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A GC-Rich Domain with Bifunctional Effects on mRNA and Protein Levels: Implications for Control of Transforming Growth Factor β1 Expression

LUIGI SCOTTO AND RICHARD K. ASSOIAN†*

Department of Biochemistry and Molecular Biophysics and Center for Reproductive Sciences, College of Physicians and Surgeons, Columbia University, New York, New York 10032

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Chimeric plasmids containing selected reporter coding domains and portions of the transforming growth factor β1 (TGF-β1) 3′ untranslated region (UTR) were prepared and used to identify potential mechanisms involved in regulating the biosynthesis of TGF-β1. Transient transfections with core and chimeric constructs containing the chloramphenicol acetyltransferase (CAT) reporter showed that steady-state CAT mRNA levels were decreased two- to threefold in response to the TGF-β1 3′ UTR. Interestingly, CAT activity was somewhat increased in the same transfectants. Thus, production of CAT protein per unit of mRNA was stimulated by the TGF-β1 3′ UTR (approximately fourfold in three cell lines of distinct lineage). The translation-stimulatory effect of the TGF-β1 3′ UTR suggested by these studies in vivo was confirmed in vitro by cell-free translation of core and chimeric transcripts containing the growth hormone coding domain. These studies showed that production of growth hormone was stimulated threefold by the TGF-β1 3′ UTR. A deletion analysis in vivo indicated that the GC-rich domain in the TGF-β1 3′ UTR was responsible for both the decrease in mRNA levels and stimulation of CAT activity-mRNA. We conclude that this GC-rich domain can have a bifunctional effect on overall protein expression. Moreover, the notable absence of this GC-rich domain in TGF-β2, TGF-β3, TGF-β4, and TGF-β5 indicates that expression of distinct TGF-β family members can be differentially controlled in cells.

Of the peptide growth factors characterized to date, transforming growth factor β1 (TGF-β1) is outstanding in its ability to evoke wide-ranging effects in a very large number of cell types. For example, TGF-β1 can either inhibit or stimulate proliferation, depending upon cell lineage and environment (33, 35, 46). Similar bifunctional effects of TGF-β1 have been observed for cell differentiation (33, 35, 46). Since receptors for TGF-β1 are widely distributed and rarely regulated (33, 52), it is likely that TGF-β1 action is controlled at the level of growth factor expression. The need for carefully regulated expression is emphasized by studies showing that bifunctional effects of TGF-β1 can result solely from changes in the extracellular concentration of the growth factor (3, 37, 51).

Studies examining control of TGF-β1 expression have shown that the human TGF-β1 promoter contains two transcription start sites and several binding domains for known transcription factors (23, 24). Phorbol ester-responsive elements seem particularly important and have been identified in both the upstream and downstream domains of the gene (23, 24, 43). Phorbol ester also up-regulates TGF-β1 gene expression posttranscriptionally in hematopoietic cell lines by selectively stabilizing the mRNA (50). Thus, activation of protein kinase C can influence the overall expression of TGF-β1 at multiple levels. Finally, production of TGF-β1 is also regulated at the translational level: freshly isolated monocytes express relatively high levels of the mRNA but fail to produce the protein (1).

There are numerous examples showing that protein biosynthesis in eukaryotes is dictated by motifs within the corresponding transcripts as well as the corresponding genes. For example, the presence of an AUUUA multimer in the 3′ untranslated region (UTR) of mRNAs is typically associated with transcript instability (45). GC-rich domains and stem-loop structures in the 5′ UTRs of mRNAs often inhibit translation (13, 16, 27, 32, 36, 39). Other studies have shown that specific *cis* elements in the 5′ and 3′ UTRs of ferritin (7, 48) and β-interferon (29) mRNAs, respectively, confer regulated translation on these transcripts. The interaction between *cis* elements in the 5′ and 3′ UTRs and *trans* factors likely dictates the stability and translatability of many mRNAs (4, 5, 13, 16, 18, 28, 40, 41).

Our previous studies on TGF-β1 gene expression showed that polyadenylation of the human TGF-β1 transcript is consistently signalled by an ATTAAA sequence rather than the nearby AATAAA consensus signal (43). Given the potential involvement of 3′ UTRs in control of protein biosynthesis (see above) and the likely contribution of translational control to production of TGF-β1 (1), we reasoned that consistent use of this atypical signal suggests a functional role for the consequent 3′ UTR in the production of TGF-β1. In the experiments reported here, we prepared chimeric transcripts in which the 3′ UTR of human TGF-β1 mRNA was positioned downstream of reporter genes. We used these chimeras to determine whether the TGF-β1 3′ UTR can influence production of mRNA or protein in vivo and in vitro.

MATERIALS AND METHODS

Cell culture. U937 (clone 7, a generous gift from Joseph DeLarco) cells were cultured in RPMI 1640 medium containing 10% heat-inactivated newborn calf serum and maintained

* Corresponding author.
† Present address: Department of Cell Biology and Anatomy and Cancer Center, University of Miami School of Medicine, Miami, FL 33101.

at a density of 0.1×10^6 to 0.5×10^6/ml. Clone 7 cells are a weakly adherent subpopulation of U937 promonocytes. NIH 3T3 and A549 cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% calf serum and heat-inactivated newborn calf serum, respectively. All media contained gentamicin. In some experiments, cells were treated with 160 nM 12-tetradecanoylphorbol 13-acetate (TPA; LC Services) for the last 40 h of the transfection procedure (see below).

Constructs for transfection. Studies in vivo used the pCDM8 expression vector (Invitrogen) with the chloramphenicol acetyltransferase (CAT) coding domain. The core CAT construct was prepared in two steps. First, a fragment containing the CAT coding domain and a small portion (25 of 96 bases) of the CAT 3′ UTR was obtained by *Hin*dIII-*Nla*IV digestion of CAT GenBlock (Pharmacia). The fragment was cloned into the *Hin*dIII-*Sma*I site of BlueScript KS+, and the *Hin*dIII-*Xba*I fragment from this clone was isolated and ligated to *Hin*dIII-*Xba*I-digested pCDM8. The ligation mixture was used to transform MC1061-P3 (Invitrogen), and the desired clone (pCAT; see Fig. 1C) was selected on the basis of restriction analysis and DNA sequencing.

