Human Activin βA Gene
IDENTIFICATION OF NOVEL 5’ EXON, FUNCTIONAL PROMOTER, AND ENHANCERS*

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Keiji Tanimoto‡, Eisaku Yoshida‡, Shunji Mita‡, Yutaka Nibu‡, Kazuo Murakami‡§, and Akiyoshi Fukamizu‡¶

From the ‡Institute of Applied Biochemistry and §Tsukuba Advanced Research Alliance (TARA), University of Tsukuba, Tsukuba, Ibaraki 305, Japan

On the basis of cDNA cloning, primer extension, and transfection experiments, we identified a novel 5’ exon of the human activin βA subunit gene, and found its enhancer and promoter regions as well as multiple transcription start sites. A series of deletion and mutation analyses of the enhancer sequences defined the 45-base pair core region (DR-1 core) containing two short elements with similarity to AP-1 (12-O-tetradecanoylphorbol-13-acetate response element; TRE) and CREB/ATF (cyclic AMP response element; CRE) binding sites, both of which were necessary for full enhancer activity. Gel shift and antibody supershift assays using DR-1 core region revealed the formation of two specific DNA-protein complexes, one of which could be partially dissociated by a competing oligonucleotide containing a single copy of the consensus TRE, but the other of which contained neither CREB/ATF nor AP-1 as major components. Although 12-O-tetradecanoylphorbol-13-acetate and cAMP induced the activin enhancer/promoter-driven CAT activity, such drug induction was obscured when either the TRE- or CRE-like elements were mutated in the native promoter context. Our results demonstrate that the promoter and enhancer regions identified here are essential for maintaining the efficient promoter activity of the human activin βA subunit gene.

Activins, originally characterized based on their ability to stimulate follicle-stimulating hormone secretion by cultures of rat anterior pituitary cells (1, 2), are members of an extensive family of growth and differentiation factors that include inhibin, transforming growth factor-β, Müllerian inhibitory substance, the fly decapentaplegic gene complex, and the product of Xenopus Vg-1 mRNA (3). Further investigations revealed multiple functions of activin, such as stimulation of hematopoiesis (4, 5), paracrine regulation of ovarian and testicular functions (6–8), modulation of nerve cell differentiation (9, 10), and mesodermal induction in early embryos (11–14). Inhibins were initially described as an inhibitory activin-like molecule, whereas activin was found to be formed by a homo- or heterodimer of the two β-subunits. These three subunits are encoded by three separate genes, and the S1 nuclease analysis in adult rat tissues has shown that the expression of mRNAs encoding these subunits (α, βA, and βB) varies by severalfold in a tissue-specific manner (16). Thus, differential subunit association results in the formation of dimers with opposing biological activities in a manner dependent on their sites of production.

Since expression of the active and inhibin subunit genes must each be precisely regulated, the identification of cis-acting elements that control the activin gene expression and the elucidation of how these elements are involved in the response to extracellular stimuli are of key importance. Recently, we reported that expression of the human activin βA gene is stimulated by the addition of 12-O-tetradecanoylphorbol 13-acetate (TPA) (17) or 8-bromo-cAMP (8-Br-cAMP) (18) and that the effects of these two drugs were synergistic in human fibrosarcoma HT1080 cells but not in either HeLa or HepG2 cells (Ref. 19 and data not shown). However, the DNA sequences that regulate expression of the gene have not been defined. Although several groups including ours have reported the cloning and sequence of the coding portion of the human activin βA gene (20–22), neither the complete structural organization of the activin βA gene nor its functional promoter or any other regulatory elements have been previously defined. We therefore report the complete structure for the activin βA subunit gene, as well as studies identifying crucial regulatory elements and transcription factors that are involved in its transcriptional control.

EXPERIMENTAL PROCEDURES
Cell Culture—Human fibrosarcoma HT1080 cells (CCL-121, American Type Culture Collection) were maintained at 37 °C and 5% CO2 in minimum essential medium (Nissui) supplemented with 10% fetal bovine serum (Flow Laboratories).
Isolation of cDNA Clones and Nucleotide Sequence Determination—Total RNA was extracted from HT1080 cells (that were treated with TPA at 100 ng/ml for 4 h) by using ISOGEN (Nippon Gene). Poly(A)+ RNA was selected by oligo(dT)-cellulose chromatography. cDNA was synthesized using random primers (6-mer; Pharmacia Biotech Inc.), followed by ligation to adapters containing EcoRI and NotI sites, digested with EcoRI, and finally ligated with EcoRI-digested, dephosphorylated AZAPII DNA (Stratagene). Screening of this library (7.5 × 106 plaques) with 32P-labeled human activin βA subunit probe (221-bp DraI

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‡ To whom correspondence should be addressed: Tel/Fax: 81-298-53-6599; Fax: 81-298-53-4605; E-mail: akif@saikku.cc.tsukuba.ac.jp.
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fragment in Fig. 1A; bases 810-1824) (22) yielded five positive plaques. All five were purified, and the nucleotide sequence of their 5'-ends was determined.

