# A Novel Low Molecular Weight Ribonucleic Acid (RNA) Related to Transforming Growth Factor $\alpha$ Messenger RNA

**Sonia B. Jakowlew, Paturu Kondaiah, Pamela J. Dillard, Michael B. Sporn, and Anita B. Roberts**

Laboratory of Chemoprevention
National Cancer Institute
Bethesda, Maryland 20892

**Human transforming growth factor $\alpha$ (TGF$\alpha$) is coded for by an mRNA of about 4800 nucleotides. The cDNA sequence demonstrates that the 50 amino acid TGF$\alpha$ is embedded in a larger 160 amino acid precursor protein. We report here that in addition to the 4800 nucleotide TGF$\alpha$ mRNA, there is a novel second RNA species of about 350 nucleotides that hybridizes to a human TGF$\alpha$ cDNA probe. This small RNA species has been found in the RNA of several human tumor cells including HT1080, A549, A431, A2058, and A673. We have demonstrated an inverse relationship between the amounts of the 4800 nucleotide TGF$\alpha$ mRNA and the 350 nucleotide novel RNA in these human cells. Restriction enzyme cleavage of a human TGF$\alpha$ cDNA probe into three separate domains consisting of a processed coding region and 5′- and 3′-preprocessed coding and untranslated regions showed that only the 3′-untranslated region hybridized to the 350 nucleotide RNA. Using sense and anti-sense single-stranded 3′-untranslated region probes, we determined that the 350 nucleotide RNA band may be composed of multiple species of RNA which are related to the anti-sense DNA strand that is opposite to the strand that codes for the 4800 nucleotide TGF$\alpha$ mRNA. (Molecular Endocrinology 2: 1056–1063, 1988)**

## INTRODUCTION

Transforming growth factor $\alpha$ (TGF$\alpha$) is a polypeptide hormone operationally defined by its ability to induce the reversible transformation of certain normal mammalian fibroblasts in culture. Treatment of such cells with TGF$\alpha$ stimulates their growth and promotes anchorage independence as measured by formation of colonies of phenotypically transformed cells in semi-solid agar (1, 2). The amino acid sequences of mouse, rat and human TGF$\alpha$ are highly conserved, sharing approximately 90% identity (3–9).

TGF$\alpha$ competes with epidermal growth factor (EGF) for binding to a common receptor (1, 10, 11), and therefore, shares many (12, 13), though not all (14–16) activities with EGF. Although TGF$\alpha$ and EGF may have originated from a common ancestral gene, TGF$\alpha$ has been much more highly conserved throughout evolution than EGF (mouse and human EGF share only 70% identity). Comparison of the amino acid sequences of mouse, rat, and human TGF$\alpha$ with the corresponding EGFs shows about 35% identity among these EGF-like polypeptides, with conservation of all six cysteine residues (17).

As is true of several other growth factors, TGF$\alpha$ is processed from a larger precursor molecule. Complementary DNA sequence analysis has shown that the 50 amino acid rat TGF$\alpha$ and human TGF$\alpha$ are proteolytically derived from a 159 and 160 residue long precursor, respectively; each is coded for by a 4500 and 4800 nucleotide long mRNA, respectively. The sequence coding for the human TGF$\alpha$ precursor has been shown to be localized on six exons (5). The precursor contains a typical leader sequence and an extremely hydrophobic domain resembling a transmembrane region (4, 6). *In vitro* translation of rat pro-TGF$\alpha$ mRNA transcripts in the presence of rough endoplasmic reticulum membrane vesicles indicates that pro-TGF$\alpha$ is synthesied as an integral membrane glycoprotein (18).

Little is known about the transcriptional elements and translational constraints that control the production of TGF$\alpha$ or its precursor *in vivo*. However, it has been shown that TGF$\alpha$ production can be driven by exogenous promoters such as the metallothionein promoter (19, 20) or retroviral long terminal repeat (20). In appropriate cell types, this abberant expression of TGF$\alpha$ can lead to transformation (21). *In situ* hybridization and immunohistochemistry demonstrate the presence of TGF$\alpha$ mRNA and protein *in vivo* in normal human skin epidermis (22) and in rodent maternal decidua adjacent to the embryo (23) with a time-dependent and tissue-specific pattern of expression.

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In this report, we present evidence that in addition to the 4800 nucleotide TGFα mRNA, there is a second 350 nucleotide RNA species found in several human tumor cells that hybridizes to human TGFα cDNA. Our results demonstrate an inverse relationship between the relative amounts of these two RNA species. We present evidence that the 350 nucleotide RNA band may be composed of multiple species of RNA that are related to the anti-sense DNA strand that is opposite to the strand that codes for the 4800 nucleotide TGFα mRNA.

