CELL REGULATION, Vol. 2, 241–249, March 1991

A second messenger RNA species of transforming growth factor β1 in infarcted rat heart

Su Wen Qian,*† Paturu Kondaiah,*
Ward Casscells,‡ Anita B. Roberts,*
and Michael B. Sporn*
*Laboratory of Chemoprevention
National Cancer Institute
‡Cardiology Branch
National Heart, Lung, and Blood Institute
Bethesda, Maryland 20892

Transforming growth factor-β1 (TGF-β1) is encoded predominantly by a 2.4-kb mRNA in most tissues. However, an additional transcript of 1.9 kb can be detected in rat heart after experimental myocardial infarction caused by ligation of the left coronary artery. This transcript level is significantly higher in infarcted heart tissue than in normal heart tissue, suggesting an important role for this mRNA species in response to injury. Structural characterization of the 1.9-kb mRNA showed that it included the entire coding sequence present in the 2.4-kb TGF-β1 mRNA, but also contained an additional nonhomologous 3′-untranslated region (UTR). The junction between the shared and unique 3′ sequence in the 1.9-kb mRNA occurred only two nucleotides before the proposed polyadenylation site of the rat TGF-β1 2.4-kb mRNA. The unique 3′-UTR and the deduced shortened 5′-UTR in the novel 1.9-kb TGF-β1 mRNA suggest different transcriptional and translational regulatory mechanisms under conditions of tissue injury.

Introduction

The transforming growth factor-β (TGF-β) gene family encodes a set of polypeptides that include five distinct TGF-βs (for review, see Roberts and Sporn, 1990). Although TGF-β was originally defined by its ability to induce normal rat kidney fibroblasts (NRK-49F) to grow and form colonies of cells in soft agar (Roberts *et al.,* 1981), subsequent studies have shown that TGF-β is present in almost all cells and can act as a growth stimulator or growth inhibitor, depending on the cell type and assay conditions. There is now substantial evidence suggesting its importance as a mediator of normal cellular physiology, in particular during formation of tissues (as in embryogenesis) and during tissue response to injury (as in inflammation and repair; for review, see Sporn and Roberts, 1989). Fundamental to the role of TGF-β in both developmental and repair processes is its ability to regulate the expression of many genes, including those encoding structural proteins such as fibronectin and its receptor or collagens type I, III, and IV (Ignotz and Massagué 1986; Roberts *et al.,* 1986; Varga *et al.,* 1987; Rossi *et al.,* 1988).

Immunohistochemical studies in rats and mice suggest an unusually large amount of intracellular TGF-β1 in cardiac myocytes, cardiac vagal ganglia, and the conducting cells of the atrioventricular node (Thompson *et al.,* 1988). The physiological role of TGF-β in normal cardiac function is unknown. Recent studies of TGF-β1 in the rat heart after experimental myocardial infarction caused by ligation of the left coronary artery have demonstrated progressive loss of TGF-β1 staining in myocytes 1–24 h after coronary ligation, but 24–48 h later, increased staining was seen in myocytes at the margin of infarcted areas. Northern blot analysis of TGF-β1 mRNA in the infarcted heart showed the appearance of a new molecular species (a 1.9-kb transcript), which hybridized with a TGF-β1 single-stranded DNA (ssDNA) probe, in addition to a marked increase in the usual 2.4-kb transcript found in most tissues (Thompson *et al.,* 1988).

To examine the structural and functional role of the 1.9-kb mRNA in infarcted heart tissue, we have cloned the rat TGF-β1 gene (Qian *et al.,* 1990) with the polymerase chain reaction (PCR). Like previously described mammalian TGF-β1 cDNAs (Derynck *et al.,* 1985, 1986, 1987; Sharples *et al.,* 1987; Van Obberghen-Schilling *et al.,* 1987; Kondaiah *et al.,* 1988), the predicted rat TGF-β1 precursor protein consists of 390 amino acid residues, and the 112-amino-acid mature TGF-β1 peptide can be released from a tetrabasic cleavage site (RHRR). In the current report we provide data on the structural characterization of the 1.9-kb mRNA, and show

† Corresponding author.

that the 1.9-kb mRNA contains a unique 3′-untranslated region (UTR) and a truncated 5′-UTR, compared with the 2.4-kb TGF-β1 mRNA. The data suggest novel transcriptional and translational regulatory mechanisms activated as a response of tissue to injury.

Results

A unique 1.9-kb mRNA species of TGF-β1 mRNA is highly expressed in infarcted rat hearts

In a previous publication (Thompson *et al.*, 1988), we described the appearance of a novel 1.9-kb mRNA that hybridized to a human TGF-β1 ssDNA probe in rat heart after experimental myocardial infarction. Considering the high level of homology between genes representing the different TGF-β isoforms (70–80%), we wished to rule out whether the 1.9-kb mRNA could be related to any TGF-β gene other than TGF-β1. Northern blot analysis (Figure 1) showed that the 1.9-kb mRNA hybridized only to a TGF-β1 ssDNA probe and not to probes corresponding to TGF-β2 or TGF-β3, strongly suggesting that it might be transcribed from the TGF-β1 gene.

The level of expression of both the 1.9- and 2.4-kb TGF-β1 mRNAs increased with time after infarction (Figure 2). The level of the 2.4-kb mRNA increased more than 6-fold 48 h after infarction compared with its level in normal rat heart. In contrast, the level of the 1.9-kb mRNA, which was undetectable in normal heart tissue, increased more than 23-fold from 6 to 48 h after infarction. In addition, there is another novel transcript of ~1.4 kb that hybridized to the TGF-β1 probe and that is observed only in infarcted heart RNA (Figure 1). The structure and biological significance of this novel transcript are unknown. Northern blot analysis showed that the levels of TGF-β2 and β3 mRNAs were quite low in rat heart and did not change after infarction (data not shown). These results suggest that expression of TGF-β1 is more closely related to

