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A novel transforming growth factor-β_1 responsive cytoplasmic *trans*-acting factor binds selectively to the 3′-untranslated region of mammalian ribonucleotide reductase R2 mRNA: role in message stability

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ABSTRACT

Ribonucleotide reductase is a highly regulated enzyme that provides the four deoxyribonucleotides required for DNA synthesis. Our studies showed that TGF-β_1 treatment of BALB/c 3T3 mouse fibroblasts markedly elevated ribonucleotide reductase R2 mRNA levels, and also increased the half-life of R2 message by 4-fold from 1.5 h in untreated cells to 6 h in treated cells. We describe a novel 75 Kd sequence-specific cytoplasmic factor (p75) that binds selectively to a 83-nucleotide 3′-untranslated region of R2 mRNA and did not bind to the 5′UTR, the coding region of the R2 message or to the 3′UTRs of other mRNAs (from *c-myc*, GM-CSF and the iron responsive element from the transferrin receptor mRNA), or to the homopolymer poly(A) sequence. p75-RNA binding activity, which requires new protein synthesis, is not present in untreated cells, but is induced following TGF-β_1 stimulation. The *in vivo* kinetics of appearance of p75 binding activity paralleled the accumulation of R2 mRNA. Insertion of the 3′-untranslated region into the chloramphenicol acetyltransferase (CAT) message confers TGF-β_1 induced stability of RNA in stably transfected cells, while the same insert carrying a deletion of the 83-nucleotide fragment had little affect on RNA levels. Furthermore, *in vitro* decay reactions that contained the 83-nucleotide RNA or deletion of this fragment caused a significant decrease in TGF-β_1 stabilization of R2 message. A model is presented of R2 message regulation in which TGF-β_1 mediated stabilization of R2 message involves a specific interaction of a p75-*trans*-acting factor with a *cis*-element(s) stability determinant within the 83-nucleotide sequence which is linked to a reduction in the rate of R2 mRNA degradation.

INTRODUCTION

Mammalian ribonucleotide reductase is composed of two dissimilar dimeric subunits, proteins R1 and R2, which are encoded by different genes. Both proteins are required to catalyze the direct reduction of ribonucleoside diphosphates to the corresponding dexoyribonucleotides, a rate limiting step in the synthesis of DNA (1,2,3). The activity of ribonucleotide reductase is cell cycle dependent (4), and S-phase specific expression of R1 and R2 mRNAs has been reported (5). Furthermore, ribonucleotide reductase is important for DNA repair (6), and appears to play a role in critical early events of tumor promotion (7,8). Gene expression in BALB/c 3T3 cells is induced by TGF-β_1 (9), and is abnormally regulated by TGF-β_1 in highly malignant fibrosarcomas (10), and together with deoxyribonucleotide levels, the activity of the enzyme has been shown to be significantly elevated during malignant cell proliferation (11), making it a useful target for the development of chemotherapeutic compounds (2,3).

Growth factors such as TGF-β_1 exert their effects by altering the expression of a discrete set of genes important for the regulation of a variety of characteristics, including cell proliferation and growth inhibition (12–15). The prevailing hypothesis is that growth factors like TGF-β_1 exert their biological modifications by inducing an altered program of gene expression through transcriptional activation of genes (16,17). There is also evidence that TGF-β_1 can affect gene expression post-transcriptionally through altering message stability. For example, TGF-β_1 acts post-transcriptionally in fibroblasts to stabilize the mRNAs of the $\alpha 1$ (I) procollagen and fibronectin genes (16,17,18). Although the mechanisms that regulate mRNA stability of different genes will have unique features, it appears that in each case specific RNA sequences, mainly located in an untranslated region (UTR) will be required for the recognition of specific protein factors (19). In this study, we have investigated the possibility that the elevation in R2 mRNA levels recently reported from our laboratory (9) can be mediated at least in part, through alterations in mRNA half-life due to direct protein interactions with R2 message. We have identified a novel cytoplasmic factor of 75 kd that binds to a 83 nucleotide (nt) 3′-UTR fragment of R2 mRNA and did not bind to the 5′UTR or coding region of R2 message. The p75-RNA binding activity is not detected in unstimulated BALB/c3T3 fibroblasts but is induced to bind to the 83 nt 3′-UTR fragment by TGF-β_1

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stimulation, and displays kinetics that parallel the expression of ribonucleotide reductase R2 mRNA. This finding suggests that p75 is involved in the regulation of R2 message metabolism. Our study has demonstrated, for the first time, that TGF-β_1 mediated stabilizations of target mRNAs likely involve inducible *trans*-acting factors.

