RNA (2002), *8*:478–496. Cambridge University Press. Printed in the USA.

DOI: 10.1017.S1355838202022586

The mRNA of the translationally controlled tumor protein P23/TCTP is a highly structured RNA, which activates the dsRNA-dependent protein kinase PKR

ULRICH-AXEL BOMMER,[1] ANTON V. BOROVJAGIN,[1,3] MARTIN A. GREAGG,[1,4] IAN W. JEFFREY,[1] PAUL RUSSELL,[1] KENNETH G. LAING,[2] MELANIE LEE,[1,5] and MICHAEL J. CLEMENS[1]

[1]Department of Biochemistry & Immunology, St. George's Hospital Medical School, London, SW17 0RE, UK
[2]Department of Medical Microbiology, St. George's Hospital Medical School, London, SW17 0RE, UK

ABSTRACT

The dsRNA-activated protein kinase PKR is involved in signal transduction pathways that mediate cellular processes as diverse as cell growth and differentiation, the stress response, and apoptosis. PKR was originally described as an interferon-inducible eIF2α kinase involved in the antiviral defense mechanism of the cell. The interaction of the kinase with specific viral RNAs has been studied in much detail, but information about cellular mRNAs, which are able to bind and activate PKR, is scarce. In search for such cellular mRNAs, we developed a cloning strategy to identify individual mRNA species from the dsRNA-rich fraction of Daudi cell poly(A)$^+$ RNA. Two out of five cDNA clones we obtained contained sequences derived from the mRNA of the translationally controlled tumor protein P23/TCTP, indicating that this mRNA is present in the dsRNA-rich fraction. Secondary structure predictions and gel electrophoretic mobility investigations on P23/TCTP transcripts confirmed the potential of this mRNA to form extensive secondary structure. A full-length P23 transcript, but not a truncated version thereof, was able to bind to PKR in vitro and in vivo. Transient transfection experiments in human 293 cells showed that coexpression of full-length P23 mRNA leads to partial inhibition of the expression of a β-galactosidase reporter gene *in trans*. Additional coexpression of a dominant negative mutant of PKR or of adenovirus VA1 RNA suppressed this inhibition, indicating that it is mediated by PKR. Studies on P23/TCTP expression in cells from PKR-knockout mice suggest that P23/TCTP mRNA translation is regulated by PKR. Hence, our results demonstrate that the mRNA of P23/TCTP may both activate PKR and be subject to translational regulation by this kinase.

Keywords: mRNA structure; PKR activation; PKR binding; translational control; translationally controlled tumor protein P23/TCTP

INTRODUCTION

Regulation of gene expression at the translational level has been demonstrated for many important cellular proteins, and various mechanisms are known to be involved in such translational regulation (reviewed in Clemens & Bommer, 1999; Willis, 1999; Sonenberg et al., 2000). A well-established negative control mechanism acts through phosphorylation of the α-subunit of initiation factor eIF2. Three distinct eIF2α kinases have been known for some time (Clemens, 1996), and a new member of this family ("PEK" or "PERK") was discovered recently (Harding et al., 1999; Ron & Harding, 2000). The most extensively studied eIF2α kinase is the double-stranded RNA-dependent protein kinase PKR. This kinase, which was originally described as part of the interferon-induced cellular antiviral defense system, has recently attracted considerable attention because of its potential tumor suppressor activity and its involvement in signal transduction pathways. PKR

Reprint requests to: Dr. Ulrich-Axel Bommer, Department of Biochemistry & Immunology, St. George's Hospital Medical School, Cranmer Terrace, London, SW17 0RE, UK; e-mail: u.bommer@sghms.ac.uk.
[3]Present address: Brown University, BioMed, Box Gj 323, Providence, Rhode Island 02912, USA.
[4]Present address: Section of Structural Biology, Institute of Cancer Research, Chester Beatty Laboratories, London, SW3 6JB, UK.
[5]Present address: Eukaryotic Transcription Laboratory, Marie Curie Research Institute, Oxted, Surrey, RH8 0TL, UK.

has been implicated in regulation of cell growth and differentiation, the stress response, and apoptosis (for recent reviews, see Clemens, 1997; Jagus et al., 1999; Tan & Katze, 1999; Williams, 1999; Kaufman, 2000; Tan et al., 2000). In addition to its well-established role in translational regulation, PKR is involved in gene regulation at the transcriptional level through effects on NF-κB and IRF (reviewed in Jagus et al., 1999; Williams, 1999; Kaufman, 2000), as well as on the level of mRNA splicing (Osman et al., 1999). Apart from being induced by interferon and activated by dsRNA, PKR can be regulated by a variety of additional mechanisms (Jagus et al., 1999; Williams, 1999; Kaufman, 2000; Tan et al., 2000).

PKR is activated by dsRNAs, which are thought to be generated as intermediates during viral replication and gene expression. As a strategy to combat host defense mechanisms, some viruses express small RNA molecules, which are able to bind to PKR and to prevent its activation. Although such interactions have been studied in much detail (Bischoff & Samuel, 1989; Robertson & Mathews, 1996), relatively little is known about cellular RNA species, which may bind to and regulate the kinase. Earlier, we observed that total poly(A)$^+$ RNA from various sources is able to activate PKR in rabbit reticulocyte lysates (Pratt et al., 1988). However no individual mRNA species had been identified within this fraction. To identify mRNAs present in the dsRNA-rich fraction of total poly(A)$^+$ RNA from human cells, we developed a cDNA cloning strategy using this RNA fraction as starting material. In this article, we report that the mRNA of the "translationally controlled tumor protein" (TCTP) is present in the dsRNA-rich fraction of cellular poly(A)$^+$ RNA.

The TCTP was originally described about two decades ago, and various synonyms have been suggested by different groups, namely "P21" (Yenofsky et al., 1982, 1983), "Q23" (Thomas & Luther, 1981; Thomas, 1986), P23 (Benndorf et al., 1988; Boehm et al., 1989), TCTP (Gross et al., 1989), and HRF (MacDonald et al., 1995). The designation TCTP was derived from the early observation that the rate of synthesis of this protein is under translational control and from the fact that the first cDNA sequence of the human homolog was obtained from a mammary tumor (Gross et al., 1989). However, there is now increasing evidence to show (1) that the synthesis of TCTP is not exclusively regulated at the translational level, and (2) that TCTP is not a tumor-*specific* protein. In fact, it is widely expressed and has been detected in most mammalian tissues investigated (Adams et al., 1995; Sanchez et al., 1997; Chung et al., 2000; Thiele et al., 2000). Comparison of P23/TCTP cDNA sequences has revealed a high degree of conservation between all eukaryotic kingdoms. However, based on sequence comparisons, no consensus sequence of known functional domains or similarity to other protein families could be identified and, in the PROSITE database, TCTP is listed as a separate protein family. Only the recent elucidation of the NMR structure of fission yeast TCTP revealed a similarity of the fold of this protein to that of the Mss4/Dss4 family of proteins, which is known to interact with Rab proteins (Thaw et al., 2001).

The nearly ubiquitous expression and high degree of sequence conservation of P23/TCTP indicate that it is an important cellular protein. However, its precise cellular function still remains elusive, even though various functional aspects have been reported. Recently, we described the properties of P23 as a tubulin-binding protein, and we identified its tubulin-binding domain (Gachet et al., 1999). We also showed that overexpression of P23 results in a slow-growth phenotype, with alterations of cell morphology and microtubule stabilization. Moreover, we observed that P23 associates with microtubules and the mitotic spindle in a cell cycle-dependent manner, indicating that it might be involved in processes important for cell proliferation. This view is in line with the early observation that the rate of P23/TCTP synthesis is rapidly induced after growth stimulation of mammalian cells (Thomas & Luther, 1981; Thomas, 1986; Benndorf et al., 1988; Boehm et al., 1989; Bommer et al., 1994) and with a recent report showing that P23/TCTP is preferentially expressed in proliferating, but not in terminally differentiated cells of the polyp *Hydra vulgaris* (Yan et al., 2000). It is also supported by the recent demonstrations in fission yeast that transcription of the TCTP gene is down-regulated at the exit from the cell cycle (Bonnet et al., 2000) and that gene-knockout of P23/TCTP yields cells compromised in the exit from and entry into the cell cycle (Y. Gachet & J. Hyams, pers. comm.) Various other functional aspects of P23/TCTP have also been reported, namely (1) its potential property as a calcium-binding protein (Haghighat & Ruben, 1992; Sanchez et al., 1997; Bhisutthibhan et al., 1999; Xu et al., 1999), (2) its potential involvement in IgE-dependent histamine and interleukin-4 release in connection with allergic disease states (MacDonald et al., 1995; Schroeder et al., 1996; MacDonald, 1997), (3) its activity as a B-cell growth factor (Kang et al., 2001), and (4) the haem- and artemisinin-binding activity of malarial TCTP (Bhisutthibhan et al., 1998; Bhisutthibhan & Meshnick, 2001).

Expression of the P23/TCTP gene is highly regulated in response to alterations of various physiological conditions, including growth induction of mammalian cells (Thomas & Luther, 1981; Thomas, 1986; Benndorf et al., 1988; Boehm et al., 1989; Bommer et al., 1994), activation of macrophages (Walsh et al., 1995), increase in cytoplasmic calcium concentration (Xu et al., 1999), circadian variations in plants (Sage-Ono et al., 1998), different stress responses (Sturzenbaum et al., 1998), and induction of apoptosis (Baudet et al., 1998). Also, the differential expression of TCTP in various testicular tissues has been reported recently (Guillaume

et al., 2001). Although there is overwhelming evidence to show that, in mammalian cells, P23/TCTP levels are regulated at the translational level (see Discussion), other reports suggest that expression of this protein is also transcriptionally regulated (Baudet et al., 1998; Sage-Ono et al., 1998; Sturzenbaum et al., 1998; Thiele et al., 1998; Bonnet et al., 2000; Yan et al., 2000). The elucidation of the gene sequence for rabbit TCTP revealed the presence of various promoter elements upstream of the transcriptional start site, although the biological importance of these elements has not yet been studied in much detail (Thiele et al., 1998). In addition to the intron-containing TCTP gene, which is localized on human chromosome 13 (MacDonald et al., 1999), there exist a large number of processed pseudogenes for TCTP, some of which have been characterized recently (Thiele et al., 2000).

The precise mechanism(s) involved in translational regulation of P23/TCTP synthesis are still largely unknown. Our earlier work showed that P23 mRNA translation correlates with activation of the cap-binding factor eIF4E (Bommer et al., 1994). As eIF4E activity is believed to be particularly important for the translational activation of mRNAs bearing a 5′-UTR rich in secondary structure (reviewed in Gingras et al., 1999; Willis, 1999), it is very likely that secondary structure is involved in keeping the P23 mRNA in an inactive state, for example, in quiescent cells. The results described in this article show that P23/TCTP mRNA is indeed a highly structured RNA molecule and suggest an additional mechanism for its translational regulation, namely, that it is able to bind to and activate PKR. In support of this conclusion, we present data indicating that P23 mRNA translation may be regulated by PKR. Thus, P23/TCTP mRNA represents one of the few cellular mRNA species known to date that are regulated through PKR activation.

RESULTS

P23 mRNA is contained in a dsRNA-rich fraction of total poly(A)$^+$ RNA

We demonstrated earlier that total poly(A)$^+$ RNA from Daudi cells is able to inhibit protein synthesis in the rabbit reticulocyte lysate system through activation of the dsRNA dependent protein kinase PKR (Pratt et al., 1988). To identify individual mRNA species present in the poly(A)$^+$ RNA preparation that might be able to activate PKR, we separated the dsRNA-rich fraction from the less structured RNA by chromatography on CF-11 cellulose. As described earlier (Clarke et al., 1990; Li & Petryshyn, 1991), this method separates single-stranded from double-stranded RNA. Both fractions were tested for their ability to inhibit globin synthesis in a cell-free RRL system and we confirmed that the dsRNA fraction was several-fold more potent in this assay compared to the ssRNA fraction (results not shown). The dsRNA-rich fraction was reverse transcribed and the resulting DNA–RNA hybrids were amplified by PCR and cloned into the pDirect vector as described in Materials and Methods. Because reverse transcription of highly structured RNAs is inefficient relatively few clones were obtained by this procedure. However the inserts of five of them were sequenced and compared with the GenBank human cDNA database. The inserts of two of the clones contained sequences identical to parts of the cDNA of the human P23/TCTP protein (Fig. 1). The clone PRDS-1 contained a sequence identical to a large part of the 5′-UTR plus a short stretch of the coding region of the mRNA, indicating that it represents a nearly full-length clone of P23 cDNA, whereas the sequence from clone PRDS-4 was found to be identical to part of the 3′-UTR. The detection of P23 sequences in two out of five sequenced clones suggests a high representation of P23 mRNA within the dsRNA-rich fraction of poly(A)$^+$ RNA. Because of the limited number of clones analyzed it is not possible to quantify the relative amount of P23 mRNA within the dsRNA-rich fraction by this approach; however, in an independent experiment using real-time RT-PCR for detection, we also found P23 mRNA in the dsRNA-rich fraction of cytoplasmic RNA from mouse embryo fibroblasts (results not shown). Thus P23/TCTP mRNAs from both human and mouse sources appear to behave as highly structured RNA molecules.

Full-length P23/TCTP mRNA has the potential to form extensive secondary structure

To assess the potential of P23/TCTP mRNA to form extended areas of secondary structure, we performed computer predictions using the RNAFOLD computer

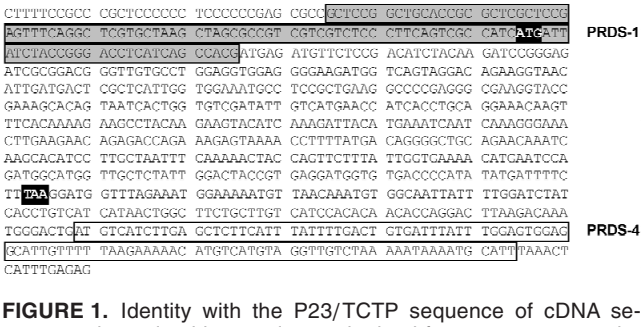


FIGURE 1. Identity with the P23/TCTP sequence of cDNA sequences determined in two clones obtained from reverse transcription of the dsRNA-rich fraction of poly(A)$^+$ RNA from Daudi cells. The complete cDNA sequence of human P23/TCTP mRNA was compiled from the first entry in the database (GenBank accession number X16064) and from a more recent entry of the complete 5′-UTR (GenBank accession number D28408). The start and stop codons are highlighted by black boxes. The sequences identical to clone PRDS-1 and clone PRDS-4 are boxed (and shaded in the case of PRDS-1).

program. As all our subsequent investigations were performed with transcripts from mouse P23 cDNA, we present here the predicted structure of mouse P23/TCTP mRNA. Figure 2 shows that this mRNA has the potential to adopt a stable secondary structure with extended double-stranded areas including all parts of the molecule.