## RESULTS

### Isolation of Hybridizing Low Molecular Weight RNA

We have found a low molecular weight RNA (LMW RNA) in total cytoplasmic RNA isolated from human fibrosarcoma HT1080 cells that hybridizes with human TGFα RNA. As shown in Fig. 2A, this LMW RNA, which has been sized to about 350 nucleotides using known RNAs as molecular weight markers, was identified initially from a blot of total HT1080 RNA probed with a

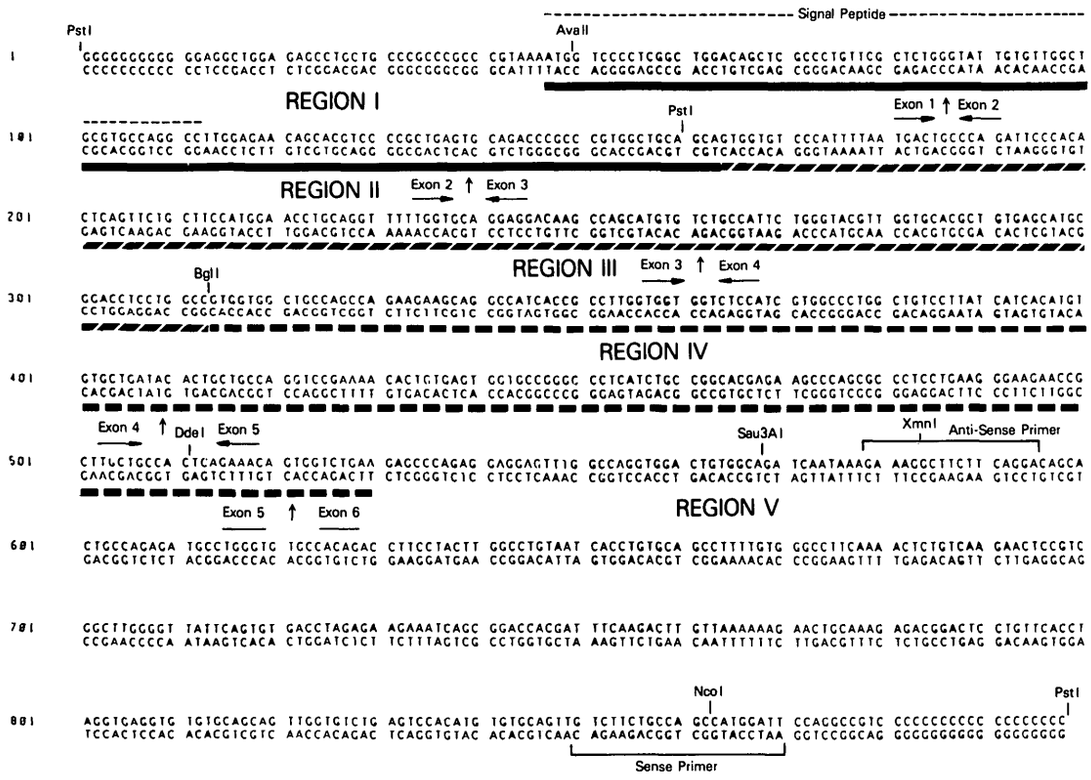


**Fig. 1.** Nucleotide Sequence of the cDNA Insert Contained in the TGFα cDNA Plasmid pTGF-C1
The nucleotides are numbered to the left. The five regions of the cDNA are designated using Roman numerals. The location of the restriction enzyme sites used to generate the fragment probes are shown above the sequence. Exon-intron boundaries are denoted by *vertical arrows* between adjacent exons. The signal peptide sequence is shown by *raised dotted lines*.

| Complementary DNA Regions | | | Fragment Probes | | |
|---|---|---|---|---|---|
| Region | | Nucleotides | Enzymes | | Nucleotides |
| I 5′-Untranslated | = | 1–46 | *Pst*I-*Ava*II | = | 1–49 |
| II 5′-Preprocessed | = | 47–163 | *Ava*II-*Pst*I | = | 50–160 |
| III Processed coding | = | 164–313 | *Pst*I-*Bgl*II | = | 161–313 |
| IV 3′-Preprocessed | = | 314–530 | *Bgl*I-*Sau*3AI | = | 314–568 |
| V 3′-Untranslated | = | 531–898 | *Sau*3AI-*Pst*I | = | 568–898 |

The 21 and 17 nucleotide sequences used as primers for primer extension of the *Sau* 3AI-*Pst* I DNA fragment are shown in brackets below the *Nco* I restriction enzyme site and above the *Xmn* I restriction enzyme site.

nick-translated complete human TGF$\alpha$ cDNA (Regions I–V, Fig. 1). From the intensity of the hybridization signal, it can be estimated that the LMW RNA is at least 10-fold more abundant than the 4800 nucleotide species previously identified as the major TGF$\alpha$ mRNA (4).