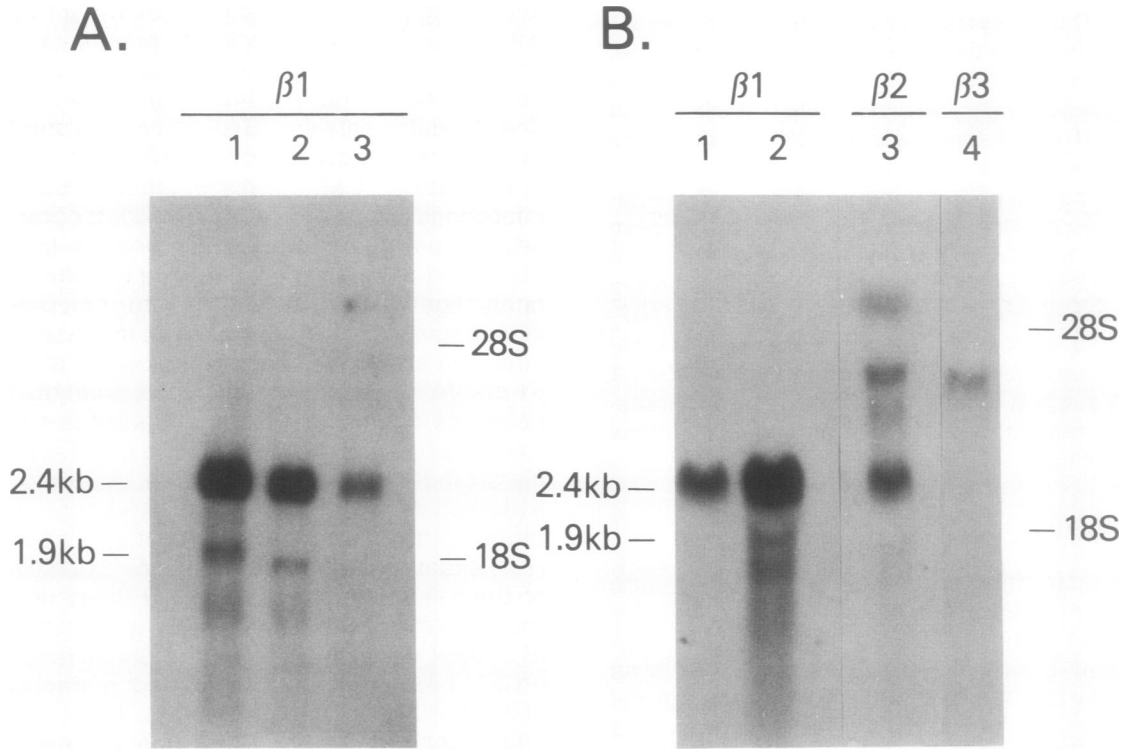

Figure 1. **The 1.9-kb mRNA highly expressed in infarcted rat heart is a TGF-β1 mRNA.** Total RNA and poly(A)+ mRNA were isolated from either infarcted or normal tissues of rat hearts. Fifteen micrograms of total RNA or 2 μg of poly(A)+ mRNA were loaded onto RNA gels and subjected to Northern blot analysis with different probes. (A) Hybridization with ^{32}P-labeled human TGF-β1 ssDNA probe. The lanes correspond to 1) poly(A)+ mRNA from the infarcted heart tissue 48 h after ligation of the left coronary artery, 2) total RNA from infarcted heart tissue 48 h after the ligation, and 3) total RNA from normal heart tissue. (B) Hybridization with ^{32}P-labeled human TGF-β1 (lanes 1 and 2), simian TGF-β2 (lane 3), or chicken TGF-β3 (lane 4) ssDNA probes. The lanes correspond to 1) total RNA from normal rat heart tissue and 2–4) total RNA from infarcted heart tissue 48 h after ligation.

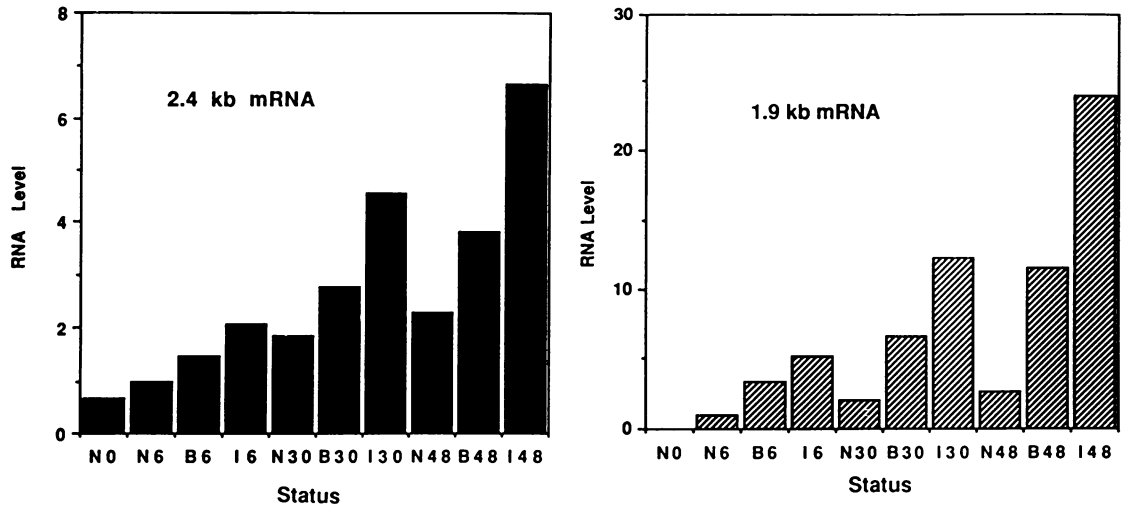

Figure 2. **The expression level of the TGF-β1 mRNAs (2.4 and 1.9 kb) increases with time after myocardial infarction.** Signals obtained from hybridization of total RNA from rat heart tissue were quantitated by densitometry. "N0, N6, B6, I6, . . . I48" indicate the status of heart tissue from which the RNA was extracted; N, B, or I stands for normal, border, or infarcted regions of the heart after the ligation, and the numbers after these letters stand for the time point (0, 6, 30, 48 h) after the ligation. All values are relative to the tracing for the signal from N6, which is standardized as 1.00.

the response of myocardial tissue to injury or repair than is expression of the other TGF-β isoforms.

