The receptor for hyaluronan mediated motility (RHAMM) gene expression is markedly elevated in fibrosarcomas exposed to transforming growth factor-β1 (TGF-β1). The half-life of RHAMM mRNA was increased by 3 fold in cells treated with TGF-β1, indicating that growth factor regulation of RHAMM gene expression at least in part involves a posttranscriptional mechanism. Our studies demonstrated that a unique 30-nucleotide (nt) region that has three copies of the sequence, GCUUGC, was the TGF-β1-responsive region in the 3′-untranslated region (3′-UTR) that mediated message stability. This region interacted specifically with cytoplasmic trans-factors to form multiple protein complexes of approximately 175, 97, 63, 26, and 17 kDa post-TGF-β1 treatment, suggesting a role for these complexes in the mechanism of action of TGF-β1-induced message stabilization. Insertion of the 3′-UTR into the chloramphenicol acetyltransferase gene conferred TGF-β1 induced stability of chloramphenicol acetyltransferase-hybrid RNA in stably transfected cells, while the same insert carrying a deletion containing the 30 nt region had no significant effect on mRNA stability. These results provide a model of RHAMM message regulation in which TGF-β1-mediated alteration of RHAMM message stability involves the up-regulation of multiple protein interactions with a 30 nt cis-element stability determinant in the 3′-UTR. This model also suggests that this 30 nt base region functions in cis to destabilize RHAMM mRNA in resting normal cells.

**EXPERIMENTAL PROCEDURES**

Cell Culture—C1, C2, and C3 fibrosarcomas derived from 10T½ cells following T24 Ha-ras transfections (17), and cells transfected with chloramphenicol acetyltransferase (CAT) hybrid RHAMM plasmids were routinely cultivated in a -minimal essential medium (Flow Laboratories) supplemented with antibiotics and 10% (v/v) fetal bovine se- rum (18). For investigating TGF-β1 effects on RNA levels and RNA-protein binding activity, cells were grown overnight in a serum-free medium containing 0.4 mg of transferrin and 0.2 mg of insulin (Sigma-Aldrich Canada) in 100 ml of a-MEM. TGF-β1 (R & D Systems Inc.) was dissolved in 1.0 mg/ml bovine serum albumin, and 4 ml HCl and was added at predetermined times. The control cells received 1.0 mg/ml bovine serum albumin, 4 ml HCl, 0.4 mg of transferrin and 0.2 mg of insulin in 100 ml of the serum-free medium. The cells were harvested from the tissue culture plates with 0.3% buffered trypsin solution after centrifugation, washed once in phosphate-buffered saline (pH 7.2), and transferred to Eppendorf tubes.

Preparation of Protein Extracts from the Cytosol and the Nucleus—Cells transfected to Eppendorf tubes were briefly centrifuged for 1 min and resuspended in hypotonic buffer (25 mM Tris-HCl (pH 7.9), 0.5 mM EDTA) and lysed by repetitive cycles of freeze-thaw. Nuclear and cytoplasmic extracts were obtained (5, 8), and protein concentrations were determined according to previously described methods (16) and imme-diately frozen on dry ice and stored at −70°C.

In Vitro Transcription—A 1.9-kilobase pair RHAMM cDNA clone (1),
containing part of the coding region and the entire 3′-UTR, was used as a template to generate PCR-amplified products. These corresponded to different segments of the 3′-UTR as shown in Fig. 2A, which spanned the following regions from nucleotides: 2082–2309 (full-length 3′-UTR), 2082–2092, 2082–2204, 2394–2435, and 2444–3004 (2). To facilitate cloning, the oligonucleotide primers (20–40 bases) complementing these regions were flanked at their 5′ and 3′ ends, by BamHI and EcoRI restriction sites, respectively. PCR parameters were: denaturation at 94 °C for 4 min, denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s, and extension at 72 °C for 2 min. Thirty cycles were used with a final extension time of 7 min. The reaction mixture contained 50 ng of DNA template, 40 pmol of DNA primers, and 5 units of Taq DNA polymerase in a total volume of 100 μL. The samples were gel-purified and subsequently extracted once with phenol/chloroform/isooamyl alcohol (25:4:1) and once with chloroform, precipitated in ethanol, resuspended in a suitable amount of Tris-EDTA (pH 7.5), and digested with the restriction endonucleases BamHI and EcoRI. Digests were heat-inactivated at 65 °C for 15 min and ligated into the pSPT18 in vitro transcription plasmid (Boehringer Mannheim) to generate a plasmid construct.

