means of three independent experiments (± S.E.).
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Therefore, it is likely that TRBP can stimulate protein synthesis through two different mechanisms, one being by heterodimerization with and inhibition of PKR, the other by favoring access of highly structured RNAs to the ribosomal and translational machinery. If this hypothesis is correct, one would expect TRBP to stimulate translation of TAR-containing RNAs in the absence of PKR. To test this, we compared the effect of TRBP on a luciferase reporter gene expressed under the control of a CMV promoter and containing or not various TAR structures at its 5'-end. Indeed, the effect of TRBP2 was analyzed on some of the TAR constructs described in Fig. 1, chosen to contain authentic (TAR), partially disrupted (BL234), extensively disrupted (SΔ3–17) or no (ΔTAR) TAR structure (Figs. 1A and 6A). Analysis was performed by transient transfection in PKR-deficient MEFs (23) with increasing amounts of pcDNA1-TRBP2. The results show that TRBP2 stimulates the reporter gene expression in all cases, albeit with different efficiencies. The highest stimulation was observed with TAR (3.8-fold), which was decreased with BL234 (2.4-fold), SΔ3–17 (1.5-fold), and ΔTAR (2.3-fold). The TAR BL234 mutant has lost its high affinity binding site for TRBP but has retained its dsRNA stem structure (containing low affinity binding sites for TRBP) whereas the TAR SΔ3–17 and ΔTAR have completely lost the TAR structure (Fig. 1A). Therefore, these data demonstrate that TRBP exerts a direct stimulation on the expression of TAR-containing RNAs in the absence of PKR. This stimulation is directly correlated to the calculated free energy of each 5'-UTR RNA. Hence, TRBP has a higher activity if the 5'-UTR is more stable except for a mild activity in the absence of TAR. Because TAR, TAR BL234, TAR SΔ3–17, and ΔTAR are translated with increasing efficiencies (Fig. 1C), we can conclude that TRBP is more active when the translation of a given RNA is more difficult to achieve. In all cases, the stimulation was dependent on the concentration of the plasmid encoding TRBP and decreased with high-concentrations of TRBP as previously observed in other cells (2, 19).

We then assessed if a specific domain in TRBP mediates the increased expression of TAR-containing RNAs. TRBP has been previously divided into fragment A (TR-A), which contains dsRBD1, fragment B (TR-B), which contains dsRBD2, and fragment C (TR-C), which contains a basic region in its C-terminal end but has no RNA or PKR-binding properties. TR-AB has both dsRBDs but not the C-terminal domain. The dsRBD1 binds TAR RNA with low affinity whereas dsRBD2 binds with high affinity (5). We cotransfected PKR/o MEFs cells with the CMV-TAR-Luc plasmid and pcDNA3 vectors expressing either TRBP2 or fragments TR-AB, TR-A, TR-B, and TR-C (Fig. 6B). In all cases, we observed an increase of the luciferase expression except with TR-C that was inhibitory or had no effect. We therefore concluded that each dsRBD will likely mediate PKR independent activation of TAR containing transcripts with AB and A having the highest activity.

**TRBP Directly Increases Translation Rather Than RNA Stability**—The increase of the expression of TAR-containing RNAs in PKR/o MEFs cells (Fig. 6) and in *in vitro* translation suggests that TRBP increases luciferase expression by enhancing, either mRNA stability and/or translation but not transcription efficiency. To determine which hypothesis is correct, PKR-deficient MEFs were transfected with the CMV-TAR-Luc and the TRBP2 plasmids as in Fig. 6. Semiquantitative RT-PCR assays were performed in parallel with luciferase measurements (Fig. 7). Whereas the luciferase activity increased with the first two TRBP concentrations (lanes 2 and 3), the level of luciferase mRNA remained unchanged, and even slightly decreased at high concentrations of TRBP. In all points, the ratio between the luciferase and the endogenous GAPDH mRNAs remained constant. These results show that the TRBP-mediated increase in luciferase activity cannot be ascribed to an enhanced level of the corresponding mRNA transcripts. We therefore concluded that the enhanced luciferase expression of TAR-containing RNAs by TRBP in the absence of PKR is due to a direct activity on translation.

**DISCUSSION**

Secondary structure of the 5'-UTR RNAs as well as cellular proteins influence the rate of translation of a given transcript (30). In this study, by using a reticulocyte lysate-based system of translation, we have shown that the secondary structure of TAR RNA affects the translation efficiency of the corresponding transcripts. RNA transcripts, starting with an authentic TAR or with TAR in which the dsRNA stem structure is retained (B123, L135), were poorly translated when compared with transcripts without TAR. In contrast, TAR mutants, in which the dsRNA structure was disrupted (SΔ3–11, SΔ3–17), were translated as the controls without TAR structure (Fig. 1, A and C). A partial restoration of translation was observed with a mutant with a different TAR upper stem-loop structure (BL234). These data reinforce the role of the TAR element as a translational inhibitor and point out the importance of the dsRNA structure of transcripts in the efficiency of translation. It has been previously reported that the secondary structure of HIV-1 TAR affects translation efficiency by preventing the accessibility of the cap structure (31), by activating the PKR-mediated phosphorylation of the initiation factor eIF-2α (12, 32) or by both mechanisms (33, 34). Our results indicate that the TAR secondary structure is a significant impediment to translational initiation.

