Murine Transforming Growth Factor-β2 cDNA Sequence and Expression in Adult Tissues and Embryos

Duncan A. Miller, Angela Lee, Ron W. Pelton, Ellson Y. Chen, Harold L. Moses, and Rik Derynck

Department of Cell Biology (D.A.M., R.W.P., H.L.M.)
Vanderbilt University School of Medicine,
Nashville, Tennessee 37232

Departments of Developmental Biology and Molecular Biology
Genentech Inc.
South San Francisco, California 94080

Murine transforming growth factor-β2 (TGF-β2) cDNAs were isolated from cDNA libraries derived from a differentiated murine embryonic carcinoma cell line, PCC3. The composite cDNA sequence is 4267 nucleotides long, including a 1217 nucleotides 5′-untranslated sequence, and encodes a murine TGF-β2 precursor of 414 amino acids with 96% identity to its human counterpart. Several consensus polyadenylation sequences are present in the 1807 nucleotides 3′-untranslated sequence. Five TGF-β2 mRNA species are observed in the developing mouse fetus and they show different patterns of expression during development. TGF-β2 mRNA expression was also examined in adult mouse tissues, in which four of the five RNA species were observed. TGF-β2 mRNAs were present in all adult mouse tissues examined, except liver, and was most abundant in placenta, the male submaxillary gland and lung. The patterns of expression suggest a physiological role for TGF-β2 both in embryonic development and in the maintenance of adult tissues. (Molecular Endocrinology 3: 1108–1114, 1989)

INTRODUCTION

The transforming growth factor-β (TGF-β2) family comprises several closely related members and an increasing number of more distant secreted polypeptides. The various members of the TGF-β family are known to be important in the control of cell proliferation and differentiation and in stimulation of extracellular matrix formation (1–3). The products of these genes should therefore be considered as major regulators of normal growth and development. The first member of this family to be purified and characterized, TGF-β1, was discovered as a factor that stimulated rodent fibroblast cell lines to proliferate in soft agar (4, 5). Complementary DNA characterization has indicated that it is synthesized in a precursor monomer form of 390 amino acid (6) with a 29 residue long N-terminal signal peptide (6, 7). The mature polypeptide, which dimerizes to form the biologically active β_1 25 kilodalton TGF molecule, corresponds to the carboxy-terminal 112 amino acid segment, cleaved from the precursor at arginine 278 (6). Additional posttranslational processing involves the glycosylation and mannose-6-phosphorylation of the precursor segment (8, 9). TGF-β1 is released by most cells in culture and by blood platelets in a latent form that can be activated by treatment with acid (10) or proteases such as plasmin (11). The latent TGF-β1 is now known to consist of dimer of the mature portion of the molecule noncovalently associated with the precursor segment (12, 13).

The existence of three additional types of polypeptides closely related to TGF-β1 has recently been determined. TGF-β2 was originally purified from porcine platelets (14) and bovine bone (15). It has similar receptor binding characteristics and biological activities to TGF-β1 (14, 16). However, TGF-β2, unlike TGF-β1, has been shown to efficiently support the mesoderm induction in Xenopus embryo explants (17). Complementary DNAs for TGF-β2 have been isolated from two human sources, prostatic carcinoma cells (18) and glioblastoma cells (19). The protein sequence deduced from cDNAs for polyergin, the simian BSC-1 cell derived growth inhibitor originally described by Holley *et al.* (20), is virtually identical to that of the human TGF-β2 (21). TGF-β3 cDNAs were recently obtained from human (22, 23), porcine (22), and chicken (24) cDNA libraries, while TGF-β4 cDNAs have as yet been isolated solely from a chicken chondrocyte library (25). The biological properties of TGF-β3 and -β4 have not been determined yet due to the lack of the purified factors. All members of the closely related TGF-β family are synthesized as precursors with a marked sequence similarity and conservation of the nine cysteine residues in their carboxy-terminal mature TGF-β monomers. All have a dibasic

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Molecular Endocrinology

cleavage site between the mature monomer and the preceding, more divergent precursor segment. TGF-β4 differs substantially in that it may lack a signal peptide (25).

