Complementary Deoxyribonucleic Acid Cloning of Bovine Transforming Growth Factor-β1

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Transforming growth factor-β1 (TGFβ1) has been purified from a number of different sources and has a broad species specificity. To deduce the complete amino acid sequence of bovine TGFβ1 we have isolated cDNA clones encoding the protein from a bovine fibropapilloma library using a human cDNA probe. Sequence analysis of two independent cDNA clones revealed that the 112 amino acids corresponding to bovine TGFβ1 are identical to those of the human and porcine proteins. This unusually high degree of conservation in the primary structure of the human and bovine proteins reflects the strong evolutionary constraints for maintenance of structure and function of the molecule. As in the human, murine, and porcine systems, the mature form of TGFβ1 is derived by proteolytic cleavage of a larger precursor. Small differences in amino acid sequence were observed in the portion of the precursor that does not include mature TGFβ1, although 92% of the residues are still conserved. A 2.25 kilobase (kb) mRNA was identified in total bovine wart and bone RNA, whereas no message was detected in polyadenylated spleen or brain RNA. In addition to the major 2.25 kb message, we observed a 1.9 kb transcript in poly(A+) RNA from wart tissue. (Molecular Endocrinology 1:693-698, 1987)

INTRODUCTION

Transforming growth factor-β (TGFβ) is a multifunctional polypeptide with widespread effects on growth, differentiation, embryogenesis, and tissue repair (for recent reviews see Refs. 1-3). It is now apparent that TGFβ is a member of an emerging gene family of regulatory peptides with similar structural features. Two closely related homodimeric forms of TGFβ exist which display receptor cross-reactivity (4). The form which was described initially, designated TGFβ1, is the predominant species. The second form, TGFβ2, is a homodimer of subunits which share 70% amino-terminal sequence homology with TGFβ1. This form has been isolated from bovine bone (known as CIF-B) (5, 6), from porcine platelets (4), and more recently from media conditioned by human glioblastoma cells (7) and human PC-3 adenocarcinoma cells (8). In addition, a rare heterodimeric form containing one TGFβ1 and one TGFβ2 chain has been identified in porcine platelets (4). These TGFβ isoproteins have thus far been found to be functionally indistinguishable. In contrast, the more distantly related members of the TGFβ gene family display a multiplicity of regulatory effects. These peptides include the inhibins (9, 10), which inhibit pituitary FSH secretion, activins (11, 12), which stimulate pituitary FSH secretion, and Mullerian inhibiting substance (13), responsible for causing regression of Mullerian ducts in the developing male reproductive tract. In addition to the mammalian genes, a Drosophila pattern gene (decapentaplegic gene complex) involved in determining dorsal-ventral specification during embryological development has been cloned, sequenced, and found to be significantly homologous to the above mentioned TGFβ family (14).

Two abundant tissue sources of TGFβ1 from which the mature active form has been purified are circulating platelets (4, 15) and demineralized bovine bone (known as CIF-A) (5, 16). While the complete sequence of human TGFβ1 is known from cDNA cloning (17), large-scale production of the recombinant molecule has been difficult to achieve. Thirty residues of bovine TGFβ1 have been determined by amino-terminal sequencing and shown to be identical to its human counterpart (16, 18). In light of the practical and economical advantages of TGFβ1 purification from bovine rather than human sources we set out to further characterize the bovine molecule by cDNA cloning and sequencing. Our findings reveal that all 112 residues of the mature protein are conserved in both species. Such a high degree of evolutionary conservation emphasizes the important physiological role for this regulatory molecule and indicates a promising alternate source of TGFβ1 for future therapeutic applications.

RESULTS AND DISCUSSION

Isolation and Characterization of TGFβ1 cDNA Clones

A bovine fibropapilloma cDNA library constructed in an Okayama and Berg cloning vector (19) was screened

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for the presence of TGFβ1-specific clones using a ^{32}P-labeled human TGFβ1 probe devoid of vector sequences. The probe consisted of a single-stranded 218 base pair (bp) sequence complementary to the 3′-end of the human TGFβ1 cDNA coding region, a region which is highly conserved in its murine homolog (20). Two positive clones were isolated from an amplified sublibrary containing 2.8–3 × 10^4 independent recombinants. cDNA inserts from the two clones were subcloned into M13 phage and sequenced using the dideoxy chain termination method of Sanger *et al.* (21). A comparison of the structure of two bovine TGFβ1 cDNA inserts with the previously described human TGFβ1 precursor cDNA is represented schematically in Fig. 1. The smaller of the two isolates, bTGF-β1 clA, contains a 1.1 kilobase (kb) insert, whereas the larger clone, bTGF-β1 clB, contains an insert of 2.1 kb. Both species share a common 3′-end sequence. The complete nucleotide sequence of bTGF-β1 clA together with the predicted amino acid sequence of bovine TGFβ1 is shown in Fig. 2.

