STRUCTURE OF THE HUMAN ACTIVIN β_A SUBUNIT GENE AND ITS REGULATION IN CULTURED CELLS

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Abbreviations

cAMP	cyclic adenosine-3',5'-monophosphate
FSH	follicle-stimulating hormone
LH	luteinizing hormone
HPLC	high performance liquid chromatography
TGF-β	transforming growth factor-β
TPA	12-*O*-tetradecanoylphorbol-13-acetate
EDF	erythroid differentiation factor
AP-1	activator protein 1
kb	kilobases
SV40	simian virus 40
bp	base pairs
cDNA	complementary DNA
SDS	sodium dodecyl sulfate
CRE	cAMP-responsive element
tk	thymidine kinase
PKC	protein kinase C
H-7	1-(5-isoquinolinesulfonyl)-2-methyl-piperadine dihydrochloride
MEM	minimum essential medium
FBS	fetal bovine serum
EDTA	disodium ethylenediamine-tetraacetate
DTT	dithiothreitol
RAV-2	Rous associated virus 2
HSV	herpes simplex virus
CAT	chloramphenicol acetyltransferase

PCR	polymerase chain reaction
RSV	Rous sarcoma virus
IGF-II	insulin-like growth factor II
rRNA	ribosomal RNA
hCG	human chorionic gonadotropin
TRE	TPA-responsive element
RT-PCR	reverse transcriptase-mediated polymerase chain reaction
IL	interleukine
TNF	tumor necrosis factor

Contents

Chapter I: Preface

The biological basis for gonadal regulation of pituitary function was formulated in 1923 by Mottran and Cramer, who observed hypertrophy of rat pituitary cells following radiation-induced testicular damage (1). In 1932, McCullaugh demonstratred that the appearance of these hypertrophied cells could be inhibited by the injection of a water-soluble testicular extracts, and he termed this hypothetical substance *inhibin* (2). Following the discovery of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), the two pituitary hormones known to regulate the development and the activity of the gonads (3), Klinefelter *et al* (4). postulated that a testicular factor, inhibin, excerted a specific negative feedback action on FSH secretion and most likely acted directly on the pituitary gland. This hypothesis was substantiated when numerous investigators demonstrated a direct suppression of peripheral FSH in animals treated with steroid-free testicular or ovarian preparations. Inhibin activity has since been observed in testicular or ovarian extracts and in cultured Sertoli or Granulosa cells from a variety of species (for review, see Ref. 5).

Although the existence of inhibin has been realized for more than 50 years, its real identity remained elusive because of the insensitive bioassay of FSH and LH. Recent use of the *in vitro* cultured pituitary cell bioassay, which measures suppression of immuno-assayable FSH, and HPLC techniques has led to the purification of inhibin (5). In 1985, several groups isolated the inhibin (Mr=32,000) from porcine follicular fluid as a heterodimeric peptide consisting of two subunits α(Mr=20,000) and β (Mr=13,000), of which the β subunit consists of two homologous forms β_A and β_B (6-9). Cloning studies showed that these subunits to be a part of precursor molecules that are processed following dimerization to form mature inhibin (10-13). The α and β subunits are partially homologous, particularly in relation to the location of seven cystein residues, and their

sequences, especially those of the β subunits, show homology to an ever increasing range of proteins in the transforming growth factor-β (TGF-β) superfamily including Müllerian inhibitory substance, bone morphogenetic proteins, and several proteins involved in development (Vg-1, Vgr-1, and decapentaplegic protein) (14, 15).

The biologic activity of inhibin was originally identified by its ability to suppress FSH release and cell content of pituitary cells in culture (Fig. 1). In side fractions during the isolation of inhibin, a protein termed *activin* was purified that, in contrast to inhibin, stimulated FSH release (16, 17). Structural studies identified that activin is formed either by a disulphide-bound homodimer of β_A and β_B subunit (Activin A and Activin B, respectively) or by a heterodimer composed of β_A and β_B subunits (Activin AB) (Fig. 2). As mentioned above, a larger group of proteins, referred to as the TGF-β superfamily, have ~30% sequence identity to TGF-β1 and seven invarient cysteins. There are two additional cystein residues conserved between activin β subunits and TGF-βs. The crystal structure of TGF-β2 determined by x-ray crystallography enables us to draw the putative structure of activins (18). Eight cysteines form four intrachain disulphide bonds and the ninth cystein, which forms an interchain disulfide bond, is involved in the stabilization of the dimer structure.

In 1987, Eto *et al.* (19) isolated a protein, from a human acute monocytic leukemia (THP-1), that exhibits extensive differentiation-inducing activity toward mouse Friend leukemia cells (F5-5). This cell line, known to provide a model system for studying erythroid differentiation, was induced to differentiate by the addition of cultured medium of THP-1 cells performed in the presence of 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Subsequent purification of this protein, termed erythroid differentiation factor (EDF), disclosed that it was the identical molecule to a dimer consisting of two inhibin β_A subunits (Activin A).

β-subunit mRNA has been identified in the gonads, bone marrow, spleen, pituitary,

and parts of the brain (20). A recent study has demonstrated that activin exerts effects on the function and/or proliferation of a variety of cells including: gonadal theca interna cells, granulosa cells, Leydig cells, hepatocytes, paraventricular oxytocin-producing cells, placental human chorionic gonadotropin- and progesterone-producing cells, pituitary somatotrophs, and corticotrophs (for review, see Ref. 21). More recently, activin has also been identified as a nerve cell survival factor (22) and a potent inducer of mesoderm formation during early embryogenesis of *Xenopus* (23-26). In addition, inhibin/activin subunit mRNAs are differently expressed during rat embryogenesis (27), suggesting a key role of this protein during vertebrate development.

Although structurally related, activins and inhibins are, for the most part, functionally antagonistic, for example, activin increases intracellular and secreted FSH, while inhibin decreases both the release and cell content of FSH (28) (Fig. 1). Thus, it appears that the α and β subunits of inhibin may have different functions, depending on their pattern of association and their site of production. Furthermore, such a surprising diversity of sites of both production and action by this protein suggests a variety of paracrine and autocrine roles in addition to their originally described endocrine functions (29). Despite this abundance of action and production sites, little is known regarding the intracellular mechanisms of activin's action and transcriptional mechanisms of gene expression.

Activin-binding sites have been identified on a number of activin-responsive cells, and chemical cross-linking studies suggested that at least three binding species (type I, type II, and type III) exist on the cell surface (30). However, as is the case for all the activin/TGF-β superfamily receptors, little is known about the structure of those proteins or about the second messenger signaling systems that they employ. Because of the lack of knowledge about an extensive biochemical characteristics of these proteins and their low abundance, molecular cloning of activin receptors has long been hampered. In 1991, Mathews and Vale (31) have cloned a family of receptors for activin using an expression cloning strategy, and

sequence comparisons have suggested that these receptors may functions as serine/ threonine-specific kinases. After that, Nakamura *et al.* (32) purified the activin receptor from the mouse embryonal carcinoma cell line and clarified that this receptor possesses protein kinase activity specific for serine, threonine, and tyrosine. To date, no transmembrane-type kinase with this specificity has been reported, suggesting that the receptors for activin and the other ligands in the family may form a new class of receptor families (33). Subsequently, additional activin receptors (34), as well as related receptor for transforming growth factor-β (35), have been cloned. However, pathways downstream from receptor binding have yet to be characterized.

Although the production of activin is expected to be strictly regulated temporally and spatially, little is known about the regulatory mechanisms of expression of this gene. Furthermore, neither the precise structure of the human activin β_A subunit gene nor the promoter sequence have been reported previously. In this thesis, to examine the regulation of the human activin β_A subunit gene, I cloned and determined the structure of the gene including the 5'-flanking region (promoter and putative regulatory regions) (chapter II), and investigated the expression of this gene in HT1080 cells, in response to TPA (chapter III) and cAMP (chapter IV), two major intracellular messengers known to modulate the important cellular events and gene expression. Finally, to localize the *cis*-regulatory elements important for the expression of this gene, I performed transfection experiments and identified the enhancer element responsive to the AP-1 transcription factor, which is known as a nuclear target for TPA action.

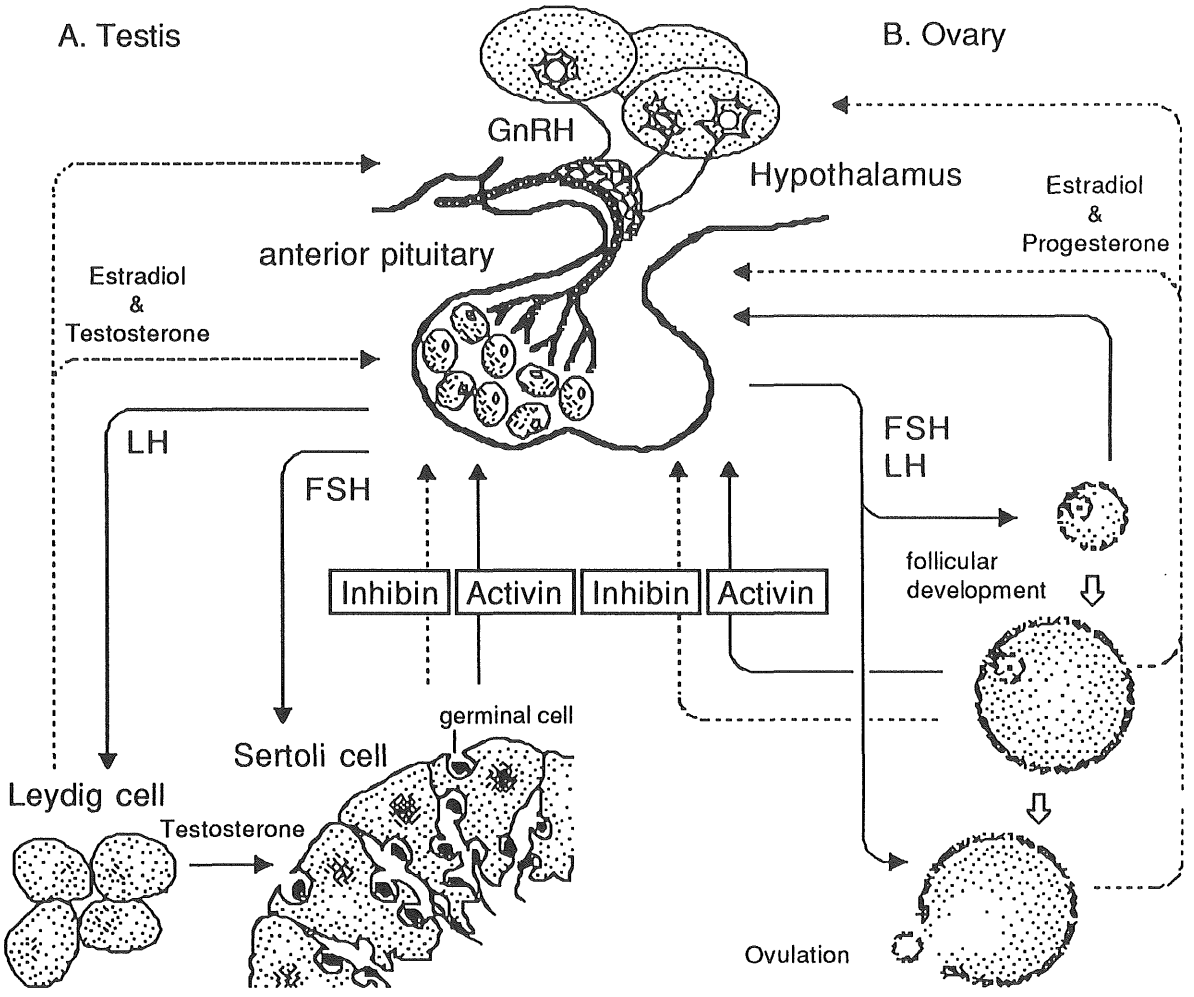


Fig. 1 Summary of hormonal control of gonadotropin-secretion (28). The hypothalamus produces GnRH which acts on the pituitary cells to secrete FSH and LH. (A) LH acts on Leydig cells to secrete testosterone which stimulates the germinal cells for spermatogenesis. Whereas FSH stimulates gonadal protein production by Sertoli cells (inhibin and activins). Testicular steroids negatively feedback to hypothalamic-hypophysical axis for GnRH-FSH and/or LH secretion. Inhibin specifically suppresses while activin enhances FSH release by the pituitary. (B) A secretion of FSH together with tonic LH stimulates the follicular development. The developed follicles secrete estradiol, progesterone, inhibins, and activins. Estradiol and progesterone, at different concentrations and/or ratios, either positively or negatively feedback to the hypothalamic hypophysial axis in regulating the secretion of FSH and LH. Inhibin specifically suppresses whereas activin enhances the secretion of FSH by the pituitary.

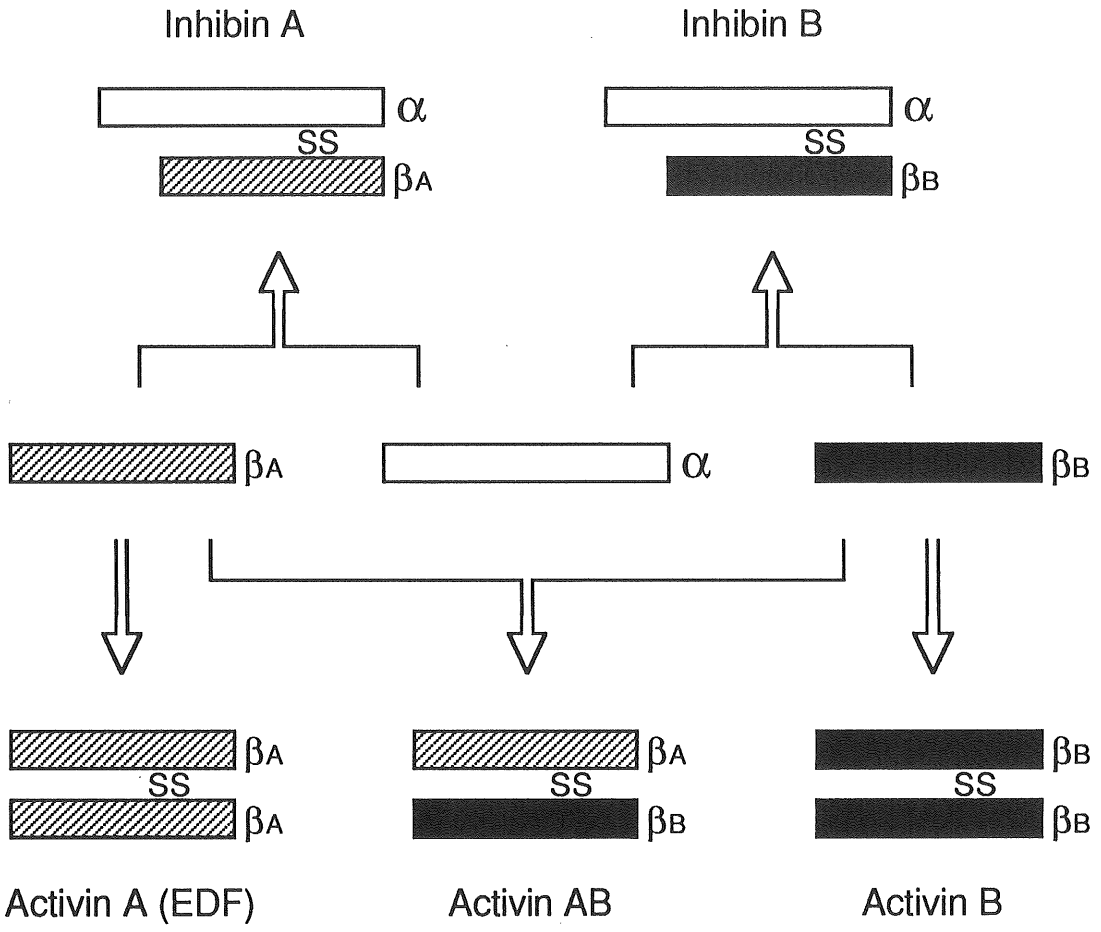


Fig. 2 Schematic representation of dimeric structures of inhibins and activins by divergent arrangements of gene products from the α, β_A, and β_B subunits through disulphide bridge. Inhibins A and B are composed of a common glycosylated α subunit (134 amino acids (aa)) and either a β_A (116 aa) or a β_B (115 aa) subunit, respectively to yield FSH suppressors. Homodimers ($\beta_A\beta_A$ and $\beta_B\beta_B$) and heterodimers ($\beta_A\beta_B$) of structurally related but distinct β subunits yield activin A, activin B, and Activin AB, respectively with opposite biological activities of those of inhibins.

Chapter II: Structure and Sequence Analysis of the Human Activin β_A Subunit Gene

Summary

I have isolated the human activin β_A subunit gene from a genomic library and analyzed the clones by restriction endonuclease mapping, Southern blotting, and DNA sequencing. The activin gene is composed of two exons interrupted by the 9-kb intron. The TATA, CCAAT, and CT-stretch sequences were found in the 5'-flanking region of the gene. An intronic sequence contaied SV40 enhancer core element in the vicinity of the exon 1. In the 3'-flanking region, eight consensus polyadenylation sequences, five ATTTA motifs, CA element consisting of $(CA)_{14}$, AP-1 binding site, and two SV40 enhancer core elements were identified. A dot matrix analysis revealed the high degree of conservation between the human and rat sequences within the 3'-flanking region, suggesting a possible functional significance.

Introduction

Inhibin, a gonadal hormone polypeptide, that specifically inhibits follicle-stimulating hormone (FSH) secretion from anterior pituitary. It is composed of two subunits, α and β, and there are two similar related forms of the β subunits, β_A and β_B, which have been identified in human (12) and other species (10, 11, 13). Dimers of the β subunits, $\beta_A\beta_A$ or $\beta_A\beta_B$, have been isolated from follicular fluid and are known as activin for their ability to stimulate FSH release from pituitary cells in culture (16,17). It has recently been demonstrated that activin ($\beta_A\beta_A$) is an identical molecule to erythroid differentiation factor (EDF) produced by human leukemia cells (19). More recently, it has become clear that the human activin has an activity inducing mesoderm in early *Xenopus* embryos (23). Therefore the β_A gene, in particular, encodes a subunit that forms either part of a hormone, inhibin, or a differentiation factor, activin. Despite the importance of this gene and recent advances in the field, little is known about the molecular mechanisms involved in the regulation of basal and inducible expression of inhibin/activin subunit genes.

The genomic DNA for inhibin α subunit has been isolated from human (36), rat (37,38), and mouse (39), and for β_B subunit from human (40), rat (37), and sheep (41). Sequence analyses clarified that the 5'-flanking region of rat and mouse α subunit gene posesses potential cAMP-responsive elements and these sequences were able to confer cAMP-responsiveness in DNA transfection analysis (38,39). The 5'-flanking sequences of the β_B subunit genes were also reported and several potential regulatory sequences were identified. In contrast, neither the precise structure, including the 5'-flanking region, nor the promoter analysis of the β_A subunit gene have been reported previously. The human activin β_A subunit gene was therefore isolated and sequenced, and results are presented in this chapter. These results will provide the basis for the regulation of activin gene expression in the following chapters.

Materials and Methods

Isolation of genomic clones for human activin βA subunit gene

The genomic DNA was extracted from human placenta, partially digested with restriction endonuclease *Sau*3AI and size fractionated on a 10-20% sucrose gradient (42). DNA fragments larger than 10 kb were ligated into λ phage Charon 28, previously cut with *Bam*HI. This material was then packaged into infectious phage particles using packaging mix (GIGAPACK GOLD) from Stratagene. The library was plated out on a lawn of *Escherichia coli* LE392, and replica plaque transfers were made onto nitrocellulose filter. Plaque hybridization was performed on a ^{32}P-labeled 360-bp *Rsa*I-*Eco*RI fragment excised from rat activin βA subunit cDNA (13). Hybridization conditions for screening were at 65°C in 5 x SSPE (42), 2 x Denhardt's reagent (42), 0.1% SDS, and 100 μg/ml denatured, fragmented salmon sperm DNA. Filter was stringently washed at 65°C in 2 x SSC (42) and 0.1% SDS. In the 3 x 10^6 phages, three positive clones were identified.