We also constructed clones (pCATβ-UTR1, pCATβ-UTR2, pCATβ-UTR3, pCATβ-UTR3A, pCATβ-UTR3B, and pCATβ-UTR4) in which all or parts of the TGF-β1 3′ UTR were positioned downstream of the CAT reporter (see Fig. 1D and 3). First, a *Pvu*II-*Hin*dIII fragment containing the entire 3′ UTR of the TGF-β1 cDNA (nucleotides 2014 to 2153, a 20-base poly(A) tail, and a *Pst*I-*Hin*dIII stuffer from pTZ19R), the *Hin*dIII-*Nla*IV fragment from CAT GenBlock, and *Hin*dIII-digested pTZ19R (Pharmacia) were ligated, and the ligation mixture was used to transform JM109. Restriction analysis allowed identification of a clone in which the TGF-β1 3′ UTR was positioned downstream of the CAT coding domain. Second, the *Hin*dIII-*Pst*I fragment from this clone was ligated to *Hin*dIII-*Pst*I-digested pCDM8, and the reaction mixture was cloned by transformation in MC1061-P3 to obtain expression vector pCATβ-UTR1 (see Fig. 1D). Third, pCATβ-UTR1 was subjected to partial digestion with *Apa*I, *Nco*I, or *Nci*I (to cut the TGF-β1 3′ UTR at nucleotides 2133, 2085, and 2042, respectively). Incubation of the *Apa*I-, *Nco*I-, and *Nci*I-linearized plasmids with *Xba*I eliminated 20-, 64-, and 112-bp fragments, respectively, of the TGF-β1 3′ UTR containing the polyadenylation signal and poly(A) tail (see Fig. 1D). Blunt ending with the Klenow fragment from DNA polymerase I and ligation of these digests preceded transformation into MC1061-P3 and allowed the cloning of pCATβ-UTR2, pCATβ-UTR3, and pCATβ-UTR3A (vectors containing the CAT coding domain and 116, 72, and 29 bases of the TGF-β1 3′ UTR, respectively; see Fig. 1D and 3).

To prepare pCATβ-UTR4 and pCATβ-UTR3B, respectively, *Nco*I-linearized pCATβ-UTR1 and *Nci*I-linearized pCATβ-UTR3 (this time cutting the TGF-β1 3′ UTR at nucleotide 2052) were blunt ended and incubated with *Hin*dIII to remove the CAT coding domain and the first 68 and 35 bases of the TGF-β1 3′ UTR. The CAT coding domain itself was then replaced by ligation of the *Hin*dIII-*Nla*IV cassette from CAT GenBlock. Desired clones were identified by restriction analysis and confirmed by DNA sequencing. The polyadenylation signal is provided by pCDM8 in all constructs, but pCATβ-UTR1 and pCATβ-UTR4 also contain the endogenous ATTAAA polyadenylation signal and 20-base poly(A) tail from TGF-β1 cDNA (43).

Finally, we constructed pCATβ-UTR3inv, which resembles pCATβ-UTR3 except that the GC-rich portion of the TGF-β1 3′ UTR is inverted. To prepare this construct, a pKSβ-UTR plasmid containing the entire TGF-β1 3′ UTR was linearized with *Nco*I, blunt ended, and incubated with *Hin*dIII to remove all but the first 72 bases of the TGF-β1 3′ UTR. The *Hin*dIII-*Nla*IV cassette from CAT GenBlock was ligated to the purified pKSβ-UTR fragment, and the ligation mixture was used to transform DH5α. We obtained a clone in which the GC-rich portion of the TGF-β1 3′ UTR was present in an antisense orientation downstream of a sense CAT coding domain. This plasmid was digested with *Bam*HI, blunt ended, and incubated with *Hin*dIII to obtain a CATβ-UTR3inv cassette which was ligated to *Xba*I blunt ended and *Hin*dIII-digested pCDM8. The ligation mixture was used to transform MC1061-P3. pCATβ-UTR3inv was identified by restriction digestion and DNA sequencing.

Transfections. Experiments designed to measure CAT enzymatic activity and mRNA levels were corrected for differences in transfection efficiency by normalizing samples to units of β-galactosidase activity or *neo* mRNA, respectively. (The instability of β-galactosidase mRNA precluded its use as a normalization vector in mRNA analyses.) In some experiments, cells were triply infected with CAT, β-galactosidase, and *neo* vectors so that enzymatic activity and mRNA levels could be determined from the same samples. Controls showed that double and triple transfectants gave identical results.

Transfections were performed with freshly prepared, CsCl-purified plasmids as previously described (15, 42), except that (i) cells (10^6 for NIH 3T3 and A549 and 10^7 for U937) were seeded in 100-mm-diameter dishes with 10 ml of the appropriate medium (see above); (ii) the preformed plasmid DNA precipitate (30 μg in 1 ml; reference 2) contained 10 to 15 μg each of the appropriate CAT vector, pCH110 (a β-galactosidase expression vector; Pharmacia), and pSV2*neo*; and (iii) transfection proceeded overnight (37°C in an atmosphere of 10% CO_2 in air) in medium containing 10% heat-inactivated fetal calf serum. Transfectants were washed twice with medium and incubated in the appropriate medium with 0.5% serum for 42 h. Samples for analysis of CAT activity were extracted as previously described (2), with 5 to 10 freeze-thaw cycles and a final extract volume of 0.04 (U937) to 0.2 (A549 and NIH 3T3) ml of buffer per 10^6 cells. Protein concentration (assessed by Coomassie binding; Bio-Rad Laboratories) and β-galactosidase activity (15) were determined for each extract to correct for variable cell recoveries and transfection efficiencies, respectively. Calculated values for total (exogenous plus endogenous) β-galactosidase activity per microgram were corrected for endogenous β-galactosidase per microgram by performing parallel analyses with mock transfectants. Finally, appropriate volumes of cell extracts (corresponding to constant units of exogenous β-galactosidase activity) were heated (65°C, 10 min) and analyzed for CAT activity by thin-layer chromatography and autoradiography (15). Controls confirmed that the CAT analyses were within the linear range with respect to CAT enzymatic activity and extract concentration. Samples for mRNA analysis were extracted in GTC-containing buffer (6, 50), and the isolated RNA was subjected to Northern (RNA) blot hybridization as previously described (42, 50).

Constructs for in vitro transcription-translation. Constructs for in vitro transcription-translation were prepared with the human growth hormone (GH) coding domain. The core GH construct was obtained by cloning the 0.8-kbp *Hin*dIII fragment from hGH-4 (a human GH cDNA; 9) into *Hin*dIII-digested Bluescript KS+ (Stratagene). The resulting

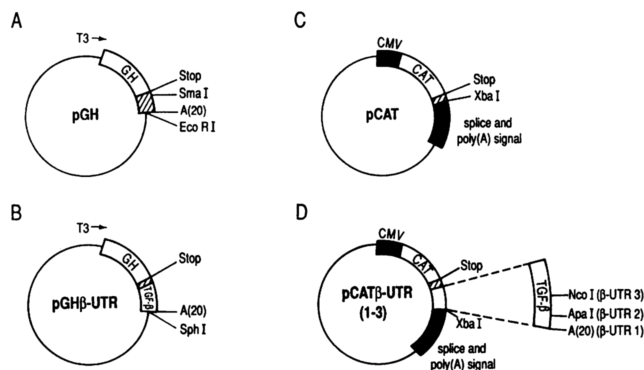

FIG. 1. Core and chimeric constructs for studies in vivo and in vitro. Shown are constructs used in vitro (A and B) and in vivo (C and D) to examine the effect of the TGF-β1 3′ UTR on production of CAT and GH reporters. Restriction sites in panels A and B indicate enzymes used to linearize the plasmids prior to in vitro transcription. The 3′ UTRs are indicated by the hatched (GH) and open (TGF-β1) boxes, respectively. A poly(A) tail (20 bases) is present in transcripts containing either the GH or TGF-β1 3′ UTR. Restriction sites in panels C and D indicate the relative positions of the 3′ end of each cloned insert. The hatched box in panels C and D represents the small portion of the CAT 3′ UTR present in each of the expression vectors. The relative positions of the cytomegalovirus (CMV) promoter and simian virus 40 splice and polyadenylation signals are shown by the closed boxes. The vectors are drawn to emphasize 3′ UTRs rather than to scale.