In brief, run-off RNA transcripts were produced by T7 polymerase activity (Boehringer Mannheim) from 1 μg of digested pSPT18 RHAMM cDNA plasmid constructs. These constructs were linearized with the HindIII restriction enzyme, at a unique site in the polylinker of the pSPT18 plasmid. These digested plasmids were then used for in vitro transcription. RNA transcripts were produced as described previously (5, 6) and 3′-end labeled with [γ-32P]-ATP using T4 polynucleotide kinase. The RNA was gel-purified and UV-cross-linked to microtiter wells placed on ice and UV-cross-linked with actinomycin D (10 μg/ml) to block transcription, in the absence (−) or presence (+) of 10 ng/ml TGF-β1, for the indicated time periods (hours).

RESULTS

Effect of TGF-β1 on RHAMM Gene Expression—Northern blot analysis using the 1.9-kilobase pair EcoRI fragment of RHAMM as a cDNA probe (1) showed that RHAMM message levels in C3 fibrosarcoma cells were significantly elevated during a 3–20-h treatment time period with 10 ng/ml TGF-β1 (Fig. 1A). To determine whether or not the TGF-β1 effect on RHAMM mRNA steady-state level results from, at least in part, changes in RHAMM message stability, Northern blot analysis of RHAMM mRNA level was performed using the total RNA obtained from TGF-β1-treated and untreated cells, in which transcription was blocked by actinomycin D (15, 16). Fig. 1B shows that there was a 3-fold increase in RHAMM message half-life in TGF-β1-treated cells in comparison with untreated cells.

Identification of Proteins Forming Complexes with a Unique cis-Element in the 3′-UTR of RHAMM mRNA—The knowledge that the 3′-UTR of other mRNAs have previously been implicated in the regulation of growth factor alterations of message stability (5, 6, 13–16) led us to test the hypothesis that TGF-β1-responsive trans-acting factors exist that can bind to a cis-element(s) of RHAMM mRNA 3′-UTR. RNA mobility shift assays were performed using radiolabeled in vitro transcribed RHAMM mRNA transcripts corresponding to the full-length 3′-UTR. As demonstrated in Fig. 2A, the 3′-UTR showed multiple RNA-protein interactions post-TGF-β1 treatment. To
identify the sequence within the 3'UTR that forms the RNA-protein complexes, we generated a series of in vitro transcribed fragments corresponding to different segments of the 3'UTR as shown in Fig. 2A (lanes 4–8), to be used in RNA binding assays as possible RNase T1 protected fragments. The data summarized in Fig. 2, A and B, indicate that the nucleotide sequence fragment with multiple protein complex interactions is located between nucleotide positions 2394 and 2435 (42 nt).

Interestingly, this 42-nt binding fragment consists of three copies of the 7-nt sequence, GCUUGCU (Fig. 2, B and C). To more precisely locate the cis-element within the 42-nt RHAMM 3'UTR fragment that forms the TGF-β1-inducible multiple RNA-protein complexes, we generated a series of synthetic oligoribonucleotides corresponding to different regions of the 42 nt. The data summarized in Fig. 2C indicate that the cis-element binding site, which forms TGF-β1 up-regulated multiple protein complexes as observed with the full-length RHAMM 3'UTR, is within the 30-nt region, nt 2400–2429.

Effect of Mutations on the cis-Element-Protein Binding Activity—To assess the contributions of the individual nucleotides to the cis-element binding activity, in an attempt to determine a consensus sequence, we focused our attention on the three copies of the sequence GCUUGCU by producing a substitution mutation within this sequence and monitoring binding activity by standard gel shift assays. The results summarized in Fig. 3 showed that UU to AA changes within this sequence, GCU-UGCU, showed no detectable binding (lane 2). Similar results were obtained with UU to GG or CC changes (lanes 3 and 5). Changes of the core U residues within the last two copies of the 7-nt sequence to CC (GCCCUGCU) resulted in no detectable binding (lane 6). Changing the G residues within the GCU-UGCU sequence to A residues also abolished binding. Interestingly, substituting the terminal U residue of the sequence GCUUGCU to A did not affect binding (lane 4), indicating that this U residue is not part of the binding motif. This observation further refines the binding requirement to a sequence consisting of GCUUGC.

