Regulation of mammalian ribonucleotide reductase R1 mRNA stability is mediated by a ribonucleotide reductase R1 mRNA 3′-untranslated region *cis–trans* interaction through a protein kinase C-controlled pathway

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Ribonucleotide reductase catalyses the reaction that eventually provides the four deoxyribonucleotides required for the synthesis and repair of DNA. U.v.-cross-linking and band-shift experiments have identified in COS 7 monkey cells an approx. 57 kDa ribonucleotide reductase R1 mRNA-binding protein called R1BP, which binds specifically to a 49-nt region of the R1 mRNA 3′-untranslated region (3′UTR). The R1BP–RNA binding activity was down-regulated by the tumour promoters phorbol 12-myristate 13-acetate (PMA; 'TPA') and okadaic acid, and up-regulated by the protein kinase C inhibitor staurosporine, in a dose-dependent fashion. Furthermore, staurosporine treatment decreased the stability of R1 and CAT (chloramphenicol acetyltransferase)/R1 hybrid mRNAs, whereas PMA and okadaic acid increased the stability of these messages, in a dose-dependent manner. In contrast, treatment of cells with forskolin, a protein kinase A inhibitor, did not alter either R1BP–RNA binding or R1 mRNA-stability characteristics. Transfectants containing R1 or CAT/R1 cDNA constructs with a deletion of the 49-nt 3′UTR sequence failed to respond in message-stability studies to the effects of PMA, staurosporine or okadaic acid. These observations indicate that a protein kinase C signal pathway regulates ribonucleotide reductase R1 gene expression post-transcriptionally, through a mechanism involving a specific *cis–trans* interaction at a 49-nt region within the R1 mRNA 3′UTR.

INTRODUCTION

Mammalian ribonucleotide reductase is responsible for the *de novo* conversion of ribonucleotides into the four deoxyribonucleotides required for the synthesis of DNA, and therefore plays a critical role in cell division and cell proliferation [1,2]. The enzyme consists of two dissimilar protein components often called R1 and R2. R1 is a dimer with an M_r of 170000 and contains binding sites for substrates and allosteric effectors, whereas R2 is a dimer with an M_r of 88000 and contains iron and a tyrosyl free radical that is essential for ribonucleotide reduction [1,3]. Enzyme activity requires both components, but different mechanisms regulate R1 and R2 during cell proliferation [1], and changes in enzyme activity and in R1 and/or R2 gene expression have been observed under a variety of environmental conditions [1–6]. The importance of ribonucleotide reductase for cell proliferation is further emphasized by observations that the mechanisms controlling ribonucleotide reductase gene expression may be altered in some malignant conditions [2–7], and are involved in the early events underlying the action of the tumour promoter phorbol 12-myristate 13-acetate (PMA; 'TPA') [1]. For example, we have recently shown that treatment of mouse 3T3 fibroblasts with PMA significantly increases the steady-state level and the stability of the R1 subunit mRNA [8]. Furthermore, we have proposed a model of R1 gene regulation in which a *cis*-element within a 49-nt sequence of the 3′-untranslated region (3′UTR) of the R1 message interacts with a cytoplasmic protein (R1BP) in a mechanism that regulates R1 message stability and is responsive to PMA treatment [8].

Although important details concerning the mode of action of PMA are still unknown, many of its diverse effects (see, e.g., [9–11]) appear to be mediated through the regulation of protein kinase C (PKC), a high-affinity receptor for PMA [12,13]. Also, it has been demonstrated that PMA can modulate the transcription of such important growth-controlling genes as c-*myc* [14,15], c-*fos* [15] and c-*sis* [16], through *cis–trans* interactions between responsive DNA elements and binding proteins. The activity of the *trans*-acting proteins (e.g. AP-1 and AP-2) can be regulated through the action of PKC in response to PMA treatment [17,18]. Although it is now clear from several recent studies that *cis–trans* interactions are also involved in post-transcriptional regulation of gene expression [19,20], relatively little is known about the signal pathway(s) that control PMA-mediated alterations in gene expression through direct modifications in the *cis–trans* reactions that regulate message-stability properties [8,21]. The present study was undertaken to provide information on this topic.

MATERIALS AND METHODS

Cell lines and culture conditions

Mouse BALB/c 3T3 cells and monkey COS 7 cells transfected with R1 or CAT (chloramphenicol acetyltransferase)/R1 hybrid constructs (Figure 1b) were routinely cultured at 37 °C in α-minimal essential medium (Flow Laboratories) supplemented with antibiotics and 10% (v/v) fetal-bovine serum (Intergene Co.) [8,22]. For determining R1 and CAT/R1 mRNA levels and R1BP–RNA binding, exponentially growing cells were treated

Abbreviations used: PMA, phorbol 12-myristate 13-acetate ('TPA'); PKC, protein kinase C; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DAG, 1,2-diacylglycerol; DMSO, dimethyl sulphoxide; SSC, 0.15 M NaCl/0.015 M sodium citrate; R1BP, ribonucleotide reductase R1 mRNA-binding protein; CAT, chloramphenicol acetyltransferase.