vector (pGH; Fig. 1A) was linearized with *Eco*RI and transcribed with T3 RNA polymerase to yield an in vitro transcript containing the GH 5′ UTR, the coding domain, the 109-base 3′ UTR, and a 20-base poly(A) tail. Alternatively, pGH can be linearized with *Sma*I prior to incubation with T3 RNA polymerase; the resulting transcript contains the stop codon and only six additional bases of the 3′ UTR (see Fig. 1A). To obtain pGHβ-UTR, a blunt-ended *Pvu*II fragment containing the entire human TGF-β1 3′ UTR, a 20-base poly(A) tail, and a pTZ19R stuffer fragment (43) was ligated to *Sma*I-digested pGH. The desired construct was cloned by transformation in JM109 and identified by restriction mapping and DNA sequencing. Linearization of the resulting plasmid with *Sph*I prior to incubation with T3 RNA polymerase yielded an in vitro transcript in which the TGF-β1 3′ UTR was positioned downstream of the GH coding domain (see Fig. 1B).

In vitro transcription-translation. Freshly prepared, CsCl-purified plasmids (42) were linearized with *Eco*RI, *Sma*I, or *Sph*I to obtain the constructs described above and in Fig. 1A. Linearized plasmids were extracted repeatedly with phenol-chloroform (to remove potentially contaminating RNase), ethanol precipitated, and dissolved (1 mg/ml) in water. All reagents were RNase free. The in vitro transcription reaction used T3 RNA polymerase and was performed similarly to standard procedures (42). In vitro transcripts were recovered from phenol-chloroform-extracted reaction mixtures by ethanol precipitation. Aliquots of water-solubilized in vitro transcripts were fractionated on formaldehyde-agarose gels (42) and analyzed by ethidium bromide staining and Northern blot hybridization (with a nick-translated probe against the GH coding domain) to ensure integrity of the mRNA and normalize yields from distinct transcription reactions. Translation of normalized in vitro transcripts (1 μg) with reticulocyte lysates (Promega) was performed in the presence of [^{3}H]leucine and RNase inhibitor as described by the manufacturer. Equal aliquots (10 μl) of the translation reaction mixtures were suspended in sodium dodecyl sulfate (SDS)-sample buffer and fractionated on SDS–10% acrylamide gels. The radiolabeled GH was visualized by fluorography. Transcript stability in the cell-free system was assessed by preparing ^{32}P-labeled in vitro transcripts (42) and incubating 2×10^5 cpm of each transcript with reticulocyte lysates. Translation proceeded under standard conditions except that unlabeled leucine replaced [^{3}H]leucine. Aliquots (10 μl) of the incubation mixture were collected at 0, 20, 40, and 60 min, diluted in 0.3 ml of buffer (10 mM Tris-HCl, 1 mM EDTA, [pH 7.5], 0.1% SDS), extracted with phenol-chloroform, ethanol precipitated, and dissolved in water. The isolated RNAs were fractionated on a denaturing agarose gel, transferred to Hybond (Amersham), and exposed to X-ray film.

RESULTS

Bifunctional effects of the TGF-β1 3′ UTR on CAT mRNA and protein levels. To identify potential regulatory roles for the 3′ UTR of TGF-β1 mRNA, we prepared constructs in which the 3′ UTR from human TGF-β1 cDNA was positioned downstream of reporter genes (Fig. 1). Constructs prepared with the CAT coding domain (Fig. 1C and D) allowed us to assess the effect of the 3′ UTR on CAT mRNA and enzymatic activity from extracts of transfected cells. Constructs prepared with the GH reporter (Fig. 1A and B) allowed analogous experiments to be performed in vitro with a rabbit reticulocyte cell-free system.

Our initial studies compared CAT activity from extracts of U937 promonocytes transiently transfected with CAT expression vectors that lacked or contained the TGF-β1 3′ UTR (pCAT and pCATβ-UTR2, respectively [Fig. 1]). As shown in Fig. 2 (right side), U937 cells transfected with pCATβ-UTR2 had consistently lower steady-state CAT mRNA levels than did U937 cells transfected with pCAT. Nevertheless, CAT activity (left side) was somewhat increased when transfectants expressed the chimeric transcript (lanes 2 and 3; pCAT versus pCATβ-UTR2, respectively). Thus, the presence of the TGF-β1 3′ UTR had a bifunctional effect on overall CAT expression: decreasing mRNA levels and increasing enzymatic activity. Taken together, these results suggested that the TGF-β1 3′ UTR stimulated production of CAT protein per unit of mRNA. By subjecting X-rays films from thin-layer chromatography and Northern blot hybridization to densitometric scanning, we were able to calculate that the TGF-β1 3′ UTR stimulated CAT activity per unit of mRNA fourfold (also refer to Table 1).

It is well established that activation of protein kinase C stimulates overall expression of TGF-β1. There are phorbol ester-responsive elements in the human TGF-β1 gene (23, 24, 43), and treatment of hematopoietic cell lines with TPA leads to selective stabilization of TGF-β1 mRNA (50). To determine whether activated protein kinase C can also regulate TGF-β1 expression by altering the activity of the TGF-β1 3′ UTR, U937 promonocytes were transfected with pCAT and pCATβ-UTR2 and portions of the transfectants were treated with TPA prior to analysis of CAT mRNA levels and enzymatic activity. Although the time period and dose we used for the TPA treatment result in both transcriptional and posttranscriptional increases in TGF-β1 gene expression (43, 50), the effects of the TGF-β1 3′ UTR on CAT mRNA levels and enzymatic activity were similar in control and TPA-treated cells (data not shown).

