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Acidic Fibroblast Growth Factor Gene 5′ Non-Coding Exon and Flanking Region From Hamster DDT1 Cells: Identification of the Promoter Region and Transcriptional Regulation by Testosterone and aFGF Protein

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Abstract Selected clones of Syrian hamster DDT1-MF2 cells are responsive to testosterone for growth. Heparin binding growth factor 1 (HBGF-1) or acidic fibroblast growth factor (aFGF) can replace testosterone (T) in the stimulation of growth in these cells. This phenomena is correlated with testosterone's ability to elevate aFGF mRNA two- to threefold in DDT1 cells. To better understand the possible mechanisms of regulation of aFGF mRNA by steroids and other growth factors, we isolated the aFGF 5′ non-coding exon and its flanking region from a EMBL3 DDT1 genomic library, using a 5′ non-coding exon 69 bp DDT1 aFGF cDNA probe. Clones spanning 30 kb of genomic DNA were isolated. After restriction mapping and DNA sequence analysis, the clones were shown to contain all of the 5′ non-coding exon included in the cDNA and approximately 10 kb of 5′ flanking region. RNase protection and primer extension assays confirmed that the 5′ non-coding exon is included in the DDT1 aFGF mRNA and that a major transcription start site is approximately 136 bp upstream of the 5′ non-coding splice junction of this exon. The 5′ flanking region DNA was inserted into pBLCAT3 reporter gene and transfected into DDT1 cells. Chloramphenicol acetyltransferase (CAT) assays demonstrated that there are promoter elements in the −1645/−392 and −392/+131 regions of the aFGF gene in the context of DDT1 cells. NIH 3T3 cells, on the other hand, show no CAT activity with these aFGF-CAT plasmids. CAT assays also demonstrated that addition of testosterone (T) or aFGF to DDT1 cells increased CAT activity threefold. This activity was mapped to −1645 to −4 bp region of this DDT1 aFGF gene promoter.

Key words: heparin binding growth factor 1, mRNA, Syrian hamster, acidic fibroblast growth factor gene, testosterone

Growth factors are hormone-like regulators of cell proliferation and/or differentiation in vivo and in vitro. They can act either in an autocrine, intracrine, or paracrine fashion. Altered responsiveness to and production of specific growth factors are important phenomena in a cells ability to grow and differentiate. Elucidation of the mechanisms that regulate the expression of growth factors in specific cell types will be important in understanding signaling pathways in cell growth.

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The heparin-binding fibroblast growth factor (HBGF) family contains seven structurally related species. The first two of these, HBGF-1 and HBGF-2, have been investigated under several names. Acidic fibroblast growth factor (aFGF) and basic FGF (bFGF) are the most common alternate names for these molecules [1–3]. The oncogene HBGF-3 (int-2) was discovered as a gene activated by mouse mammary tumor virus [4–7]. The HBGF-4 (hst/KS3) and HBGF-5 (FGF-5) oncogenes were identified by their ability to transform NIH 3T3 cells [8–13]. The most recent additions to the family are HBGF-6, which was isolated from a mouse cosmid library using a human hst probe and has the ability to transform NIH 3T3 cells [20] and

HBGF-7 or KGF, which is an epithelial cell-specific growth factor [15].

aFGF and bFGF are associated with several functions including stimulation of mitogenesis, acceleration of wound repair, plasminogen activator activity, angiogenesis and mesoderm induction, chemotaxis, bone growth, and neurite extension [1,16–19]. In the Syrian hamster DDT1-MF2 tumor cell line (DDT1), either aFGF or bFGF can replace androgen (testosterone) in stimulating growth [21–24]. Syrian hamster DDT1 cells are derived from an androgen dependent leiomyosarcoma of the ductus deferens [25,26]. Selected clones of DDT1 cells have been shown to be sensitive to and have receptors for androgens and glucocorticoids [27–30]. Northern analysis and *in situ* hybridization studies on DDT1 cells demonstrated a two- to fivefold increase in aFGF mRNA in cells treated with testosterone (21,31). A hamster aFGF cDNA from a lambda gt11-DDT1 library and a 38 kb genomic clone (cos 7) were isolated from a DDT1 cosmid library [31]. The cDNA clone includes all the coding sequence and about 100 bp each of the 5′ and 3′ non-coding regions. The genomic DNA (cos 7) contains the three coding exons and the 3′ non-coding region. However, 81 bp of 5′ non-coding region in the cDNA sequence was unaccounted for in this cosmid genomic clone. Analysis of cos 7 DNA showed a consensus splice site at the position where the similarity between the cDNA and genomic clones stopped. This suggested that most of 5′ non-coding region was contained in exon(s) which were upstream of cos 7. In order to pursue studies on the effects of testosterone (T) and growth factors on transcription of the DDT1 aFGF gene, this non-coding exon and flanking region was isolated.

