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TAR RNA-binding protein is an inhibitor of the interferon-induced protein kinase PKR

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ABSTRACT **A cDNA encoding a double-stranded-RNA (dsRNA)-binding protein was isolated by screening a HeLa cell cDNA expression library for proteins that bind the HIV-1 Rev-responsive-element RNA. The cDNA encoded a protein that was identical to TRBP, the previously reported cellular protein that binds the transactivation response element (TAR) RNA of human immunodeficiency virus type 1. TRBP inhibited phosphorylation of the interferon-induced ribosome-associated protein kinase PKR and of the eukaryotic translation initiation factor eIF-2α in a transient-expression system in which the translation of a reporter gene was inhibited by the localized activation of PKR. TRBP expression in HeLa cells complemented the growth and protein-synthesis defect of a vaccinia virus mutant lacking the expression of the dsRNA-binding protein E3L. These results implicate TRBP as a cellular regulatory protein that binds RNAs containing specific secondary structure(s) to mediate the inhibition of PKR activation and stimulate translation in a localized manner.**

Global regulation of eukaryotic mRNA translation is mediated by protein phosphorylation events, most notably at the translation initiation step, that involve eukaryotic initiation factor 2 (eIF-2). eIF-2 is a heterotrimer composed of three nonidentical subunits, α (36 kDa), β (38 kDa), and γ (52 kDa), that forms a complex with the initiator Met-tRNA and GTP and binds to the 40S ribosomal subunit to form a 43S species. After mRNA binding, the 60S ribosomal subunit associates with the complex to generate the 80S species concomitant with the hydrolysis of GTP to GDP and release of GDP-bound eIF-2α. Phosphorylation of the eIF-2α subunit prevents the GTP exchange and eIF-2 recycling and arrests translation (1, 2). A ribosome-associated protein kinase, PKR, phosphorylates eIF-2α upon activation by double-stranded (ds) RNA (3–6). PKR is induced by interferon treatment (7–9) and mediates the antiviral and antiproliferative effects of interferon (10, 11) and modulates cellular differentiation (12) and stress response (10). Although some viral RNAs and proteins that regulate PKR activation have been identified (8, 13–17), there is a paucity of information regarding cellular regulators of PKR. A 15-kDa protein, dRF, induces reversible inhibition of PKR autophosphorylation in undifferentiated cells by preventing dsRNA binding (18). PKR activation was inhibited in v-*ras*-transformed cells by a thermolabile, diffusible factor that was not bound by dsRNA (19). A 58-kDa cellular protein that inhibits eIF-2α phosphorylation by activated PKR has been identified in influenza virus-infected cells (20). Poliovirus infection induces either a cellular protease or a proteolytic activator that degrades the endogenous PKR (21).

We have characterized a dsRNA-binding protein that was isolated from HeLa cells on the basis of selective reactivity toward the Rev-responsive element (RRE) RNA of human immunodeficiency virus type 1 (HIV-1). The candidate protein, RBF, was identical to the previously reported (22) HIV-1 TAR (transactivation response) RNA-binding protein TRBP. TRBP was a potent inhibitor of dsRNA-mediated activation of PKR. TRBP also complemented the replication-defective phenotype of vaccinia virus deleted for the expression of the dsRNA-binding protein E3L. These findings suggest that TRBP represents a class of cellular RNA-binding proteins that may antagonize interferon-mediated translational control mechanisms.

MATERIALS AND METHODS

Expression Plasmids for Studying the Effects of TRBP on PKR Activation in Cultured Cells. The expression vectors pMTVA− and pD61 used to monitor localized activation of PKR have been described (15). Plasmids expressing the vaccinia virus E3L protein or the wild-type eIF-2α coding region were constructed by inserting an *Eco*RI-linkered PCR-amplified 570-bp E3L coding fragment of the vaccinia genome or a 1.6-kb human cDNA containing the eIF-2α coding region at the single *Eco*RI site in pMTVA−, respectively. In the pD61-2αwt expression plasmid, the eIF-2α coding region had been cloned into pD61 (15). The TRBP insert from the *Escherichia coli* expression plasmid was engineered to introduce a AUG start codon and upstream Kozak box and *Sal* I restriction site and remove the 28-residue LacZ tag by M13 mutagenesis (Bio-Rad). The mutagenized TRBP insert was cloned as a *Sal* I–*Xba* I insert downstream of the Rous sarcoma virus (RSV) long terminal repeat (LTR) promoter in the eukaryotic expression vector RSV.5. For negative control, TRBP was cloned in an antisense polarity downstream of the cytomegalovirus immediate early promoter.