MATERIALS AND METHOD

Cell cultures and preparation of cytoplasmic lysates

BALB/c3T3 mouse fibroblasts were routinely maintained at 37°C in α-minimal essential medium (MEM) supplemented with antibiotics and 10% (V/V) fetal bovine serum (FBS) as previously described (20). For growth factor experiments, cells were grown overnight in α-MEM with 10% FBS and the medium was replaced by a serum free medium which contained 0.4 mg transferin and 0.2 mg insulin (Sigma) in 100 ml MEM (10). Transforming growth factor-β_1 (TGF-β_1) (R and D Systems Inc., Minneapolis, MN) was dissolved in 1.0 mg/ml bovine serum albumin (BSA) and 4 mM hydrochloric acid (HCL). 10 ng/ml TGF-β_1 was added at predetermined times. The control plates received 1.0 mg/ml BSA and 4 mM HCL. The cells were harvested from the tissue culture plates with 0.3% buffered trypsin solution after centrifugation, washed once in phosphate buffered saline and transferred to eppendorf tubes. After brief centrifugation for 1 min cells were resuspended in hypotonic buffer (25 mM Tris−HCL (pH 7.9), 0.5 mM ethylene diaminetetraacetic acid (EDTA)) and lysed by repetitive cycles of freeze-thaw. Nuclei were removed by centrifugation at 15000×g at 4°C for 15 min in a microfuge, and cytoplasmic extracts were quickly frozen on dry ice and stored at −70°C. Protein concentrations were determined by the Bradford method (Bio-rad). Transcription and translation were inhibited by the addition of 10 μg/ml actinomycin D (Act D) and 10 μg/ml cycloheximide respectively.

Northern analysis and half-life measurements

Total cellular RNA was extracted from the TGF-β_1 treated and untreated cells by a rapid RNA method (21). For half-life measurements, transcription was inhibited by addition of 10 μg/ml Act D. RNA was isolated at different times and analyzed for R2 and CAT mRNA. Briefly, washed harvested cells were pelleted in a 1.5 ml microfuge tube (5 min, 800 g), supernatant was removed by aspiration and the cell pellet was resuspended in 200 μl of cold 10 mM Tris−HCL (pH 7.5), 0.15 M NaCl, 1.5 mM $MgCl_2$, 0.65% NP-40. After vortexing and centrifugation at 15000×g for 10 min at 4°C, the cytoplasmic lysate was transferred to a fresh microfuge tube containing 200 μl of 7 M urea, 1% sodium dodecyl sulphate (SDS), 0.35 M NaCl, 10 mM EDTA, 10 mM Tris−HCL (pH 7.5) plus 400 μl phenol/chloroform/iso-amylalcohol (50/50/1) and vortexed vigorously. The deproteinized cytoplasmic extract was separated from the phenol phase by centrifugation for 5 min at 15000×g and the RNA was recovered by precipitation with 1.0 ml 95% ethanol and chilling at −20°C. Thirty μg of total cellular RNA were electrophoresed through 1% formamide agarose gels and blotted onto Nytran nylon membranes. The blots were prehybridized and probed with random primer ^{32}P-labeled R2 cDNA and the Pvu11−Sca1 fragment of CAT coding sequence as previously described (18). Hybridized probe was stripped from the blots by washing twice for 30 min each in 50% formamide in 0.1×SSC (1×SSC is 0.15 M NaCl, 15 mM sodium acetate pH 7.0) at 75°C and then rinsed in 0.1×SSC. Loading of RNA was determined by probing with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (7,23). Autoradiograms were scanned densitometrically on a LKB laser densitometer coupled to a line printer and the peaks corresponding to the bands were measured by computer integration. R2 message signals were corrected for RNA content in each sample by comparison to the intensity of GAPDH bands, and the percentage of R2 mRNA signal was plotted logarithmically against time. The best-fit lines were drawn to determine R2 message half-life.

Plasmid construction and *in vitro* transcription

A summary of the construction of expression plasmids is shown (Figure 3). The 5′-UTR plus coding region of mouse R2 cDNA (22) was generated by digesting R2 cDNA with Sdu1 which was subsequently blunt-ended and subcloned into the Sma1 site of pSPT18 SP6/T7 (p3′UTRX) *in vitro* transcription vector (Boeringer Mannheim). The 3′UTR was removed from R2 cDNA by digestion with Fok1 to generate a 966 nucleotide (nt) fragment. This fragment was blunt-ended and then subcloned into Sma1 site of p3′UTRX to create p3′UTR1. The plasmid p3′UTR1 was digested with EcoR1 at nt position 1861 and also at another EcoR1 site in the polylinker. This removed a 627 nt fragment from the 3′UTR to generate p3′UTR2. Digestion of 3′UTR with Sau1 at two unique positions—1435 and 1944 generated p3′UTR3. Restriction of p3′UTR4 with Sau1 at position 1944 generated p3′UTR4. To locate the region containing the TGF-β_1 inducible RNA-protein binding activity, p3′UTR2 was restricted by Sau1 and religated. The resulting plasmid, p3′UTR5 contained a 83 nt fragment. To transcribe sense RNA transcript labeled probes, all the plasmids with the exception of p3′UTR5 were linearized with Hind III in the polylinker, followed by transcription with T7 polymerase activity (24). Corresonding anti-sense transcripts could be produced using SP6 polymerase activity. To produce radiolabeled transcripts, unlabeled CTP was reduced to 25 μM and ^{32}P-CTP was added (3000 Ci/mMol, Amersham) according to the manufacturer's procedure (Boeringer Mannheim), to produce RNA transcripts with specific activity of approximately 3×10^8 cpm/μg RNA (24). The riboprobes were purified by gel electrophoresis through 5−12% acrylamide, 8 M urea, eluted at 37°C in 2.5 M ammonium acetate, 1% SDS, 1 mM EDTA and were ethanol-precipitated to recover the RNA (25).