We report here the isolation and mapping of EMBL3 DDT1 genomic clones which include this entire 5′ non-coding exon for the DDT1 aFGF mRNA and approximately 10 kb of 5′ flanking region. Subclones of DDT1 aFGF cDNA and aFGF genomic DNA were used in ribonuclease protection assays (RPA) to confirm that the non-coding exon was indeed part of the DDT1 aFGF mRNA and estimate the transcription start sites. Primer extension assays were also utilized to estimate the transcription start site(s). Chloramphenicol acetyltransferase (CAT) assays were employed to detect the presence of promoter activity in the 5′ flanking region. Our studies show that when testosterone (T) or aFGF is added to transfected DDT1 cells, CAT activity is increased about threefold using a CAT plasmid containing the 5′ non-coding exon and about 1.7 kb of 5′ flanking region. Also two regions in the 5′-flanking region were identified with promoter activity by deletion analysis and transfection of 5′ flanking-CAT constructions into DDT1 cells. The DDT1 aFGF promoter showed a degree of cell specificity.

METHODS AND MATERIALS

Plasmid Constructs

The plasmid pSKH240 was subcloned by excising a BamHI fragment from a DDT aFGF cDNA [31] in the SK vector and then religating the remaining vector-insert. It contains 231 bp of the 5′ end of the DDT1 cDNA. CAT constructs were made by inserting hamster DDT1-MF2 (DDT1) genomic restriction enzyme fragments into the vector pBLCAT3 [32]. Figure 6A shows the lineage of clones used in these studies. The fragments were generated from pKS3.0, a 3 kb Ban 1 fragment from DDT1 genomic clone 46 (Fig. 1) that was subcloned into pBLCAT3 to make pCAT-3.0. The 1.7 kb fragment was removed from the pKS3.0 plasmid with BamHI and BgIII and subcloned into the BamHI site of pBLCAT3 and referred to as pCAT-1.7. The 523 or 135 bp fragments were made by cutting the remaining vector-insert with AccI or StuI, respectively, and religating back together to produce pCAT-400 (−392/+131) and pCAT+1 (−4/+131).

Probes

A twice gel purified 69 bp aFGF specific probe to the 5′ non-coding region was generated by cutting a DDT1 aFGF cDNA with Ban 1 and EcoRI and then used to screen the DDT1 genomic library [31]. The probe was labeled with [α-^{32}P]dCTP (300 Ci/mmole) by random priming [33], using a kit from Pharmacia (Piscataway, NJ) and its specific activity was $>10^8$ cpm/μg DNA.

Ribo-probe RP1 was made from pSKH240 linearized with EcoRI and transcribed with RNA polymerase T3. RP2 was produced from pKS3.0 linearized with BspMI and transcribed by RNA polymerase T7. The RNA probes were prepared using 5 μCi [α-^{32}P]UTP (800 Ci/mmole) and a Stratagene (San Diego, CA) transcription kit.