DNA Transfection and Analysis of Expression. COS-1 monkey kidney cells were transfected by a DEAE-dextran procedure (23). Forty-two hours later, the cells were labeled with [^{35}S]methionine (100 μCi/ml; >1000 Ci/mmol; Amersham; 1 Ci = 37 GBq) for 30 min in methionine-free minimal essential medium. Phosphorylation was measured after a 4-hr labeling with [^{32}P]phosphate (200 μCi/ml) labeling at 42 hr posttrans-

Abbreviations: CEF, chicken embryo fibroblast; DHFR, dihydrofolate reductase; dsRNA, double-stranded RNA; eIF-2, eukaryotic initiation factor; HIV, human immunodeficiency virus; LTR, long terminal repeat; RRE, Rev-responsive element; RSV, Rous sarcoma virus; TAR, transactivation response element; TRBP, TAR RNA-binding protein.

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fection. Cell extracts were prepared by lysis in Nonidet P-40 lysis buffer and analyzed by SDS/PAGE after immunoprecipitation (15). Steady-state levels of eIF-2α were measured in cell extracts by SDS/PAGE under reducing conditions and electroblotting to nitrocellulose. Filters were incubated with rabbit anti-eIF-2α antibody and developed by chemiluminescence (Amersham). RNA filter blotting of total RNA with ^{32}P-labeled dihydrofolate reductase (DHFR), chicken β-actin, or TRBP cDNA hybridization probes was as described (15).

Vaccinia Virus Complementation. The E3L gene of vaccinia virus (Copenhagen strain) was mutagenized by homologous recombination and insertion of a bacterial β-galactosidase gene (J.T., unpublished data). E3L deletion mutant (ΔE3L, also known as vp1080) was plaque purified and propagated on chicken embryo fibroblasts (CEFs). Complementation experiments were done in HeLa cells by a transfection/infection protocol. Subconfluent monolayers in 60-mm dishes were transfected for 8 hr with 20 μg of plasmid DNA by the calcium phosphate procedure. Forty-eight hours later, the cells were infected with the wild-type or the ΔE3L virus at a multiplicity of infection of 30 in 0.25 ml of Dulbecco's modified Eagle's medium at 37°C for 1 hr. After removal of the inoculum, the cells were rinsed five times and incubated for 6 hr for protein labeling and 24 hr for virus production assays. Virus production was assayed by plaque titration of cell-associated virus on CEFs.

RESULTS

TRBP Inhibits dsRNA Activation of PKR in Cultured Cells. Two recombinant plasmids expressing HeLa cell proteins that bound the HIV-1 RRE RNA were isolated by affinity screening of a cDNA expression library. A common open reading frame of 344 aa in these cDNAs was identical to TRBP (22). TRBP displayed affinities for a broad range of natural and synthetic RNAs, including HIV RRE and TAR RNAs, with a 12-bp G·C-rich dsRNA constituting the minimal binding motif (data not shown). TRBP shared significant sequence homology with several cellular and viral RNA-binding proteins, including the dsRNA-binding domain near the N terminus (aa 10–77) of dsRNA-dependent PKR (24), the C-terminal 67 aa (aa 118–184) of the vaccinia virus dsRNA-binding E3L protein (25), the *Xenopus* dsRNA-binding protein X lrbpa, and the *Drosophila* Staufen protein, confirming a recent report (26).

TRBP had no homology with any of the 11 kinase domains of PKR or other ATP- or GTP-dependent protein kinases. Purified TRBP had no measurable phosphorylation activity with or without dsRNAs (data not shown). Interferon-inducible PKR is normally activated by low amounts of viral dsRNA or poly(I)·poly(C) to phosphorylate itself (3, 27, 28). Autophosphorylation results in a conformational change in the enzyme, enabling it to phosphorylate other substrates such as histones and eIF-2α, but not other molecules of PKR (2, 3, 29, 30). Excess amounts of dsRNA irreversibly inactivate PKR (3, 5, 31). TRBP inhibited dsRNA-induced phosphorylation of PKR *in vitro*, and this inhibition was reversed by the addition of excess dsRNA (data not shown).