Southern blot analysis

Human genomic and phage DNAs were digested with various restriction enzymes, electrophoresed on a 0.7% agarose gel, and transferred to GeneScreen Plus (nylon) membrane (DuPont). Hybridization was carried out at 65°C for 16 h in 1 M NaCl, 1% SDS, 10% dextran sulfate, 100 μg/ml denatured salmon sperm DNA, and 1 x 10^6 cpm/ml labeled probes. The five hybridization probes derived from phage clones were indicated as follows: probe 1, 311-bp *Alu*I fragment; probe 2, 650-bp *Bam*HI/*Bgl*II fragment; probe 3; 1200-bp *Bgl*II/*Bam*HI fragment; probe 4, 950-bp *Bam*HI/*Eco*RI fragment; probe 5, 412-bp *Hin*cII fragment.

DNA sequencing

DNA restriction fragments from the phages containing the human activin β_A subunit gene were subcloned into pUC19 and pUC119, and sequenced by the dideoxy-chain termination methods (43). All the deduced exons and the intronic sequences reported in the present study were determined on both strands.

Computer-assisted analysis

Exon 2 and the part of the 3'-untranslated regions of human and rat (13) activin β_A subunit genes were compared. Dot-plots were obtained using a computer program, HARPLT2 (SDC-GENETYCS) based on that described by Staden (44).

Results

Isolation and characterization of the human activin βA subunit gene

Three overlapping phage clones containing the human activin βA subunit gene were isolated from human genomic library (Fig. 3). To examine whether the cloned genes were rearranged, the cloned and genomic DNAs were analyzed by Southern blot hybridization with the region-specific probes 1 to 5, those which expected to cover the entire human activin βA subunit gene (Fig. 4A). A single hybridizing fragment of human genomic DNA was found in each restriction digest (Fig. 4C). The same hybridizing pattern was also detected in the recombinant phage DNAs (Fig. 4B), indicating that there is no gross rearrangement during the cloning procedure. This also suggested that there is a single gene encoding activin βA subunit in the human genome.

Nucleotide sequence analysis of the human activin βA subunit gene

Nucleotide sequence determination of the human activin βA subunit gene (Fig. 5) and comparison of this sequence with previously reported human activin βA subunit cDNA sequences (12,45) gave the map of two exons and a 9-kb intron that interrupts the first nucleotide of the 130th amino acid codon. This organization and the size of the exons are almost identical to those reported by others (36,46). Furthermore, no differences are found between the cDNA and exon sequences in the protein-coding regions of the activin βA subunit.

Characteristic features of sequences of the activin βA gene are listed in table 1, a part of which are indicated in Fig. 3.

Features of the 5'-flanking region

Because most regulatory elements for gene expression are located in the 5' flanking

region, DNA sequence of 1857 nucleotides 5'-upstream to the ATG initiation codon were determined (Fig. 5). This region possesses a classical TATA box, which is known to be the binding site for TFIID (47). It has been demonstrated that the binding of this factor to TATA box enables the cell to initiate transcription with accuracy (48). At 56 nucleotides 5' of the TATA box, the CAAT box was also found, which is critical for transcription of numerous cellular and viral genes (49). Mason *et al.* (40) have determined the structure of human activin β_B subunit gene and identified the extensive GC-rich sequences containing the eight GC boxes, the biding site for the transcription factor Sp1 (50), and three cAMP-responsive elements (51) in its 5'-flanking region. No such sequences were present at the 5'-flanking region of the β_A subunit gene.

Another interesting feature of this flanking region is a 263-bp pyrimidine-rich sequence consisting of 83 CT dinucleotide repeats. *In vitro* studies have shown that under superhelical torsion, extended arrays of CT nucleotide repeats can form triplestranded H-DNA (52). Furthermore, it has been reported that a set of 10 CT dinucleotide repeats from a *Drosophila* heat shock gene promoter is capable of binding a specific nuclear protein (53). At present, however, it should be emphasized that there is no evidence that the 5'-flanking region of the human activin β_A subunit gene could be utilized as an *in vivo* promoter.

Sequence of the intron

In eukaryotes, exon/intron junctional structures are well-conserved. Introns are bounded at the 5' end by a conserved sequence of 9 nucleotides, (A/C)AGGT(A/G)AGT. They are bounded at the 3' end by another sequence which is pyrimidine-rich with the consensus sequence, Py-Py-Py-Py-Py-Py-NCAGG (54). The junctional sequences of exon and intron of the human activin β_A subunit gene shows a striking homology to these consensus sequences.

The SV40 enhancer core sequence, GTGGAATG, was found in the 9-kb intron. The activity of such enhancer sequences appears to be relatively independent of the position or orientation of the sequence (55). Indeed, this type of enhancer in the first intron of the human α1 (type I) collagen gene has been shown to activate its own and heterologous SV40 promoters (56). The presence of the enhancer core sequence located in the intron of the activin gene might indicate some functional significance to that of collagen.

Analysis of the 3'-flanking region

Two classes of AT-rich sequences were identified in the 3'-flanking region of the human activin β_A subunit gene. One of which is the eight ATTTA motifs that have been proposed as a mRNA-destablizing element (57). The other sequences are eight polyadenylation signals, AATAAA or ATTAAA, which play an important role in the processing of eukaryotic mRNA maturation (58).

A stretch of $(CA)_{14}$ is located between the second and third polyadenylation signals. Poly (dC-dA) copolymers are ubiquitous component of eukaryotic genomes (59). An additional study has shown that these copolymers can form two different DNA conformations (B and Z forms) depending on enviromental factors (60). There is evidence that such elements enhance gene transcription in a manner similar to that of viral enhancers which also have the potential to assume Z-DNA conformations (61). Similar to the viral enhancers, their activity is relatively independent of position or orientation, although they seem to be more effective when they are closer to the promoter (62).

In addition to the above sequences, two SV40 enhancer core sequences and consensus AP-1 binding site were also found. The latter sequence is recognized by transcription factor AP-1 which is encoded by the proto-oncogene c-*jun* and is known to act as an inducible enhancer capable of activating transcription in response to TPA (63).

Gene expression can be controlled through the interaction of a regulatory protein with

specific DNA sequences (64). If the 3'-flanking regions are involved in such regulatory mechanisms, it should be possible to define them by comparing the 3'-flanking regions of the same gene among different species. To compare the 3'-flanking region of the human activin β_A subunit with that of the rat activin β_A subunit (13), a dot matrix analysis were performed. As shown in Fig. 6, an extended diagonal line of dots denotes that the human and rat sequences are remarkably homologous. In particular, the similar location of CA repeats, SV40 enhancer core sequence, and AP-1 binding site between human and rat is noteworthy. The high degree of conservation in the sequence of the 3'-flanking region suggests that this region could be of functional significance.

Discussion

The human β_A gene was shown to consist of two exons which contain all of the coding sequences; this is analogous to the genes for the human α and β_B subunits (36,40,46). The conservation of genomic structure suggests a common origin for these three related proteins. Interestingly, the genomic structure of the related TGF-β and Mullerian inhibiting substance genes has diverged considerably since these members of TGF-β superfamily are encoded on seven and five exons, respectively (65,66).

Inspite of the structural and functional similarities of activin β_A and β_B subunits regarding FSH secretion from pituitary, the differences between the 5'-flanking sequences of these two subunit genes might imply an independent regulation of both genes. Three putative cAMP-responsive elements (CRE) were identified in the human β_B gene (40) but not in the β_A gene. Follicle maturation is under the control of LH and FSH, which uses cAMP as an intracellular second messenger. Furthermore, the inhibin (consists of α and one of β subunits) is produced primarily by ovarian granulosa and testicular Sertoli cells, and its production is stimulated by FSH and LH. These observations are well consistent with the presence of CRE in the 5' regulatory region of the human β_B gene. However, also the β_A subunit mRNA is expressed in rat ovarian tissues (20), suggesting that the β_A and β_B genes are differently regulated by cAMP, such as posttranscriptional mechanisms.

Neither α nor β_B subunit gene flanking sequence (37,39,40,41) contains classical TATA or CAAT box elements which may appear at 20-30 and 40-100 nucleotides, respectively, upstream from the transcription start site of many protein coding genes (54). In contrast, these sequences were found in the 1866 bp of the 5' flanking region of the β_A subunit gene (Table 1). However, results from primer extension (Fig. 8 in chapter III) and Northern blot analysis (data not shown) indicated that no transcription initiated at 20-30 and 40-100 nucleotides, respectively, downstream from these sequences, suggesting that they

do not serve as promoters for the transcription of β_A subunit gene. The promoters for the genes lacking for TATA box have been divided into two classes. One class contains GC-rich sequences, which are found primarily in housekeeping and growth-related genes (67). These genes characteristically contain several transcription start sites spread over a fairly large region and several potential binding sites for the transcription factor Sp1 with the sequence GGGCGG (GC box) (50). The rat and human β_B subunit gene promoters belong to this class. The second class of promoters without TATA boxes is not GC rich. These promoters are usually not constitutively active but are regulated during differentiation or development. Many of these genes initiate transcription at one or a few tightly clustered sites (68). Which class of promoter the β_A subunit gene possesses could not be determined only by the sequence information.

In this chapter, I have analyzed the structure of the human activin β_A subunit gene and its DNA sequence. Southern blot analysis indicated that there is a single copy of the β_A subunit gene in the human genome. In the process of determining the nucleotide sequence of activin β_A subunit gene, several sequences analogous to potential regulatory elements and multiple polyadenylation signals were identified. The functional significance of these sequences will be experimentally determined in the following chapters.

Table 1 Sequence features of the human activin β_A subunit gene

Feature	From	To	Comments
CCAAT	446	450	CAAT box
TATAAA	512	518	TATA box
CT repeat	1337	1599	
ATG	1858	1860	Initiation codon
CAGGTTGGT	2243	2251	Donor site (= 5'-splice junction)
TCATCTTCAGG	3517	3527	Acceptor site (= 3'-splice junction)
GTGGAATG	2698	2705	SV40 enhancer core element
TAG	4417	4419	Termination codon
CA repeat	4933	4960	
ATTAAA	4521	4526	Polyadenylation signals
or	4565	4570	
AATAAA	5071	5076	
	6596	6601	
	6768	6773	
	6773	6778	
	7204	7209	
	7941	7946	
ATTTA	5399	5403	mRNA-destabilizing elements
	6234	6238	
	6445	6449	
	7259	7263	
	7469	7473	
TGAGTCAG	5621	5628	AP-1 binding site
GTGGATTG	5654	5661	SV40 enhancer core elements
	8055	8062	

All the sequences are on the coding strand.

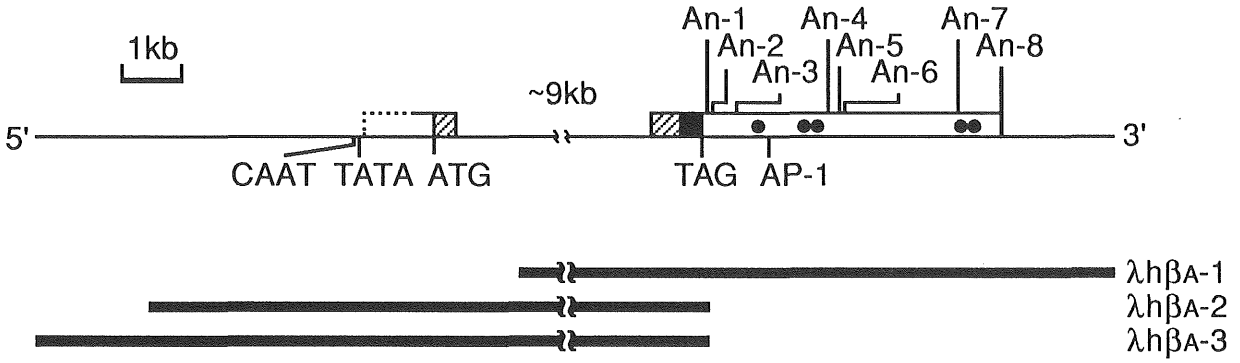


Fig. 3 Schematic representation of the human activin βA subunit gene. Three overlapping clones were isolated from a *Sau*3AI partially digested DNA library from human placenta DNA. The first line is the joined segment corresponding to the whole phage clones. Exons are represented by boxes, and intron and flanking regions by thin lines. The hatched boxes indicate the precursor sequences. The positions of TATA and CAAT boxes, translation initiation codon (ATG), termination codon (TAG), and AP-1 binding site (AP-1) are shown. Consensus polyadenylation signals (An-1 to An-8) are indicated in the 3'-untranslated region. The positions of ATTTA motifs are indicated by solid circles. The extent of individual phage clones are indicated at the bottom.

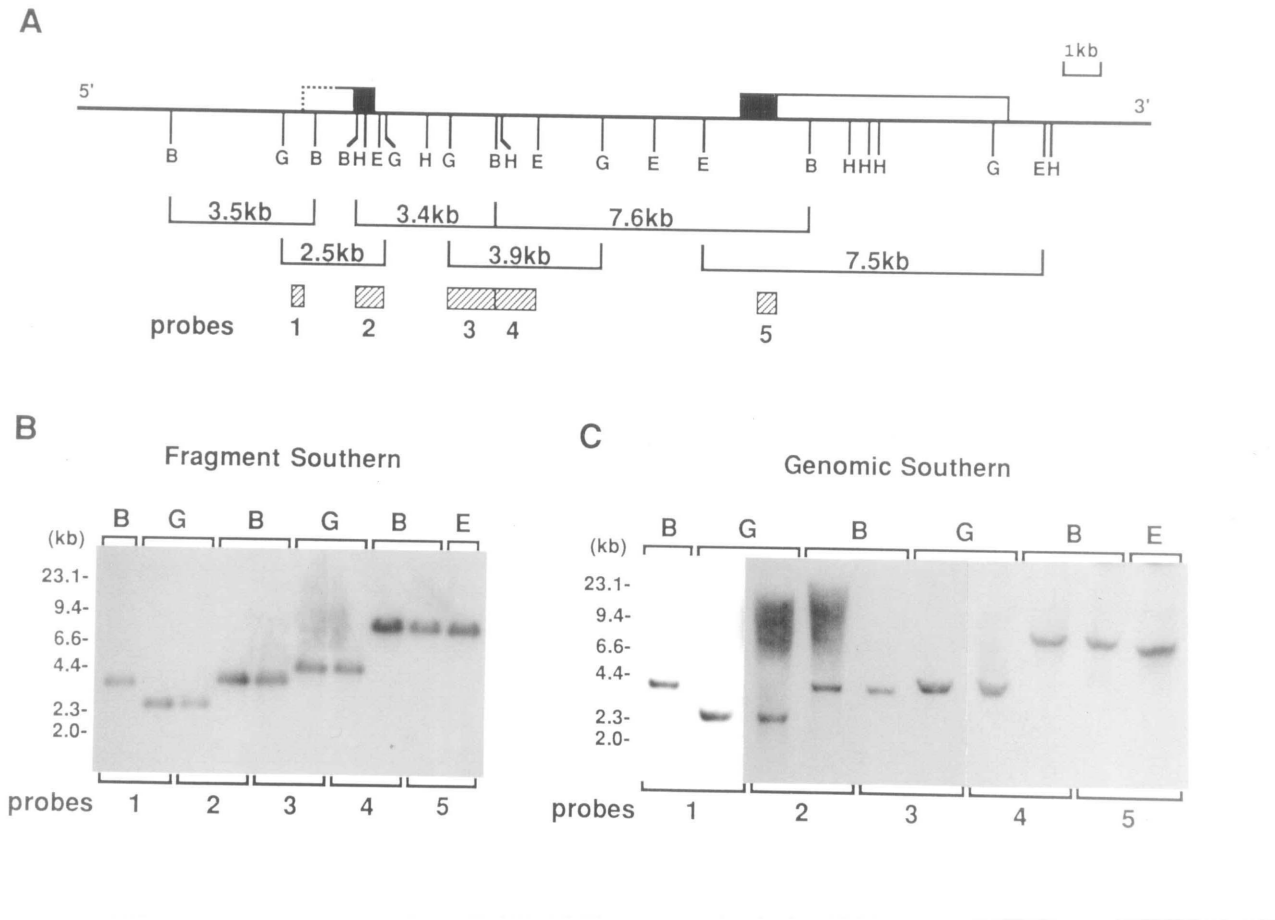


Fig. 4 Southern blot analysis. (A) Restriction map of the human activin βA subunit gene. Non-coding and coding exons are indicated by open and filled-in boxes, respectively. Restriction sites are represented as follows: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; G, *Bgl*II. The positions of the region-specific probes used for Southern blots are shown at the bottom of the panel. (B) Fragment Southern blot. Cloned phage DNAs containing the human activin βA subunit gene were completely digested with *Bam*HI (B), *Bgl*II (G) or *Eco*RI (E). The digests were hybridized with probes shown in panel A. Relative sizes in kilobases (kb) are indicated. (C) Genomic Southern blot. Panel C is identical to panel B except that genomic DNA from human placenta (10 μg/lane) was used for the blot.

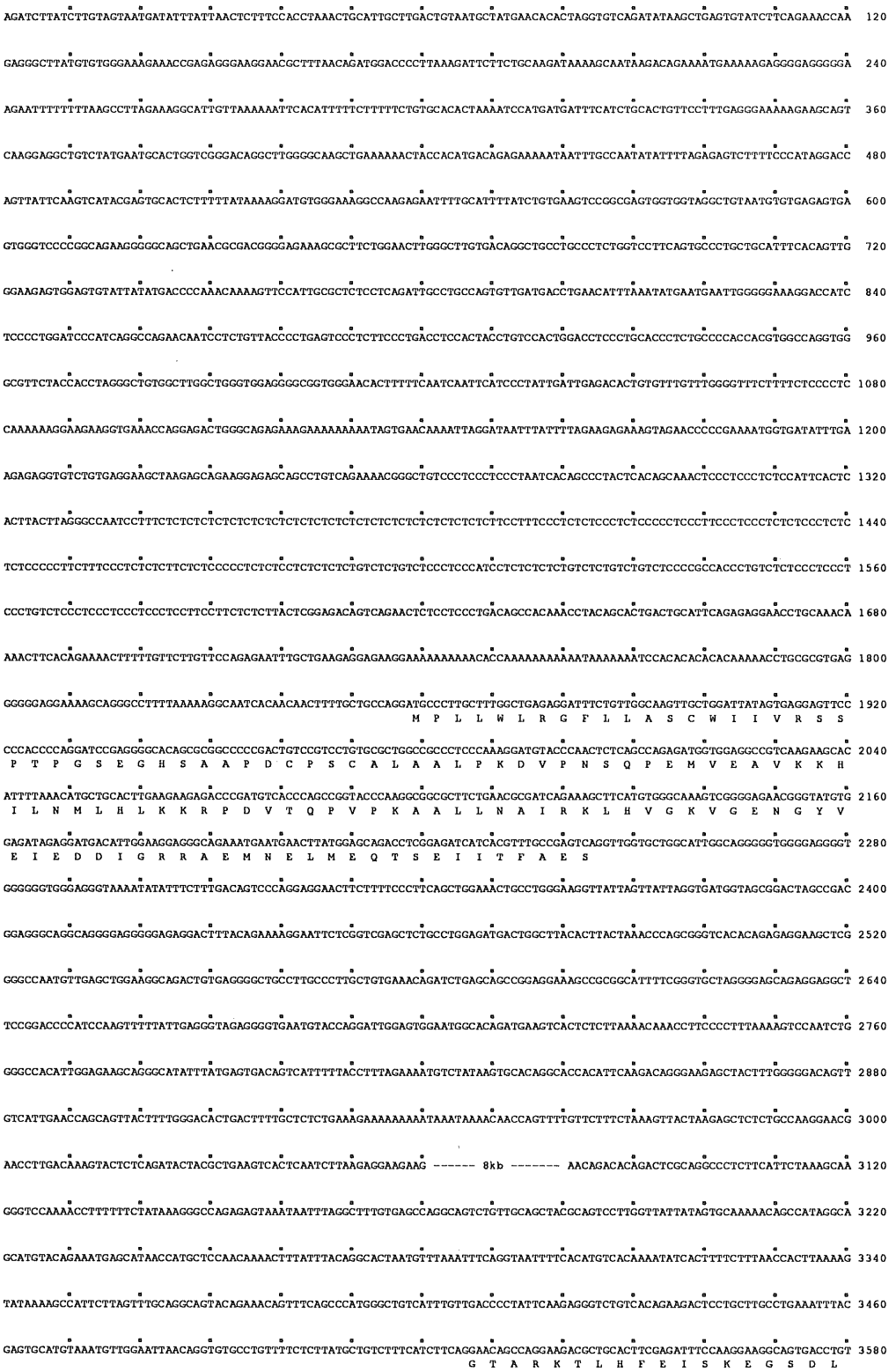


Fig. 5 The nucleotide sequence of the coding strand of the genomic DNA, along with the deduced amino acid sequences of the human activin βA subunit.