To test the hypothesis that P23/TCTP mRNA is highly structured, we performed comparative gel mobility assays. Electrophoresis in polyacrylamide gels under non-denaturing conditions has been employed previously to assess the structure content of RNA molecules (see, e.g., Gast et al., 1996; Brinegar et al., 1997) The following in vitro transcripts from P23/TCTP cDNA constructs were investigated in this assay: full-length mRNA and truncated mRNAs lacking the 5′-UTR, the 3′-UTR or both 5′- and 3′-UTR. For comparison, control RNA species were included that are known either to adopt a high degree of secondary structure (the structured part of the 5′-UTR of EMC virus RNA) or to be unstructured

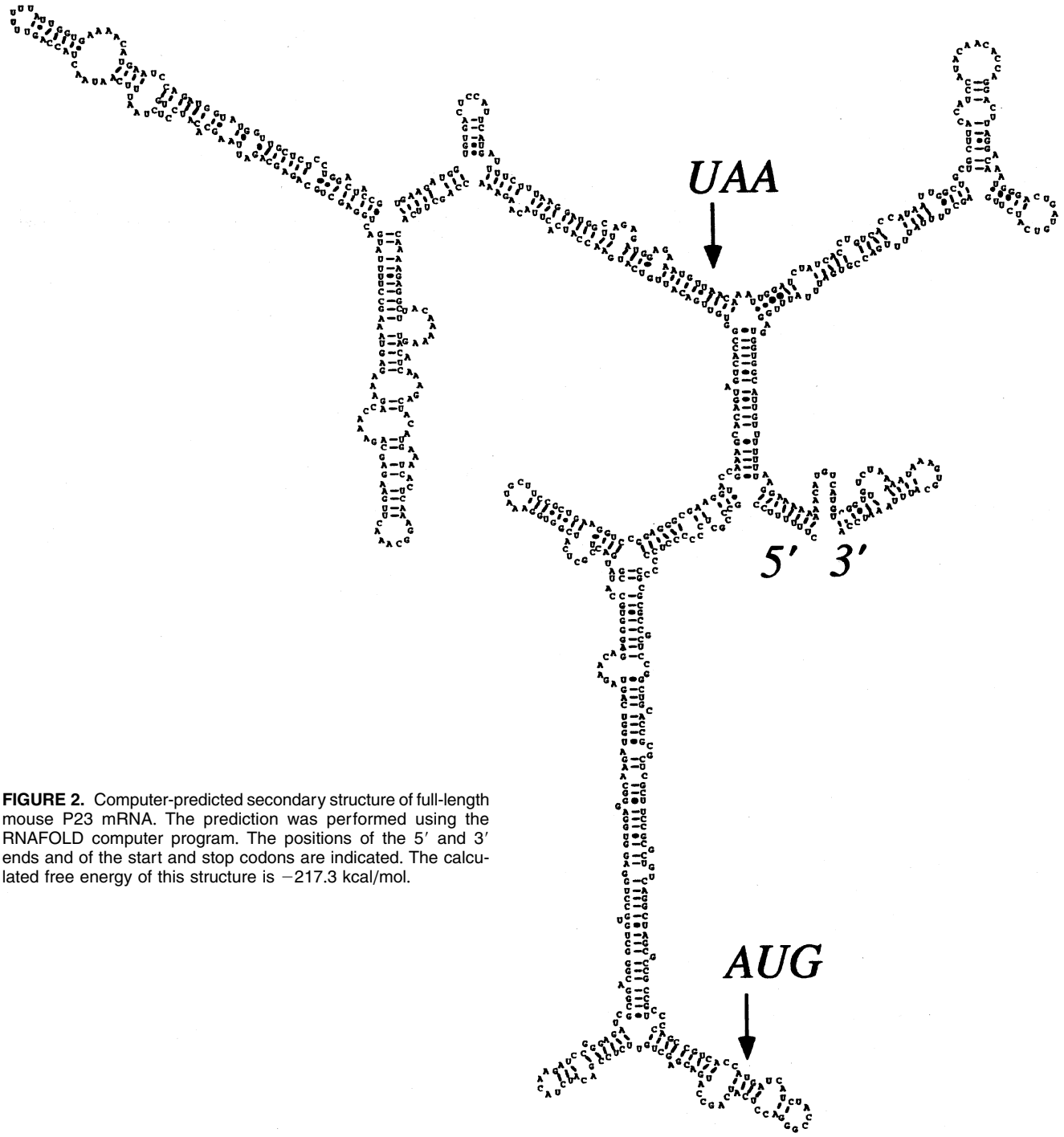


FIGURE 2. Computer-predicted secondary structure of full-length mouse P23 mRNA. The prediction was performed using the RNAFOLD computer program. The positions of the 5′ and 3′ ends and of the start and stop codons are indicated. The calculated free energy of this structure is −217.3 kcal/mol.

(Alfalfa-mosaic virus RNA 4; Langereis et al., 1986). We compared the mobility of radiolabeled transcripts of these cDNA constructs in 8% gels under both denaturing and nondenaturing conditions (Fig. 3) The results obtained from the denaturing gel show that the relative mobilities of the different RNA species in this electrophoretic system reflect only the differences in size. In contrast, under nondenaturing conditions, the relative mobilities of the RNAs deviate considerably from those observed under denaturing conditions. The migration of the control RNAs in the nondenaturing gel shows that the mobility of the structured RNA (EMCV-RNA 5′-UTR) is reduced compared to that of the nonstructured one, the larger AlMV-RNA 4. The full-length P23 mRNA shows an even lower mobility than that of the EMCV-RNA 5′-UTR. In contrast, the truncated versions of P23 mRNA display an increased mobility. The differences in mobility between the full-length P23/TCTP mRNA and the truncated versions cannot be attributed simply to differences in size, as can be seen from the results of the denaturing gel. By far the highest mobility is displayed by the transcript representing only the coding region of P23 mRNA, suggesting that this part of the molecule does not form extended secondary structure on its own. Thus, the gel mobility studies confirm the computer prediction in suggesting that full-length P23 mRNA is able to adopt a high degree of structure in vitro and that both UTRs contribute importantly to the overall structure of the molecule.

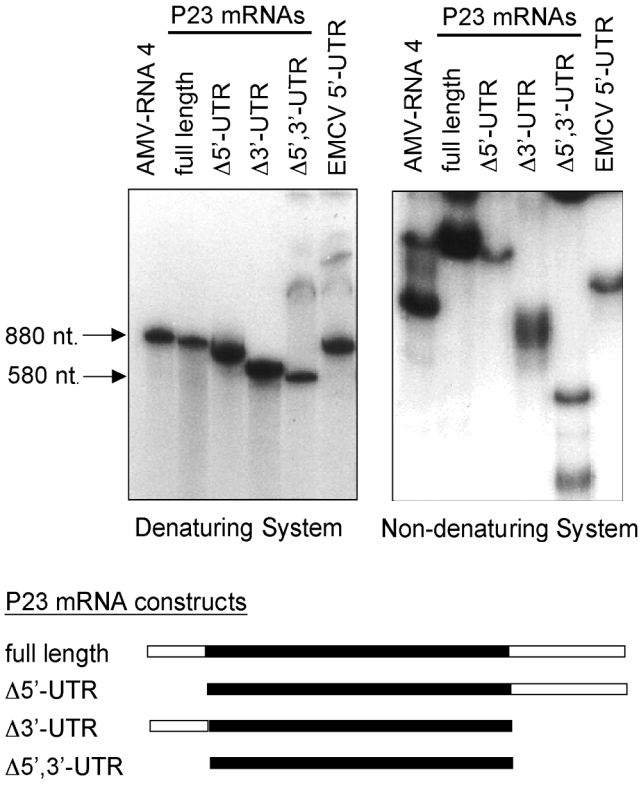


FIGURE 3. Full-length P23 mRNA migrates as a highly structured RNA in an electrophoretic mobility assay. ^{32}P-labeled RNA transcripts from a full-length P23 cDNA clone (860 nt) and from clones deleted in the 5′-UTR (790 nt), in the 3′-UTR (640 nt) and in both (585 nt), were run on 8% polyacrylamide gels under denaturing and nondenaturing conditions. For comparison, the highly structured portion of the EMC virus RNA 5′-UTR (777 nt) and the unstructured alfalfa mosaic virus RNA 4 (880 nt) were run in parallel. (Loading: 20,000 cpm of transcript per lane.) The P23 mRNA constructs are represented by bars (drawn to scale) below the figure (filled bar: coding region).

P23/TCTP mRNA binds to PKR

The potential of P23 mRNA to form extended regions of secondary structure suggested that it may be able to bind to and activate PKR. To investigate this question, we assessed the ability of P23 mRNA to bind to PKR in two different in vitro assays: (1) the coprecipitation of in vitro translated [^{35}S]methionine-labeled PKR by biotinylated RNA bound to streptavidin-coated beads, and (2) the PKR-dependent retention of ^{32}P-labeled P23 mRNA on nitrocellulose filters. As, according to the results of the gel mobility assay (Fig. 3), the full-length P23 mRNA and the truncated construct (P23-Δ3′5′) differ extremely in their ability to form secondary structure, we compared the activity of these two RNA transcripts in both types of PKR binding assays.

To investigate binding of radiolabeled PKR to biotinylated P23 mRNA transcripts, a truncated version of PKR, comprising the first 268 amino acids, which include both RNA binding domains, was transcribed. The resulting transcript was translated in the wheat germ system in the presence of [^{35}S]methionine. An aliquot of the translation mix was then incubated with the biotinylated transcripts (P23-FL and P23-Δ3′5′), and the RNA was recovered by binding to streptavidin-coated agarose beads. The result of this assay (Fig. 4A) shows that biotinylated full-length P23 mRNA is able to precipitate labeled PKR, whereas the coding region of P23 mRNA on its own is unable to bind PKR. This is in good agreement with the result of the filter-binding assay (Fig. 4B), which shows that full-length P23 mRNA has a much higher affinity for PKR than the P23-Δ3′5′ transcript. Thus, the results of both assays independently demonstrate that full-length P23/TCTP mRNA is able to bind to PKR in vitro.

P23/TCTP mRNA activates PKR in a transient transfection assay

To address the question of whether P23/TCTP mRNA activates PKR in vivo, we performed transient transfection assays. Previously, PKR has been shown to inhibit the expression of reporter genes *in trans* (Terenzi et al., 1999), and this observation has been exploited in transient transfection assays to demonstrate both activation (Wu & Kaufman, 1996) and inhibition (Wu et al., 1996; Wu & Kaufman, 1997; Kumar et al., 1999) of PKR in vivo. Therefore, we investigated whether the

cotransfection of a plasmid expressing TCTP mRNA would inhibit the expression of a β-galactosidase reporter gene *in trans*. To this end, we cotransfected into 293 cells the pcDNA3 plasmid driving the expression of full-length P23/TCTP mRNA from the CMV promoter, together with the pCMV-β-gal plasmid at a threefold excess of effector over reporter plasmid (unless stated otherwise). As controls, we cotransfected either the vector alone (pcDNA3), or a corresponding plasmid driving the expression of the truncated P23 mRNA (P23-Δ3′5′), which did not bind to PKR (Fig. 4). The results show that full-length P23/TCTP mRNA is indeed able to inhibit reporter gene expression in a concentration-dependent manner (Fig. 5A). In contrast, cotransfection of the control plasmids did not affect reporter gene expression, whereas addition of poly(I,C) to the medium resulted in a 90% inhibition of β-gal activity, indicating that the assay system is sensitive to dsRNA.

To establish that the observed inhibition of reporter gene expression is, in fact, mediated by PKR activation, we also performed triple transfection assays with the additional inclusion of plasmids directing the expression of various effectors of PKR (Fig. 5B). This experiment demonstrated that the inhibition of β-gal activity, as observed in the presence of P23-FL mRNA compared to the truncated version (P23-Δ3′5′) or the empty vector, can be prevented by coexpression of the PKR inhibitor VA1 RNA or a dominant negative mutant of PKR. However, a mutant version of VA1 RNA, unable to prevent PKR activation (Mellits & Mathews, 1988), did not relieve the inhibition of β-gal expression induced by P23/TCTP mRNA. Coexpression of wild-type PKR inhibited the reporter gene expression by 90% in each case, indicating that the system was responsive to PKR activation.