Molecular cloning during the past few years has identified several proteins that show structural and some sequence similarity to TGF-β1, yet are more distantly related than the other TGF-β species. These include Müllerian inhibiting substance (26), the α and βA and βB chains of the inhibins or activins (27, 28), the bone morphogenetic proteins 2A, 2B and 3 (29), the *Drosophila* decapentaplegic gene (30), the *Xenopus* vg-1 (31), and the mammalian vgr-1 (32). The cDNAs of all these genes predict conservation of seven of the nine cysteines found in the mature TGF-β peptides along with other sequence similarities in the corresponding regions.

The existence of multiple genes in the closely related TGF-β family suggests differential, cell-type specific expression in the adult and during embryonic development. Differential expression of TGF-β1, TGF-β2, and TGF-β3 has been demonstrated in cultured cells (22). TGF-β2 and -β3 expression in cell lines is more restricted than the more ubiquitous TGF-β1 (6). Only TGF-β1 gene expression has been examined during mouse embryonic development by *in situ* hybridization (33, 34). These studies show relatively high expression levels of TGF-β1 mRNAs in hematopoietic cells and in developing bone (33, 34).

TGF-β2 expression may be of significant importance in mammalian embryogenesis. Thus we have isolated murine TGF-β2 cDNAs in order to carry out studies on TGF-β2 expression during mouse fetal development. The cDNA sequence and its derived polypeptide sequence for the entire murine TGF-β2 precursor are presented. While highly conserved during evolution, the mature mouse TGF-β2 monomer shows less similarity to its human counterpart than was observed in the case of TGFβ1. Expression of the TGF-β2 gene was detected in mouse embryos from day 10.5 to 17.5 *post coitum* (pc) with higher mRNA levels during the later stages. The placenta and adult lung tissue and male submaxillary gland also exhibited high levels of TGF-β2 expression. The multiple mRNAs observed by Northern hybridizations may be subject to differential regulation.

RESULTS AND DISCUSSION

Isolation and Characterization of Murine TGF-β2 cDNAs

Two cDNA libraries derived from murine PCC3 teratocarcinomas, induced for differentiation by retinoic acid for either 5 or 7 days, were screened for the presence of mTGF-β2 cDNA phage. Two × 10^6 phage were screened by hybridization using a ^{32}P-labeled human TGF-β2 cDNA as probe, which led to the identification of 34 hybridizing phage. The recombinant phage, which had cDNA inserts of a length between 0.8 and 3.0 kilobase pair (kbp) (data not shown), were analyzed for the completeness of their TGF-β precursor coding sequences by dot blot hybridization using two oligonucleotides corresponding to conserved regions in either the known mature TGF-β sequences or in the TGF-β precursor sequences, close to their N-termini (see *Materials and Methods*). Additional dot blot hybridizations were carried out using oligonucleotide probes, which are highly specific for the precursor regions of TGF-β1, -β2, and -β3. These analyses indicated that 28 cDNAs hybridized with the mature TGF-β probe, 16 of which also hybridized with the 5′ most probe for the conserved precursor region. Twenty three cDNAs hybridized with the TGF-β2 precursor specific probe, while none hybridized to the TGF-β1 or -β3 specific probes. These results suggested that most of the cDNAs contained complete coding sequences presumably for murine TGF-β2. Three of the cDNAs were selected for nucleotide sequence analysis on the basis of these results. These cDNAs, mTGF-β2-5, mTGF-β2-9, and mTGF-β2-27, were 2473, 1072 and 3151 base pair (bp) in length respectively. Their combined nucleotide sequence and the derived polypeptide sequence for the murine TGF-β2 precursor are shown in Fig. 1.