It was possible to isolate the LMW RNA using at least two methods of extraction. Cell lysis using NP-40 and separation of nuclei from cytoplasmic extract (24) was more efficient at recovering the LMW RNA species than homogenization in a urea-containing buffer and precipitation using LiCl (25) (Fig. 2A) as has been shown for other LMW RNAs (26). The method of cell lysis using NP-40 was used to isolate the LMW RNA for this study. No attempt was made to quantify absolutely the recovery of the LMW RNA and TGF$\alpha$ RNA although the relative amounts of the two RNAs did not change upon successive extractions, nor were they dependent on the degree of confluency of the HT1080 cells (data not shown). The LMW RNA is not a lysis product or degradation product of the 4800 nucleotide species since lysis of HT1080 cells for a longer time (15 min rather than 3 min) did not result in an increase in the relative level of the LMW RNA (Fig. 2A). It could be demonstrated that the LMW RNA was indeed RNA and not DNA by digesting the RNA with alkali; after this treatment, no hybridization of TGF$\alpha$ cDNA was seen to either the 4800 or 350 nucleotide species (Fig. 2A). Finally, chromatography of heat-denatured RNA on oligo d(T)-cellulose suggested that the LMW RNA, like TGF$\alpha$ mRNA, was polyadenylated (Fig. 2B).

The LMW RNA was separated from TGF$\alpha$ mRNA and other high molecular weight RNAs by sucrose density gradient centrifugation in the presence of formamide. The LMW RNA eluted predominantly in fraction 1 and TGF$\alpha$ mRNA predominantly in fraction 3 (Fig. 2B). In addition, we observed at least three other minor RNA species that could hybridize to TGF$\alpha$ cDNA; these RNAs migrate at about 1, 0.7, and 0.5 Kb, each hybridizing about 100-fold less than the LMW RNA.