To determine whether the observed effects of P23/TCTP mRNA expression on reporter gene expression are exerted at the translational level, we performed real-time RT-PCR experiments to quantify the β-galactosidase mRNA levels in 293 cells transfected as in Figure 5A. The results indicate that the amount of β-gal mRNA is not negatively affected by coexpression of either of the two P23 mRNA constructs; in fact, we rather observed slight increases in the mRNA levels relative to cells transfected with β-gal alone (Fig. 5C). In parallel real-time RT-PCR experiments, we also measured the relative P23/TCTP mRNA levels in 293 cells from the different transfection assays. We employed a primer-probe combination that detects the coding region of the mouse P23 mRNA, that is, the transcripts originating from both transfected plasmids, but not the endogenous human P23/TCTP mRNA. These experiments revealed that the level of the truncated P23 mRNA is around 50% lower than that of the full-length transcript. It is possible that this difference is due to a decreased stability of the truncated version compared to

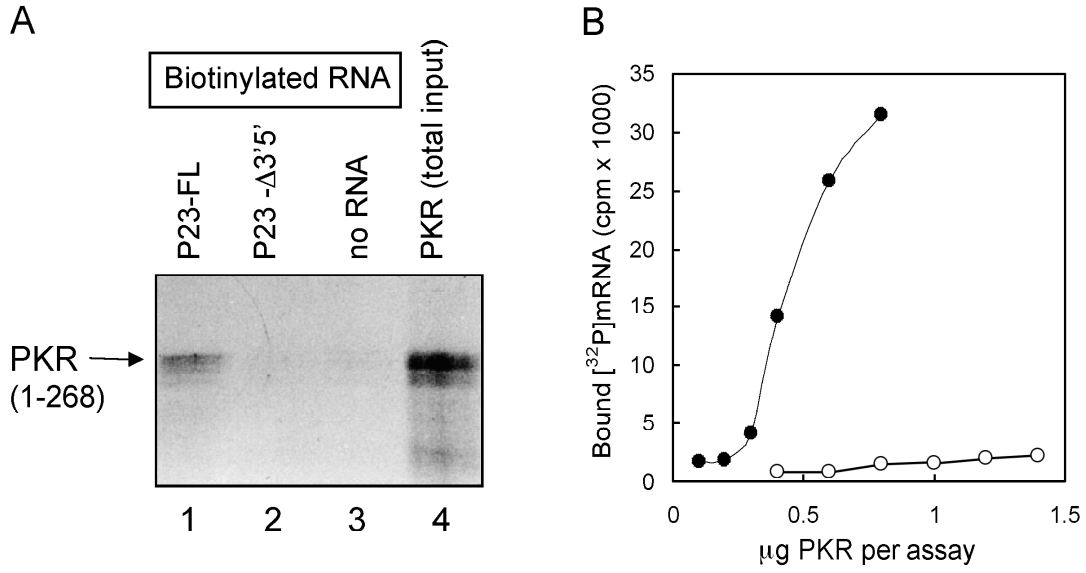


FIGURE 4. Full-length P23 mRNA binds to PKR. **A:** Binding of [^{35}S]methionine-labeled PKR to biotinylated P23 mRNA. The plasmid pLTheo containing the cDNA sequence for the N-terminal 268 amino acids of human PKR, inclusive of the two RNA-binding domains, was transcribed in vitro. The capped transcript was translated in the wheat germ translation system in the presence of [^{35}S]methionine and 1 μL of the translation mix was then incubated with the following biotinylated unlabeled RNA transcripts: P23-FL (lane 1) and P23-Δ3′5′ (lane 2). The transcripts were recovered on streptavidin-coated agarose beads, and the binding of labeled PKR was assessed by SDS-gel electrophoresis and subsequent fluorography. Lane 3: control assay without biotinylated RNA; lane 4: complete translation mix. **B:** Binding of ^{32}P-labeled P23/TCTP mRNA to PKR. ^{32}P-labeled transcripts corresponding to full-length P23 mRNA (filled circles) or to P23-Δ3′5′ mRNA (open circles) were assayed for their ability to bind to PKR in a filter-binding assay. PKR was expressed in a Baculovirus system and purified from Sf-9 cell extracts. The indicated amounts of purified protein were incubated in the presence of 40,000 cpm ^{32}P-labeled RNA transcript per assay, filtered through nitrocellulose filters and the retained radioactivity counted.

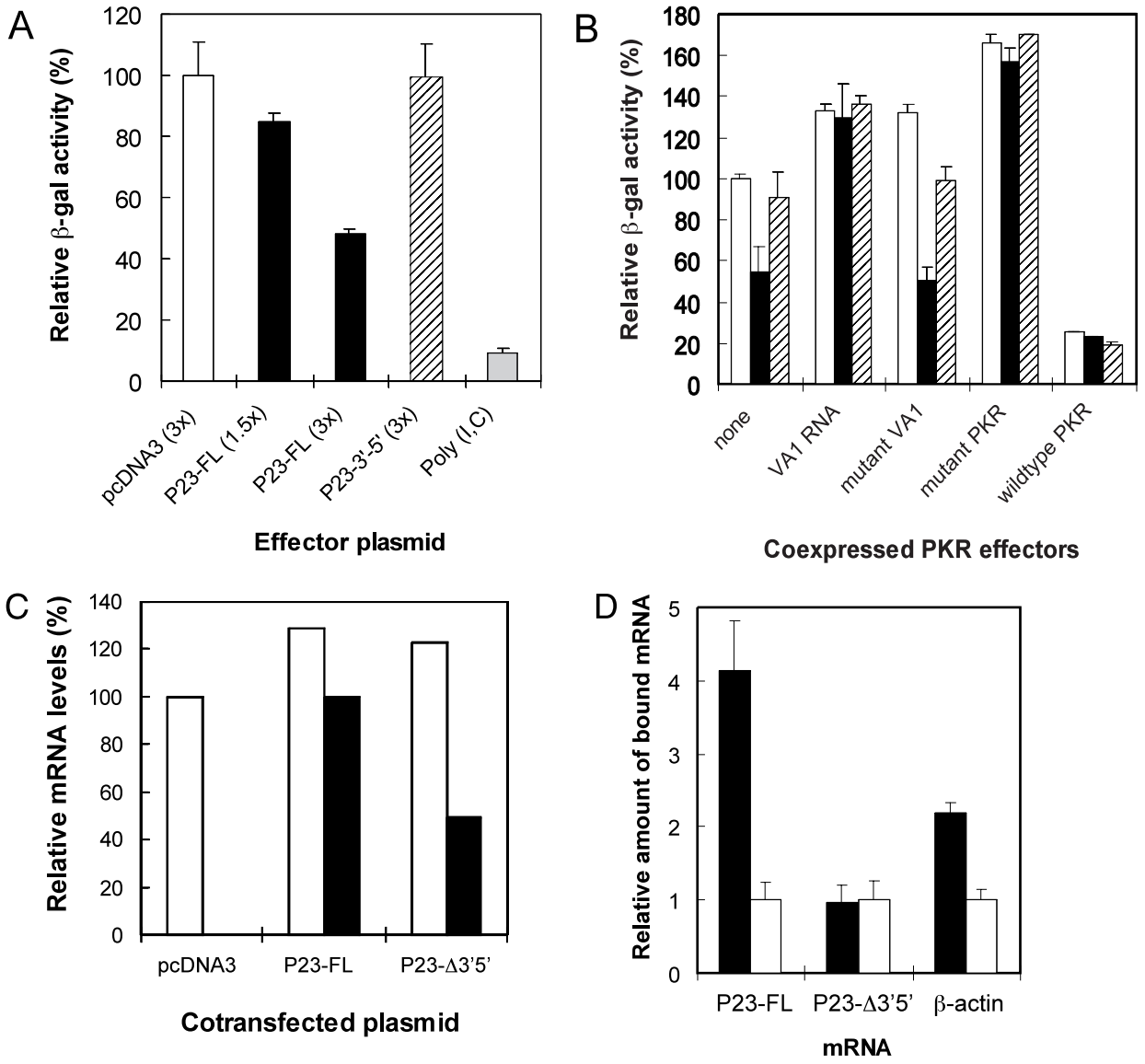


FIGURE 5. (*Figure continues on facing page.*)

that of the structured full-length mRNA. However, it is unlikely that the difference in reporter gene expression between the 293 cells transfected with the different P23 cDNA constructs can be accounted for by only a 50% reduction in P23-Δ3′5′ mRNA levels, as we have never observed any inhibition of β-galactosidase activity in cells expressing P23-Δ3′5′ at various concentrations, compared to the vector control transfections.

To seek additional evidence for the potential of full-length P23/TCTP mRNA to activate PKR, we addressed the question of whether the mRNA was actually bound to PKR in vivo using a coimmunoprecipitation assay. Transfections were carried out essentially as described for Figure 5A, except that the reporter gene plasmid was omitted in this case; cytoplasmic extracts were prepared and incubated with protein G–Sepharose beads preloaded with an anti-PKR antibody, which effectively precipitated PKR (results not shown). The beads were washed and incubated with SDS and proteinase K, and the RNA was recovered and treated with DNase I. The level of P23 mRNA present in these RNA preparations was quantified by real-time RT-PCR. The results show that full-length P23 mRNA, but not the Δ3′5′ version, is bound to the beads at a fourfold excess relative to the minus antibody control (Fig. 5D). This figure represents an average from three independent experiments. For a comparison, we also probed the RNA samples for the endogenous β-actin mRNA. In this case, we observed only a twofold higher amount of RNA bound in comparison to the minus antibody control. At this point, we cannot exclude the possibility that actin mRNA is also able to bind weakly to PKR.

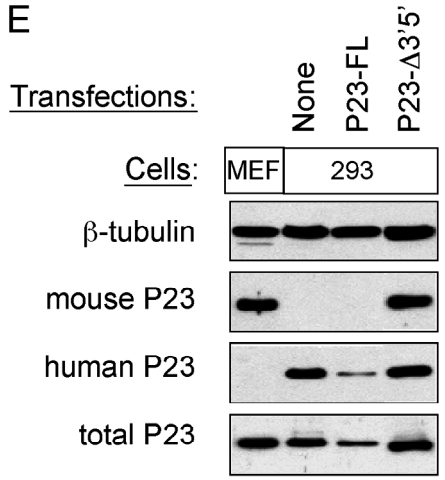


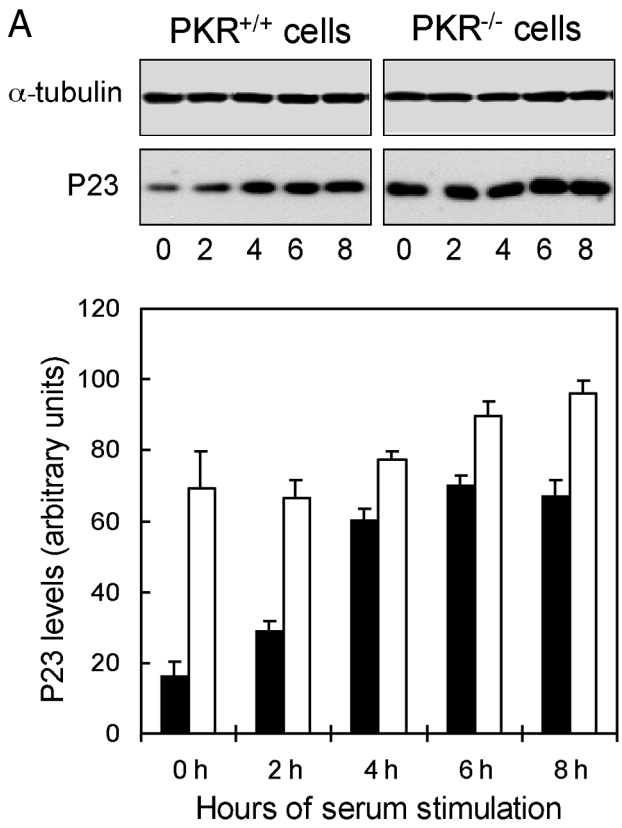
FIGURE 5. Full-length P23/TCTP mRNA binds to and activates PKR in transiently transfected 293 cells. **A:** Full-length P23 mRNA inhibits the expression of a reporter gene *in trans*. 293 cells were transiently transfected with the reporter plasmid pCMV-β-gal, together with one of the following effector plasmids: P23-FL (filled bars), P23-Δ3′5′ (hatched bar), or the empty vector, pcDNA3 (open bar). At 40 h posttransfection, cell extracts were prepared and assayed for β-galactosidase activity (plotted as percent of the pcDNA3 control). The effector plasmids indicated below the graph were cotransfected with the plasmid pCMV-β-gal at different relative amounts: Figures in brackets indicate the *x*-fold excess of the effector plasmid over the reporter gene plasmid. The right bar represents β-gal expression in 293 cells incubated in the presence of 0.1 mg/mL poly(I,C). **B:** Negative effectors of PKR prevent the inhibition of reporter gene expression induced by full-length P23/TCTP mRNA. The pCMV-β-gal plasmid was cotransfected with a threefold excess of one of the following effector plasmids: pcDNA3 (open bars), P23-FL (filled bars), or P23-Δ3′5′ (hatched bars). In addition, a plasmid encoding either VA1 RNA, an inactive mutant form of VA1 RNA, a dominant negative mutant form of PKR or wild-type PKR was added to the transfection mixture at an equal amount compared to the reporter gene plasmid. β-gal activity was assayed as for **A**. **C:** Reporter gene mRNA levels are not affected by P23/TCTP mRNA expression. Cells were cotransfected with the plasmid pCMV-β-gal and a threefold excess of effector plasmid as described for **A**. At 40 h posttransfection, total cytoplasmic RNA was prepared, and relative mRNA levels for β-galactosidase (open bars) and P23 mRNA (filled bars) were determined by real-time RT-PCR experiments in separate reactions. (Note that the figures are only comparable for each type of mRNA individually.) PCR reactions were performed in duplicate and the average figures are plotted as percent of the relevant control value. **D**: Full-length P23 mRNA coimmunoprecipitates with PKR. 293 cells were transfected with the plasmids driving the expression of P23-FL or P23-Δ3′5′ mRNAs. At 40 h posttransfection, cell extracts were prepared and immunoprecipitated on protein G–Sepharose beads preloaded with a monoclonal anti-PKR antibody (filled bars) or without antibody (open bars), as indicated. RNA was prepared from the immunoprecipitates and analyzed for the presence of P23 and β-actin mRNAs by real-time RT-PCR. RNA levels are plotted for each type of mRNA relative to the corresponding minus antibody control. **E**: Overexpression of full-length P23 mRNA, but not of P23-Δ3′5′ mRNA, results in repression of P23 translation. P23/TCTP protein levels were assessed by western blotting in human 293 cells either nontransfected or transfected with the expression plasmids for P23-FL mRNA or the truncated P23-Δ3′5′ mRNA, both derived from the mouse cDNA sequence. Blots were probed with antibodies specific for mouse or human P23, or with a P23 antibody detecting the proteins from both species ("total P23"), and with a β-tubulin antibody for a loading control. The left lane shows the blot of an extract from mouse embryo fibroblasts (MEF).

Taken together, these data demonstrate that full-length but not the truncated version of P23/TCTP mRNA is able to bind to and activate PKR in vivo.