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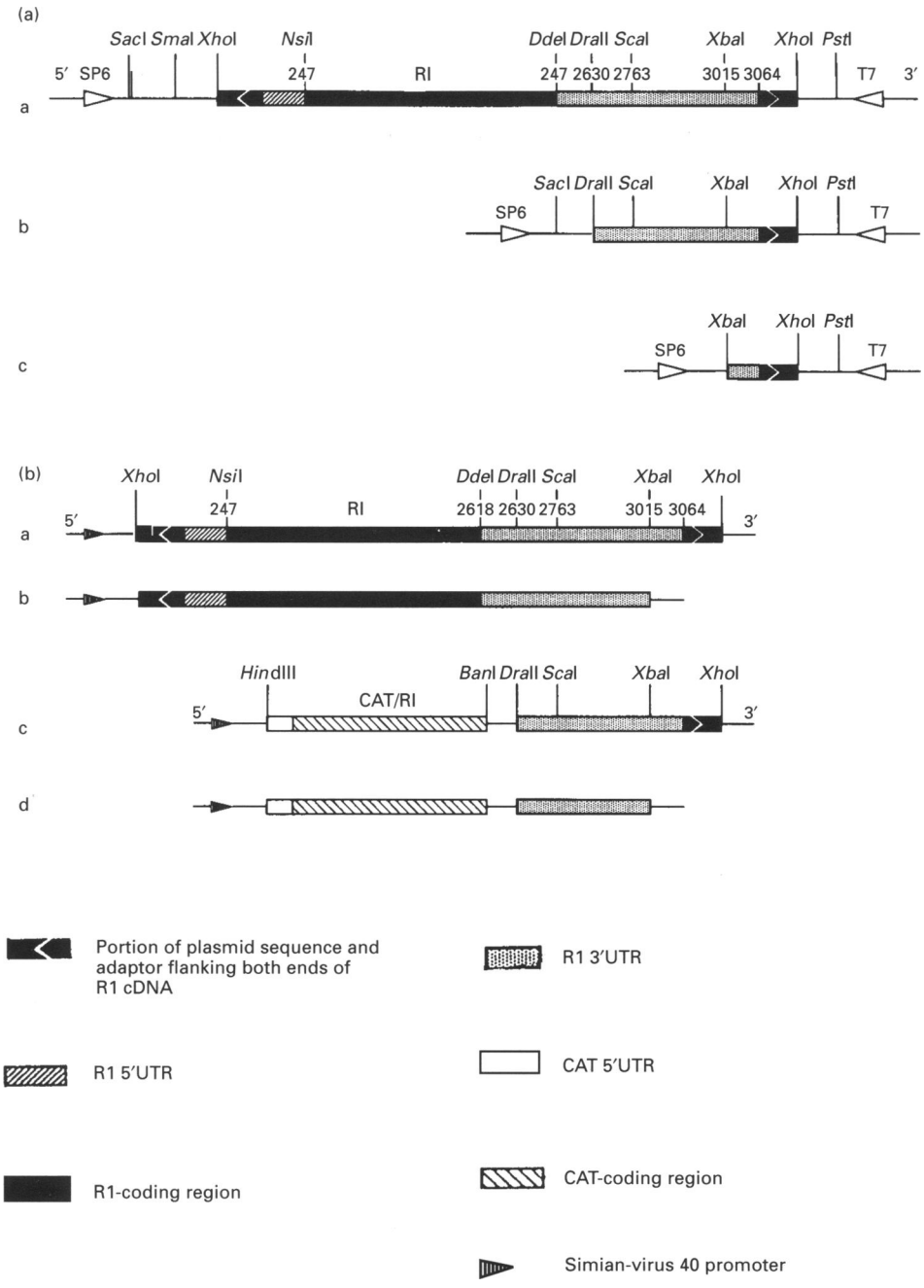

Figure 1 Plasmid constructs

The sites for restriction endonucleases and numbers referring to nucleotide positions are shown above the diagrams. **(a)** Transcription plasmids containing the full-length R1 cDNA, the R1 3′UTR and the 49-nt 3′UTR sequence are shown. The construct pSPT19-R1 (a) containing the full-length R1 cDNA was made by inserting the 3.1 kb *XhoI*/*XhoI* cDNA fragment from pCD-R1 into an *in vitro* transcription vector, pSPT19 SP6/T7. Using this construct pSPT19-R13e (b) and pSPT19-R13e49 (c) containing the 3′UTR and the 49-nt 3′UTR downstream sequence respectively were produced as described in the Materials and methods section. **(b)** The R1 and CAT/R1 hybrid constructs used for transfection of monkey COS 7 cells are shown. The diagrams represent the R1 cDNA-related constructs, pR1mc and pR13D2 (a and b), and the CAT/R1 hybrid constructs, pCAT-R13 and pCAT-R132 (c and d) that were made using the pECE expression vector as described in the Materials and methods section. Construct a (pR1mc) contains the full-length R1 cDNA downstream of a simian-virus-40 promoter. Construct c (pCAT-R13) contains a full-length CAT-coding region and a downstream full-length R1 3′UTR. The 49-nt 3′TR deletion constructs, pR13D2 and pCAT-R132 (b and d), were constructed from pR1mc (a) and pCAT-R13 (c) respectively. These constructs were transfected into COS 7 cells using the calcium phosphate method described in the Materials and methods section.

