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# Identification of a novel TGF-β-regulated gene encoding a putative zinc finger protein in human osteoblasts

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## ABSTRACT

**The TGF-β family of growth factors has been extensively studied and found to play major roles in bone physiology and disease. A novel, TGF-β-inducible early gene (TIEG) in normal human fetal osteoblasts (hFOB) has been identified using differential-display PCR. Using this differentially expressed cDNA fragment of TIEG to screen a hOB cDNA library, a near full-length cDNA for this gene was isolated. Northern analyses indicated that the steady-state levels of the 3.5 kb TIEG mRNA increased within 30 min of TGF-β treatment of human osteoblasts and reached a maximum of 10-fold above control levels at 120 min post-treatment. This regulation was independent of new protein synthesis. Computer sequence analyses indicates that TIEG mRNA encodes for a 480 amino-acid protein. The TIEG protein contains three zinc finger motifs, several proline-rich *src* homology-3 (SH3) binding domains at the C-terminal end, and is homologous in this region to the zinc finger-containing transcription factor family of genes. A growth factor/cytokine-specific induction of TIEG has been shown. TIEG expression in hFOB cells was highly induced by TGF-β and bone morphogenetic protein-2 (BMP-2), with a moderate induction by epidermal growth factor (EGF), but no induction by other growth factors/cytokines was observed. In addition to osteoblastic cells, high levels of TIEG expression were detected in skeletal muscle tissue, while low or no detectable levels were found in brain, lung, liver or kidney. Because TIEG is an early induced putative transcription factor gene, and shows a growth factor induction and tissue specificity, its protein product might play an important role as a signalling molecule in osteoblastic cells.**

## INTRODUCTION

Transforming growth factor-β (TGF-β) is thought to play an important role in human bone cell physiology (1). It is highly concentrated in bone, produced by both normal human bone-forming osteoblasts (hOB) and bone-resorbing osteoclasts (OC), has major effects on hOB and OC activities and is possibly involved in diseases of bone (1,2). The production and activation of TGF-β is regulated by estrogen, parathyroid hormone, glucocorticoids and other important bone regulatory agents including TGF-β itself (3–6). Moreover, it is possible that TGF-β plays a role in the coupling between osteoblasts and osteoclasts. TGF-β is known to have multiple effects on bone-specific genes in cultured osteoblastic cells. TGF-β has been shown to increase type I collagen, osteopontin and alkaline phosphatase synthesis (7–10). Furthermore, TGF-β decreases osteocalcin synthesis in osteoblasts in culture. The regulation of most of these genes by TGF-β has been measured after 18–24 h of growth factor treatment. To understand the early mechanism of action of this growth factor in osteoblasts, we have recently shown that TGF-β induces *c-fos* and *jun-B* mRNA levels within 2 h of growth factor treatment (6). To further examine the molecular mechanism of action of this growth factor on OB growth and differentiation, we have applied the differential-display PCR technique described by Liang and Pardee (11) and Liang *et al.* (12). We compared the complimentary DNA fragments from differentially expressed human fetal osteoblastic cell (hFOB) mRNAs following a 60 min treatment with either vehicle, or $10^{-8}$ M TGF-$\beta_1$. This analysis resulted in the identification of a novel TGF-β-inducible gene which is described in this paper.

## METHODS

### Cell culture and Northern analysis

The new immortalized human fetal osteoblastic (hFOB) cell line, hFOB 1.19, was recently developed and described previously (13). The hFOB cells were routinely grown in DMEM:F12 (1:1) with 10% FBS serum-containing media. At the time of the experiment the cells were plated onto 100 mm culture dishes and allowed to grow to near confluency. At this time, the cells were washed twice with serum-free media and incubated in 10 ml of 1% serum-containing media for 48 h. The serum-starved cells were treated with TGF-$\beta_1$ (2 ng/ml) for different time periods and the cells were processed for a total RNA isolation using the guanidinium/caesium chloride method. Total RNA (8–15 μg) was used for Northern analysis. The Northern blots were probed

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with $^{32}$P-labelled TIEG cDNA. Primary cultures of normal adult human osteoblasts (hOB) were grown in culture as mentioned above and the cells were also treated with TGF-$\beta_1$ for various time periods and the Northern analysis was performed as mentioned above.