1.9- and 2.4-kb TGF-β1 mRNAs encode the identical protein

Using techniques based on PCR, we cloned a rat TGF-β1 cDNA containing the complete rat TGF-β1 coding region (390 amino acids) and 412 bp of the 5′-untranslated region (UTR; Qian *et al.,* 1990). To determine the degree of overlap of the 2.4- and 1.9-kb mRNA species, we used three different TGF-β1 probes from either the 5′-UTR (upstream of the ATG, position −412 to −75, 338 bp), the proregion of the protein (position 1–789, 789 bp), or the region encoding the mature form of TGF-β1 (position 835–1173, 339 bp) for Northern blot analysis. The 1.9-kb mRNA showed positive hybridization with each of these probes (data not shown), suggesting that it is homologous to the 2.4-kb TGF-β1 mRNA species in each of these regions. To determine the actual sequence of the 1.9-kb mRNA, we size-fractionated total mRNA extracted from infarcted heart on an agarose gel, on the basis of the approximation that the 1.9-kb mRNA is the same size as 18S RNA. The purity of the size-fractionated 1.9-kb mRNA was confirmed by Northern blot analysis with the TGF-β1 probe, and this RNA was reverse-transcribed to cDNA with an oligo (dT) primer. The resultant mixture of cDNAs was then amplified by the use of PCR. Two different PCR products were cloned and sequenced (Figure 3): the first one was a fragment (789 bp) amplified with primers P1 and P2, representing the sequence from the start codon ATG (position 1) to position 789 and covering most of the TGF-β1 proregion; the second one was a fragment (630 bp) amplified with primers P3 and P4, representing the sequence from position 543 of the proregion to the TGA stop codon and including the complete coding region of the mature form of TGF-β1. The sequences of these two overlapping pieces of cDNA were identical to those of the rat TGF-β1 gene, allowing us to conclude that the 1.9-kb mRNA is an alternate transcript of the TGF-β1 gene.

1.9-kb mRNA contains a unique 3′-UTR

Having demonstrated that the 1.9-kb mRNA is an alternate transcript of the TGF-β1 gene, we next determined what differences might exist between the two TGF-β1 mRNA species (2.4 and 1.9 kb). A λZAP cDNA library was constructed from mRNA of infarcted heart tissue using oligo (dT) and random primers. Screening of the library with the rat TGF-β1 probe resulted in identification of 22 positive clones. Four of these clones contained 140 bp of 3′-UTR plus a poly(A)+ tail. The 3′-UTR has a typical polyadenylation site (ATTAAA) located 13 bp upstream of the poly(A)+ tail and is very similar to the 3′-UTR in human TGF-β1 mRNA reported recently

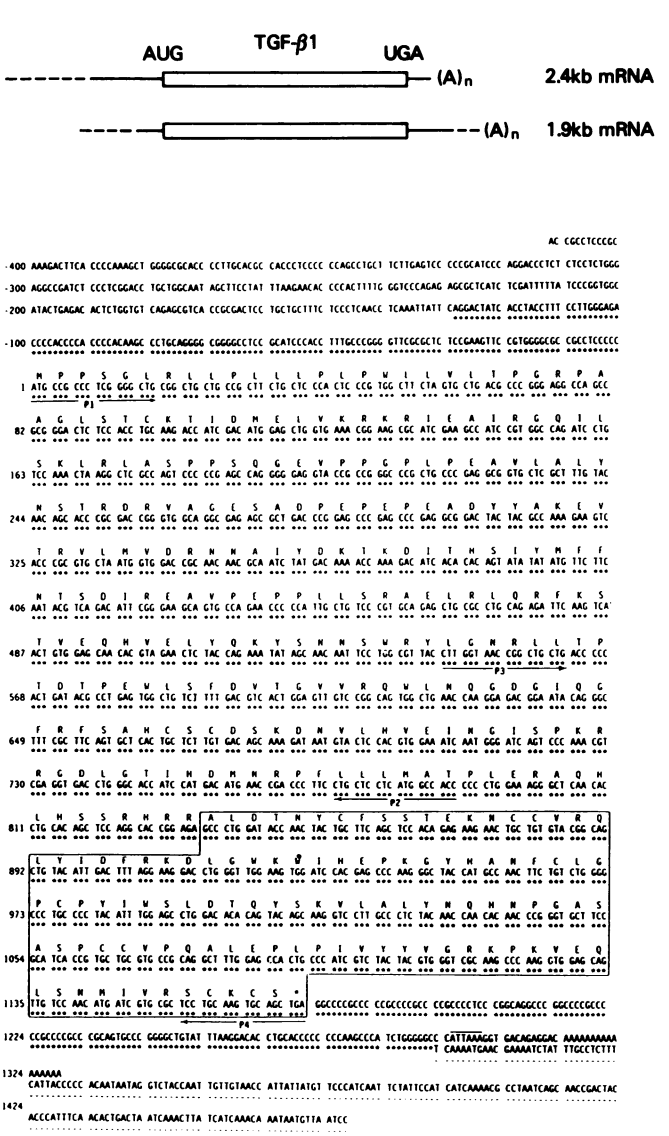

Figure 3. **Nucleotide sequences of the 1.9- and 2.4-kb TGF-β1 mRNAs.** (A) A schematic illustration of the structure of the 1.9- and 2.4-kb TGF-β1 transcripts. The uncharacterized regions are shown by dotted lines. (B) The nucleotide sequence of the cDNAs from the 2.4- and 1.9-kb TGF-β1 mRNA was determined by the dideoxy-chain termination method. The nucleotides were numbered from the start codon (ATG). The top line represents the cDNA sequence from the 2.4-kb mRNA; the lower line represents the cDNA sequence from the 1.9-kb mRNA. * indicates the same nucleotide. The mature form of TGF-β1 (112 aa) is boxed in. Primers (P1, P2, P3, and P4) used for PCR amplification of the 1.9-kb mRNA are underlined with their orientation indicated; the two corresponding PCR fragments (P1 to P2 and P3 to P4) were cloned in a Bluescript plasmid and sequenced. The postulated polyadenylation site (ATTAAA) in the 2.4-kb mRNA is overlined with a solid line. The unique 3′-UTR in the 1.9-kb mRNA is shown by a dotted line.

by Scotto *et al.* (1990). Most interestingly, we also found two clones each containing a longer 3′-UTR that extends 306 bp beyond the termination codon TGA but that does not reach a poly(A)+ tail. Both types of clones share the first 119 bp after the termination codon TGA, but diverge just 2 bp before the postulated polyadenylation site (ATTAAA) that is present in the shorter 3′-UTR, but not found at the same site of the longer 3′-UTR (Figure 3).