**Plasmid Constructions**—All of the fragments used in the constructions were excised from the genomic clone (22) containing the human activin 5'-upstream region. pBlACAT19 was constructed as follows. A 1015-bp Dral fragment (bases 810-1824; Ref. 22) was ligated with HindIII linker, digested with EcoT141I and HindIII to generate an 852-bp fragment (bases 973-1824). This DrallEcoT141I fragment (bases 1-975) were inserted in the BglII/HindIII sites of pUCSV0CAT (23). To make plhACAT35, a 3.5-bk BamHI fragment (bases -847) was blunt-ended by the fill-in reaction using the Klenow fragment (TaKaRa), ligated with HindIII linker, digested with HindIII, and inserted in the HindIII site of pUC0CAT (24). A 2.7-kb HincII fragment was excised from the pRSV-c-Jun (25) vector (bases 973-1356) and inserted in the HindIII site of pUC0CAT to make pHACAT45. Construction of pUCSV0CAT and pUCSV3CAT was described previously (23).

For construction of 5'-deletion mutants of the activin 5'-upstream region, the 3.5-bk BamHI fragment (the same as that used for construction of phACAT35) was inserted in the BamHI site of pBluescriptII KS(+) (Stratagene) in the reverse orientation. This plasmid was linearized with XbaI and PstI restriction enzymes, partially digested with exonuclease BAL31 (TaKaRa) for various lengths of time, treated with Klenow fragment to form blunt ends, and ligated in the presence of BglII linker. DNAs were transferred into Escherichia coli HB101. After determination of the 5'-deleted ends by DNA sequencing, the fragments were isolated (sites were phosphatased unless otherwise indicated) with a previously described activin gene at position 1 (22). The deleted fragments were inserted in the BglII site, located upstream of the 1.9-bk BglII-HindIII fragment, of phACAT19 to make pHACAT30, -29, -28, -26, and -24.

The putative enhancer (DR-1; -275 to -89, Fig. 1A) and promoter (FR-2; -88 to +103) elements were synthesized by polymerase chain reaction (PCR) using pBlACAT30 as a template. Oligonucleotides used to produce these fragments were synthesized on a Milligen/Bioresearch Cyclone™ Plus oligonucleotide synthesizer. Each primer contains either BglII (in sense primers) or BamHI (in antisense primers) recognition sites. The positions of the synthetic oligonucleotides were as follows: 1S (-275/-257), 1A (-89/-106), 2S (-91/-76), 2A (+104/+86). The truncated or mutated DR-1 fragments were also synthesized by PCR. The positions of the synthetic oligonucleotides used were as follows: 1S(-180/-162), 1S2 (-158/-141), 1S3 (-151/-133), 1A1 (-114/-130), 1A2 (-140/-157), 1M1 (-158/-136), 1M1C (-114/-134), 1M2 (-114/-146). The oligonucleotides used for site-directed mutagenesis were dM1, dM1C, and dM1 for D, A, C, and D mutations, respectively. The DR-1 fragment and its derivatives, cut with BglII and BamHI, were inserted upstream of the 5'-deletion construct (Fig. 5A) or pUC0CAT (Figs. 4 and 6) (17), which contains the CAT gene driven by the herpes simplex virus thymidine kinase (TK) promoter.