P23/TCTP mRNA translation may be regulated by PKR

Activation of PKR by P23/TCTP mRNA raises the question of whether the expression of the P23/TCTP protein itself is affected by PKR. It has previously been suggested that individual mRNAs may bind and locally activate PKR, thereby preventing their own translation (De Benedetti & Baglioni, 1984). We therefore investigated whether the P23 protein is actually expressed from the two different P23 plasmids. To distinguish between the endogenous human and the ectopically expressed mouse protein, we employed antibodies, which are able to differentiate between these two proteins. Western blotting experiments demonstrate that the mouse P23 protein is readily expressed from the truncated mRNA, but not at all from the corresponding full-length transcript (Fig. 5E). This result shows (1) that the truncated transcript is fully functional in vivo, and (2) that the full-length transcript, despite displaying a higher steady-state level (Fig. 5C), is severely inhibited in its translational activity. The latter is consistent with the possibility that local activation of PKR by full-length P23 mRNA results in inhibition of P23 translation. A partial inhibitory effect *in trans* of full-length P23 mRNA on the translation of the endogenous human P23/TCTP mRNA (Fig. 5E, bottom panels) is also consistent with PKR activation. However, we cannot exclude the possibility that other translational control mechanisms also play a part in regulating the activity of P23 mRNA (see Discussion).

We have also investigated whether loss of PKR expression interferes with the regulation of P23 synthesis, as it normally occurs in fibroblasts. A well-documented example of translational regulation of P23/TCTP synthesis is early growth-dependent induction (Thomas, 1986; Benndorf et al., 1988; Boehm et al., 1989), resulting in a rapid increase of P23 protein level after growth stimulation of quiescent cells. To investigate the potential involvement of PKR in the regulation of P23/TCTP, we monitored the levels of P23 protein by western blotting in mouse embryo fibroblasts derived from PKR-knockout ($PKR^{-/-}$) mice and from wild-type animals ($PKR^{+/+}$) after serum starvation and restimulation (Fig. 6A). The wild-type cells typically displayed a low level of the P23 protein after serum-starvation, which increased rapidly after restimulation of cells to about a fourfold higher level. In contrast, in $PKR^{-/-}$ cells, P23/TCTP levels remained high during serum starvation and increased only marginally (1.5-fold) and at a later time point after serum addition. Remarkably, at each time point, P23 levels are higher in $PKR^{-/-}$ cells compared to $PKR^{+/+}$ cells. These ob-



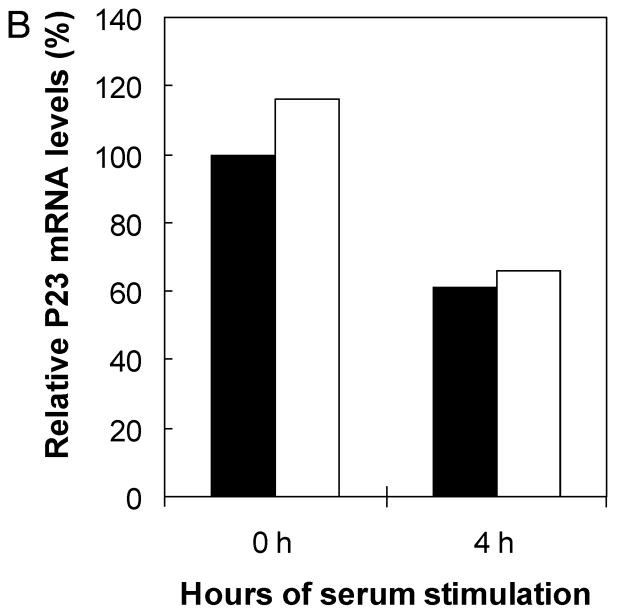
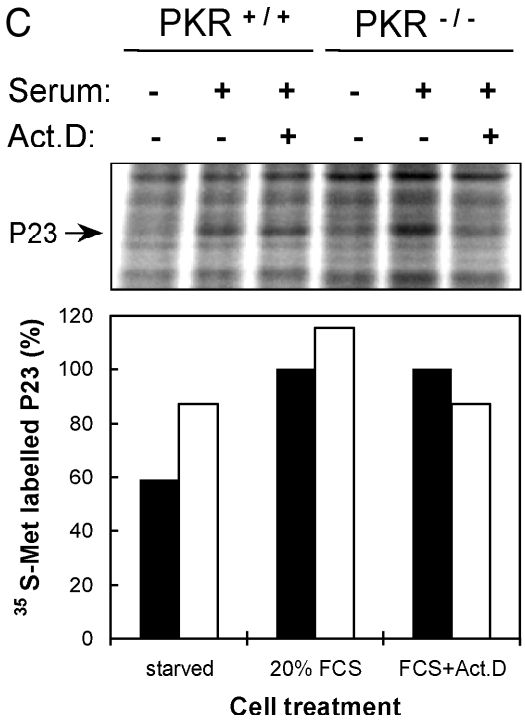


FIGURE 6. Loss of PKR expression interferes with the translational regulation of P23/TCTP synthesis. **A:** P23/TCTP protein levels are down-regulated during quiescence in wild-type but not in PKR-knockout cells. The levels of P23/TCTP protein were assessed by western blotting in MEFs derived from PKR-knockout mice ($PKR^{-/-}$) and from wild-type mice ($PKR^{+/+}$) after 20 h of serum starvation and restimulation for the indicated periods of time. Ten micrograms of protein was loaded per lane and the western blots were probed with antibodies against P23 and α-tubulin. The histogram shows the relative intensity of the P23/TCTP signals after quantification and normalization against the tubulin signals (filled bars: $PKR^{+/+}$ cells, open bars: $PKR^{-/-}$ cells). **B:** P23 mRNA levels are similar in $PKR^{+/+}$ and $PKR^{-/-}$ cells. Relative P23 mRNA levels were measured by real-time RT-PCR on 2.5 ng each of total cytoplasmic RNA prepared from $PKR^{+/+}$ cells (filled bars) and $PKR^{-/-}$ cells (open bars) after serum starvation (0 h) and after stimulation with 20% serum for 4 h. PCR reactions were performed in duplicate and the average figures plotted as percent of the value from serum-starved $PKR^{+/+}$ cells. **C:** The rate of P23/TCTP synthesis is subject to growth-dependent translational control in wild-type but not in PKR-knockout cells. The relative rate of P23/TCTP synthesis in $PKR^{+/+}$ and $PKR^{-/-}$ cells was assessed by [^{35}S]methionine incorporation. Cells were serum starved for 20 h and either not stimulated or restimulated with 20% FCS alone or in the presence of actinomycin D, as indicated. Thirty minutes after serum addition, cells were subjected to [^{35}S]methionine labeling for 1 h. Cell extracts were separated by SDS-gel electrophoresis and the radioactivity pattern of the dried gels was analyzed and quantified in a phosphorimager. The top panel shows the relevant part of a representative gel. The identity of the P23 band was verified by western blotting. The bottom panel shows the normalized P23 signals (average from three experiments) in $PKR^{+/+}$ cells (filled bars) and $PKR^{-/-}$ cells (open bars) under the conditions indicated at the top of the figure.



servations indicate that PKR is likely to be involved in reducing the rate of TCTP synthesis in quiescent cells, as this down-regulation is prevented in the cells derived from PKR-null mice.

To rule out the possibility that the differences in P23/TCTP expression between the two cell types are due to differential transcription or mRNA stability, we quantified P23 mRNA levels in PKR-knockout and wild-type cells by means of real-time RT-PCR. These experiments showed that P23 mRNA levels remain the same between $PKR^{+/+}$ and $PKR^{-/-}$ cells, both under serum-starvation and refeeding conditions (Fig. 6B). The ap-

parent 30% decrease of the P23 mRNA level in both cell lines after serum stimulation can be attributed to the increase in ribosome numbers at 4 h, resulting in a relative decrease of the mRNA in total cytoplasmic RNA. In addition, we performed [^{35}S]methionine labeling studies to monitor the rate of P23 synthesis in the two cell lines under serum-starvation and refeeding conditions (Fig. 6C). These studies showed that in wild-type cells there is about a twofold increase in the rate of P23 synthesis after serum stimulation, and that this increase is not prevented by the transcription inhibitor actinomycin D. We conclude that, in PKR$^{+/+}$ cells, regulation of P23/TCTP expression occurs indeed at the translational level. In contrast, in PKR$^{-/-}$ cells, the rate of P23/TCTP synthesis is less inhibited by serum starvation (Fig. 6C), as it is reflected in the corresponding protein levels (Fig. 6A). In these cells, there is a small increase (1.3-fold on average) in the rate of P23 synthesis after serum stimulation, but this is inhibited by actinomycin D, suggesting that it reflects an increase at the transcriptional level. This is consistent with the observed small increase in P23 protein level occurring at later times (Fig. 6A). These studies therefore indicate that PKR is important for the translational silencing of P23 mRNA in wild-type fibroblasts under serum-starvation conditions.

DISCUSSION

The mRNA of P23/TCTP is a highly structured RNA molecule

The data in this article indicate that the mRNA for P23/TCTP is an example of a cellular mRNA, which both activates PKR and is subject to translational regulation by the protein kinase. Earlier evidence suggesting that the synthesis of the TCTP is regulated at the translational level includes the following: (1) P23 mRNA is one of the abundant mRNAs in translationally inactive mRNP particles (Yenofsky et al., 1982, 1983); (2) when cells are stimulated from quiescence into proliferation, the growth-dependent increase in P23 protein synthesis occurs much earlier than in the case of transcriptionally regulated proteins and is not blocked by transcription inhibitors (Thomas, 1986; Boehm et al., 1989); (3) the considerably higher rate of P23 synthesis observed in growing versus nongrowing Ehrlich ascites tumor cells is not paralleled by higher P23 mRNA levels (Boehm et al., 1989). Moreover, the sequence of P23 mRNA displays features typical of translationally controlled mRNAs, including a 5′-terminal oligopyrimidine tract (5′-TOP) and a 5′-UTR with a high CG-content (about 80%). The latter is suggestive of a high degree of secondary structure (Gray & Hentze, 1994), in line with the result reported here, that P23 mRNA is highly represented in the dsRNA-rich fraction of poly(A)$^+$ RNA.

To investigate the potential of P23/TCTP mRNA to form secondary structure, we performed structure predictions using the RNAFOLD computer program (Fig. 2). The result showed that P23 mRNA is indeed able to adopt a complex secondary structure with extended base-paired areas involving about two-thirds of the molecule. As it was not the aim of this study to establish the validity of a particular secondary structure model, we did not perform RNase or chemical cleavage analyses. Instead, to obtain experimental evidence for the presence of secondary structure, we performed gel mobility investigations. The results clearly demonstrated that full-length P23 mRNA behaves as a highly structured RNA in nondenaturing gels (Fig. 3) This conclusion is supported by the following additional observations: (1) In an earlier RNase-protection assay, it was shown that hybridization with total poly(A)$^+$ RNA from Ehrlich ascites tumor cells protected both a radiolabeled 5′-terminal 285-nt fragment of P23 mRNA and the corresponding antisense RNA construct equally well (Boehm et al., 1991); (2) in northern blot analyses, P23 mRNA was found to be recognized by an antisense probe and, to a lesser extent, also by a sense probe (A.V. Borovjagin & U.-A. Bommer, unpubl. observation). Both results indicate self-complementarity of the P23 mRNA.

When comparing the relative mobilities of the different P23 mRNA constructs, it appears that the 3′-UTR makes a greater contribution than the 5′-UTR. This may be simply due to the larger size of the 3′-UTR (about 200 nt compared to 100 nt of the 5′-UTR), but it is also in agreement with the predicted secondary structure model (Fig. 2). However, more detailed RNA structure investigations will be necessary to clarify the precise contributions of the two untranslated regions to the overall secondary structure. Also, it should be noted that the gel mobility assay assesses the overall conformation of the molecule and this may reflect both secondary and tertiary structure.

A recently published article reports the existence of two different P23/TCTP mRNA species in rabbit (Thiele et al., 2000). Although the ratio between the two species varies between different tissues, the shorter mRNA is always more abundant. This matches our own observation obtained by northern blots on different rat tissues (A. Borovjagin & U.-A. Bommer, unpubl. result). The longer TCTP mRNA species in rabbit was shown to arise from alternative transcription termination, 320 nt downstream of the usual termination site (Thiele et al., 2000). The potential impact of this 3′-terminal extension on the structure of the molecule is presently unknown. The biological significance of the existence of two TCTP mRNA species is also an open question, but it is interesting that different processed pseudogenes have been identified in rabbit that are derived from both types of mRNA (Thiele et al., 2000).

P23/TCTP mRNA binds to and activates PKR

Our finding that P23 mRNA is represent in the dsRNA-rich fraction of Daudi cell poly(A)$^+$ RNA, which, as a whole, is able to activate PKR in rabbit reticulocyte lysates (Pratt et al., 1988), raised the question of whether P23/TCTP mRNA actually binds to and activates PKR. Our binding experiments show that full-length P23 mRNA binds to PKR with high affinity, whereas transcripts comprising just the coding region of the same RNA do not display significant PKR-binding activity in these assays (Fig. 4). This observation mirrors the conclusion from the gel mobility experiments, which suggests that the coding region of the mRNA on its own does not possess much secondary structure.

To test whether binding of P23/TCTP mRNA to PKR results in activation of the kinase, we initially performed in vitro assays of PKR activity. These experiments showed that addition of P23 mRNA transcripts resulted both in PKR autophosphorylation (A. Elia & A.V. Borovjagin, unpubl. observation) and in inhibition of globin mRNA translation in the RRL system (M. James & U.-A. Bommer, unpubl. result) However, an inherent problem with this type of experiment lies in the difficulty of producing transcripts that are free of dsRNA contaminants that might activate PKR in these assays (Mellits et al., 1990). For this reason, we chose another strategy and employed transient transfection experiments to test the ability of P23/TCTP mRNA to activate PKR. We used 293 cells for these investigations, as these cells display high transfection efficiency and contain significant amounts of endogenous PKR, even in the absence of stimulation by interferon. Our results clearly demonstrate that full-length P23/TCTP mRNA (but not the truncated form, devoid of the 5′- and 3′-UTRs) is an effective inhibitor of reporter gene expression *in trans* and that this inhibition is due to activation of PKR (Fig. 5A,B) We also established (1) that this effect is exerted at the translational level (Fig. 5C), (2) that full-length P23 mRNA is indeed bound to PKR under these conditions (Fig. 5D), and (3) that translation of full-length P23 mRNA itself is severely inhibited by its own overexpression in these cells (Fig. 5E). The involvement of PKR in these effects on translation would be expected to result in an increase in eIF2α phosphorylation. Surprisingly, western blot experiments employing two different phospho-specific antibodies against eIF2α failed to show a consistent increase in the levels of phosphorylated eIF2α in 293 cells transfected with the P23-FL plasmid (M. Bushell & U.-A. Bommer, unpubl. observation). However, it is possible that the activation of PKR is of a local nature and is not sufficiently widespread to alter the global phosphorylation state of eIF2α. Furthermore, our studies with PKR-knockout cells indicate that a substantial amount of eIF2α phosphorylation is contributed by other kinases (results not shown). Thus, despite the failure to show an effect on global eIF2α phosphorylation, our results overwhelmingly demonstrate that the mRNA of the mammalian translationally controlled protein P23/TCTP binds to and activates the dsRNA-dependent protein kinase PKR.