Gel mobility assay

Binding reactions were carried out as previously described (24) with 10−30 μg of cytoplasmic protein extract and $1-5\times10^4$ cpm of R2 mRNA in 10% glycerol, 12 mM Hepes (pH 7.9), 15 mM KCl, 0.25 mM EDTA and 0.25 mM dithiothreitol (DTT), 5 mM $MgCl_2$, and *E.coli* tRNA (200 ng/μl) in a total volume of 15 μl at 30°C for 10 min. Forty units of RNase T1 (to digest unprotected riboprobe) were added and the reaction was incubated at 37°C for 10 min followed by the addition of 5 mg/ml heparin for an additional 10 min on ice to remove unspecific binding. Samples were subjected to electrophoresis on 7% native polyerylamide gels in 0.25×TBE (Tris-borate EDTA) buffer. The gel was dried and used for autoradiograms. In some assays, cytoplasmic extracts were incubated at 20 min at room temperature with different compounds (proteinase K and competitor mRNAs).

Ultraviolet (UV) light cross-linking of RNA–protein complexes

RNA-protein binding reactions were carried out using 30 μg of cytoplasmic protein extract and 5×10^4 cpm of labeled RNA per reaction. Following the addition of heparin, samples were placed on ice and irradiated for 15 min. in a UV-stratalinker chamber (Stratagene) apparatus, set at 25 mJ (energy level). The samples were then boiled in Laemmli buffer for 3 min. and separated by electrophoresis on 7.5% SDS-polyacrylamide gels. The gels were dried and exposed to Kodak X-AR films at −70°C.

In vitro RNA decay assay

The *in vitro* RNA decay reactions were modified from previously described methods (26,27). Briefly, the RNA decay reactions were performed in a total volume of 30 μl at 37°C using 0.7 A_{260} units of polysomes in 10 mM Tris−HCL (pH 7.5), 100 mM potassium acetate, 5 mM magnesium acetate, 2 mM dithiothreitol, 10 mM creatine phosphate, 1 μg creatine phosphokinase, 1 mM ATP, 0.4 mM GTP, 0.1 mM spermine and 8 units of RNasin (Promega), supplemented with 30 μg of S130 (post-polysomal supernatant fractions as previously described, 26) from TGF-β_1 treated and untreated cells. Specific competitor RNA (83 nt) was added where noted (see Figure 7C). After incubation of 37°C, total endogenous RNA was prepared by phenol extraction, and Northern blot analyses were performed (see Materials and Methods). RNA loading was determined by stripping the blot and reprobing for a stable transcript such as GAPDH mRNA. For *in vitro* decay reactions where exogenous R2 riboprobes were added, RNasin was not added. Aliquots from the reaction mixtures were quenched by the addition of 20 μl phenol at different times. After phenol and chloroform extractions, each sample was mixed with 5 μl of loading buffer (90% Formamide, 1×TBE, 0.02% bromophenol blue, 0.02% Xylene Cyanol), and the RNA was resolved by electrophoresis on a 10% sequencing gel. Gels were then dried and subjected to autoradiography and densitometric analyses.

CAT constructs and transfection

The chloramphenicol acetyltransferase (CAT) coding gene fragment with deleted poly(A) signal which renders the CAT mRNA unstable (28) was derived from the plasmid pSVlacO CAT. The CAT gene was then ligated into the BglII site of the PECE plasmid (29) with intact poly(A) signal. The R2 3′-UTR cDNA without the polyadenylation signal was inserted into a Sma1 site adjacent to the CAT coding sequence. CAT hybrid DNA constructs were cotransfected with the selectable marker plasmid pSVneo at 10:1 into BALB/c 3T3 mouse fibroblasts using the cell phect kit method (Pharmacia) according to the manufacturer's instructions. Stably transfected cells were selected with 400 μg of G418 (Gibco Laboratories) per ml of media, beginning 48 h after the addition of the DNA-calcium phosphate precipitate to the cells. Colonies (>30) were pooled and expanded into cell lines for use in experiments after subsequently screening for CAT expression and copy numbers.

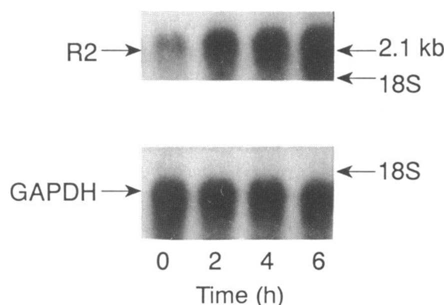


Figure 1. Effect of TGF-β_1 on R2 mRNA steady state level. R2 mRNA levels in BALB/c 3T3 fibroblasts incubated in the absence (control) or presence of 10 ng/ml TGF-β_1 for 2, 4 and 6 h. Total cellular RNA blots were probed with GAPDH (cDNA) as a control for loading. The position of R2 and GAPDH message and a ribosomal marker is indicated. R2 message levels were increased by 3.2, 4.3 and 6.5 fold following 2, 4 and 6 h TGF-β_1 treatment respectively. Similar results were obtained in 3 independent experiments.