The Murine TGF-β2 cDNA Sequence

The sequence shown in Fig. 1 is 4267 nucleotides long and encodes a murine TGF-β2 precursor of 414 amino acids. This coding sequence corresponds to the longest open reading frame and could be assigned easily on the basis of the known human or simian TGF-β2 precursor sequence. The 5′-untranslated sequence of 1217 nucleotides contains many stop codons in all three different reading frames. One ATG triplet which could function as an initiator codon is present at position 119, but is followed by an in frame stop codon after six triplets. The presence of this short open reading frame may reduce the translational efficiency of the downstream located TGF-β2 precursor coding sequence. However, the large distance between the stop codon at position 140 and the start codon at position 1218 suggests that this reduction in translational initiation efficiency may not be as severe as if the distance were much shorter (35). The few hundreds of 5′-untranslated sequence immediately preceding the start codon are relatively rich in A and T nucleotides, making it likely that there is only a low degree of secondary structure in this region. This in turn may make the start codon easily accessible for initiation of translation by the ribosomal subunits and may thus result in a relatively high translational efficiency. This is in striking contrast with the TGF-β1 sequence, in which the start codon is surrounded by G-C rich sequence and is likely buried in a region with a high level of secondary structure (6). The sequence shown in Fig. 1 has a 3′-untranslated sequence of 1807 nucleotides and ends with a short stretch of A-residues. It is possible that these do not constitute part of the poly(A) tail at the end of the 3′-untranslated region in the mRNA, since there is no

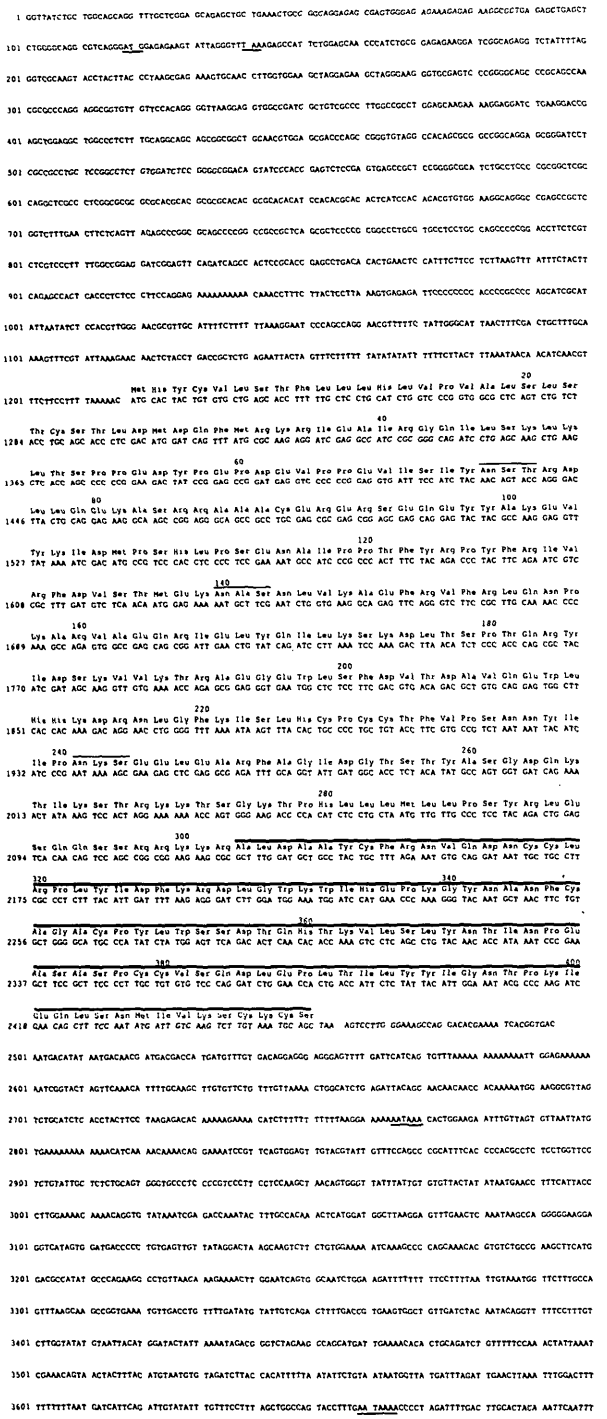

Fig. 1. Complementary DNA Sequence and Predicted Amino Acid Sequence of the murine TGF-β2 Precursor