To inquire whether exogenous TRBP can modulate PKR activation in living cells, we employed a transient expression system (15) that exploits the ability of the plasmid pD61 to express an mRNA which elicits localized PKR activation during transient transfection of COS-1 cells, resulting in increased eIF-2α phosphorylation and specific inefficient translation of the DHFR reporter gene cloned into that plasmid. Cotransfection of pD61 with a vector that expresses a gene product inhibiting PKR activation, such as adenovirus VAI RNA (32), vaccinia virus E3L protein (33), or reovirus σ3 polypeptide (34), increases the translational efficiency of the DHFR mRNA derived from pD61.

To monitor the effect of TRBP expression in this system, the RSV.5 vector, or the vector encoding TRBP, pRSV-TRBP, was cotransfected with pD61. As controls, pD61 was cotransfected with either p2α-wt, which encoded the wild-type eIF-2α and had no effect on PKR activation (35), or pE3L, which encoded a potent inhibitor of PKR activation. After 42 hr, cells were metabolically labeled with [^{35}S]methionine, and cell extracts were prepared for SDS/PAGE. RNA was prepared from plates that were transfected in parallel and evaluated by Northern blot analysis. Cotransfection of pD61 with p2α-wt resulted in detectable levels of DHFR protein synthesis in the total cell extract (Fig. 1*A*, lane 2, arrow) and significant levels of DHFR mRNA (Fig. 1*B*, lane 1). Cotransfection of pD61 with pE3L increased the DHFR protein expression 3-fold (Fig. 1*A*, lane 3) above that observed in cells cotransfected with pD61 and pMTVA− vector (Fig. 1*A*, lane 1), although similar levels of DHFR mRNA were expressed (Fig. 1*B*, lanes 1 and 2). Since the vectors p2α-wt and pE3L contained DHFR cDNA sequence within the 3′ end of the transcript, these plasmid-derived mRNAs were also detected by hybridization to a DHFR probe (15). The ≈25-kDa E3L protein was readily detected in the total protein lysates of these cotransfectants (Fig. 1*A*, lane 3, dot). A smaller species, of ≈20 kDa (Fig. 1*A*, lane 3, dot) represents an internally initiated (at the 38th codon) translation product (15, 33). While the relative abundance of the two E3L species was variable, both of them bound RNA (H.-W.C. and B.L.J., unpublished data). Incidentally, another viral dsRNA-binding protein, the reovirus σ3 protein, is also expressed as two species, a full-length 40-kDa protein and an N-terminally truncated form that is initiated at a methionine codon at position 38 (K. L. Denzler and B.L.J., unpublished data).

Cotransfection with control RSV.5 vector did not result in increased DHFR synthesis from pD61 (Fig. 1*A*, lane 4), whereas cotransfection with RSV-TRBP increased DHFR protein synthesis 10-fold (Fig. 1*A*, lane 5). An additional negative control, which employed a cytomegalovirus promoter-linked TRBP antisense plasmid, also failed to enhance DHFR expression (data not shown). The ≈43-kDa TRBP was not readily visualized in the crude lysates of pRSV-TRBP cotransfectants (Fig. 1*A*, lane 5), presumably because it was masked by the abundant actin band. However, TRBP expression in these cells was quite obvious when the electrophoresed lysates were immunoblotted with anti-TRBP antibodies (Fig. 1*A*, lane 10) and was correlated with the presence of TRBP mRNA detected by RNA filter hybridization (Fig. 1*B*, lane 4). After normalization to actin mRNA, a portion of the increased DHFR synthesis in the RSV-TRBP transfectants was estimated to be due to the 3-fold increase in the DHFR mRNA level (Fig. 1*B*, lane 4). Therefore, expression of TRBP enhanced the translational efficiency of DHFR mRNA 3-fold, similar to the levels observed in the E3L transfectants.

