Defining a Novel cis-Element in the 3'-Untranslated Region of Mammalian Ribonucleotide Reductase Component R2 mRNA

cis-trans-INTERACTIONS AND MESSAGE STABILITY*

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Mammalian ribonucleotide reductase is a highly regulated activity essential for DNA synthesis and repair. The 3'-untranslated region (3'-UTR) of mammalian ribonucleotide reductase R2 mRNA has been implicated in the tumor promoter, 12-O-tetradecanoylphorbol-13-acetate-mediated stabilization of mouse BALB/c 3T3 R2 message. We investigated the possibility that the 3'-UTR contains regulatory information for R2 mRNA turnover. Using 3'-end-labeled RNA in gel shift and UV cross-linking analyses, we detected in the 3'-UTR a novel 9-nucleotide cis-element, 5'-UCGUUGUCGU-3', which interacted with a widely distributed cellular cytosolic protease-sensitive factor(s) in a sequence-specific manner to form a 45-kDa R2 binding protein complex. The binding activity was redox-sensitive and down-regulated by 12-O-tetradecanoylphorbol-13-acetate and okadaic acid in a dose-dependent manner. Insertion of a 154-base pair fragment containing the cis-element led to markedly reduced accumulation of chloramphenicol acetyltransferase hybrid mRNA relative to the same insert carrying a series of G → A mutations within this element that eliminated binding. We suggest that the 9-nucleotide region functions as a destabilizing element. These results provide a model for ribonucleotide reductase gene expression through a novel and specific mRNA cis-trans-interaction involving a phosphorylation signal pathway that leads to changes in the stability of R2 message.

Mammalian ribonucleotide reductase is composed of two dissimilar dimeric protein components, proteins R1 and R2, which are required to catalyze the direct reduction of ribonucleoside diphosphates to the corresponding deoxyribonucleotides, a rate-limiting step in the synthesis and repair of DNA (1, 2). The importance of ribonucleotide reductase for cell proliferation is further emphasized by the observation that the mechanisms controlling ribonucleotide reductase gene expression may be altered in some malignant conditions (3). Ribonucleotide reductase appears to be important in an aberrant growth factor signal pathway in Ha-ras-transformed cells (5). Clearly, obtaining more information about the mechanisms that control ribonucleotide reductase gene expression is important for understanding DNA synthesis, DNA repair, and cell proliferation (2, 6).

The regulation of mRNA stability has emerged as an important control mechanism of gene expression. Although the mechanisms that alter mRNA stability of different genes have unique features, it appears that in each case specific RNA sequences, mainly located in the 3'-untranslated regions (3'-UTRs) of mRNAs, are required for the interactions with specific protein factors (7–13). For example, ribonucleotide reductase R2 mRNA stability is elevated by TPA (8). Although important details concerning the mode of action of TPA are still unknown, the prevailing hypothesis is that TPA exerts its diverse effects through the activation of protein kinase C, a high affinity target for TPA (14–16). Although it is now evident that TPA can affect gene expression posttranscriptionally through altering message stability (8, 9, 17, 18), relatively little is known about the molecular mechanisms of the cis-elements and trans-factors involved. We have recently shown that a 20-nt fragment (Fokl-HgI) in the 3'-UTR of R2 mRNA is involved in the TPA-mediated stabilization of R2 message (8). The present study was intended to define the unique TPA-responsive cis-element in the 3'-UTR that leads to alterations in R2 mRNA stability.

EXPERIMENTAL PROCEDURES

Cell Culture—Mouse BALB/c 3T3, 10T 1⁄2, COS7, C1 and C2 fibrosarcoma, human HeLa S3, and cells transfected with chloramphenicol acetyltransferase (CAT) R2 3'-UTR hybrid plasmids were routinely cultured in minimal essential medium supplemented with antibiotics and 10% (v/v) fetal bovine serum (19). For determining CAT/R2 3'-UTR hybrid mRNA levels and the cis-element binding activity, exponentially growing cells were treated for different times with various concentrations of TPA (Sigma), okadaic acid (Upstate Biotechnology Inc., Lake Placid, NY), and dimethyl sulfoxide (Me2SO). Me2SO was used to dissolve TPA and OKA; control cells received medium containing 0.01% Me2SO alone. Cells were also treated with 10 μg/ml actinomycin D (Sigma) to block transcription and were harvested as described previously (20).