Striking homology was observed between bTGF-β1 clA and the human TGFβ1 cDNA which extends from a polyadenylation signal (ATTAAA), 12 bp upstream from the poly(A) tract, to the G-tail flanking the 5′-end of the insert. Several porcine cDNAs recently isolated and characterized in our laboratory also contain the ATTAAA sequence 12 bp upstream from the site of polyadenylation (Kondaiah, P., E. Van Obberghen-Schilling, R. L. Ludwig, A. B. Roberts, M. B. Sporn, and R. Dahr, in preparation). In the cloning procedure used to construct both the bovine and porcine cDNA libraries, the plasmid DNA vector serves as the primer for first- and second-strand cDNA synthesis, and nuclease treatment of the products is avoided, thereby ensuring that the 3′-end of the mRNA is represented in the cDNA clone. Although AATAAA is the most common consensus polyadenylation signal, ATTAAA is functional in a number of genes from many species (22). Interestingly, ATTAAA is also present in the human TGFβ1 gene (Fig. 1) where it is preceded by a G-C rich 3′-untranslated sequence that is highly homologous with the corresponding bovine and porcine cDNAs. It has been previously reported that in the human TGFβ1 gene an AATAAA hexanucleotide located 500 bp downstream from the termination codon was likely to be the polyadenylation signal. However, this assignment of the more distal signal was based on sequence analysis obtained from a genomic clone since none of the 70 human TGFβ1 cDNAs isolated from different oligo(dT)-primed cDNA libraries contained more than a few nucleotides of the 3′-noncoding region (17). It is likely that the ATTAAA located 145 bp downstream of the termination codon is the authentic polyadenylation signal in the human system as well.

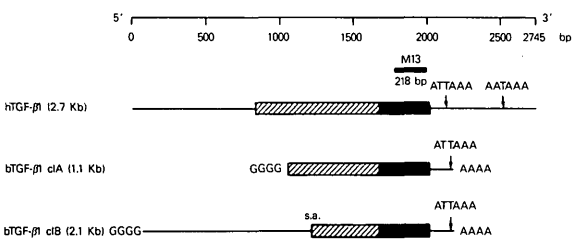

Fig. 1. Diagram of Human and Bovine TGFβ1 cDNAs

Structures of two bovine TGFβ1 cDNAs are represented schematically beneath that of the previously reported human cDNA (17). Bars indicate the protein-coding regions; the area representing mature processed TGFβ1 is filled in. Untranslated sequences are represented by lines. ATTAAA and AATAAA indicate the positions of potential polyadenylation signals. The 5′-half of bTGF-β1 clB, which shares no sequence homology with the bovine or human cDNA inserts, terminates in the putative splice acceptor (s.a.) consensus dinucleotide AG.

The first nucletide of bTGF-β1 clA corresponds to the second base in the Glu 75 codon of human TGFβ1 precursor cDNA. Thus, bTGF-β1 clA appears to be missing the first 223 nucleotides of the coding region and the 5′-untranslated region, which spans 841 bp in the human TGFβ1 cDNA. The three potential N-linked glycosylation sites present in the human TGFβ1 precursor are conserved in the bovine protein, as well as the dibasic residues comprising the proteolytic processing site for generation of the mature polypeptide chain from the 112 carboxyl-terminal residues (Fig. 2).

Inspection of the nucleotide sequences of the two bovine cDNAs revealed identity over 1 kb of 3′-end sequence. The 5′-end of bTGF-β1 clB, however, showed no sequence homology with either bTGF-β1 clA or human TGFβ1 cDNA sequence (not shown). Sequence divergence in the larger species occurs at a nucleotide, indicated by an arrow in Fig. 2, which corresponds exactly to the intron 1/exon 2 junction recently described in the human TGFβ1 gene (23). In addition, the 5′-sequence in bTGF-β1 clB which flanks the homologous segment terminates with the dinucleotide AG, a highly conserved eukaryotic mRNA splice acceptor sequence (24, 25). Thus, it is likely that bTGF-β1 clB represents an incompletely processed polyadenylated mRNA which lacks the first exon.