There are a few reports on other cellular mRNAs that are able to activate PKR. These include the mRNA of PKR itself (Thomis & Samuel, 1993) and the 3′-UTR of α-tropomyosin mRNA (Davis & Watson, 1996). The latter displays tumor-suppressor properties when expressed in different cell lines (Rastinejad & Blau, 1993; Rastinejad et al., 1993), consistent with the possibility that this activity is mediated by activation of PKR (Clemens, 1997). Another candidate mRNA has been reported very recently. The 3′-UTR of myotonic dystrophy protein kinase (DMPK) mRNA contains CUG repeat sequences, which are hugely amplified in myotonic dystrophy patients. These CUG repeats were shown to form secondary structure and to activate PKR in vitro (Tian et al., 2000). Similarly, in Huntington's disease, an increase in CAG repeats of huntingtin mRNA has been linked to the ability of this mRNA to bind to PKR, consistent with an increase in PKR activity in autopsy material from brains of affected individuals (Peel et al., 2001).

It is interesting that several of the mRNAs shown previously to activate PKR code for proteins involved in muscle function or development, as it was recently shown that PKR is likely to be involved in the regulation of myogenic differentiation (Kronfeld-Kinar et al., 1999; Salzberg et al., 2000). Activation of PKR has also been related to the differentiation of mouse embryonic 3T3-F442A cells (Petryshyn et al., 1997). Poly(A)$^+$ RNA from these cells contains a small fraction of dsRNA-rich RNA that activates PKR in homologous cell extracts (Li & Petryshyn, 1991), and recently an RNA species, called R-RNA, has been shown to activate PKR (Petryshyn et al., 1997). Two other types of cellular RNAs have been found to be able to bind to PKR, namely, the Alu RNAs (Chu et al., 1998) and the Y-RNAs (M. James, G. Pruijn, & M.J. Clemens, unpubl. observation). In the former case, binding of the Alu RNAs to PKR results in inhibition of the enzyme.

Translation of P23/TCTP mRNA may be regulated by PKR

The mechanisms involved in regulating the translational efficiency of P23/TCTP mRNA have not yet been studied in much detail. As mentioned earlier, P23/TCTP is an early growth-induced protein. The two classical control mechanisms involved in growth-dependent translational activation of specific mRNAs comprise (1) the multiple phosphorylation of the ribosomal protein S6 through the p70^{S6K} protein kinase, which has been implicated in the translational activation of mRNAs bearing a 5′-TOP (reviewed in Fumagalli & Thomas, 2000;

Meyuhas & Hornstein, 2000); and (2) the activation of the cap-binding initiation factor eIF4E through phosphorylation and concomitant inactivation of the eIF4E binding proteins. The latter mechanism is believed to preferentially activate mRNAs rich in secondary structure (reviewed in Gingras et al., 1999). As P23 mRNA contains a 5′-TOP and is also rich in secondary structure, it is likely that both mechanisms are involved in its translational activation. Both these translational control mechanisms are regulated through mTOR (mammalian target of rapamycin) downstream of the PI-3 kinase signaling pathway (Kleijn et al., 1998; Gingras et al., 1999; Raught & Gingras, 1999; Fumagalli & Thomas, 2000), and indeed rapamycin prevents the growth-induced recruitment of P23 mRNA into polysomes (H.B.J. Jefferies & G. Thomas, pers. comm.) and delays the accumulation of the P23 protein after growth-stimulation of fibroblasts (Y. Gachet & U.-A. Bommer, unpubl. observations) We have also shown that the rate of P23 synthesis is related to eIF4E activity or expression levels (Bommer et al., 1994). Thus, in comparison to other 5′-TOP mRNAs, which are not regulated through eIF4E activation (Shama et al., 1995), P23/TCTP mRNA appears to represent a special case, in that its translational efficiency may be regulated both by the $p70^{S6K}$ pathway and by eIF4E activation.

In this article, we describe yet another mechanism of translational regulation, which impinges on P23/TCTP mRNA, that is, negative regulation through activation of PKR. This conclusion is supported by the following observations: (1) Activation of PKR by expression of full-length P23 mRNA is accompanied by a severe inhibition of P23 mRNA translation (Fig. 5C,E); (2) down-regulation of the rate of P23 synthesis (Fig. 6C) and of P23 protein levels (Fig. 6A), which occurs during serum starvation in normal cells, is abolished in fibroblasts derived from PKR-knockout mice; (3) The observed effects on P23/TCTP expression occur at the *translational* level, as can be concluded from the comparison of P23 mRNA (Fig. 5C) and protein levels (Fig. 5E) in the transfected 293 cells and from the [^{35}S]methionine labeling experiments (Fig. 6C). In addition, we have observed in cell-free translation experiments that full-length P23 mRNA is poorly translated under conditions when PKR is activated, for example, in the rabbit reticulocyte lysate (RRL). However, it is very well translated in the RRL system in the presence of the PKR inhibitor 2-aminopurine, or in the wheat germ system where PKR is absent (results not shown).

Repression of P23 mRNA translation imposed by PKR activation is likely to be mediated by eIF2α phosphorylation. To determine whether there is a possible correlation between a low rate of P23 synthesis and enhanced eIF2α phosphorylation, we monitored eIF2α phosphorylation levels in PKR-knockout and wild-type cells under the same conditions as described in Figure 6A. In both cell lines, the alteration of eIF2α phosphorylation followed essentially the same pattern, that is, a high level during starvation, an initial drop after serum stimulation to about 30% (at 2 h), and adjustment to about 50% at later time points. The only difference between the two cell lines was that, in the PKR-knockout cells, the overall phosphorylation level of eIF2α was about 20% lower at each time point (results not shown). This finding indicates that the contribution of PKR to the overall degree of eIF2α phosphorylation is relatively small in comparison to that of other eIF2α kinases. It also shows that it is impossible to correlate overall eIF2α phosphorylation levels with the specific inhibition of an individual mRNA species by PKR, which in any case may well occur in a localized manner. However, we also performed experiments on mouse embryo fibroblasts derived from "gene knock-in" mice homozygous for a Ser-to-Ala mutation in amino acid position 51 of the eIF2α gene, which renders this initiation factor nonphosphorylatable by PKR and other eIF2α kinases (Scheuner et al., 2001). These cells displayed the same phenotype as PKR-knockout cells with regard to P23/TCTP expression levels during serum starvation and refeeding (U.-A. Bommer, V. Tilleray, M. Clemens, D. Scheuner, & R. Kaufman, unpubl. result), indicating that eIF2α phosphorylation is indeed essential for the PKR-dependent down-regulation of P23 synthesis during serum starvation.

If P23 mRNA is able to bind to and activate PKR and thereby to inhibit its own translation, this raises the question of how and under which cellular conditions such a PKR-mediated translational block is relieved. Our observations on P23/TCTP levels in wild-type and PKR-knockout cells suggest that, in the former, PKR is bound to P23 mRNA and active under serum starvation conditions, but is inactivated upon serum stimulation of the cells. One possibility is that unwinding of secondary structure, brought about by activation of the eIF4F factor complex, is involved in this case. However, different mechanisms acting directly on the PKR enzyme might be implicated as well. Several cellular control mechanisms regulating PKR activity have been described in recent years (for reviews, see Clemens & Elia, 1997; Williams, 1999; Kaufman, 2000; Tan et al., 2000) and it will be important to determine which of these is involved in reversing the PKR-mediated inhibition of P23/TCTP synthesis.

Regulation of PKR activity has been implicated in a variety of cellular conditions, including alteration in intracellular calcium levels (reviewed in Nairn & Palfrey, 1996; Brostrom & Brostrom, 1998). With regard to P23/TCTP synthesis, the calcium-dependent alterations of PKR activity are of particular interest, as the P23/TCTP protein has been repeatedly reported to display calcium-binding properties in a Ca^{2+}-overlay assay. This was demonstrated for the human protein (Sanchez et al., 1997), for TCTP from monkey cells (Xu et al., 1999), and for protozoan homologs of P23/TCTP (Haghighat

& Ruben, 1992; Bhisutthibhan et al., 1999). We obtained the same result with the recombinant mouse protein (U.-A. Bommer & L. Ruben, unpubl. results). Interestingly, the induction of P23/TCTP expression after depletion of ER Ca^{2+} stores has been reported recently, both at the transcriptional and the translational level (Xu et al., 1999). Earlier papers described the activation of PKR upon depletion of ER Ca^{2+} stores (Prostko et al., 1995; Srivastava et al., 1995). These two apparently incompatible observations would appear to rule out the possibility that PKR regulates P23 mRNA translation under ER stress conditions. However, the observed calcium-induced activation of PKR mainly occurs over short time periods, whereas long-term adaptation to ER stress actually results in a decrease rather than an increase of eIF2α phosphorylation (Prostko et al., 1992). In addition, most of the investigations on the PKR response to alterations in cytoplasmic Ca^{2+} levels were performed before the discovery of the ER-associated eIF2α kinase PEK/PERK (Shi et al., 1998; Harding et al., 1999), which was recently shown to be essential for the cellular response to ER stress, inclusive of translational regulation (Harding et al., 2000). Moreover, PERK-knockout in mice led to postnatal damage of the pancreas and progressive diabetes mellitus (Harding et al., 2001). As both kinases, PEK/PERK and PKR, utilize the same molecular mechanism to inhibit protein synthesis, it is often difficult to distinguish between the involvement of one or the other in certain cellular stress responses (reviewed in Ron & Harding, 2000). In fact, a recent study (Kimball et al., 2001) and some earlier observations (reviewed in Nairn & Palfrey, 1996) are incompatible with the view that PKR is involved in Ca^{2+}-dependent regulation of protein synthesis. Therefore, from our present knowledge, a potential involvement of PKR in Ca^{2+}-dependent alterations of P23/TCTP synthesis cannot be judged conclusively, and further work will be necessary to address this issue directly.

As PKR mediates the cellular response to a variety of stress conditions (reviewed in Jagus et al., 1999; Williams, 1999; Kaufman, 2000; Tan et al., 2000), it would be interesting to establish whether regulation of P23/TCTP synthesis is part of such types of stress response mediated by PKR. Presently, there are only few observations on the relation of TCTP synthesis to stress. Apart from the well-documented inhibition under serum starvation (see discussion above and Fig. 6A,C), we observed an immediate cessation of P23 synthesis after exposure of mammalian cells to heat shock (Bommer et al., 1994). On the other hand, there are also cases where TCTP synthesis and stress are positively related, as described above (Sturzenbaum et al., 1998; Xu et al., 1999). Thus, to establish a potential involvement of PKR in stress-mediated regulation of P23/TCTP synthesis, it will be necessary to investigate individual cases of cellular stress responses in more detail.

In summary, we have shown here that a cellular mRNA that encodes a translationally regulated protein is able to bind to and stimulate the activity of the dsRNA-activated protein kinase PKR in vivo and in vitro. This ability to regulate PKR resides in the untranslated regions of the mRNA. Our data suggest that the P23 mRNA is able to activate PKR in a localized fashion in vivo and that such activation can lead to the inhibition of P23 expression at the translational level. This mechanism may provide a paradigm for the regulation by PKR of the synthesis of other proteins encoded by highly structured mRNAs, which are capable of interacting with and activating the protein kinase.

MATERIALS AND METHODS

Cloning and sequencing of mRNAs from a dsRNA-rich fraction of poly(A)$^+$ RNA

To identify cellular mRNAs present in the dsRNA-rich fraction, total poly(A)$^+$ RNA from Daudi cells was chromatographed on Whatman CF-11 cellulose as described earlier (Clarke et al., 1990; Li & Petryshyn, 1991). Briefly, the RNA was adsorbed in STE buffer (20 mM Tris-HCl, pH 6.8, 100 mM NaCl, 1 mM EDTA) containing 35% ethanol, and the ssRNA fraction was eluted with the same buffer containing 15% ethanol. The dsRNA-rich fraction was obtained by elution with STE buffer in the absence of ethanol. This RNA was then reverse transcribed using a modified oligo-dT primer containing an extra 12 nt [designed to allow later cloning into the pDirect vector (Clontech)]. The resulting cDNA:RNA hybrid product was purified using a Chroma Spin-100 column, and a 3′-terminal poly(G)-tail was added to the cDNA strands in a terminal transferase reaction. The cDNAs were then amplified in a PCR reaction using modified oligo-dT and oligo-dC primers. The PCR products were purified using a Chroma Spin-100 column and incubated with T4 DNA polymerase in the presence of dTTP to achieve exonucleolytic degradation of the 3′ terminal ends of the DNA up to the first T encountered. The resulting single-stranded overhangs allowed direct cloning into the pDirect vector by annealing. The DNA was transformed into Sure™ cells (Stratagene), and white colonies were selected. Approximately 50% of the clones contained inserts and five of these were sequenced.