The deduced 414 amino acid precursor sequence is shown above the nucleotide sequence. The mature TGF-β2 sequence is boldly overlined and is cleaved from the rest of the precursor after the multibasic sequence. The three potential N-glycosylation sites are thinly overlined. An ATG triplet (position 119) and the in frame stopcodon (position 140) in the 5′ untranslated sequence are underlined, as are two potential polyadenylation signals (AATAAA) in the 3′-untranslated sequence.

recognizable polyadenylation signal sequence shortly preceding this 3′-end of the sequence. On the other hand, there are two potential polyadenylation signals (AATAAA, Ref. 36), located at positions 2763 and 3659. Murine TGF-β2-5 and mTGF-β2-9 both had a sizable poly(A) tail shortly after the polyadenylation signal at position 2763, indicating the functionality of this signal. It is similarly also possible that the AATAA sequence at position 3659 is a functional polyadenylation signal. Differential polyadenylation could explain at least in part the size heterogeneity of the TGF-β2 mRNAs (18, 19; see also below). The 1807 nucleotides long murine 3′-untranslated sequence is in marked contrast with the much shorter polyadenylated 3′-sequence of 737 nucleotides long in the human TGF-β2 mRNA. This much longer sequence in the case of the murine cDNA is due to the mutation of the human polyadenylation signal AATAAA into the probably nonfunctional AACAAA sequence (position 3227 in Fig. 1; Tamm, J., A. Lee, R. Derynck, unpublished data), thus leading to the requirement to use a more downstream located polyadenylation signal in the gene. Comparison of the human and murine TGF-β2 cDNA sequences also shows that the 5′ and 3′-untranslated regions are clearly much less conserved than the highly similar coding sequences (data not shown).

The Murine TGF-β2 Precursor: Comparison with the Human Sequence

Alignment for homology between the murine and the human TGF-β2 precursor sequences (Fig. 2) shows that both are 414 amino acids long and that there is a high degree of sequence conservation. It has been established that the human and simian TGF-β2 precursor sequences are identical (18, 19, 21). On the basis of the experimentally determined N-terminus of porcine (14) and simian (21) TGF-β2 and of the homology with TGF-β1 (6, 18, 19, 21), it can be assumed that the mature murine TGF-β2 monomer corresponds to the C-terminal 112 amino acids of the precursor and is cleaved from the precursor after five basic residues (Figs. 1 and 2). There are only three amino acid differences between the human and murine mature TGF-β2 monomers. While this similarity emphasizes a high degree of conservation, it is striking that this is less conservation than in the case of TGF-β1 with only one conservative amino acid change between the mouse and human counterparts (37), and of TGF-β3 with an identical mouse and human sequence (unpublished data). It is likely that the N-terminal signal peptide of the mature TGF-β2 precursor is cleaved following residue 19, thus leaving a Leu-residue at the N-terminus of the precursor. This can be assumed on the basis of the experimentally determined N-terminus of the simian TGF-β1 precursor (7) and of the polypeptide sequence