Effects of TRBP on eIF-2α Phosphorylation. The inhibitory effects of TRBP on the local activation of PKR were also evaluated by measuring the translation ability and the phosphorylation status of eIF-2α. For this purpose, the eIF-2α expression vector, pD61-2αwt, was cotransfected with RSV-TRBP. eIF-2α expression from this vector allows the evaluation of the overall level of synthesis and the phosphorylation state of eIF-2α by measurement of [^{35}S]methionine and [^{32}P]phosphate incorporation into the protein. The 2αwt mRNA transcribed from this plasmid elicits PKR activation, resulting in the repressed synthesis of the plasmid-coded eIF2α that gets hyperphosphorylated. pD61-2αwt was separately cotransfected with the control vectors pMTVA− or RSV.5, as well as the expression plasmids pE3L or RSV-

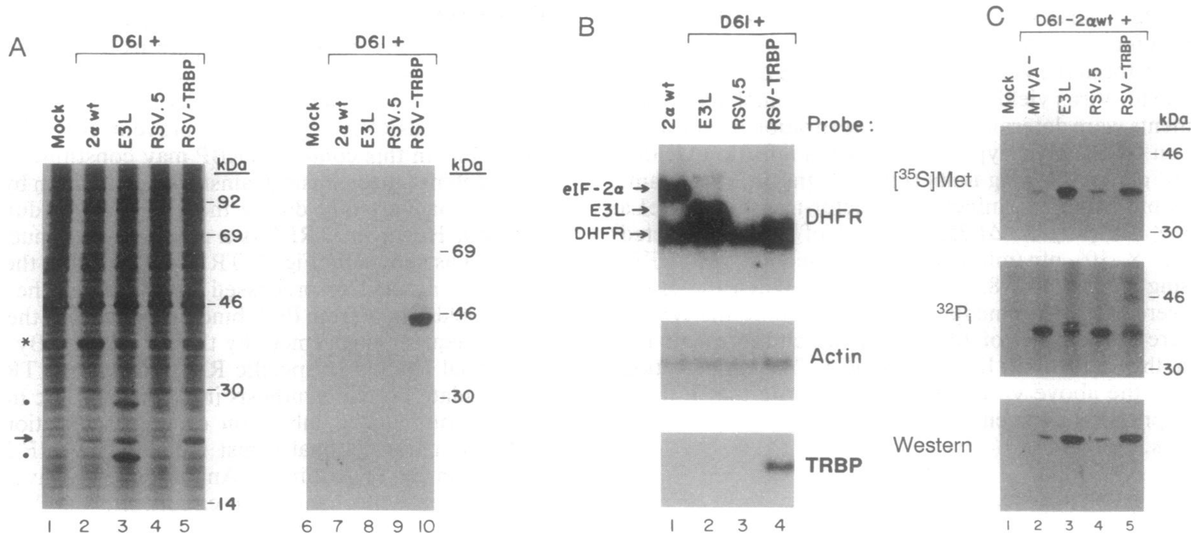

FIG. 1. TRBP expression enhances translation of plasmid-derived mRNA. COS-1 cells were cotransfected with equal amounts (8 μg) of pD61 and the following plasmid DNAs: pMTVA− (15); p2αwt (15); pE3L (33); pRSV.5; and pRSV-TRBP. (*A*) After 42 hr the cells were labeled with [35S]methionine and harvested for SDS/PAGE and fluorography with EN3HANCE (NEN). Electrophoretic migration of E3L (dots), DHFR (arrow), and eIF-2α (asterisk) is indicated. Aliquots of cell extracts were analyzed by SDS/PAGE (reducing conditions) and immunoblotting using polyclonal anti-TRBP antiserum raised in rabbits (lanes 6–10). Labels above the lanes refer to the respective transfections. (*B*) From parallel duplicate plates, RNA was harvested for Northern blot hybridization to a radiolabeled DHFR probe, which was mixed with the 2αwt probe, the actin probe, or the TRBP probe as indicated. (*C*) Phosphorylation of eIF-2α in cultured cells is inhibited by TRBP. The eIF-2α expression vector (D61-2αwt) was cotransfected with either pMTVA−, pE3L, RSV.5, or RSV-TRBP. After 42 hr the cells were incubated for 30 min with [35S]methionine and extracts were prepared for immunoprecipitation with anti-human eIF-2α antibody and analysis by SDS/PAGE (*Top*). Cells from parallel dishes were incubated with [32P]phosphate for 4 hr and extracts were prepared for immunoprecipitation and SDS/PAGE (*Middle*). Samples were also analyzed by Western immunoblotting using anti-human eIF-2α antibody (*Bottom*).