### Differential display PCR

Differential display PCR was performed following the method described earlier by Liang and Pardee and Liang *et al.* (11,12). Mainly, the protocol described in GenHunter kit (Brookline, MA) for differential display PCR was followed. Total RNA was isolated from hFOB 1.19 that were treated with either vehicle (0.25% BSA in PBS) or $10^{-8}$ M TGF-$\beta_1$ (60 min) using the guanidinium isothiocyanate and caesium chloride method (14). The total RNA was treated with RNase-free DNase to remove any DNA contamination. DNA free RNA (0.2 µg) from control and TGF-$\beta_1$-treated cells was used as a template for first strand cDNA synthesis in the presence of 10 µM $T_{12}$ MG, T12 MC, T12 MA and T12 MT primers, MMLV-reverse transcriptase, reverse transcriptase buffer and 250 µM dNTP mix. The synthesized first strand cDNA was used as a template in the next PCR reaction. In a 0.5 ml microfuge tube the following were added: 2 µl of 10× PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl, pH 8.3), dNTP mix (25 µM), 5′-random primer (2 µM), $T_{12}$ MN mix (same as used in cDNA synthesis), 1 µCi of dCTP (3000 Ci/mmol), 2 µl of template cDNA and 1 U of *Taq* DNA polymerase (Perkin Elmer). PCR was performed as follows: 94°C, 30 s; 40°C, 2 min; 72°C, 30 s for 40 cycles. After the PCR was done 6 µl of the sample was run on a 6% urea:acrylamide sequencing gel. The dried gel was exposed to an X-ray film and the autoradiogram was analyzed for the differentially expressed genes. After identifying the band of interest from the autoradiogram, the gel was superimposed over the autoradiogram and the band was cut out. The DNA was eluted from the gel by soaking the gel in 100 µl of TE buffer for 10 min and then boiling for 10 min. The DNA was precipitated using glycogen and ethanol. The precipitated DNA was dissolved in a small volume of $dH_2O$. A portion of this DNA was used as a template in the second PCR along with the same 5′- and 3′-primers used in the first PCR. The amplified DNA obtained was analyzed in a 1.5% agarose gel. Once the DNA was found to be pure without any other contaminating bands, the DNA was purified to remove the dNTP and the protein and this was used as a probe in Northern analyses and cDNA library screening.

### cDNA library screening

To obtain the full-length cDNA, the hOB cDNA library was screened using the 350 bp of the 3′-end of TIEG. The hOB cDNA library was kindly provided by Dr Marian Young at NIH. The cDNA library was constructed using poly-A$^+$ RNA obtained from a 55-year-old female who had hip replacement surgery. Using the 350 bp DNA as a probe, ~200 000–400 000 plaques were screened. In the primary screen, three positive clones were obtained. The three clones were plated for secondary screen, but only one seemed to be a potential positive clone. The tertiary screen confirmed this, since it was 100% positive. Since the cDNA library was constructed in a lambda ZAPII vector, the *in vivo* excision of pBluescript plasmid was obtained from lambda ZAPII vector. The pBluescript plasmid was digested with *Eco*RI restriction enzyme to liberate the cDNA insert from the vector and a 2.9 kb insert was obtained. Both strands of 2.9 kb cDNA were then completely sequenced using fmol PCR sequencing kit and also by an automated sequencer of our institutional molecular biology core facility. There was perfect homology between the 350 bp ddPCR DNA fragment and the 3′-end of the cDNA. The 2.9 kb DNA sequence was analyzed for homologies with known genes in the GenBank using the University of Wisconsin GCG Program Fast A.