To demonstrate the presence of the novel extended 3′-UTR in TGF-β1 mRNA, we carried out a Northern blot analysis on total RNA from infarcted heart tissue, using as a probe the unique portion of the 3′-UTR extension (position 1293–1477, 185 bp) present only in the longer 3′-UTR. Only the 1.9-kb mRNA, not the 2.4-kb mRNA, hybridized to this probe (Figure 4). PCR amplification from infarcted heart RNA also showed the presence of the alternate 3′-UTR (Figure 5). Two of the PCR bands (1, 2) resulted from amplification of the TGF-β1 coding region to the extended 3′-UTR (position 986–1477, 492 bp; 986–1454, 469 bp), while a third band (3) was from the TGF-β1 coding region only (position 986–1173, 187 bp). All of these PCR-amplified DNA fragments showed positive signals when they were hybridized with an oligonucleotide probe (5′ GGTCGCAAGCCCAAGGTGGAG 3′, position 1111–1131) located within the PCR amplification region (data not shown). The relative amounts of the three PCR bands corre-

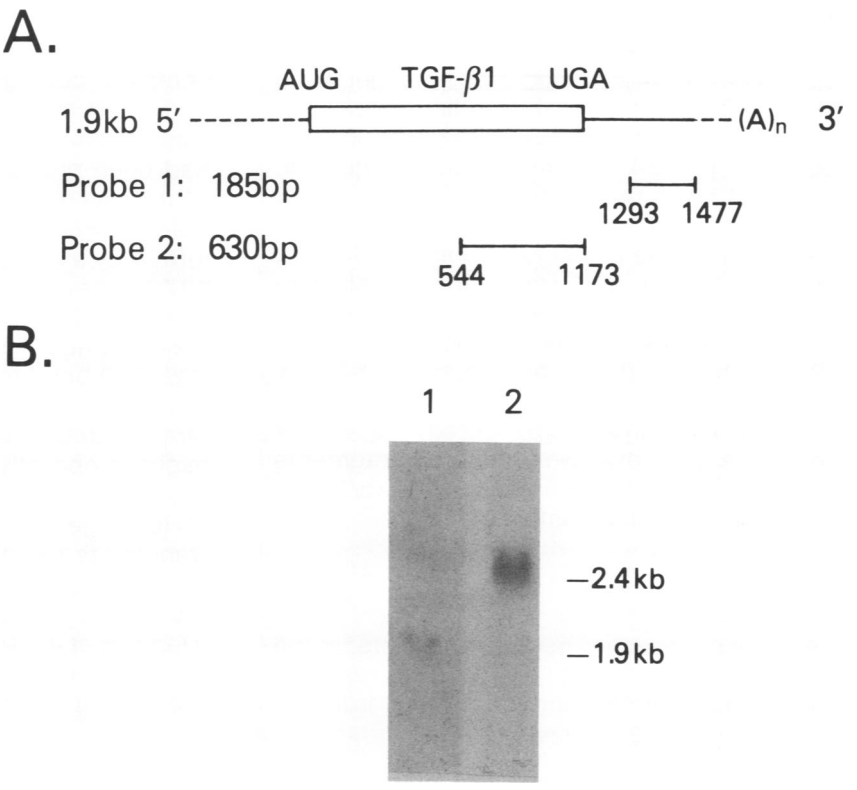

Figure 4. **The unique 3′-UTR hybridizes only to the 1.9-kb mRNA.** Twelve micrograms of total RNA from infarcted heart tissue (48 h) were loaded on each lane of a RNA gel and subjected to hybridization. (A) Illustration of the positions of the probes: probe 1 represents the unique 3′-UTR, while probe 2 represents the TGF-β1 coding region (P3 to P4, shown in Fig. 3). Both probes were obtained by PCR amplification and labeled by ^{32}P-nick translation. (B) RNA hybridized with probe 1 (lane 1) and probe 2 (lane 2).

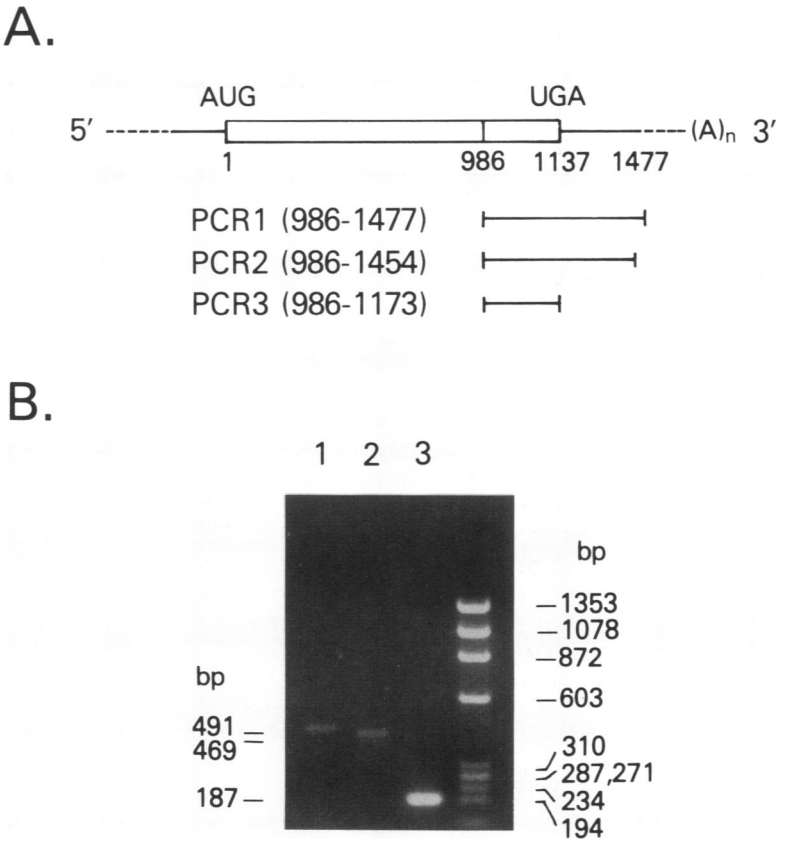

Figure 5. **PCR amplification of RNA from infarcted rat hearts shows the presence of the unique TGF-β1 3′-UTR.** Total RNA from infarcted heart tissue (48 h) was primed with oligo (dT) to make the first strand of the cDNA, and the resultant RNA/DNA mixture (1 μg) was used for PCR amplification. (A) Diagram illustrating the 3 PCR reactions. PCR 1 and PCR2 were carried out with primers located at the coding region and the extended 3′-UTR, whereas PCR 3 employed primers located only at the extremes of the coding region. (B) Lanes 1–3 show the expected PCR fragments (491, 469, and 187 bp) corresponding to PCR 1, 2, and 3. Equal amounts of PCR mixture (10 μl) were loaded on agarose gel for analysis. Right lane: molecular weight markers.