TRBP. After 42 hr, cells were labeled with [35S]methionine for 30 min to measure protein synthesis and duplicate plates were labeled for 4 hr with [32P]phosphate to measure phosphorylation of eIF-2α. Equivalent amounts of 35S- and 32P-labeled cell extracts were immunoprecipitated with a rabbit polyclonal antibody directed against eIF-2α and were analyzed by SDS/PAGE. eIF-2α was expressed at very low levels when pD61-2αwt was cotransfected with pMTVA− or RSV.5 (Fig. 1*C Top*, lanes 2 and 4) or the cytomegalovirus promoter-linked TRBP antisense plasmid (data not shown). Coexpression of TRBP increased eIF-2α synthesis 7-fold (Fig. 1*C Top*, lane 2 vs. lane 5). The TRBP-induced increase in the eIF-2α synthesis from the pD61 vector was comparable to that observed with E3L coexpression (Fig. 1*C Top*, lane 3). eIF-2α phosphorylation was monitored by comparing the steady-state level of eIF-2α determined by Western blot analysis with the amount of 32P incorporation. Analysis of 32P-labeled immunoprecipitates (Fig. 1*C Middle*) showed that in presence of E3L, phosphorylation of eIF-2α was reduced by a factor of 2 compared with the control level (lane 3 vs. lane 2), but when corrected for the increased steady-state level of eIF-2α (Fig. 1*C Bottom*, lane 3), the percentage of phosphorylated eIF-2α actually decreased by a factor of 46. With a similar correction for the steady-state level (Fig. 1*C Bottom*, lane 5), TRBP expression (Fig. 1*C Middle*, lane 5) decreased the percentage of phosphorylated eIF-2α by a factor of 20. This experiment shows that TRBP is an inhibitor of eIF-2α phosphorylation in living cells.

TRBP Complements the Replication-Defective Phenotype of Vaccinia Virus E3L Mutant. Since TRBP was nearly as efficient as the vaccinia virus E3L protein in inhibiting PKR-mediated phosphorylation of eIF-2α, we inquired whether TRBP would functionally replace the E3L gene product in ΔE3L, a vaccinia virus mutant that lacked the E3L gene (Fig. 2). ΔE3L replicates poorly in HeLa cells but well in CEFs. ΔE3L-infected HeLa cells were blocked for both viral and host cell protein synthesis (Fig. 2). However, when HeLa cells were transfected with an E3L expression plasmid 48 hr prior to infection with the ΔE3L mutant, the profile of the labeled proteins resembled that of wild-type infection (Fig. 2, lane 5). The 110-kDa protein seen in the ΔE3L infections represents the β-galactosidase expressed from the corresponding gene insertion at the E3L locus of the vaccinia virus genome. Transfection with the vector alone was with-

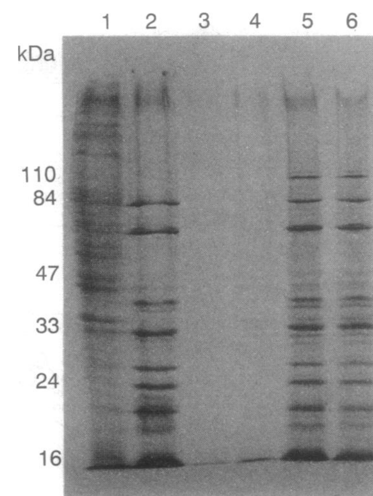

FIG. 2. TRBP coexpression restores the viral protein synthesis of a replication-defective vaccinia virus E3L mutant. Subconfluent HeLa cell monolayers were transfected by the calcium phosphate method with no DNA (lanes 1–3), vector plasmid pMT2VA− (lane 4), E3L expression plasmid pMT-E3L (lane 5), or TRBP expression plasmid pRSV-TRBP (lane 6). At 48 hr after DNA transfection, the cultures either were mock infected (lane 1) or were infected with the wild-type vaccinia virus (lane 2) or the E3L deletion mutant ΔE3L (lanes 3–6) at a multiplicity of infection of 30. Six hours after infection, the cells were incubated for 1 hr with [35S]methionine (200 μCi/ml) and detergent-treated lysates were prepared. The labeled proteins were resolved by SDS/PAGE and visualized by autoradiography.

out any effect (lane 3). Under similar conditions of transfection and infection, RSV LTR-linked TRBP expression plasmid also restored the viral protein synthesis in ΔE3L-infected cells (lane 6). Virus yields from the various complementation experiments were determined by plaque titration of HeLa cell lysates on CEFs. Wild-type vaccinia virus infection yielded 6.2×10^8 plaque-forming units (pfu)/ml; the ΔE3L mutant, 1.0×10^5 pfu/ml; ΔE3L infection of vector-transfected HeLa cells, 8×10^5 pfu/ml; ΔE3L infection of E3L-transfected cells, 1.5×10^8 pfu/ml; and ΔE3L infection of TRBP-expressing HeLa cells, 8.3×10^7 pfu/ml. When the crude stocks were assayed immediately after infection, the typical titers were of the order of 10^5 pfu/ml, which represents the virus in the inoculum. If we assume a 20% transfection efficiency, the above virus yields demonstrate that E3L or TRBP expression compensated for the ΔE3L defect of vaccinia virus.