**RESULTS**

**Cloning and Sequence Analysis of the 5'-untranslated Region of cDNA for Human Activin βA**—The complete genomic structure (including a description of the number and organization of exons and introns) of the activin βA subunit gene has not been reported. Although more extended sequences lying 5’ to the translated region of the βA gene have been reported for the mouse (32), rat (33), and human (34) cDNAs, the corresponding genomic sequences have not been defined. We previously cloned the gene for the human activin βA subunit and determined the nucleotide sequence extending from 1857 bp 5’ to the translation start codon (22). However, a computer-assisted homology search did not identify significant identities between either rodent 5'-untranslated region and this region of the human genomic sequence (not shown). We have considered the possibility that a previously unidentified 5’ exon might exist and that this hypothetical exon lay even further upstream of the activin βA-coding sequence. To test this hy-
Fig. 1. Structure and nucleotide sequence of the human activin bA gene. A, nucleotide sequence of the 5′-flanking region, exon 1, and a part of intron 1 and exon 2 of the human activin bA gene. The intron sequences are shown in lowercase letters. Open circles indicate the positions of the transcription start sites determined by the primer extension analysis, as shown in Fig. 2. The nucleotide number +1 is assigned to the predominant transcription initiation site. The 5′-ends of different cDNA clones are indicated by closed circles. The positions of the antisense primers, PR3PX and HBA-6, used for primer extension (Fig. 2) are indicated by arrows. B, schematic representation of intron/exon organization of the human activin bA gene. The open and solid boxes indicate the noncoding and coding exons, respectively. The positions of the transcription initiation codon (ATG) and termination codon (TAG) are shown. Consensus polyadenylation signals (vertical lines) are indicated in the 3′-untranslated region.

To determine the transcription start site of the human activin A gene, we conducted primer extension analysis using two different primers, complimentary to sequences within exon 1 or 2 (Fig. 2A), and these yielded two major and several minor extension products that were in good agreement with each other (lanes 4 and 7 in Fig. 2B) and with the RNase protection assays (data not shown). No extension product was formed when yeast tRNA (lane 5) or RNA from noninduced HT1080 cells (lanes 3 and 6) were used. When the size of the products (Fig. 2B, lane 4) was compared with sequence ladders primed using the same primer on a high resolution sequence gel, multiple transcription start sites were detected (Fig. 2C, lane 2). Comparing the data obtained by both primer extension and RNase protection analyses, the band having the strongest intensity (an A located 143 bases 5′ to the initiation codon) was assigned position +1 (Figs. 1A and 2C).

We determined the sequences of the putative 5′-flanking regions and found several nucleotide consensus motifs that are involved in transcriptional regulation of many genes (Fig. 1A). Possible TATA boxes (TATAAA and TACAAA) were found 29 bp upstream from the first nucleotide of the ATG initiation region, although of varying length. Comparison of these untranslated sequences with our previously reported genomic sequences (22) for the human activin A gene, respectively, Introns and 5′-flanking region are indicated by thin lines. B, primers specific for exon 1 (PR3PX, lanes 2–5) and exon 2 (HBA-6; lanes 6 and 7) were hybridized with 20 μg of poly(A)+ RNA from TPA-treated (lanes 4 and 7) or nontreated (lanes 3 and 6) HT1080 cells or with 20 μg of yeast tRNA (lane 5) and extended with reverse transcriptase. Products were analyzed on an 8 M urea, 5% polyacrylamide gel. C, the extended products shown in lanes 3 and 4 in B were electrophoresed in lanes 1 and 2, respectively, adjacent to a DNA sequencing ladder (lanes A, C, G, and T) by using the same primer as in the primer extension reaction. Arrows indicate the positions of extended products in lane 2. The major cap site is circled and represents nucleotide +1.

sequences (Fig. 1A) (36).

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We determined the sequences of the putative 5′-flanking regions and found several nucleotide consensus motifs that are involved in transcriptional regulation of many genes (Fig. 1A). Possible TATA boxes (TATAAA and TACAAA) were found 29 and 47 bp, respectively, upstream of the major transcription start sites determined by primer extension analysis, a typical
promoter feature of many eukaryotic genes. In addition, CCAAT boxes, another element often found in RNA polymerase II-governed promoters, are present at positions 2270, 2103, and 260. GATA and CACCC box binding sites, which are common structural and functional features of the promoters of many genes, are also present (37). Two putative AP-1 (c-Jun homodimer or c-Jun-c-Fos heterodimer) binding sites (TPA response element; TRE) (T1 site, 2201 to 2195; T2 site, 2153 to 2147) (38, 39), one lymphokine-specific sequence (40, 41), one PEA-3 binding sequence (42–44), one cyclic AMP response element (CRE)-like site (C site, −123 to −116) (45), and a consensus AP-2 binding site (46) were also present.