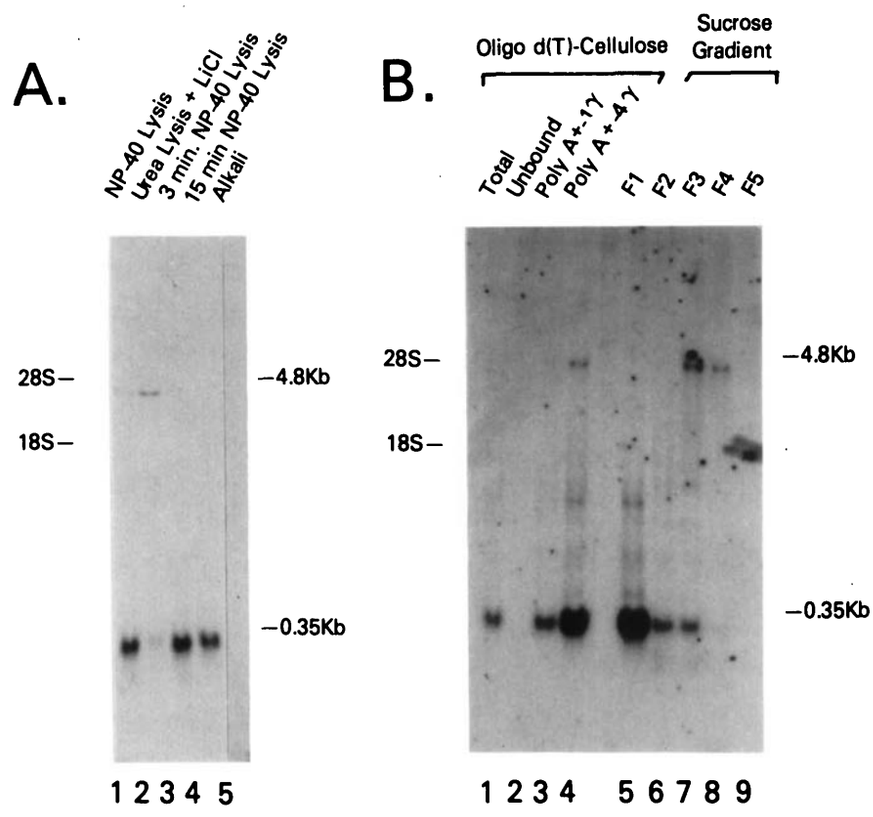


**Fig. 2.** Isolation of the LMW RNA and TGF$\alpha$ mRNA from HT1080 Cells

A, RNA was isolated from subconfluent cells using lysis with Nonidet P-40 or homogenization in urea and LiCl. Lysis using Nonidet P-40 was carried out for either 3 or 15 min and is labeled as such above the RNA lanes. RNA (15 $\mu$g) was digested with 1 N NaOH overnight at 37 C and then precipitated with ethanol (labeled as alkali). Fifteen micrograms of RNA were subjected to electrophoresis on a 1.2% agarose-formaldehyde gel, transferred to a Nytran filter, and hybridized to plasmid pTGF-C1 labeled with [$^{32}$P] by nick-translation. B, Poly(A)$^+$ RNA was isolated from total RNA using oligo d(T)-cellulose. Total RNA (15 $\mu$g); unbound, poly(A)$^-$ flow-through RNA (15 $\mu$g); bound, poly(A)$^+$ RNA (1 and 4 $\mu$g) were subjected to electrophoresis, transferred, and hybridized as in A. Poly(A)$^+$ RNA (100 $\mu$g) was sedimented through linear sucrose gradients containing denaturing formamide, fractionated, and precipitated with ethanol. One microgram of RNA from each fraction was electrophoresed, blotted, and hybridized as before. The RNA fractions are numbered in order of increasing molecular weight.

### Presence of 350 Nucleotide RNA in Human Cells

We have detected the LMW RNA in several human tumor cells in addition to HT1080 fibrosarcoma cells (Fig. 3). The RNA is present in the A375, A2058, and FemX II melanomas; in the A549 lung carcinoma; in the A673 rhabdomyosarcoma; and in the A3827 renal carcinoma. With the exception of the A673 rhabdomyosarcoma, the 4800 nucleotide TGFα mRNA is also present in these tumor cells. However, the LMW RNA is not detected in the T24 bladder carcinoma while TGFα mRNA is present. Neither the LMW RNA nor TGFα mRNA is detected in normal lung LL47 and WI-38 cells, WI-38-SV40 cells, or in HeLa cells.

In the human tumor cells in which both the LMW RNA and the TGF-alpha RNA have been detected, there is an inverse relationship between the levels of each RNA present as measured by the intensity of hybridization (Fig. 3). This is most evident in comparison of the relative degree of hybridization of these two species in HT1080 and A2058 cells. Densitometric scanning shows that the level of the LMW RNA is approximately 20-fold higher than the level of TGFα mRNA in HT1080 cells; whereas, in A2058 cells, which have 12-fold more TGFα mRNA than HT1080 cells, the level of the LMW RNA is approximately one tenth the level of TGFα mRNA. This inverse relationship between the levels of the LMW RNA and TGFα mRNA is further demonstrated in A549, A375, A3827, and FemX II cells where there are intermediate levels of the two RNA species. In none of the cells are high levels of both transcripts ever concordant. The absolute level of the LMW RNA varies widely in the cells examined from the highest level in HT1080 cells to the lowest level in A2058 cells. No LMW RNA is detected in T24, RD-P52, MCF-7, and T47-D cells.

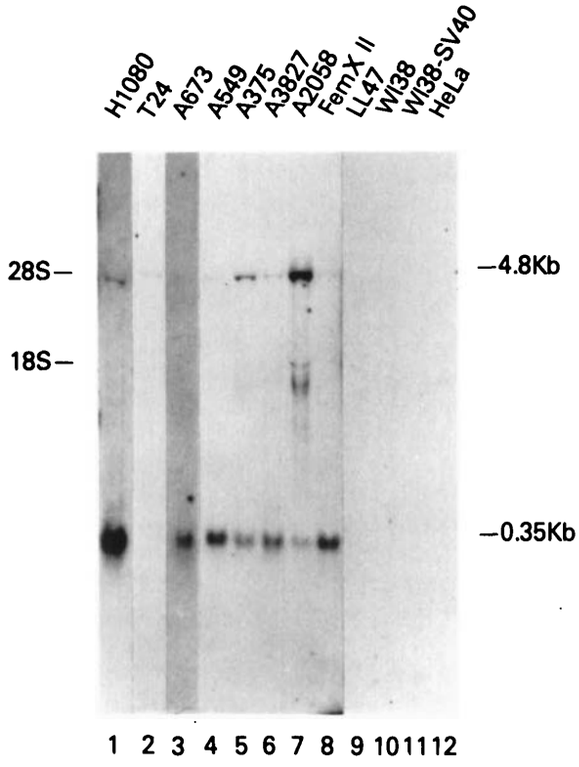


**Fig. 3.** Northern Hybridization of Human Cell RNAs.

Total RNA (15 μg) extracted from confluent cells using lysis with Nonidet P-40, was subjected to electrophoresis on a 1.2% agarose-formaldehyde gel, transferred to a Nytran filter, and hybridized to plasmid pTGF-C1 labeled with [$^{32}$P] by nick translation. The cells used are labeled above each lane of RNA.