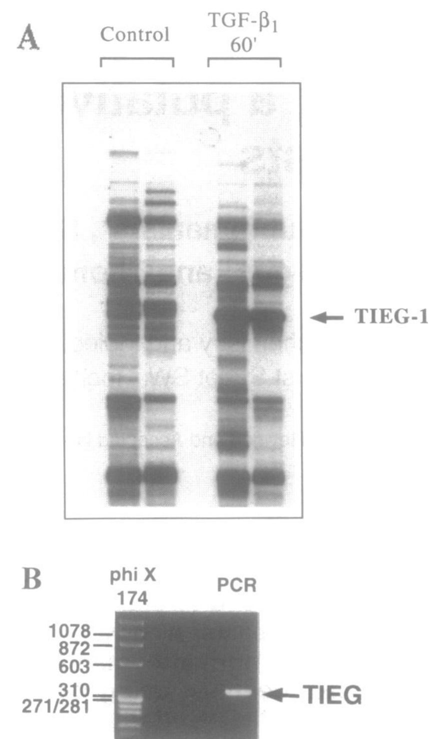



**Figure 1.** Differential display PCR analysis. (**A**) Near confluent human fetal osteoblastic (hFOB) cells were serum-starved with DMEM + F12 media containing 1% FBS for 48 h. The serum-starved cells were stimulated with TGF-$\beta_1$ (2 ng/ml) for 60 min and the control cells were treated with 0.25% BSA in PBS for 60 min. Total RNA was isolated from control and treated cells. The RNA was treated with DNase to remove any DNA contamination and 0.2 µg was used in cDNA synthesis in the presence of T12 MN primer mix and MMLV-reverse transcriptase. A portion of the cDNA in the presence of T12 MN mix (as a 3′-primer), 5′-CCTGTAATCC-3′ (as a 5′-primer), [$^{32}$P]dCTP and dNTP mix was PCR amplified and the products were analyzed in a 6% urea-acrylamide gel. Samples from each lane were obtained from independent PCR reactions from different RNA samples. The gel was dried and exposed to X-ray film. (**B**) The differentially displayed cDNA was PCR amplified and 500 ng of the DNA was separated on a 1.5% agarose gel along with DNA size markers.

## RESULTS

When differential-display PCR was performed from hFOB cell RNA, a 350 bp cDNA fragment (3′-end of the gene) was highly expressed in hFOB cells which were treated with TGF-β for 60 min compared to control (untreated) cells (Fig. 1). The cDNA fragment was cut and purified from the sequencing gel and used as a probe for Northern analyses. RNA was isolated from hFOB cells, treated with TGF-$\beta_1$ for various time periods, as fractionated on glyoxal–agarose gels, and probed with the TIEG cDNA.

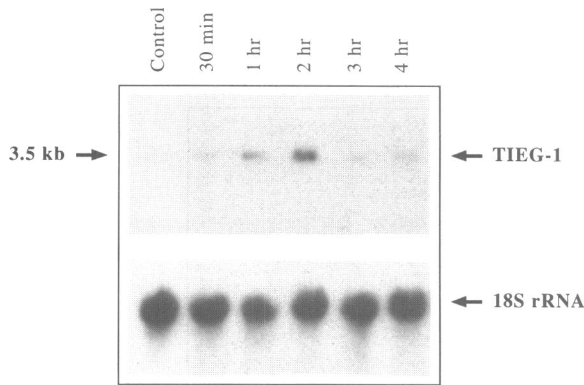
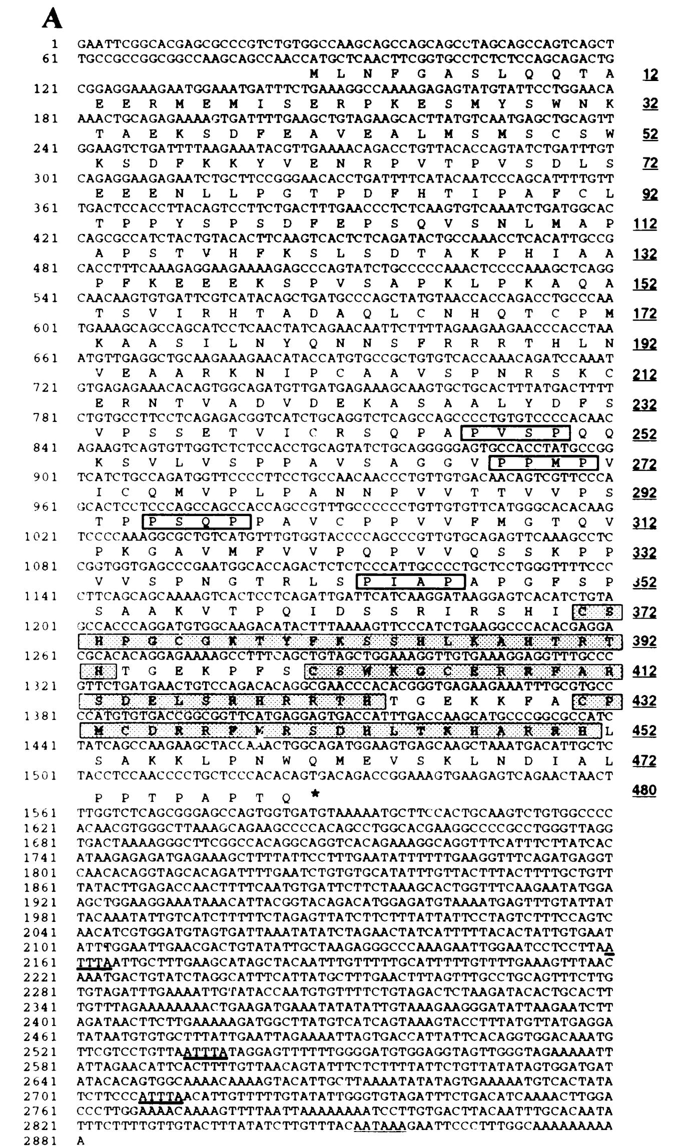