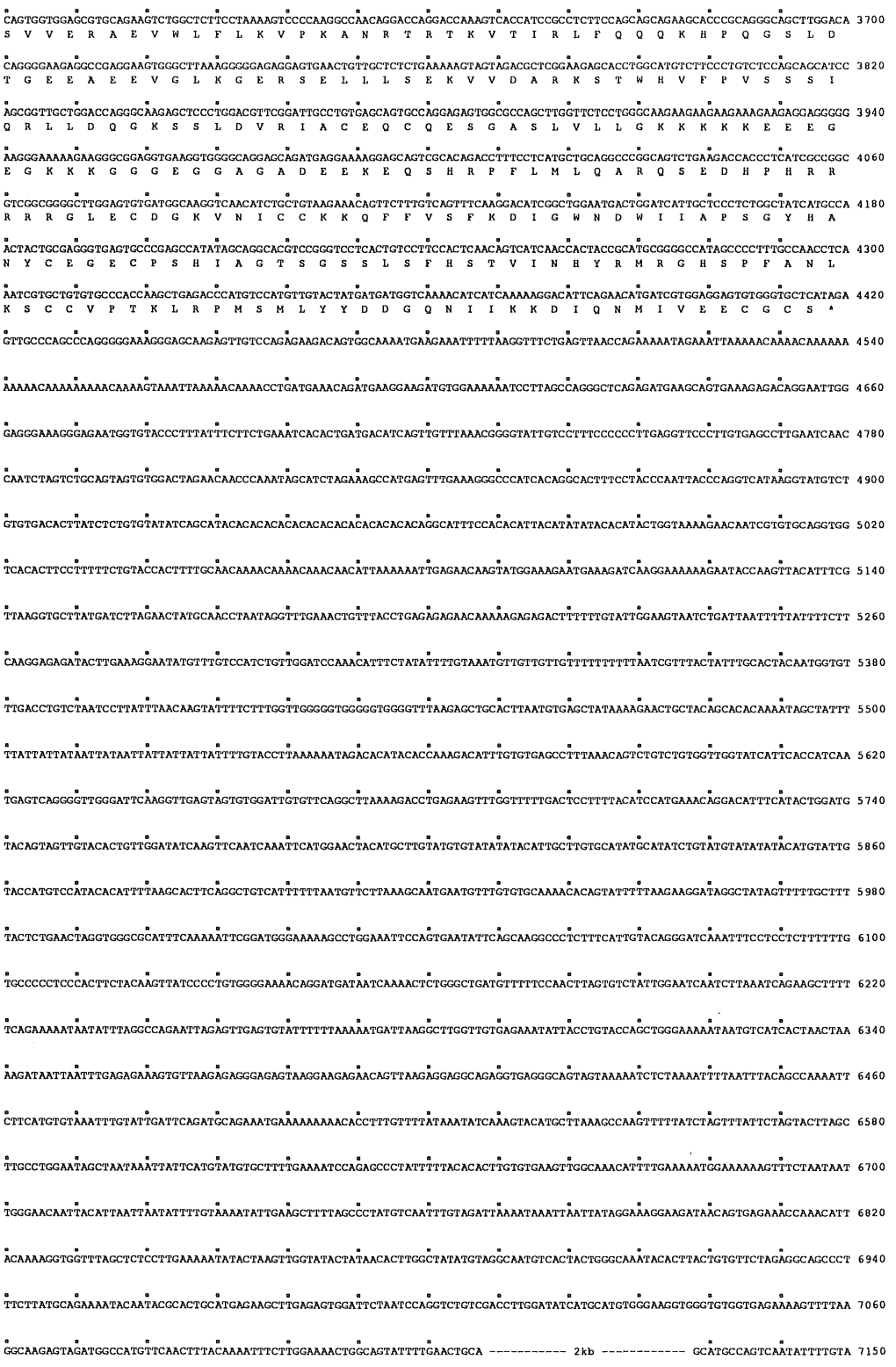


Fig. 5 – Continued

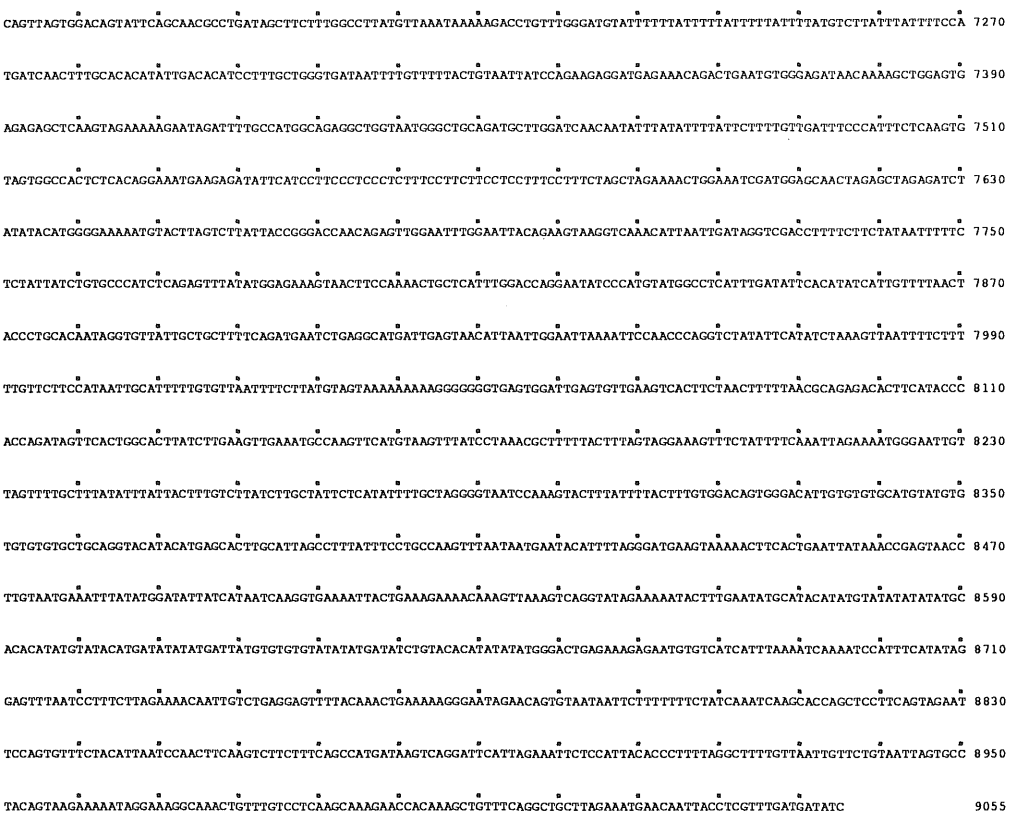


Fig. 5 – Continued

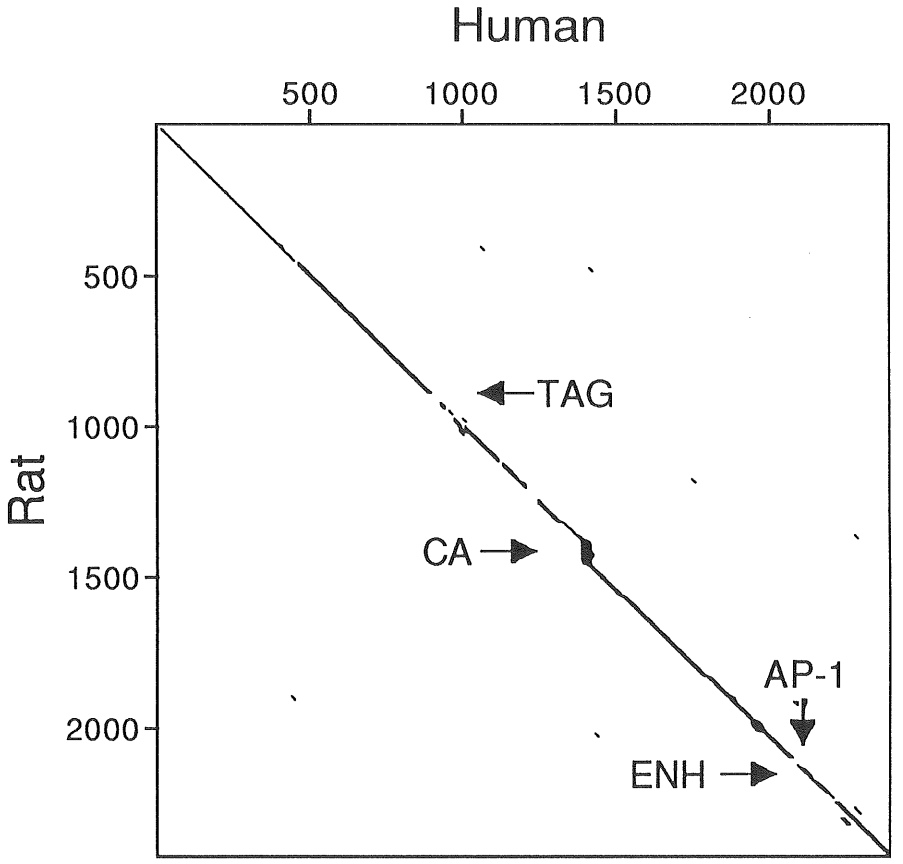


Fig. 6 Comparison of the 3'-untranslated regions of the human (horizontal axis) and rat (vertical axis) activin βA subunit gene. The human sequence determined in this chapter was aligned for maximal match with the rat sequence using the HARPLT2 program (SDC-GENETYCS). The parameters used were a span length of 20 and a proportional matching score of 16. Positions of the termination codon (TAG), CA repeat (CA), AP-1 binding site and SV40 enhancer core sequence (ENH) are shown.

Chapter III: Possible Roles of the 3'-Flanking Sequences of the Human Activin βA Subunit Gene in Its Expression

Summary

Tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) stimulates an increase in erythroid differentiation activity in human fibrosarcoma HT1080 cells. Here, I demonstrated that this process involves a rapid accumulation of five species of activin βA/erythroid differentiation factor mRNA, following protein kinase C activation and that variation in size of the activin transcripts is due to multiple 3' ends, presumably reflecting an alternative polyadenylation. In transiently transfected HT1080 cells, a 97-bp DNA fragment containing an AP-1 consensus sequence (TGAGTCA) located in the 3'-flanking region of the activin gene was capable of activating the heterologous herpes simplex virus thymidine kinase (tk) and SV40 early promoters, and a co-transfected c-Jun enhanced these fusion promoter activities. The deletion of TGAG sequences from AP-1 element in the 97-bp DNA sequence context abolished its c-Jun-mediated activation from the tk promoter even in HT1080 cells overexpressing stably transfected c-Jun. Co-transfected adenovirus E1A products repressed the tk promoter activity enhanced by activin AP-1 element itself or in concert with transiently transfected c-Jun, indicating that the putative AP-1 sequence acts as an activator element, depending upon c-Jun activity. These results suggest that the 3'-flanking DNA sequences of the human activin βA subunit gene plays an important role in its expression.

Introduction

Tumor-promoting phorbol esters, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), induce a variety of changes in cell morphology, metabolism, and gene expression. These alterations are largely a consequence of the activation of protein kinase C, the principal cellular target of TPA. As various extracellular stimuli, including antigens, hormones, and growth factors, utilize the protein kinase C signal transduction pathway, the changes in gene expression mediated by these agents overlap with those induced by TPA.

It has recently been demonstrated that activin/EDF activity is induced by TPA in human fibrosarcoma HT1080 cells (69). Additionally, I have found two kinds of *cis*-acting sequence elements, eight hexanucleotide-sequence clusters (AATAAA or ATTAAA) and a potential activator protein (AP)-1 element (TGAGTCA), in the 3'-flanking region of the human activin β_A subunit gene (see chapter II). The former sequences have been demonstrated to play an important role in mRNA processing and polyadenylation (58). The latter sequence has originally been identified as an activator element (70), which binds to transcription factor AP-1, a homodimer of the product of proto-oncogene c-*jun* or a heterodimer of the products of proto-oncogenes c-*jun* and c-*fos* (71). These facts, therefore, prompted me to examine the effect of TPA on the human activin β_A subunit gene expression and possible roles of the activin 3'-flanking sequences in its expression in HT1080 cells. In this chapter, I show that stimulation of multiple species of activin mRNA accumulation by TPA in HT1080 cells is mediated by protein kinase C (PKC) activation and that the differential polyadenylation of the human activin β_A subunit gene results in its mRNA size-heterogeneity. Furthermore, I demonstrate a role of activin AP-1 sequence in heterologous promoter activation as an activator element, depending upon c-Jun activity.

Materials and Methods

Materials

12-*O*-tetradecanoylphorbol-13-acetate (TPA) was purchased from Nacalai Tesque, Inc. and dissolved in absolute ethanol at a concentration of 100 μg/ml. 1-(5-isoquinolinesulfonyl)-2-methyl-piperadine dihydrochloride (H-7) (72) was obtained from Seikagaku Kogyo Co., Ltd. and dissolved in distilled water at a concentration of 10 mM. All other materials are from standard sources unless noted.

Cell culture

Human HT1080 fibrosarcoma cells (American Type Culture Collection, CCL-121) were maintained at 37°C and 5% CO_2 in minimum essential medium (MEM, Nissui Pharmaceutical Co. Ltd.) supplemented with 10% fetal bovine serum (FBS, Flow Laboratories), 0.1 mM nonessential amino acids, and 2 mM L-glutamine.

Northern and Southern blot analyses

Total RNA was isolated from HT1080 cells as described by Gough (73). Poly(A)$^+$ RNA was purified by oligo(dT)-cellulose chromatography. Total or poly(A)$^+$ RNAs were denatured with 1 M glyoxal and 50% dimethyl sulfoxide, electrophoresed on a 1.2% agarose gel, and transferred to GeneScreen Plus (nylon) membrane (Dupont).

Human genomic DNA was obtained from placenta as described in chapter II. Ten micrograms of DNA were digested with various restriction enzymes to completion, electrophoresed on 0.7% agarose gel, and transferred to nylon membrane. Filters were prehybridized for 30 min at 60°C in a solution consisting of 1% sodium dodecyl sulfate (SDS), 1 M NaCl, and 10% dextran sulfate. Hybridization was performed for 16 h at 60°C in the same solution containing 300 μg/ml denatured salmon sperm DNA and 1 x 10^6

cpm/ml of the ^{32}P-labeled probes. Filters were washed twice with 2 x SSC (1 x SSC=0.15 M NaCl and 0.015 M sodium citrate) for 5 min at room temperature, twice with 2 x SSC with 1% SDS for 30 min at 60°C, and twice with 0.1 x SSC for 30 min at room temperature. Autoradiograms were performed with an intensifying screen at -70°C.

The region-specific hybridization probes excised from the human activin βA genomic clone were as follows: probe 5, 412-bp *Hin*cII fragment; probe 6, 512-bp *Pst*I-*Bam*HI fragment; probe 7, 684-bp *Dra*I fragment; probe 8, 890-bp *Pst*I fragment; probe 9, 317-bp *Sph*I-*Pst*I fragment; and probe 10, 750-bp *Eco*RV-*Hin*dIII fragment.

Primer extension

The 5'-ends of the activin transcripts were determined by extension of an oligonucleotide primer 5'-GCAAAAGTTGTTGTGATTGC-3' complementary to nucleotides 1831 to 1850 of the activin βA subunit genomic sequence (see chapter II). The primer was 5'-end labeled using [γ-^{32}P]-ATP and T4 polynucleotide kinase to a specific activity of 2 x 10^8 cpm/μg. The labeled primer (5 x 10^5 cpm) was annealed with 10 μg of RNA from human placenta or 5 μg of RNA from HT1080 cells, in the presence of 10 mM Tris-HCl (pH 7.9), 1 mM EDTA, and 250 mM KCl in 10 μl total volume. The annealed primer was then extended by the addition of 24 μl of a mixture consisting of 10 mM Tris-HCl (pH 8.7), 10 mM $MgCl_2$, 5 mM DTT, 100 μg/ml actinomycin D, 0.4 mM of dNTPs, and 10 units of Rous associated virus (RAV-2) reverse transcriptase (TAKARA), as described by Bodner *et al.* (74). The extension products were precipitated in ethanol, denatured, and subjected to electrophoresis on a 5% (w/v) polyacrylamide/8 M urea gel, in parallel with size markers comprising ^{32}P-labeled *Hin*cII- or *Hae*III-digested φX174 DNA.

Plasmid constructions

To construct p0/tk-CAT, the herpes simplex virus (HSV) thymidine kinase (tk)

promoter sequences (75) from nucleotides -109 to -80 flanked by *Xba*I and *Eco*RI compatible ends was chemically synthesized and ligated to sequence at nucleotides -79 to +19, isolated as an *Eco*RI-*Pst*I fragment from the pMC1neo (76). The resultant fragment was cloned into the *Xba*I/*Pst*I sites of pUC19, excised with *Xba*I and *Hin*dIII, and inserted into the *Xba*I/*Hin*dIII sites of promoter-less chloramphenicol acetyltransferase (CAT) vector, pUCSV0cat (77).

To construct pUCSV3GT-CAT, pSV2cat (78) was digested with *Sph*I, blunt-ended, and ligated to *Bgl*II linker. The DNA was digested with *Bgl*II and *Hin*dIII to generate a 204-bp fragment carrying the 21-bp repeated sequences (GC box) and TATA box. This fragment was inserted into the *Bgl*II/*Hin*dIII sites of pUCSV0CAT.

A 97-bp activin βA subunit fragment carrying a consensus sequence of AP-1 binding site (5'-TGAGTCA-3') was excised from the genomic clone containing the human activin βA gene with *Dra*I and *Dde*I (positions 5584 and 5680, respectively) (see chapter II), blunt-ended, ligated to *Bgl*II (5'-end) and *Bam*HI (3'-end) linkers, and digested with *Bgl*II and *Bam*HI. Two copies of this fragments were inserted into the *Bgl*II sites of pUCSV3GT-CAT and p0/tk-CAT to construct pβA/AP-1/GT-CAT and pβA/AP-1/tk-CAT, respectively.

To construct pβA/AP-1mut/tk-CAT, the 97-bp *Bgl*II-*Bam*HI activin βA subunit fragment was subcloned into the *Bgl*II/*Bam*HI sites of the modified version of pBluescriptII/KS(+) (*Sma*I site is converted to *Bgl*II site by linker insertion). Using this plasmid as a template, site directed mutagenesis was performed by overlap extension method using PCR (79,80). The sequences of mutagenic primers were as follows; upper strand primer: 5'-TCATTCACCATCAATCAGGGGTTGGGATTC-3' (positions 5607 to 5640 of the activin βA subunit gene with deletions at positions 5621 to 5624) and lower strand primer: 5'-GAATCCCAACCCCTGATTGATGGTGAATGA-3' (complementary to upper-strand primer). 5' and 3' primers were designated to flank the multi-cloning site of

the vector. The PCR product was excised with *Bgl*II and *Bam*HI, gel-purified, and inserted into the *Bgl*II site of p0/tk-CAT. Mutations were verified by DNA sequencing.

pUC-E1A expressing E1A products was constructed as follows. Adenovirus type 2 genomic DNA (81) was digested with *Bal*I, ligated to *Sac*I linker, and digested with *Sac*I to generate a 1502-bp fragment (between positions 270 to 1771). This fragment carrying the E1A promoter and the E1A structural gene was ligated to the *Sac*I site of pUC19. To construct the control plasmid pUC-E1Apr., pUC-E1A was digested with *Sac*I and *Sau*3AI to generate a 357-bp fragment (between positions 270 and 626) carrying the E1A promoter. This fragment was ligated to the *Sac*I/*Bam*HI sites of pUC19.

pRSV-c-Jun, in which the human c-Jun cDNA was linked to the long-terminal repeat of Rous sarcoma virus, was kindly provided by Professor Michael Karin (University of California, San Diego) (82).