P23/TCTP cDNA plasmids

All P23/TCTP cDNA constructs were based on the mouse cDNA sequence. The primary clones originate from the laboratories of Drs. G. Brawerman (P23-GB) and M. Gaestel (P23-G3/31/13), the former being the only original clone of a full-length P23/TCTP mRNA available at that time. The latter one had been mutagenized to introduce a *Nde*I site at the start codon and a *Bam*HI site immediately downstream of the stop codon. The resulting coding region was subcloned into the vector pT7.7 to yield the plasmid *P23-MG1* suitable for bacterial expression of the protein. All further constructs for the generation of full-length P23 mRNA transcripts and of truncated versions thereof were based on the vector pT7/T3α-18 (Gibco). For technical reasons, it was necessary to

reconstruct the full P23 cDNA sequence in the following way: (1) The *Nde*I-*Bam*HI fragment comprising the coding region of P23/TCTP mRNA was subcloned from P23-MG1 together with an *Eco*RI-*Nde*I linker into the pT7/T3α-18 vector. From this construct (*P23-AB1*), the *Eco*RI-*Kpn*I fragment was removed and replaced by the *Eco*RI-*Kpn*I fragment from the plasmid P23-MG2, a derivative of the original clone P23-GB. (A unique *Kpn*I-site is located 285 nt downstream of the AUG codon.) This step yielded the construct *P23-AB2* comprising the complete 5′-UTR, the coding region, and an additional sequence upstream of the actual 5′-UTR, which originated from the original cloning procedure of P23-GB. (2) The P23-AB2 plasmid was cleaved with *Eco*RI and *Bss*HI, thereby removing the additional sequence together with the first 30 nt of the 5′-UTR. The missing first 30 nt of the P23/TCTP mRNA sequence, inclusive of the *Eco*RI and *Bss*HI sites, were synthesized as a pair of complementary DNA oligos, annealed, and ligated into P23-AB2 plasmid. The resulting construct (*P23-AB3*) contained the complete 5′-UTR and coding region of P23/TCTP cloned in an *Eco*RI site immediately downstream of the T7 polymerase start site. An additional construct lacking the first 30 nt of P23 mRNA (*P23-AB4*) was generated by blunt-ending and religation of plasmid P23-AB2 without insertion of the oligos. (3) The constructs P23-AB3 and P23-AB4 were still devoid of the 3′-UTR of P23 mRNA. To complete the full-length mRNA sequence, both plasmids were digested with *Kpn*I and *Xba*I, the latter site being located in the downstream polylinker. A *Kpn*I-*Xba*I fragment comprising the P23 coding region from the *Kpn*I site plus the complete 3′-UTR was excised from a subclone of the construct P23-G3/31/13, and inserted into the corresponding sites of plasmids P23-AB3 (→ *P23-AB5*) and P23-AB4 (→ *P23-AB8*). The identity of the full-length constructs was checked by sequencing, and the start of the 5′-UTR was verified by primer extension analysis.

To be able to subclone the 5′-UTR of the cDNA separately, a *Nde*I site was introduced at the start codon of construct P23-AB5 by PCR-based mutagenesis. The obtained full-length P23 construct with the *Nde*I site (*P23-MG8*) and another one containing only the P23 coding region beginning with a *Nde*I site (*P23-MG12*) were used in some experiments. For eukaryotic expression of the full-length P23/TCTP mRNA and of the coding region alone, the inserts of plasmids P23-MG8 and P23-MG12 were subcloned into the vector pcDNA3 (Invitrogen) under the control of the CMV promoter. The resulting plasmids are referred to as *pcDNA3-P23-FL* and *pcDNA3-P23-Δ3′5′*, respectively, or briefly as P23-FL and P23-Δ3′5′.

Transcription reactions

Transcriptions were carried out according to standard protocols. For the production of unlabeled RNA transcript, a 50-μL reaction mixture contained 40 mM Tris-HCl, pH 8, 50 mM NaCl, 8 mM $MgCl_2$, 2 mM spermidine, 15 mM DTT, 1.6 mM of each rNTP, 20 U RNAguard, 1 U pyrophosphatase, 2 μg linearized DNA template, and 20 U T7 RNA polymerase. The reaction mixture was incubated for 90 min at 37 °C, then a further 20 U of T7 RNA polymerase were added and the incubation was continued for another 20 min. The DNA was digested with RNase-free Dnase and the transcript was precipitated with LiCl/isopropanol and dissolved in DEPC-treated water. For quantitation, the RNA was trace-labeled with [^{3}H]UTP if required. For capped transcripts, 1.92 mM cap analog was added to the reaction mixture containing only 0.48 mM GTP, and after 90 min of reaction, another 1 mM GTP was added before allowing the reaction to proceed for another 20 min.

^{32}P-labeled RNA transcripts were prepared in 25-μL reaction mixtures containing 0.16 mM UTP and 50 μCi [^{32}P]UTP instead of 1.6 mM UTP. The concentrations of DNA template and T7 RNA polymerase were raised twofold compared to the standard reaction. The ^{32}P-labeled RNA transcripts for the gel mobility assay were prepared using the TransProbe T kit (Pharmacia). Biotinylated transcripts were prepared essentially as unlabeled ones with the exception that 0.16 mM biotinylated UTP was added to the reaction mixture.

Computer prediction of mRNA secondary structure

Predictions of the potential secondary structure of full-length mouse and human P23 mRNA were performed using the RNAFOLD program based on the Zuker algorithm provided by the Human Genome Project computing facilities (http://www.hgmp.mrc.ac.uk/).

RNA electrophoresis

The relative mobility of different RNA species was investigated by polyacrylamide gel electrophoresis under both nondenaturing and denaturing conditions. The samples were run on 8% PAA gels containing 0.2% bisacrylamide with TBE as running buffer. In the denaturing system, 7 M urea was included and electrophoresis was performed at high current to achieve a temperature of about 50 °C. The samples were boiled and chilled immediately before loading. The nondenaturing system was run without urea for 4–5 h at 10 mA at 4 °C. Samples of ^{32}P-labeled RNA (about 20,000 cpm per lane) were prepared in 20 mM Tris-HCl, pH 7.6, 150 mM K-acetate, 5 mM Mg-acetate, 0.1 mM spermidine, incubated for 5 min at 30 °C and for 5 min on ice. The samples were loaded in the presence of 50% glycerol and tracking dyes. After the run, the gels were dried and exposed for autoradiography. As controls, a highly structured portion of the EMCV RNA 5′-UTR [nt 378 to 1155, transcribed from plasmid pTE-17 (Borovjagin et al., 1991; Evstafieva et al., 1991)] and the unstructured Alfalfa Mosaic Virus RNA 4 (cDNA clone provided by Dr. I.N. Shatsky) were run in parallel.

In vitro PKR-binding assays

Precipitation of ^{35}S-labeled PKR with biotinylated P23 mRNA

This assay was performed essentially as described earlier for binding of proteins to U1 RNA (Scherly et al., 1989; Boelens et al., 1993). A capped transcript comprising the coding sequence for the N-terminal 268 amino acids of PKR was generated by transcription of the plasmid pLTheo (linearized with *Bam*HI) using T7-RNA polymerase. The plasmid was constructed and kindly provided by Dr. K. Laing. The tran-

script was translated in a wheat germ extract according to the manufacturer's recommendations (Promega) in the presence of [^{35}S]methionine. Biotinylated RNA transcripts from the plasmids P23-MG8, corresponding to full-length P23/TCTP mRNA (P23-FL), and P23-MG12, representing the truncated version of P23 mRNA, P23 were obtained as described above. The binding reaction was performed in a total volume of 20 μL containing 20 mM HEPES-KOH, pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, 0.05% NP-40, 0.1 μg/μL yeast tRNA, 20 ng biotinylated RNA, and 1 μL of the translation mix containing the [^{35}S]methionine-labeled N-terminal PKR fragment. After incubation for 1 h at room temperature, 20 μL of streptavidin-agarose beads were added, the total volume was made up to 0.5 mL with the same buffer without RNA, and the incubation was continued for another hour on an end-over-end mixer. The beads were collected by centrifugation, washed three times with 0.5 mL buffer and the proteins were eluted with SDS sample buffer. Following SDS PAGE, the gel was incubated in Amplify (Amersham), dried, and exposed for autoradiography.

Filter binding assay

Binding of ^{32}P-labeled P23 mRNA transcripts to PKR was measured by retention on nitrocellulose filters essentially as described earlier (Xiao et al., 1994). Mutant PKR was expressed in the Baculovirus system and purified from Sf-9 cell extracts by ammonium sulphate fractionation and chromatography on DEAE and phosphocellulose columns (Sharp et al., 1993). Increasing amounts of PKR were incubated for 15 min at 30 °C with 40,000 cpm of ^{32}P-labeled RNA in 25-μL reaction mixtures made up in binding buffer containing 10 mM Tris-HCl, pH 7.5, 75 mM KCl, 0.8 mM Mg-acetate, and 1 mg/mL BSA. The reaction mixtures were passed through a Millipore nitrocellulose membrane (0.45 μm pore size) using a 96-well filtration plate. The filters were washed twice with 200 μL of the binding buffer without BSA, dried, and the bound radioactivity determined.

Transient transfection experiments

Activation of PKR was investigated in transient transfection experiments by measuring the inhibition of reporter gene expression *in trans* using two different P23/TCTP mRNA constructs. Human 293 cells were seeded at a density of 9×10^4 cells per well in a 24-well plate and incubated overnight in DMEM containing 10% FCS, antibiotics, and nonessential amino acids (Life Technologies). The cells were then transfected with a mixture of the reporter gene plasmid (pCMV-β-gal, Promega) and one of the following effector plasmids using the calcium phosphate precipitation method: (1) pcDNA3-P23-FL, (2) pcDNA3-P23-Δ3′5′, or (3) empty control vector (pcDNA3). A total amount of 1.5 μg of plasmid DNA was added to each well and the ratio of reporter to effector gene plasmids was generally kept at one in three. In cases where different quantities of effector plasmid were applied, the total amount of effector was made up by addition of the appropriate quantity of the empty control vector. Where indicated, the PKR activator poly (I,C) was added to the medium at a final concentration of 0.1 mg/mL throughout the complete transfection experiment. In the experiment shown in Figure 5B, one of the following additional plasmids was included in the transfection mixture at an equal amount compared to the reporter gene plasmid: (1) pSP6-VA1, expressing wild-type VA1 RNA; (2) pSP6-LS1, expressing a truncated version of VA1 RNA inactive in suppressing PKR activation (generated by Dr. K. Mellits); (3) pCMV-m6PKR, expressing the catalytically inactive mutant (296 K $\rightarrow$ R) form of PKR, or (4) pCMV-PKR, expressing wild-type PKR. These plasmids were kindly provided by Dr. S. Goodbourn. Each transfection was performed in triplicate. The DNA precipitates were left on the cells for 8 h; thereafter, the medium was replaced and the incubation continued for another 36 h. The cells were then harvested, washed in PBS, and resuspended in 30 μL 0.1 M Tris-HCl, pH 7.5, per sample. Cell extracts were prepared by three cycles of freeze-thawing and vortexing for 20 s per cycle. The homogenates were centrifuged for 10 min at 20,000 $\times$ *g* and the supernatants were assayed for β-galactosidase activity using the ONPG assay. Protein concentrations were determined using the Bradford assay and BSA as standard protein. ONPG assays were performed in 96-well plates using a constant amount of protein (usually 1 or 2 μg) in a total assay volume of 200 μL. The plates were incubated for 30 min at 37 °C and the absorbance was read at 414 nm in a microtiter plate reader. After subtraction of background readings (extracts from untransfected cells), the relative β-galactosidase activity was calculated as absorbance units per microgram of total cell protein. The figures show one out of two or three independent experiments.

mRNA quantification

Preparation of cytoplasmic RNA

For the purpose of RNA quantification, coimmunoprecipitation, and western blot analyses, transient transfections were scaled up to the size of 6-cm tissue culture dishes. Total cytoplasmic RNA was prepared according to standard protocols and DNase I-treated to remove remaining plasmid DNA.

Coimmunoprecipitation experiments

The binding of full-length P23/TCTP mRNA and the truncated version (P23-Δ3′5′) to PKR in 293 cells was investigated by coimmunoprecipitation using the monoclonal antibody against human PKR developed by Laurent et al. (1985). A 2.5-μL sample of this antibody was bound to 20 μL protein G-Sepharose beads in IP buffer (see below), without additions. The beads were washed three times in 400 μL wash buffer (IP buffer containing 0.4 M NaCl and 0.1 M KCl) and once with IP buffer. Cell extracts were prepared from transfected 293 cells in IP buffer (20 mM HEPES, pH 7.6, 40 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 10% glycerol, 0.2% NP-40) containing the following additions: 1 mM DTT, 0.1 mM PMSF, and a protease inhibitor cocktail tablet (Complete Mini, minus EDTA; Boehringer Mannheim). The total cell extract obtained from a 6-cm petri dish (about 0.6 mg of protein) was incubated with the preloaded beads in a total volume of 400 μL IP buffer containing 16 U of RNase inhibitor (Ambion) and 0.1 mg/mL yeast tRNA. Incubation was on a rotating wheel for 3 h at 4 °C. The beads were washed three times in 400 μL wash buffer. For the first wash, yeast tRNA

was added at a concentration of 0.1 mg/mL. After washing, the beads were resuspended in 350 μL IP buffer and 5 μg tRNA were added as carrier. The beads were then incubated with 0.1% SDS for 5 min at 70 °C, followed by a proteinase K treatment for 15 min at 37 °C on a rotating wheel. The beads were removed and the RNA recovered by phenol/chloroform extraction and ethanol precipitation. Finally, the RNA samples were subjected to DNase I treatment prior to analysis by real-time RT-PCR.

RNA quantification by real-time RT-PCR

Relative levels of P23/TCTP and β-gal mRNA, respectively, were determined by real-time RT-PCR on cytoplasmic RNA preparations, using an ABI Prism 7700 sequence detection system and the following reagents. The primers and probe for P23/TCTP were based on the mouse cDNA sequence (GenBank accession number X06407). The forward primer, 5′-TGA TCA TCT ACC GGG ACC TCA-3′, covered positions +2 to +22 (relative to the first nucleotide of the start codon), and the reverse primer, 5′-GCG ATC TCC CGG ATC TTG TA-3′, nt +52 to +71. An additional forward primer (5′-CTA GCG CCG CCG TCC-3′) covering positions −27 to −12 was designed to specifically distinguish the P23 mRNA containing the 5′-UTR from the truncated version devoid of the UTRs. The probe, 5′-(6-carboxyl-fluorescein [FAM]) AGC CAT GAC GAG CTG TTC TCC GAC A(6-carboxyl-tetramethyl-rhodamine [TAMRA])-3′, covered nucleotides +25 to +49. To quantify the β-gal mRNA, the following primers and probe were generated: forward primer, 5′-GCT GGC TGG AGT GCG ATC-3′, reverse primer, 5′-GTT GGT GTA GAT GGG CGC AT-3′, probe, 5′-(6-carboxyl-fluorescein [FAM]) TCC TGA GGC CGA TAC TGT CGT CGT C(6-carboxyl-tetramethyl-rhodamine [TAMRA])-3′. The probes were purchased from MWG Biotech UK, and the primers were obtained from Sigma-Genosys UK. The probe and primers for β-actin, as documented elsewhere (Greenhead et al., 2000), were kindly provided by Drs. P. Watts and R. Shattock. Total cytoplasmic RNA samples were prepared as described above. A full-length P23/TCTP mRNA transcript and the pCMV-β-gal plasmid, respectively, were used as positive controls, and yeast tRNA served as a negative control RNA. The RT-PCR reactions were performed in 96-well Micro-Amp plates (PE Applied Biosystems) using the OneStep RT-PCR kit from Qiagen, UK. This kit contains the Omniscript Reverse Transcriptase, which is suitable to transcribe particularly structured RNA molecules. The reverse transcription reaction was performed for 30 min at 50 °C followed by a 15-min PCR activation step at 95 °C and by 40 amplification cycles consisting of a 15-s dissociation step at 95 °C and a 30-s annealing and elongation reaction at 60 °C. Initial optimization experiments demonstrated that this system operates over a wide dynamic range and is able to discriminate against the human P23/TCTP mRNA sequence endogenous to 293 cells and between full-length mouse P23 mRNA and the truncated form deleted in the 5′-UTR.

