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Analysis of Activin A Gene Expression in Human Bone Marrow Stromal Cells

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Abstract Activin A, a member of the TGF-β superfamily, plays roles in differentiation and development, including hematopoiesis. Our previous studies indicated that the expression of activin A by human bone marrow cells and monocytes is highly regulated by inflammatory cytokines and glucocorticoids. The present study was undertaken to investigate the regulation of activin A gene expression in the human bone marrow stromal cell lines L87/4 and HS-5, as well as in primary stromal cells. Northern blots demonstrated that, like primary stromal cells, the cell lines expressed four activin A RNA transcripts (6.4, 4.0, 2.8, and 1.6 kb), although distribution of the RNA among the four sizes varied. The locations of the 5′ ends of the RNAs were investigated by Northern blots and RNase protection assays. The results identified a transcription start site at 212 nucleotides upstream of the translation start codon. In addition, luciferase expression assays of a series of deletion constructs were used to identify regulatory sequences upstream of the activin A gene. A 58 bp upstream sequence exhibits promoter activity. However, severalfold higher expression requires a positive element consisting of an additional 71 bp of the upstream region. Promoter activity was also identified between 2.5 and 3.6 kb upstream of the start codon. These findings suggest that expression of activin A at the transcriptional level follows complex patterns of regulation. J. Cell. Biochem. 70:8-21, 1998. © 1998 Wiley-Liss, Inc.

Key words: activin A; bone marrow stromal cells; gene regulation; promoter activity

Activin A is a cytokine within the transforming growth factor-β (TGF-β) superfamily [see Vale et al., 1990, for review]. It is composed of two β_A subunits; the β_A subunit also constitutes a component of inhibin A. Activin A is expressed in many tissues and has diverse functions. It stimulates follicle-stimulating hormone production from the anterior pituitary [Ling et al., 1986; Vale et al., 1986], induces differentiation of ovarian granulosa cells [Sugino et al., 1988], slows proliferation of plasmacytoma cells [Brosh et al., 1995] and hepatocytes [Yasuda et al., 1993], inhibits neuronal differentiation [Hashimoto et al., 1990], and supports the survival of neuronal cells [Schubert et al., 1990]. Actions of activin A in hematopoiesis include stimulation of erythropoiesis [Broxmeyer et al., 1988; Eto et al., 1987; Yu et al., 1987], expression in response to inflammatory cytokines [Shao et al., 1992; Takahashi et al., 1992; Uchimaru et al., 1995], and suppression of the effects of some inflammatory cytokines [Brosh et al., 1995; Yu et al., 1997; reviewed in Yu and Dolter, 1997].

Although the functions of activin A have been studied extensively, little is known about the regulation of activin A gene expression. An understanding of activin A expression will be important for understanding the multiple autocrine and paracrine effects of activin on cell growth and differentiation, and hence its potential as an anti-inflammatory and/or anticancer agent. Previously, activin A expression was detected in human monocytes and primary bone marrow stromal cells [Shao et al., 1992] and in murine bone marrow cell lines [Yamashita et al., 1992]. Granulocyte-macrophage colony-stimulating factor, lipopolysaccharide (LPS), and interferon γ stimulated activin A expression in monocytes, while interleukin 1 (IL-1), tumor necrosis factor α (TNF-α), and LPS stimulated its expression in marrow stromal cells [Shao et al., 1992]. In addition, 12-O-tetradecanoyl phorbol-13-acetate (TPA) stimulated activin A expression, whereas glucocorticoids in-

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hibited expression in bone marrow stromal cells [Shao et al., 1998] and in monocytes [Yu et al., 1996]. Stimulation of bone marrow stromal cells by TNF-α resulted in an increase in the rate of activin A gene transcription and had no effect on stability of the mRNA [Shao et al., 1998]. However, the molecular basis for the regulation of activin A transcription has not been elucidated. In the present study, we have investigated the expression of activin A in human bone marrow stromal cells in order to identify a putative promoter region and transcription start sites.

MATERIALS AND METHODS

Cells

Human primary bone marrow stromal cells were obtained as described previously [Shao et al., 1992]. Briefly, bone marrow was collected from nonleukemic patients who underwent diagnostic bone marrow aspiration. Bone marrow was centrifuged at 200*g* for 4 min, and the buffy coat was then diluted with HCC-5100 myeloid long-term bone marrow culture medium (Terry Fox Lab, Vancouver, BC) to 2–3 $\times$ 10^7 cells per 8 ml. These cells were cultured for 2–3 weeks, with weekly feeding, and then subcultured for use in experiments. Human stromal cell lines L87/4 [Thalmeier et al., 1994] and HS-5 [Roecklein and Torok-Storb, 1995] were provided by Karin Thalmeier and Beverly Torok-Storb, respectively. L87/4 cells were cultured in long-term bone marrow culture medium, and HS-5 cells were cultured in RPMI containing 10% fetal calf serum (FCS). HT1080 human fibrosarcoma cells (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's minimum essential medium (DMEM) containing 10% FCS.

Immunocytochemical Analysis

Marrow stromal cells were subcultured in chamber slides before fixation in methanol at 0°C for 10 min. Cells were then preincubated in a blocking solution of phosphate-buffered saline, pH 7.4, containing 1.5% normal goat serum for 30 min at room temperature. The cytochemical immunoassay was performed by incubating the slides with affinity-purified anti-β_A antiserum, which was raised in rabbits immunized with conjugated β_A peptide (amino acids 81–113) [Vaughan et al., 1989]. The slides were incubated with antiserum diluted 1:2,000 in blocking solution at room temperature for 2 h. For a control, the antiserum was preadsorbed with the synthetic peptide β_A (81-113) for 30 min before incubation with the slides. After incubation, the slides were washed for 10 min in buffer and processed by the avidin–biotin procedure with tetranitroblue tetrazolium as the substrate [Robb, 1983]. Afterward, the slides were washed for 5 min and counterstained with 0.25% metanil yellow and 0.1% Nuclear Fast Red.

Plasmids

phβ_A4.5 [Petraglia et al., 1990] was used as a source of genomic activin A upstream sequences (a gift of Hélène Meunier, University of Toronto, Ontario, Canada). Subclones of this plasmid were inserted into a luciferase expression plasmid, p19Δluc (a gift from Alan McLachlan, The Scripps Research Institute, La Jolla, CA) [Raney et al., 1990]. pRKβ_A (a gift from Irene Smith, Genentech, South San Francisco, CA) contains the cDNA coding sequence of activin A and was used for generating probes for Northern hybridization. Plasmids were prepared by alkaline lysis, followed by purification through a CsCl gradient or an anion-exchange column (Qiagen, Santa Clarita, CA).

Oligonucleotides

Oligonucleotides were synthesized by the DNA Core Laboratory at The Scripps Research Institute. The following oligonucleotides were used for polymerase chain reaction (PCR) or DNA sequencing: F1, GACTAAGCTTGACTGTAATGCTATGAAC; R1, TTGGTACCTTTTCGGGGGTTCTACTTTC; F2, GAGTAAGCTTGGTGATATTTGAAGAGAGG; R2, GCAGGTACCTGGCAGCAAAAGTTGTTG; F3, TGCGGATATCCATTCACTCACTTACTTAG; R3, TATGGTACCTTGTTTGCAGGTTCCTCTC; R4, AAGAGAGAAGGAAGGAGGGA; J1, GGACAAGCTTCGGACTTCACAGATAAAATGC; J2, GATCAAGCTTCAAGTCATACGAGTGC; J3, TGTAAAGCTTAGTGTCTCAATCAATAGG; J4, CATCAAGCTTTTCGGGGGTTCTACTTTC; R5, TGTCAGGGAGGAGAGTTC. 1S, TGATTCCAATGTTTTTCTA and PR3PX, CTTTCTGGTCCCCACT [Tanimoto et al., 1996].