Establishment of stable cell lines overexpressing human c-Jun

Stably transformed cell line (HT-cJun) was derived by co-transfection of 15 μg of pRSV-c-Jun with 3 μg of pSV2-neo into HT1080 cells by the calcium phosphate coprecipitation method. Colonies of cells expressing the *neo* gene were selected by resistance to G418 (Geneticin, Sigma Chemical Co.; 480 μg/ml). Approximately 50-100 colonies were pooled to provide bulk transfected cultures.

Transfection assays

HT1080 cells were plated in MEM supplemented with 10% FBS at 2 x 10^5 cells per 60 mm diameter dishes 20 h prior to DNA transfection. Three hours before transfection, the dishes received fresh medium. Cells were transfected with the different CAT-constructs by the calcium phosphate coprecipitation method, exposed to the the precipitate for 6 to 8 h, washed twice in phosphate buffered saline, and kept in fresh medium for 24 to 36 h. Cells

were collected and cell extracts were prepared by freezing and thawing, and CAT activity was determined as described by Gorman *et al.* (78). CAT activity was quantitated using a FUJIX BIO-imaging Analyzer BAS2000 (FUJI Photo Film).

Results

Effect of TPA on activin βA accumulation in HT1080 cells

Addition of TPA to HT1080 cells leads to an increase in EDF/activin activity (69). To examine effect of TPA on levels of activin βA mRNA using this cell line, total RNA was isolated and analyzed by Northern hybridization. Multiple activin mRNAs (6.4, 4.9, 4.3, 3.0, and 2.0 kb) were observed maximally at a concentration of 100 ng/ml (Fig. 7A). Figure 7B showed a detailed time course for the appearance of activin mRNA during TPA treatment (100 ng/ml). Five species of activin mRNA were seen, with sizes of 6.4, 4.9, 4.3, 3.0, and 2.0 kb, and the maximum level of activin mRNA accumulation occurred about 3 h after treatment of the cells with TPA. The mRNA levels then gradually decreased and returned to background levels by 48 h.

The above results raised the possibility that accumulation of activin βA mRNA caused by TPA is mediated by PKC activation. To test whether PKC activation is necessary for the induction of activin mRNA by TPA, HT1080 cells were stimulated with TPA for 4 h in the presence of H-7 which has been reported to be the most potent and selective PKC inhibitor with K_i of 6 μM (72). As shown in Fig. 7C, increasing concentrations of H-7 (5 to 50 μM) were effective to block the induction of activin mRNA. This suggested a possible involvement of PKC activation in the induction pathway of activin mRNA accumulation.

Identification of multiple start sites of the activin βA subunit transcripts

I postulated that the observed multiple transcripts could arise mainly from two possible mechanisms: (a) multiple sites of transcription initiation; or (b) alternative polyadenylation. First, I determined the transcription start sites of the human activin βA subunit gene by extension of a synthetic oligonucleotide (see Materials and Methods) (Fig. 8) and for this analysis, RNA derived from HT1080 cells and human placenta was used as templates because human placenta is one of the major source of activin βA subunit mRNA (chapter

IV). Two major primer-extended products were observed in human placenta total RNA (Fig. 8, lane 2), with length corresponding to transcription start sites at positions 1627 and 1646 (see chapter II). Additional minor bands were also found in poly(A)$^+$RNA extracted from human placenta (lane 3) and from TPA-induced HT1080 cells (lane 5). No detectable primer-extended product was seen in poly(A)$^+$RNA from uninduced HT1080 cells (lane 4). This experiment indicated that the transcription start sites are clustered within the short DNA regions between about 250 to 180 bp 5' to the predicted start site of translation. These results, therefore, led me to consider that the transcription from the multiple start sites did not contribute largely to the generation of multiple activin β_A transcripts.

Generation of multiple activin mRNAs by differential polyadenylation

In chapter II, I have reported a single gene for human activin β_A subunit, which consists of two exons interrupted by a 9-kb intron, and identified possible polyadenylation sites, AATAAA or ATTAAA, located in the 3'-flanking region (the relative location of these sites is indicated in Fig. 9). Thus, I next tested the above second possibility that multiple species of human activin mRNA could be generated from the single gene by the use of the different polyadenylation sites. For this purpose, Northern blots were performed to poly(A)$^+$RNA prepared from TPA-stimulated HT1080 cells, using the six DNA fragments located among the putative polyadenylation signals as region-specific probes (probes 5 to 10) (Fig. 9A). As shown in Fig. 9B, probe 5 hybridized with 6.4-, 4.9-, 4.3-, 3.0-, and 2.0-kb activin mRNAs. Compared with probe 5, probe 6 detected the 6.4-, 4.9-, 4.3-, and 3.0-kb species of mRNA, but not the 2.0-kb species of mRNA. This is the expected results since probe 6 is located 3' to the polyadenylation signal for the 2.0-kb activin mRNA. Moving further 3'-end, the next fragment (probe 7) detected the 6.4-, 4.9-, and 4.3-kb mRNAs, but not the 3.0-kb mRNA. A more 3' fragment, probe 8 detected the 6.4- and 4.9-kb mRNAs, as did probe 9; and finally, probe 10 did not detected any species

of activin mRNA. By Southern blot analysis, I confirmed that the probe 10 exists at the expected location 3' to the eighth polyadenylation site (Fig. 9C). These results supported my second idea that the variation in size of activin β_A mRNA originated from the single gene may, at least in part, reflect alternative polyadenylation.

Property of the 3'-flanking DNA sequence in the activin gene as an activator element

I have found an AP-1 consensus sequence, TGAGTCA, located between the second and third polyadenylation signals in the 3'-flanking region of the human activin β_A subunit gene (see chapter II). This sequence element is well known to be required for basal and enhanced activities of certain promoters by the action of transcription factors AP-1 composed of c-Jun/c-Fos or c-Jun/c-Jun (71). To determine whether this consensus sequence of the activin gene could function as an activator element, a 97-bp DNA fragment containing the AP-1 site was cloned into a chloramphenicol acetyltransferase (CAT) expression vector driven by either tk or SV40 early promoters. The doublet DNA fragments were inserted upstream of CAT-coding gene (Fig. 10A) and each CAT expression vector with or without the 97-bp DNA fragment was transiently transfected into HT1080 cells. As shown in Fig. 10B, the tandem DNA fragments activated CAT transcription from SV40 and tk promoters at 3.1 and 5.4 folds, respectively, as compared to the basal CAT activity derived from each control plasmid. These results suggested that the fusion virus promoter activities are potentiated by endogenous AP-1 transcription factors through the activin AP-1 element.

In an attempt to explore if this increase was mediated by AP-1, co-transfection analysis was performed. A c-Jun expression vector (pRSV-c-Jun) was co-transfected with each reporter plasmid containing the doublet AP-1 sites in HT1080 cells. As controls, CAT plasmids possessing either SV40 or tk promoters were used. As demonstrated in Fig. 10B,

co-transfection with pRSV-c-Jun led to a significant increase in CAT activity from the two reporter constructs harboring AP-1 sites as compared to control vectors lacking the *cis*-acting elements.

To examine the functional importance of activin AP-1 sites in the 97-bp fragment context upon the promoter activation mediated by c-Jun, a mutated AP-1 site with TGAG deletion from TGAGTCA was made by oligonucleotide mutagenesis and inserted as the doublet 93-bp fragments in front of the tk promoter (Fig. 10A). In addition, HT1080 cells were stably transformed by co-transfection of pRSV-c-Jun with pSV2-neo, selected G418-resistent colonies, and established cell lines (HT-cJun) overexpressing the transfected c-Jun. Using HT-cJun cells, tk-CAT constructs with either the wild-type AP-1 sites (pβA/AP-1/tk-CAT) or mutated AP-1 sites (pβA/AP-1mut/tk-CAT), or without AP-1 sites (p0/tk-CAT) were transiently transfected and CAT activity was assayed. Fig. 10C represented a typical set of experiments and showed that CAT activity was abolished in HT-cJun cells transfected with tk-CAT harboring the mutated AP-1 sequences, although the enzyme activity was highly expressed in the cells transfected with tk-CAT possessing the wild-type AP-1 sequences. These results indicates that the 97-bp DNA fragment located in the 3'-flanking region of the human activin βA subunit gene could serve as a basal activator and demonstrated that activin AP-1 sequences in the 97-bp DNA context contribute largely to the tk promoter activation mediated by c-Jun. However, it is noted that activin AP-1 element failed to activate transcription from the tk promoter in response to TPA in HT1080 cells, even under serum-starved condition as well as under 10% FBS condition (data not shown).

Repression of c-jun activity by adenovirus E1A products

It has been shown that adenovirus E1A represses transcription of the collagenase gene *via* its AP-1 sequence element by abolishing the *trans*-activation function of the DNA-

bound AP-1 (83). Thus, I tested whether E1A can inhibit expression of the tk promoter containing activin AP-1 element by co-transfection experiments in HT1080 cells. When the construct containing only tk promoter was used as a control, E1A (pUC-E1A) had little effect on the promoter activity (Fig. 11). In contrast, both the basal promoter activity driven by AP-1 element and the induced promoter activity by the transiently transfected c-Jun (pRSV-c-Jun) were effectively repressed by E1A. This repression by co-transfection of pRSV-c-Jun with pUC-E1A was not due to an inhibition of RSV promoter activity by E1A, because it has been demonstrated that E1A did not repress RSV promoter activity (83). These findings further supported the preceding result that the putative AP-1 sequence present in the 3'-flanking region of the human activin β_A subunit gene acts as a functional activator element, depending upon c-Jun activity.

Discussion

I have revealed that TPA induces rapid accumulation of the five activin mRNA species in HT1080 cells, with sizes of 6.4, 4.9, 4.3, 3.0, and 2.0 kb. Examination of nucleotide sequences in the 3'-flanking region of the human activin βA subunit gene provided a structural basis for possible usage of differential hexanucleotide-polyadenylation signals, AATAAA or ATTAAA, those of which are natural variants of functional polyadenylation signals (58). In addition, primer extension data indicate that activin βA transcription initiates at clustered sites within the short DNA region between about 250 to 180 bp 5' to the start of translation. Thus, major variations in activin message size would not be explained by differences in the size of the 5' end. Northern blot analyses suggested that all five activin βA mRNA species are polyadenylated based on the fact that they are present in poly(A)$^+$ selected RNA. Examination using region-specific DNA probes derived from sequences located between respective polyadenylaiotn signals also demonstrated a consecutive loss of hybridization from the smallest to the largest activin transcripts. The loss of message hybridization to each successive region-specific DNA probe corresponded to message sizes expected, if the differences in its sizes were the result of alternative polyadenylation. Taken together with these observations, I conclude that the variation in size of human activin transcripts is caused by multiple 3' ends, presumably due to alternative polyadenylation using the eight possible hexanucleotide motifs. At present, however, the functional significance of both polyadenylation sequences, AATAAA and ATTAAA, upon efficiency of activin mRNA processing is not precisely defined.

It has been shown that the alternative 3' mRNA polyadenylation often affects not only an efficiency of RNA processing, but also its physiological activity. For example, the three IGF-II mRNAs of 4-, 2.2-, and 1.2-kb species in rat, were found to be generated from a single gene, due to the use of different polyadenylation signals in BRL-3A cells (84) and

the 1.2-kb mRNA was translated into IGF-II precursor in cell-free systems (85). Although the 4-kb IGF-II mRNA species was polyadenylated and localized in the cytoplasm, the largest mRNA molecule did not direct synthesis of the precursor protein (84). These findings suggest that the different 3'-untranslated sequences on IGF-II mRNAs could influence translation efficiency. In the case of the human activin β_A subunit gene, however, whether differential processing at the 3' end of the gene plays a physiological role in its bioactivity remains to be determined.

AP-1 recognition sequences, TGA(C/G)TCA, were originally identified as an enhancer element required for optimal basal activity of the human metallothionein IIA and SV40 early promoters (70). To assess the functional role of activin AP-1 element, four types of experiments were performed as follows: (a) activation of tk and SV40 early promoters by fusing the 97-bp DNA fragment containing AP-1 element located in the 3'-flanking region of the activin gene, (b) enhancement of the induced fusion promoter activity by c-Jun, (c) reduction of the fusion tk promoter activity by the internal deletion of AP-1 sequences from the 97-bp DNA sequence context, and (d) repression of both the induced and enhanced fusion tk promoter activity by adenovirus E1A gene products. The first experiment suggests that the 97-bp DNA fragment containing AP-1 element is capable of activating tk and SV40 early promoters, probably due to the action of endogenous AP-1, in transiently transfected HT1080 cells. The second experiment confirms the first suggestion by showing that these potentiated promoter activities by the fused doublet DNA fragments are further enhanced by transiently transfected c-Jun. In the third experiment, the deletion of TGAG sequences from activin AP-1 sequences (TGAGTCA) was unable to confer promoter inducibility on the 97-bp DNA sequence context even in HT-cJun cells where the transfected c-Jun stably overexpresses. In the fourth experiment, a transient co-transfection analysis using adenovirus E1A gene products revealed that activation of tk promoter by the 97-bp DNA segment itself or in concert with co-transfected c-Jun was repressed by E1A.

This is consistent with the fact that E1A represses transcription of the collagenase gene *via* its AP-1 site (83). Therefore, the results from these four experiments led me to conclude that the activin AP-1 site-containing DNA segment could act as an activator element, depending upon c-Jun activity.

Activation of PKC is well known to contribute largely to the alteration in gene expression following administration of TPA (86). The fact that the response of activin mRNA accumulation to TPA can be blocked by an effective inhibitor of PKC, H-7, provides evidence that PKC activation is a necessary step for the activin gene expression through the intracellular signalling mechanism. AP-1 consensus sequences are shown to be one of nuclear target elements for the signal transduction pathway stimulated by activators of PKC such as TPA (63). Surprisingly, however, the 97-bp DNA segment of the human activin β_A subunit gene failed to further activate the heterologous tk and SV40 early promoters when HT1080 cells were treated with TPA (data not shown), suggesting that activin AP-1 element itself is insufficient to mediate stimulation of the activin gene expression by TPA. This implicates that activin AP-1 element is required for the basal expression of the activin gene in HT1080 cells but not for TPA response. In fact, two similar situations have been reported that AP-1 element in the human stromelysin gene promoter (87) and the rat JE gene promoter (88) is necessary for their basal expression but not for TPA response of these genes. From these observations, one interesting aspect of the inability of activin AP-1 element to activate promoters in response to TPA is supposed as follows: TPA cancels a negative regulatory mechanism which might normally function to repress the AP-1-mediated transcription of the activin gene, thereby a combination of activin AP-1 element and additional sequences is needed for its gene activation in response to TPA.

In this chapter, I have presented three major conclusions: (a) PKC activation is required for activin mRNA accumulation in response to TPA, (b) variation in size of the activin

transcripts is due to multiple 3' ends, presumably reflecting an alternative polyadenylation, and (c) activin AP-1 element located in the 3'-flanking region could serve as an activator, depending upon c-Jun activity.

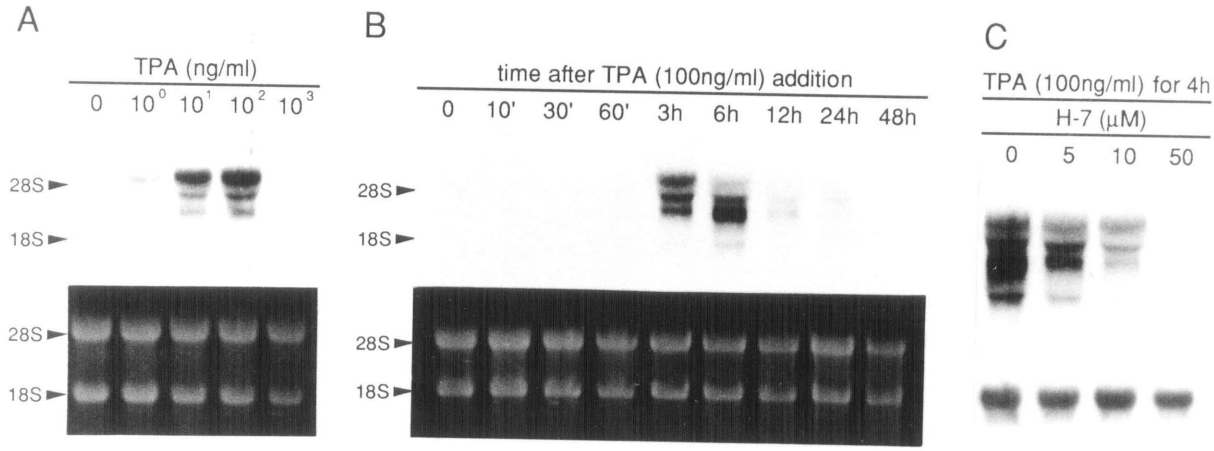


Fig. 7. Accumulation of human activin βA subunit mRNA in TPA-induced HT1080 cells. (A) Effect of TPA concentration on level of activin mRNA. HT1080 cells were maintained in growth medium (MEM, 10% FBS) until subconfluent. The cells were then treated with indicated concentrations of TPA for 4 h. Total RNA was prepared and 15 μg of RNA from each sample were electrophoresed on a 1.2% agarose gel. After transfer to Nylon membrane, human activin βA subunit mRNA was detected using probe 5. Total RNA was stained with ethidium bromide as a control of the amount of RNA applied. The relative migration of 28S and 18S rRNA is indicated on the left vertical axis. (B) Time course induction. The cells were grown until subconfluent and then treated with TPA (100 ng/ml) for different times as indicated. Northern blot analysis was performed as described above. (C) Effect of H-7 on accumulation of TPA-induced activin mRNA. The cells were grown until subconfluent and then exposed to TPA (100 ng/ml) in the absence or presence of indicated concentrations of H-7 for 4 h. Cells were harvested and total RNA was extracted. Northern blot analysis was performed as described above. Expression of β-actin gene (89) is shown as an internal control.