Identification of Sequences Required for Activin \(\beta_A\) Transcription—To identify the sequences responsible for controlling the expression of the human activin \(\beta_A\) gene, CAT constructs that contained various lengths of the 5'flanking sequences of this gene were generated (Fig. 3A). A DraI restriction site, lying 36 bp 5' to the translation start site and within the mature mRNA (Fig. 1A), was used as the 3'-end of each construct. A series of the 5'-deletion mutants were transiently transfected into HT-cJun cells (17) because the levels of activity expressed by these transiently transfected constructs were too low to be determined accurately. The promoterless plasmid pUCSV0CAT was used as a background control, and pUCSV3CAT (a reporter directed by the SV40 enhancer-promoter region) was used as a positive control. As shown in Fig. 3B, deletion of activin \(\beta_A\) sequences up to position 3.0 kb to the gene (in ph\(\beta_A\) ACAT30) resulted in a 2-fold increase in CAT activity relative to the parental full-length plasmid (ph\(\beta_A\) ACAT45). This suggests the presence of negative regulatory region(s) within 4.5 and 3.0 kb of the gene. Deletion of the next (approximately) 100 bp (ph\(\beta_A\) ACAT29) led to a dramatic reduction in activity (18% of ph\(\beta_A\) ACAT30). Further deletion of the next 100 bp (ph\(\beta_A\) ACAT28) reduced activity to background levels.

To test whether these same regions are also active without c-Jun expression, some representatives of this deletion series...
were stably transfected into the HT1080 cells and analyzed for CAT activity (Fig. 3C). Deletion of the activin βA gene sequences from −4.5 to −3.0 kb resulted in an overall 4.8-fold increase in CAT activity, again suggesting the existence of negative regulatory elements within this region that affect both basal and c-Jun-induced transcription. Further deletion of 5′ sequences (e.g. in phβACAT29) severely reduced reporter gene activity (13% of the phβACAT30 activity). phβACAT28 exhibited no activity above background. These results showed that a positive regulatory element, located between the region described by constructs phβACAT30 and phβACAT28, is required for expression of the activin A gene. We designated this region DR (distal region)-1 (Fig. 3A).

To ascertain whether or not DR-1 fulfilled the requirements of an enhancer, a DNA fragment corresponding to sequences lying between −275 and −89 (designated DR-1; nucleotide numbering as shown in Fig. 1A) was synthesized by PCR and then fused to a CAT reporter gene construct that was transcriptionally directed by the herpes simplex virus TK promoter (Fig. 4A). These test constructs were then transiently transfected into HT-cJun cells. CAT activities were normalized to that obtained with the enhancerless vector, p0/tk-CAT, which was arbitrarily assigned the value 1. The addition of the DR-1 fragment in the sense and antisense orientations produced a 19- and 17-fold stimulation of the TK promoter (data not shown). These results show that the 187-bp DR-1 fragment functions as a powerful enhancer in HT-cJun cells.

Identification of Activin βA Promoter—Since the DR-1 fragment alone was unable to confer transcription to a cis-linked reporter gene (data not shown), further deletion analysis of more proximal regions to the transcription initiation sites, placed under the control of the DR-1 enhancer, was performed to determine whether any additional segments of this 5′ region contributed to gene activity. Removing DR-1 from phβACAT30 completely abolished CAT activity (phβACAT28), and as anticipated, the addition of DR-1 (in the reverse orientation) to phβACAT28 (pDR1(−)/CAT28) partially restored this activity (Fig. 5a). However, DR-1 was unable to restore the activity of constructs containing more significant deletions (e.g. in constructs pDR1(−)/CAT26, pDR1(−)/CAT24, and pDR1(−)/CAT19; Fig. 5a), suggesting that the region of the activin A gene described by the end points lying between phβACAT28 and phβACAT26 is also required for transcription. We designated this region PR (proximal region)-2 (−88 and +103, nucleotide numbering as in Fig. 1A; Fig. 5b, top).

To further characterize the PR-2, this DNA region was prepared by PCR and analyzed for activity after transfection into HT-cJun cells. As shown in Fig. 5B, PR-2 alone had little CAT activity (PR2(+)/CAT) in comparison with the negative control. When the DR-1 enhancer was included in the construct with the sense-oriented PR-2 fragment (pDR1(−)/PR2(+)/CAT), a significant stimulation in activity was observed. However, the reverse-oriented PR-2 fragment in the same construct (pDR1(−)/PR2(−)/CAT) gave background levels of activity. Transcriptional enhancement was also observed using the heterologous SV40 enhancer (pSVE(+)/PR2(+)/CAT). These results thus demonstrate that PR-2 contains the activin βA gene promoter.