Polymerase Chain Reaction

PCR was performed using the GeneAmp PCR System 9600 (Perkin Elmer Cetus, Foster City, CA) with 2.5 units *Taq* DNA polymerase, approximately 0.1 nmol of each of two primers

and approximately 50 ng of *Sma*I-linearized phβ$_A$4.5 DNA in a total reaction volume of 100 μl containing 1× reaction buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$) and 200 μM dNTPs. Amplification was carried out with an initial incubation at 94°C for 1 min, followed by 25 cycles consisting of 1 min at 94°C, 1 min at 48°–55°C and 1 min at 72°C, with a final incubation at 72°C for 7 min.

Cloning

phβ$_A$4.5 was used as the source of activin A upstream sequences through both restriction digests and PCR (Fig. 1). phβ$_A$2.5 was derived from phβ$_A$4.5 after digestion with *Bam*HI (which also cuts within the multiple cloning site of the vector), gel purification and religation (Fig. 1). Primers F1 and R1 were used to generate a 1.1 kb fragment by PCR containing *Hin*dIII and *Kpn*I sites at the ends (Fig. 1). This fragment was ligated to *Hin*dIII–*Kpn*I-digested p19Δluc to create pluc1.1 (see Fig. 7A) and into pBSSK+ (Stratagene, La Jolla, CA) to create pBS1.1. Similarly, pluc0.7 was created by ligating a *Hin*dIII–*Kpn*I-flanked PCR fragment generated using primers F2 and R2 to p19Δluc (Figs. 1, 7A). PCR using primers F1 plus J4, F1 plus J1, and J2 plus J3 was performed to generate 1.1 kb, 511 bp, and 564 bp fragments, respectively, with *Hin*dIII sites at their ends for ligation into the *Hin*dIII site of pluc0.7 to construct plasmids pluc1.1/0.7, pluc511/0.7, and pluc564/0.7, respectively (Figs. 1, 7A). The orientations of the *Hin*dIII fragments in these clones were confirmed by restriction mapping. The 340 bp *Bam*HI–*Kpn*I fragment from pluc1.1 was treated with T4 DNA polymerase to blunt the ends and ligated to pluc0.7 cut with *Hin*dIII and blunted with Klenow to create pluc340/0.7 (Figs. 1, 7A). In addition, pluc1.1up contains the *Eco*RI–*Sac*I far upstream fragment from phβ$_A$4.5 (Fig. 1). This 1.1 kb fragment was first ligated into the *Eco*RI–*Sac*I sites of pBSSK+ to construct pBS1.1up. The fragment was then cut from this plasmid with *Hin*dIII and *Sac*I and ligated into p19Δluc to create pluc1.1up.

Five luciferase construct subclones (Figs. 1, 8A) and two Bluescript construct subclones of pluc0.7 were made as follows. A 486 bp *Hae*III fragment from pluc 0.7 was ligated to the *Sma*I site of p19Δluc to create pluc486 (see Fig. 8A). pluc0.7 was digested with *Bsm*I, blunted with Klenow and digested with *Hin*dIII to generate a 468 bp fragment that was ligated to the *Hin*dIII–*Sma*I sites of p19Δluc to generate pluc468 (see Fig. 8A). PCR using primers F3 and R3 generated a 370 bp fragment with *Eco*RV–*Kpn*I ends (Fig. 1). After digestion, this fragment was ligated to the *Sma*I–*Kpn*I sites of p19Δluc to produce pluc370 (see Fig. 8A) and to the *Eco*RV–*Kpn*I sites of pBSSK+ to produce pBS370. A 324 bp fragment was cut from pluc0.7 with *Hae*III and *Bsm*I, blunted with Klenow and ligated into the *Sma*I site of p19Δluc to create pluc324 (see Fig. 8A). Primers F3 and R4 were used in a PCR to produce a 288 bp fragment that was ligated into the *Sma*I site of p19Δluc to generate pluc288 (see Fig. 8A). pBS200 contains the 200 bp *Ear*I fragment in the *Sma*I site of pBSSK+ (Fig. 1).

Deletion subclones of pluc468 were constructed using exonuclease BAL31 (Fig. 8A). pluc468 was linearized with *Hin*dIII and then digested with BAL31 for varying amounts of

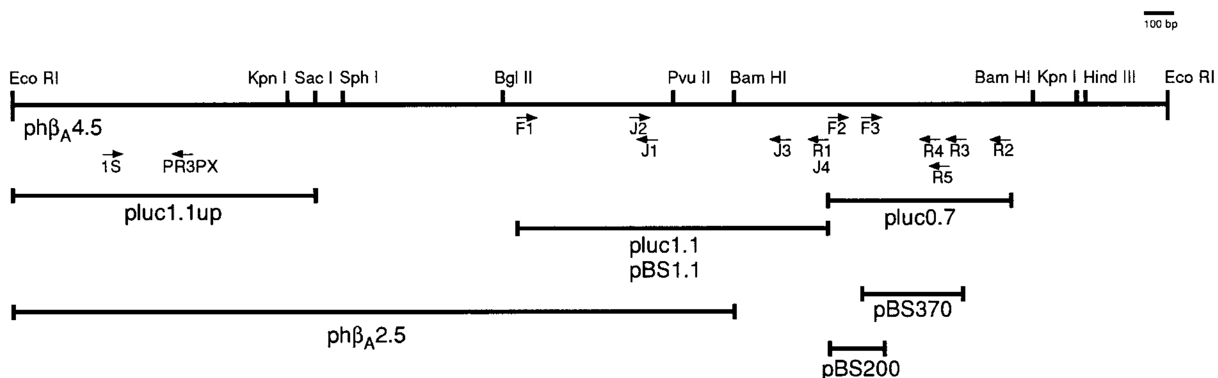

Fig. 1. Structure of the activin A upstream sequences in phβ$_A$4.5. Fragments that were subcloned for pluc1.1up, pluc1.1, pBS1.1, pluc0.7, phβ$_A$2.5, pBS200, and pBS370 are indicated. Arrows, locations of primers used for PCR and DNA sequencing.

time. The ends were blunted with T4 DNA polymerase and Klenow, and the upstream activin fragment was liberated by digestion with *Kpn*I (which cuts within the multiple cloning site of the vector). The upstream activin fragments were gel isolated and ligated to the *Sma*I–*Kpn*I sites of p19Δluc. Automated DNA sequencing was performed by the DNA Core Laboratory using the R5 primer to identify the deletion end points.

RNA Preparation

Bone marrow stromal cells were stimulated with 10 nM TNF-α or 10 ng/ml IL-1α,or both, for 6–9 h, as indicated. HT1080 cells were stimulated with 100 ng/ml TPA for 4 h. Then, total RNA was prepared using RNAzol B according to the manufacturer's instructions (Tel-Test, Friendswood, TX).

Northern Blots

Total RNA (15 μg) was electrophoresed on 1.2% formaldehyde-agarose gels and blotted to Hybond-N membrane (Amersham, Arlington Heights, IL) in 20× SSC (1× SSC is 0.15 M NaCl, 15 mM sodium citrate, pH 7.0) overnight. After ultraviolet cross-linking (UV Crosslinker FB-UVXL-1000, Fisher Biotech, Pittsburgh, PA), the membrane was prehybridized in 50% formamide/5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM sodium phosphate, pH 7.7, 1 mM EDTA)/5× Denhardt's (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin)/ 0.5% sodium dodecyl sulfate (SDS)/10% dextran sulfate at 42°C for 1 h. The coding sequence probe consisted of the 0.8 kb *Bam*HI–*Pst*I fragment from pRKβA, whereas upstream probes consisted of the 0.85 kb *Bgl*I–*Bam*HI fragment from phβ_A4.5 and the 1.1 kb *Hin*dIII–*Kpn*I fragment from pBS1.1. The fragments were labeled in random-primed labeling reactions using the Random Primed DNA Labeling Kit (Boehringer Mannheim, Indianapolis, IN) and purified by passing through a Bio-Spin 6 chromatography column (Bio-Rad Laboratories, Hercules, CA). The probes were added to the prehybridization solution at 2×10^6 cpm/ml and hybridized at 42°C overnight. The membranes were washed three times in 2× SSC/1% SDS at room temperature for 10 min, followed by two washes with 0.1× SSC/1% SDS at 42°C for 15 min. The membranes were exposed to Kodak XAR-5 film or a Molecular Dynamics phophorimager screen for 4 days.