**Figure 2.** Northern analyses of TIEG in hFOB cells. The hFOB cells were serum-starved in 1% serum containing media for 48 h. The serum-starved cells were treated with TGF-$\beta_1$ for various time periods as shown on top of each lane. Total RNA was isolated and 15 µg of RNA from each time point was used for Northern analysis. The differentially displayed cDNA from TGF-$\beta_1$ treated cells (TIEG-1) was used as a probe for the Northern blot.

The differentially-expressed TIEG mRNA was minimally detectable in control (vehicle treated) cells, but a rapid and transient increase was observed at 30 min of TGF-$\beta_1$ treatment. There was a maximal (>10-fold above control) level of expression measured at 2 h post-treatment. The steady-state levels of this 3.5 kb mRNA returned to control levels following 3 h of TGF-$\beta_1$ treatment (Fig. 2). To obtain the full-length cDNA, a normal human osteoblast-like cell cDNA library was screened using the 350 bp of the 3′-end of TIEG. The library screening resulted in the isolation of a 2.9 kb cDNA encoding a 480 amino-acid protein, and having a long 3′-untranslated region. The cDNA sequence and predicted amino acid sequences are shown in Figure 3A. The highly conserved zinc finger motifs and other amino acids are shown in Figure 3B.

To determine whether the induction of TIEG by TGF-β is dependent on new protein synthesis, hFOB cells were treated with TGF-$\beta_1$ together with the protein synthesis inhibitor, cycloheximide (10 µg/ml for 2 h), and Northern analyses were performed using the labelled full-length cDNA. A super-induction of TIEG mRNA was observed at 2 h of treatment (Fig. 4A). This strongly suggests that the increase in TIEG mRNA by TGF-β treatment is a primary response and is independent of new protein synthesis, but may be under the negative control of a protein repressor. It is also possible that cycloheximide inhibits the nucleases that are involved in degradation of TIEG mRNA. This possibility is supported by the fact that TIEG mRNA contains three AUUUA motifs in 3′-untranslated region of TIEG, which are found in *c-fos* and *c-myc* nuclear proto-oncogene mRNAs and are signals for rapid degradation (15). As shown in Figure 4A, the TIEG mRNA returns to near control levels by 3–4 h of TGF-β post-treatment.