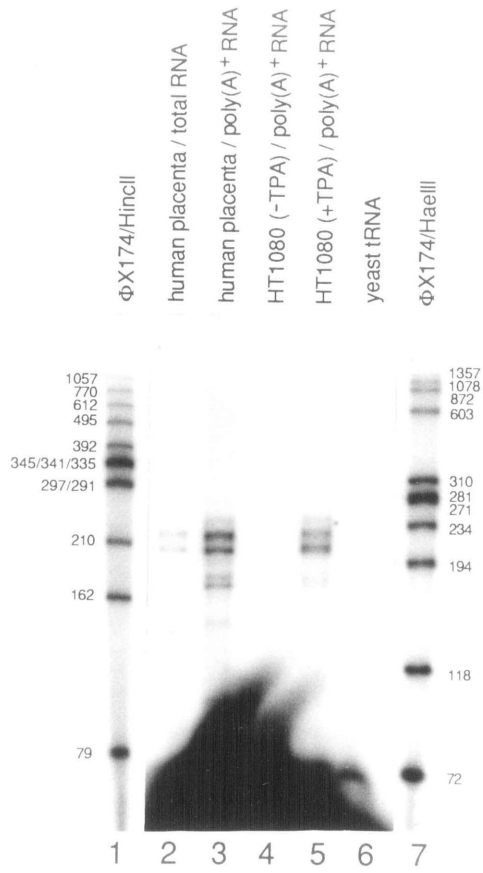
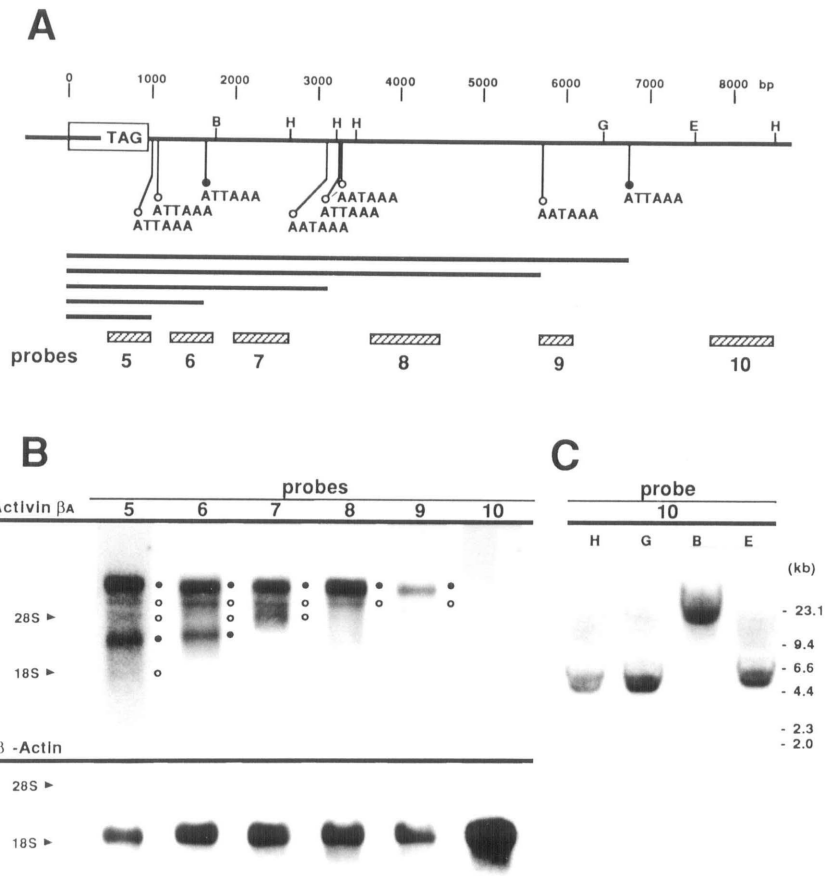


Fig. 8. Determination of the transcription start sites of the activin βA subunit gene by primer extension analysis. A radiolabeled oligonucleotide corresponding to positions -8 to -27 of activin coding sequences was hybridized, at 55°C for 1 h, with 10 μg of total (lane 2) or poly(A)$^+$RNA (lane 3) from human placenta and with 5 μg of poly(A)$^+$RNA from TPA (100 ng/ml for 4 h)-treated (lane 5) or non-treated (lane 4) HT1080 cells. Yeast tRNA (10 μg) was used as a negative control (lane 6). Following hybridization, the primer was extended by RAV-2 reverse transcriptase at 37°C for 45 min. The extended cDNA was analysed on a 5% denaturing polyacrylamide gel followed by autoradiography. Size markers (in nucleotides) were derived from φX174 DNA cut with *Hin*cII or *Hae*III.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- 9.4
- 6.6
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- 2.3
- 2.0

Fig. 9. Multiple polyadenylation signals in the 3'-flanking region of the human activin βA subunit gene. (A) Structure of the 3'-flanking region of the activin gene is shown with partial restriction enzyme map. The protein coding region in exon 2 is represented by open box with its termination codon (TAG). Positions of the polyadenylation sites (AATAAA or ATTAAA) are indicated by vertical lines with open or solid circles. Predicted transcripts are indicated by bold horizontal lines. The probes used for Northern and Southern blot analyses are shown at the bottom of the panel. Restriction enzymes sites: B, *Bam*HI; H, *Hin*dIII; G, *Bgl*II; E, *Eco*RI. (B) Northern blot analysis. Four micrograms of poly(A)$^+$RNA from TPA-induced HT1080 cells (100 ng/ml for 4 h) were denatured, electrophoresed on a 1.2% agarose gel, and transferred to Nylon membrane. The RNA was analyzed using series of ^{32}P-labeled probes (probes 5 to 10) derived from the genomic clones of the activin gene. Probes are indicated above each panel. Open and solid circles to the right of each lane indicate the transcripts originated from the corresponding polyadenylation sites (see panel A). After a set of experiments, filters were washed five times with 0.01 x SSC, 0.01% SDS at 90°C for 3 min and rehybridized with ^{32}P-labeled β-actin probe. Expression of β-actin gene is shown as an internal control of the quality and quantity of RNA applied. The positions of 28S and 18S rRNA markers are indicated. (C) Southern blot analysis. Genomic DNA (10 μg/lane) from human placenta was digested to completion with *Hin*dIII (H), *Bgl*II (G), *Bam*HI (B), and *Eco*RI (E), electrophoresed on a 0.7% agarose gel, and transferred to Nylon membrane. Filter was then hybridized with probe 10. Markers are provided by λ/*Hin*dIII fragments on the right of the panel.

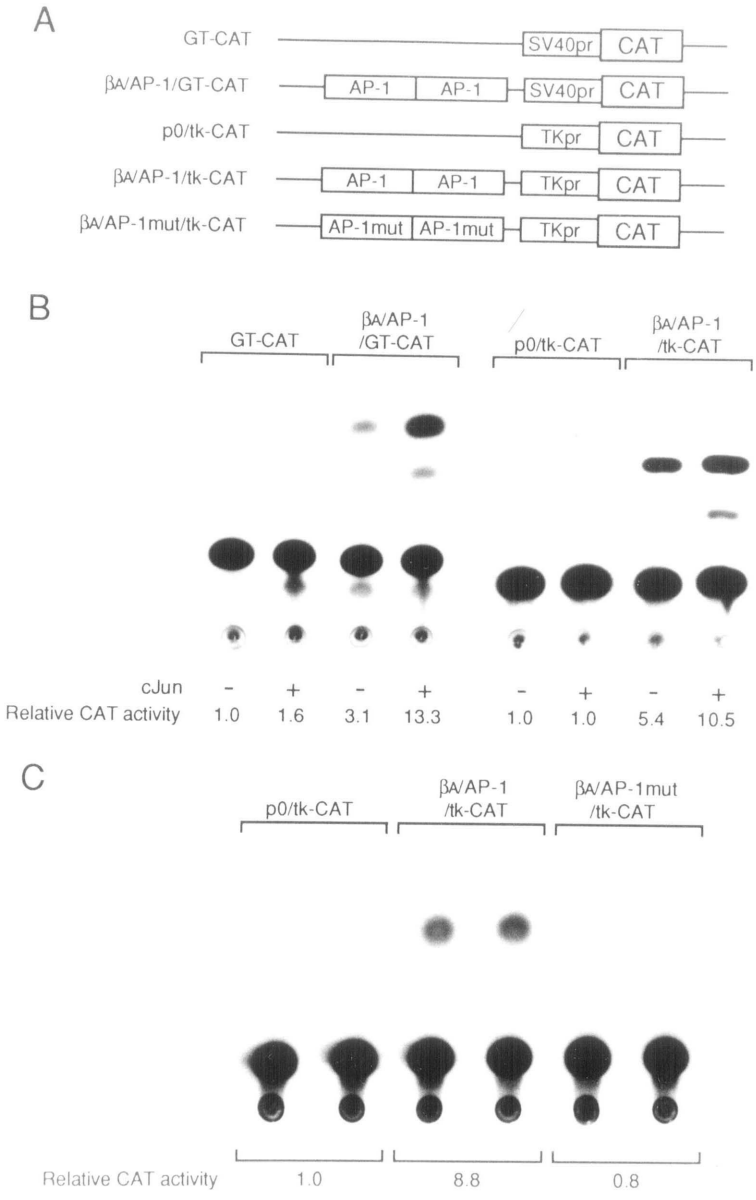

A
GT-CAT
SV40pr
CAT
βA/AP-1/GT-CAT
AP-1
AP-1
SV40pr
CAT
p0/tk-CAT
TKpr
CAT
βA/AP-1/tk-CAT
AP-1
AP-1
TKpr
CAT
βA/AP-1mut/tk-CAT
AP-1mut
AP-1mut
TKpr
CAT
B
GT-CAT
βA/AP-1
/GT-CAT
p0/tk-CAT
βA/AP-1
/tk-CAT
cJun - + - + - + - +
Relative CAT activity 1.0 1.6 3.1 13.3 1.0 1.0 5.4 10.5
C
p0/tk-CAT
βA/AP-1
/tk-CAT
βA/AP-1mut
/tk-CAT
Relative CAT activity 1.0 8.8 0.8

Fig. 10. Inducibility of AP-1 element by c-Jun. (A) Structure of the constructs to test the role of activin AP-1 element. GT-CAT (pUCSV3GT-CAT) and p0/tk-CAT are the basic constructs containing the minimal promoter regions of SV40 (SV40pr) or HSV-tk (TKpr) gene fused to a bacterial chrolamphenicol acetyltransferase (CAT) gene. βA/AP-1/GT-CAT and βA/AP-1/tk-CAT are derivatives of the basic constructs in which the doublet AP-1 elements derived from the 3'-untranslated region of the activin gene are inserted 5' to the basal promoters. βA/AP-1mut/tk-CAT is the same construct as the βA/AP-1/tk-CAT except that four nucleotides crucial for AP-1 binding activity have been deleted from the AP-1 consensus sequences. (B) *Trans*-activation experiments. HT1080 cells were cotransfected with 4 μg of reporter plasmid illustrated above and 1 μg of pRSV-c-Jun (+) or pUC19 (-). After 36 h, cells were harvested and CAT assay was performed as described in Materials and Methods. Relative CAT activities were determined by averaging several independent experiments and comparing them to the basal level activities obtained with the basic constructs without AP-1 elements. (C) Effect of deletion mutation on c-Jun responsiveness of AP-1 element. HT-cJun cells, stably transformed HT1080 cells with plasmid pRSV-c-Jun, were transiently transfected with 3 μg of reporter plasmids indicated above each lane. After 24 h, cells were harvested and CAT assay was performed as described in Materials and Methods. Relative CAT activities were determined by averaging four independent experiments and comparing them to the activities obtained with p0/tk-CAT.

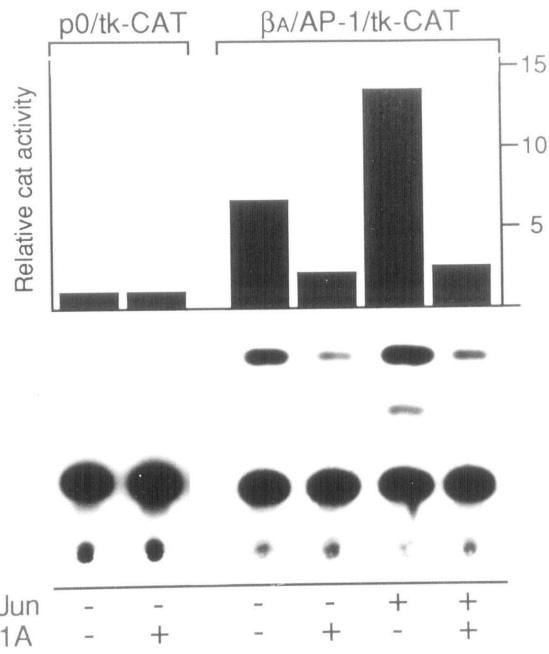


Fig. 11. Effect of E1A products on c-Jun-induced activation of AP-1 element. HT1080 cells were cotransfected with 4 μg of reporter plasmids (p0/tk-CAT or βA/AP-1/tk-CAT) and 1 μg of expression vectors coding for either c-Jun (cJun) or E1A products (E1A). In experiments, a 4:1 ratio of reporter plasmid to each of the expression vectors was used and the total amount of DNA was maintained constant at 6 μg by the addition of plasmid pUC19 or pUCE1A-pr containing only E1A promoter region. CAT assay was performed as described in Materials and Methods.

Chapter IV: Regulation of Activin β_A mRNA Level by cAMP

Summary

I demonstrated the presence of five species of the activin β_A mRNA in human placenta and one major RNA associated with four minor RNAs of the activin in the fetal membrane. The effect of 8-bromo-cAMP (8-Br-cAMP) on accumulation of activin β_A subunit mRNA was investigated in human fibrosarcoma HT1080 cells. Although low levels of the activin mRNA were detectable in the untreated cells, the one main RNA species was predominantly accumulated by 8-Br-cAMP. Generation of one major activin mRNA in the fetal membrane and cAMP-treated HT1080 cells is proposed to be due to a cell-specific alternative polyadenylation.

Introduction

Cellular activities including gene expression are under the influence of a variety of external stimuli, those of which from the cell surface to the nucleus is mediated by two types of second messengers; cyclic AMP (cAMP) and diacylglycerol. Inhibin activity has been reported in the human placenta (90), and human chorionic gonadotropin (hCG) has shown to regulate the secretion of inhibin from a primary culture of human placental trophoblasts in a cAMP-dependent manner (91). Although the presence of mRNAs for human inhibin α subunit (92) and rat activin βA subunit (20) was established in placenta, expression of β subunits has not been investigated in human placenta.

Data from several laboratories showed that FSH, a major regulator of inhibin production in Sertoli cells , increases steady state α, but not βB, mRNA levels *in vivo* and *in vitro* (93-97). Because the FSH receptor is a G-protein-coupled receptor that upon binding to FSH can stimulate adenyl cyclase activity (98), the actions of FSH on Sertoli cell inhibin production are believed to be regulated *via* the cAMP-mediated pathway. Furthermore, 8-Br-cAMP has been shown to induce rat inhibin α and βB mRNA levels in primary granulosa (38) and Sertoli cells (99,100). Although it has been shown that EDF/activin A activity is induced by 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) in human leukemia THP-1, HL-60 (45,101), and human fibrosarcoma HT1080 cells (chapter III), the regulation of the human activin βA subunit gene by cAMP remaines unclear.

In this chapter, I found activin βA mRNA in human placenta and fetal membrane, and examined the effects of cAMP on levels of the activin βA subunit mRNA in HT1080 cells.

Materials and Methods

Cell Culture

Human fibrosarcoma HT1080 (CCL121) and cervical carcinoma HeLa (CCL2) cells were obtained from American Type Culture Collection. These cells were maintained at 37°C in minimum essential medium (GIBCO or SIGMA) supplemented with 10% fetal bovine serum (FBS), nonessential amino acids (0.1mM), and L-glutamine (2mM).

Northern blot analysis

Total RNA was isolated from HT1080 and HeLa cells as described (73) and from human placenta and fetal membrane by the guanidium-cesium chloride method (102). Total RNA denatured with 1 M glyoxal and 50% dimethyl sulfoxide was electrophoresed on a 1.2% agarose gel and transferred to GeneScreen Plus membrane. Filters were prehybridized for 30 min at 60°C in a solution consisting of 1% sodium dodecyl sulfate (SDS), 1 M NaCl, and 10% dextran sulfate. Hybridization proceeded for 16 h at 60°C in the same solution containing 300 μg/ml denatured salmon sperm DNA and the ^{32}P-labeled 412-bp *Hin*cII (probe 5), 512-bp *Pst*I/*Bam*HI (probe 6), or 684-bp *Dra*I (probe 7) DNA fragments excised from the human activin βA genomic clone. These region-specific DNA probes can distinguish between the transcripts generated from the different polyadenylation signal sequences of the human activin βA subunit gene. Filters were washed twice with 2 x SSC (1 x SSC = 0.15M NaCl and 0.015M sodium citrate) for 5 min at room temperature, twice with 2 x SSC with 1% SDS for 30 min at 60°C, and twice with 0.1 x SSC for 30 min at room temperature. Autoradiography was performed with an intensifying screen at -70°C.

Results

Identification of activin βA mRNA in human placenta

It has been demonstrated that human inhibin and its α subunit mRNA are present in placenta (90-92) and that rat activin βA mRNA is produced mainly by the placenta (20). To examine whether the human activin βA subunit gene is expressed in the placenta and fetal membrane, total RNA was extracted and subjected to Northern blot analysis (Fig. 12). In placenta, the 6.4- and 3.0-kb activin mRNAs were found as the predominant species. The 2.0-kb RNA was the next most abundant, and the 4.3- and 4.9-kb RNAs were also detectable. On the other hand, the 3.0-kb RNA was the major band in the fetal membrane, and the 6.4-, 4.9-, 4.3- and 2.0-kb RNA levels were very low. No significant hybridization was seen to RNA from HeLa cells. These results suggested that activin could be also locally produced in human placenta cells.

Effect of cAMP on activin βA mRNA accumulation in HT1080 cells

An earlier work has suggested that cAMP plays an important role in inhibin production in the primary cytotrophoblast layer cells of placenta (91) and that 8-Br-cAMP stimulates inhibin α and βB mRNA levels in primary granulosa (38) and Sertoli cells (99,100). To investigate the effect of cAMP on βA mRNA levels, HT1080 cells were used as model systems, where EDF/activin A activity is known to be stimulated by TPA (chapter III). The cells were exposed to various concentrations of 8-Br-cAMP for 24 h, and total RNA was extracted and analyzed by Northern hybridization. As shown in Fig. 13A, this cell line produced the 3.0-kb activin mRNA as the major species, at a concentration of 1 mM 8-Br-cAMP, with lesser effects in cells exposed to 1 to 100 nM. Treatment of HT1080 cells with 1 mM 8-Br-cAMP by 24 h increased level of the major activin transcript (3.0 kb) associated with the four minor species (6.4, 4.9, 4.3, and 2.0 kb) (Fig. 13B).

I have shown that there is only one activin βA subunit gene in human (chapter II) and

the different size of βA subunit mRNA is the result of using different polyadenylation sites (chapter III). To examine by Northern blot analysis whether the major transcripts of the activin βA gene in the fetal membrane of placenta and cAMP-treated HT1080 cells were processed from the same polyadenylation site, two region-specific probes were used; probes 6 and 7 can recognize the mRNA sequences between the second and third polyadenylation signals and between the third and fourth polyadenylation signals of the activin βA subunit gene, respectively. Fig. 14 suggested that the main 3.0-kb activin mRNA is generated by the use of the third polyadenylation signal for mRNA processing.

Discussion

In the present study, by Northern blot analysis, I could detect the five RNA species (6.4, 4.9, 4.3, 3.0, and 2.0kb) for activin β_A subunit in human placenta and one major RNA species (3.0 kb) associated with the four minor ones (6.4, 4.9, 4.3, and 2.0kb) in both the fetal membrane and cAMP-induced HT1080 cells. Taken together with the fact that hCG stimulates production of inhibin in primary cultures of human trophoblasts of placenta in a cAMP-dependent manner (91), the present results raise the possibility that cAMP acts as a second massenger that includes the gene expression of human activin β_A in human placental and HT1080 cells.

Generally, cAMP regulates many cellular processes, including pattern of gene expression. The effects of cAMP on gene expression are complex and have been shown to involve transcriptional control. A number of studies have demonstrated that the transcription of many eukaryotic genes is included by increases in cellular levels of cAMP through the interactions of specific transcription factors with highly conserved cAMP-responsive elements (CREs) (103). However, I could not find such a consensus sequence in the 5'-flanking region of the human activin β_A subunit gene by sequence analysis (chapter II). In addition to the transcriptional regulation, cAMP has shown to play a crucial role in mRNA stability (104-106). Indeed, α subunit mRNA in rat Sertoli cells is regulated by cAMP both transcrptionally and posttranscriptionally (100). In view of these observation, two plausible mechanisms could be considered about the generation of observed mRNA-size heterogeneity of human activin β_A subunit; one is the multiple polyadenylation processing and the other is the difference in stability of mRNA species.

Analysis of the determinants of 3' mRNA maturation has demonstrated an important role in processing and polyadenylation for the consensus sequences, AATAAA or ATTAAA, present at the end of the 3'-untranslated region of a large number of genes

(107). Indeed, I have identified eight possible polyadenylation sites in the 3'-flanking region of the human activin βA subunit gene (chapter III) and the current study shows that the third polyadenylation site is involved in the generation of 3.0-kb activin mRNA in the fetal membrane of human placenta and cAMP-stimulated HT1080 cells. Thus, it is likely that the multiple polyadenylation sites may contribute to the posttranscriptional regulation of the human activin βA subunit gene.