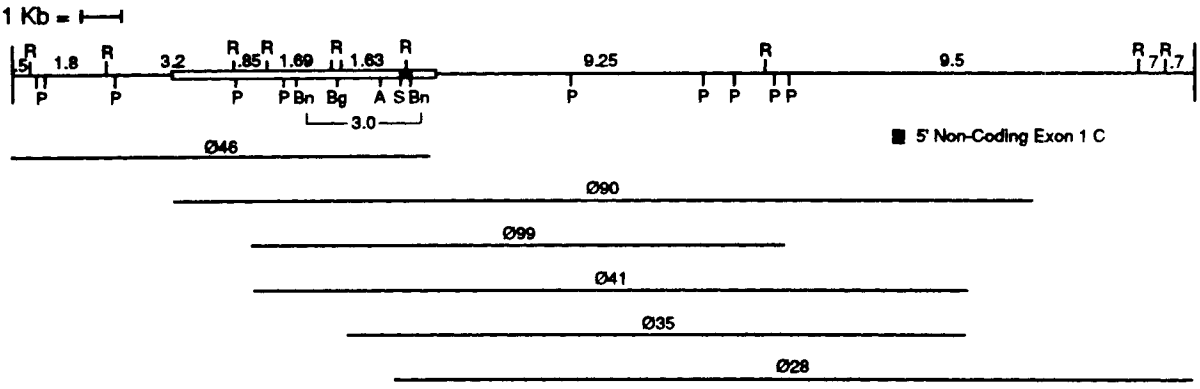


Fig. 1. Syrian hamster DNA containing the 5′ non-coding and flanking regions of the aFGF gene utilized in DDT1-MF2 cells A representative diagram of the aFGF gene DNA, containing ~10 kb of 5′ flanking, the 5′ non-coding (NC) region, and approximately 19 kb of DNA 3′ of the DDT1 5′ NC exon R = EcoR1, P = PstI, Bg = Bg1II, A = AccI, Bn = Ban 1 □, area completely sequenced at least twice, ■, 5′ non-coding exon identical to the sequence in DDT1 aFGF cDNA [31], Ø 46 through Ø 28, representation of overlapping phage clones isolated from DDT1 λEMBL library

The specific activities of the probes were $> 10^8$ cpm/μg RNA.

Genomic Library Construction and Screening

The DDT1 genomic library was generated using lambda EMBL3/BamHI digested vector (Stratagene, San Diego). DDT1 DNA was partially digested by Mbo I into 10–40 kb pieces and ligated into the BamHI digested EMBL3 arms. The DNA was packaged with Gigapack Gold phage extracts and then infected into P2PLK-17 *E. coli* cells (Stratagene). A total of 2×10^6 EMBL3 plaques were screened with the 69 bp 5′ non-coding region probe.

Mapping and Sequencing the Genomic Clones

The DDT1 genomic clones isolated from the EMBL library were subjected to Southern analysis using several restriction enzymes [34,35]. The 69 bp aFGF probe was used to determine which fragments contain the non-coding exon and help generate a restriction map of the 5′ flanking region. This data was also used to prepare the DDT1 genomic DNA for sequencing by subcloning restriction enzyme fragments into the bluescript SK-vector (Stratagene). Sequences were obtained using Applied Biosystems (ABI, Foster City, CA) 370A DNA sequencer. Reactions were performed as described in the ABI kits for Taq dye primer cycle sequencing and Sequenase dye primer sequencing using double stranded templates. Supplemental sequence information was produced by ^{35}S dideoxy chain termination method for double stranded templates using T7 DNA polymerase (United States Biochemical, Cleveland, OH) [37,38].

The DNA sequence has been submitted to GenBank with the Accession No. L06092.

RNA Preparation and RNase Protection

Total DDT1 RNA was obtained using guanidine isothiocyanate methods, described as the RNAsoyl/method (Cinna/Biotex, Houston, TX) or by centrifugation over a 2 ml cushion of 5.7 M CsC1 + 25 mM NaOAc for 2 h at 42 K rpm in a SW55 rotor [34]. The RNase protection assay was performed as previously described with the following modifications [34]. Hybridization was in the same buffer as used in primer extension at 50% formamide: 42°C, 0.4 M NaCI. After hybridization, 30 μg of total DDT1 RNA was digested with a RNA digestion buffer containing 40 ng/ml ribonuclease A (Sigma, St. Louis, MO) and 2 ng/ml ribonuclease T1 (Sigma) for 30 min at 37°C. This is referred to as 1× RNase (see Fig. 4). Approximately 3×10^5 cpm of RNA probe was used per reaction (Sp. Act. 4×10^8/μg), prepared using Stratagene transcription kit and ^{32}P-CTP.