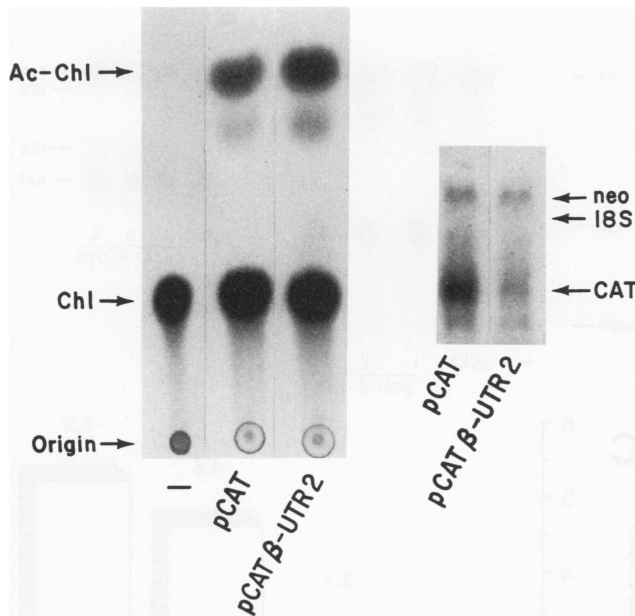

FIG. 2. The TGF-β1 3′ UTR affects CAT mRNA levels and CAT activity in transfected cells. Shown are CAT activities (left side) and CAT mRNA levels (right side) obtained from extracts of U937 cells that had been transiently transfected with CAT vectors that lacked (pCAT) or contained (pCATβ-UTR2) the TGF-β1 3′ UTR. The negative control (−) shows the mobility of unreacted chloramphenicol (Chl). Samples for analysis of CAT activity and CAT mRNA levels were normalized to β-galactosidase activity and *neo* mRNA levels, respectively; see Materials and Methods. Total RNA was purified from transfected cells and analyzed by Northern blot hybridization. Filters were prehybridized (2 h at 55°C) in 50% formamide–6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7])–5× Denhardt's reagent–0.1% SDS, 0.2 mg of low-molecular-weight DNA per ml–0.2 mg of tRNA per ml. The hybridization proceeded (20 h at 55°C) in 50% formamide–6× SSPE–1× Denhardt's reagent–0.1% SDS–10% dextran sulfate–0.2 mg of low-molecular-weight DNA per ml–0.2 mg of tRNA per ml containing ^{32}P-labeled riboprobes (2×10^6 cpm/ml) against the coding regions of CAT or *neo* cDNA. Filters were washed at 70°C in 0.1× SSPE–0.2% SDS. Ac, acetyl.

We also performed these transfection experiments with two other cell lines of distinct lineages (A549 epithelial cells and NIH 3T3 fibroblastic cells). The results obtained with the three cell lines we tested were similar and are summarized in Table 1. CAT mRNA levels were reduced 2- to 3-fold and CAT enzymatic activity was stimulated approximately 1.5-fold by the presence of the TGF-β1 3′ UTR. The combined effect of the TGF-β1 3′ UTR on CAT protein production per unit of mRNA was approximately fourfold in each of the three cell lines tested. These results indicate that the activity of the TGF-β1 3′ UTR is likely detectable in many cell types. Consistent with the results of Fig. 2B, treatment of A549 and NIH 3T3 cells with TPA did not alter the level of CAT activity or mRNA expression observed with pCAT or pCATβ-UTR2 (data not shown). Given the three cell types tested, we observed this bifunctional effect of the TGF-β1 3′ UTR on CAT mRNA levels and CAT activity in at least 12 separate experiments. The fourfold stimulation of CAT activity per unit of mRNA that we found is the most representative value; the range was three- to sixfold.

The bifunctional effects of the TGF-β1 3′ UTR map to the GC-rich domain. The TGF-β1 3′ UTR has domains that are highly conserved in several species (10, 11, 20, 26, 44, 49). Figure 3A shows nucleotides in the 3′ UTR of human TGF-β1 mRNA that are well conserved in monkey, bovine, porcine, murine, and chicken TGF-β1 transcripts (indicated by capital letters). Although there are several small regions of conservation (including the AUUAAA polyadenylation signal and an AUUUA motif), this UTR can also be viewed as being composed of two large, well-conserved domains (GC rich and non-GC rich [underlined and overlined, respectively]). The chimeric CAT–TGF-β1 transcript used in the experiments reported above contained both of these domains (nucleotides 2134 to 2153 were deliberately deleted to avoid introducing a polyadenylation signal that would not be present in pCAT). However, to determine the potential roles of these distinct UTR domains, we compared CAT mRNA levels and activity in extracts of U937 cells that had been transiently transfected with vectors containing the 3′ UTR GC-rich domain (pCATβ-UTR3), both conserved domains in the 3′ UTR (pCATβ-UTR2), and the entire 3′ UTR (pCATβ-UTR1) of human TGF-β1 mRNA (Fig. 1D and 3A).

As shown in Fig. 4, CAT mRNA levels (relative to *neo* signal intensities) were decreased and CAT activity was somewhat stimulated by all three of the UTR-containing vectors compared with the pCAT control. When these

TABLE 1. Bifunctional effect of the TGF-β1 3′ UTR detected in several cell types[a]

Cell line	Construct	CAT activity	CAT mRNA level	CAT activity-CAT mRNA ratio	Fold stimulation
U937	CAT	4,066	6.61	615	1
	CATβ-3′ UTR	6,924	3.07	2,255	3.7
A549	CAT	1,896	0.80	2,370	1
	CATβ-3′ UTR	3,063	0.28	10,939	4.6
NIH 3T3	CAT	5,706	2.25	2,536	1
	CATβ-3′ UTR	8,729	0.72	12,123	4.8

[a] U937, A549, and NIH 3T3 cells were transiently transfected with pCAT and pCATβ-UTR2 (see Materials and Methods for details of cotransfection) prior to analysis of CAT activities and CAT mRNA levels as described in the legend to Fig. 2. Signal intensities were quantitated by densitometric scanning of X-ray films, and total areas are shown as CAT activities (normalized to β-galactosidase activity) and CAT mRNA levels (normalized to *neo* signal intensities). The ratio of CAT activity to CAT mRNA was calculated for each sample. Finally, the translation-stimulatory effect of the TGF-β1 3′ UTR (fold stimulation) was calculated relative to that of pCAT. The apparent difference in CAT activity and CAT mRNA level between cell types is a consequence of the variable exposure times used for autoradiography; it does not reflect actual differences in CAT activity or CAT mRNA expression.

A

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GGuCCCGCCC CGCCCCGCCC CgCCCcGGCA GGCCCGGCCC CaCCCCGCCC cGCCcccgcu gcCuugCCca

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ugGGGGCUGU AUUUAAGGAC AcCGUGCCC CAAGCCCACcU GGGgcCccAU UAAAGaUgGa gAGAGG(A_{20})

↓ pCATβ-UTR3 ↓ pCATβ-UTR2 ↓ pCATβ-UTR1

B

2086

caugGGGGCUGUAUUUAAGGACAcCGUGCCCCAAGCCCACcUGGGgcCccAUUAAAGaUgGagAGAGG(A_{20})

↓ pCATβ-UTR4

C

2018 2089

GGuCCCGCCCCGCCCCGCCCCgCCCcGGCAGGCCCGGCCCCaCCCCGCCCcGCCcccgcugcCuugCCcaug

↓ pCATβ-UTR3A ↓ pCATβ-UTR3B

FIG. 3. Sequence conservation in the 3′ UTR of human TGF-β1 mRNA. (A) Nucleotide sequence of the human TGF-β1 3′ UTR. Uppercase letters indicate bases in human TGF-β1 mRNA that are conserved in at least four of the five other species studied to date (10, 11, 20, 26, 44, 49). Two large conserved domains are underlined and overlined. Arrows indicate the 3′ nucleotide present in the three pCATβ-UTR vectors. (B) Non-GC-rich region of the TGF-β1 3′ UTR in pCATβ-UTR4. (C) GC-rich TGF-β1 UTR sequence in pCATβ-UTR3 and its subdivision into the 5′ and 3′ portions that make up pCATβ-UTR3A and pCATβ-UTR3B, respectively (underlined). Identical sequences in the subdomains of panel C are overlined. Nucleotide numbers are based on the full-length cDNA sequence (10).