**B**

| | 371 … 447 |
|---|---|
| TIEG | CSHPGCGKTYFKSSHLKAHTRTHTGEKPFSCSWKGCERRFARSDELSRHRRTHTGEKKFACPMCDRRFMRSDHLTKH |
| SPR-2 | CHIPGCGKVYGKTSHLRAHLRWHSGERPFVCNWMYCGKRFTRSDELQRHRRTHTGEKKFVCPECSKRFMRSDHLAKH |
| Sp3 | CHIPGCGKVYGKTSHLRAHLRWHSGERPFVCNWMYCGKRFTRSDELQRHRRTHTGEKKFVCPECSKRFMRSDHLAKH |
| SPR-1 | CHIEGCGKVYGKTSHLRAHLRWHTGERPFICNWMFCGKRFTRSDELQRHRRTHTGEKRFECPECSKRFMRSDHLSKH |
| Sp1 | CHIQGCGKVYGKTSHLRAHLRWHTGERPFMCTWSYCGKRFTRSDELQRHKRTHTGEKKFACPECPKRFMRSDHLSKH |
| BTEB | CDYPGCTKVYTKSSHLKAHLRTHTGEKPYKCTWEGCDWRFARSDELTRHYRKHTGAKPFQCGVCNRSFSRSDHLALH |
| MUS Krp | CGHEGCGKSYSKSSHLKAHLRTHTGEKPYACSWDGCDWRFARSDELTRHYRKHTGHRPFCCGLCPRAFSRSDHLALH |
| Sp2 | CHIPDCGKTFRKTSLLRAHVRLHTGERPFVCNWFFCGKRFTRSDELQRHARTHTGDKRFECAQCQKRFMRSDHLTKH |
| WT-1 | CAYPGCNKRYFKLSHLQMHSRKHTGEKPYQCDFKDCERRFSRSDQLKRHQRRHTGVKPFQCKTCQRKFSRSDHLKTH |

**Figure 3.** cDNA sequence of TIEG. (**A**) Nucleotide and deduced amino-acid sequence of TIEG cDNA. The M denotes the translational start site and the stop codon is shown with an asterisk. The shaded amino-acids show the three zinc-finger motifs. The proline-rich region *src* homology-3 (SH3) binding domains are shown in boxes. The underlined nucleotides indicate the polyadenylation signal sequence and AUUUA motifs. The numbers on the left denote the nucleotides and the numbers on the right refers to the amino-acid residues. (**B**) A diagram showing the homology of the zinc-finger region of the TIEG protein to other members of this family. The conserved cysteine and histidine in the zinc-finger motif are shown in bold letters. The highly conserved amino-acids in TIEG and other gene family are underlined. The numbers denote the amino-acid positions of TIEG. TIEG, TGF-β inducible early gene; SPR-2, human GT box binding protein; Sp3, human Sp3 protein; SPR-1, human GT box binding protein; Sp1, human transcription factor Sp1; BTEB, human GC-box binding protein; MUSKrp, mouse erythroid Krueppel-like transcription factor; Sp2, human Sp2 protein; WT-1, human Wilm's tumor zinc-finger protein.