sponded well to the ratio of the 1.9-kb/2.4-kb mRNAs in infarcted rat heart. S1 mapping analysis with a ^{32}P-end labeled probe starting from the 3′-UTR extension and including part of the TGF-β1 mature form coding region (position 930–1477, plus 24 bp from the vector sequence, 572 bp) showed two protected fragments (363 bp and 548 bp) in RNA from infarcted heart, whereas only one protected fragment (363 bp) was detectable in RNA from normal heart (Figure 6). The shorter fragment (363 bp) represents protection of the shorter 3′-UTR of the 2.4-kb mRNA, whereas the longer fragment (548 bp) represents protection of the extended 3′-UTR of the 1.9-kb mRNA. As expected, the ratio of the intensity of the two protected fragments corresponded to the ratio of the 1.9-kb/2.4-kb mRNAs in normal and infarcted heart.

Discussion

The studies presented here demonstrate for the first time that the 1.9-kb mRNA initially reported in rat heart after experimental myocardial infarction is a second transcript of the TGF-β1 gene. This 1.9-kb TGF-β1 mRNA includes the complete coding information found in the 2.4-kb TGF-β1 mRNA; no alternate splicing pattern was observed in the TGF-β1 coding region, as had been previously reported in porcine TGF-β1 mRNAs (Kondaiah *et al.*, 1988). However, the 1.9-kb mRNA is distinguished from the 2.4-kb mRNA by a unique 3′-UTR that extends at least 187 bp beyond the polyadenylation site (ATTAAA) of the 2.4-kb transcript; in addition, the 1.9-kb species is estimated to be missing at least 680 bp of the 5′-UTR of the 2.4-kb TGF-β1 mRNA species. These findings suggest that different transcriptional regulatory mechanisms might be operative during the response of heart tissue to injury or repair.

Heterogeneity at the 3′ end of the rat TGF-β1 transcripts has not previously been described. From the genomic DNA data, it is known that the human TGF-β1 gene consists of seven exons (Derynck *et al.*, 1987). Whereas the first five exons of the mouse TGF-β1 gene are identical to the corresponding exons of the human TGF-β1

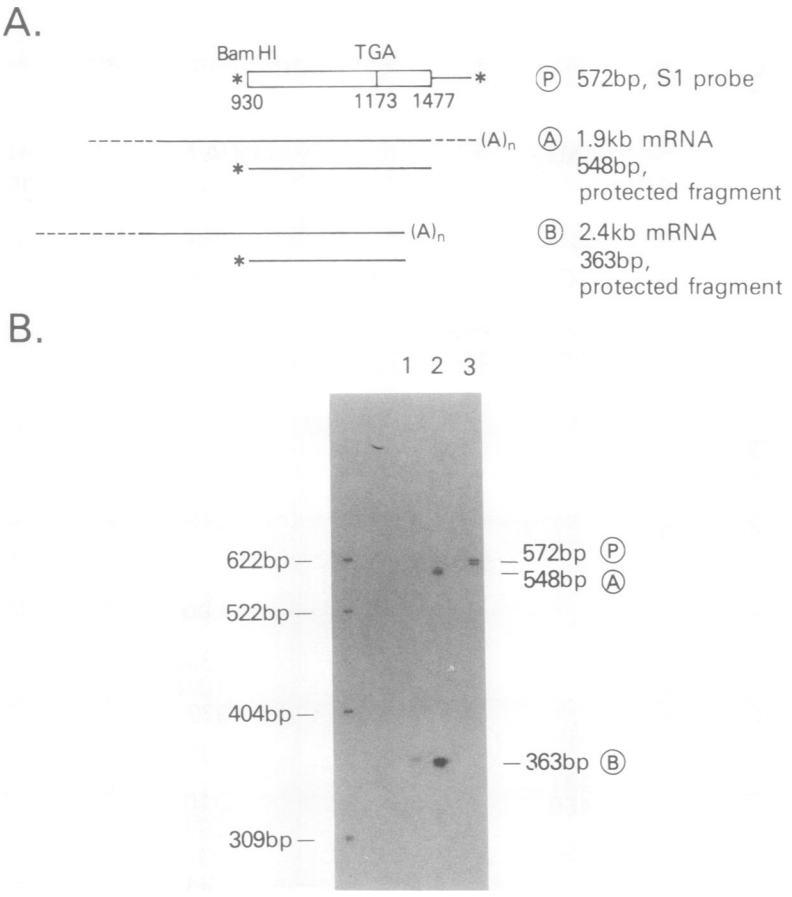

***Figure 6.* S1 nuclease protection analysis of the two transcripts of the TGF-β1 gene in infarcted heart tissue.** An S1 probe (P) was generated by ^{32}P end-labeling of the *Bam*HI fragment from position 930 to 1477 plus 24 bp of vector sequence. Seventy-five μg of total RNA from heart tissue were subjected to S1 nuclease protection assay as described under Materials and methods. Lane 1: protected fragment (B, 363 bp) from RNA from normal heart tissue; lane 2: protected fragments (A, 548 bp; B, 363 bp) from RNA from infarcted heart tissue at 48 h after the ligation; lane 3: S1 probe (P), 572 bp; left lane: molecular weight markers.

gene, the remaining two exons (6th and 7th exon) have not been characterized (Geiser *et al.*, 1991). Recent studies by Scotto *et al.* (1990) show that only one polyadenylation signal (ATTAAA) at position 2136 is utilized in the transcription of the human TGF-β1 gene. Our results show that the 2.4-kb rat TGF-β1 mRNA has the same polyadenylation signal (ATTAAA) at a position analogous to that found in human TGF-β1 mRNA. We have not seen a similar polyadenylation signal sequence in clones corresponding to the 1.9 kb TGF-β1 mRNA. However, we postulate that a similar polyadenylation site will be found downstream of the identified 3′ sequence. Further studies on genomic cloning of the rat TGF-β1 gene will answer these questions.