DISCUSSION

We have shown that TRBP is an efficient competitive inhibitor of dsRNA-dependent PKR activation in cultured cells. TRBP reversed the translational inhibition of the reporter DHFR transcript by localized activation of PKR and stimulated the expression of eIF-2-α from the same reporter plasmid. Although phosphorylation of eIF-2α represents the major control of global protein synthesis at the initiation step (1), differential mRNA affinities for limiting amounts of eIF-2α can result in a gene-specific translation control when eIF-2α phosphorylation is enhanced. The best example of this type of control is that of GCN4 in yeast (37). During amino acid starvation of yeast, activation of GCN2 by uncharged tRNAs results in eIF-2α phosphorylation such that GCN4 is translated at the expense of upstream open reading frames (38). The yeast GCN2 mechanism is distinct from the global control of mammalian protein synthesis by the dsRNA-activated kinase PKR. Induction of PKR synthesis primes the translational apparatus for repressive control by PKR activation by dsRNA structures in the mRNAs. Cytoplasmic expression of proteins such as TRBP can sequester the dsRNA regions and prevent generalized PKR activation. In this context, TRBP may constitute an endogenous defense mechanism against PKR activation by dsRNA released inadvertently during the cell cycle or during viral infection. However, TRBP is a predominantly nuclear protein, and as shown by Fig. 3, TRBP may bind to the dsRNA regions of nascent or processed transcripts in the nucleus, and "shield" them from PKR binding throughout their transit and subsequent engagement by the polysomes. By virtue of differential binding to specific RNA structures, TRBP may thus regulate protein synthesis in a gene-specific manner.

Interferon-induced inhibition of viral replication results partly from translational arrest mediated by eIF-2α phosphorylation by PKR kinase. Animal viruses have evolved several defense mechanisms to circumvent the PKR activation pathway. They include expression of virus-coded competitive inhibitors of PKR activation, such as the reovirus σ3 (16, 34) and vaccinia virus E3L (14) proteins and small RNA inhibitors of PKR, such as the adenovirus VAI RNA (8, 17), and virus-coded eIF-2α homologs, such as the vaccinia virus K3L protein (15, 33). TRBP overexpression complemented the ΔE3L vaccinia virus mutant. Loss of E3L function debilitates vaccinia virus in some but not all cell lines, suggesting that cellular proteins such as TRBP may be abundant in certain cell types or be easily recruited by the vaccinia virus as the E3L surrogate.

Translational mechanisms also regulate cell growth. Overexpression of the mRNA cap-binding protein eIF4E induced malignant transformation (39). The antiproliferative activity of interferons is probably mediated by PKR activation. Expression of mutant forms of PKR led to oncogenic transformation of NIH 3T3 mouse fibroblasts (36, 40) resulting