FIG. 4. The GC-rich domain of the TGF-β1 3′ UTR has bifunctional effects on CAT mRNA levels and CAT activity. Shown are the effects of distinct domains in the TGF-β1 3′ UTR on CAT activities (A) and CAT mRNA levels (B). U937 cells were transfected with pCAT, pCATβ-UTR1, pCATβ-UTR2, and pCATβ-UTR3 (refer to Fig. 3 for the 3′ UTR domains present in each vector). The negative control (−) and cotransfection methods for normalization of CAT activities and CAT mRNA levels are described in the legend to Fig. 2 and in Materials and Methods. Panel C shows a bar graph in which the primary data for each construct have been converted to fold stimulation (relative to pCAT) as described in the footnote to Table 1. This experiment used TPA-treated U937 cells. Chl, chloramphenicol; Ac, acetyl.

primary data were converted to fold stimulation (CAT activity per unit of mRNA; Fig. 4C), the results showed that (i) the presence of the entire TGF-β1 3′ UTR did not enhance CAT activity relative to pCATβ-UTR2 (compare bars 2 and 3) and (ii) the stimulatory effect of the pCATβ-UTR2 was mimicked completely by a chimera containing only the first conserved domain (compare bars 3 and 4). Comparison of these results with the TGF-β1 3′ UTR sequence (Fig. 3A) shows that the bifunctional effects of the TGF-β1 3′ UTR on CAT mRNA levels and CAT activity per unit of mRNA localize to the hallmark GC-rich domain located immediately downstream of the stop codon. This general result was observed in five separate experiments. Note that the somewhat reduced stimulation of CAT activity per unit of mRNA observed with pCATβ-UTR1 in this experiment was not reproducible (e.g., Fig. 5C).

To confirm the results of Fig. 4, we transfected cells with a CAT–TGF-β1 chimera that lacked (pCATβ-UTR4; Fig. 3B) the GC-rich domain of the TGF-β1 3′ UTR. As shown in Fig. 5, CAT activity was somewhat increased (relative to pCAT) in transfectants expressing pCATβ-UTR4 mRNA (panel A) but the steady-state CAT mRNA level (panel B) was high in this transfectant and resembled the levels typical of pCAT rather than pCAT–TGF-β chimeras containing the intact 3′ UTR (pCATβ-UTR1 in Fig. 5) or the GC-rich domain (pCATβ-UTR3 in Fig. 4). Thus, when these data were converted to CAT activity per unit of mRNA (panel C), the results showed that the non-GC-rich domain of the TGF-β1 3′ UTR did not significantly contribute to either the mRNA-inhibitory or the translation-stimulatory effect of the intact 3′ UTR.

We also transfected cells with CAT vectors containing the 5′ and 3′ portions of the TGF-β1 3′ UTR GC-rich domain (pCATβ-UTR3A and pCATβ-UTR3B, respectively [Fig. 3C]) and compared their effects with those obtained with the chimera containing the entire GC-rich domain (pCATβ-UTR3). As shown in Fig. 6, CAT activity was stimulated (panel A) and CAT mRNA levels (relative to *neo* mRNA) were similarly reduced (panel B) relative to pCAT when transfectants expressed the intact GC-rich domain, the 5′ portion of the GC-rich domain, or the 3′ portion of the GC-rich domain. Refer to panel C for the calculations of

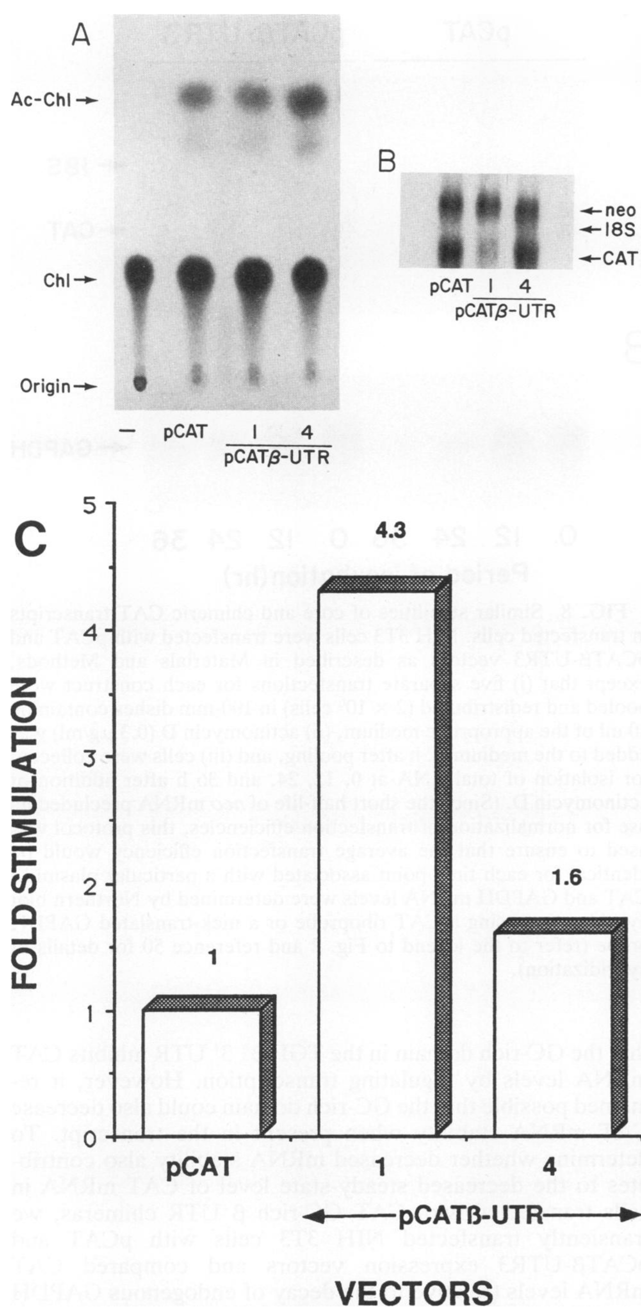

FIG. 5. Relative inactivity of a pCAT–TGF-β1 3′ UTR chimera that lacks the GC-rich domain. Comparison of CAT activities (A) and CAT mRNA levels (B) obtained from extracts of U937 cells transfected with pCAT, pCATβ-UTR1 (the chimera containing a complete TGF-β1 3′ UTR), and pCATβ-UTR4 (a chimeric construct lacking the GC-rich domain of the TGF-β1 3′ UTR). Refer to Fig. 3 for the 3′ UTR domains present in each vector. The negative control (−) and cotransfection methods for normalization of CAT activities and CAT mRNA levels are described in the legend to Fig. 2 and in Materials and Methods. Panel C shows a bar graph in which the primary data for each construct have been converted to fold stimulation (relative to pCAT) as described in the footnote to Table 1. This experiment used TPA-treated U937 cells. Note that both pCATβ-UTR1 and pCATβ-UTR4 have the same poly(A) tail sequences (refer to Materials and Methods). Chl, chloramphenicol; Ac, acetyl.