Fig. 2. DNA sequence of DDT1 aFGF 5′-flanking region, 5′ non-coding region (Exon 1 C), and part of the 1st intron The GT at +137 is the beginning of Intron 1 The transcription start site is indicated as +1 Several candidate regulatory DNA elements are indicated (see Table I for details) *, RPA are sites protected in the RN protection assay The +1 site was determined from primer extension assays

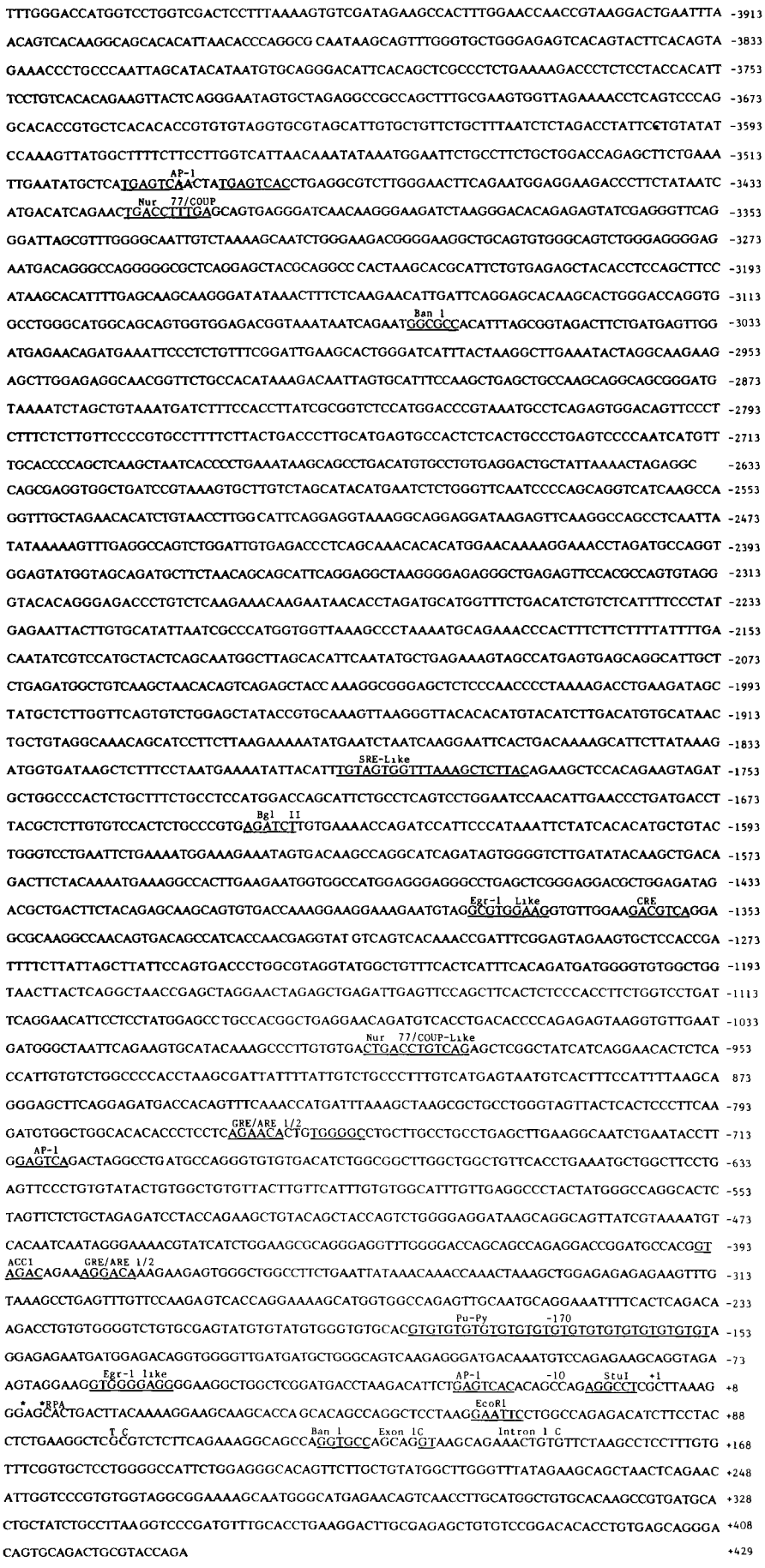

AP-1
Nur 77/COUP
Ban I
SRE-Like
Bgl II
Egr-1 Like
CRE
Nur 77/COUP-Like
GRE/ARE 1/2
AP-1
ACC1
GRE/ARE 1/2
Pu-Py
-170
Egr-1 like
AP-1
-10
StuI
+1
RPA
EcoRI
T C
Ban I
Exon 1C
Intron 1 C

Figure 2.