Analysis of DR-1 Enhancer—A search of the DR-1 region for known consensus binding sites for transcription factors identified several potential sequences, such as two TRE (AP-1 binding sequences, T1 and T2)-like and CRE (CREB/ATF binding sequences, C)-like sequences (Fig. 1A). It was reported that the CRE is efficiently trans-activated by cJun/AP-1 and that some of the CRE/ATF transcription factors heterodimerize with c-Jun to bind to the CRE (47). Deletion mutations were targeted to determine whether the three potential T1, T2, and C sites (Fig. 1A and 6A) individually or combinatorially contributed to DR-1 enhancer function. A series of 5′- and/or 3′-deletion mutants from the DR-1 enhancer fragment were placed in the sense orientation 5′ to the TK gene promoter (p0/tk-CAT), and their contribution to transcriptional activity was tested in transiently transfected HT-cJun cells (Fig. 6A).
Deletion of site T1 (pDR1(−180:−89)/tk-CAT) from the parental construct (pDR1(−275:−89)/tk-CAT) moderately reduced the enhancer activity of DR-1. Nevertheless, the same deletion had a statistically insignificant effect on enhancer activity when introduced into pDR1(−275:−114)/tk-CAT (compared with pDR1(−180:−114)/tk-CAT). Further deletion of sequences at the 5′-end (in pDR1(−151:−89)/tk-CAT) reduced the enhancer activity to 16% of the original activity, which is probably attributable to destruction of sequence T2, 5′-TGTCCTCA-3′, lying between −153 and −147. Results examining the activity of the 3′-deletion mutants indicated that sequences up to nucleotide −114 had no significant role in enhancer activity of DR-1 (constructs pDR1(−275:−114)/tk-CAT and pDR1(−180:−114)/tk-CAT). However, deletion of 26 nucleotides 5′ from position C reduced enhancer activity to less than 18% of the original level (pDR1(−275:−140)/tk-CAT). A DNA fragment that contains both T2 and C sites (pDR1(−158:−114)/tk-CAT) exhibited significant enhancer activity. In summary, this detailed mutational analysis identified the core enhancer sequences of DR-1 as lying between nucleotide positions −158 and −114, and we subsequently refer to this region as the DR-1 core.

To directly demonstrate a functional role for the two putative sequence motifs in the activity of the DR-1 core enhancer, we deleted three and seven nucleotides from each of the putative binding sites for transcription factors (as well as from the region between the sites) using PCR site-directed mutagenesis (Fig. 6B). Four different deletion mutants for DR-1 core enhancer were examined: one for the T2 site (ΔT), one for the C site (ΔC), one for the double mutant containing both of them (ΔCT), and one for the intervening sequence (ΔI). Each of these mutants was cloned 5′ to the TK promoter (in the p0/tk-CAT reporter plasmid), and their individual effects on transcriptional activity were tested after transfection into HT1080 cells. As shown in Fig. 6C, mutation of sequences lying between these two elements (ΔI; pDR1(coreΔI)/tk-CAT) modestly increased enhancer activity, whereas the ΔT mutant (pDR1(coreΔT)/tk-CAT) reduced CAT activity to about 22% of the wild type enhancer level, but still well above the activity of the TK promoter alone. In contrast, the activities of the ΔC and ΔCT mutants (pDR1(coreΔC)/tk-CAT and pDR1(coreΔCT)/tk-CAT) virtually completely abolished enhancer activity. These results suggest that a combined action of T2 and C sequences is important for enhancer activity of the DR-1 core fragment, although the T2 function may be dependent on the presence of site C.

Detection of Specific DNA/Protein Interactions within DR-1—To examine the ability of the T2 and C elements to interact with trans-acting factors, the DR-1 core fragment was examined in gel shift assays with nuclear extracts prepared from HT1080 cells (Fig. 7). After electrophoresis of the products of binding reactions in polyacrylamide gels, two complexes, designated C1 and C2, were identified (lanes 2 and 11). Formation of both complexes was efficiently inhibited after adding an excess of unlabeled DR-1 core fragment (lanes 3 and 4). To test whether the T2 and C sites were also critical for protein-DNA complex formation, the ΔT and ΔC mutations were individually introduced into the DR-1 core fragment. When these mutant fragments were used in competition experiments, only the DR-1 core fragment with ΔT mutation (core-ΔT) was able to efficiently compete for protein binding in both complexes (lane 5), indicating that the core-ΔT fragment retains binding activity to both complexes. On the other hand, the DR-1 core fragment harboring the ΔC mutation failed to compete with C2 complex formation (lane 6).