It has been shown that posttranscriptional regulation of gene activity by control of mRNA stability may be a common mechanism operating for many of those growth factors, lymphokines, cytokines, and protooncogenes (57). In their 3'-untranslated regions, many of these transiently expressed genes are known to contain the conserved ATTTA motif, which is proposed to play a role in selective degradation of mRNAs (57). In fact, I have identified the putative three ATTTA motifs located between the third and fourth polyadenylation sites of the human activin βA subunit gene (chapter II). It is likely that treatment of HT1080 cells with cAMP decreases the stability of the 6.4-, 4.9-, and 4.3-kb activin βA mRNAs, because they have the three common ATTTA motifs in their 3'-untranslated regions. Consequently, the 3.0-kb activin mRNA would be seemingly accumulated as a predominant species from the use of the third polyadenylation site. Recent studies have identified that a single (45,101) and multiple (101) species mRNAs for the activin are present in THP-1 and HL-60 cells, respectively. These observations may support my hypothesis that the size-heterogeneity of the activin transcripts is due to a cell type specific alternative polyadenylation. Further study will be required for studying the regulatory functions of the polyadenylation and ATTTA sequences.

In cultured Rat Granulosa cells, FSH and forskolin (activator of PKA pathway) were reported to stimulate the inhibin but not activin production. In contrast, GnRH and TPA (activator of PKC pathway) stimulated activin, and to a lesser degree, inhibin production (108). It is also reported that ACTH and dibutyryl cAMP (activator of PKA pathway)

increased the level of inhibin α subunit mRNA, and slightly β_A subunit mRNA, in cultured fetal and adult adrenal cells. On the other hand, TPA increased only the β_A mRNA level (109). Taken together, it is suggested that the PKA signalling pathway favors formation of αβ inhibin dimers while the PKC pathway favors formation of β-subunit activin dimers. The β_A subunit mRNA induced by cAMP may, therefore, contributes mainly to the inhibin production *in vivo*. My observations in expression of β_A subunit gene treated with TPA and cAMP are quantitatively consistent with these results because much higher level of β_A subunit mRNA were accumulated when treated with TPA.

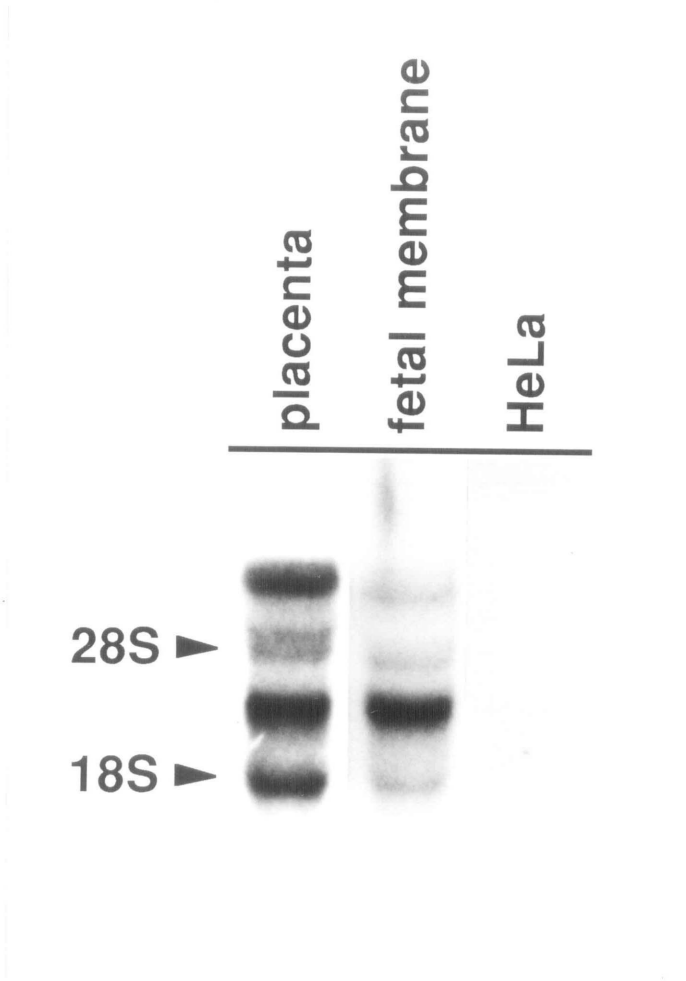


Fig. 12. Expression of the human activin βA subunit gene. Twenty μg of total RNAs isolated from human placenta, fetal membrane, and HeLa cells were hybridized to probe 5. The size of the RNA markers are indicated.

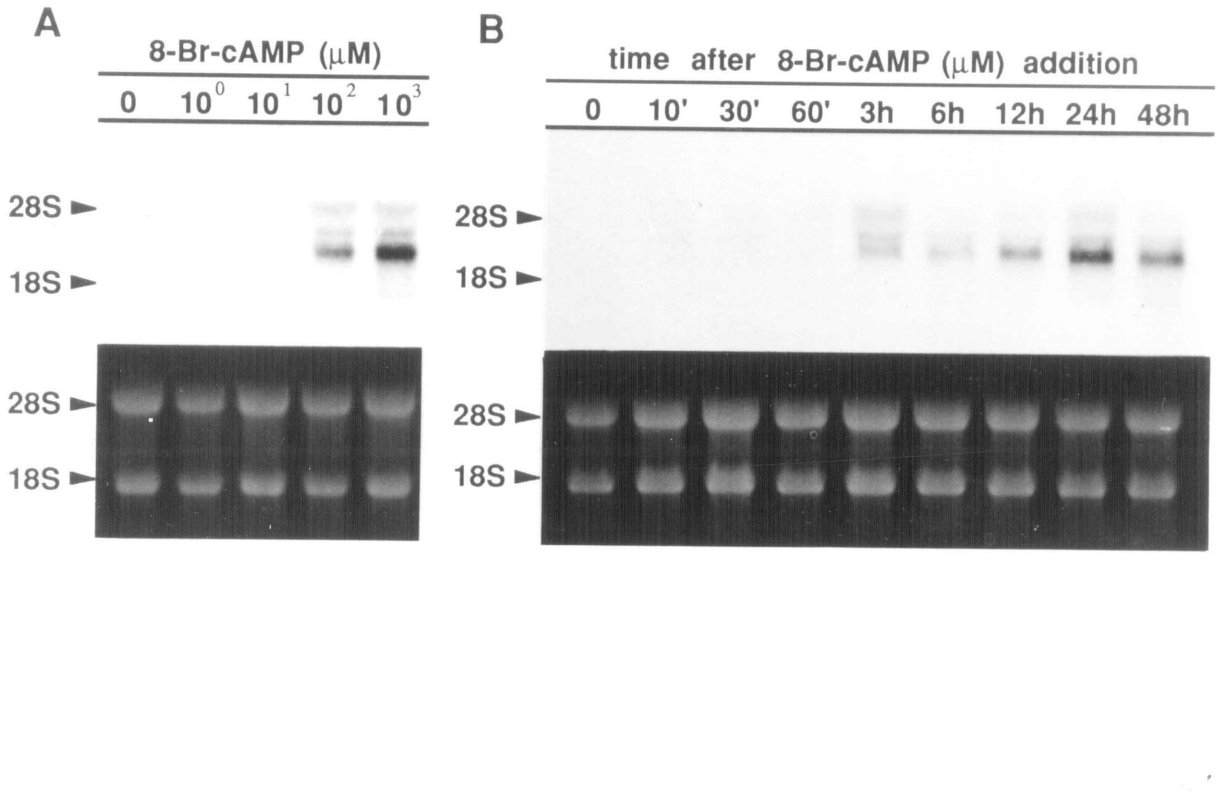


Fig. 13. Accumulation of human activin βA subunit mRNA in cAMP-induced HT1080 cells. (A) Effect of cAMP concentration on level of human activin βA subunit mRNA. HT1080 cells were maintained in growth medium (MEM, 10% FBS) until subconfluent and treated with indicated concentrations of cAMP for 24h. Total RNA (15μg) was hybridized to probe 5. (B) Time course of induction. The cells were grown until subconfluent and treated with cAMP (1mM) for different times as indicated. Total RNA was stained with ethidium bromide as a control of the amount of RNA applied. The relative migration of the 28S and 18S rRNA is indicated on the left vertical axis.

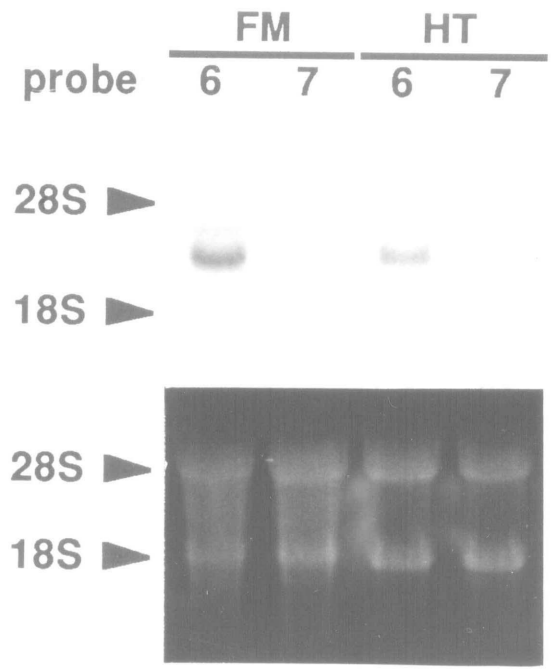


Fig. 14. Northern blot analysis of RNAs from human fetal membrane (FM) and cAMP-treated HT1080 cells (HT). Total RNA was prepared from fetal membrane and cAMP-treated (1mM, 24h) HT1080 cells. Twenty μg (FM) and 15 μg (HT) of samples were hybridized to probe 6 or 7 as described in Materials and Methods. Total RNA was stained with ethidium bromide as a control of the amount of RNA applied. The positions of 28S and 18S rRNA markers are indicated.

Chapter V: Characterization of a Distal Enhancer Element Essential for the Expression of the Human Activin βA Subunit Gene in Human Fibrosarcoma HT1080 Cells

Summary

To identify *cis*-acting elements required for basal and AP-1-induced transcription of the human activin βA subunit gene, the promoter activity of its 5'-flanking region was analyzed by transient and stable transfection assays. The fusion genes were constructed by inserting the 5'-flanking region of the human activin gene upstream from the bacterial chloramphenicol acetyl transferase (CAT) gene and were introduced into human fibrosarcoma HT1080 cells. I thus found the enhancer element, 188-base pair (bp) fragment located approximately 2.7-kilobase (kb) pairs upstream from the transcription initiation sites, that enhances the transient expression of the CAT reporter gene under the elevated amount of AP-1 protein expressed from pRSV-cJun. Stable transfection analysis revealed that this element was also required for basal transcription of this gene. Using the fusion genes containing putative enhancer elements under the control of the heterologous thymidine kinase (TK) promoter, this element was confirmed to direct the AP-1 responsiveness. Sequence analysis identified several potential binding sites for AP-1 and CREB/ATF transcription factors within this region. A deletion mutagenesis of these regions dramatically reduced the transcriptional activities in both transient and stable transfection experiments. These results suggest that the interaction between the ubiquitous factor(s) containing the AP-1 and the distal enhancer element is essential to the maximal expression of the human activin βA subunit gene.

Introduction

Since expression of the activin βA subunit gene needs to be exquisitely regulated as described in chapter I, the identification of transcription factors that interact with the activin promoter and the elucidation of how these factors alter the expression of this gene are of key importance. I have found that the expression of the human activin βA subunit gene is activated by 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (chapter III) and cAMP (chapter IV) in human fibrosarcoma HT1080, but neither in HeLa nor HepG2 cells (data not shown). However, the DNA sequences and transcription factors that mediate these activation have not been defined. Recent studies suggest that intracellular messengers may regulate gene expression by altering the phosphorylation state of transcription factors (110). Members of one family of DNA-binding proteins known as CREB/ATF are activated by cyclic AMP (cAMP) kinase (protein kinase A) (111). The phosphorylation of CREB/ATF by protein kinase A is required for CREB/ATF-inducible gene expression (112). Several other transcription factors (AP-1, AP-2, and NF-κB) are activated by phorbol esters such as TPA, which activate protein kinase C (113-115). It is reported that the dephosphorylation of c-Jun (a component of AP-1) enables it to bind to a TPA-responsive element (TRE) (116).

I have determined the structure of the human activin βA subunit gene with its 5'-flanking sequences (chapter II). To identify regions of the activin βA subunit gene promoter that are important for basal and agent-induced transcription, HT1080 cells were transiently transfected with activin-CAT fusion genes and an enhancer element that appears to be responsive for AP-1 transcription factor was identified. Within this region, sequence and deletion analyses found the several potential binding sites for AP-1, one of which is CRE-like and the others are TRE-like sequences. Further site-directed mutagenesis experiments revealed that both TRE- and CRE-like sites were necessary for full function of the

enhancer. These results suggest that the cooperating interaction of these elements are important in controlling the basal and the induced transcription of the activin β_A subunit gene and that the transcription factor AP-1 is involved in this process.

Materials and Methods

Cell Culture

Human fibrosarcoma HT1080 (CCL-121, American Type Culture Collection), HeLa, and HepG2 cells were maintained at 37°C and 5% CO_2 in minimum essential medium (MEM, Nissui Pharmaceutical Co. Ltd.) supplemented with 10% fetal bovine serum (FBS, Flow Laboratories), 0.1 mM nonessential amino acids, and 2 mM L-glutamine.

Plasmid Constructions

All the fragments used in the constructs in this paragraph (illustrated in Fig. 15) were excised from the genomic clone (chapter II) containing the activin 5'-upstream region. A 3.5-kb *Bam*HI fragment was blunt-ended by the fill-in reaction using the Klenow fragment (Takara Shuzo, Kyoto, Japan), ligated with *Hin*dIII linker, digested with *Hin*dIII, and inserted in the *Hin*dIII site of pUC0CAT (117) to make phβACAT35. phβACAT19 was constructed as follows. A 1015-bp *Dra*I fragment (807/1822) was ligated with *Hin*dIII linker, digested with *Eco*T14I and *Hin*dIII to generate 850-bp fragment (972/1822). This fragment and a 972-bp *Bgl*II-*Eco*T14I fragment (1/972) was inserted in the *Bgl*II/*Hin*dIII sites of pUCSV0CAT. To make phβACAT45, the 3.5-kb *Hin*dIII-*Bgl*II fragment from the phβACAT35 and the *Bgl*II-*Hin*dIII fragment (1/1822) from the phβACAT19 were inserted in the *Hin*dIII site of pUC0CAT. A 2.1-kb *Bam*HI fragment, which is the 5'-end segment of the λhβA-3 (chapter II), was inserted in the *Bgl*II site of pUCSV0CAT to generate phβACAT22. phβACAT66 was constructed by cloning the 4.5-kb *Hin*dIII fragment from the phβACAT45 into the *Hin*dIII site of phβACAT22. The orientation of all the fragments in these CAT constructs is as in the activin genome. Construction of pUCSV0CAT and pUCSV3CAT was described previously (77).

For construction of 5'-deletion mutants of the activin 5'-upstream region, the 3.5-kb

*Bam*HI fragment (same as that used for construction of phβACAT35) was inserted in the *Bam*HI site of pBluescriptII KS(+) (Stratagene) in reversed orientation. This plasmid was linearlized with *Xba*I and *Pst*I restriction enzymes, partially digested with exonuclease BAL31 (Takara) for various length of time, treated with Klenow fragment (Takara) to form blunt ends, and ligated in the presence of *Bgl*II linker. DNAs were transferred into *Escherichia coli* HB101. After determination of the 5'-deleted ends by DNA sequencing, the fragments were excised with *Bgl*II (sites were provided by linker sequence and the activin gene at position 1 (chapter II)). The deleted fragments were inserted in the *Bgl*II site, located upstream of the 1.9-kb *Bgl*II-*Hin*dIII fragment, of phβACAT19. The plasmid phβACAT45/3Δ166 was constructed as follows. The 2.6-kb *Bam*HI-*Bgl*II fragment, which is localized at the 5'-upstream segment of phβACAT45, was inserted in the *Bgl*II site of pUCSV0CAT to generate phβACAT26. The 1279-bp *Bsm*I fragment (377/1656) was blunt-ended, subcloned into the *Sma*I site of pUC119 in the sense orientation, excised with *Eco*T14I and *Hin*dIII as a 684-bp fragment (972/1656), and inserted in the *Bgl*II/*Hin*dIII sites of phβACAT26 together with the 972-bp *Bgl*II-*Eco*T14I fragment (1/972).

The nucleotide sequence of the DR-21 enhancer fragment was tentatively numbered from 1' to 188' because the nucleotide sequence of the DNA segment located between about -2.3 kb and -1857 bp (at *Bgl*II site in Fig. 15) to the translation start site was not determined. The putative enhancer elements, DR-2, DR-1, and DR-21, were synthesized by polymerase chain reaction (PCR) using phβACAT29Δ as a template under reaction conditions recommended by the supplier of the *Taq* polymerase (Perkin-Elmer). One or two copies of these fragments, cut with *Bgl*II and *Bam*HI, were inserted in the *Bgl*II site of p0/tk-CAT, which contains the CAT gene driven by herpes simplex virus thymidine kinase (TK) promoter, in the sense or antisense orientation (Fig. 17A). The truncated or mutated DR-21 fragments were also synthesized by PCR. After digestion with *Bgl*II and

*Bam*HI, the amplified fragments were inserted in the *Bgl*II site of p0/tk-CAT in a sense orientation as a single copy (Fig. 18B and Fig. 19A). The positions and sequences of the synthetic oligonucleotides used to produce DR-21 fragment and its derivatives were as follows: 2S (1'/19'), 5'-AGTAGATCTGATTCCAATGTTTTTCTA-3'; 2D (64'/81'), 5'-AGTAGATCTCACCCAGTAGTGTCTCA-3'; 2D2 (96'/114'), 5'-AGTAGATCTTTCC-ATTAGATAATGAA-3'; 2D3 (118'/135'), 5'-AGTAGATCTGAAATGTCTCACACTG-G-3'; 1S (125'/143'), 5'-AGTAGATCTCACACTGGAAAAACCA-3'; 1A (188'/170'), 5'-TGCGGATCCTTTGTTTGGGATTGGTTG-3'; 1D (162'/146'), 5'-TGCGGATCC-ATGACATCAGCGGATG-3'; 1D2 (136'/119'), 5'-TGCGGATCCAGTGTGAGACA-TTTC-3'; 2A (124'/105'), 5'-TGCGGATCCATTTCATAATTCATTATCT-3'; 2D3M (118'/140'), 5'-AGTAGATCTGAAAT•••TCACACTGGAAAAA-3'; 1DM (162'/142'), 5'-TGCGGATCCAT•••ATCAGCGGATGACTG-3'; and 1DM2 (162'/130'), 5'-TGCGG-ATCCCATGACATCAGCGGA•••••••TTTTTCCAGTG-3'. Each primer, containing 17 to 26 nt of DR-21 specific sequences and either *Bgl*II (in sense primers) or *Bam*HI (in antisense primers) recognition sites and three additional nucleotides, were synthesized on a MilliGen/Bioresearch Cyclone™ Plus oligo-nucleotide synthesizer. The 5'-end positions of each primer were illustrated in Fig. 18B by broken vertical lines. Dots in the primers 2D3M, 1DM, and 1DM2 denote the deleted nucleotides from the wild type sequences and the oligonucleotides used for site-directed mutagenesis were 2D3M, 1DM, and 1DM2 for ΔT, ΔC, and ΔP mutants, respectively. The constructs were verified by DNA sequencing by the dideoxy chain termination method.

Stable Transfections

HT1080 cells were co-transfected with a 5:1:1 molar ratio of the activin 5'-flanking region-CAT chimeric constructs, pSV2neo (118), and pCH110, a plasmid that contains the *Escherichia coli* β-galactosidase gene controlled by the SV40 promoter (119) (21 μg of

total DNA/100 mm diameter dish, 1 x 10^6 logarithmically growing cells) by the calcium phosphate coprecipitation method. After selection with 800 μg/ml G418 for 8 to 12 days, the resistant cells were pooled and analyzed for CAT activity. One-fifth of the cell extract was assayed for β-galactosidase activity to monitor the transfection efficiency.

CAT Assay

Transient transfections and the CAT assay were performed as described in Materials and Methods in chapter III.