for different times with 0.1 μM PMA (Sigma), 30 nM staurosporine (Sigma), 0.1 μM okadaic acid (Upstate Biotechnology Inc., Lake Placid, NY, U.S.A.) and 15 μM forskolin (Sigma) respectively. Dimethyl sulphoxide (DMSO) was used to dissolve the compounds; control cells received medium containing 7 μM DMSO alone. To measure dose effects of the above compounds on R1 and CAT/R1 message stabilities and R1BP–RNA binding activity, proliferating cells were treated for 2 h (R1) or 4 h (CAT/R1) respectively with DMSO (3.5, 7 and 14 μM), PMA (0.01, 0.1 and 0.3 μM), staurosporine (0.01, 0.03 and 0.1 μM),

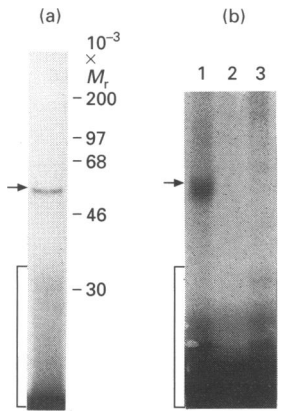

Figure 2 Monkey-cell R1BP binds to mouse R1 mRNA

(a) Protein–RNA binding reactions were performed by incubation of ^{32}P-labelled full-length R1 mRNA with cell extract from pR1mc-transfected monkey COS 7 cells, followed by u.v. cross-linking and electrophoresis on a 7% SDS/polyacrylamide gel, as described in the Materials and methods section. The complex (arrow) had an M_r of about 57000. The long vertical square bracket shows the digested RNA probe. **(b)** Band-shift analysis with pR1mc-transfected COS 7 cell extract and portions of the mouse R1 3′UTR, an *Xba*I/*Xba*I fragment containing the downstream 49-nt 3′UTR sequence (lane 1), a *Dra*II/*Sca*I fragment containing the upstream 133-nt 3′UTR which lacks the 49-nt sequence in lane 1 (lane 2) and a *Dra*II/*Xba*I fragment containing almost all the 3′UTR sequence except the 49-nt sequence (lane 3). The preparation of the above transcripts and the band-shift assay are described in the Materials and methods section. The 49-nt sequence has been shown elsewhere [8].

okadaic acid (0.01, 0.1 and 1 μM) and forskolin (5, 15 and 50 μM) respectively. Cells were also treated with 10 μg/ml actinomycin D (Sigma) to block transcription [8,20].

In vitro transcription of plasmids

In vitro transcription plasmid constructs containing different regions of R1 cDNA were prepared using an *in vitro* transcription vector pSPT19 SP6/T7 (Boehringer Mannheim, Mannheim, Germany). The construct pSPT19-R1 (Figure 1a, construct a) contains the entire R1 cDNA sequence. After linearization of the construct with *Pst*I, *Nsi*I or *Dra*II, sense transcripts of full R1, R1 5′UTR or 5′UTR plus coding region were generated. The construct pSPT19-R13e (Figure 1a, construct b) contains only R1 3′UTR. This construct was digested with *Pst*I, *Sca*I or *Xba*I to generate transcripts corresponding to the full R1 3′UTR, the 3′UTR 133-nt or the 3′UTR 385-nt sequence respectively. The construct pSPT19-R13e49 (Figure 1a, construct c) contains the downstream 49 bp sequence of the 3′UTR.

In vitro transcription of the plasmid constructs was performed using an SP6/T7 transcription kit (Boehringer, Mannheim) to produce transcripts by SP6 polymerase from 0.5 μg of plasmid construct DNA as described previously [8]. Briefly, mRNA was transcribed using SP6 RNA polymerase at 37 °C for 1 h in the presence of [^{32}P]CTP (3000 Ci/nmol; Amersham International), nucleotides, RNasin and buffer conditions as described by the manufacturer. After transcription, template DNA was digested with DNAase I. After extraction and precipitation, RNA transcripts were purified by electrophoresis through a 4% native polyacrylamide gel, eluted, precipitated with ethanol, and reconstituted in diethyl pyrocarbonate-treated water.

Analysis of protein–RNA interactions

Binding reactions were performed to detect protein–RNA complex formation using 20 μg of cytoplasmic protein prepared as described by Malter [23], incubated with 1.5×10^4 c.p.m. of ^{32}P-labelled transcript at 30 °C for 30 min in a final volume of 10 μl. Unprotected probe was digested by incubation with 50 units of RNAase T_1 (BRL) for 30 min. To exclude non-specific protein binding, heparin (5 mg/ml) was added, and the mixture was incubated at room temperature for an additional 10 min. RNA–protein complexes were analysed with 7% native polyacrylamide gels.

For u.v. cross-linking of RNA and protein, the reaction mixtures were put on ice immediately after the addition of heparin and irradiated by u.v. light at 2500 μJ for 15 min, using a UV-Stratalinker chamber apparatus (Stratagene). The samples were then resolved by electrophoresis with a 7%-SDS/polyacrylamide gel under reducing conditions [8]. Protein concentrations were determined by the Bio-Rad protein assay kit (Bio-Rad) according to the manufacturer's instructions.