Because the 1.9-kb TGF-β1 mRNA contains the complete TGF-β1 coding region and a longer 3′-UTR, it follows that its 5′-UTR must be shorter than that of the 2.4-kb TGF-β1 mRNA. Our attempts to complete the cloning of the 5′-UTR of this novel TGF-β1 mRNA have been unsuccessful, presumably because of the extremely GC rich zones present in this region; however, on the basis of the sequence data available, it can be estimated that the 5′ end begins downstream of position 680 of the 2.4-kb mRNA species. A translational inhibitory element located in the 5′-UTR at nucleotide −792 to −764 (start codon as nucleotide 1) has been described in the human TGF-β1 gene (S.-J. Kim, as-yet-unpublished observations). On the basis of the presumed loss of the translational inhibitory sequence in the 5′-UTR of the 1.9-kb mRNA, the highly increased level of expression of this mRNA species in infarcted heart tissue could be interpreted as an "emergency response" to injury. Thus, the increased levels of TGF-β1 protein in the infarcted heart may be partly contributed by the more facilitated translation of the 1.9-kb TGF-β1 mRNA relative to the 2.4-kb mRNA species. The unique 3′-UTR may also have effects on translation. However, because the 2.4-kb TGF-β1 mRNA is still the predominant species of TGF-β1 transcripts in infarcted heart tissue, the increased levels of TGF-β1 may also result from the elevated 2.4-kb TGF-β1 mRNA. We intend to address these questions directly in future investigations.

Immunohistochemical studies have demonstrated that TGF-β1 expression is markedly enhanced after experimental myocardial infarction (Thompson *et al.*, 1988; Casscells *et al.*, 1990). Recent studies of Lefer *et al.* (1990) suggest that this increased TGF-β1 expression might be associated with repair of the myocardium. They showed that intravenous administration of TGF-β1 after ligation and immediately before reperfusion of the left coronary artery of a rat diminished several parameters of cardiac injury, including circulating levels of creatine kinase and tumor necrosis factor α. We would like to propose that the highly elevated level of the 1.9-kb TGF-β1 mRNA might play a role in TGF-β1-mediated cardioprotection.

The presence of a 1.9-kb TGF-β1 mRNA has been observed not only in infarcted rat heart tissue but also in a variety of other mouse and rat tissues (Akhurst *et al.*, 1988; Braun *et al.*, 1988; Czaja *et al.*, 1989a,b; Thompson *et al.*, 1989; Manthey *et al.*, 1990), as well as in human cells such as Burkitt lymphoblasts and peripheral blood lymphoblasts activated by mitogens (Derynck *et al.*, 1985). Interestingly, the level of the 1.9-kb TGF-β1 mRNA was most often elevated after tissue injury, as in rat liver after partial hepatectomy (Braun *et al.*, 1988) or exposure to carbon tetrachloride (Czaja *et al.*, 1989a) or streptococcal cell walls (Manthey *et al.*, 1990); elevated levels were also seen in mouse liver infected with *Schistosoma* (Czaja *et al.*, 1989b) and in mouse skin after topical application of phorbol esters (Akhurst *et al.*, 1988). The increased TGF-β1 expression observed in rat liver after treatment with carbon tetrachloride corresponded with increased expression of the mRNAs for several extracellular matrix proteins (procollagens type I, III, and IV; Nakatsukasa *et al.*, 1990). Collectively, these observations strongly suggest that the 1.9-kb TGF-β1 mRNA may play an important role in the response of a variety of tissues to injury and in subsequent repair processes.

Materials and methods

Heart tissue preparation

The left coronary artery of Sprague-Dawley rats was ligated under ether anesthesia by the procedure described (Selye *et al.*, 1960). Rats were killed 6, 24, 30, and 48 h after surgery. Both infarcted and uninfarcted cardiac tissues were obtained from these animals; hearts from unoperated rats served as controls. Infarction was confirmed by inspection of the color and lack of motion of the anterior part of the left ventricle, as well as by ST-segment elevation and Q-waves on precordial electrocardiograms. Only hearts meeting all these criteria were included. The hearts were removed by the use of sterile techniques, frozen in liquid nitrogen, and stored at −70°C for subsequent RNA analysis.

RNA isolation and Northern analysis

Total RNA from heart tissue was isolated by extraction in guanidinium isothiocyanate (MacDonald *et al.*, 1987). Polyadenylated RNA was prepared by selection on an oligo (dT)-cellulose column (Jacobson, 1987). For Northern blot anal-

ysis, RNA was separated on 1.2% agarose gels containing 2.2 M formaldehyde, and transferred to Nytran membranes (Schleicher & Schuell, Keene, NH) in 1.0 M NH_4Ac. Northern hybridization was performed according to the method of Church and Gilbert (1984) in a buffer containing 1% bovine serum albumin (BSA), 0.5 M sodium phosphate (pH 7.0), 7% sodium dodecyl sulfate (SDS), and 0.01 M EDTA at 65°C. The blots were washed two times in 40 mM sodium phosphate buffer (pH 7.0), 0.5% BSA, 5% SDS, and 0.01 M EDTA for 10 min at room temperature and four times in 40 mM sodium phosphate buffer (pH 7.0), 1% SDS, and 0.01 M EDTA for 10 min at 65°C. Autoradiograms were quantitated with an LKB (Piscataway, NJ) scanning densitometer.