RNase Protection Assay

RNA probes were made by in vitro transcription using the MAXIscript kit (Ambion, Austin, TX) according to the manufacturer's instructions, followed by gel purification on a 5% acrylamide/8 M urea gel and elution in 0.5 M ammonium acetate, 1 mM EDTA, 0.2% SDS at 37°C for 1–2 h with shaking. T7 RNA polymerase was used to synthesize [α-^{32}P]-UTP-labeled transcripts from linearized plasmid templates. pBS370 (nucleotides −547 to −177 relative to the start codon) was linearized with *Eco*RV. pBS200 (nucleotides −664 to −465) was linearized with *Xba*I. pBS1.1 (for transcription of nucleotides −671 to −1011) was linearized with *Bam*HI. phβ_A2.5 was linearized with *Pvu*II (for transcription of nucleotides −1011 to −1233).

RNA was prepared from cells treated with 10 nM TNF-α for 6–7 h. RNA probes were mixed with 10 μg RNA in 2× hybridization buffer (Continental Scientific, San Diego, CA), heated at 85°C for 5 min and hybridized at 55°C overnight. RNase digestion was carried out in 10 mM Tris–HCl, pH 7.5, 300 mM NaCl, 5 mM EDTA, containing 5 μg/ml (0.33 Kunitz U/ml) RNase A and 100 U/ml RNase T1 at 37°C for 30 min, followed by the addition of an equal volume of stop buffer (Continental Scientific) for 10 min at room temperature. The samples were precipitated with an equal volume of isopropanol, resuspended in loading buffer and run on a 5% polyacrylamide/8 M urea gel. The gel was exposed to Kodak XAR-5 film overnight.

Luciferase Assays

As an internal control, 5 μg pSVβ-gal (Promega, Madison, WI) was cotransfected with 10 μg luciferase expression plasmid into $2–7 \times 10^6$ L87/4 or HT1080 cells in 0.25–0.3 ml Opti-MEM (Life Technologies). DNA was introduced into the cells by electroporation using a Bio-Rad Gene Pulser at 960 μF and 200 V. The cells were then incubated in fresh medium in 60 mm dishes for 20–28 h. Cells were lysed in 350 μl 0.1 M potassium phosphate, pH 7.8/1 mM DTT/ 0.5% Triton X-100 at 4°C and centrifuged to remove cellular debris. Luciferase expression was measured using the Monolight 2010 Luminometer (Analytical Luminescence, Ann Arbor, MI). A total of 100 μl of cell extract plus 100 μl 0.1 M potassium phosphate, pH 7.8/15 mM $MgSO_4$/5 mM ATP/1 mM DTT was used for each sample, which was injected with 100 μl of 1 mM

luciferin (Analytical Luminescence Laboratories) by the luminometer before measurement of light output for 10 s.

β-galactosidase assays were used as internal controls. A total of 30 μl of cell extract was added to 100 μl of 0.1 M sodium phosphate, pH 7.5/880 μg/ml O-nitrophenyl-β-D-galactopyranoside/1 mM $MgCl_2$/45 mM β-mercaptoethanol and incubated at 37°C for 1–5 h. Absorbance at 405 nm was measured in the UV Max kinetic microplate reader (Molecular Devices, Menlo Park, CA). All samples were measured in duplicate and expressed as luciferase luminescence per β-galactosidase absorbance.

RESULTS

Activin A Is Expressed in Fibroblastoid Cells of Bone Marrow

Expression of activin A was previously observed in human primary bone marrow stromal cells through Northern and Western analyses [Shao et al., 1992]. Immunochemical staining of activin A in these stromal cells was performed in this study by incubating slides containing primary marrow cells with affinity-purified rabbit anti-β_A antiserum raised with β_A peptide (81–113) [Vaughan et al., 1989]. Visualization of the antigen–antibody complex was carried out by the avidin-biotin-glucose oxidase procedure [Robb 1983]; the presence of immune complexes indicates the expression of activin A by these fibroblastoid cells in the bone marrow stromal preparation (Fig. 2). As a control, when antiserum was preadsorbed with the synthetic peptide used for immunization, β_A (81–113), for 30 min before incubation with samples, no staining was observed (not shown). Antisera against an inhibin α peptide (amino acids 1–26) consistently failed to label any marrow stromal cells in similar analyses (not shown), indicating that the β_A staining represents activin A rather than inhibin A.

Activin A RNA Is Expressed as Four Species in Primary Bone Marrow Stromal Cells and in Bone Marrow Stromal Cell Lines

Bone marrow contains a heterogeneous population of cells, including fibroblasts, endothelial cells, adipocytes, osteoclasts and monocytes/macrophages [reviewed in Dorshkind, 1990]. The above results suggest that only a subset of cells expresses activin A. We therefore investigated the expression of activin A in two established fibroblastoid bone marrow stromal cell lines, L87/4 and HS-5, in comparison with primary bone marrow stromal cells. Expression of activin A mRNA was detected by Northern blot in both cell lines and in primary stromal cells (Fig. 3). Stimulation of the cells with the proinflammatory cytokines TNF-α and IL-1α greatly increased the levels of expression. As seen previously for primary stromal cells and monocytes [Shao et al., 1992], four sizes of activin A RNA were observed: 6.4, 4.0, 2.8, and 1.6 kb. However, the relative amounts of each size of RNA varied in the three cell types. In particular, the largest band (6.4 kb) was predominant in primary stromal cells, whereas it was observed to a lesser degree in HS-5 and especially in L87/4 cells (Fig. 3). Primary stromal cells may therefore contain a population of cells not represented by the cell lines which is the major source of the 6.4 kb mRNA.

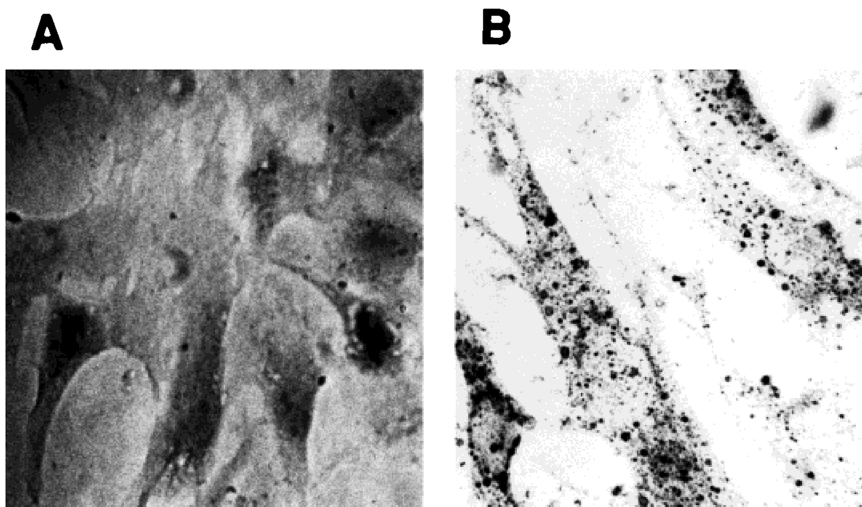

Fig. 2. Phase-contrast microscopy and immunocytochemical staining of human marrow stromal cells. Human stromal cells at 21 days of culture were examined. **A:** Phase-contrast microscopy. **B:** Immunochemical staining for activin A.