RESULTS

Effect of TGF-β_1 on R2 gene expression

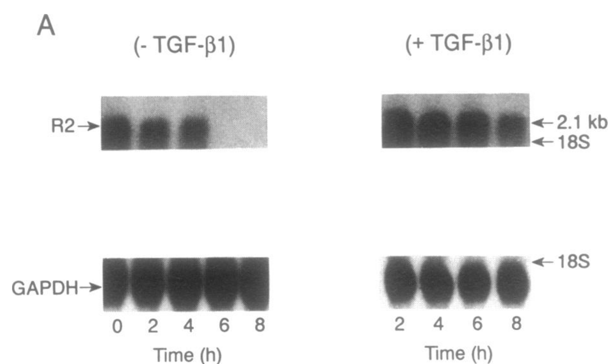
The effects of TGF-β_1 treatment on ribonucleotide reductase R2 gene expression in proliferating BALB/c 3T3 fibroblasts were determined by Northern blot analysis (Figure 1). In keeping with previous observations (9), significant elevations in R2 mRNA were observed. For example, in the experiments shown in Figure 1, R2 message elevations of 3.2, 4.3 and 6.5 fold were observed after 2, 4 and 6 hours, respectively.

Effect of TGF-β_1 on R2 message stability

To determine whether the TGF-β_1 induced increase in ribonucleotide reductase R2 mRNA steady state levels involved alterations in message stability, we compared R2 mRNA half-life in untreated and TGF-β_1 treated BALB/c3T3 mouse fibroblasts. Message turn-over rates were estimated by inhibiting transcription with 10 μg/ml actinomycin D and monitoring the decline in message level with time. The results from these experiments showed that TGF-β_1 treatment increased R2 message half-life by a factor of 4 from 1.5 h in untreated cells to 6 h in treated cells (Figures 2A and B). Similar results were also obtained in experiments in which both transcription and translation were inhibited (data not shown).

Novel RNA-binding factor interacts with R2 3′UTR message

To test the hypothesis that *trans*-acting factors exist that can bind to *cis*-element(s) of R2 message, an *in vitro* transcription vector pSPTSP6/T7 with the R2 3′UTR cDNA ligated into the Sma1 site of the transcription vector was used to generate the constructs shown in Fig. 3. RNA gel retardation and UV cross linking assays gave negative results for the 5′-UTR plus coding region (Figure 3C). The 3′-UTR showed a TGF-β_1 inducible RNA−protein complex bands (Figure 4) which corresponded to a 75 kd (p75) UV cross-linked protein (Figure 5). Sense RNA labeled transcripts of the deletion constructs derived from the 3′-UTR were synthesized and used in RNA-gel shift and UV cross-linking assays as described (see Materials and Methods). The results of these experiments are summarized as shown in Figure 3C. The results indicated that the nucleotide sequence fragment with the inducible protein mRNA binding activity is located between restriction enzyme sites at EcoRI-SauI (nucleotide positions 1861−1944 of R2 cDNA). This 83 nt cDNA sequence was then transcribed to the corresponding riboprobe as indicated (Fig. 3D). RNA band shift assays with the 83 nt riboprobe gave similar RNA−protein complexes (Fig. 4) and 75 kd UV cross-



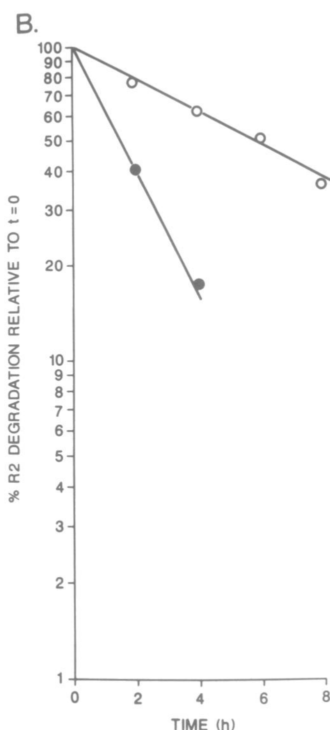


Figure 2. Effect of TGF-β_1 on R2 mRNA half-life. **(A)** BALB/c 3T3 cells were incubated with actinomycin D (10 μg/ml) to block transcription and in the presence of 10 ng/ml (+TGF-β_1) or absence (−TGF-β_1) for the indicated times (h). Total cellular RNA was isolated and probed for R2 message and GAPDH message (as a control for loading). **(B)** Autoradiographs of the Northern blots were quantitated by laser densitometry and the results (the average of 3 independent experiments) presented as a logarithmic plot of R2 message remaining at the indicated times relative to the 0 (h) level. The data presented showed that TGF-β_1 increased the half-life of R2 message by a factor of 4 from approximately 1.5 h in untreated cells to 6 h in TGF-β_1 treated cells; (●) and (○) shows data used to draw the best-fit lines for half-life measurements in the absence and presence of TGF-β_1 respectively.

linked protein as the R2 3′UTR message following TGF-β_1 stimulation.