### Hybridization of Human TGFα cDNA Regions to HT1080 RNA

To localize the LMW RNA to a region of TGFα cDNA, HT1080 RNA was hybridized to probes made from each of the five regions of TGFα cDNA as outlined in *Materials and Methods* (see also Fig. 1), namely to the 5′-untranslated region (Region I), 5′-preprocessed coding region (Region II), processed coding region (Region III), 3′-preprocessed coding region (Region IV), and 3′-untranslated region (Region V). As expected, each of the probes hybridized to the 4800 nucleotide TGFα mRNA to about the same degree (data not shown). However, only Region V, corresponding to the 3′-untranslated region of TGFα cDNA, hybridized to the LMW RNA (Fig. 4A). To rule out the possibility that hybridization of Region V, which contains a GC-rich tail of the cDNA, might be an artifact, hybridization of a gel-purified *Xmn* I-*Nco* I fragment (Region V) which does not contain the GC-tail sequence, was demonstrated. Identical results were obtained in four other cell lines examined: T24 bladder carcinoma cells and A673, A204 and RD-P52 rhabdomyosarcoma cells (Fig. 4A). Using poly(A)$^+$ RNAs, we were able to detect hybridization of probes corresponding to Region V and also complete TGFα cDNA to the LMW RNA in T24 cells, which had not been detected using total RNA (Fig. 4A).

### Hybridization of Human TGFα cDNA Regions to Human DNA

Complementary DNA fragments corresponding to Regions III and V of the TGFα cDNA sequence were individually hybridized to genomic DNA from HT1080, T24, A673, and RD-P52 cells that had been cleaved with *Eco*RI, *Bam*HI, and *Hin*dIII (Fig. 4B). Both regions hybridized to HT1080, T24, and RD-P52 cell genomic DNA in a simple band pattern consisting of one or two bands. The same band patterns were obtained using stringent (50% formamide, 42 C) or less stringent (40% formamide, 37 C) hybridization conditions and stringent (0.2× SSC, 65 C) and less stringent (4× SSC, 42 C) washing conditions. This suggests that the LMW RNA and TGFα mRNA are probably coded for by the same genomic DNA region. It is uncertain whether only one or multiple fragments of the same size are being detected in these bands.