Additional Northern blots were performed in order to identify the region(s) in which transcription of activin A RNA initiates. As summarized in Figure 4, five overlapping probes were used in this analysis, covering the region from −1857 to +72 relative to the translation start codon. Three of the probes were positive for hybridization, and these hybridized to all four mRNAs, suggesting nearby transcription start sites for all four. These results suggest that the 5' ends of the mRNAs are located between −671 and −202.

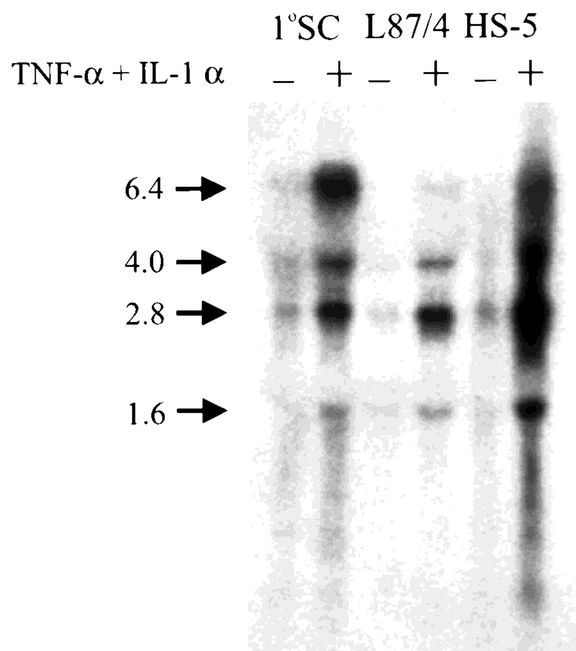

Fig. 3. Expression of activin A mRNA in bone marrow stromal cells. Total RNA was prepared from primary bone marrow stromal cells (1°SC), L87/4 and HS-5 stromal cell lines, untreated or treated with 10 nM TNF-α and 10 ng/ml IL-1αfor 8–9 h. 15 μg of total RNA was electrophoresed, blotted, and probed for activin A coding sequences generated from pRKβ_A.

Activin A RNA Is Expressed From a Single Transcription Start Site

RNase protection assays were performed to identify transcription start sites using RNA from primary stromal cells, L87/4 and HS-5 cells. Four upstream probes were used: −177 to −547 (transcribed from pBS370), −465 to −664 (transcribed from pBS200), −671 to −1011 (transcribed from pBS1.1), and −1011 to −1233 (transcribed from phβ_A2.5) to investigate the possibility of multiple transcription start sites (Fig. 5). This possibility is suggested by the observation of four different sizes of mRNA. However, only the probe from −177 to −547 was protected in these assays (Fig. 5), giving rise to a single band of approximately 36 nucleotides, corresponding to an mRNA 5' end at −212. The lack of protection of the other probes indicates that activin A RNA does not include the sequences in these regions (−465 to −664, −671 to −1011, −1011 to −1233).

Figure 6 shows the sequence of a portion of the 1857 nucleotides reported upstream of the activin A gene [Tanimoto et al., 1991; N.L. Frigon, Jr., and J. Yu, unpublished data]. The only

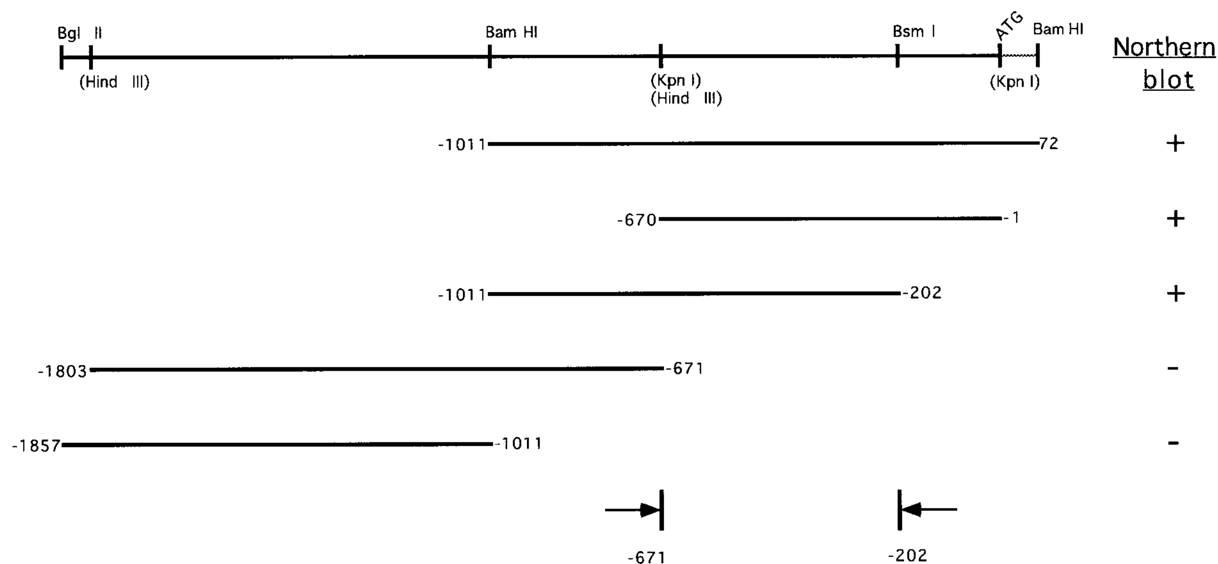

Fig. 4. Summary of Northern blots using overlapping probes from the activin A upstream region. Diagram of the activin A upstream region is shown at the top with the locations of the five probes that were used shown below. Each probe either hybridized to all four activin A mRNA bands, or to none of them, as indicated by "+" or "−".

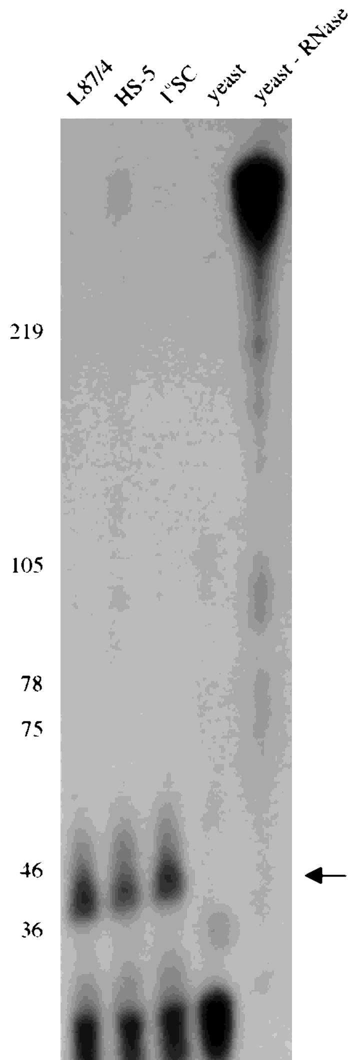

Fig. 5. Mapping of activin A transcription start sites by the RNase protection assay. 10 μg of total RNA from L87/4, HS-5 and primary stromal cells which were treated with 10 nM TNF-α and yeast RNA as a control, was hybridized to the RNA probe generated from pBS370, digested with RNase, electrophoresed on a 5% polyacrylamide/8 M urea gel, and autoradiographed. "yeast–RNase", control with yeast RNA in which RNase was omitted from the RNase digestion reaction. Results with three other RNA probes which were negative are not shown (probes generated from pBS200, pBS1.1, and phβ$_A$4.5).