Kinetics of p75-binding activity following TGF-β_1 treatment

If the TGF-β_1 dependent protein, p75 is involved in the regulation of mRNA metabolism, then the kinetics of p75-RNA binding might be expected to approximate the expression of R2 mRNA expression (Figure 1). To test this possibility, cells were treated with 10 ng/ml TGF-β_1 for various time periods. TGF-β_1 inducible RNA−protein complex formation was observed after 3 h (Figure 4, Lane 3). Inducible p75 binding activity and RNA protein complex formation was not observed with untreated cell extracts (Figure 4A, Lane 1). The corresponding UV crosslinking assay, using 3′-UTR as riboprobe demonstrated the presence of a 75 Kd protein−RNA binding activity following TGF-β_1 stimulation, but absent in untreated cells (Figure 5). The UV crosslinking assay also showed the presence of a 45 Kd protein present in both untreated and TGF-β_1 treated cells. This 45 Kd protein, which may serve as a useful control for loading is associated with the R2 message when the 3′-UTR is used as a probe but not with the 83 nt riboprobe (data not shown). The induction of p75 binding activity following TGF-β_1 treatment requires new protein synthesis, since cycloheximide diminished the induction of RNA−protein complex formation with time (Figure 4B, Lanes 4, 5 and 6).

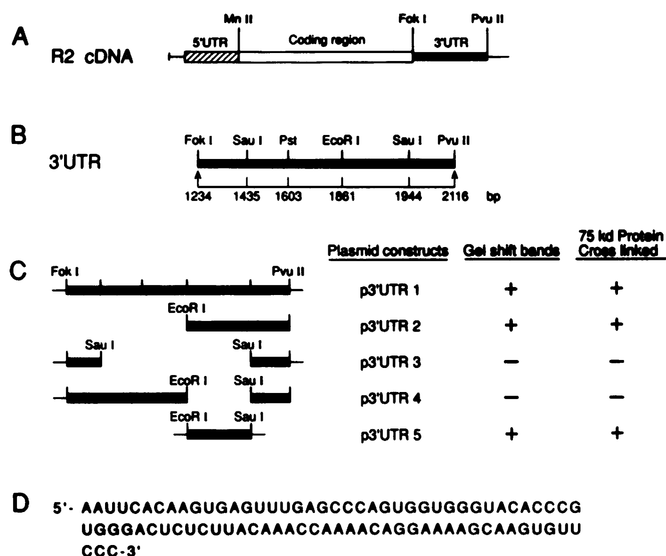


Figure 3. Restriction map of ribonucleotide reductase R2 cDNA. Schematic representation of the restriction enzyme map of mouse ribonucleotide reductase R2 cDNA: **(A)** Full length, **(B)** 3′-untranslated region and **(C)** deletion constructs generated from the 3′-UTR cloned into pSPT18SP6/T7 vector (p3′UTR-X) as described (see Materials and Methods). These constructs were used for *in vitro* transcription. Run-off transcripts were produced by the activity of T7 polymerase after linearization of the corresponding cDNAs. The labeled RNA probes were then used in RNA band shift and UV cross-linking assays (Figure 3C). The positive and negative signs indicate the presence and absence respectively of TGF-β_1 inducible RNA−protein complexes and the corresponding 75 kd UV cross-linked protein **(D)**. RNA nucleotide sequence of the 83 nt *cis* element(s) fragment.

Specificity of p75 binding

Specificity of R2 message-p75 binding activity was demonstrated by showing that other unlabeled competitor RNA transcripts: c-*myc* 3′UTR (human) IRE, Poly(A) and granulocyte macrophage colony stimulating factor GM-CSF (3′UTR) were poor competitors (Figure 4C) on the other hand unlabeled 83 nt and R2 3′UTR effectively competed out the formation of the RNA−protein complexes (Figure 4B, Lanes 2, 3). Pretreatment of cell lysate with proteinase K (2.5 mg/ml) eliminated evidence of TGF-β_1 inducible RNA−protein complex (Figure 4C) indicating the polypeptide component of these RNA−protein complexes.