Plasmid construction and cell transfections

The cloned R1 cDNA [24] contains 242 bp of 5′UTR, 2379 bp of coding region and 443 bp of 3′UTR. A fragment containing the entire mouse R1 cDNA was derived from the plasmid pCD-R1 [24] with *Xho*I digestion. pR1mc (construct a of Figure 1b) was generated by inserting this *Xho*I-digested fragment into a *Sal*I site in the polylinker of the pECE expression vector [25] in the sense orientation under control of a simian-virus-40 promoter. The R1 cDNA 3′UTR deletion construct, pR13D2 (construct b of Figure 1b), was made by digestion of pR1mc with *Xba*I to remove a *Xba*I/*Xba*I fragment containing a downstream 49 bp sequence in the R1 3′UTR, followed by ligation using T4 DNA ligase (BRL).

For the preparation of CAT/R1 hybrid constructs, a *Hin*dIII/*Ban*I fragment containing part of the CAT 5′UTR and the full sequence (715 bp) of the coding region was removed from the pSV1acOCAT plasmid [26], and inserted into the *Bgl*II site in the polylinker of the pECE plasmid. The full-length R1 cDNA sequence was then inserted into the polylinker at the *Sal*I site, and a fragment containing the 5′UTR and coding region of R1 cDNA was deleted with *Hin*dIII and *Dra*II to generate the construct pCAT-R13 (construct c of Figure 1b). The R1 3′UTR of CAT-R13 was located downstream of the CAT-coding region. pCAT-R132 (construct d of Figure 1b) was prepared by digesting pCAT-R13 with *Xba*I to remove the 49 bp fragment at the 3′end of R1 cDNA, followed by re-ligation [27].

Transfection of COS 7 cells was carried out by the calcium phosphate method described previously [28,29]. Briefly, plasmid DNA constructs were co-transfected with selectable marker plasmid, pSV2neo [30], at a 30 :1 ratio. Stably transfected COS 7 cells were selected with 400 μg of G418 (Gibco Laboratories)/ml of medium, starting 48 h after addition of the plasmid/calcium phosphate precipitate to the cells. After about 4 weeks of culture, colonies were pooled and expanded. Northern-blot analysis [3–6,8] and CAT assays [8,31] were performed to screen for stable transfectants.

RNA isolation and Northern-blot analysis

Total RNA was extracted from proliferating cells by the method described by Gough [32]. Transcription was inhibited by addition of 10 μg of actinomycin D/ml of medium [8,20]. A 20 μg portion of total cellular RNA was electrophoresed through 1% formaldehyde/agarose gels, followed by transfer to Zeta-probe nitrocellulose membranes (Bio-Rad). RNA blots were pre-hybridized for 5 min and probed for 16–24 h in formamide hybridization solution containing $(1–2) \times 10^6$ c.p.m./ml of a ^{32}P-

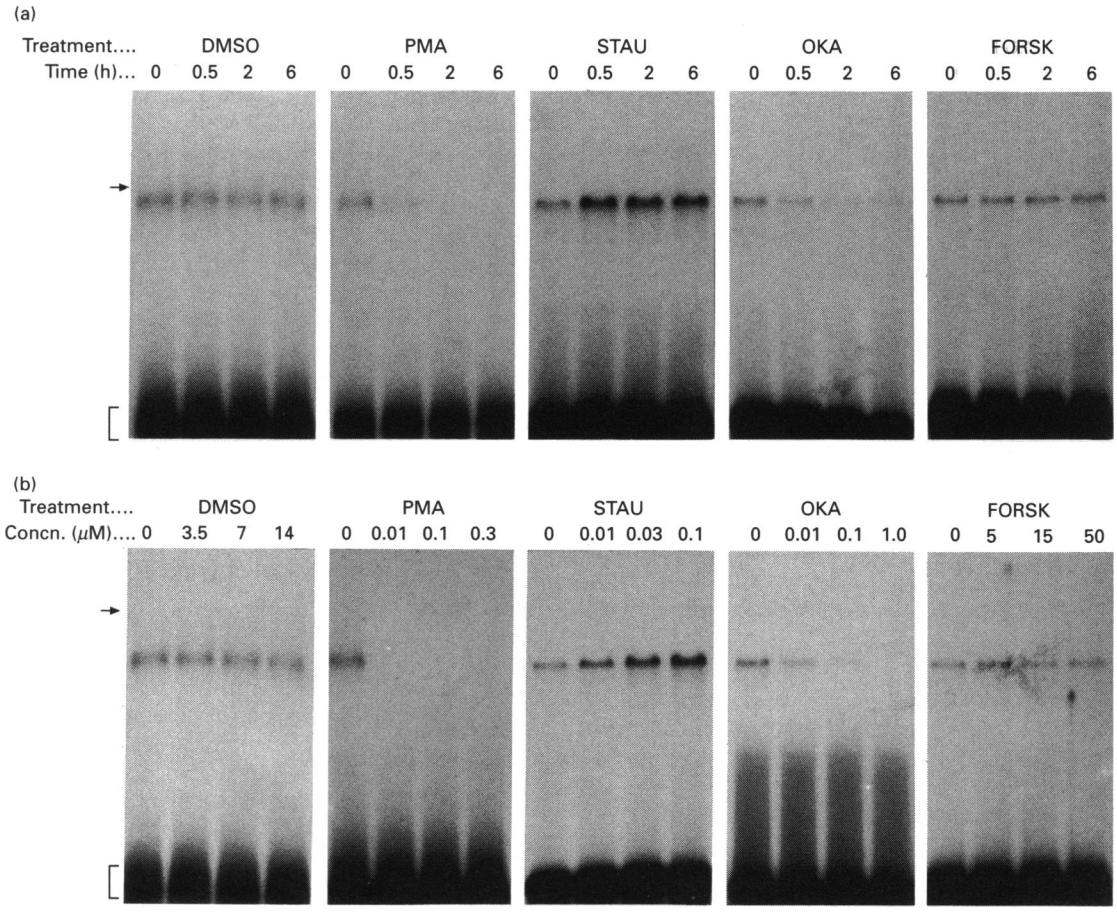

Figure 3 (a) Effects of phosphorylation modifying agents on R1BP–RNA binding activity and (b) dose effects of phosphorylation-modifying agents on R1BP–RNA binding activity