Expression of Bovine TGFβ1 mRNA

Expression of a major TGFβ1 mRNA species of approximately 2.5 kb has been observed in a wide variety of both normal and transformed cells in culture (17, 20). Constitutive expression of the factor by cultured cells may be related to the stimulatory action of TGFβ1 on matrix protein formation (26, 27) required by cells to adapt to tissue culture conditions. In soft tissues the TGFβ1 message is not uniformily present, although bone has been shown to produce relatively high levels of TGFβ1 (5). We compared the expression of TGFβ1 mRNA in bone cells, and in brain, spleen, and wart tissue by Northern analysis (Fig. 3). No detectable signal was found in polyadenylated RNA from adult brain or spleen (lanes 1 and 2). A major TGFβ1 message of approximately 2.25 kb was detectable in wart poly(A)+ RNA (lane 3) and in total bone cell and fibropapilloma RNA (lanes 4 and 5, respectively). In the wart polyadenylated RNA from which our library was made the TGFβ1 mRNA appeared as a doublet with the major band (upper) migrating with the 2.25 kb species found in other tissues. The lower band was not detected in total RNA from a different wart; however, poly(A) selection

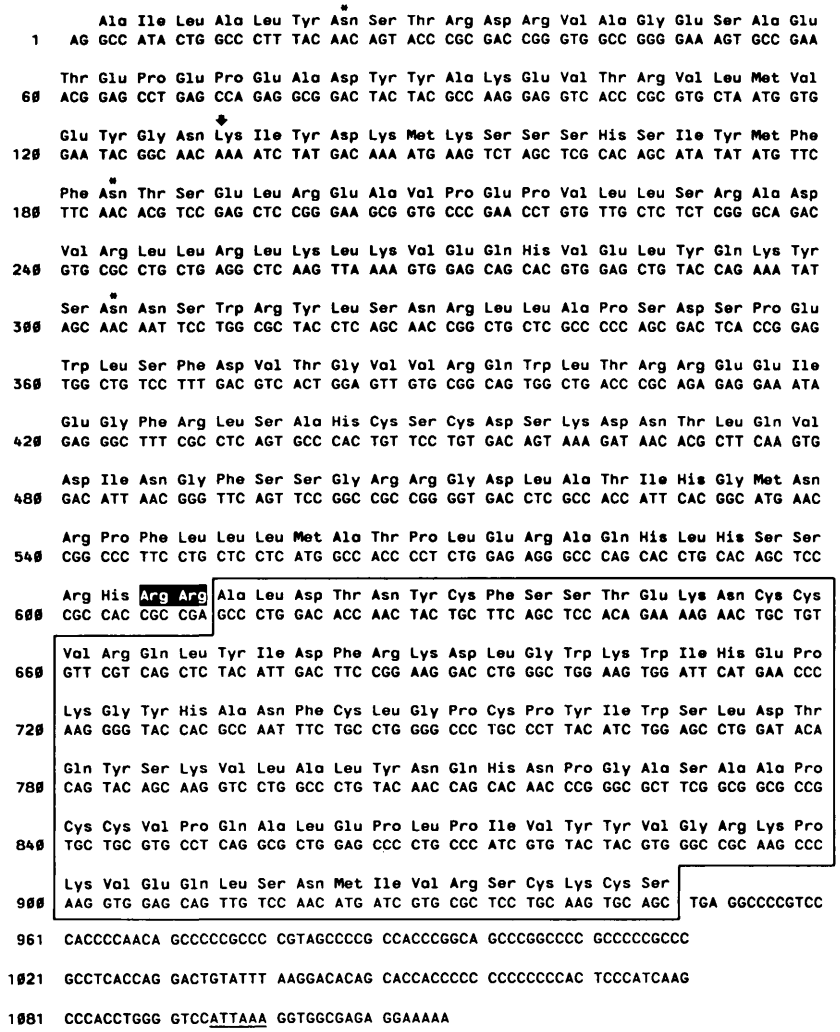

Fig. 2. Nucleotide and Predicted Amino Acid Sequence of bTGF-β1 clA

Nucleotide and deduced amino acid sequence of the bovine cDNA bTGFB-clA is shown with nucleotides numbered at the beginning of each line. By analogy with the known human sequence, the first in-frame codon corresponds to residue 75 of the precursor molecule. Potential N-linked glycosylation sites are denoted by (*). The 112 amino acid residues of mature TGFβ1 (boxed) are preceded by the proteolytic processing site, highlighted with reverse formatting. The putative polyadenylation signal, ATTAAA, is underlined.

may be required for its detection as it comigrates with 18S ribosomal RNA. Since the large bovine cDNA clone containing an intron sequence at the 5′-end is about 2.1 kb, we tested whether the shorter mRNA transcript represented this species. To do so, a single-stranded M13 probe complementary to the nonhomologous 5′-region was made and hybridized to a Northern blot of poly(A)-selected wart RNA. The probe did not hybridize detectably to any species on the Northern blot of wart RNA, whereas it did hybridize to the corresponding sequence in the cDNA insert analyzed on a Southern blot (data not shown). This finding suggests that the large cDNA clone represents a relatively rare precursor RNA. We are presently investigating the nature of the smaller (~1.9 kb) mRNA species which may involve tissue-specific splicing of the primary TGFβ1 transcript.