Since the T2 and C sites were similar to the authentic TRE (25) and CRE (47), respectively, we investigated whether the DR-1 core binding complexes contained these factors. As shown in Fig. 7, a 200-fold molar excess of a single copy of consensus TRE-containing oligonucleotide (AP-1(s)) (lanes 8 and 13) was able to compete for C1 complex formation only weakly, indicating that this complex includes members of the AP-1 family. However, the DR-1 core fragment appears to compete much more effectively than does TRE oligonucleotide for the formation of complex C1 (compare lanes 3, 4, and 8), suggesting that this complex does not consist principally of AP-1. This was also confirmed in supershift experiments (lane 9) using a polyclonal antibody that recognizes Jun family proteins (c-Jun, JunB, and JunD). Only the formation of complex C1 was perturbed by the inclusion of antibody to the DNA association reaction, but only to a very limited extent. The formation of both complexes was also unaffected by the addition of a CRE competitor (lanes 7 and 12); similarly, the addition of a monoclonal antibody against CREB/ATF family proteins (ATF-1, CREB-1, and CREM-1) left the two complexes essentially unaffected (lane 15). Parallel experiments showed that anti-Jun family and anti-CREB/ATF family antibodies effectively supershifted the
complexes formed on legitimate CRE (lanes 16–19) and TRE (lanes 20–23) probes, respectively. Interestingly, a tandem duplicated copy of the TRE oligonucleotide (200-fold molar excess of AP-1(w), lane 14) effectively eradicated C2 complex formation, although an 800-fold molar excess of AP-1(s) did not (data not shown). No competition with either complex was observed when a 200-fold molar excess of unlabeled oligonucleotides containing consensus binding sites for Sp-1, NF-1, and C/EBP were added to the reactions (data not shown).

**Induction of Activin βA Gene Transcription by TPA and cAMP**—We previously reported that expression of the human activin βA gene in HT1080 cells was induced by both TPA and 8-Br-cAMP and that the effects of these two drugs were synergistic (17–19). We postulated that this induction might be mediated at the transcriptional level, possibly through a DR-1 element. To investigate this possibility, both pDR1(+)/PR2(+)/CAT and pPR2(+)/CAT were transiently transfected into HT1080 cells, followed by treatment with one or both of the inducing agents. An autoradiogram of one representative experiment is shown in Fig. 8A, and in support of the hypothesis that DR-1 mediates the inductive responsiveness of the activin βA gene, the expression of pDR1(+)/PR2(+)/CAT, but not PR2(+)/CAT (data not shown), was stimulated by TPA (2.3-fold) or 8-Br-cAMP (2.2-fold) or both (5.5-fold). Furthermore, a duplicated DR-1 fragment directing expression from a heterologous (TK gene) promoter showed a synergistic responsiveness to induction by both drugs (Fig. 8B). These results demonstrated that the DR-1, including T2 and C sites, and the PR-2 are both necessary and sufficient for induction of the activin βA gene by TPA and/or 8-Br-cAMP in HT1080 cells.

Site-directed mutagenesis was performed to determine whether T2 and C sites were functionally relevant to the induction of activin βA gene transcription by TPA and/or 8-Br-cAMP (Fig. 9). The pDRI1(+)/ΔT/PR2(+)/CAT construct containing the 3-bp T2 site deletion produced 80% lower basal activity in comparison with the wild-type response (pDR1(+)/PR2(+)/CAT). However, this mutant also responded significantly (albeit slightly weaker) to TPA induction (1.9-fold), 8-Br-cAMP induction (1.9-fold), and the additive effects mediated by the addition of both TPA and 8-Br-cAMP (3.4-fold) in the native promoter context. Basal expression produced from the construct containing the 3-bp C site deletion (pDR1(+)/ΔC/PR2(+)/CAT) was reduced by 95% in comparison with the wild-type construct; as with the ΔT mutant, however, a modest inductive response to drug treatment was still retained. The three constructs were also found to respond positively to elevated AP-1 levels mediated by co-transfection with a c-Jun expression vector.

**DISCUSSION**

On the basis of the results presented here, the structural organization of the human activin βA gene was shown to consist of three exons interrupted by two introns (Fig. 1B), which is totally different from the composition of the human α or βB subunits, since both have only two exons (20, 21, 34, 42). When we sequenced and compared the 5′-flanking regions of both the human (Fig. 1) and mouse βA (4) genes, the DR-1 and PR-2 regions were found to be highly similar in their nucleotide sequence. This evolutionary conservation suggests that these conserved regions play an important role in the regulation of activin A expression in both species. In addition, the T2 and C sites in the DR-1 core (Fig. 3B) are also conserved in both the human and mouse activin βA promoters at the same positions. In contrast, the sequences of the 5′-flanking region of the human βA gene is different from that of α (rat (48–50) and mouse (51)) and βB (human (35), rat (49, 52), and sheep (53)) subunit genes. The human, rat, and ovine activin βB subunit genes have no consensus TATA and CAAT boxes but instead consist of a series of GC boxes, a typical promoter for housekeeping genes.