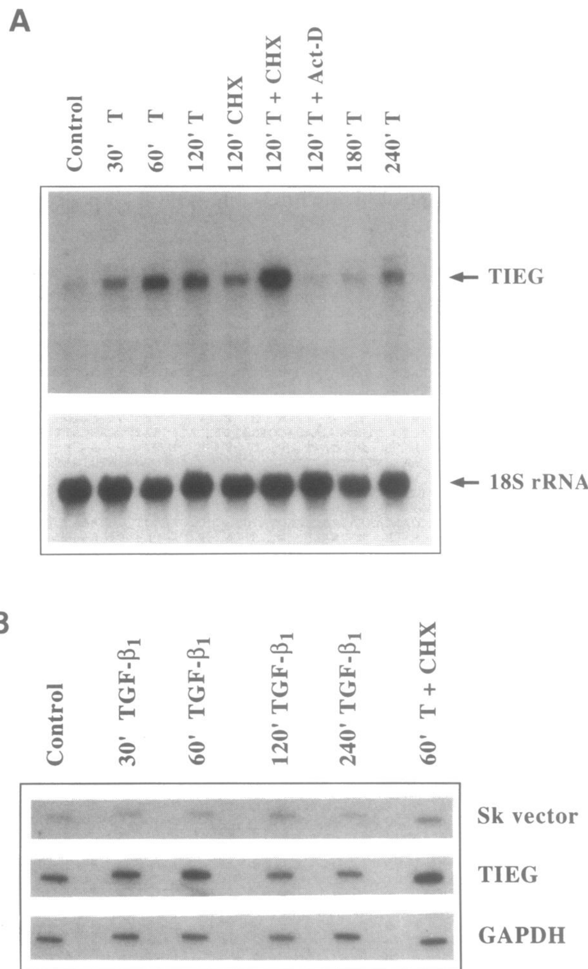



**Figure 4.** Northern analyses of TIEG mRNA levels after TGF-$\beta_1$, cycloheximide and actinomycin-D treatment and transcriptional regulation of TIEG in hFOB cells. (**A**) Serum-starved confluent hFOB cells were treated with TGF-$\beta_1$ (2 ng/ml), cycloheximide (10 μg/ml) and actinomycin-D (1 μg/ml) for various time periods as indicated on top of each lane. The control cells were treated with 0.25% BSA in PBS for 2 h. Total RNA was isolated from control and treated and 8 μg of the RNA was used for Northern analyses. The blots were probed for TIEG mRNA and 18S rRNA. (**B**) Serum-starved hFOB cells were treated with TGF-$\beta_1$ (2 ng/ml) for various time periods, nuclei were isolated and transcripts were elongated *in vitro* in the presence of [$^{32}$P]UTP. The labelled transcripts were hybridized to the slot-blotted DNA and processed for autoradiography.

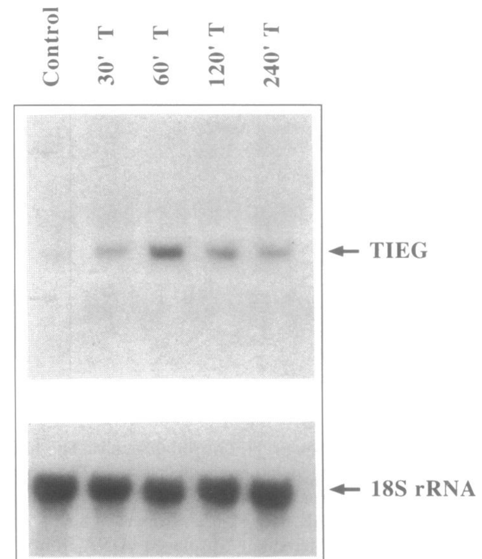



**Figure 5.** Northern analyses of TIEG mRNA in adult human osteoblast-like cells. Confluent adult human osteoblast-like (hOB) cells were serum-starved for 48 h and treated with TGF-$\beta_1$ (2 ng/ml) for various time periods as shown on top of each lane. Total RNA was isolated from control (vehicle treated for 2 h) and TGF-treated cells, and 8 μg of the RNA was used for Northern analyses. The blots were probed for TIEG and 18S rRNA.

When the RNA synthesis inhibitor, actinomycin-D (1 μg/ml), was included with the TGF-β treatments during this same interval, the increase in TIEG mRNA was totally abolished (Fig. 4A). These results suggest that the induction of TIEG mRNA by TGF-β is mediated, at least in part, at the level of transcription. In fact, nuclear run-on transcriptional analysis indicate that the TGF-β regulation of TIEG expression occurs at the level of transcription (Fig. 4B).

To determine whether TIEG expression is regulated by TGF-β in normal adult osteoblastic cells, hOB cells were treated for various time periods and Northern analyses performed in the RNA. These data show that a progressive increase in TIEG mRNA is observed with a maximal increase at 1–2 h of TGF-β treatment (Fig. 5).

To determine whether other members of TGF-β superfamily have any effect on TIEG mRNA levels, hFOB cells were treated with bone morphogenetic protein-2 (BMP-2) at a concentration of 100 ng/ml for various time periods, and analyzed by Northern analysis. Figure 6 shows that the TIEG mRNA levels increased at 1–2 h of BMP-2 treatment. These data indicate that the other members of TGF-β family also regulate the expression of TIEG in these cells.