(**a**) The pR1mc-transfected COS 7 cells were transcriptionally blocked (10 μg/ml actinomycin D) and treated with PMA, staurosporine (STAU), okadaic acid (OKA) or forskolin (FORSK). Treatment times (h) are indicated above the panels, and the concentrations used are described in the Materials and methods section. Cross-linking assays with the *in vitro*-transcribed full-length R1 mRNA (see Figure 1a, construct a) and the cytosolic proteins extracted from the variously treated cells were carried out, followed by electrophoresis of the reaction mixture using a 7%-SDS/PAGE gel, to determine the effects of the compounds on R1BP–RNA binding activity (arrow). Cells treated with DMSO, the solvent for the compounds, were used as a negative control. The digested free probe is indicated by the square brackets. (**b**) Exponentially growing pR1mc-transfected COS 7 cells were transcriptionally blocked (10 μg/ml actinomycin D) and treated with PMA, staurosporine (STAU), okadaic acid (OKA) or forskolin (FORSK) for 2 h at the concentrations (μM) indicated above each lane. Cross-linking assays were performed with ^{32}P-labelled full-length R1 mRNA and cytosolic protein to determine R1BP–RNA binding activity (arrow) under various treatment conditions. Cells treated with DMSO and PMA alone were used as negative and positive controls respectively. Compared with the negative control, treatment with staurosporine up to 0.1 μM had a significant promoting dose effect on the formation of the R1BP–RNA complex, whereas increasing okadaic acid concentration up to 1 μM resulted in total abolishment of complex formation, which is similar to PMA action on the binding activity. R1BP–RNA binding activity was not affected by treatment with forskolin. Square brackets in the Figure indicate the location of free nucleotides.

labelled R1 *Nco*I/*Nco*I cDNA fragment from pCD-R1, or a CAT *Hin*dIII/*Ban*I cDNA fragment from pSVlacOCAT. cDNA probes were labelled by the nick-translation method using [^{32}P]dCTP (Amersham International). Hybridized probe was stripped from blots by washing the membrane twice for 20 min at 95 °C in the solution containing 0.5% SDS (w/v) and 0.1 × SSC (0.015 M NaCl/0.0015 M sodium acetate) and then rinsed with distilled water three times (Bio-Rad Literature Bulletin no. 292). RNA loading was detemined by probing with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA [4–6,8].

Quantification of autoradiograms

Autoradiograms were quantified by scanning densitometry using a model 620 video densitometer (Bio-Rad) coupled to a line printer. All densitometric values presented in this investigation were corrected for RNA loading by taking into account densitometric determinations of GAPDH mRNA levels by probing the same membranes with labelled GAPDH cDNA. Measurements of the relative quantities of ^{32}P-labelled RNA or protein–RNA migration bands in each sample were performed as described previously [8].

RESULTS

Monkey COS 7 cell extract contains a protein that binds a 49-nt sequence within the mouse R1 mRNA 3′UTR

Using full-length mouse R1 message transcribed from pSPT19-R1 (Figure 1a, construct a) and COS 7 cell extract we observed, in gel motility assays by u.v. cross-linking, that a cytosolic protein with an M_r of approx. 57000 was bound to mouse R1 message (Figure 2a). Different riboprobes of R1 mRNA were used to show that this protein, called R1BP (R1 mRNA-binding protein) [8], bound to the R1 message at the 3′UTR. Figure 2(b) shows that the monkey R1BP bound to a mouse R1 mRNA 49-

nt 3′UTR sequence that has been identified in *cis–trans* interactions in recent studies with mouse cell extract [8]. These observations imply that the *cis–trans* interaction involving the 49-nt sequence in the R1 3′UTR and a cytosolic protein, R1BP, is conserved in monkey and mouse.

A protein kinase C signal pathway is involved in the regulation of R1BP–RNA binding activity

Since PMA is a potent PKC stimulator [12,13], and we have shown previously that PMA treatment decreases the binding between R1BP and the 49-nt 3′UTR sequence [8], we tested the hypothesis that a phosphorylation pathway is critically involved in mediating the PMA effects observed on R1BP–RNA binding activity (Figure 2). Full-length R1 transcripts and cytosolic extracts from pR1mc-transfected COS 7 cells (Figure 1b, construct a), were used in gel mobility assays. Cells were treated for various times with staurosporine (a PKC inhibitor), okadaic acid (an inhibitor of phosphoserine/phosphothreonine protein phosphatases 1 and 2A) or forskolin (a stimulator of protein kinase A activity) [5,33]. Transcription was blocked by treatment with actinomycin D [8,20]. Control experiments included the treatment of cells with DMSO, which had no effect on protein–RNA binding activity in u.v.-cross-linking experiments, or with PMA, which dramatically decreased the binding activity (Figure 3a), in agreement with previous studies [8]. Interestingly, the cross-linking experiments showed that staurosporine treatment for only 30 min markedly increased protein–RNA binding activity (Figure 3a). These results suggest that a direct relationship exists between PKC activity and the interaction of R1BP with R1 mRNA. Similar to the effects of PMA on protein–RNA binding activity, treatment of cells with okadaic acid for only 30 min also significantly reduced R1BP–RNA binding (Figure 3a). Therefore, both stimulation of PKC by PMA and inhibition of phosphatases by okadaic acid have similar effects at the level of protein–R1 mRNA binding activity. These PKC-related effects appear to be specific, since treatment with forskolin, a stimulator of the PKA signal pathway, did not alter R1BP–RNA binding activity (Figure 3a).