Sequence Conservation of TGFβ1

Previously, N-terminal amino acid sequence analyses of the mature homodimeric form of TGFβ1 purified from bovine kidney (18) and bone (16) have shown that residues 1–30 of the bovine factor are identical to the published human sequence. The present study allows us to extend that observation to all 112 residues which comprise the mature growth factor. Primary structures of the human, bovine, porcine, and murine TGFβ1 precursors are aligned in Fig. 4 to illustrate the homologies. The mature polypeptide is conserved 100% across human, bovine, and porcine species, and differs by only one residue in murine TGFβ1. Overall homologies with human TGFβ1 in the unprocessed portion of the molecule are about 86% for murine (residues 1–278), and 92% for porcine (residues 1–278) and bovine (residues 75–278) TGFβ1; the middle one-third of the TGFβ1 precursor shows the most divergence. It is noteworthy that the cellular fibronectin recognition sequence, RGD-X (28), is present in the precursor of all four species (residues 244–247). The high degree of interspecies conservation of TGFβ1 reveals strong resistance to the pressure of natural selection and implicates an important physiological role for the molecule. Furthermore,

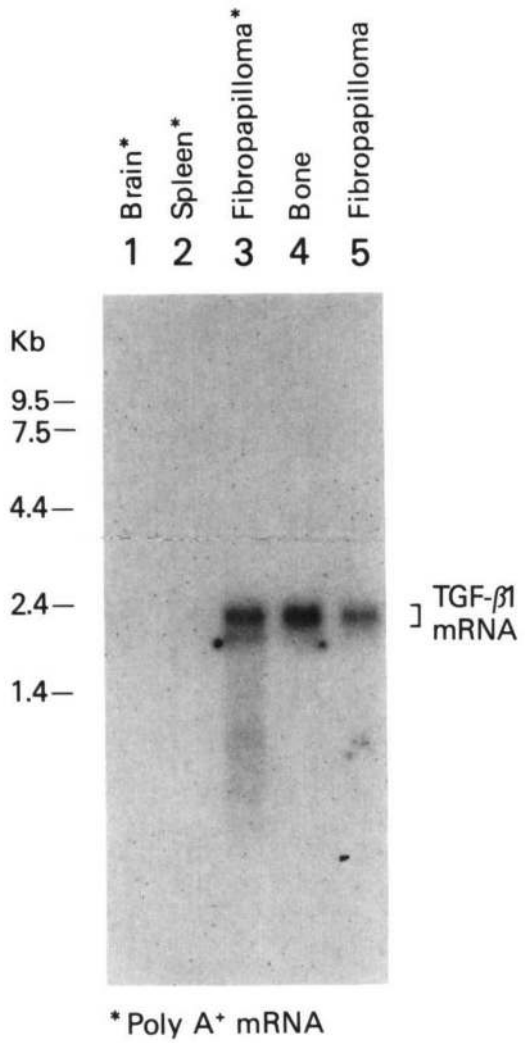

Fig. 3. Northern Analysis of Bovine RNA

RNA from the various bovine sources was isolated and Poly(A) selected (where indicated by *), electrophoresed on a 1% agarose gel, and transferred to GeneScreen as described in *Materials and Methods*. Hybridization was performed with a ^{32}P-labeled probe specific for the mature protein-coding region of TGFβ1. Position of molecular weight markers are shown on the left, and the position of TGFβ1 mRNA is indicated by a bracket.

the identical amino acid sequence accounts for the equal biological potency and immunoreactivity of the human and bovine proteins (16, 29). Concerning the sequence conservation of other bovine growth factors for which the primary structure is known, insulin-like growth factor-I (IGF-I) is identical to human IGF-I, whereas three out of 67 residues are different in bovine and human IGF-II (30). Insulin displays a considerable degree of sequence homology (94%) (31) between the two species. Eleven out of 140 residues are different in bovine and human acidic fibroblast growth factor (FGF) (32, 33) *vs*. two differences in the 146 amino acid form of basic FGF (34, 35). In contrast to these highly homologous proteins, considerably less conservation (65%) has been observed for bovine and human GH (36) and interleukin-2 (37, 38).