To identify cis-acting DNA elements responsible for transcription of the human activin βA gene, fusion genes were constructed with progressive 5′-deletions linked to a reporter"
sequences for whose mutation partially overlaps with the putative binding site. While mutation of the C site (C) completely abolished core enhancer activity, mutation of the T2 site (T) reduced, but did not abolish, enhancer activity. Although Dorsal element has a positive regulatory element for the activin gene, factors in HT1080 cells and suggest that the complex C1 is not cell type-specific and that the DR-1 enhancer can also mediate a transcriptional activation in HeLa and HepG2 cells (data not shown). Factor(s) that confers cell-type specificity to the activin βA gene promoter likely interacts with a region outside the sequences defined in the present studies, or the gene could be regulated post-transcriptionally.

In DNA binding competition experiments (Fig. 7), complex C1 formation was greatly diminished by including either core-ΔT or core-ΔC competitor oligonucleotides. These results show that neither the T2 nor C sites are required for C1 complex formation on the DR-1 core. Unlabeled consensus AP-1 oligonucleotides (both single and double sites) displaced the binding of factor(s) to DR-1 core (C1 complex) only partially. An antibody that recognizes multiple members of Jun family proteins (c-Jun, JunB, and JunD) moderately interfered with C1 complex formation. Furthermore, bacterially expressed c-Jun protein, which is a component of the AP-1, also reacted with the DR-1 core probe (data not shown). Taken together, these results provide significant evidence that the DR-1 core enhancer is recognized by proteins of the AP-1 family of transcription factor(s) in HT1080 cells and suggest that the complex C1 is actively involved in the regulation of activin βA gene expression. However, the data also show that the Jun family and ATF proteins themselves cannot account for most of the DNA complex formation with the DR-1 core elements.

To determine whether proteins in nuclear extracts prepared from HT1080 cells could specifically interact with DR-1 core sequences, gel shift assays were conducted. These studies defined two specific protein-DNA complexes (Fig. 7). However, these binding activities were not cell type-specific, since they were found in all cell lines tested (HeLa, HepG2, T98G, and A172 cells; data not shown). These results are entirely consistent with those from stable transfection experiments, showing that the basal transcriptional activity expressed from the largest reporter construct is not cell type-specific and that the DR-1 enhancer can also mediate a transcriptional activation in HeLa and HepG2 cells (data not shown). Factor(s) that confers cell-type specificity to the activin βA gene promoter likely interacts with a region outside the sequences defined in the present studies, or the gene could be regulated post-transcriptionally.
activin demonstrates that a nuclear protein binding to the C site even in
which contains a mutation in T2 but an intact C site, directed
present nearby. On the other hand, pDR1(+/T/PR2(+)/CAT)
of expression (Figs. 9 and 10)
functions as a
to be indispensable for enhancer function. In other words, T2
expression of the activin
suggests that certain extracellular ligands may regulate the
responsive site(s) within the activin enhancer and promoter
determined, the identification of functional c-Jun/AP-1-
extracellular ligands (56). Although the precise location is still
formcomplexC1.Thec-Jun/AP-1isaprimarynucleartargetof
respectively) used in these constructs still retain the ability to
form complex C1. The c-Jun/AP-1 is a primary nuclear target of
receptor-mediated signal transduction pathways activated by
extracellular ligands (56). Although the precise location is still
determined, the identification of functional c-Jun/AP-1-
responsive site(s) within the activin enhancer and promoter
suggests that certain extracellular ligands may regulate the
expression of the activin βA subunit gene.

pDR1(+)/ΔC/PR2(+)/CAT, which contains a mutation in the C
site but an intact T2 site, directed almost background levels of
expression (Figs. 9 and 10A), indicating that site C appears
to be indispensable for enhancer function. In other words, T2
functions as a cis-acting DNA element only when site C is
present nearby. On the other hand, pDR1(+)/ΔT/PR2(+)/CAT,
which contains a mutation in T2 but an intact C site, directed
low, but nonetheless significant, levels of expression. This
demonstrates that a nuclear protein binding to the C site even in
the absence of a functional T2 site weakly trans-activates the
activin βA promoter. Thus, we can speculate that T2 site
binding protein(s) may take part in C2 complex formation, utilizing
the C site binding factor(s) as a scaffold. There is no discrep-
ancy between this model and the experimental observation
that the core-ΔT competitor effectively disrupted the C2
complex (Fig. 7). Since the site C binding factor(s) would hold a
dominant position hierarchically in complex formation in this
model, a C2 complex would never form in the absence of the C
site binding factor(s) (Fig. 10B). The gel shift assay result using
monomeric versus dimeric AP-1 sites as competitors also sup-
ports this model (Fig. 7); tandem duplicated and properly spaced
consensus AP-1 binding sites may substitute for the binding sites
in C2 complex formation in the DR-1 core (Fig. 10B).