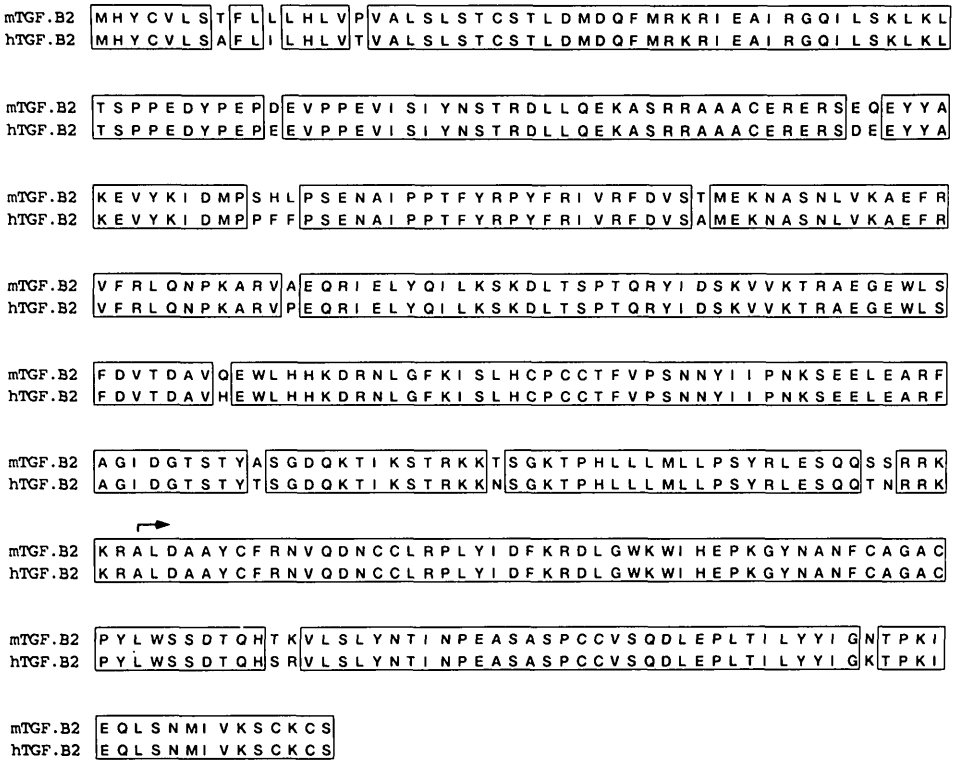

Fig. 2. Structural Similarity between the Murine (m) and Human (h) Amino Acid Sequences of the TGF-β2 Precursors
The human (19) and simian (21) TGF-β2 precursor sequences are identical. Identical residues are boxed. The N-termini of the mature TGF-β2 species start at the arrow.

homology in this region (Fig. 2). The precursor segment of the murine TGF-β2 sequence contains three potential N-linked glycosylation sites, which are also present in the corresponding human precursor (Fig. 1). No N-glycosylation sites are present in the mature TGF-β2 sequence nor in any other known mature TGF-β sequence. Sequence comparison between the human and murine TGF-β1 precursors revealed that there was a much lower degree of sequence conservation in the middle third of the precursor, compared to the N- and C-terminal thirds (37). Such area of relaxed homology is not present in the TGF-β2 precursors, which are very similar all over the polypeptide sequence.

TGF-β2 mRNA Expression During Fetal Development

The developmental expression of TGF-β2 in total mouse embryos was evaluated using Northern hybridization of polyadenylated mRNAs. RNAs were prepared from embryos between 10.5 and 17.5 days pc. The hybridization results revealed the expression of TGF-β2 mRNAs at all stages tested (Fig. 3). Five different transcript sizes were observed: 7.0 kilobases (kb), 6.0 kb, 5.0 kb, 4.0 kb, and 3.5 kb. The 7.0 kb, 6.0 kb, and 4.0 kb mRNAs increase in the mouse fetus during the developmental period studied, with the 6.0 kb mRNA species being the predominant species. The 5.0 kb TGF-β2 transcript gradually increases during development through day 15.5 day pc and then decreases by day 17.5. The 3.5 kb mRNA is present only in RNA from the 17.5 day fetus (Fig. 4). These results thus suggest a differential regulation for these different TGF-β2 mRNA species during fetal development. The nature of these different transcripts remains to be fully characterized. However, it is apparent from the results of the cDNA characterization, described above, and from the presence of multiple potential polyadenylation signals in the 3′-untranslated sequence (Fig. 1), that part of this heterogeneity is due to differential polyadenylation. In addition, there may be heterogeneity in the 5′-untranslated sequences, possibly due to use of different promoters. It has been reported that some heterogeneity exists for the TGF-β1 transcripts due to different transcriptional initiation sites (38). Alternative splicing of TGF-β2 mRNAs may also take place. It has been shown that the insertion of an additional exon occurs in one of the TGF-β2 mRNA species and may result in a longer TGF-β2 percursor segment (39). The differential regulation of the different mRNA species throughout embryonic development may be a reflection of the fact that these individual transcripts may have somewhat different functions or have differences in their stability.