TATA box is located at −1346 relative to the start codon. As indicated in Figure 4, Northern blots using far upstream probes (−1857 to −1011 and −1803 to −671) failed to hybridize to activin A RNA from primary stromal cells, L87/4 and HS-5 cells, suggesting that the TATA box at −1346 is not functional. Instead, activin A expression is presumably directed by a TATA-less promoter located in some other region of the sequence.

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-1407  ATATTTTAGA GAGTCTTTTC CCATAGGACC AGTTATTCAA GTCATACGAG
-1357  TGCACTCTTT TTATAAAAGG ATGTGGGAAA GGCCAAGAGA ATTTTGCATT
-1307  TTATCTGTGA AGTCCGGCGA GTGGTGGTAG GCTGTAATGT GTGAGAGTGA
       ..................................................
 -707  TAATTTATTT TAGAAGAGAA AGTAGAACCC CCGAAAATGG TGATATTTGA
 -657  AGAGAGGTGT CTGTGAGGAA GCTAAGAGCA GAAGGAGAGC AGCCTGTCAG
 -607  AAAACGGGCT GTCCCTCCCT CCCTAATCAC AGCCCTACTC ACAGCAAACT
 -557  CCCTCCCTCT CCATTCACTC ACTTACTTAG GGCCAATCCT TTCTCTCTCT
 -507  CTCTCTCTCT CTCTCTCTCT CTCTCTCTCT CTCTCTCTCT TCCTTTCCCT
 -457  CTCTCCCTCT CCCCCTCCCT TCCCTCCCTC TCTCCCTCTC TCTCCCCCTT
 -407  CTTTCCCTCT CTCTTCTCTC CCCCTCTCTC CTCTCTCTCT GTCTCTGTCT
 -357  CCCTCCCATC CTCTCTCTCT GTCTCTGTCT GTCTCCCCGC CACCCTGTCT
 -307  CTCCCTCCCT CCCTGTCTCC CTCCCTCCCT CCCTCCTTCC TTCTCTCTTA
 -257  CTCGGAGACA GTCAGAACTC TCCTCCCTGA CAGCCACAAA CCTACAGCAC
                  <-------------------------- R5
 -207  TGACTGCATT CAGAGAGGAA CCTGCAAACA AAACTTCACA GAAAACTTTT
 -157  TGTTCTTGTT CCAGAGAATT TGCTGAAGAG GAGAAGGAAA AAAAAAACAC
 -107  CAAAAAAAAA AATAAAAAAA TCCACACACA CACAAAAACC TGCGCGTGAG
  -57  GGGGGAGGAA AAGCAGGGCC TTTTAAAAAG GCAATCACAA CAACTTTTGC
   -7  TGCCAGGATG
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Fig. 6. Activin A gene upstream sequence [Tanimoto et al., 1991; N.L. Frigon, Jr., and J. Yu, unpublished data]. Numbering is relative to the translation start codon, which is underlined. A TATA box and a CT-rich region are boxed. Arrow, primer R5, used for DNA sequencing.

Activin A Promoter Activity Is Located Within 0.7 kb of the Start Codon

In order to identify the putative promoter, the activin A gene upstream region was analyzed using luciferase expression assays. PCR products containing nucleotides −1803 to −671 and −670 to −1 were generated and cloned into the luciferase expression vector, p19Δluc (i.e., pluc1.1 and pluc0.7 in Fig. 7A). These constructs were electroporated into L87/4 cells and luciferase assays were used to measure the promoter activity of each cloned fragment. Luciferase activity was normalized to β-galactosidase activity from a control plasmid, pSVβ-gal. The 1.1 kb clone, pluc1.1, showed little to no promoter activity relative to the negative control, p19Δluc (Fig. 7B), whereas the 0.7 kb clone, pluc0.7, showed significant promoter activity (Fig. 7B). Clones containing portions of the 1.1 kb upstream region adjacent to the 0.7 kb fragment were then constructed (Fig. 7A). The additional sequences from the 1.1 kb fragment in clones pluc1.1/0.7, pluc511/0.7, pluc564/0.7 and pluc340/0.7 conferred little to no increase in promoter activity relative to pluc0.7 (Fig. 7B).

The 0.7 kb Upstream Sequence Contains a Promoter, Positive Element, and Negative Element

Five subclones containing portions of the 0.7 kb sequence (pluc486, pluc324, pluc468, pluc370 and pluc288) were also constructed

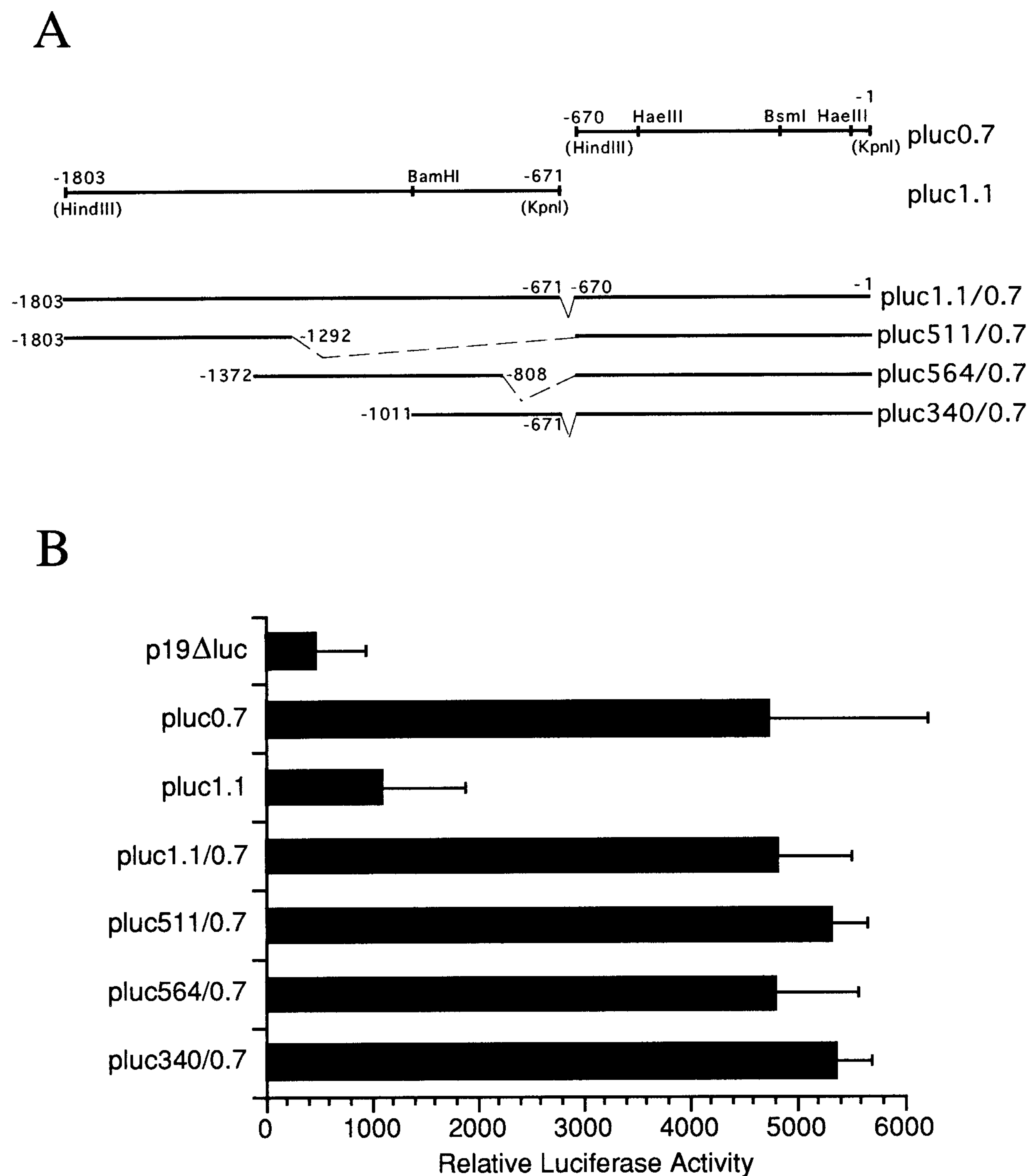

Fig. 7. Expression of activin A promoter constructs in L87/4 cells. **A:** The structure of activin A upstream sequences subcloned 5′ to the luciferase gene in p19Δluc. Restriction sites in parentheses refer to sites that were introduced by PCR. **B:** Luciferase expression. L87/4 cells were transiently transfected with the indicated constructs and assayed for luciferase activity. Activity is expressed as luciferase activity relative to β-galactosidase activity. Expression was measured in duplicate for three independent experiments.