1.9-kb mRNA size fractionation

The 1.9-kb mRNA was size fractionized on a 1.5% agarose gel with a buffer of 50 mM boric acid, 5 mM sodium sulfate, 1 mM EDTA, and 50 μg/ml ethidium bromide. mRNA from heart tissue (25 μg) was heated at 68°C for 10 min and loaded onto the gel with 10% glycerol and the running buffer. To isolate the 1.9-kb mRNA, we cut a small window in front of the 1.9-kb mRNA band (same size as 18S RNA) after the gel had run sufficiently long to separate the 28S and 18S RNAs. The gel pieces were removed from the window, and the space was then filled with low melting point (LMP) agarose gel in the running buffer; the gel was electrophoresed further until the 1.9-kb mRNA band had migrated into the LMP gel window. The LMP gel pieces were melted at 68°C for 5 min and extracted with phenol/chloroform; then the RNA was precipitated with ethanol. The size of the fractionationed 1.9-kb mRNA was confirmed by Northern blot analysis with a ssDNA probe to human TGF-β1.

PCR amplification

Total RNA or polyadenylated RNA from rat heart tissue was primed with oligo (dT) to make the first strand of the cDNA. Twenty micrograms of total RNA or 2 μg of poly(A)+ RNA were dissolved in 25 μl H_2O, heated at 70°C for 5 min, and placed on ice. Reverse transcription was performed at 37°C for 1 h by adding 25 μl of a reaction mixture containing 10 μl of 5× reverse transcriptase buffer (BRL), 0.5 μl of oligo dT (12–18 mer, 1 mg/ml), 2.5 μl of 10 mM dNTPs, 30 units of RNasin, 2 μl of RNase-free BSA (2.5 mg/ml), 5.0 μl of actinomycin D (0.5 mg/ml), and 2.5 μl of M-MLV reverse transcriptase (200 U/μl, BRL). The reaction was stopped by heating the sample at 95°C for 5 min. After extraction with phenol/chloroform and ethanol precipitation, the RNA/DNA pellet was dissolved in 40 μl H_2O. The PCR reaction was performed by the use of 1–4 μl of the RNA/DNA mixture with the PCR reagent kit from Perkin Elmer Cetus (Norwalk, CT). Variable annealing temperatures (42–58°C) were used for different reactions based on the GC composition of oligonucleotide primer. The amplified PCR fragments were cloned directly into the plasmid pBluescript KSII+ (Stratagene, La Jolla, CA) for sequencing.

cDNA cloning and sequencing of the 1.9-kb mRNA

cDNA was constructed from mRNA of infarcted rat heart tissue using an Amersham (Arlington Heights, IL) cDNA synthesis kit with oligo (dT) and random primers. The λZAP vector (Stratagene) was used for the cDNA library construction. The library was screened using a ^{32}P-labeled probe of 630 bp DNA fragment corresponding to position 543–1173 in the coding region of the rat TGF-β1 cDNA. The probe was constructed by PCR techniques and labeled by nick-translation. Hybridizations were performed at 42°C by the use of 2×10^6 cpm/ml ^{32}P-labeled probe in 50% formamide, 5× SSC, 9× Denhardt's solution, 50 mM sodium phosphate (pH 7.0), 1% glycine, 0.1% SDS, and 100 μg/ml denatured salmon sperm DNA. Filters were washed two times in 2× SSC and 0.1% SDS at room temperature, then three times in 0.5× SSC and 0.1% SDS at 55°C, and exposed to X-ray films. The positive clones containing inserts in λZAP vectors were recircularized to form a phagemid by adding helper phage R408 for in vivo excision. Nucleotide sequences of the insert fragment were determined on both strands of the clones by the Sanger dideoxy chain termination method, using sequenase (United States Biochemicals, Cleveland, OH) in combination with synthetic oligonucleotide primers.

S1 mapping

Total RNA (75 μg) was co-precipitated in ethanol with 6×10^5 cpm of a ^{32}P end-labeled DNA probe representing the *Bam*HI fragment from position 930 to 1477 plus 24 bp of the vector sequence. Hybridization was performed by dissolving the precipitate in 40 μl of hybridization buffer (80% formamide, 20 mM tris(hydroxymethyl)aminomethane [Tris], pH 7.4, 0.4 M NaCl, 1 mM EDTA, 0.05% SDS), heating at 75°C for 15 min, and incubating overnight at 55°C. Three hundred fifty microliters of S1 nuclease buffer (0.3 M NaCl, 3.3 mM $ZnSO_4$ and 60 mM NaOAc, pH 4.5) containing 2 μg of denatured salmon sperm DNA and 150 U of S1 nuclease (Boehringer Mannheim, Indianapolis, IN) was added to the DNA-RNA hybrid and incubated at 37°C for 1 h. The protected fragments were extracted by phenol/chloroform before ethanol precipitation. The pellet was dissolved in 3 μl H_2O and 6 μl of gel loading buffer (95% formamide, 10 mM EDTA, 10 mM NaOH, 0.02% 5-Bromophenol blue, 0.02% xylene cyanol FF), denatured by heating at 95°C for 5 min, and analyzed on a 6% sequencing gel.

Acknowledgments

We thank R. Derynck for the original human TGF-β1 cDNA clone and A. Geiser, J. Burmester, and T. Winokur for many helpful suggestions.

Received: November 19, 1990.
Revised and accepted: January 8, 1991.

References

Akhurst, R.J., Fee, F., and Balmain, A. (1988). Localized production of TGF-β mRNA in tumor promoter-stimulated mouse epidermis. Nature *331,* 363–365.

Braun, L., Mead, J.E., Panzica, M., Mikumo, R., Bell, G.I., and Fausto, N. (1988). Transforming growth factor-β mRNA increases during liver regeneration: a possible paracrine mechanism of growth regulation. Proc. Natl. Acad. Sci. USA *85,* 1539–1543.

Casscells, W., Bazoberry, F., Speir, E., Thompson, N., Flanders, K.C., Kondaiah, P., Ferrans, V.J., and Sporn, M.B. (1990). Transforming growth factor-β1 in normal heart and in myocardial infarction. Ann. NY Acad. Sci. *593,* 148–161.

Church, G.M., and Gilbert, W. (1984). Genomic sequencing. Proc. Natl. Acad. Sci. USA *81,* 1991–1995.

Czaja, M.J., Flanders, K.C., Biempica, L., Klein, C., Zern, M.A., and Weiner, F.R. (1989a). Expression of tumor necrosis factor-α and transforming growth factor-β1 in acute liver injury. Growth Factors *1,* 219–226.

Czaja, M.J., Weiner, F.R., Flanders, K.C., Giambrone, M.A., Wind, R., Biempica, L., and Zern, M.A. (1989b). In vitro and in vivo association of transforming growth factor-β1 with hepatic fibrosis. J. Cell Biol. *108,* 2477–2482.