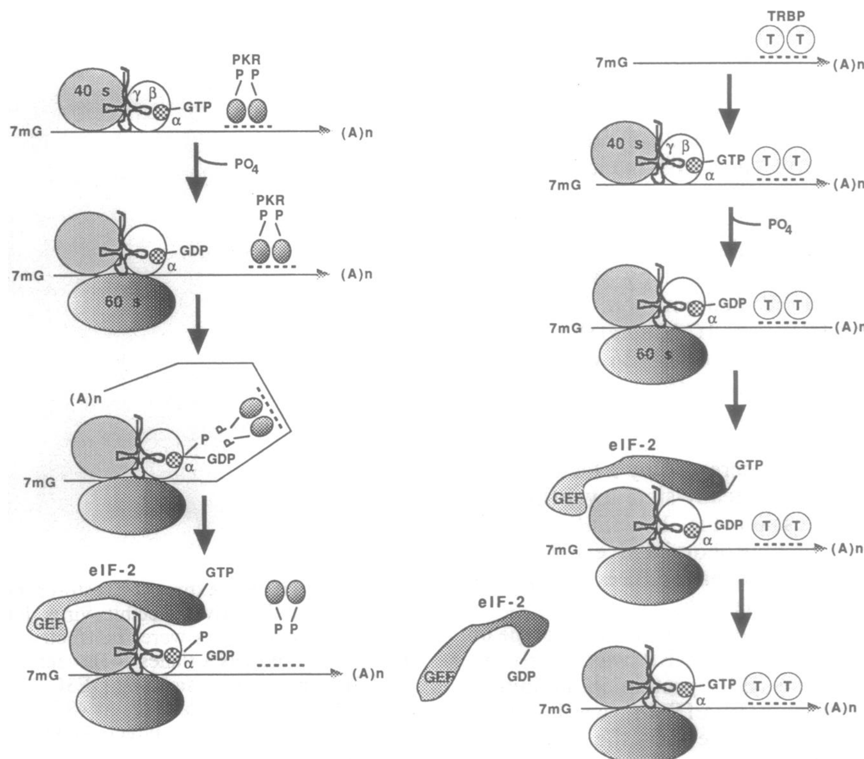

FIG. 3. Model depicting possible gene-specific translation control by TRBP. dsRNA regions in the mRNA (shown as an interrupted line) elicit activation of polysome-associated PKR, which phosphorylates the α subunit of eIF-2 in the initiation complex and thereby prevents GDP/GTP exchange with the guanine nucleotide-exchange factor (GEF). RNA-binding proteins such as TRBP that recognize specific dsRNA structures can insulate these mRNAs against translation repression due to localized activation of PKR.

from the unregulated function of eIF-2α. A similar phenomenon can be envisioned for proteins such as TRBP if they inhibit PKR in the global context. But cellular dsRNA-binding proteins can also induce profound physiological changes by acting locally. For instance, the mRNAs for specific gene products such as growth factors, protooncogenes, and tyrosine kinases such as c-Sis (41), c-Myc (42), and Lck (43) are translated poorly and have extensive secondary structures at their 5′ ends that may elicit localized PKR activation and translational repression. Proteins such as TRBP that may bind different subsets of dsRNAs and relieve the translational block of selected oncogenes present novel mechanisms of viral and cellular regulation. The dsRNA-binding proteins that regulate the PKR activation may act in concert with the RNA helicases such as eIF4E and eIF4F (44, 45) that unwind the 5′ end of the mRNAs to promote ribosome binding (46).

The roles of TRBP in HIV replication have not been defined. TRBP coexpression augmented the expression of a chloramphenicol acetyltransferase gene linked to HIV LTR or the simian virus 40 promoter and the magnitude of trans-activation by the HIV Tat protein (22). However, we consistently failed to demonstrate any modulation of basal HIV LTR transcription or Tat transactivation by TRBP (unpublished data). In a recent report Gatignol *et al.* (47) have shown that nascent HIV TAR transcripts preferentially associate with endogenous TRBP, but they have not interpreted this finding in terms of their earlier claims of TRBP effects on LTR transcription or Tat transactivation (22). We believe that the association of endogenous TRBP with the HIV TAR RNA in living cells simply reflects the intrinsic affinity of this nuclear protein for highly structured RNAs and may have no bearing on the nuclear events. TRBP failed to relieve the effects of cis negative RNA elements on HIV-1 Gag expression and did not alter the Rev effect on HIV transcripts containing RRE sequence. TRBP is one among three RRE RNA-binding nuclear factors that have been identified so far, including the 56-kDa protein and the interferon-induced 9.27 gene product. The 9.27 protein is presumed to be a Rev antagonist (48), whereas the 56-kDa protein may be a Rev helper factor (49). The 9.27 protein and the 56-kDa protein are induced by interferon treatment and HIV infection, respectively, whereas TRBP expression is modulated by neither treatment (data not shown). However, it is possible that TRBP may be recruited by RRE or *crs* RNAs or by RRE and Rev to facilitate localized polysomal suppression of PKR under certain physiological conditions, such as interferon induction or cytokine activation. Although there have been disagreements about whether HIV-1 TAR RNA activates or inhibits PKR *in vitro* (50, 51), a recent report has suggested that TAR RNA prevents the localized activation of PKR in living cells (52). In this case, it is of interest to examine whether TRBP is recruited by the polysome-associated TAR transcripts.

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