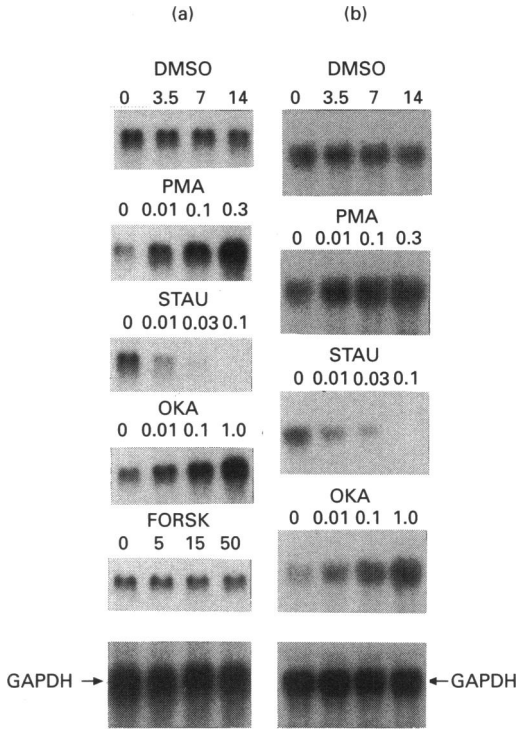

Figure 4 (a) Dose effects of phosphorylation-modifying agents on R1 mRNA levels and (b) dose effects of phosphorylation-modifying agents on CAT/R1 hybrid message levels

(**a**) Exponentially growing pR1mc-transfected COS 7 cells were treated with 10 μg/ml actinomycin D to block transcription, and the cells were treated with PMA, staurosporine (STAU), okadaic acid (OKA) or forskolin (FORSK) for 2 h at the concentrations (μM) indicated at the top of each lane. Northern-blot analysis of R1 mRNA was carried out following the extraction of total RNA. GAPDH cDNA was used to determine loading, and a representative result is shown at the bottom of the Figure. (**b**) Exponentially growing COS 7 cells transfected with pCAT-R13 were transcriptionally blocked with 10 μg/ml actinomycin D, and treated with PMA, staurosporine (STAU) or okadaic acid (OKA) for 4 h at the concentrations (μM) indicated at the top of each lane. After Northern-blot analysis of CAT/R1 message, the same membrane was probed with GAPDH for loading, and a representative result is provided at the bottom. Densitometric analysis showed that the effects of the various compounds on R1 and CAT/R1 message were similar.

Dose-dependent effects on R1BP–RNA binding activity and R1 mRNA stability by staurosporine and okadaic acid

If staurosporine and okadaic acid alter protein–RNA binding activity and message-stability properties, presumably through a PKC signal pathway, then their effects should be concentration-dependent. Figures 3(b), 4(a) and 4(b) show that this is correct. For example, increasing concentrations of staurosporine lead to corresponding elevations in R1BP–RNA binding activity in pR1mc-transfected cells, while increasing concentrations of okadaic acid results in a dose-dependent decline in binding activity that is similar to the effect obtained with PMA-treated cells (Figure 3b). As expected from earlier u.v.-cross-linking results (Figure 3a) and message-stability experiments (Figure 4), concentrations of forskolin as high as 50 μM did not affect R1BP binding to R1 mRNA (Figure 3b). As an estimate of message-stability characteristics following transcription inhibition with actinomycin D [8,20], COS 7 cells transfected with either pR1mc or pCAT-R13 were treated with various concentrations of the phosphorylation-modifying compounds for several hours and the levels of R1 message (Figure 4a) or CAT/R1 message (Figure 4b) were determined by Northern-blot analysis [8,34]. Dose-dependent effects on message-stability properties were observed with PMA, staurosporine and okadaic acid treatments. In keeping with earlier observations (Figure 3b), treatment with a variety of forskolin concentrations did not appear to alter the stability characteristics of R1 mRNA (Figure 4a). Indeed, attempts to modify the message-stability properties of R1 by exposing cells directly to cyclic AMP in the form of a biologically stable analogue (8-bromo cyclic AMP) as high as 0.8 mM also had no effect (results not shown).