Preparation of Protein Extracts from the Cytosol and the Nucleus—Cells transferred to Eppendorf tubes were briefly centrifuged for 1 min, resuspended in hypertonic buffer (25 mM Tris-HCl, pH 7.9, 0.5 mM EDTA), and lysed by repetitive cycles of freeze-thaw. Nuclei and cytoplasmic extracts were obtained as described previously (8), and protein concentrations were determined by the Bio-Rad protein assay kit as described by Bio-Rad.

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1 The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; UTR, untranslated region; CAT, chloramphenicol acetyltrans-
In Vitro Transcription—A summary of the construction of the in vitro transcription plasmid p3'-UTR containing the full-length 3'-UTR of R2 cDNA from mouse TA3 cells has been previously described (8). In brief, run-off RNA transcripts were produced by T7 polymerase activity (Boehringer Mannheim) from 1 μg of digested cDNA plasmids as described previously (21). RNA transcripts were produced and extracted to a specific activity of approximately 3 × 10^6 cpm/μg of RNA (10).

3'-End Labeling of Synthetic RNA Oligonucleotides—The RNA oligonucleotides purchased from Dalton Chemical Laboratories Inc. (Toronto, Canada) were synthesized on the Applied Biosystems ABI model 392 DNA/RNA synthesizer using phosphoramidite RNA monomers according to the manufacturer’s instructions. The ribonucleotides were labeled at their 3'-ends by a previously described method (22). The 3'-end-labeled RNA oligonucleotides were gel-purified, extracted, reconstituted in RNase-free water (10), and stored at −70°C.

RNA Mobility Shift Assay—Binding reactions were performed as described previously (21) with 10–40 μg of cytosolic and nuclear protein extract and 50 × 10^3 cpm of 32P-labeled RNA transcript or oligoribonucleotides. However, 50 units of RNase T1 (Boehringer Mannheim) were added when the R2 3'-UTR was used (but not for binding reactions with the 3'-end-labeled oligoribonucleotides). In some assays cytoplasmic extracts were preincubated with compounds such as diamide and N-ethylmaleimide for 10 min before the addition of the riboprobe.

UV Cross-linking Analyses—RNA-protein binding reactions were carried out as described above. Following the addition of heparin, samples were placed in microtiter wells placed on ice and UV-cross-linked for 10 min in a Stratalinker Chamber (Stratagene), at a 250-mJ energy level. The samples were separated by electrophoresis on a 10% SDS-polyacrylamide gel (21). The RNA-protein complexes may dissociate to some degree during electrophoresis, resulting in a diffuse banding pattern (23).

Northern Blot Analysis and Half-life Measurements—Total cellular RNA was extracted from drug-treated and untreated mouse BALB/c 3T3 transfectants by a rapid method of RNA preparation (24). For half-life measurements, the cells were treated with 10 μg/ml actinomycin D. RNA was isolated at different times and analyzed for CAT and CAT/R2 3'-UTR hybrid transcripts. Twenty μg of total cellular RNA was electrophoresed through 1% formamide-agarose gels and blotted onto Nytran nylon membranes. The blots were prehybridized and probed with the 32P-labeled HindIII-Bam fragment of CAT cDNA, derived from the plasmid pSvIacCAT (8). Determination of sample loading was performed by probing with glyceraldehyde-3-phosphate dehydrogenase cDNA (5). The mRNA half-life was estimated as described previously (7, 10).

Plasmid DNA Constructs and Transfection—The plasmid pCAT and the pCAT/R2 3'-UTR hybrid construct were similarly derived as described previously (8), with the notable exception that in this study the expression vector pECK polyadenylation signal fragment (25) was not deleted. This confers an increased half-life upon the pCAT transcript. Transfection of mouse BALB/c 3T3 cells was carried out by the calcium phosphate method as described previously (26), using the plasmid pSV2neo as a selectable marker. Northern blot analysis (7, 10) and CAT assays (27) were performed to screen for permanently transfected cell lines.