Previous investigations of the regulation of the human activin
βA gene by various drugs, including phorbol ester (TPA)
and 8-Br-cAMP, were based on assays for the biological activity
or mRNA levels of activin (4, 18, 57). In this report, we describe
the first identification of the enhancer and promoter for this
genome, as well as the possible basis for the effects of these drugs
on transcription from this locus. Transcriptional activity of the
activin βA enhancer-promoter-CAT fusion constructs in
HT1080 cells significantly increased after treatment with TPA
or 8-Br-cAMP (Fig. 8). Hence, the increase in activin βA mRNA
level was due, at least in part, to an increase in activin βA gene
template activity. The discrepancy between the relative in-
crease in levels of mRNA (19) and gene transcription rates
implies that the stability of mRNA may be affected by the drug
treatment.

TPA and cAMP responsiveness of several genes is mediated
via the common AP-1 binding site (referred to as a TRE, or
TPA-responsive element) and/or cAMP response element
(CRE), respectively (47). The CREs identified in the promoters
of several c-AMP-responsive genes (43, 58, 59) have been shown
to interact with the nuclear factors CREB/ATF (60, 61).
A CRE-like sequence identified within the DR-1 core region
(5′-TGATGTCA-3′) differs from the consensus sequence at one
nucleotide (5′-TGACGTCA-3′) (Fig. 1A). However, this same
sequence has been reported to be functional CRE in the human
retinoic acid receptor type β gene (62). Although transfection
analyses disclosed the importance of this CRE-like se-
quence in maintaining DR-1 enhancer activity (Figs. 6 and 9),
neither competitor oligonucleotides nor monoclonal antibodies
specific for CREB/ATFs interfered with the formation of com-
plexes on the DR-1 core probe in the gel shift assay, suggesting
that these complexes do not contain CREB/ATF transcription
factors.

In the transfection experiments reported here, enhancer-
promoter-CAT fusion constructs with mutations at TRE-like
(pDR1(+)/ΔT/PR2(+)/CAT) and CRE-like (pDR1(+)/ΔC/PR2(+)/
CAT) sites lowered basal expression by 80 and 95%, respec-
tively, and yet TPA and/or 8-Br-cAMP responsiveness was
somewhat retained in both constructs (Fig. 9). There can be no
doubt that these particular cis-acting elements play a role in
maintaining normal, basal activin βA gene expression and are
probably responsible in part for the inducibility. However, in
the absence of TRE-like and CRE-like sites in each construct,
other sequences must function as drug-responsive elements.
One possibility is that another TRE-like site (T1), which was
left intact in the site-directed mutant T2 and C constructs, may
provide an alternative site through which TPA and/or cAMP
inducibility might be mediated. Because AP-1 recognizes not
only TRE, but also CRE, and expression of the protooncogenes
c-fos and c-jun/AP-1 is induced via the cAMP pathway (63), it is
possible that the expression of the activin βA gene by cAMP
may also require the c-Jun/AP-1 system. Of special note within
the context of the DR-1 enhancer, it has also been reported that
a PEA-3 binding site in conjunction with the AP-1 element
within an enhancer and promoter has been shown to be in-
volved in phorbol ester induction, and that PEA-3 and AP-1
motifs function synergistically and cooperatively for transcrip-
tional activation (42–44). The cis-acting DNA elements that
are known to mediate cAMP activation also include the AP-2
element (46). Although this motif was identified in the PR-2
region (Fig. 1A), the pPR2(+)/CAT construct was not respon-
sive to 8-Br-cAMP induction (data not shown).

In this report, we show experiments that allow us to
completely describe the primary structure of the human activin βA
gene. We also detail and delineate two critical regulatory ele-
ments involved in phorbol ester induction, and that PEA-3 and AP-1

![Hypothetical model of the function upon T and C sites.](image)
ments, the promoter and upstream enhancer, that are necessary for basal and efficient expression of the gene. Finally, we provide functional evidence that transcriptional regulation of the human activin βA gene by TPA and 8-BrcAMP involves both of these 5'-flanking DNA sequence elements. Further study of the functional significance of the T2 site, C site, and other putative regulatory elements within and beyond the DR-1 region promises to provide insight into the exquisite control of inhibin and activin production in vitro and in vivo.

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