To determine if the induction of TIEG mRNA in hFOB cells is specific to the TGF-β family of growth factors, cells were treated with different growth factors and cytokines, such as EGF, PDGF, IGFs, FGF, TNF-α, IL-6, and IL-1β. The results of this study are shown in Figure 7A. It is evident that TGF-β is the major inducer of TIEG mRNA levels with a lesser effect by EGF, while the remaining growth factors/cytokines had a minimal effect on TIEG expression.

To investigate whether TIEG expression is specific to osteoblasts or is expressed in other human tissues, multi-tissue Northern analysis was performed. The Northern blot contained an equal amount of poly-A$^+$ RNA from different human tissues which was normalized to β-actin mRNA levels. The blot was probed with TIEG cDNA, and the results are shown in Figure 7B. Skeletal muscle showed the highest level of TIEG mRNA relative to β-actin. Heart, placenta and pancreas had moderate amounts of TIEG mRNA, while liver tissue showed minimal expression of this gene. Other tissues, e.g., brain, lung and kidney had no detectable levels of the TIEG mRNA. As expected, both heart and skeletal muscle showed expression of α-actin mRNA in addition to β-actin. The above results demonstrate a partial tissue specificity of TIEG expression.

## DISCUSSION

We have identified a novel TGF-β-inducible early gene in human osteoblasts. The regulation of this gene occurs as early as 30 min

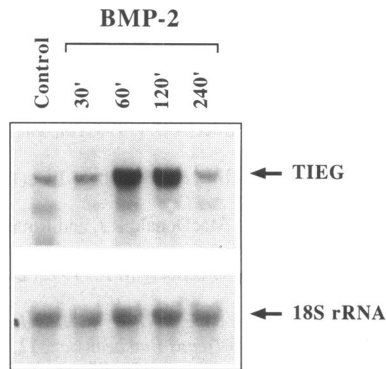



**Figure 6.** Northern analyses of TIEG mRNA levels after BMP-2 treatment. Confluent hFOB cells were serum-starved for 48 h and treated with BMP-2 (100 ng/ml) for various time periods as shown on top of each lane. Total RNA was isolated from control (vehicle treated for 2 h) and BMP-2-treated cells and 8 μg of the RNA was analyzed for Northern analyses. The blots were probed for TIEG mRNA and 18S rRNA.