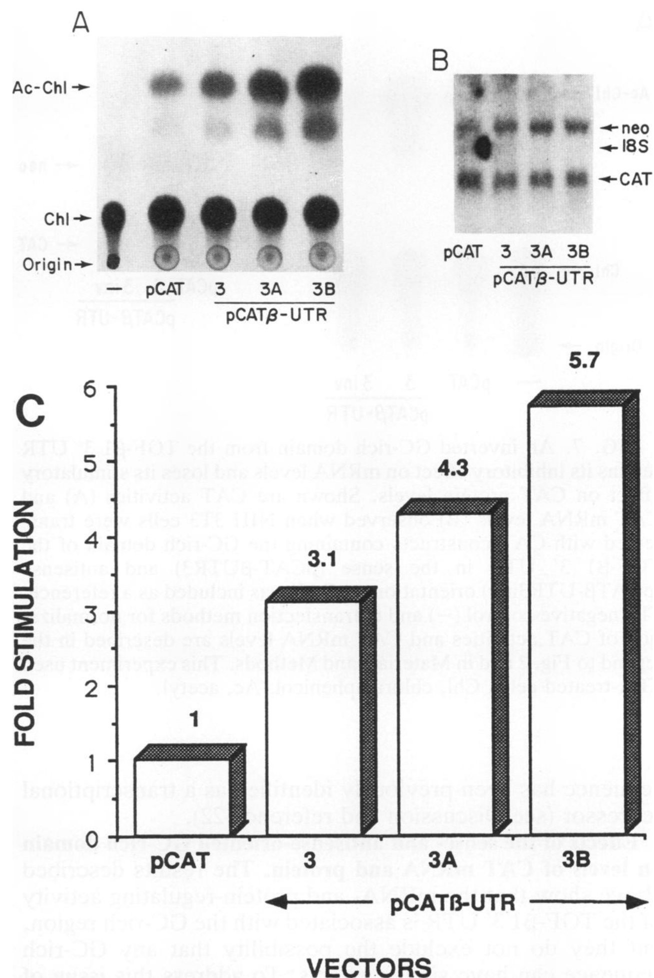

FIG. 6. Similar bifunctional effects associated with the 5′ and 3′ portions of the GC-rich domain of the TGF-β1 3′ UTR. Shown are CAT activities (A) and CAT mRNA levels (B) observed when NIH 3T3 cells were transfected with CAT constructs containing the 5′ (pCAT-βUTR3A) and 3′ (pCATβ-UTR3B) portions of the GC-rich domain of the TGF-β1 3′ UTR. pCAT was included as a reference. The negative control (−) and cotransfection methods for normalization of CAT activity and CAT mRNA levels are described in the legend to Fig. 2 and in Materials and Methods. Panel C shows a bar graph in which the primary data for each construct have been converted to fold stimulation (relative to pCAT) as described in the footnote to Table 1. Chl, chloramphenicol; Ac, acetyl.

CAT activity per unit of mRNA. Corresponding results were obtained in transiently transfected U937 cells (data not shown), and CAT activity was 20 to 30% higher with pCATβ-UTR3B than with pCATβ-UTR3A in five of seven separate transfections (similar to the results shown in Fig. 6A).

Notwithstanding the slight difference in CAT activity observed with the pCATβ-UTR3A and pCATβ-UTR3B chimeras, it is clear that both the 5′ and 3′ portions of the GC-rich domain have similar effects. Note that the slightly reduced stimulatory effect obtained with the pCATβ-UTR3 chimera in this experiment was not reproducible (e.g., Fig. 4C). Interestingly, analysis of the two subdomains in chimeras pCATβ-UTR3A and pCATβ-UTR3B revealed a highly conserved 20-base sequence (overlined in Fig. 3C), and this

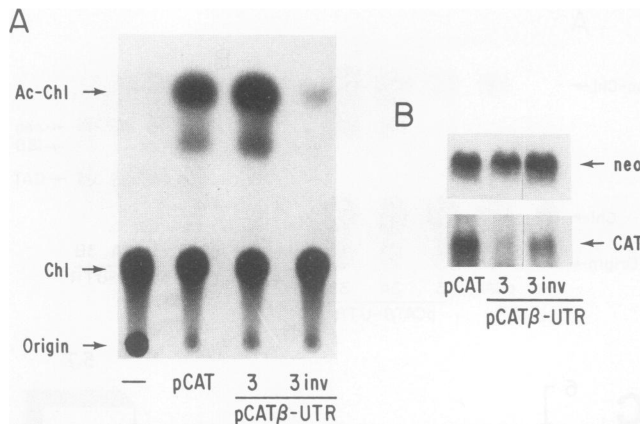

FIG. 7. An inverted GC-rich domain from the TGF-β1 3′ UTR retains its inhibitory effect on mRNA levels and loses its stimulatory effect on CAT protein levels. Shown are CAT activities (A) and CAT mRNA levels (B) observed when NIH 3T3 cells were transfected with CAT constructs containing the GC-rich domain of the TGF-β1 3′ UTR in the sense (pCAT-βUTR3) and antisense (pCATβ-UTR3inv) orientations. pCAT was included as a reference. The negative control (−) and cotransfection methods for normalization of CAT activities and CAT mRNA levels are described in the legend to Fig. 2 and in Materials and Methods. This experiment used TPA-treated cells. Chl, chloramphenicol; Ac, acetyl.

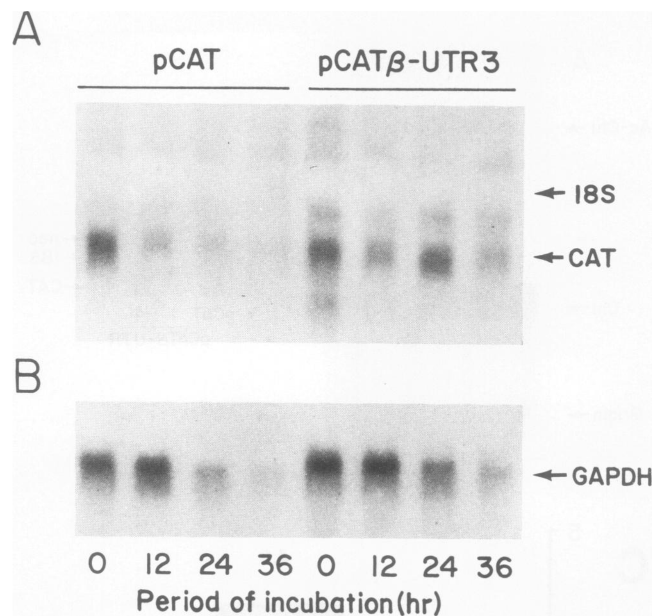

FIG. 8. Similar stabilities of core and chimeric CAT transcripts in transfected cells. NIH 3T3 cells were transfected with pCAT and pCATβ-UTR3 vectors as described in Materials and Methods, except that (i) five separate transfections for each construct were pooled and redistributed (2×10^6 cells) in 100-mm dishes containing 10 ml of the appropriate medium, (ii) actinomycin D (0.3 μg/ml) was added to the medium 12 h after pooling, and (iii) cells were collected for isolation of total RNA at 0, 12, 24, and 36 h after addition of actinomycin D. (Since the short half-life of *neo* mRNA precluded its use for normalization of transfection efficiencies, this protocol was used to ensure that the average transfection efficiency would be identical for each time point associated with a particular plasmid.) CAT and GAPDH mRNA levels were determined by Northern blot hybridization using a CAT riboprobe or a nick-translated GAPDH probe (refer to the legend to Fig. 2 and reference 50 for details of hybridization).

sequence has been previously identified as a transcriptional repressor (see Discussion and reference 22).