Derynck, R., Jarrett, J.A., Chen, E.Y., Eaton, D.H., Bell, J.R., Assoian, R.K., Roberts, A.B., Sporn, M.B., and Goeddel, D.V. (1985). Human transforming growth factor-β complementary DNA sequence and expression in normal and transformed cells. Nature *316,* 701–705.

Derynck, R., Jarrett, J.A., Chen, E.Y., and Goeddel, D.V. (1986). The murine transforming growth factor-β precursor. J. Biol. Chem. *261,* 4377–4379.

Derynck, R., Rhee, L., Chen, E.Y., and Tilburg, A.V. (1987). Intron-exon structure of the human transforming growth factor-β precursor gene. Nucleic Acids Res. *15,* 3188–3189.

Geiser, A.G., Kim, S.-J., Roberts, A.B., and Sporn, M.B. (1991). Characterization of the mouse transforming growth factor-β1 promoter and activation by the Ha-*ras* oncogene. Mol. Cell. Biol. *11,* 84–92.

Ignotz, R.A., and Massagué, J. (1986). Transforming growth factor-beta stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. J. Biol. Chem. *261,* 4337–4345.

Jacobson, A. (1987). Purification and fractionation of poly(A)+ RNA. In: Methods in Enzymology, ed. S.P. Colowick and N.O. Kaplan, New York: Academic Press, 254–261.

Kondaiah, P., Van Obberghen-Schilling, E., Ludwig, R.L., Dhar, R., Sporn, M.B., and Roberts, A.B. (1988). cDNA cloning of porcine transforming growth factor-β1 mRNAs: evidence for alternate splicing and polyadenylation. J. Biol. Chem. *263,* 18313–18317.

Lefer, A.M., Tsao, P., Aoki, N., and Palladino, M.A. (1990). Mediation of cardioprotection by transforming growth factor-β. Science *249,* 61–64.

MacDonald, R.J., Swift, G.H., Przybyla, A.E., and Chirgwin, J.M. (1987). Isolation of RNA using guanidinium salts. In: Methods in Enzymology, ed. S.P. Colowick and N.O. Kaplan, New York: Academic Press, 219–227.

Manthey, C.L., Allen, J.B., Ellingsworth, L.R., and Wahl, S.M. (1990). In situ expression of transforming growth factor beta in streptococcal cell wall-induced granulomatous inflammation and hepatic fibrosis. Growth Factors *4,* 17–26.

Nakatsukasa, H., Nagy, P., Evarts, R.P., Hsia, C.-C., Marsden, E., and Thorgeirsson, S.S. (1990). Cellular distribution of transforming growth factor-β1 and procollagen types I, III, and IV transcripts in carbon tetrachloride-induced rat liver fibrosis. J. Clin. Invest. *85,* 1833–1843.

Qian, S.W., Kondaiah, P., Roberts, A.B., and Sporn, M.B. (1990). cDNA cloning by PCR of rat transforming growth factor-β1. Nucleic Acids Res. *18,* 3059.

Roberts, A.B., Anzano, M.A., Lamb, L.C., Smith, J.M., and Sporn, M.B. (1981). New class of transforming growth factors potentiated by epidermal growth factor. Proc. Natl. Acad. Sci. USA *78,* 5339–5343.

Roberts, A.B., and Sporn, M.B. (1990). The transforming growth factor-βs. In: Peptide Growth Factors and Their Receptors. Handbook of Experimental Pharmacology, ed. M.B. Sporn and A.B. Roberts, New York: Springer Verlag, 95/1, 419–472.

Roberts, A.B., Sporn, M.B., Assoian, R.K., Smith, J.M., Roche, N.S., Wakefield, L.M., Heine, U.I., Liotta, L.A., Falanga, V., Kehrl, J.H., and Fauci, A.S. (1986). Transforming growth factor-beta: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. Proc. Natl. Acad. Sci. USA *83,* 4167–4171.

Rossi, P., Roberts, A.B., Roche, N.S., Karsenty, G., Sporn, A.B., and de Crombrugghe, B. (1988). A nuclear factor-1 binding site mediates the transcriptional activation of a type I collagen promoter by transforming growth factor-β. Cell *52,* 405–414.

Scotto, L., Vaduva, P.I., Wager, R.E., and Assoian, R.K. (1990). Type β1 transforming growth factor gene expression: a corrected mRNA structure reveals a downstream phorbol ester responsive element in human cells. J. Biol. Chem. *265,* 2203–2208.

Selye, H., Bajusz, E., Grasso, S., and Mendell, P. (1960). Simple techniques for the surgical occlusion of coronary vessels in the rat. Angiology *11,* 398–407.

Sharples, K., Plowman, G.D., Rose, T.M., Twardzik, D.R., and Purchio, A.F. (1987). Cloning and sequence analysis of simian transforming growth factor-β cDNA. DNA *6,* 239–244.

Sporn, M.B., and Roberts, A.B. (1989). Transforming growth factor-β: multiple actions and potential clinical applications. J. Am. Med. Assoc. *262,* 938–941.

Thompson, N.L., Bazoberry, F., Speir, E.H., Casscells, W., Ferrans, V.J., Flanders, K.C., Kondaiah, P., Geiser, A.G., and Sporn, M.B. (1988). Transforming growth factor beta-1 in acute myocardial infarction in rats. Growth Factors *1,* 91–99.

Thompson, N.L., Flanders, K.C., Smith, J.M., Ellingsworth, L.R., Roberts, A.B., and Sporn, M.B. (1989). Expression of transforming growth factor-β1 in specific cells and tissues of adult and neonatal mice. J. Cell Biol. *108,* 661–669.

Van Obberghen-Schilling, E., Kondaiah, P., Ludwig, R.L., Sporn, M.B., and Baker, C.C. (1987). Complementary deoxyribonucleic acid cloning of bovine transforming growth factor-β1. Mol. Endocrinol. *1,* 693–698.

Varga, J., Rosenbloom, J., and Jimenez, S.A. (1987). Transforming growth factor-β (TGF-β) causes a persistent increase in steady-state amounts of type I and type III collagen and fibronectin mRNA in normal human dermal fibroblasts. Biochem. J. *247,* 587–604.