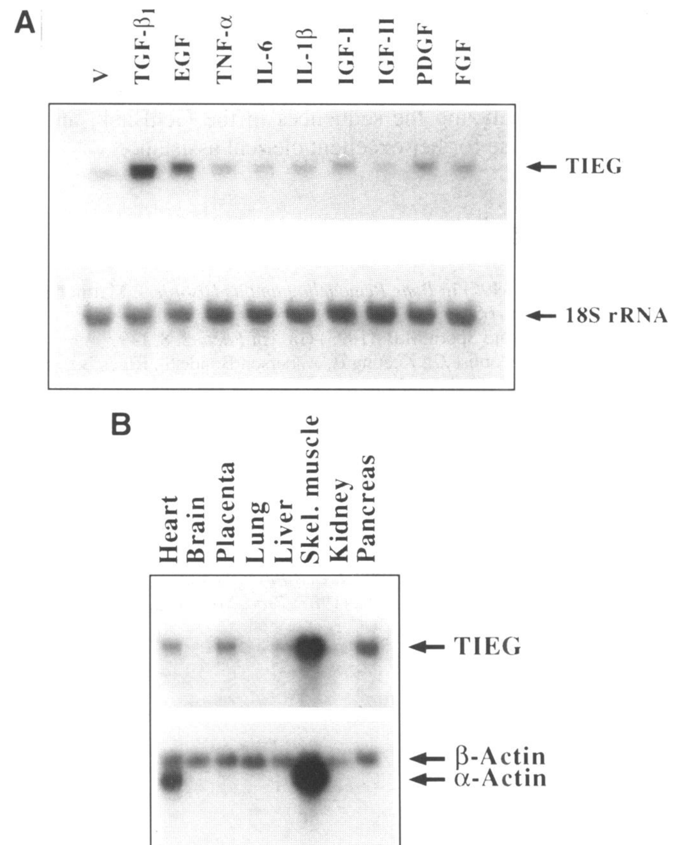



**Figure 7.** Growth factor and tissue specificity of TIEG expression.(**A**) Serum-starved confluent hFOB cells were treated with different growth factors: TGF-$\beta_1$ (2 ng/ml), EGF (20 ng/ml), TNF-α (10 U/ml), IL-6 (10 ng/ml), IL-1β (10 U/ml), IGF-1 (3.5 ng/ml), IGF-II (3.5 ng/ml), PDGF (5 ng/ml), and FGF (10 ng/ml) for 90 min. Total RNA was isolated from vehicle (V-treated with 0.25% BSA in PBS for 90 min) and growth factor-treated cells and 10 μg of the total RNA was used for Northern analyses. The blots were probed for TIEG mRNA and 18S rRNA. (**B**) Multi-tissue Northern blot containing 2 μg of poly-$A^+$ (purchased from Clontech, Palo Alto, CA) isolated from the indicated tissues. The blot was probed with TIEG cDNA and human β-actin cDNA.

of TGF-β treatment and is independent of new protein synthesis. We have demonstrated that the induction of TIEG mRNA in hFOB cells were restricted to TGF-β and EGF, whereas other growth factors had a minimal effect. TIEG mRNA levels in hFOB cells were induced by BMP-2, a member of TGF-β superfamily. In addition, TIEG mRNA levels show a tissue specificity with osteoblast and muscle cells displaying the highest levels. Since these cells arise from common progenitor cells in marrow stroma, it is possible this factor is involved in this lineage family. These observations combined with the fact that TGF-β and BMP-2 are involved in osteoblast growth and differentiation (1), the induction of TIEG by these factors may allude to the involvement of TIEG in osteoblast function or the function of cells with the osteoblast lineage. Thus, it is possible that this early regulated gene may represent a key regulatory factor involved in the action of the general TGF-β family members on target cells.

The cDNA sequence analyses of TIEG indicated that this gene contains three zinc finger motifs at the C-terminal region of the protein. This region of the protein is homologous to zinc finger-containing transcription factors like Sp1, Sp3, Wilm's tumor protein, GT box binding protein and other zinc finger proteins. In contrast, the N-terminal region of the protein was found to be unique showing no homology to any genes in the GenBank. The zinc finger-containing domains were first identified in *Xenopus* transcription factor TFIIIA (16) and are known to bind nucleic acid (reviewed in 17,18). Similarly, Lemaire *et al.* (19) have isolated a mouse Krox-24 gene which encodes a zinc finger-containing, transcription factor-like gene which is regulated by serum and purified growth factors similar to that of TIEG. Further, Galera *et al.* (20) have reported another zinc finger protein gene, *c-krox*, which is highly expressed in the skin and is an important regulator of type I collagen. Because TIEG has homology with other transcription factor genes only in the zinc finger region, these genes might have evolved from a common ancestral gene and then diversified differently depending on the functional demands.

The transcription factor CTF/NF-1 is known to contain proline-rich domains in the C-terminal region which are responsible for transcriptional activating function (21). Interestingly, the C-terminal region of TIEG contains a similar proline-rich region (24% proline residues within 125 amino acids) analogous to that of CTF/NF-1 transcription factors. Proline-rich regions are known to bind Src homology-3 (SH3) domains (22). Yu *et al.* (23) have reported a proline motif of PXXP that can bind SH3 domains are highly conserved among numerous genes. The C-terminal region of TIEG gene contains four PXXP motifs. These proline motifs in TIEG protein may associate with SH3 domains of *src* tyrosine kinases which may be involved in the signal transduction processes.

Because the TIEG gene is rapidly, but transiently, induced in osteoblastic cells following TGF-β treatment, it may be an important early signalling molecule for TGF-β . Therefore, it is possible that the TIEG gene represents a key regulatory gene which mediates the effects of TGF-β on target cell (i.e., osteoblast) growth and function.

## ACKNOWLEDGEMENTS

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