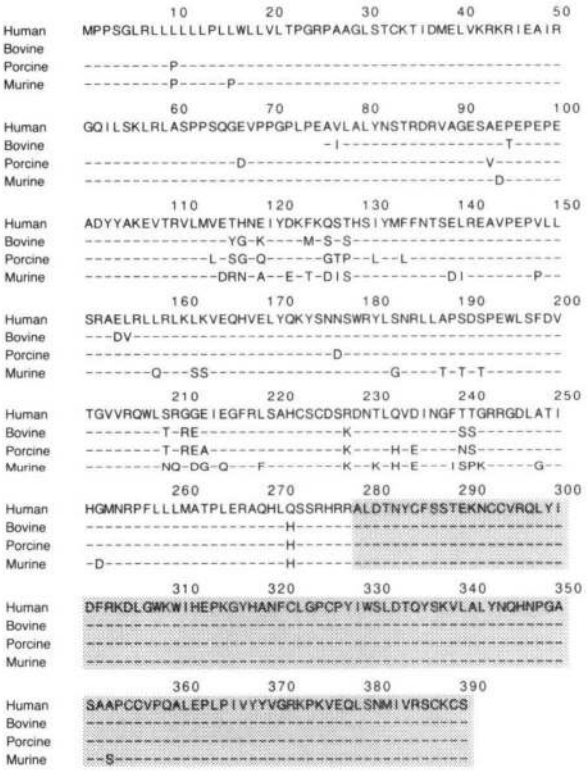

Fig. 4. Comparison of TGFβ1s

Amino acid sequences of TGFβ1 precursor from human (23), bovine, porcine (48), and murine (20) sources are aligned for maximal homology; sequences could be aligned without introduction of gaps. Residue numbers are indicated above the one letter amino acid codes, and dashes represent residues identical to those of the human sequence. The C-terminal 112 residues comprising mature TGFβ1 are shaded.

MATERIALS AND METHODS

General Methods

Unless otherwise stated, all methods were as described by Maniatis *et al*. (39). Reagents were of the highest grade commercially available.

cDNA Library Screen

Construction of the cDNA library from polyadenylated bovine fibropapilloma RNA according to the method of Okayama and Berg (40) has recently been reported (19). Colony hybridization after chloramphenicol amplification of plasmid DNA was performed essentially as described by Maniatis *et al*. (39) using a 218 bp single-stranded probe complementary to the mature protein-coding region of a human TGFβ1 cDNA [provided by R. Derynck (17)]. The probe was prepared by primer extension on a single-stranded M13 template containing a 243 bp (*pvu*II) insert from the 3′-end of the human cDNA. A specific oligonucleotide within the insert was extended using the Klenow fragment of DNA polymerase, and the resulting product was digested with *Xba*I at a unique site in the polylinker region to remove M13-specific sequences. The single-stranded DNA probe was isolated from a 5% acrylamide gel run under denaturing conditions.

DNA Sequence Analysis

Complementary DNA restriction fragments were subcloned into M13 mp18 and 19 (41) and sequenced according to the

Sanger dideoxy chain-termination method (21) modified to use [α-^{35}S]dATP (42). Sequence ambiguities were resolved by using Reverse Transcriptase (SEQUEL-SS/RT kit from IBI, New Haven, CT). Klenow Large fragment was purchased from BRL (Gaithersburg, MD), labeled dATP was from Dupont/New England Nuclear (Boston, MA), (1000 Ci/mmol), and ^{35}S Nucleotide Reagents Kit from Pharmacia (Piscataway, NJ). Both the universal primer and oligonucleotides complementary to the derived insert sequence (kindly synthesized by Shinichi Watanabe) were used as sequencing primers. Data were analyzed using the IBI/Pustell DNA and protein sequence analysis system.

Northern Analysis

Bovine spleen and brain poly(A)+ RNA was purchased from Clontech (Palo Alto, CA). Bone cell RNA from primary cultures of fetal bone (43) was a gift of Marion F. Young. Bovine fibropapilloma tissue was obtained from fibropapillomas induced by inoculation with bovine papillomavirus type I (44). Total RNA was extracted from wart tissue by the guanidinium thiocyanate/cesium chloride gradient method of Chirgwin *et al.* (45) as modified by Freeman *et al.* (46) and poly (A)-selected by oligo d(T)-cellulose affinity chromatography (39). RNA was separated for Northern blot analysis on 1% agarose gels containing 0.66 M formaldehyde and transferred to GeneScreen (Dupont/New England Nuclear). Hybridization was carried out as described by Church and Gilbert (47). An ethidium bromide-stainable RNA ladder (BRL) was used to determine molecular weights.

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