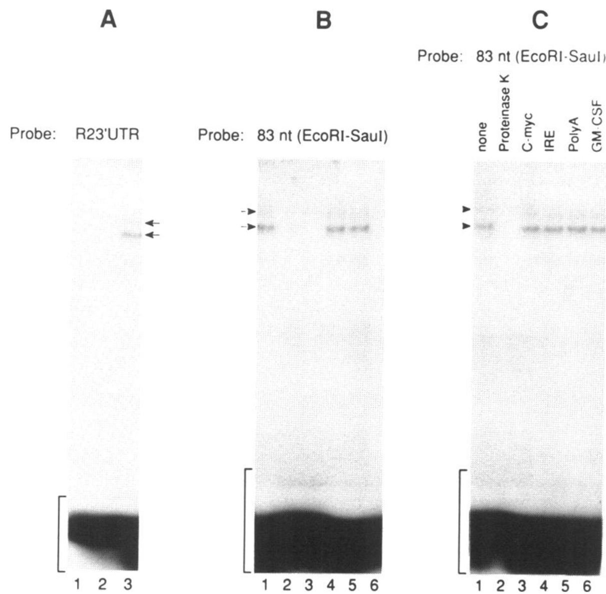


Figure 4. Identification of an RNA-binding activity that binds to 3'UTR R2 mRNA. RNA gel shift mobility assays were performed in the presence of 30 μg cytoplasmic protein lysate and 10^4 cpm ^{32}P-labeled 83 nt (EcoR1−SauI) or R2 3'UTR (SauI−PvuII at nt positions, 1435−2116) RNA probes. The arrow indicates the position of the RNA−protein band shift complexes. **(A)** Lanes: (1) untreated cell lysate as control, (2−3) cell lysate prepared from cells treated with 10 ng/ml TGF-β_1 for 2 and 3 h, respectively, **(B)** Lanes (1) 83 nt riboprobe, control (2−3) 1000× molar excess of specific competitors 83 nt and R2 3'UTR respectively (4−6) 10 ng/ml TGF-β_1 treatment in the presence of 10 μg/ml cycloheximide for 1, 2, 3 h respectively. **(C)** Lanes (1) TGF-β_1 treated cell lysate (2) Pretreatment of TGF-β_1 treated cell lysates with 2.5 mg/ml proteinase K, Lanes (3−6) Pretreatment of TGF-β_1 treated cell lysates with 1000×molar excess of competitor RNA transcripts: c-*myc* (3'UTR) IRE, poly A and GM-CSF (3'UTR) respectively.

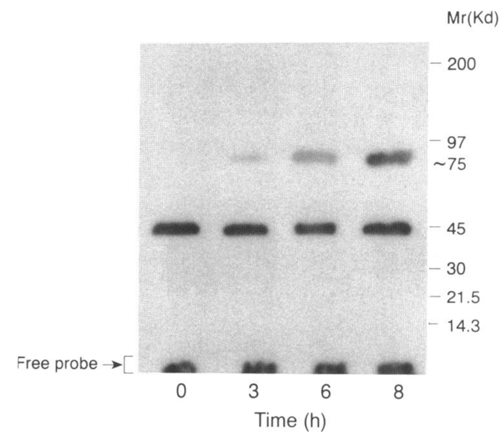


Figure 5. UV cross-linking and kinetics of p75 RNA-binding activity following TGF-β_1 stimulation. Following successive incubations with RNAse T1 and heparin, the reaction mixtures (see Materials and Methods) were UV cross-linked at 25 mJ for 15 min in a stratalinker, a UV cross-linking apparatus (Stratagene), and were subsequently separated by electrophoresis on 10% SDS-polyacrylamide gel. The binding reactions were carried out in the presence of 10^6 cpm ^{32}P labeled R2 3'-UTR probe and 30 μg cytoplasmic protein lysate from untreated cells (0 h) as control, and 10 ng/ml TGF-β_1 treated cells for 3, 6 and 8 h. The presence of the 45 kd protein in untreated cell lysate (control) is only observed for the R2 3'-UTR probe but not with the 83 nt probe (data not shown). Furthermore, the binding activity for the 45 kd protein is not affected by TGF-β_1 stimulation, and is a useful control for loading. Position of the molecular weight standard (M_r) in kilodaltons is indicated on the right.

Kinetics of *in vitro* decay reactions

To determine whether the *in vivo* TGF-β_1 mediated stabilization of R2 message (Figure 2) involves the TGF-β_1 inducible protein (p75) detected *in vitro*, we investigated the effects of polysomal and post-polysomal fractions prepared from TGF-β_1 treated and untreated cells, on endogenous R2 message degradation in an *in vitro* system used previously to study RNA degradation (26). To determine whether the *in vitro* decay system contained RNA-binding proteins that influence R2 message turnover, the specific competitor (83 nt RNA fragment) or non specific competitor (c-*myc* 3'UTR) was added to the cell free system. If the competitor sequestered a regulatory factor, the stability of R2 message might be expected to decrease in the presence of TGF-β_1 stimulation. The data presented in Figures 6A and B show that R2 message degradation was significantly reduced with cell lysates from TGF-β_1 treated cells when compared to control lysates from untreated cells. Furthermore, pre-incubation of cell lysates from TGF-β_1 treated cells with the specific competitor RNA (83 nt RNA) caused a destabilization of R2 message (Figure 7C). These results indicate that titrating out the p75 protein is analogous to the absence of TGF-β_1 stimulation. The c-*myc* 3'-UTR RNA (non-specific competitor) had no effect on RNA message stabilization (data not shown) demonstrating the sequence specificity of TGF-β_1 mediated R2 message stabilization. These results are consistent with our hypothesis that TGF-β_1 inducibility of p75 binding activity affected the normal R2 message turnover. These data do not however directly implicate the involvement of the 83 nt RNA in R2 message turnover. To test this possibility further, we carried out an *in vitro* decay reaction previously described (27), by supplementing the polysomes with exogenous R2 riboprobe. This enabled us to determine the effect of an internal deletion of the 83 nt fragment on R2 message decay. The data in Figures 7A and B showed that TGF-β_1 stimulation stabilized the exogenous R2 message. However, with an internal deletion of the 83 nt RNA fragment, R2 message was destablized even in the presence of TGF-β_1 stimulation (Figure 8B). This result indicates that the deletion of the 83 nt RNA is analogous to preventing the binding of p75 to R2 mRNA or lack of TGF-β_1 induction of p75-binding activity. By using only polysomes in the *in vitro* decay reactions there was no significant difference observed between TGF-β_1 treated and untreated cells (data not shown). This indicates that the p75 binding protein is not ribosomal bound. The *in vitro* decay reactions showed specificity as there was no difference between the half-life of GAPDH or c-*myc* mRNAs in fraction from TGF-β_1 treated and untreated cells.