TGF-β2 mRNA Expression in Different Tissues

TGF-β2 mRNA expression was also evaluated in different tissues and organs of adult mice (Fig. 4). All RNA

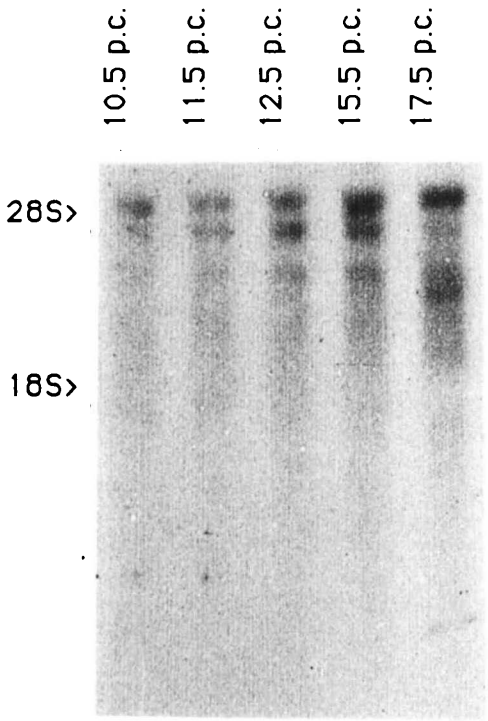

Fig. 3. Northern Blot Analysis of TGF-β2 mRNA Expression during Fetal Mouse Development

Five micrograms of polyadenylated RNA was electrophoresed per lane. The ages of the mouse fetuses from which the RNAs were isolated, are indicated above each lane. The positions of the 18S and 28S ribosomal RNAs are marked.

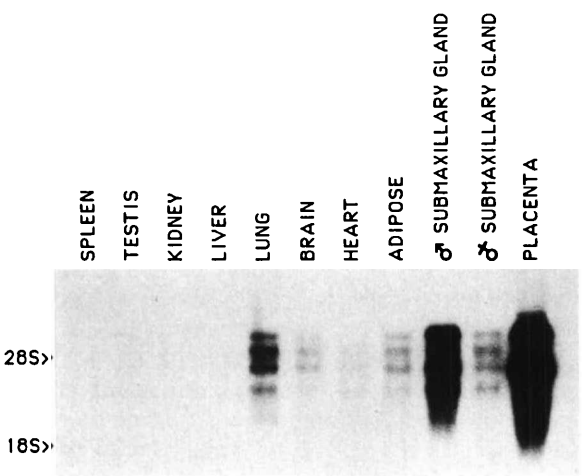

Fig. 4. Northern Blot Analysis of TGF-β2 mRNAs in Adult Mouse Tissues and Organs

Four micrograms of polyadenylated RNA from tissues and organs from CF-1 mice was electrophoresed in each lane. The RNA source is denoted above each lane. The positions of the 18S and 28S ribosomal RNAs are marked.

samples were from tissues from male CF-1 mice, except for the placenta and submaxillary gland RNAs isolated from female mice. Again, transcripts of different sizes were observed: 6.0 kb, 5.0 kb, 4.0 kb, and 3.5 kb. Thus, four of the mRNA species were identical as observed in the fetal RNAs (see above). These four RNA species have also previously been detected in several cell lines (18, 19, 22). The TGF-β2 mRNAs were detected in all tissues examined with the exception of liver, after a longer exposure of the Northern blot (data not shown). Clearly demonstrated in Fig. 4 is also that the level of TGF-β2 mRNA expression in the submaxillary gland is significantly higher in the male than in the female. This much higher level of TGF-β2 mRNA expression is similar to the results previously described for epidermal growth factor (40) and nerve growth factor (41) protein levels, substantiating a higher level of growth factor expression in the male submaxillary gland.

The studies presented here show the need for a more detailed analysis of the TGF-β2 gene expression. The potential differential regulation of each TGF-β2 mRNA species emphasizes the need to differentiate between these TGF-β2 transcripts, which will require the characterization of their structural differences. Once we are able to distinguish these various mRNA species individually, several questions can be addressed regarding their regulation of their transcription, mRNA stability and cell and tissue specific localization. The detailed localization during fetal development and in adult tissues will require histological analysis by *in situ* hybridization and immunohistochemistry.