Protein analyses

Cell culture

P23/TCTP protein levels were monitored by western blotting in mouse embryo fibroblasts (MEFs) derived from PKR-knockout mice in comparison to MEFs from a corresponding wild-type strain. These cell lines (provided by the laboratory of Dr. C. Weissmann) were cultivated in DMEM containing streptomycin/penicillin, nonessential amino acids, 0.1 mM β-mercaptoethanol, and 10% FCS. To investigate the growth-dependent regulation of P23 levels, cells were grown to confluency in 15-cm petri dishes, serum starved (0.5% FCS) for 20 h and restimulated by the addition of 20% FCS for 2, 4, 6, and 8 h. The cells were harvested, cytoplasmic cell extracts prepared, and the protein concentration determined using the Bradford assay and BSA as standard protein. To monitor protein levels in 293 cells after transfection of P23 cDNA constructs, cells were transfected in 6-cm dishes as described above and the proteins were quantified in cell extracts by western blotting.

Western blotting

Equal amounts of protein per lane were loaded on to SDS-gels (usually 12.5% in acrylamide). Blots were probed with the relevant antibody and with a monoclonal anti-α (or anti-β) tubulin antibody (Amersham-Pharmacia) for a loading control, and were developed using the ECL system (Amersham-Pharmacia). The signals of the autoradiographs were quantified using the ImageQuant software (Molecular Dynamics). The signals were corrected for the loading control. Usually, the values for three experiments were averaged and plotted with the standard deviation, using the Excel-97 computer program. The generation of polyclonal antipeptide antibodies against mouse P23/TCTP (Bommer et al., 1994) and polyclonal antibodies against the recombinant mouse P23/TCTP (Gachet et al., 1999) have been described earlier. In most cases, an antibody (Q6) reacting with mouse and human P23 was used. For the purpose of distinction between the endogenous human and the ectopically expressed mouse protein in transfected 293 cells (Fig. 5E), the antibody P6 (specific for mouse P23) and a monoclonal antibody against human P23/TCTP (provided by Dr. J.-C. Sanchez, Geneva) were employed. A phospho-specific antibody against eIF2α (Research Genetics) and a monoclonal antibody detecting total eIF2α (Scorsone et al., 1987) were used to investigate eIF2α phosphorylation levels.

[^{35}S]Methionine incorporation

To investigate the relative rate of P23/TCTP synthesis, [^{35}S]methionine incorporation experiments were performed on wild-type and PKR$^{-/-}$ cells. The cells were grown to confluency in 6-well plates and serum starved (0.5% FCS) for 20 h. After preincubation in low-methionine (10 μM) medium for 1 h, cells were either left untreated or restimulated with 20% dialyzed FCS in the presence or absence of 5 μM actinomycin D. Thirty minutes after addition of the growth signal, the cells were pulsed for 1 h with 80 μCi [^{35}S]methionine per milliliter of medium and chased for another 20 min with DMEM containing the normal concentration of unlabeled methionine and the appropriate stimulus. Cell extracts were prepared, counted for radioactivity, and equal amounts (10^6 cpm per lane) analyzed on 16-cm-long SDS gels. The gels were either dried directly or electrotransferred onto PVDF membranes prior to scanning in a STORM phosphorimager and

quantification of the appropriate bands using the ImageQuant software (Molecular Dynamics). Western blotting and alignment with the radioactivity pattern confirmed the identity of the P23 band. The relative [^{35}S]methionine incorporation into P23/TCTP was calculated after subtraction of a background value from the same lane and after normalization for the signal of a protein in the vicinity that is not growth regulated. The figures obtained from three experiments were averaged.

NOTE ADDED IN PROOF

While this article was in revision, a paper was published describing interferon-γ mRNA as another case of a cellular mRNA that activates PKR and inhibits its own translation (Ben-Asouli et al., *Cell*, 2002, *108*:221–232).

ACKNOWLEDGMENTS

We thank Miss Dipali Patel for plasmid preparations and cell transfection studies. We appreciate the help of Dr. M. Bushell for initial studies to quantify eIF2α phosphorylation levels and of Dr. M. Rau (University of Sussex) for generating capped mRNA transcripts. Dr. R. Harbottle (London) was instrumental in performing RNA secondary structure predictions and Dr. Y. Gachet helped to establish the cultivation of a recombinant Baculovirus in insect Sf9 cells for the production of mutant PKR, initially provided by Dr. D. Gewert (formerly Wellcome Laboratories, Beckenham, UK). The provision of valuable plasmids and other materials from various colleagues is highly appreciated: Dr. M. Gaestel (formerly Max-Delbrueck-Centrum, Berlin) provided the original mouse P23 cDNA clones. We thank Dr. S. Goodbourn for helpful discussions and for providing the plasmids encoding VA1 RNA, an inactive mutant of VA1 RNA, wild-type PKR, and a dominant negative mutant of PKR. PKR-knockout and wild-type control cells were obtained from the laboratory of Dr. C. Weissmann (Zürich).

This work was funded by project grants from the Wellcome Trust (to U.A.B. and M.J.C.) and from the Leukaemia Research Fund (to M.J.C.), with further support from the Cancer Prevention Research Trust. A.V.B. was the recipient of a Postdoctoral Fellowship from the Royal Society and M.L. was supported by a Ph.D. studentship from St. George's Hospital Medical School.

Received December 28, 2000; returned for revision February 13, 2001; revised manuscript received December 21, 2001

REFERENCES

Adams MD et al. 1995. Initial assessment of human gene diversity and expression pattern based upon 83 million nucleotides of cDNA sequence. *Nature 377*:3–174.

Baudet C, Perret E, Delpech B, Kaghad M, Brachet P, Wion D, Caput D. 1998. Differentially expressed genes in C6.9 glioma cells during vitamin D-induced cell death program. *Cell Death Differ 5*:116–125.

Benndorf R, Nuernberg P, Bielka H. 1988. Growth phase-dependent proteins of the Ehrlich ascites tumor analyzed by one- and two-dimensional electrophoresis. *Exp Cell Res 174*:130–138.

Bhisutthibhan J, Pan XQ, Hossler PA, Walker DJ, Yowell CA, Carlton J, Dame JB, Meshnick SR. 1998. The *Plasmodium falciparum* translationally controlled tumor protein homolog and its reaction with the antimalarial drug artemisinin. *J Biol Chem 273*:16192–16198.

Bhisutthibhan J, Philbert MA, Fujioka H, Aikawa M, Meshnick SR. 1999. The *Plasmodium falciparum* translationally controlled tumor protein: Subcellular localization and calcium binding. *Eur J Cell Biol 78*:665–670.

Bhisutthibhan J, Meshnick SR. 2001. Immunoprecipitation of [(3)H]dihydroartemisinin translationally controlled tumor protein (TCTP) adducts from *Plasmodium falciparum*-infected erythrocytes by using anti-TCTP antibodies. *Antimicrob Agents Chemother 45*: 2397–2399.

Bischoff JR, Samuel CE. 1989. Mechanism of interferon action. Activation of the human P1/eIF-2 alpha protein kinase by individual reovirus s-class mRNAs: s1 mRNA is a potent activator relative to s4 mRNA. *Virology 172*:106–115.

Boehm H, Benndorf R, Gaestel M, Gross B, Nuernberg P, Kraft R, Otto A, Bielka H. 1989. The growth-related protein P23 of the Ehrlich ascites tumor: Translational control, cloning and primary structure. *Biochem Int 19*:277–286.

Boehm H, Gross B, Gaestel M, Bommer UA, Ryffel G, Bielka H. 1991. The 5′-untranslated region of p23 mRNA from the Ehrlich ascites tumor is involved in translation control of the growth related protein p23. *Biomed Biochim Acta 50*:1193–1203.

Boelens WC, Jansen EJ, van Venrooij WJ, Stripecke R, Mattaj IW, Gunderson SI. 1993. The human U1 snRNP-specific U1A protein inhibits polyadenylation of its own pre-mRNA. *Cell 72*:881–892.

Bommer UA, Lazaris-Karatzas A, De Benedetti A, Nurnberg P, Benndorf R, Bielka H, Sonenberg N. 1994. Translational regulation of the mammalian growth-related protein P23: Involvement of eIF-4E. *Cell Mol Biol Res 40*:633–641.

Bonnet C, Perret E, Dumont X, Picard A, Caput D, Lenaers G. 2000. Identification and transcription control of fission yeast genes repressed by an ammonium starvation growth arrest. *Yeast 16*: 23–33.

Borovjagin AV, Ezrokhi MV, Rostapshov VM, Ugarova TY, Bystrova TF, Shatsky IN. 1991. RNA–protein interactions within the internal translation initiation region of encephalomyocarditis virus RNA. *Nucleic Acids Res 19*:4999–5005.

Brinegar JD, Hampel A, Alter GM, Cruz P. 1997. The secondary structure of a fourteen-nucleotide fragment of the hairpin ribozyme. *Biochem Biophys Res Commun 232*:444–448.

Brostrom CO, Brostrom MA. 1998. Regulation of translational initiation during cellular responses to stress. *Prog Nucleic Acid Res Mol Biol 58*:79–125.

Chu WM, Ballard R, Carpick BW, Williams BR, Schmid CW. 1998. Potential Alu function: Regulation of the activity of double-stranded RNA-activated kinase PKR. *Mol Cell Biol 18*:58–68.

Chung S, Kim M, Choi W, Chung J, Lee K. 2000. Expression of translationally controlled tumor protein mRNA in human colon cancer. *Cancer Lett 156*:185–190.

Clarke PA, Sharp NA, Arrand JR, Clemens MJ. 1990. Epstein–Barr virus gene expression in interferon-treated cells. Implications for the regulation of protein synthesis and the antiviral state. *Biochim Biophys Acta 1050*:167–173.

Clemens MJ. 1996. Protein kinases that phosphorylate eIF2 and eIF2B, and their role in eukaryotic cell translational control. In: Hershey JWB, Mathews MB, Sonenberg N, eds. *Translational control*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 139–162.

Clemens MJ. 1997. PKR—A protein kinase regulated by double-stranded RNA. *Int J Biochem Cell Biol 29*:945–949.

Clemens MJ, Bommer UA. 1999. Translational control: The cancer connection. *Int J Biochem Cell Biol 31*:1–23.

Clemens MJ, Elia A. 1997. The double-stranded RNA-dependent protein kinase PKR: Structure and function. *J Interferon Cytokine Res 17*:503–524.

Davis S, Watson JC. 1996. In vitro activation of the interferon-induced, double-stranded RNA-dependent protein kinase PKR by RNA from the 3′ untranslated regions of human alpha-tropomyosin. *Proc Natl Acad Sci USA 93*:508–513.

De Benedetti A, Baglioni C. 1984. Inhibition of mRNA binding to ribosomes by localized activation of dsRNA-dependent protein kinase. *Nature 311*:79–81.

Evstafieva AG, Ugarova TY, Chernov BK, Shatsky IN. 1991. A complex RNA sequence determines the internal initiation of encephalomyocarditis virus RNA translation. *Nucleic Acids Res 19*: 665–671.

Fumagalli S, Thomas G. 2000. S6 phosphorylation and signal transduction. In: Sonenberg N, Hershey JWB, Mathews MB, eds. *Translational control of gene expression*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 695–727.

Gachet Y, Tournier S, Lee M, Lazaris-Karatzas A, Poulton T, Bommer UA. 1999. The growth-related, translationally controlled protein P23 has properties of a tubulin binding protein and associates transiently with microtubules during the cell cycle. *J Cell Sci 112*:1257–1271.

Gast F-U, Kempe D, Spieker RL, Saenger HL. 1996. Secondary structure probing of potato spindle tuber viroid (PSTVd) and sequence comparison with other small pathogenic RNA replicons provides evidence for central noncanonical base-pairs, large A-rich loops, and a terminal branch. *J Mol Biol 262*:652–670.

Gingras AC, Raught B, Sonenberg N. 1999. eIF4 initiation factors: Effectors of mRNA recruitment to ribosomes and regulators of translation. *Annu Rev Biochem 68*:913–963.

Gray NK, Hentze MW. 1994. Regulation of protein synthesis by mRNA structure. *Mol Biol Rep 19*:195–200.

Greenhead P, Hayes P, Watts PS, Laing KG, Griffin GE, Shattock RJ. 2000. Parameters of human immunodeficiency virus infection of human cervical tissue and inhibition by vaginal virucides. *J Virol 74*:5577–5586.

Gross B, Gaestel M, Bohm H, Bielka H. 1989. cDNA sequence coding for a translationally controlled human tumor protein. *Nucleic Acids Res 17*:8367.

Guillaume E, Pineau C, Evrard B, Dupaix A, Moertz E, Sanchez JC, Hochstrasser DF, Jegou B. 2001. Cellular distribution of translationally controlled tumor protein in rat and human testes. *Proteomics 1*:880–889.

Haghighat NG, Ruben L. 1992. Purification of novel calcium binding proteins from *Trypanosoma brucei*: Properties of 22-, 24- and 38-kilodalton proteins. *Mol Biochem Parasitol 51*:99–110.

Harding HP, Zhang Y, Ron D. 1999. Protein translation and folding are coupled by an endoplasmic reticulum-resident kinase. *Nature 397*:271–274.