The ability of TAR-CAT transcripts to induce eIF-2α phos-
phorylation (Fig. 5) was expected as a result of the activation of the reticulocyte endogenous PKR by the dsRNA TAR structure (12, 32). Therefore, it was surprising to observe that the CAT transcript, that has no TAR structure, was similarly capable of inducing eIF-2α phosphorylation to a level comparable to that achieved with TAR-CAT RNA and with similar kinetics (Fig. 5). The phosphorylation of eIF-2α may occur from activation of other eIF-2α kinases such as the hemin-controlled repressor HRI (35). Indeed, a measurable low level of phosphorylated eIF-2α can be observed after incubation during 30 min of the control lysates incubated in the absence of RNA transcript (Fig. 5B, lane 1). It is also possible that the in vitro transcript preparations contain traces of dsRNA (36). Indeed, very low amounts of dsRNA are known to activate PKR in reticulocyte lysates, as seen by the control experiment carried out with poly(I)-poly(C) (Fig. 5B, lane 12). Hence, since TAR-CAT and CAT transcripts were prepared under similar conditions, this potential dsRNA contamination cannot account for the difference in the translational behavior of TAR and CAT transcripts. Another explanation is that long RNAs most often have double-stranded regions in their structure, which might be sufficient to activate PKR. Indeed, folding of the first 500 nt of CAT and TAR-CAT RNAs on the Mfold server showed 18 and 20 alternative putative structures and the most stable of each have several double-stranded regions besides TAR when present (data not shown). These multiple structures probably can activate PKR and, as a consequence, eIF-2α will become phosphorylated. Whatever the case, our results indicate that translation of CAT RNA is not impaired by the accumulation of phosphorylated eIF-2α and that the difference in translation efficiency between the two RNAs must have a different origin.

The stable structure of TAR RNA located at the 5′-end of the transcript is the most plausible explanation to a decreased translation. The translational initiation complex may be more difficult to assemble with this structure rather than with a less stable structure and will require the activity of additional proteins to increase the recruitment of ribosome subunits and translational factors. The direct relationship between the RNA stability of the 5′-UTRs and the decrease in translation supports this hypothesis (Fig. 1).

The TAR dsRNA structures are present at the 5′-end of all HIV-1 mRNAs and with PKR. In the latter case, TRBP has been shown to directly bind PKR in the absence of dsRNA and to reverse the inhibitory effect of PKR on HIV replication (17). In agreement with that, we have recently identified TRBP as a partner for PKR in the screening of a human cDNA library with PKR using the yeast two-hybrid assay.3 TRBP reverses the inhibitory effect of PKR on HIV-1 replication (17) and on HIV-1 LTR expression in a reporter assay (18). TRBP can also reverse the PKR-mediated control of yeast growth (19). The ability of TRBP to inhibit PKR is believed to occur by antagonizing the translational block imposed by PKR, but no direct evidence has been demonstrated in an in vitro translation assay. Our initial aim was to study the effect of TRBP on the translation of TAR-containing RNA, in the context of dsRNA-mediated PKR activation. TRBP increased specifically translation of TAR-CAT RNA but not the translation of CAT RNA, which proves its ability to antagonize a translational block. In this context, incubation of the reticulocyte lysate with TAR-CAT or CAT RNAs led to similar increase in eIF-2α phosphorylation, suggesting identical activation of PKR (Fig. 5A).

Furthermore, a purified preparation of TRBP blocked the phosphorylation of eIF-2α to the same extent, whether it was triggered by TAR-CAT or CAT messengers (Fig. 5B). Finally, TRBP increased specifically the translation efficiency of the TAR-CAT RNA but did not modify translation of CAT transcripts (Figs. 3 and 4). Taken together, these results strongly suggest that TRBP increases TAR-CAT translation through a mechanism independent from PKR inhibition.

To verify this hypothesis, the activity of TRBP was assayed in a PKR-free context. We performed reporter gene assays in PKR-deficient MEFs (23), to demonstrate that TRBP has a direct stimulatory effect on the expression of the TAR-containing RNAs (Fig. 6). We measured the expression of CMV-Luc plasmids containing the authentic TAR, TAR mutated in the upper stem-loop region (BL234), TAR deleted in the bottom stem (S33–17) or in the absence of TAR (ATAR). These TAR sequences are representative of the most stable, relatively stable and weakly stable 5′ RNA structure, respectively (Fig. 1).

A better stimulation effect of TRBP on TAR-Luc plasmid, followed by the BL234 mutant, by the S33–17 mutant suggests that the TRBP-mediated stimulation requires the dsRNA structure of the RNA. The small increase in the absence of TAR remains unexplained and may be due to specific RNA structure not seen in the folding or to a low TRBP activity on all RNAs. Because luciferase assays represent all expression steps between transcription and translation, we analyzed whether the mRNA level was affected. An absence of variation of the Luc mRNA in an RT-PCR assay showed that neither the transcription rate, nor the stability was affected by TRBP. The remaining and most likely explanation is a direct activity of TRBP on translation. This effect can be due to either an unfolding of the TAR structure that increases its accessibility to ribosomes or to a direct activity on the translational machinery. A destabilization of the TAR RNA structure has been previously observed with TRBP peptides that bind the RNA (6) but the similar activity of both dsRBDs (TR-AB, TR-A, and TR-B in Fig. 6) suggests that only low affinity binding is necessary to mediate this function. Therefore, the overall effect of the protein may be a destabilization of the stable RNA structure to allow efficient translation. These results demonstrate that the ability of TRBP to stimulate TAR RNA containing structures is not solely mediated by its ability to inhibit the translational block imposed by PKR, but also by a PKR-independent activity on translation.

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