(Fig. 8A). The level of expression for pluc 486 (−526 to −40 relative to the start codon) was similar to that for pluc324 (−526 to −202), implying that the region between −202 and −40 does not contribute much to activin A gene expression. The pluc468 subclone (−670 to −202) produced a much higher level of expression than any of the other clones (4.8-fold relative to the activity of pluc0.7), whereas pluc324 gave much lower, although significant expres-

sion (Fig. 8B). Therefore, the region from −670 to −526 contains a strong positive element that contributes to gene expression. The much higher level of activity exhibited by pluc468 relative to pluc0.7 suggests that the region between −202 and −1 contains a negative element. Because the sequence from −202 to −40 does not appear to affect expression (compare pluc486 and pluc 324), the negative element is presumably located between −40 and −1. However, it will be important to determine whether the negative effect is due to a specific transcriptional element or whether it is an effect of the leader sequence on translation of the luciferase RNA. In particular, secondary structure within the leader can reduce the efficiency of translation of mRNA [Kozak, 1991]. The pluc370 subclone (−547 to −177) expressed luciferase at a relatively high level of expression, while pluc288 (−547 to −259) produced little to no expression. Therefore, the region between −259 and −177 is important for expression and may be capable of exhibiting promoter activity. Similar results were obtained from transient expression assays in K562 erythroleukemia cells (data not shown).

To define the promoter region more closely, the pluc468 subclone was treated with BAL31 from the 5′ end in order to construct additional expression subclones. Five subclones were obtained with 5′ end points ranging from −600 to −337 (Fig. 8A); all five were at least as active as the original 0.7 kb clone, but were lower than pluc468 (Fig. 8C). Therefore, the region between −337 and −202 contains the core promoter, but the region between −670 and −600 contains an element that enhances the promoter activity. The previous experiments (Fig.

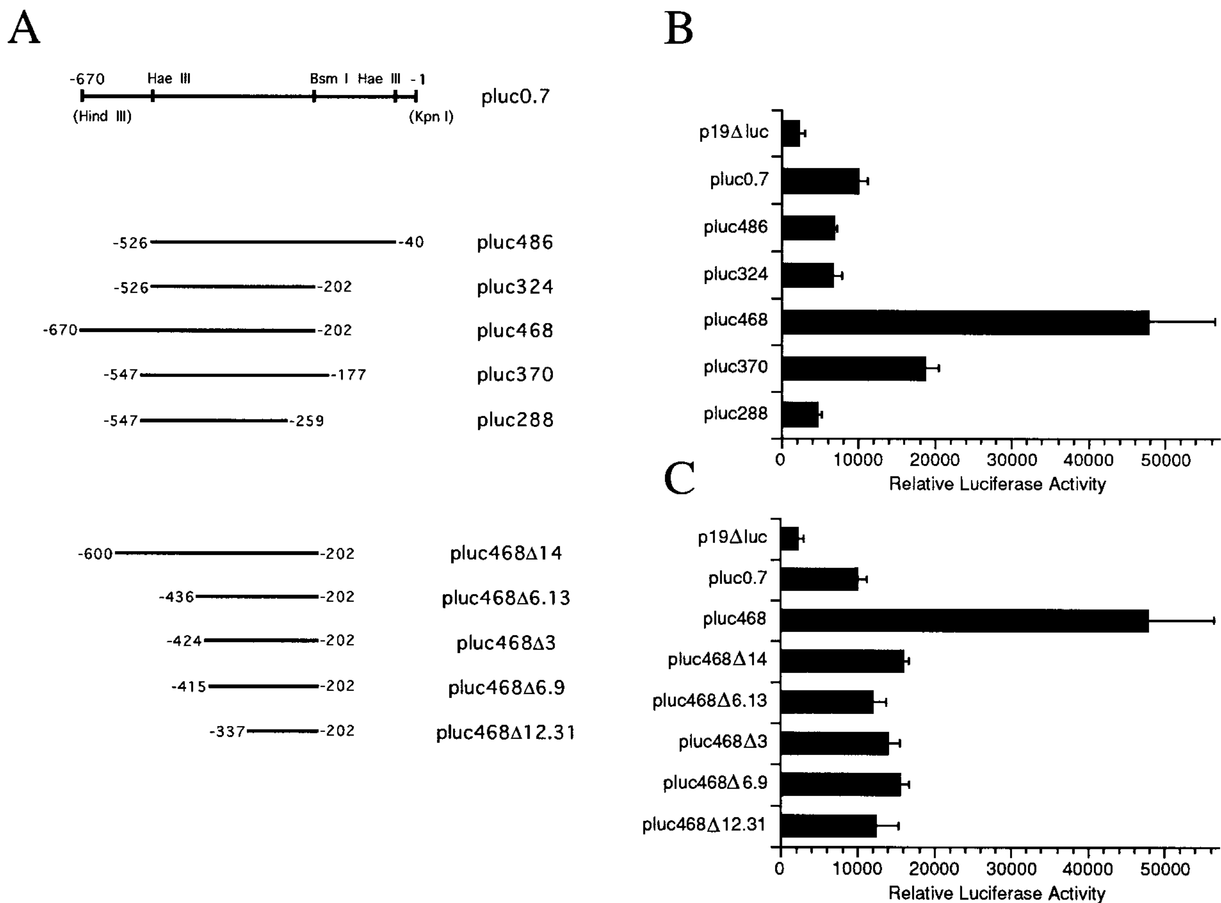

Fig. 8. Expression of activin A promoter construct subclones in L87/4 cells. **A:** Subclone fragments cloned upstream of the luciferase gene in p19Δluc. Restriction sites in parentheses refer to sites that were introduced by PCR. **B:** Luciferase expression of the pluc0.7 subclones. Expression was measured in duplicate for three independent experiments. Activity is expressed as luciferase activity relative to β-galactosidase activity. **C:** Luciferase expression of the pluc0.7 BAL31 deletion subclones. Expression was measured as in B.

8B) identified promoter activity between −259 and −177; therefore, the promoter activity is expected between −259 and −202.

Additional Promoter Is Located Far Upstream From the Start Codon

Tanimoto and colleagues have proposed that activin A is expressed from a promoter located 2.9 kb upstream of the start codon in a fibrosarcoma cell line, HT1080 [Tanimoto et al., 1996]. They propose that this far upstream promoter is spliced to the near upstream region of activin A. To test the activity of this distal promoter in bone marrow stromal cells, we constructed the luciferase expression clone pluc1.1up containing nucleotides between 3.6 kb and 2.5 kb upstream of the start codon (Fig. 1). pluc1.1up produced significant luciferase activity in L87/4 and in HT1080 cells (Fig. 9), indicating that this distal promoter may be active in bone marrow stromal cells and fibrosarcoma cells. However, pluc468 also gave a high level of expression in L87/4 and especially high in HT1080 cells; therefore, both promoters may be active in both cell types. TPA stimulates activin A expression in marrow stromal cells [Shao et al., 1997; Takahashi et al., 1992] and in HT1080 fibrosarcoma cells [Tanimoto et al., 1993]. Therefore, its ability to stimulate the luciferase expression clones was tested. TPA stimulation of pluc468 and pluc1.1up expression in L87/4 and HT1080 cells was marginal (data not shown). Consequently, both core promoters may be insufficient to respond to TPA in vivo.