Modulation of R1BP–RNA binding activity by staurosporine and okadaic acid affects R1 and CAT/R1 mRNA stability

COS 7 cells were transfected with pR1mc and pCAT-R13 constructs (Figure 1b, constructs a and c), treated with actinomycin D to block transcription [8,20], and exposed to PMA, staurosporine, okadaic acid or forskolin. Northern-blot analyses were performed to determine the half-lives of R1 message (Figure 5) and CAT/R1 mRNA (results not shown). The results of these experiments revealed that staurosporine exposure decreased R1 and CAT/R1 mRNA turnover rates by about 3.3- and 3.1-fold respectively. Treatment with okadaic acid increased R1 and

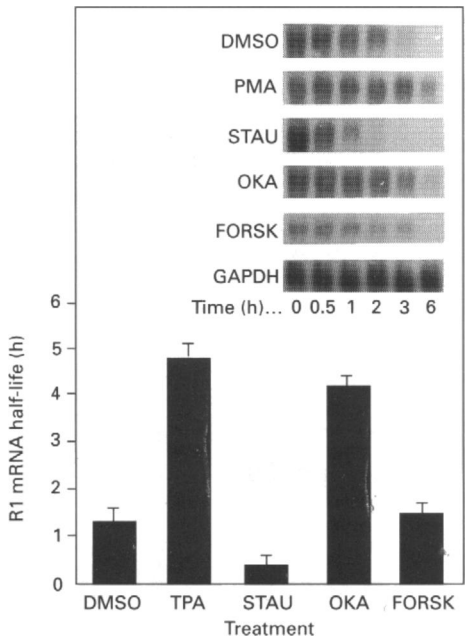

Figure 5 Effects of phosphorylation-modifying agents on the half-life of R1 mRNA in COS 7 cells

Exponentially growing cells transfected with pR1mc were transcriptionally blocked (10 μg/ml actinomycin D) and treated for the various times (h) as indicated with 0.1 μM PMA, 0.03 μM staurosporine (STAU), 0.1 μM okadaic acid (OKA) or 15 μM forskolin (FORSK). Cells treated with 7 μM DMSO were used as a negative control. The Northern-blot analysis of mouse R1 mRNA half-life in monkey COS 7 cells was performed as described in the Materials and methods section. GAPDH cDNA was used to probe the same membrane to estimate the sample loading, and a representive result is shown.

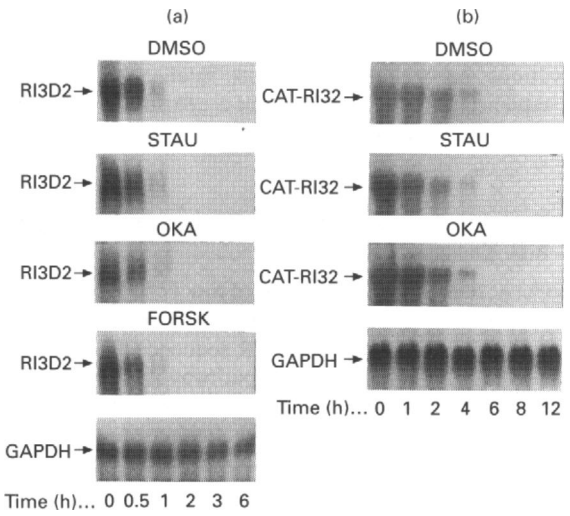

Figure 6 Turnover of R1 and CAT/R1 messages lacking a 49-nt 3′UTR sequence after treatment with phosphorylation-modifying agents

Exponentially growing COS 7 cells transfected with the 49-nt deletion constructs, pR13D2 (**a**) and pCAT-R132 (**b**), were transcriptionally blocked with 10 μg/ml actinomycin D and treated for various times (indicated in h at the bottom of this Figure) with phosphorylation-modifying agents, at the concentrations described in the Materials and methods section. Northern-blot analysis of the mRNA half-lives were carried out after the extraction of total RNA. A GAPDH cDNA was used to determine loading, and a representive result is provided at the bottom of (**a**) and (**b**). The half-lives of R13D2 (0.5 h) and CAT-R132 (1.5 h), detected after treatment with DMSO (control), were not significantly altered after treatment with the various compounds.

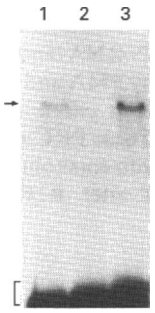

Figure 7 Effects of phosphorylation-modifying agents on mouse R1BP–RNA binding activity

BALB/c 3T3 cells were transcriptionally blocked (10 μg/ml actinomycin D) and treated for 2 h with 7 μM DMSO (negative control, lane 1), 0.1 μM PMA (lane 2) and 0.03 μM staurosporine (lane 3). Cross-linking assays were carried out with the *in vitro*-transcribed full-length R1 mRNA (Figure 1a, construct a) and cytosolic proteins were extracted from treated cells as described above. In comparison with the negative control (lane 1), mouse 3T3 cell R1BP–RNA binding activity (arrow) was inhibited by PMA treatment (lane 2) and increased by staurosporine treatment (lane 3). The square bracket indicates the location of free nucleotides.

CAT/R1 message stability by approx. 3.2- and 2.5-fold respectively. Similar to the observations made with okadaic acid, PMA treatment increased the half-lives of R1 and CAT/R1 mRNA turnover rates by 3.7- and 2.6-fold respectively. Interestingly, an inverse relationship between the effects of staurosporine, okadaic acid and PMA on message stability (Figure 5) and R1BP–R1 mRNA binding activity (Figure 3a) was observed. Also, as expected from the u.v.-cross-linking results (Figure 3a), treatment of cells with forskolin did not have a significant effect on R1 message half-life. These observations are consistent with the notion that the PKC signal pathway is intimately linked to R1 message stability through a mechanism that directly regulates protein–mRNA binding activity.