Characterization of the cis-Element Binding Activity—Standard RNA gel shift assays were used to investigate the properties of the TGF-β1-mediated RHAMM mRNA-protein binding activity. The results summarized in Fig. 4A showed that pre-incubation of cytoplasmic extracts from C3 TGF-β1-treated cells with proteinase K (40 units/ml) or 0.1% SDS abolished the
the oligonucleotide sequences shown along the R2 protein (R1BP, R2BP) binding lar expression of the TGF- 

cis

ductase R1 and R2 binding protein mRNA (32, 33). The

3'-UTR of lymphokine and cytokine mRNAs (32, 33). The

mRNA. Using the C3 cell line, various 3'-UTR-CAT plasmid

formation of the multiple RNA protein complexes (lanes 2 and 3). When RNA binding reactions were performed with nuclear extracts instead of cytosolic extracts, the TGF-β1 up-regulation of RNA-protein complexes was not observed (lanes 4 and 5). These results demonstrated the polypeptide component of these complexes and suggested that the proteins involved in complex formation are confined to the cytoplasm. The results in Fig. 4B showed that the related sequences, the RHAMM 3'-UTR self-competitor, the 30-nt cis-element fragment, and three copies of the sequence GCUCUGCUCUUGCUUGCU (lanes 6 and 7), ribonucleotide reductase R1 and R2 binding protein cis-elements, respectively. Potential competitor sequences were included at a 10-fold molar excess. C, cellular expression of the TGF-β1-inducible multiple RNA-protein complexes. Lane 1, RNA binding reactions of the RHAMM cis-element riboprobe only in the absence of cell lysate (control). Lanes 2-5, RNA gel shift assays were carried out with cell extracts from C1 and C2 fibrosarcomas untreated (−) or treated (+) with 10 ng/ml TGF-β1 for 4 h.

To investigate the possibility of the role of the TGF-β1-responsive cis-element in the stabilization of RHAMM mRNA in vivo, we have assessed the contribution of the 3'-UTR of RHAMM mRNA to the stability of a heterologous CAT mRNA. Using the C3 cell line, various 3'-UTR-CAT plasmid constructs shown in Fig. 6 were stably transfected. The half-lives of the CAT hybrid transcripts were determined in the absence and presence of TGF-β1 treatment as described previously (5, 6). The results showed that in the control experiments there was no significant difference between the half-life of the CAT transcript in the absence or presence of TGF-β1 treatment (Fig. 6A). Northern blot analysis showed a markedly significant difference between the half-life of CAT RH1 in the presence and absence of TGF-β1 stimulation. These results indicated that treatment of cells with 10 ng/ml TGF-β1 increased the half-life of CAT mRNA with the RHAMM 3'-UTR by a factor of about 5 relative to message from untreated cells (Fig. 6B). Interestingly, no changes in half-lives were observed after TGF-β1 stimulation with CAT RH2 mRNA, which has a 3'-UTR deletion of a 295-bp fragment containing the 30-nt TGF-β1-responsive cis-element binding site (Fig. 6C). Furthermore, deletion of a 450-bp fragment (nt 2684–3133) downstream of this element, pCATRH3, did not abolish the TGF-β1-induced stabilization of CAT hybrid mRNA (Fig. 6D).