The PCR (40 cycles) was performed in a 100-μl total volume, and the reaction mixtures contained 50 ng of DNA template, 40 pmol of DNA primers, and 5 units of Taq DNA polymerase. The samples were gel-purified and subsequently digested with the restriction endonucleases, Sall/XbaI. DNA digests were heat-inactivated at 65°C for 15 min and ligated into the pCAT plasmid to generate pCAT/R2 normal and pCAT/R2 mutant. The nucleotide sequence of these fragments was confirmed by DNA sequencing (Sequenase). The wild-type DNA fragment (R2 normal) containing 154 base pair of the 3'-UTR of R2 mRNA (nt positions 1224–1378), with the putative R2 binding protein complex (R2BP) cis-element, was generated by PCR with the plasmid pCD10 (8), harboring the R2 cDNA and the overlap extension primers, 5'-ACAACCACGTGACGCTGACTTCTAAGTAACTC-3' and 5'-CGCGACAGATCCGAGTGAGTGAGTAACTC-3. Similarly, oligonucleotides, 5'-ACCACACGTCGACGCTGACTTCTAAGTAACTC-3' and 5'-CGCGACAGATCCGAGTGAGTGAGTAACTC-3' were used as primers to generate the 154-base pair DNA fragment of the R2 3'-UTR containing the mutated cis-element (R2 mutant). The underlined sequences above represent the Sall and XbaI linker sequences.
similar RNA-protein complex shifts as the full-length 3'-UTR of R2 mRNA (Fig. 1C). The RNA fragment, 5'-UCUGUGUCUC-3', is identified as the only binding site within the 20-nt fragment, shown by the formation of the RNA-protein complex, when the corresponding riboprobe was incubated with cytoplasmic extracts from mouse BALB/c 3T3 cells, respectively. B, in the competition study, 100 nM excess of the competitor unlabeled RNA transcripts, R2 3'-UTR (20 nt), R2 3'-UTR, and c-myc 3'-UTR were incubated with 20 μg of cytosolic extract from mouse BALB/c 3T3 cells, before the addition of the radiolabeled 9-nt R2 cis-element probe (50 x 10^3 cpm). The RNA-protein complex formation was analyzed by UV cross-linking analyses. C, gel shift assays were performed using 20 μg of cytoplasmic extract from different cell lines and the R2 cis-element probe.

led to inhibition of the formation of the R2BP (Fig. 2A, lanes 1 and 2). The addition of 100 nM excess of the human c-myc mRNA 3'-UTR fragment (12) did not prevent the RNA-protein complex formation (Fig. 2B, lane 3). Since the c-myc mRNA 3'-UTR used in this experiment contains four copies of the AU-rich cis-element (11), this result demonstrates the sequence-specific binding of protein(s) to the cis-element. RNA gel shift assays were performed with 30 μg of cytosolic protein extract from BALB/c 3T3 cells, 10T½ mouse fibroblasts, C1 and C2 mouse fibrosarcoma cells derived from 10T½ cells following T24-H-ras transfection (29), human HeLa S3 cells, or COS7 monkey kidney fibroblasts. Fig. 2C shows that regardless of the origin and species of cells, the cis-element formed similar patterns of RNA-protein complex band shifts. This suggests that we have identified a widely distributed cellular protein(s) capable of interacting with the R2 3'-UTR cis-element.

Redox-sensitive Binding Activity of the cis-Element—There are precedents in the literature for redox-sensitive binding of protein(s) to nucleic acid (23). We investigated the possibility that there were similar interactions with the R2 9-nt cis-element. We carried out a study of the effects of various reducing and oxidizing agents on the formation of R2BP in standard gel shift assays. Incubation of mouse BALB/c 3T3 cell extract with increasing concentrations of the reductant dithiothreitol significantly altered the cis-element binding activity (Fig. 3A, lanes 2–4). When 2-mercaptoethanol was added in increasing amounts, the intensity of the bands further increased (Fig. 3A, lanes 5 and 6). These observations suggest that BALB/c 3T3 cell extract had some ribonuclease activity in the oxidized form that could be reduced to achieve greater RNA binding. However, this binding activity was abolished with the preincubation of the cell extract with N-ethylmaleimide (which alkylates and thus blocks free sulfhydryl groups). Treatment of the cell extract with increasing concentrations of diamide (which catalyzes the oxidation of free sulfhydryls) inhibits formation of the cis-element-protein complex. This inhibition by diamide was dose-dependent (Fig. 3B, lanes 2–4). However, inhibition produced by 100 mM diamide was slightly reversed by 2% 2-mercaptoethanol, whereas the bind-
After preincubation of cytosolic extract with 50 mM diamide, binding activity was completely restored by 2% 2-mercaptoethanol, indicating that the reduced activity was due to disulfide bond formation. Supplementary bases are indicated by dots.