MATERIALS AND METHODS

Isolation of mTGF-β2 cDNAs

Two λgt10 based cDNA libraries derived from the murine embryonic carcinoma cell line PCC3, induced for differentiation using retinoic acid for either 5 or 7 days, were obtained from Dr. F. Poirier (NIMR Mill Hill, London, England). The phage libraries were screened for the presence of murine TGF-β2 cDNAs using a 2.2 kbp *Eco*RI fragment of a human TGF-β2 cDNA (Tamm, J., A. Lee, and D. Derynck: unpublished data) as a ^{32}P-labeled (42) hybridization probe using standard procedures (43). The hybridizations of the nitrocellulose filters were carried out overnight using high stringency conditions (22). The subsequent washes were 0.5× SSC, 0.1% sodium dodecyl sulfate (SDS) at 42 C. Thirty four individual hybridizing recombinant phage were isolated.

Characterization of cDNAs

DNA was isolated from each recombinant hybridizing phage and characterized by dot blot hybridization using five different ^{32}P-end-labeled oligonucleotides. One of these (5′GGATCTAGGCTGGAAGTGGATCCACGAACCCAAGGGCTACAATGC - CAACTTCTG) corresponded to amino acids 335-352 in the human TGF-β2 precursor sequence (18, 19), a conserved sequence in the different mature TGF-β polypeptide sequences (22, 23). An other one (5′ATGCGCAAGAGGATCGAGGCGATCCGCGGGCAGATCCTGAGCAAGCTGAAGC - TCACCAGTCCCCCA) was specific for the precursor and corresponded to amino acid 33–54 in the human TGF-β2 precursor (18, 19), the most conserved region in all three TGF-β precursors (22, 23). Finally, three oligonucleotides were used that were highly specific for the precursors for either TGF-β1, -β2 or -β3, as determined by their polypeptide sequence comparisons (22). These oligonucleotides were: 5′GAGCCGTGGAGGGGAAATTGAGGGCTTTCGCCTTAGCGCC, corre-

sponding to amino acid 209-221 in the human TGF-β1 precursor (6), 5′CAGAAAACTATAAAGTCCACTAGGAAAAAAAA-CAGTGGGAAGACC, corresponding to residue 267-278 in the human TGF-β2 precursor sequence (18, 19), and 5′ATC-AAATTCAAAGGCGTGGACAATGAGGATGAC, which corresponds to residues 246-257 in the human TGF-β3 precursor (22). The nitrocellulose filters were hybridized with the radiolabeled probes in a 20% formamide hybridization buffer (6, 22) for 12–16 hr at 42 C and washed with 0.5× SSC, 0.1% SDS at 42 C. The individual mouse TGF-β2 cDNA inserts were subcloned into the unique *Eco*RI site of pUC119 and were sequenced by the dideoxy chain termination method.

RNA Preparation and Northern Hybridization

Mouse embryos were obtained from pregnant C57 Black/DBA mice at different stages of embryonic development. Noon of the day of plug was considered as 0.5 days pc. All extra-embryonic membranes were removed from the embryos. Tissues were obtained from 5- to 6-week-old CF-1 mice. Tissues and embryos were frozen in liquid nitrogen immediately after removal. RNA was extracted from tissues by the guanidinium thiocyanate method as described (42). Polyadenylated RNA was isolated by adsorption to oligo d(T)-cellulose (42). Northern hybridizations were carried out under high stringency conditions (6) using a ^{32}P-labeled (41) mTGF-β2 riboprobe, corresponding to nucleotides 1511-1953 (Fig. 1). Washes after the hybridizations were in 0.1× SSC, 0.1% SDS at 68 C.

Acknowledgments

We thank Dr. F. Poirier (NIMR Mill Hill, London, UK) for generously providing the cDNA libraries and Dr. B. Hogan (Vanderbilt University, Nashville, TN) for the fetal RNA samples.

Received March 17, 1989. Accepted April 18, 1989.

Address correspondence to: D. A. Miller, Department of Cell Biology, Vanderbilt School of Medicine, Nashville, Tennessee 37232.

This research was supported by NIH Grant CA-42572 (to D.A.M., H.L.M.) and NIH Grant T32-GM07347 (to R.W.P.).

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