DISCUSSION

In the present study, we have demonstrated for the first time that alterations in message stability through specific cis-trans interactions play an important role in the TGF-β1-induced elevation of RHAMM mRNA. We have identified a novel 30-nt cis-element, 5'-GCUCUGCUCUUCUCGGCUUGCUAGCCUUGCU-3' (nt 2400–2429), in the 3'-UTR of RHAMM mRNA, which interacts with multiple cytosolic protease sensitive factors in fibrosarcomas exposed to TGF-β1, to form five major RNA-protein complexes of approximately 175, 97, 63, 26, and 17 kDa. These results showed that in the control experiments there was no significant difference between the half-life of the CAT transcript in the absence or presence of TGF-β1 treatment (Fig. 6A). Northern blot analysis showed a markedly significant difference between the half-life of CAT RH1 in the presence and absence of TGF-β1 stimulation. These results indicated that treatment of cells with 10 ng/ml TGF-β1 increased the half-life of CAT mRNA with the RHAMM 3'-UTR by a factor of about 5 relative to message from untreated cells (Fig. 6B). Interestingly, no changes in half-lives were observed after TGF-β1 stimulation with CAT RH2 mRNA, which has a 3'-UTR deletion of a 295-bp fragment containing the 30-nt TGF-β1-responsive cis-element binding site (Fig. 6C). Furthermore, deletion of a 450-bp fragment (nt 2684–3133) downstream of this element, pCATRH3, did not abolish the TGF-β1-induced stabilization of CAT hybrid mRNA (Fig. 6D).
the cis-element with the trans-acting proteins and suggest that
the sequence 5'-GCUUGC-3' is important for binding activity.
It is not known if the nucleotides in the 30-nt cis-element
observed not to be directly involved in protein binding in these
studies play some other roles in message regulation. Further
studies will more precisely define their potential roles and also
determine the relative importance of the three 5'-GCUUGC-3'
motifs within the 30-nt cis-element sequence, with regard to
protein binding and eventually in models of message stability
regulation. Knowledge of a cis-element binding motif for TGF-
β1-responsive mRNA-binding proteins may alert us to regula-
tory sequences present in candidate 3'-UTR that are posttranscrip-
tionally regulated by TGF-β1 (34, 35). The appearance of
the RNA-protein complexes following TGF-β1 treatment of C3
cells was time-dependent and was also observed with cytosolic
extracts from the TGF-β1-treated ras-transformed fibrosarco-
mas, C1 and C2.

Insertion of the 3'-UTR of RHAMM next to a heterologous
CAT sequence resulted in a marked increase in the stability of
CAT hybrid mRNA following TGF-β1 treatment, supporting
the concept that the RHAMM 3'-UTR is directly involved in the
process that mediates TGF-β1-induced stabilization of RHAMM mRNA. This view is consistent with the finding that
an internal deletion within the RHAMM 3'-UTR, containing the
30 nt cis-element, abolished the TGF-β1-induced increase in
CAT hybrid mRNA half-life, and suggests that the RHAMM
cis-element is necessary to mediate the TGF-β1 effects on mes-
sage stability. Further evidence of the importance of the 30 nt
cis-element was the observation that another deletion within
the 3'-UTR downstream of the cis-element sequence did not
prevent the TGF-β1 induced stabilization of CAT hybrid mRNA.

The results of this investigation are consistent with a model
in which the 30-nt cis-element functions as a message destabi-
lider, and the TGF-β1-responsive proteins act as stabilizing
factors by binding to the cis-element sequence, leading to a
reduced rate of RHAMM mRNA degradation. It will be impor-
tant in future studies to further refine the minimal cis-element
and to determine how the binding of the TGF-β1-responsive
proteins alter message-targeted nuclease activity in ras-trans-
formed cells expressing RHAMM mRNA.

Acknowledgment—We are grateful to Arthur Chan for technical
assistance.

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Fig. 6. The role of the 3'-UTR in TGF-β1-mediated stabilization of CAT hybrid mRNA. C3 fibrosarcoma cells were stably transfected with
pCAT (A) and various pCAT hybrid RHAMM constructs: pCATRH1 (B), pCATRH2 (C), and pCATRH3 (D). The numbers refer to the nucleotide
positions of the various restriction enzyme sites. PL, is the polylinker region. The half-lives (hours) of the corresponding transcripts from these
plasmid constructs were determined in the absence (−) and presence (+) of TGF-β1 (30 ng/ml) treatment. The results are the average of two
independent experiments. The RHAMM (long tail arrow) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (short tail arrow) transcripts
are indicated.
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Transforming Growth Factor-\(\beta\) Stimulates Multiple Protein Interactions at a Unique \textit{cis}-Element in the 3'-Untranslated Region of the Hyaluronan Receptor RHAMM mRNA

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\textit{J. Biol. Chem.} 1996, 271:15279-15284.
doi: 10.1074/jbc.271.25.15279

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