In order to test for the possibility that activin A RNA in bone marrow stromal cells is spliced in the same manner as suggested for HT1080 cells [Tanimoto et al., 1996], Northern blots containing total RNA from bone marrow stromal cells stimulated with TNF-α and from HT1080 cells stimulated with TPA were probed with upstream sequences. Figure 10A shows the results using a probe from −391 to −177 relative to the start codon, which is located within the putative upstream intron proposed by Tanimoto et al. (approximately −2742 to −144 relative to the start codon). Varying extents of hybridization were observed to all four sizes of mRNA. This demonstrates that the RNA is not strictly spliced in the manner they described. Figure 10B shows a blot probed with a fragment approximately 2.8 kb upstream from the start codon (−59 to +68 in [Tanimoto et al., 1996]). Some hybridization to activin RNA is

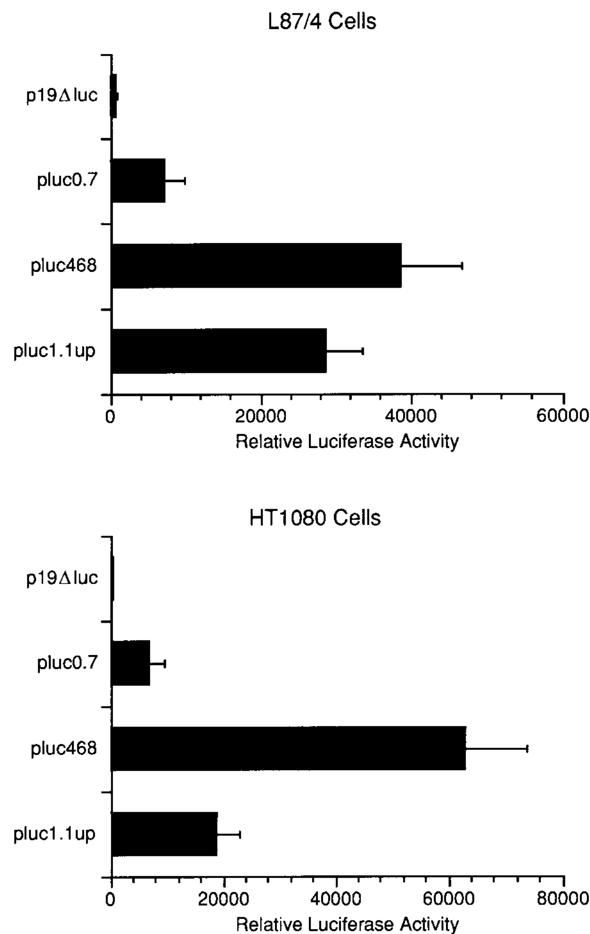

Fig. 9. Expression of activin A promoter construct subclones in L87/4 and HT1080 cells. Activity is expressed as luciferase activity relative to β-galactosidase activity. Expression was measured in duplicate for three independent experiments.

observed with this probe. These results in combination with the previous Northern blots (Fig. 4) and consideration of the RNA sizes suggest that upstream splicing of the RNA may occur to a slight extent. However, the far upstream probe produced more than 10-fold lower signals for each band than the near upstream probe, except for the 6.4 kb band in primary stromal cells. Therefore, a small percentage of the RNA was generated from the far upstream promoter and/or the RNA is less stable. These observations reinforce the idea that there are cell-specific differences in expression of activin A mRNAs.

DISCUSSION

The results presented here show that bone marrow stromal cells express activin A from a promoter located within 300 nucleotides of the start codon. Earlier studies found that human

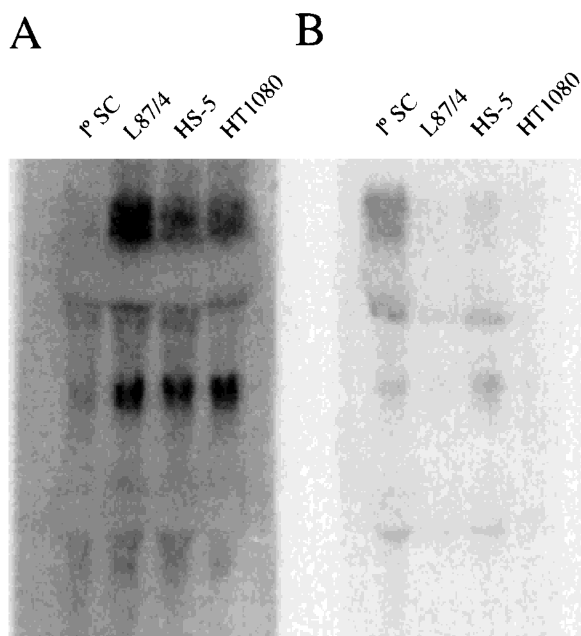

Fig. 10. Expression of RNA from the activin A upstream region in bone marrow stromal cells and HT1080 fibroblastoma cells. Northern blots were performed as in Figure 3. The exposure time for the two blots was identical. **A:** Total RNA was probed with the near-upstream fragment (−391 to −177). **B:** Total RNA was probed with the far-upstream fragment (−2.8 kb).

primary marrow stromal cells express activin A mRNA in four sizes [Shao et al., 1992; Yamashita et al., 1992], although the coding region of activin A constitutes only 1.3 kb [Mason et al., 1986]. The current study shows that two established human stromal cell lines also express these four RNAs, but in varying amounts. Different transcription start sites may be responsible for the existence of different transcript sizes, although others [Tanimoto et al., 1993] have identified several polyadenylation sites at the 3' end of the gene, which may account for much of the size difference. Northern blots and RNase protection assays were performed to identify the 5′ ends of the RNAs. RNase protection assays identified a 5' end at −212, consistent with the primer extension results of others [Tanimoto et al., 1993].

A promoter-regulatory region for the activin A gene was identified within 700 nucleotides of the start codon. Promoter activity was found in a 136 bp region between −337 and −202, and a positive element consists of 71 bp located between −670 and −600, based on the data for the BAL31 deletion clones. A comparison of the promoter activities of subclones pluc370 and pluc288 identifies promoter activity between −259 and −177. Therefore, activin A promoter activity is likely to reside within the region from −259 to −202 as shown schematically in Figure 11A. In addition, a negative element appears to reside between −40 and −1 (Fig. 11A). Similar to the activin B and inhibin α genes in rat [Feng et al., 1989], this putative promoter region does not contain a TATA or CAAT box, but it does contain several potential Sp1 consensus sites, as well as three small overlapping inverted repeats that may be important for activin A expression. In addition, a CT-rich region present between −521 and −259 is conserved in the bovine sequence [Thompson et al., 1994].