The predicted structure of a 77-base segment of the R2 3'-UTR containing the cis-element is shown in Fig. 4B. The secondary structure was determined by computer-generated analysis of a 77-nt base sequence (nt positions 1225–1302) of the R2 mRNA. This structure predicts one possible stem-loop configuration where the cis-element sequence is located in the middle of the loop. The presence of this stem-loop structure is further supported by UV cross-linking analyses using 3'-end-labeled probes. Lane 1, human GM-CSF 3'-UTR (20 nt) as a control for cytosolic protein loading. Lane 2, 3'-UTR (20 nt) of the GM-CSF plus wild-type 9-nt sequence. Lane 3, 3'-UTR (20 nt) of the GM-CSF plus mutant 9-nt R2 cis-element. Lane 4, the consensus cis-element sequence for optimal R2BP formation, where N denotes an A, G, U, or C residue. NUCUUGUGCU GAGAGCUU UGGUGGUU UCGUGGUGA GAGUGGCU GAGUGGCU GAGUGGCU

The effects of various mutations in the cis-element binding site were assessed by gel shift assays. Mutations within the cis-element of mouse R2 3'-UTR mRNA (bases 1245–1253) demonstrated that certain disruptions completely prevented R2BP formation (Fig. 4C). For example, mutation of any one of the core U residues to A, G, or C residues abolished binding activity, but mutation of the flanking U residues to A, G, or C residues did not prevent binding activity. In addition, mutations of C residues to G residues and vice versa within this motif abolished binding activity (Fig. 4C). These results are consistent with UV cross-linking data. For example, results from UV cross-linking analyses showed that the 9-nt cis-element (5'-UCGUUGUGCU-3') formed a 45-kDa R2BP (Fig. 4D) but not the mutant fragment, 5'-UCGAGACGU-3' (Fig. 4D). Furthermore, to examine cytosolic protein loading, a 3'-end-labeled human GM-CSF 3'-UTR (20 nt) fragment (12) was added to the binding reaction mixtures. The results from the cross-linking analyses showed that RNA-protein complexes with migration bands of 30 and 35 kDa were cross-linked to the GM-CSF 3'-UTR, as previously reported (12) and that the loading in the various lanes appeared to be approximately equal, as determined by densitometry (Fig. 4D).

Binding of the R2BP Correlates with Reduced mRNA Stability—To assess directly the functional role of the R2BP in the degradation of R2 mRNA, a 154-base pair R2 DNA PCR fragment (nt positions 1224–1378) was inserted into the mouse BALB/c 3T3 cells. Northern analyses were performed by probing for CAT hybrid transcript and also glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as a control for loading. The results are the average of two independent experiments.

The results showed that the CAT hybrid mRNA containing the normal cis-element binding site exhibited a half-life markedly shorter than that of the mRNA with the mutated insert. For example, the half-life of CAT/R2M was increased about 3-fold relative to CAT/R2N (Fig. 5C). The half-life of CAT/R2M mRNA was 1.6 h compared with that of CAT/R2M mRNA of 0.5 h. These results are consistent with the hypothesis that the R2BP plays a role in the degradation of R2 mRNA.
elements that may be associated with R2 message stabilization (8). We have identified a putative novel 9-nt cis-element, 5-UCGUGUGCU-3' in the 3'-UTR of R2 mRNA, which interacts with a cytosolic protease-sensitive factor(s) to form a 45-kDa R2BP. The binding activity of the cis-element was sequence-specific. Similar to several previously published mRNA cis-elements (33), there is only one copy of the 9-nt cis-element within the R2 message. The cis-element binding site showed a similar band shift complex in a variety of cells of different species, suggesting a common pathway for the regulation of R2 mRNA degradation. Mutagenesis studies indicated that there are relatively stringent primary sequence requirements for formation of the R2BP.

Our results indicate that the cis-element binding activity is redox-sensitive, because formation of the cis-element-protein complex was abolished by treatment of cell extracts with N-ethylmaleimide and diamide; however, the inhibition of RNA-protein complex formation by diamide was reversed by 2% mercaptoethanol, suggesting a possible sulphydryl switch mechanism. The present study connects, for the first time, the action of the tumor promoters TPA and okadaic acid and phosphorylation signal-controlled pathway(s) in a mechanism(s) leading to message stability that is associated with down-regulation of the R2BP activity. As expected, treatment of cells with okadaic acid, which exerts its effects by causing a net increase in the prevailing levels of phosphorylated proteins, resulted in the down-regulation of the cis-element binding activity. Such a mechanism has been proposed for the posttranscriptional regulation of ribonucleotide reductase R1 mRNA stabilization (20). Our data indicated that insertion of a PCR-amplified DNA fragment containing the wild type protein binding site into a heterologous CAT gene caused message destabilization, whereas mutations introduced into the normal cis-element that eliminated formation of the R2BP had no significant effect on the stability of the CAT hybrid RNA. This result is consistent with the idea that the in vivo message destabilization of the R2 mRNA is mediated by the R2BP in cells, and down-regulation of the binding activity of this complex leads to a decrease in the degradation of R2 mRNA. We conclude that the cis-element functions as a destabilizing sequence.

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