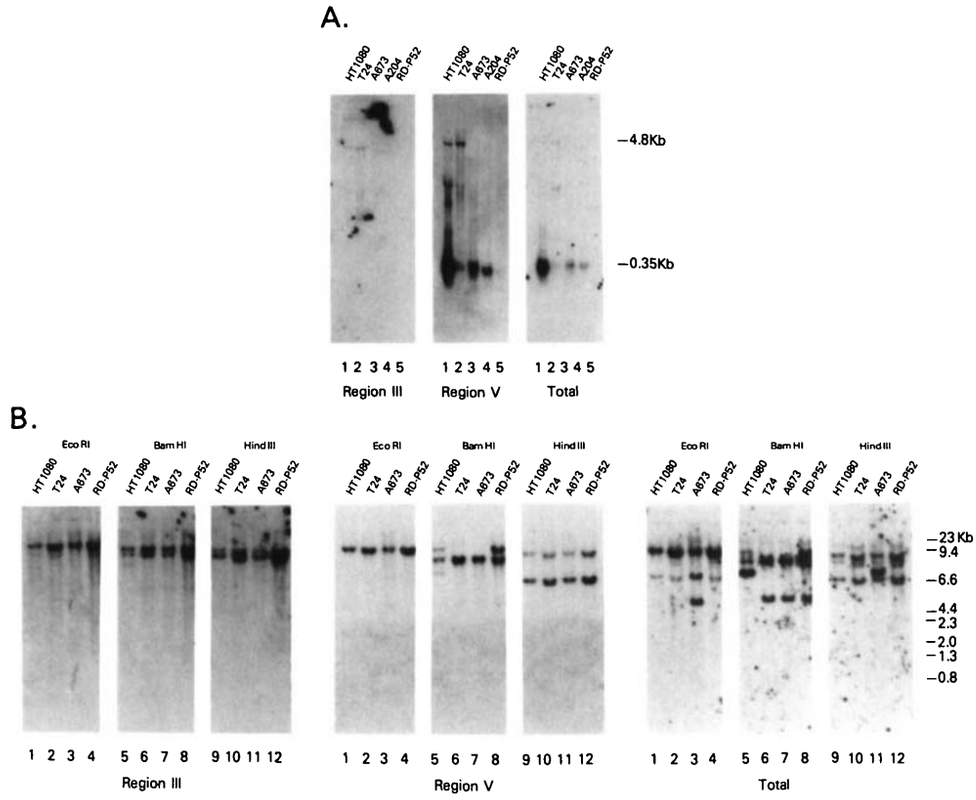


**Fig. 4.** Hybridization of Human TGF$\alpha$ cDNA Regions to Human RNAs and DNAs
A, Human TGF$\alpha$ cDNA was fragmented using specific restriction enzymes into Regions III and V. Five micrograms of poly(A)$^+$ RNA extracted from human HT1080, T24, A673, A204, and RD-P52 cells were electrophoresed, blotted, and hybridized with nick-translated double-stranded cDNA fragments (Regions III and V). Regions III and V and total correspond to the cDNA fragments that were used for hybridization. B, Genomic DNA was extracted from HT1080, T24, A673, and A204 cells. Fifty micrograms of each DNA were digested with *Eco*RI, *Bam*HI, or *Hind*III and 10 $\mu$g each DNA were electrophoresed and blotted onto Nytran in three separate blots. Each blot was hybridized with nick-translated $^{32}$P-labeled double-stranded cDNA fragment regions III and V and total TGF$\alpha$ cDNA as described in A.

### Hybridization of Sense and Anti-Sense Human TGF-$\alpha$ cDNA Probes to HT1080 RNA

The *Sau*3AI-*Pst*I DNA fragment of Region V of TGF$\alpha$ cDNA was subcloned into M13 mp18 to generate a single-stranded DNA probe with higher specific activity than that which could be obtained using nick-translation of the gel-purified fragment (27). Hybridization of this single-stranded probe (the 3′-sense probe) was detected to HT1080 TGF$\alpha$ mRNA but not to the LMW RNA (Fig. 5). The *Sau* 3AI-*Pst* I DNA fragment was also subcloned into M13 mp19 to generate an anti-sense single-stranded probe. In contrast to the results obtained with the sense strand, this anti-sense DNA probe hybridized only to HT1080 LMW RNA but not to TGF$\alpha$ mRNA. This suggests that the LMW RNA is related to the anti-sense DNA strand that is opposite to the strand that codes for the TGF$\alpha$ mRNA.

## DISCUSSION

In this report, we describe a novel LMW RNA species that can hybridize to human TGF$\alpha$ cDNA and that is related to the anti-sense DNA strand that is opposite to the strand that codes for TGF$\alpha$ mRNA. In eukaryotes, the function of the noncoding DNA strand that is opposite the transcribed strand is presumably to serve as a structure to participate in double-stranded, semi-conservative DNA replication. However, a number of recent reports have shown that the anti-sense DNA strand may have other roles in addition to replication. For example, the Gart locus of *Drosphila melanogaster*, known to encode three purine pathway enzymatic activities, has been shown to contain an entire gene encoding a cuticle protein contained within the first Gart intron and transcribed from the opposite DNA strand (28). A second Drosophila locus which codes for dopa decarboxylase has been shown to share an 88 nucleotide region at its 3′-end with the 3′-end of a transcript arising from an unknown gene on the opposite strand (29). A region of mouse DNA from BALB/cTS-A-3T3 cells has been identified at which processed mRNAs from two adjacent convergent transcription units overlap by 133 nucleotides at their 3′-untranslated regions (30). A genomic locus in the rat has been identified that encodes gonadotropin-releasing hormone and a second

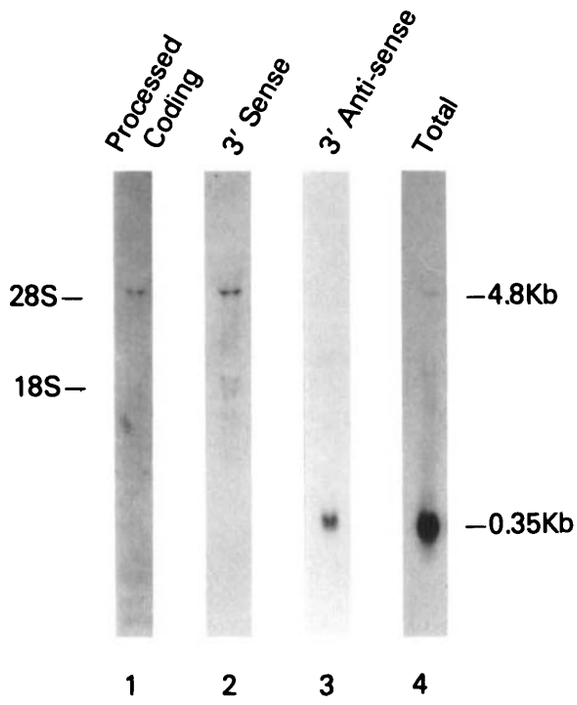


**Fig. 5.** Hybridization of Sense and Anti-Sense Human TGFα cDNA Probes to HT1080 RNA

Both the coding and noncoding DNA strands of the *Sau*3AI-*Pst*I DNA fragments of Region V of TGFα cDNA were subcloned into M13 mp18 and mp19, uniformly labeled with [$^{32}$P] by primer extension using DNA polymerase I Klenow fragment, and hybridized to total RNA (15 μg) that had been electrophoresed and blotted onto Nytran filters. The processed coding region of TGFα cDNA (Region III) and total TGFα cDNA were nick-translated and hybridized to identical blots of HT1080 RNA. Each blot contains one lane of total RNA.