The R1BP–49 nt interaction is necessary for staurosporine and okadaic acid R1 message-stability regulation

To study the role of R1BP–49 nt interactions in the regulation of R1 mRNA turnover, COS 7 cells transfected with the 49-nt 3′UTR deletion constructs, pR13D2 and pCAT-R132 (Figure 1b, constructs b and d), were blocked at transcription, treated with the phosphorylation-modifying compounds staurosporine, okadaic acid or forskolin, and message half-lives were determined by Northern-blot analysis [8,34]. In contrast with studies carried out with R1 and CAT/R1 message constructs containing the 49-nt 3′UTR, no significant effects on the stabilities of R1 or CAT/R1 messages lacking the 49-nt sequence were detected after treatment with these compounds (Figure 6). These observations indicate the importance of the 49-nt region in the R1 message-stability alterations mediated by staurosporine and okadaic acid and are consistent with previous results reported for PMA [8].

The pathway of R1BP–RNA binding activity in monkey cells is shared by mouse cells

Treatment of BALB/c mouse 3T3 cells with phosphorylation-modifying agents followed by u.v. cross-linking demonstrated that the pattern of R1BP–RNA binding activity was similar to

that observed with monkey COS 7 cells. As shown in Figure 7, treatment of mouse cells with PMA inhibited R1BP–RNA binding, whereas staurosporine treatment increased the binding activity. Okadaic acid treatment also decreased binding activity, and forskolin did not appear to have an effect on R1BP–RNA binding (results not shown). These results suggest that the mechanism underlying R1BP–R1 mRNA interactions that affect R1 message stability use common pathways in monkey and mouse cells, and possibly those of other mammalian species.

DISCUSSION

PMA is a potent biological response modulator with a diverse range of effects on cells, *in vivo* and in cell culture [9–11]. Many of the pleiotropic effects of PMA and related tumour promoters occur at the cell membrane. Studies have shown that the membrane-associated receptor for PMA is the multifunctional serine/threonine-specific protein kinase PKC, which is regulated by intracellular levels of free Ca^{2+} and 1,2-diacylglycerol (DAG) [35,36]. PMA appears to act as a DAG analogue and induces a direct activation of PKC in intact cells [37]. Indeed, the variety of responses elicited by PMA-induced PKC activation appears, in part, to be due to the existence of a PKC gene family [38–40]. There are two general ways in which PMA, through a PKC pathway, may modify gene expression. One involves transcription, where *cis–trans* interactions at the DNA level is regulated by PKC in response to PMA treatment [17,18]. At the post-transcriptional level several studies have demonstrated changes in message-stability characteristics after exposure to PMA (see, e.g., [8,21]). The present study connects, for the first time, the action of the tumour promoters PMA and okadaic acid [41,42] and the PKC signal pathway to a post-transcriptional mechanism of message stability mediated by a specific *cis–trans* reaction at the 3′UTR of R1 mRNA. We also show that this tumour promoter responsive reaction involves a protein of approx. 57000, R1BP, and a sequence within a 49-nt region of the R1 3′UTR. Furthermore, the PKC signal pathway appears to be specifically involved in this regulation, since forskolin, a PKA pathway effector, did not alter R1 message-stability properties, or the R1BP–R1 mRNA interaction. In keeping with these findings, we have also observed that treatment of cells with 8-bromo cyclic AMP, like forskolin treatment, had no effect on R1 message stability, whereas treatment of cells with bisindolylmaleimide GF 109203X, another inhibitor of PKC activity [43], leads to increased R1BP–R1 mRNA binding activity and decreased R1 message stability (results not shown).

The effects of staurosporine and okadaic acid on message stability and on R1BP–RNA binding activity are in agreement with observations obtained with PMA [8]. These results support a general model in which dissociation of R1BP from the R1 mRNA 3′UTR is linked to an increase in the stability of R1 message, whereas increasing the binding activity leads to mRNA destabilization. The exact role of the R1BP–mRNA complex in the regulation of R1 mRNA stability is not yet clear. However, R1BP may be acting as a targeting protein for R1 mRNA degradation, or it may be part of a ribonuclease complex directly participating in message turnover. It is also important to note that other regions besides the 49-nt sequence within the R1 3′UTR may also have a role to play in message stability. This is suggested by our observation that R1 and CAT/R1 messages containing deletions of the 49-nt sequence exhibited shorter half-lives (Figure 6) relative to messages containing the full R1 3′UTR (Figure 5). This possibility is not surprising, and is supported by other studies, where, for example, in addition to the repeated AUUUA pentameter in the c-*myc* mRNA 3′UTR, a second sequence (in the coding region) is also involved in message turnover.

Interestingly, the effects of PMA, staurosporine and okadaic acid on message stability and R1BP–RNA binding activity in monkey and mouse cells are similar, suggesting that the PKC regulation pathway for R1 message stability described in the present study is common to these two species and may be conserved in mammals.

In conclusion, the present results indicate that a protein kinase C pathway is linked to the post-transcriptional regulation of ribonucleotide reductase R1 gene expression by a mechanism involving an R1BP–mRNA 3′UTR *cis–trans* interaction.

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