Requirement of the 3'UTR (83 nt RNA) in TGF-β_1 induced stabilization of CAT mRNA

If the 83 nt RNA fragment is involved in the TGF-β_1 mediated stabilization of R2 message observed *in vivo* (Figure 2), this fragment might be expected to transfer the TGF-β_1 response onto a heterologous gene. We have assessed the role of the 3'UTR on the stability of cytoplasmic mRNA in a stably transfected murine fibroblast system through the use of CAT hybrid deletion constructs. As shown in Figure 8A, the 3'UTR increased the TGF-β_1 mediated stabilization of CAT hybrid mRNA by a factor of about 3.3. However, no significant difference in CAT hybrid mRNA half-life between TGF-β_1 treated and untreated

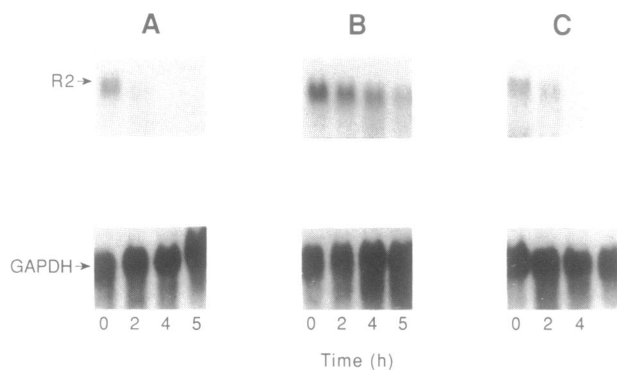


Figure 6. Effect of competitor RNA on *in vitro* R2 decay. The *in vitro* decay reactions were supplemented with 30 μg of S130/fractions from exponentially growing cells. RNA (20 μg) was electrophoresed and the blot was then hybridized separately to ^{32}P labeled probes of R2 cDNA (see Material and Methods) and coding region of mouse GAPDH. The blot was autoradiographed. Reaction times in (hours) are shown below the lanes. **(A)** untreated cells, **(B)** 10 ng/ml TGF-β_1 stimulation for 6 h, and **(C)** pre-incubation of TGF-β_1 treated cell lysates with 3 μg of the 83 nt sense RNA (specific competitor) for 30 min. Estimated half-lives of R2 message determined were: (A) 1. h, (B) 4 h, and (C) 1.5 h. Similar results were obtained in 3 independent experiments.

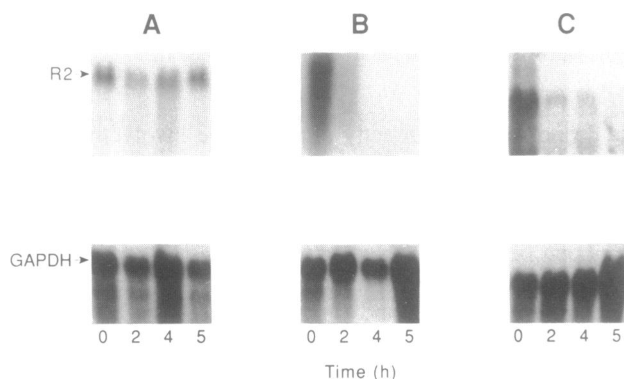


Figure 7. Effect of the 83 nt internal deletion on R2 decay. The *in vitro* decay reactions were supplemented with 30 μg of S130 fractions from exponentially growing cells and 10^4 cpm of exogenous R2 and GAPDH ^{32}P labeled riboprobes. RNA was separated by electrophoresis on 10% sequencing gels. The gels were dried and autoradiographed. Reaction times (hours) are shown below the lanes: **(A)** 10 ng/ml TGF-β_1 stimulation for 6 h., **(B)** untreated cells, **(C)** 10 ng/ml TGF-β_1 stimulation for 6 h, and in the presence of exogenous R2 riboprobe with the 83 nt RNA fragment deleted from the 3'UTR. Estimated half-lives of R2 message: (A) > 4 h, (B) 1.1 and (C) 50 min. Similar results were obtained in 3 independent experiments.

cells was detected with a 3'-UTR containing an internal deletion of the 83 nt RNA (Figure 8B). These results suggest that the 83 nt RNA contains a TGF-β_1 responsive cis-element(s) involved in R2 message stabilization.

DISCUSSION

Several studies have indicated that growth factor regulation of gene expression may involve posttranscriptional mechanisms, presumably by altering processing, transportation or stability of target mRNAs. However, very little is known about the posttranscriptional mechanism(s) underlying growth factor