gene of unknown function which overlap and occupy both strands of the same piece of DNA (31). The 5′-untranslated region of chicken fast muscle myosin heavy chain mRNA has identity with a significant portion of the 3′-untranslated region of both translational control (tc) RNAs 102 A and B (32). The 3′-untranslated regions of chicken embryo cardiac myosin heavy chain and myosin light chain mRNAs have identity with muscle-specific 7S RNA (33).

The data presented in this report show for the first time the existence of an LMW RNA(s) complementary to a portion of a growth factor mRNA. We have localized the area of identity of the LMW RNA to Region V of TGFα mRNA and have shown that the LMW RNA hybridizes to the anti-sense strand of the 3′-untranslated region of TGFα cDNA, specifically to the 330 nucleotide *Sau* 3AI-*Pst* I fragment. RNA blot hybridization shows that the LMW RNA does not migrate as a tight band, but instead, migrates as a diffuse band, suggesting that the LMW RNA may be composed of more than one species with slightly different molecular weights.

In prokaryotes and viruses, the formation of double-stranded RNA by the hybridization of complementary transcripts is important in the regulation of gene expression and replication. Such a mechanism has been shown for the inhibition of translation of the ompF (34) and IS10 transprosase (35) genes, and the control of replication of the plasmid *Col* E1 by inhibiting priming (36–38). In eukaryotes, the introduction of anti-sense RNA or vector constructs expressing anti-sense RNA into mammalian cells, has been used to inhibit gene expression (39, 40). Given the inverse relationship between expression of the LMW RNA and TGFα mRNA, it is tempting to speculate that the LMW RNA may be a natural anti-sense RNA whose function is to regulate transcription or translation of TGFα mRNA (41–44). An overlap between these two RNAs may raise the interesting possibility that a sense/anti-sense hybrid might exist *in vivo* that could have regulatory implications for the expression of one or both RNAs. Further studies on the transcription, translation, and cloning of the LMW RNA may answer some of these questions.

## MATERIALS AND METHODS

### General Methods

All methods were as described by Maniatis *et al.* (45) unless otherwise stated.

### Cells

All cells with the exception of the human A2058 melanoma cells (kindly supplied by Dr. J. DeLarco) and the human *Fem* XII melanoma cells (kindly supplied by Drs. A. C. Morgan and J. W. Pearson) were obtained from the American Type Tissue Culture Collection. Human cells were grown as follows: HT1080 fibrosarcoma, A549 lung carcinoma, A673 rhabdomyosarcoma, A204 rhabdomyosarcoma, RD-P52 rhabdomyosarcoma, A375 melanoma, A2058 melanoma, and A3827 renal carcinoma in Dulbecco's-Vogt modified Eagles' medium, WI-38 diploid lung, WI-38-SV40 diploid lung, transformed with Simian virus-40 and HeLa epitheloid cervix carcinoma in Dulbecco's-Vogt modified Eagle's medium supplemented with additional glucose; T24 bladder carcinoma in McCoy's medium; and FemX II melanoma in RPMI 1640 medium. In addition, all media were supplemented with 10% fetal calf serum and 50 μg/ml penicillin and streptomycin.

### RNA Isolation, Purification and Northern Analysis

RNA was isolated by two methods: 1) by lysing washed cells in cold 10 mM NaCl, 1 mM $MgCl_2$, 10 mM Tris-HCl (pH7.5), and 0.5% NP-40 for 3–5 min, pelleting nuclei, and extracting with phenol-chloroform (24), and 2) by homogenizing cells in urea-sodium dodecyl sulfate (SDS) and precipitating with LiCl following a modified protocol by Auffray and Rougeon (25). Poly(A)$^+$ RNA was selected from total RNA using oligo d(T)-cellulose (Collaborative Research, Waltham, MA) (46) and sedimented through linear 5–22% sucrose gradients containing 70% formamide, 3 mM EDTA, and 3 mM HEPES (47) for 36 h at 20 C and 39,000 rpm in a SW41 rotor. RNA was separated for Northern blot analysis on 1.2% agarose gels containing 0.66 M formaldehyde, and transferred to Nytran (Schleicher & Schuell, Keene, NH). Hybridization was performed according to Maniatis *et al.* (45) using 50% formamide, 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50 mM sodium phosphate (pH 7.0), 1% glycine, 0.05% each of BSA, Ficoll and polyvinylpyrrolidone, 1 μg/ml yeast tRNA,

and 100 μg/ml sonicated, denatured calf thymus DNA at 42 C for 48 h. The blots were washed one time in 2× SSC-0.1% SDS at 42 C for 15 min, four times in 0.2× SSC-0.1% SDS for 30 min each time at 42 C and then two times at 0.2× SSC-0.1% SDS for 45 min each time at 65 C.