Results

Identification of an upstream region required for transcription

To identify the sequences responsible for controlling the expression of the human activin βA subunit gene, CAT constructs which contained variable length of the 5'-flanking sequence of this gene were introduced into HT1080 cells (Fig. 15). As I have found that the activin βA subunit gene utilizes multiple transcription start sites, clustered between about 250 to 180 bp 5' to the start site of translation (chapter III), a *Dra*I restriction site, 36-bp 5' to the translation start site, was used to form a 3'-end of each fragment, except for phβACAT35. phβACAT66 and phβACAT45 which contained the activin 5' upstream segments of about 6.6-kb and 4.5-kb DNA together with multiple transcription start sites, respectively, gave significant levels of basal CAT activities. These were enhanced by the co-transfection of a c-Jun/AP-1 expression vector, pRSV-cJun. However, no CAT activity was detected in HT1080 cells transfected with a construct phβACAT19, containing about 1.8-kb DNA segment along with transcription start sites, or with constructs containing the 5'-flanking region in the opposite orientation, even in the presence of AP-1 (Fig. 15 and data not shown). Furthermore, no detectable level of CAT activity was observed with phβACAT35, which assumed to lack the proximal promoter region of the phβACAT45. Thus, the element responsible for basal and AP-1-induced expression of this gene seems to be located within the *Bgl*II/*Bam*HI DNA fragment between about -4.5-kb and -1.9-kb to the translation start site and this element is suggested to possess an enhancer property. In transfected HeLa and HepG2 cells, these constructs showed remarkably similar profile of CAT expression (data not shown). I thus assume that the human activin βA subunit gene may possess an additional element, directing its cell specific expression, in the region further upstream or 3'-downstream.

5'-Deletion analysis of the activin promoter

To precisely search for sequence elements required for AP-1-induced high level expression of the activin βA subunit gene, 5'-deletions of the activin promoter were constructed (Fig. 16A) and transiently transfected into HT-cJun cells, HT1080 cells stably transfected with pRSV-cJun (Fig. 16B). The promoterless plasmid pUCSV0CAT was used as a background reference and pUCSV3CAT was used as a positive control including the SV40 enhancer-promoter region. As shown in Fig. 16B, deletion to the construct phβACAT29Δ resulted in a two-fold increase in CAT expression relative to the undeleted construct, phβACAT45. This result suggests the presence of negative regulatory elements within this region. Deletion to the construct phβACAT28.5Δ reduced the promoter-CAT hybrid gene expression to 18% of phβACAT29Δ. Further deletion to phβACAT28.1Δ decreased CAT activity to the background level demonstrated in the negative control, pUCSV0CAT. A significant decline in CAT activity was not observed with deletion constructs extending further downstream. To test whether these elements are also required for basal expression of this gene, some representatives were stably transfected into the HT1080 cells and analyzed for CAT activity (Fig. 16C). Deletion from phβACAT45 to phβACAT29Δ resulted in a 4.5-fold increase in basal CAT activity, again suggesting an existence of negative regulatory elements. Further deletion of 5'-upstream region to phβACAT28.5Δ severely reduced the basal promoter activity to the level of 15% of the construct phβACAT29Δ. phβACAT28.1Δ exhibited a CAT activity similar to that obtained with phβACAT19. Note that a residual activity was still detected with phβACAT28.1Δ and phβACAT19 as compared with phβACAT45/3Δ166, lacking almost part of the 5'-untranslated region and some of the transcription start sites. Similar results were obtained with transiently and stably transfected HeLa and HepG2 cells (data not shown). Taken together, these results suggested that a proximal promoter region exists around the transcription start sites and that positive regulatory elements, located between the 5'-end

points of phβACAT29Δ and phβACAT28.1Δ, possess an enhancer property. I designated the latter regions DR (Distal Region)-2 and DR-1 (Fig. 16A).

Characterization of the Distal Region (DR) Enhancer

To test such enhancer activity in the context of a heterologous promoter, DNA fragments, corresponding to the sequences between 1' and 124' (DR-2), between 125' and 188' (DR-1), and between 1' and 188' (designated DR-21), were synthesized by PCR so that there was a *Bgl*II restriction site at the 5'-end and a *Bam*HI site at the 3'-end, and fused to the CAT gene driven by the herpes simplex virus TK promoter (Fig. 17A and 18A). CAT activity obtained with the enhancerless vector, p0/tk-CAT, was arbitrary given the value 1. Addition of either DR-1 or DR-2 fragment in the sense or antisense orientation produced an only 2 to 3-fold increase in the CAT activity in transiently transfected HT-cJun cells (Fig. 17B). In contrast, DR-21 fragment strongly activated the TK promoter (17 to 19-fold) in both orientations. Furthermore, duplicated copies of this DR-21 fragment enhanced the activation dramatically (69 to 82-fold). I have also examined the activity of the DR-21 fragment at a position downstream of the CAT gene and obtained 4.5-fold activation of the TK promoter (data not shown). These results clearly demonstrated that the 188-bp DR-21 fragment is able to increase the activity of the heterologous TK promoter and that this element functions as a classical enhancer in HT-cJun cells. In addition, reduction of the enhancer activity by splitting the DR-21 fragment into two subfragments may provide two plausible explanations; two or more separate interacting elements are present within the DR-21 enhancer or the essential sequence motif is present at the border. In former cases, synergistic interactions between those elements should exist to provide a powerful activation mechanism.

To characterize the AP-1 responsiveness of the DR-21 enhancer, phβACAT45, pDR21/tk-CAT(+), and pDR21/tk-CAT(W+) were transfected into HT1080 cells together

with increasing amounts of pRSV-cJun. As shown in Fig. 17C, all of these were *trans*-activated by cJun/AP-1, while the enhancerless construct p0/tk-CAT was unresponsive (data not shown). These results demonstrated that the DR-21 fragment could confer AP-1 responsiveness on the TK promoter, though the maximal *trans*-activation was apparent at a considerably lower ratio of activator to reporter DNA. It should be noted that the activin promoter, phβACAT45, was *trans*-activated to a similar degree to the constructs pDR21/tk-CAT, suggesting that the DR-21 element indeed functions in the native promoter context. However, it must be emphasized that it remains to be established whether normal endogenous levels of AP-1 act on the DR-21 enhancer (see Discussion). In either cases, the *trans*-activation experiments demonstrate that the DR-21 fragment is an AP-1-responsive enhancer capable of functioning in the native and the heterologous promoter contexts.

Sequence and Deletion analyses of the DR-21 Enhancer element

A search of the DR-21 region for known consensus sequences of binding sites for transcription factor cJun/AP-1 identified several potential sites, four TPA responsive elements (TRE) or AP-1 binding sequences (designated as *AP-1A* to *AP-1D*) and a cyclic AMP responsive element (CRE) or CREB/ATF binding sequence (designated as *CRE*) (Fig. 18A). It is reported that the CRE is efficiently *trans*-activated by cJun/AP-1 (120) and that some of the CREB/ATF transcription factors heterodimerize with c-Jun to bind to the CRE (111, 121, 122). In further analysis of the sequence requirements for the DR-21 enhancer activity, deletion mutations were targeted systematically to eliminate these potential binding sites to determine whether the sites contributed to the enhancer function. A series of 5'- and/or 3'-deletion mutants from the DR-21 enhancer fragment were placed in the sense orientation in front of the TK gene promoter in the p0/tk-CAT reporter plasmid and their transcriptional activity was tested in transiently transfected HT-cJun cells (Fig. 18). When the oligonucleotide 1A was used as a 3'-end primer for PCR, deletions of *AP-*

1A and *AP-1B* sites from the 5'-ends slightly reduced the enhancer activity of the DR-21 fragment (compare DR-21, 21D, and 21D2). Nevertheless, the same deletions had little effect on the enhancer activity when the oligonucleotide 1D was used as a 3'-end primer (compare DR-21d, 21Dd, and 21D2d). Further deletion of sequences after nucleotide 125' at the 5'-end (DR-1) reduced the enhancer activity to 16% of the original activity and this may be attributable to destruction of the *AP-1C* sequence, 5'-TGTCTCA-3', at positions 123' to 129'. Results with the 3'-deletion mutants indicated that the sequence up to nucleotides 163' had no significant role in the activity of the DR-21 enhancer (DR-21d, 21Dd, and 21D2d). However deleting 26 more nucleotides upstream from that position reduced the enhancer activity to less than 18% of the original level (DR-21d2, 21Dd2, and D2d2). This region contains the sequence 5'-TGATGTCA-3' at positions 153' to 160', which resembles the binding sites for CRE-binding protein CREB/ATF and this variant is reported to function in the context of retinoic acid receptor β2 promoters (123). Interestingly, the remaining activity was little affected by further deletion up to sequence number 124' (compare DR-21d2 and DR-2) which removed the 5 nucleotides from the *AP-1C* sequence. This result, together with that obtained with 5'-deletion mutants, proposed the hypothesis that the *AP-1C* element functions only when the *CRE* element exists. In summary, I identified the enhancer core sequence in the DR-21 region which contained at least two sequence motifs both of which are essential for the enhancer activity of the DR-21 fragment.

Mutational Analysis of the DR-21 Enhancer

Examination of the nucleotide sequences in the two sequence motifs described above revealed two well-known sequence motifs for transcription factor's binding sites. One is the TRE (binding site for AP-1) and the another is the CRE (binding site for CREB/ATF transcription factors). Diagnostically, the loss of one copy of this sequence from the DR-21 sequence resulted in a significant loss of enhancer activity, as was found in the 5' and the

3' deletion mutants (Fig.18). In order to demonstrate a functional role for these two motifs, three nucleotides were deleted from the consensus binding sites for each transcription factor by using mutant PCR primers (see Materials and Methods and Fig. 19). Three different internal deletion mutants for DR-21core enhancer, one for the *AP-1C* site (ΔT), one for the *CRE* site (ΔC), and the double mutant containing combination of them (ΔCT), were placed in front of the TK promoter in the p0/tk-CAT reporter plasmid and their transcriptional activity was tested in HT-cJun cells. As shown in Figure 19B, the ΔT reduced the CAT activity to about 22% of the wild type (CORE) enhancer, but still higher than that obtained with TK promoter construct (TK). In contrast, the activities of the ΔC and ΔCT enhancers were abolished completely. These results suggested that the combination of *AP-1C* element and the *CRE* is important for efficient activation of the activin promoter. In other words, it is likely that both the *AP-1C*- and the *CRE*-binding proteins are important and should be bound to the DR-21core enhancer element simultaneously.

Discussion

The 5'-flanking region of the activin βA subunit gene lacks a TATA or CAAT consensus sequence immediately 5' to the multiple initiation sites, the proximal sequences contain CT-rich sequences and a perfect consensus sequence for retinoic acid control element, 5'-GCCACCC-3' (chapter II and III). I have previously found that TATAAA and CCAAT sequences exist 1348 and 1412 bp 5' to the translation start site, respectively (chapter II), but these sequences were unlikely to function because I could not detect any transcripts by Northern blot analysis using region-specific DNA probes within this gene segment (data not shown). Furthermore, reverse transcriptase-mediated polymerase chain reaction (RT-PCR) was performed to exclude the possibility that an intronic sequences, which could not be detected by primer extension analysis, exist between transcription and translation start sites. The results again demonstrated that the region downstream of the CT-rich sequence overlaps the multiple transcription start sites and suggested that this region contains core promoter elements (data not shown). Despite these observations, phβACAT19 which contains this region could not direct the transcription of this gene in HT1080 cells. Therefore, I extended the promoter region to further 5' upstream and could detect the transcriptional activity with phβACAT45.

Next, I tested the responsiveness of this construct to TPA because human activin A and its mRNA was reported to be induced by TPA in HT1080 cells (69 and chapter III). A somewhat unexpected observation was that co-transfection of phβACAT45 with pRSV-cJun into HT1080 cells could significantly *trans*-activate the CAT activity (Fig. 15) but addition of TPA could not, even when the transfected cells were serum deprived (data not shown). Furthermore, I was unable to confirm the cell specificity of the human activin promoter activity because the same results were obtained with HeLa and HepG2 cells which do not express the activin gene when stimulated by TPA (data not shown). I thus

assume that the human activin gene may possess an additional DNA element, mediating the TPA responsiveness and cell specificity, in the region outside my construct. Another possibility is that these may be regulated post-transcriptionally. One intriguing example is that the mRNA for TGF-β1 is selectively degraded in U937 promonocytes and this degrading system is completely blocked by treatment with TPA (124).

To identify *cis*-acting elements responsible for the expression of the human activin βA subunit gene, fusion genes were constructed with progressive 5' deletions linked to the bacterial CAT reporter gene and their promoter activities were examined in transiently transfected HT1080 cells. The levels of CAT activities expressed in these constructs were too low to be determined accurately in the absence of elevated level of AP-1, which is, at least in part, due to the low transfection efficiency. As Northern blot analysis revealed that the significant level of c-*jun* mRNA is accumulated in HT1080 cells (data not shown) and the strong basal transcriptional activity was observed with phβACAT45 when stably introduced into HT1080 cells (Fig. 16C), I assumed that the involvement of c-Jun/AP-1 transcription factor in the activin gene expression is evident. Therefore unless noted, HT-cJun cells were used in subsequent experiments to facilitate the detection of CAT activity. The presence of a positive regulatory element within the activin promoter, located at about -2.7 kb from the transcription start sites, was suggested by the observation that deletion of this region resulted in more than 90% decrease of the CAT activity in HT1080, HeLa, and HepG2 cells. Further stable transfection experiments could confirm this notion. In addition, the complete absence of the CAT activity obtained with stably transfected phβACAT45/3Δ166, lacking immediately downstream region containing the predicted transcription start sites, implied that the CAT activity measured in my transfection assay resulted from proper transcription initiation.

A DNA segment corresponding to this sequence was able to activate the ubiquitous TK-promoter in a position- and orientation-independent manner, indicating that this element

possesses an enhancer property. Sequence analysis of this region, termed DR-21, has revealed several potential binding sites for *trans*-acting factors. Further deletion and mutational analyses identified the enhancer core sequence and the nucleotides critical for enhancer and basal transcriptional activity (Fig. 18 and Fig. 19).

c-jun is one of the primary response genes (genes whose induction can occur without intervening protein synthesis) and its product, AP-1, is a primary nuclear target of receptor-mediated signal transduction pathways activated by extracellular ligands (125). The identification of functional AP-1 binding (responsive) sites within the activin promoter and the demonstration that cJun/AP-1 *trans*-activates these sequences suggest that certain extracellular factors may regulate the expression of the activin β_A subunit gene. One class of physiological activators that could potentially utilize the AP-1 to regulate activin gene expression is likely to be the pleiotropic cytokines, interleukine (IL) -1, IL-6, and tumor necrosis factor (TNF). In fact, it is reported that the activin A gene expression is induced by TNF-α and interleukin-1β in bone marrow stromal cells (126).

The CREs identified in the promoters for the α subunit of the glycoprotein hormones (127, 128) and somatostatin (129) have been shown to interact with the nuclear factors CREB/ATF (130, 131). Hormonal stimulation of cAMP production results in the activation of protein kinase A and subsequent phosphorylation of CREB/ATFs. This phosphorylation directly activates transcription and may also affect the binding affinity of CREB/ATFs for CREs (125). CRE sequence identified within the DR-21core element (5'-TGATGTCA-3') differs from the consensus sequence (5'-TGACGTCA-3'). However, this sequence has been reported to be functional CRE as a naturally occurring variant found in the human retinoic acid receptor type β gene (123). I have previously reported that the activin β_A subunit mRNA levels in HT1080 cells were elevated by cAMP treatment (chapter IV) and that this mRNA accumulation required *de novo* protein synthesis (data not shown). Furthermore, follicle-stimulating hormone (FSH),which is a major regulator of inhibin production but does not increase the steady-state mRNA levels of β subunits, was shown

to induce the expression of proto-oncogene c-*fos* and c-*jun*/AP-1 in Sertoli cell primary cultures *via* cAMP (132). Taken together, it is also possible that the induction of the βA subunit gene expression by cAMP may require the binding of AP-1 to the DR-21 enhancer element, although the DR-21core element linked to the TK promoter was unresponsive to this agent in my present experimental conditions (data not shown).

Studies with the wild-type and mutated DR-21core element suggest a role for both *AP-1C* and *CRE* in the enhancer activity. For example, mutations of the *CRE* (ΔC) completely abolished the enhancer activity. On the other hand, mutation of the *AP-1C* (ΔT) sequence reduced by 75% but did not completely abolish the enhancer activity. These data suggest that *CRE*-binding protein plays a dominant role in the enhancer function. One possible model explaining the nature of the *AP-1C-CRE* interaction would be that the binding of an unknown protein(s) to the *CRE* sequence causes *cis* effect, changing the DNA structure of the *AP-1C* sequence and increasing its affinity for the AP-1 or the AP-1 containing complex. If this is the case, excess quantity of AP-1 may interfere with this ordered assembly, because the AP-1 is known to bind the *CRE* sequence (120), and will result in the decreased transcription, as I observed in figure 17C.

In conclusion, I have identified the enhancer sequence DR21, which is involved in the response to AP-1 and in the basal expression of the activin βA subunit gene. This enhancer activity does not seem to be cell type-specific, suggesting that it is actively involved in the regulation of transcription of the activin gene. This enhancer is active when linked to the phβACAT28Δ construct and the heterologous TK-promoter, however inactive when combined with phβACAT19 construct. This observation provide evidence that some other regulatory regions, located between the 5'-end point of these constructs, are required for efficient transcription of this gene. In this respect, I am now looking for such a region to understand the molecular basis of activin βA subunit gene expression as a whole.

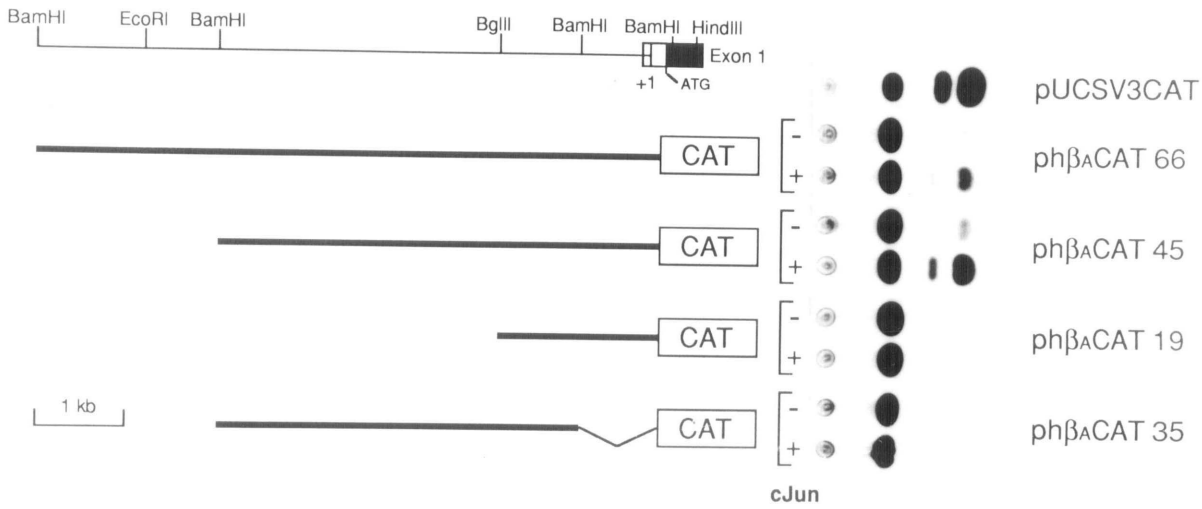


Fig. 15. AP-1-dependent induction of the activin βA subunit gene expression. The restriction map of the 5'-flanking region of the human activin βA subunit gene is shown at the top. The solid box indicates coding sequences and the open box the transcribed, noncoding region. DNA fragments from the 5'-upstream region of the gene were fused to the promoterless CAT gene, pUCSV0CAT. To analyze basal promoter activity and AP-1 responsiveness, HT1080 cells were transfected with 3μg of plasmid DNA, together with 3μg of pUC19 plasmid (-) or with 3μg of c-Jun expression vector (+). CAT assay was performed as described in Materials and Methods and a typical result from five independent experiments is shown. The scale is indicated by the 1 kb bar.