Effects of the sense- and antisense-oriented GC-rich domain on levels of CAT mRNA and protein. The results described above show that the mRNA- and protein-regulating activity of the TGF-β1 3′ UTR is associated with the GC-rich region, but they do not exclude the possibility that any GC-rich sequence can have similar effects. To address this issue of sequence specificity, we prepared a construct (pCATβ-UTR3inv) in which the 72-base GC-rich portion of the TGF-β1 3′ UTR was cloned in an inverted orientation downstream of the standard sense-oriented CAT reporter. Thus, transfection of cells with pCAT-βUTR3 and pCATβ-UTR3inv should yield transcripts that differ in the sequence (but not in the size or percent G + C) of the GC-rich 3′ UTR. These two constructs (and the pCAT reference plasmid) were transiently transfected into NIH 3T3 cells; we compared CAT mRNA levels and CAT activity.

As shown in Fig. 7A, CAT activity in extracts from cells transfected with pCATβ-UTR3inv was strikingly reduced compared with the activity from extracts transfected with pCATβ-UTR3 or pCAT. This result is in marked contrast to those obtained from CAT chimeras having the GC-rich portion of the TGF-β1 3′ UTR in a sense orientation as described above. Interestingly, the parallel analysis for CAT mRNA levels (Fig. 7B) showed that steady-state CAT mRNA levels were similarly reduced relative to pCAT (five- and threefold as determined by densitometric scanning and normalized to *neo* mRNA signal intensities) when cells were transfected with pCATβ-UTR3 or pCATβ-UTR3inv, respectively. Thus, the GC-rich portion of the TGF-β1 3′ UTR could be present in a sense or antisense orientation without elimination of its inhibitory effect on CAT mRNA levels. In contrast, the stimulatory effect of the GC-rich domain on production of CAT protein per unit of mRNA was lost when the sequence was present in an antisense orientation.

Studies by others (22) and the results of Fig. 7 and indicate that the GC-rich domain in the TGF-β1 3′ UTR inhibits CAT mRNA levels by regulating transcription. However, it remained possible that the GC-rich domain could also decrease CAT mRNA stability when present in the transcript. To determine whether decreased mRNA stability also contributes to the decreased steady-state level of CAT mRNA in cells transfected with CAT–GC-rich β UTR chimeras, we transiently transfected NIH 3T3 cells with pCAT and pCATβ-UTR3 expression vectors and compared CAT mRNA levels (relative to the decay of endogenous GAPDH [glyceraldehyde-3-phosphate dehydrogenase] mRNA) at 0, 12, 24, and 36 h after exposure of transfectants to actinomycin D. As shown in Fig. 8, CAT and CATβ-UTR3 mRNAs were degraded similarly within the 36-h period examined. In fact, pCATβ-UTR3 mRNA may have been slightly more stable. Thus, decreased stability does not contribute to the decreased steady-state CAT mRNA levels observed in cells transfected with CAT–TGF-β1 3′ UTR chimeras.

Translation-stimulatory effects of the TGF-β1 3′ UTR in reticulocyte lysates. The results we obtained in vivo indicate that the GC-rich domain in the TGF-β1 3′ UTR can stimulate translation of the CAT reporter. However, this conclusion is complicated by the need to correct for different steady-state mRNA levels in transfectants expressing the core and chimeric mRNAs. Thus, to examine directly the potential translation-stimulatory effect of the TGF-β1 3′ UTR, we

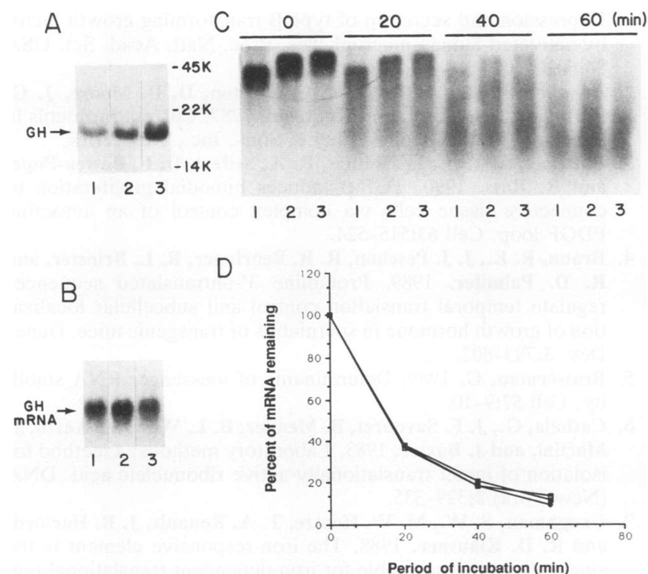

FIG. 9. TGF-β1 3′ UTR stimulates translation of GH in vitro. Shown is the effect of the TGF-β1 3′ UTR on translation and mRNA levels with a GH reporter and rabbit reticulocyte lysates. In vitro transcripts containing the GH coding domain and differing in their 3′ UTRs were prepared as described in Materials and Methods. Equal amounts of the in vitro transcripts were translated with rabbit reticulocyte lysates, and the amount of GH present in 10 μl of each reaction mixture was assessed by SDS-acrylamide gel electrophoresis and fluorography (A). An equal aliquot of each in vitro transcript was also analyzed by Northern blot hybridization (with a nick-translated GH coding domain probe) to confirm transcript integrity and compare amounts of mRNA translated (B). Panel C shows time-dependent degradation of the ^{32}P-labeled in vitro transcripts during incubation under the same conditions used for in vitro translation (1 h at 30°C). Lanes 1 to 3, respectively, for panels A to C, show results obtained with in vitro transcripts that lacked a 3′ UTR (*Sma*I-linearized vector), contained the GH 3′ UTR (*Eco*RI-linearized vector, or contained the TGF-β1 3′ UTR (*Sph*I-linearized vector). The X-ray film from panel C was subjected to densitometric scanning to generate the graph in panel D. Data in panel D are presented relative to the initial signal intensity for each transcript as follows: □, GH coding domain alone; ◇, coding domain with the GH 3′ UTR; ■, coding domain with the TGF-β1 3′ UTR.

determined the effect of this domain on translation in vitro by using transcripts that contained the GH coding domain and (i) lacked a 3′ UTR, (ii) contained the GH 3′ UTR, or (iii) contained the TGF-β1 3′ UTR. The in vitro transcripts were translated in a reticulocyte cell-free system, aliquots of the translation products were fractionated on SDS-acrylamide gels, and the amount of GH produced was assessed by fluorography.