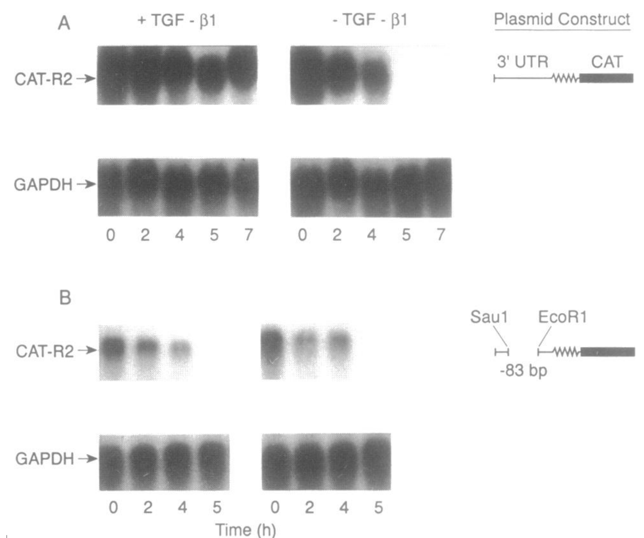


Figure 8. Role of 3'UTR in TGF-β_1 mediated stability of cytoplasmic mRNA. Mouse BALB/c 3T3 cells were stably transfected with CAT/R2 3'UTR hybrid DNA plasmid constructs: **(A)** The 3'UTR R2 cDNA was inserted adjacent to the CAT coding sequence, and **(B)** The 83 nt RNA-p75 binding fragment was deleted from the 3'UTR. Exponentially grown cells were incubated with actinomycin D (10 μg/ml) to block transcription in the presence and absence (control) of 10 ng/ml TGF-β_1 for the indicated time periods, shown below the lanes. Total cellular RNA was isolated and probed for CAT and GAPDH message. Autoradiographs of the Northern blots were quantitated by laser densitometry. Estimated half-lives of CAT hybrid mRNAs were: (A) TGF-β_1:5.4 h, control:1.6 h, (B) TGF-β_1:2.8 h, control:2.2. Similar results were obtained in 3 independent experiments.

modulation of gene expression. In this study, TGF-β_1 treatment of BALB/c 3T3 cells increased the half-life of ribonucleotide reductase R2 message by 4 fold from 1.5 h in untreated cells to 6 h in treated cells. This observation is consistent with our finding that TGF-β_1 treatment of BALB/c3T3 fibroblasts caused a marked elevation of R2 mRNA levels by 4.3 and 6.5 fold at 4 and 6 h respectively. These results of course do not rule out the possibility and perhaps likelihood that TGF-β_1 may modulate ribonucleotide reductase gene expression by altering transcription as well (10).

Interestingly, R2 message was found to contain a previously undescribed 83 nt fragment (derived from R2 cDNA EcoR1/Sau1 fragment) located in the proximal portion of R2 3'UTR. This fragment binds a specific TGF-β_1 responsive cytoplasmic protein, with a molecular weight of 75 kd (p75) as determined by UV cross-linking and SDS-polyacrylamide gel electrophoresis. This 83 nt RNA also appears to be unique because it does not contain any known RNA-protein binding sequences such as the AUUUA pentamer (30,31), the iron response element (IRE) (32) or the ornithine decarboxylase 5'-UTR conserved sequence that binds a 58 kd protein (25). In addition, specificity of p75 binding activity was demonstrated by the observation that other 3'UTR RNA sequences were poor competitors. The kinetics of appearance of p75 binding activity following TGF-β_1 stimulation showed a significant increase in binding activity after 3 h, which correlated with the accumulation of R2 message. The induction of p75 binding activity following TGF-β_1 stimulation requires new protein synthesis since cycloheximide diminished the formation of the TGF-β_1 inducible RNA-protein complex. Thus, the correlation between p75 binding activity, the induction

of R2 gene expression and the observation that cycloheximide and proteinase K block induction of p75 binding is consistent with factor p75 as an induced regulatory protein. Our data, from the *in vitro* decay assays, showed that R2 message degradation was significantly reduced in postpolysomal fractions from TGF-β_1 treated cells when compared to untreated cells. However, preincubation of postpolysomal fractions from TGF-β_1 treated cells with the specific competitor RNA (83 nt RNA) caused a destabilization of R2 message. No effect on R2 mRNA stability was observed *in vitro* when a non-specific competitor RNA (human c-*myc* 3′-UTR) was used (data not shown). To account for these observations, we suggest that the 83 nt RNA specific competitor fragment titrated out the p75 *trans*-acting factor or inhibited its binding to R2 message. Furthermore, internal deletion of the 83 nt RNA from R2 message caused destabilization of exogenous R2 mRNA in the presence of postpolysomal fractions from TGF-β_1 treated cells. This result indicates that the deletion of this fragment is analogous to preventing the binding of p75 to R2 message. In addition to this *in vitro* degradation system, we also examined the role of the 3′UTR in message stabilization *in vivo*. The TGF-β_1 effect on message stability was transfered onto a heterologous gene (CAT) by the 3′-UTR. However, deletion of the 83 nt RNA from the 3′UTR showed no significant affect on CAT message half-life in the presence of TGF-β_1, supporting the view that the 83 nt RNA contains a TGF-β_1 responsive *cis*-element(s). Although the 83 nt region is necessary for message stability, it may not be sufficient on its own, and our results do not rule out the possibility that other sequences within the R2 3′UTR (not included in the 83 nt fragment) may also play a role in message stability. We are presently attempting to map with greater precision the *cis*-element(s) in the 83 nt sequence, and to subsequently purify and characterize the novel p75 *trans*-acting factor, so that it can be used to test posttranscriptional models of growth factor control of gene expression and provide further insight into the mechanism responsible for regulating message stability.

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