Harding HP, Zeng H, Zhang Y, Jungries R, Chung P, Plesken H, Sabatini DD, Ron D. 2001. Diabetes mellitus and exocrine pancreatic dysfunction in perk−/− mice reveals a role for translational control in secretory cell survival. *Mol Cell 7*:1153–1163.

Harding HP, Zhang Y, Bertolotti A, Zeng H, Ron D. 2000. Perk is essential for translational regulation and cell survival during the unfolded protein response. *Mol Cell 5*:897–904.

Jagus R, Joshi B, Barber GN. 1999. PKR, apoptosis and cancer. *Int J Biochem Cell Biol 31*:123–138.

Kang HS, Lee MJ, Song H, Han SH, Kim YM, Im JY, Choi I. 2001. Molecular identification of IgE-dependent histamine-releasing factor as a B cell growth factor. *J Immunol 166*:6545–6554.

Kaufman RJ. 2000. The double-stranded RNA-activated protein kinase PKR. In: Sonenberg N, Hershey JWB, Mathews MB, eds. *Translational control of gene expression*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 503–527.

Kimball SR, Clemens MJ, Tilleray VJ, Wek RC, Horetsky RL, Jefferson LS. 2001. The double-stranded RNA-activated protein kinase PKR is dispensable for regulation of translation in response to either calcium mobilization from the endoplasmic reticulum of essential amino acid starvation. *Biochem Biophys Res Commun 280*:293–300.

Kleijn M, Scheper GC, Voorma HO, Thomas AA. 1998. Regulation of translation initiation factors by signal transduction. *Eur J Biochem 253*:531–544.

Kronfeld-Kinar Y, Vilchik S, Hyman T, Leibkowicz F, Salzberg S. 1999. Involvement of PKR in the regulation of myogenesis. *Cell Growth Differ 10*:201–212.

Kumar KU, Srivastava SP, Kaufman RJ. 1999. Double-stranded RNA-activated protein kinase (PKR) is negatively regulated by 60S ribosomal subunit protein L18. *Mol Cell Biol 19*:1116–1125.

Langereis K, Mugnier M-A, Cornelissen BJC, Pinck L, Bol JF. 1986. Variable repeats and poly(A)-stretches in the leader sequence of alfalfa mosaic virus RNA 3. *Virol*ogy *154*:409–414.

Laurent AG, Krust B, Galabru J, Svab J, Hovanessian AG. 1985. Monoclonal antibodies to an interferon-induced Mr 68,000 protein and their use for the detection of double-stranded RNA-dependent protein kinase in human cells. *Proc Natl Acad Sci USA 82*:4341–4345.

Li J, Petryshyn RA. 1991. Activation of the double-stranded RNA-dependent eIF-2 alpha kinase by cellular RNA from 3T3-F442A cells. *Eur J Biochem 195*:41–48.

MacDonald SM. 1997. Human recombinant histamine-releasing factor. *Int Arch Allergy Immunol 113*:187–189.

MacDonald SM, Paznekas WA, Jabs EW. 1999. Chromosomal localization of tumor protein, translationally-controlled 1 (TPT1) encoding the human histamine releasing factor (HRF) to 13q12 → q14. *Cytogenet Cell Genet 84*:128–129.

MacDonald SM, Rafnar T, Langdon J, Lichtenstein LM. 1995. Molecular identification of an IgE-dependent histamine-releasing factor. *Science 269*:688–690.

Mellits KH, Mathews MB. 1988. Effects of mutations in stem and loop regions on the structure and function of adenovirus VA RNAI. *EMBO J 7*:2849–2859.

Mellits KH, Pe'ery T, Manche L, Robertson HD, Mathews MB. 1990. Removal of double-stranded contaminants from RNA transcripts: Synthesis of adenovirus VA RNAI from a T7 vector. *Nucleic Acids Res 18*:5401–5406.

Meyuhas O, Hornstein E. 2000. Translational control of TOP mRNAs. In: Sonenberg N, Hershey JWB, Mathews MB, eds. *Translational control of gene expression*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 671–693.

Nairn AC, Palfrey HC. 1996. Regulation of protein synthesis by calcium. In: Hershey JWB, Mathews MB, Sonenberg N, eds. *Translational control of gene expression*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 295–318.

Osman F, Jarrous N, Ben Asouli Y, Kaempfer R. 1999. A *cis*-acting element in the 3′-untranslated region of human TNF-alpha mRNA renders splicing dependent on the activation of protein kinase PKR. *Genes & Dev 13*:3280–3293.

Peel AL, Rao RV, Cottrell BA, Hayden MR, Ellerby LM, Bredesen DE. 2001. Double-stranded RNA-dependent protein kinase, PKR, binds preferentially to Huntington's disease (HD) transcripts and is activated in HD tissue. *Hum Mol Genet 10*:1531–1538.

Petryshyn RA, Ferrenz AG, Li J. 1997. Characterization and mapping of the double-stranded regions involved in activation of PKR within a cellular RNA from 3T3-F442A cells. *Nucleic Acids Res 25*:2672–2678.

Pratt G, Galpine A, Sharp N, Palmer S, Clemens MJ. 1988. Regulation of in vitro translation by double-stranded RNA in mammalian cell mRNA preparations. *Nucleic Acids Res 16*:3497–3510.

Prostko CR, Brostrom MA, Malara EM, Brostrom CO. 1992. Phosphorylation of eukaryotic initiation factor (eIF) 2 alpha and inhibition of eIF-2B in GH3 pituitary cells by perturbants of early protein processing that induce GRP78. *J Biol Chem 267*:16751–16754.

Prostko CR, Dholakia JN, Brostrom MA, Brostrom CO. 1995. Activation of the double-stranded RNA-regulated protein kinase by depletion of endoplasmic reticular calcium stores. *J Biol Chem 270*:6211–6215.

Rastinejad F, Blau HM. 1993. Genetic complementation reveals a novel regulatory role for 3′ untranslated regions in growth and differentiation. *Cell 72*:903–917.

Rastinejad F, Conboy MJ, Rando TA, Blau HM. 1993. Tumor suppression by RNA from the 3′ untranslated region of alpha-tropomyosin. *Cell 75*:1107–1117.

Raught B, Gingras AC. 1999. eIF4E activity is regulated at multiple levels. *Int J Biochem Cell Biol 31*:43–57.

Robertson HD, Mathews MB. 1996. The regulation of the protein kinase PKR by RNA. *Biochimie 78*:909–914.

Ron D, Harding HP. 2000. PERK and translational control by stress in the endoplasmic reticulum. In: Sonenberg N, Hershey JWB, Mathews MB, eds. *Translational control of gene expression*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 547–560.

Sage-Ono K, Ono M, Harada H, Kamada H. 1998. Dark-induced accumulation of mRNA for a homolog of translationally controlled tumor protein (TCTP) in *Pharbitis*. *Plant Cell Physiol 39*:357–360.

Salzberg S, Vilchik S, Cohen S, Heller A, Kronfeld-Kinar Y. 2000.

Expression of a PKR dominant-negative mutant in myogenic cells interferes with the myogenic process. *Exp Cell Res 254*:45–54.
Sanchez JC, Schaller D, Ravier F, Golaz O, Jaccoud S, Belet M, Wilkins MR, James R, Deshusses J, Hochstrasser D. 1997. Translationally controlled tumor protein: A protein identified in several nontumoral cells including erythrocytes. *Electrophoresis 18*:150–155.
Scherly D, Boelens W, van Venrooij WJ, Dathan NA, Hamm J, Mattaj IW. 1989. Identification of the RNA binding segment of human U1 A protein and definition of its binding site on U1 snRNA. *EMBO J 8*:4163–4170.
Scheuner D, Song B, McEwen E, Liu C, Laybutt R, Gillespie P, Saunders T, Bonner-Weir S, Kaufman RJ. 2001. Translational control is required for the unfolded protein response and in vivo glucose homeostasis. *Mol Cell 7*:1165–1176.
Schroeder JT, Lichtenstein LM, MacDonald SM. 1996. An immunoglobulin E-dependent recombinant histamine-releasing factor induces interleukin-4 secretion from human basophils. *J Exp Med 183*:1265–1270.
Scorsone KA, Panniers R, Rowlands AG, Henshaw EC. 1987. Phosphorylation of eukaryotic initiation factor 2 during physiological stresses which affect protein synthesis. *J Biol Chem 262*:14538–14543.
Shama S, Avni D, Frederickson RM, Sonenberg N, Meyuhas O. 1995. Overexpression of initiation factor eIF-4E does not relieve the translational repression of ribosomal protein mRNAs in quiescent cells. *Gene Expr 4*:241–252.
Sharp TV, Xiao Q, Jeffrey I, Gewert DR, Clemens MJ. 1993. Reversal of the double-stranded-RNA-induced inhibition of protein synthesis by a catalytically inactive mutant of the protein kinase PKR. *Eur J Biochem 214*:945–948.
Shi Y, Vattem KM, Sood R, An J, Liang J, Stramm L, Wek RC. 1998. Identification and characterization of pancreatic eukaryotic initiation factor 2 alpha-subunit kinase, PEK, involved in translational control. *Mol Cell Biol 18*:7499–7509.
Sonenberg N, Hershey JWB, Mathews MB. 2000. *Translational control of gene expression*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.
Srivastava SP, Davies MV, Kaufman RJ. 1995. Calcium depletion from the endoplasmic reticulum activates the double-stranded RNA-dependent protein kinase (PKR) to inhibit protein synthesis. *J Biol Chem 270*:16619–16624.
Sturzenbaum SR, Kille P, Morgan AJ. 1998. Identification of heavy metal induced changes in the expression patterns of the translationally controlled tumor protein (TCTP) in the earthworm *Lumbricus rubellus*. *Biochim Biophys Acta 1398*:294–304.
Tan SL, Katze MG. 1999. The emerging role of the interferon-induced PKR protein kinase as an apoptotic effector: A new face of death? *J Interferon Cytokine Res 19*:543–554.
Tan SL, Katze MG, Gale MJ Jr. 2000. Translational reprogramming during Influenza virus infection. In: Sonenberg N, Hershey JWB, Mathews MB, eds. *Translational control of gene expression*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 933–950.
Terenzi F, deVeer MJ, Ying H, Restifo NP, Williams BR, Silverman RH. 1999. The antiviral enzymes PKR and RNase L suppress gene expression from viral and nonviral based vectors. *Nucleic Acids Res 27*:4369–4375.
Thaw P, Baxter NJ, Hounslow AM, Price C, Waltho JP, Craven CJ. 2001. Structure of TCTP reveals unexpected relationship with guanine nucleotide-free chaperones. *Nat Struct Biol 8*:701–704.
Thiele H, Berger M, Lenzner C, Kuhn H, Thiele BJ. 1998. Structure of the promoter and complete sequence of the gene coding for the rabbit translationally controlled tumor protein (TCTP) P23. *Eur J Biochem 257*:62–68.
Thiele H, Berger M, Skalweit A, Thiele BJ. 2000. Expression of the gene and processed pseudogenes encoding the human and rabbit translationally controlled tumour protein (TCTP). *Eur J Biochem 267*:5473–5481.
Thomas G. 1986. Translational control of mRNA expression during the early mitogenic response in Swiss mouse 3T3 cells: Identification of specific proteins. *J Cell Biol 103*:2137–2144.
Thomas G, Luther H. 1981. Transcriptional and translational control of cytoplasmic proteins after serum stimulation of quiescent Swiss 3T3 cells. *Proc Natl Acad Sci USA 78*:5712–5716.
Thomis DC, Samuel CE. 1993. Mechanism of interferon action: Evidence for intermolecular autophosphorylation and autoactivation of the interferon-induced, RNA-dependent protein kinase PKR. *J Virol 67*:7695–7700.
Tian B, White RJ, Xia T, Welle S, Turner DH, Mathews MB, Thornton CA. 2000. Expanded CUG repeat RNAs form hairpins that activate the double-stranded RNA-dependent protein kinase PKR. *RNA 6*:79–87.
Walsh BJ, Gooley AA, Williams KL, Breit SN. 1995. Identification of macrophage activation associated proteins by two-dimensional gel electrophoresis and microsequencing. *J Leukocyte Biol 57*:507–512.
Williams BR. 1999. PKR; a sentinel kinase for cellular stress. *Oncogene 18*:6112–6120.
Willis AE. 1999. Translational control of growth factor and proto-oncogene expression. *Int J Biochem Cell Biol 31*:73–86.
Wu S, Kaufman RJ. 1997. A model for the double-stranded RNA (dsRNA)-dependent dimerization and activation of the dsRNA-activated protein kinase PKR. *J Biol Chem 272*:1291–1296.
Wu S, Rehemtulla A, Gupta NK, Kaufman RJ. 1996. A eukaryotic translation initiation factor 2-associated 67 kDa glycoprotein partially reverses protein synthesis inhibition by activated double-stranded RNA-dependent protein kinase in intact cells. *Biochemistry 35*:8275–8280.
Wu SY, Kaufman RJ. 1996. Double-stranded (ds) RNA binding and not dimerization correlates with the activation of the dsRNA-dependent protein kinase (PKR) *J Biol Chem 271*:1756–1763.
Xiao Q, Sharp TV, Jeffrey IW, James MC, Pruijn GJM, vanVenrooij WJ, Clemens MJ. 1994. The La antigen inhibits the activation of the interferon-inducible protein kinase PKR by sequestering and unwinding double-stranded RNA. *Nucleic Acids Res 22*:2512–2518.
Xu A, Bellamy AR, Taylor JA. 1999. Expression of translationally controlled tumor protein is regulated by calcium at both the transcriptional and post-transcriptional level. *Biochem J 342*:683–689.
Yan L, Fei KY, Bridge D, Sarras MP, Jr. 2000. A cnidarian homologue of translationally controlled tumor protein (P23/TCTP) *Dev Genes Evol 210*:507–511.
Yenofsky R, Bergmann I, Brawerman G. 1982. Messenger RNA species partially in a repressed state in mouse sarcoma ascites cells. *Proc Nat Acad Sci USA 79*:5876–5880.
Yenofsky R, Cereghini S, Krowczynska A, Brawerman G. 1983. Regulation of mRNA utilization in mouse erythroleukemia cells induced to differentiate by exposure to dimethyl sulfoxide. *Mol Cell Biol 3*:1197–1203.