### DNA Southern Analysis

Human tumor cell genomic DNA digests (10 μg) were electrophoresed on 0.8% agarose gels in a buffer containing Tris-acetate-EDTA according to Maniatis *et al.* (45). The gel was blotted onto Nytran filter paper by the procedure of Southern (48) using 10× SSC.

### Oligonucleotides

Oligonucleotides were synthesized by using an Applied Biosystems model 380A DNA synthesizer. They were purified by gel electrophoresis using polyacrylamide-urea gels before use for priming.

### Gel Isolation of DNA Fragments

Plasmid DNA was isolated according to the method of Ish-Horowitz and Burke (49), digested with the appropriate restriction enzymes (New England Biolabs, Boston, MA), and fractionated by electrophoresis through a preparative 1% low melting point agarose gel (FMC-Marine Colloids, Rockland, ME) containing 5 μg/ml ethidium bromide. The DNA was visualized under UV light and the appropriate DNA fragment was excised, heated to 65 C to melt the agarose, extracted with an equal volume of 0.5 M NaCl-saturated phenol (65 C) followed by phenol-chloroform (1:1) and chloroform, before precipitating the DNA with ethanol.

### Isolation of Genomic DNA

Genomic DNA was prepared by lysing cells in a solution containing 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.2% SDS, and 100 μg proteinase K/ml (Boehringer/Mannheim, Indianapolis, IN) at 37 C for 16 h. The DNA was extracted with phenol-chloroform three times and then with ether three times. The DNA was treated with RNase A at 37 C for 2 h followed by treatment with proteinase K plus 0.2% SDS at 37 C for 2 h. The DNA was then extracted three times with phenol-chloroform and precipitated with ethanol.

### Fragmentation of TGFα cDNA

Human TGFα cDNA was fragmented at nucleotides 1 and 49 with *Pst*I and *Ava*II (Region I, Fig. 1); at nucleotides 49 and 160 with *Ava*II and *Pst*I (Region II); at nucleotides 1 and 160 with *Pst*I (Regions I and II); at nucleotides 160 and 313 with *Pst*I and *Bgl*I (Region III); at nucleotides 313 and 568 with *Bgl*I-*Sau*3AI (Region IV); and at nucleotides 568 and 898 with *Sau*3AI and *Pst*I (Region V).

### Subcloning Plasmid DNA Fragments into M13

The *Sau*3AI-*Pst*I DNA fragment (Region V) was subcloned into the *Bam*HI-*Pst*I sites of M13 mp18 to generate the sense orientation and was subcloned into the same sites in M13 mp19 to generate the anti-sense orientation according to the method of Messing (50).

### Radioactive Probes

Double-stranded DNA probes were prepared by nick-translation (51) and had specific activities of $1–3 \times 10^8$ cpm/μg. Single-stranded DNA probes were prepared by the primer extension method of Burke (27). A 21 nucleotide long oligonucleotide (CAGAAGACGGTCGGTACCTAA) was used to prime synthesis of the 330 nucleotide long sense *Sau*3AI-*Pst*I single-stranded DNA fragment (Region V) and a 17 nucleotide long oligonucleotide (GAAAGGCTTCTTCAGGA) was used to prime synthesis of the 330 nucleotide long anti-sense *Sau*3AI-PstI single-stranded DNA fragment (Region V) (see Fig. 1). After labeling, the DNA was digested with appropriate restriction enzymes [*Eco*RI for the sense *Sau*3AI-*Pst*I DNA fragment (Region V) and *Hin*dIII for the anti-sense *Sau*3AI-*Pst*I DNA fragment (Region V)] to give probes of 316 nucleotides for the sense fragment and 327 nucleotides for the anti-sense fragment. The fragment was isolated from the larger template strand by electrophoresis in a 5% polyacrylamide-8 M urea gel and located by autoradiography. The gel region containing the fragment was excised and the DNA was eluted into water overnight at 37 C.

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Address requests for reprints to: Dr. Sonia B. Jakowlew, Laboratory of Chemoprevention, Building 41, Room B902, National Institutes of Health, Bethesda, Maryland 20892.

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