As shown in Fig. 9A, the absence (lane 1) or presence (lane 2) of the GH 3′ UTR had only a slight effect on production of GH (especially in view of the small differences in the amounts of mRNA added to the system; compare lanes 1 and 2 of Fig. 9B). In contrast, significantly more GH was produced by translation of the chimeric (GH–TGF-β1 3′ UTR) transcript in this system (compare lanes 1 and 3 in Fig. 9A and B). The average stimulatory effect of the TGF-β1 3′ UTR was threefold (with a range of two- to fourfold in four separate experiments). In contrast, there was no reproducible effect of the GH 3′ UTR on translation of the GH reporter. Since the 3′ UTRs of GH and TGF-β1 mRNA are similar in size (see Materials and Methods), the translation-stimulatory effect of the TGF-β1 3′ UTR does not result from a nonspecific increase in transcript size.

In related studies, we synthesized the same three in vitro transcripts in the presence of [^{32}P]UTP, added them to reticulocyte lysates, and determined their relative stability during the 60-min in vitro translation reaction. As shown in Fig. 9C and D, the in vitro transcripts were equivalently degraded by the rabbit reticulocyte lysate. Thus, under conditions in which core and chimeric mRNA levels were equivalent, the TGF-β1 3′ UTR still stimulated production of reporter proteins and the magnitude of this effect was similar to that calculated from the in vivo experiments.

DISCUSSION

We identified a GC-rich region in the downstream domain of the TGF-β1 gene and mRNA that has bifunctional effects on control of overall protein expression. When we used this domain as a cassette with reporter coding domains, we found that steady-state mRNA levels were reduced in the presence of the GC-domain whereas production of protein per unit of mRNA was stimulated. These effects were detected in three cell lines of distinct lineages, indicating that the results reported here are likely applicable to several cell types. The translation-regulating activity of this domain was not altered by exposure to TPA, and this result distinguishes transcriptional from translational stimulation of TGF-β1 expression.

An AUUUA sequence (derived from nucleotides 2098 to 2102 of the TGF-β1 3′ UTR) is present in the 3′ UTR of pCATβ-UTR2, our reference chimera, and the presence of a AUUUA motif is typically associated with transcript instability (45). Note, however, that we found decreased steady-state mRNA levels with pCATβ-UTR chimeras (e.g., pCATβ-UTR3; Fig. 4) that lack the AUUUA motif and no decrease in steady-state mRNA levels with a chimera (pCATβ-UTR4; Fig. 5) that contains the AUUUA motif. Thus, the single-copy AUUUA in the TGF-β1 3′ UTR is not responsible for the decreased steady-state mRNA expression observed with our chimeric mRNAs. In fact, this result agrees with other studies showing that single-copy AUUUA motifs are insufficient to destabilize transcripts (5).

Considering the similar stabilities of transcripts containing and lacking the GC-rich portion of the TGF-β1 3′ UTR, the decreased steady-state mRNA levels observed in U937 cells transfected with pCATβ-UTR vectors likely result from decreased transcription of the CATβ-UTR chimeras. Interestingly, the sequence GGGCGGGGCG has been identified in the β-actin promoter as the binding site for a transcriptional repressor (22), and its complementary sequence, CGCCCCGCCC, is present in multiple copies within the GC-rich domain of the TGF-β1 3′ UTR that is associated with decreased mRNA levels in our transfectants. Moreover, this GC-rich domain has similar inhibitory effects on CAT mRNA levels when present in a sense or antisense orientation. This result would be predicted if the domain functioned as a transcriptional repressor.

In addition to the effects on transcript levels that we found, our data show that protein production (assessed as CAT activity in vivo and GH levels in vitro) was increased by the TGF-β1 3′ UTR; deletion analysis in vivo showed that the GC-rich domain was also responsible for this effect. In our transfection experiments, the full translation-stimulatory effect of the TGF-β1 3′ UTR was observed after normalization of CAT activities to the differing steady-state CAT mRNA levels. We believe that these calculations are reasonable, since a similar translation-stimulatory effect was de-

tected with rabbit reticulocyte lysates, a system in which the TGF-β1 3′ UTR had no effect on mRNA levels throughout the incubation period tested (Fig. 9).

Interestingly, both the 5′ and 3′ portions of the GC-rich domain have the ability to stimulate CAT activity per unit of mRNA. This raises the possibility that the CGCCCCGCCC motif described above is transcription inhibitory in the gene yet translation stimulatory in the transcript. Note that in contrast to the results we obtained when examining CAT mRNA levels, the translation-stimulatory effect of the GC-rich domain of the TGF-β1 3′ UTR requires that it be present in the sense orientation. Thus, a particular sequence, rather than a GC-rich stretch in general, is required to generate the effects on production of CAT per unit of mRNA that we observed. We are now trying to identify the mechanism by which this GC-rich sequence evokes its translation-stimulatory effects.

In addition to the GC-rich domain in its 3′ UTR, there is a large GC-rich domain in the 5′ UTR of TGF-β1 mRNA (10). Consistent with several studies on upstream GC-rich domains, results obtained by Kim et al. (discussed in reference 38) indicate that this 5′ GC-rich domain inhibits translation. Thus, opposing translational effects may be associated with the 5′ and 3′ UTRs of TGF-β1 mRNA. Interestingly, transcription of the TGF-β1 gene is also regulated by positively and negatively acting *cis* elements in the upstream and downstream domains of the gene (23, 24, 43). We suggest that the relative contributions of these inhibitory, stimulatory, and bifunctional (this report) elements on transcription and translation of the growth factor are likely dictated by temporal or cell-specific expression of appropriate *trans* factors. Interestingly, the 5′ GC-rich domain is absent from human TGF-β1 mRNAs originating from the second start site in the gene (23, 24). Two similarly positioned transcription start sites are present in the murine TGF-β1 gene (14), and we have previously shown that murine germ cells express a small TGF-β1 transcript exclusively (53). Other studies indicate that a similarly truncated transcript is expressed in injured heart tissue (38). Thus, temporal or cell-specific use of alternative transcription start sites may also play a role in dictating overall translatability of TGF-β1 mRNA.

The 3′ UTR GC-rich domain of TGF-β1 mRNA is well conserved in TGF-β1 (10, 11, 20, 26, 38, 44, 49) and absent from the transcripts for TGFβ-2, TGFβ-3, TGFβ-4, and TGFβ-5) (8, 12, 17, 19, 21, 25, 31, 34, 47, 53). Thus, our data suggest that translational control of distinct TGF-β family members should differ. Similarly, control of TGF-β1 gene transcription differs significantly from that of TGF-β2 and TGF-β3 (reference 30 and discussion therein). Taken together, these results suggest that redundancy in the TGF-β family may reflect, at least in part, the desirability for differential controls on production of these proteins. This suggestion, in turn, is consistent with studies showing that individual TGF-β family members have distinct expression patterns in vivo (33).

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