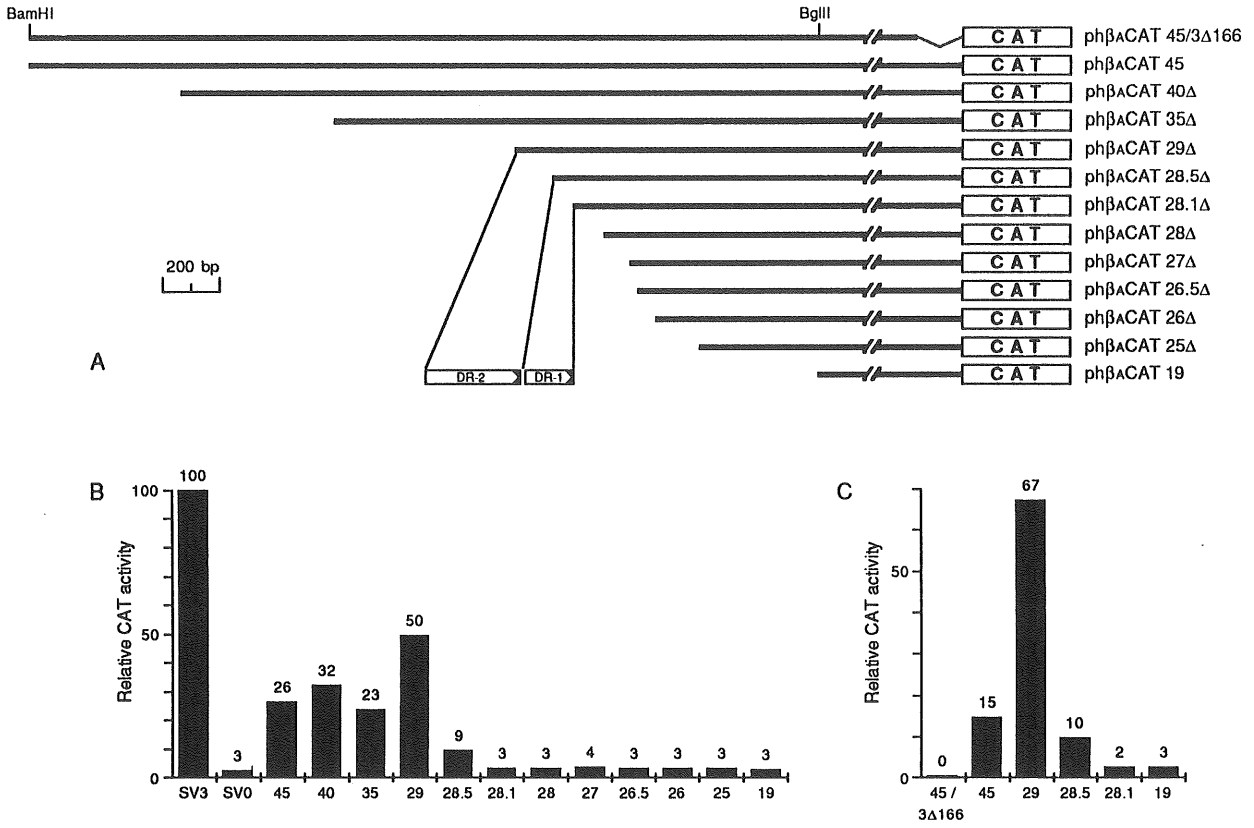
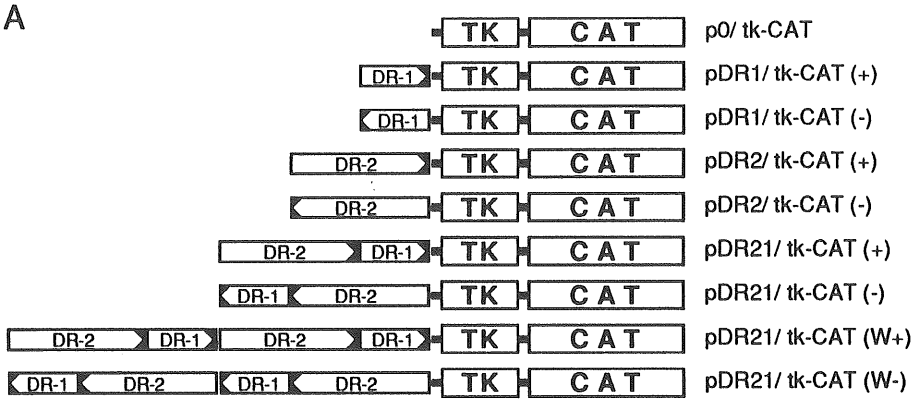
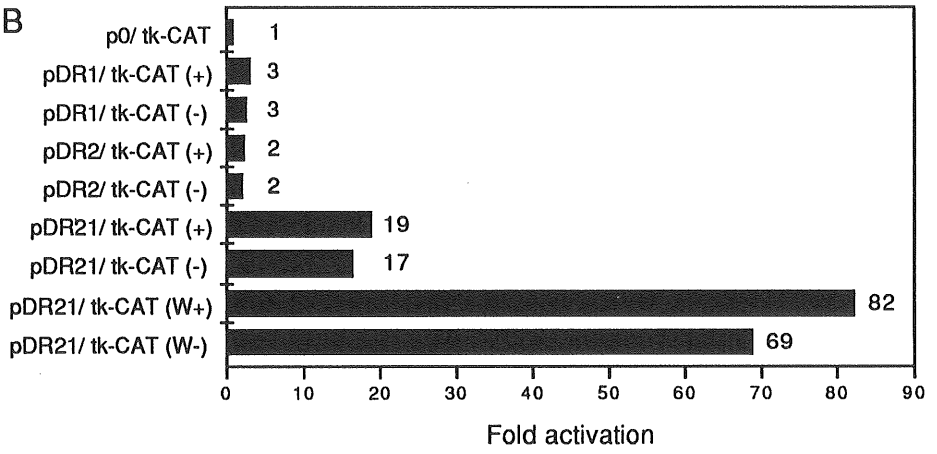


Fig. 16. 5'-Deletion analysis of the human activin βA subunit gene regulatory sequence. *A*, schematic representation of the activin 5'-upstream region-CAT fusion genes. 5' progressive deletions and a 3' deletion (phβACAT 45/3Δ166) of the fragment from the phβACAT 45 were fused to the promoterless CAT gene, pUCSV0CAT. Location of the distal regions (DR-1 and DR-2) used for further analysis is shown. The scale is indicated by the 200bp bar. *B*, AP-1-induced expression of the deletion constructs. HT-cJun cells were transiently transfected with 6μg of fusion genes. pUCSV3CAT, which contains both SV40 enhancer and promoter, and pUCSV0CAT were used as positive and negative controls, respectively. Following 36h incubation, cells were harvested, lysed, and the cytosolic fractions were assayed for CAT activity as described in Materials and Methods. Relative CAT activities were determined by averaging eight independent experiments with very similar results and comparing them to the activity obtained with pUCSV3CAT (assigned a value of 100). *C*, Basal expression of the deletion constructs. HT1080 cells were stably transfected with a series of activin/CAT fusion genes (15μg) and pSV2neo (3μg). After selection with 0.8 mg/ml G418 for 2 weeks, cell lysates were prepared and analyzed for CAT activity. Results are expressed as relative CAT activities compared to that obtained with phβACAT29Δ and the values are averages of at least two independent experiments.


A
TK
CAT
p0/ tk-CAT
DR-1
pDR1/ tk-CAT (+)
pDR1/ tk-CAT (-)
DR-2
pDR2/ tk-CAT (+)
pDR2/ tk-CAT (-)
pDR21/ tk-CAT (+)
pDR21/ tk-CAT (-)
pDR21/ tk-CAT (W+)
pDR21/ tk-CAT (W-)


B
p0/ tk-CAT
pDR1/ tk-CAT (+)
pDR1/ tk-CAT (-)
pDR2/ tk-CAT (+)
pDR2/ tk-CAT (-)
pDR21/ tk-CAT (+)
pDR21/ tk-CAT (-)
pDR21/ tk-CAT (W+)
pDR21/ tk-CAT (W-)
1
3
3
2
2
19
17
82
69
0 10 20 30 40 50 60 70 80 90
Fold activation

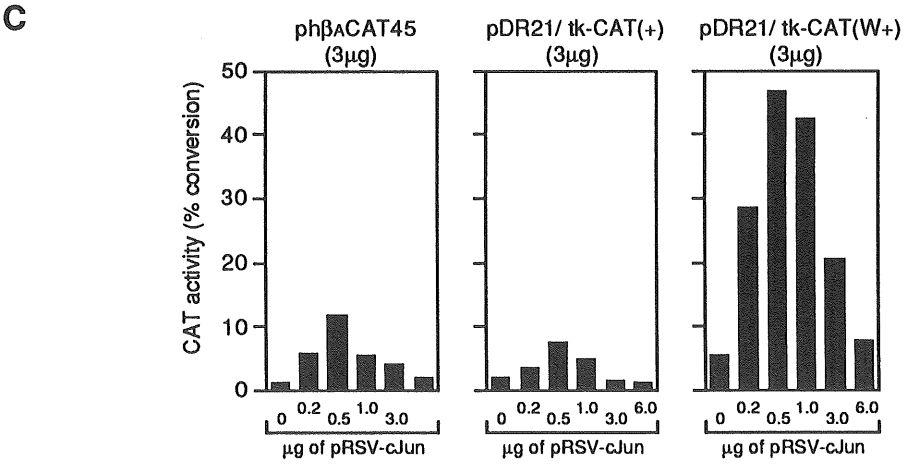

C
phβACAT45 (3μg)
pDR21/ tk-CAT(+) (3μg)
pDR21/ tk-CAT(W+) (3μg)
CAT activity (% conversion)
0 10 20 30 40 50
0 0.2 0.5 1.0 3.0
0 0.2 0.5 1.0 3.0 6.0
μg of pRSV-cJun

Fig. 17. Activation of heterologous TK promoter by DR21 enhancer fragment. *A*, Construction of a series of DR/TK-CAT plasmids. One or both of the DR elements (DR-1 and DR-2) were inserted in the sense or antisense orientation, upstream of the p0/tk-CAT, bearing the herpes simplex virus thymidine kinase gene promoter. *B*, Enhancer activity of the DR-21 fragment. HT-cJun cells were transiently transfected with 6μg of plasmids. After 36 h, cell lysates were prepared and analyzed for CAT activity as described in Materials and Methods. Enhancer activities are expressed relative to the CAT activity obtained with p0/tk-CAT (assigned a value of 1). The values represent the average of at least four independent experiments. *C*, Transactivation of the DR-21 enhancer by AP-1. HT1080 cells were cotransfected with 3μg of each reporter plasmid and indicated amounts of pRSV-cJun. Total amounts of DNA were maintained constant by the addition of plasmid pUC19. The CAT assay was performed as described above. Results are expressed as percent conversion of [^{14}C] chloramphenicol to its acetylated forms and the values are averages of two independent experiments.

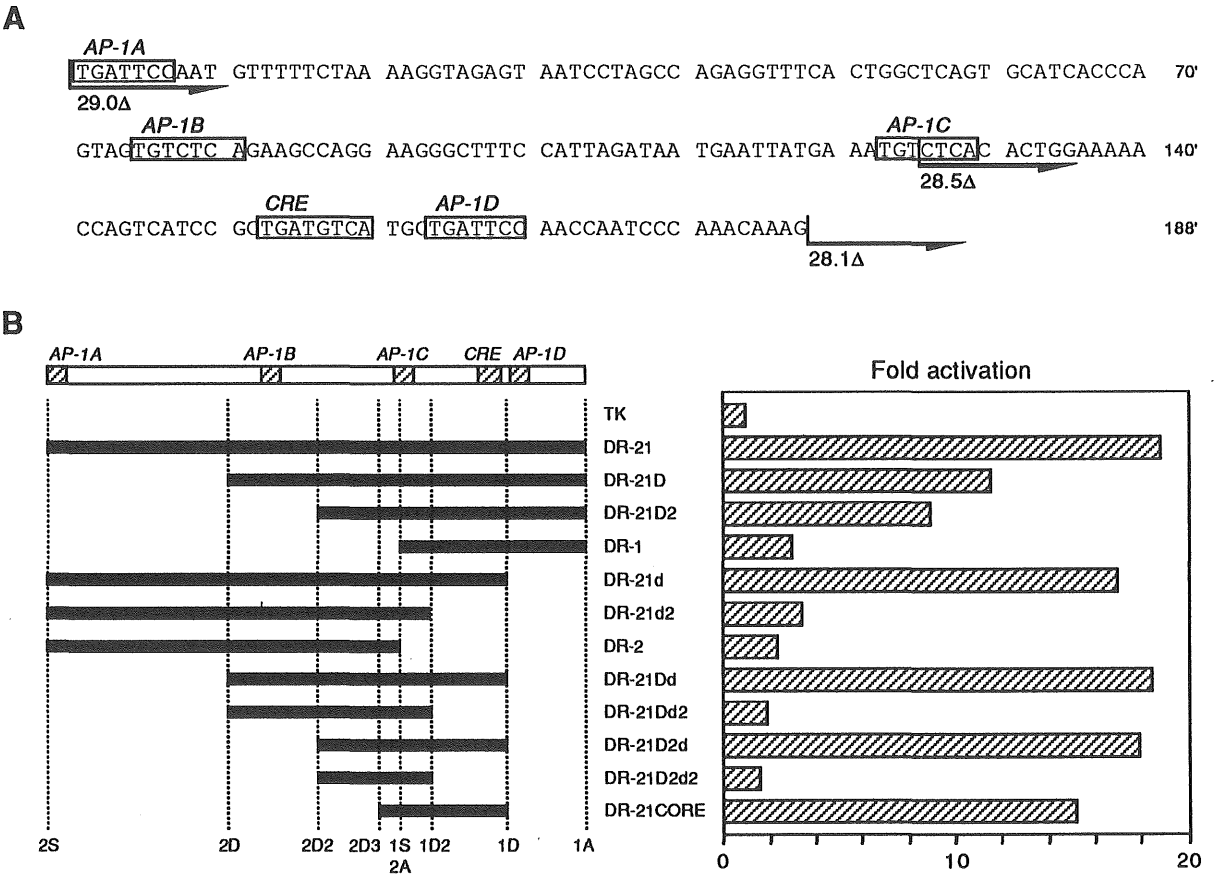


Fig. 18. Identification of core element in the DR-21 enhancer fragment. *A*, Nucleotide sequence of the DR-21 enhancer fragment. The arrows indicate the deletion site positions shown in Fig.16 and the open boxes locate sequence motifs suggesting potential protein binding sites for AP-1 (*AP-1A* to *AP-1D*) and CREB/ATF (*CRE*; cAMP responsive element). *B*, CAT assay of constructs containing truncated DR-21 fragment. Subfragments of the DR-21 enhancer (solid bars) were synthesized using PCR and inserted upstream of the TK promoter in p0/tk-CAT. The vertical broken lines indicate the positions of the 5'-terminal of the primers used to synthesize the fragments. HT-cJun cells were transfected with the 6μg of indicated constructs. After 36h, cell lysates were prepared and analyzed for CAT activity as described in Materials and Methods. Results are expressed as fold activation compared to that obtained with p0/tk-CAT (TK).

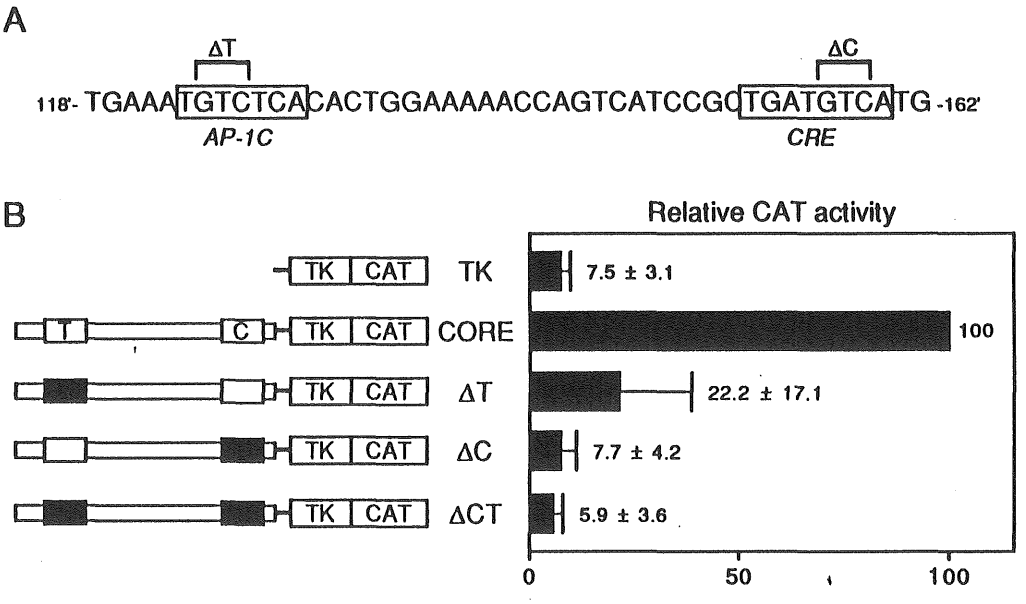


Fig. 19. Mutational analysis of the DR-21core enhancer activity. *A*, Schematic representation of the DR-21core (118'/162') enhancer region and the positions of deleted nucleotides. *B*, The wild-type (CORE) fragment and its mutated derivatives, in which either one of the *AP-1C* (ΔT) and *CRE* (ΔC) or combination of them (ΔCT) were disrupted, were fused to the p0/tk-CAT (TK). HT-cJun cells were transiently transfected with the 6μg of each constructs and the CAT assays were performed as described in Materials and Methods. The level of activation is depicted as a percentage of the level obtained with the wild-type construct (CORE, 100%). The mean ± S.E. of at least six independent experiments is shown.

Chapter VI: Concluding Remarks

Considerable progress has been made in recent years in the identification and analysis of regulatory elements involved in the control of gene expression. As a first step to analyze regulatory mechanisms of activin gene expression, I cloned the gene for activin β_A subunit along with its 5'-flanking region from the human placenta genomic library. Sequence, primer extension, and Northern blot analyses disclosed the gene structure which consisted of two exons and a 9-kb intron and which contained multiple transcription start sites and polyadenylation signals. Next, I performed Northern blot analysis with the cloned gene as a probe to demonstrate agent-induced expression of this gene in a human fibrosarcoma cell line, HT1080 and observed TPA- and cAMP-dependent activation. Finally, transient and stable transfection experiments were conducted to localize the regulatory element required for its proper expression. A 4.5-kb segment upstream to the transcription start sites was thus identified as a promoter region. However, this region neither respond to the agent-treatments nor direct the cell-specific expression in the present experimental conditions. This suggests that *cis*-acting DNA elements necessary for transducing TPA and/or cAMP action reside outside the 4.5-kb of 5'-flanking DNA segment present in the fusion construct or that factors of such signalling pathways were impaired by the transfection procedure. It is also possible that the posttranscriptioal regulation or surrounding chromatin structure is involved in these regulatory mechanisms. A function of chromatin structure in the control of gene activity is suggested by several observations, including the repression of transcription by nucleosomes and histone H1, and specific loss of positioned nucleosomes during agent induction (133). To discriminate which is the case, the transgenic study will be a powerful tool as an *in vivo* assay system. The widespread use of transgenic technology has provided valuable and novel insights into a variety of complex systems including gene expression. I am now generating transgenic mice using the cloned human

activin gene with an expectation of getting informative findings concerning the activin gene expression. Such mouse, expected to overexpress activin A, will be also useful to confirm the biological activities of activin concluded on the basis of *in vitro* experiments.

The activin/inhibin genes are regulated by multiple hormones and growth factors, and their expression varies according to cell or tissue type. Further investigations will focus on determining which DNA elements and nuclear factors are responsible not only for the multifactorial induction but also for cell-type specific differences in the expression pattern of the activin gene. I expect that the results presented in this thesis will contribute to understanding the functions and regulatory mechanisms of expression of members of activin/inhibin gene family.

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