TNF-α,IL-1, or TPA treatment of marrow stromal cells stimulates expression of the endogenous activin A gene [Shao et al., 1992; Shao et al., 1998; Takahashi et al., 1992]. TNF-α action is typically effected by NF-κB [Duh, et al., 1989; Lowenthal et al., 1989; Osborn, et al., 1989], but no consensus binding sites are present within the 1857 nucleotides upstream of the activin A gene start codon [Tanimoto et al., 1991; N. L. Frigon, Jr., and J. Yu, unpublished data] nor are any of these sites present within the sequence reported by Tanimoto et al. [1996] surrounding the putative promoter located 3 kb upstream of the start codon. Conversely, the glucocorticoids dexamethasone and hydrocortisone decrease expression of activin A in bone marrow stromal cells [Shao et al., 1997]. Despite the in vivo responses to TNF-α,IL-1, TPA, dexamethasone, and hydrocortisone, the luciferase expression constructs did not respond to treatment (data not shown). These clones presumably lack the regulatory region(s) containing these response elements. A comparison of human and bovine sequences shows that the sequence between −259 and −202 is identical (Fig. 11B). Comparison with the available chicken sequence (−244 to −202) showed 87% identity (Fig. 11B). These similarities suggest that the sequence in this region has an important function, consistent with the identification of promoter activity between −259 and −202.

Tanimoto and colleagues [1996] isolated activin cDNA clones from HT1080 fibrosarcoma cells which lack 2.6 kb of genomic upstream sequence, presumably an intron. They postulated a promoter-regulatory region for the activin A gene approximately 3 kb upstream from the start codon. We also found that this region exhibits promoter activity in bone marrow stro-

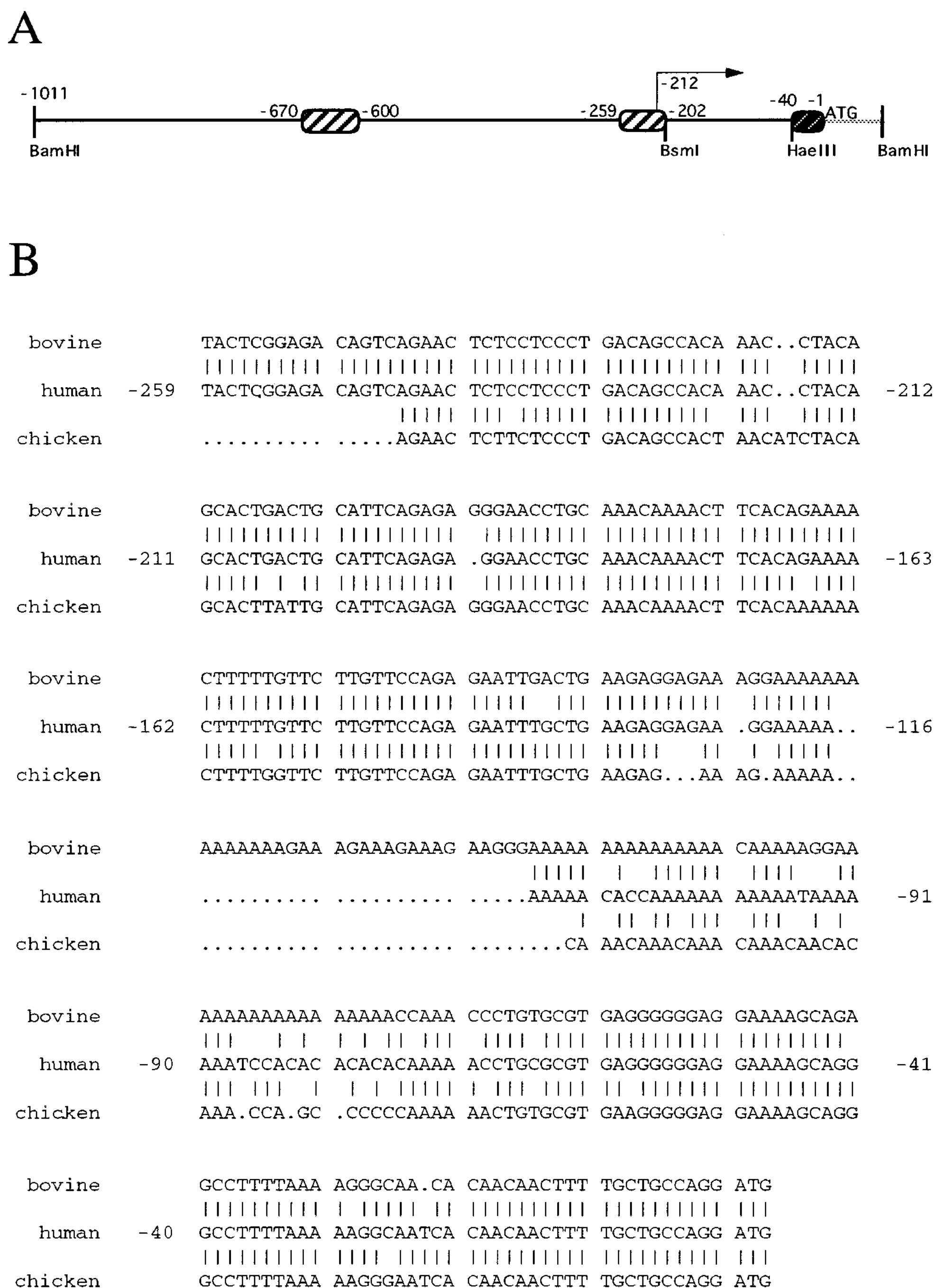

Fig. 11. Activin A gene near upstream region. **A:** Diagram of the upstream region with positive element and promoter marked with hatched white ovals. The putative negative element is marked with a hatched black oval. The transcription start site (arrow) and the start codon (ATG) are also indicated. **B:** Comparison of human [Tanimoto et al., 1991; N.L. Frigon, Jr., and J. Yu, unpublished data], bovine [Thompson et al., 1994], and chicken (J.X. Huang, J. Lough, and R. B. Runyan, GenBank accession number U26946) activin A gene upstream sequences. The numbers correspond to the human sequence relative to the start codon.

mal cells. However, we observed hybridization of total RNA from HT1080 fibrosarcoma cells to a near upstream probe (−391 to −177), which is located within the putative upstream intron. This observation indicates the existence of mRNA containing the putative intron. Therefore, some activin mRNA synthesized by HT1080 cells is presumably not spliced in the upstream region. The data suggest that HT1080 cells synthesize activin RNA from at least two

very different transcription start sites, resulting in upstream splicing for one message but not the other. Overall, the similar hybridization patterns with the near and far upstream probes in Northern blots suggest that the size differences of the RNAs are mostly due to differences in length at the 3′ end and that upstream splicing generates a 5′ end that is similar in length to the mRNAs that do not undergo upstream splicing. Another possibility is that some upstream splicing may occur using a splice site which is different from that described previously [Tanimoto et al., 1996]. In addition, a previous study of activin A cDNAs from human ovary reported a sequence [Mason et al., 1986] that matches the activin A genomic sequence and therefore is not spliced in the upstream region. This finding is consistent with the Northern blot data presented here, which show that this putative intron sequence is present within some mature activin A mRNAs. It is possible that both promoters are functional in all cell types, as suggested by our luciferase assay and Northern blot data, or there may be tissue-specific differences in the use of one or the other promoter. Both possibilities have been observed in the case of the murine GATA-1 gene, which is expressed from two promoter regions [Ito et al., 1993; Migliaccio et al., 1996]. The proximal promoter, nearest the start codon, is primarily expressed in hematopoietic cells [Tsai et al., 1991], whereas a distal promoter, approximately 8 kb upstream from the proximal promoter, is used by Sertoli cells of the testes [Ito et al., 1993]. Migliaccio et al. [1996] found that promoter usage varied during hematopoietic stem cell differentiation. In a similar way, activin A promoter usage may depend both on the tissue and on the condition of the cells in terms of cytokine stimulation and state of differentiation. Furthermore, activin A has numerous effects on a variety of cell types. Multiple RNAs and multiple promoters may ultimately lead to differences in the levels of activin A protein expressed. Different amounts of protein can have a profound effect on cells. For example, activin can induce the formation of different tissues during development in vitro, depending on the dose [Green and Smith, 1990]. Further studies are needed to test these hypotheses.

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