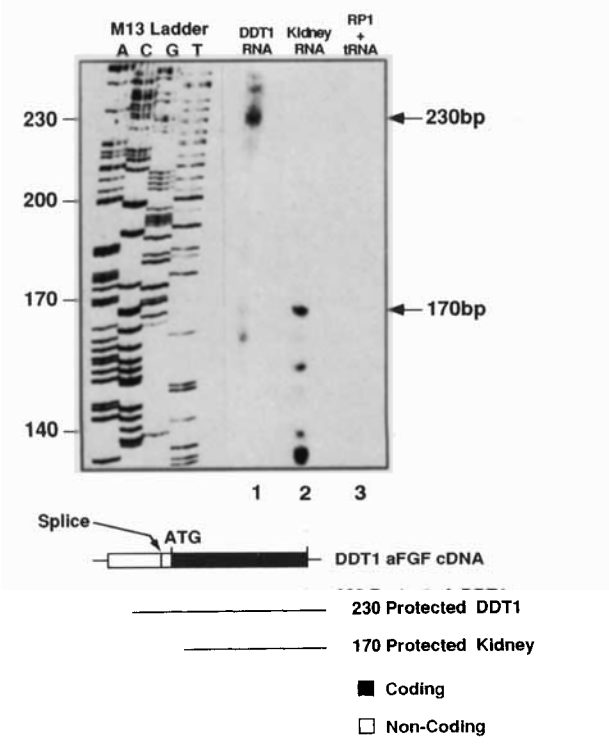


Fig. 3. The RNase protection assay using riboprobe RP1 containing DDT1 5′ NC region and part the ATG containing coding exon 2 [31]. **Lane 1:** 30 μg total DDT1 RNA. **Lane 2:** Total normal hamster kidney RNA (30 μg). **Lane 3:** RP1 + 10 μg tRNA.

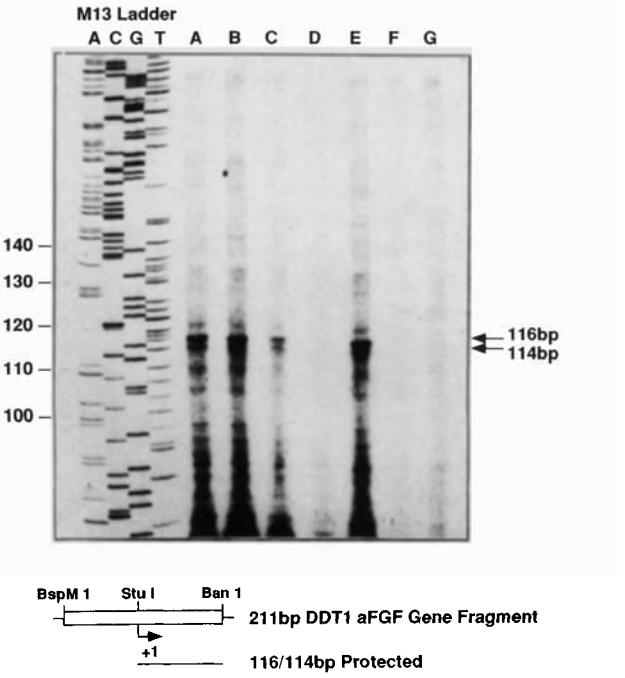


Fig. 4. The RNase protection assay using riboprobe RP2 containing complimentary 5′ flanking and 5′ non-coding RNA sequences. **Lane A:** Total DDT1 RNA (15 μg) 1× RNase. **Lane B:** Total RNA (30 μg) 1× RNase. **Lane C:** Total RNA (30 μg) 5× RNase (see Methods). **Lane D:** Total RNA (30 μg), 10× RNase. **Lane E:** Total DDT1 RNA (15 μg) from cells transfected with pCAT-1.7, 1× RNase. **Lane F:** NIH 3T3 total RNA (30 μg) 1× RNase. **Lane G:** Probe RP2 with tRNA, 1× RNase.

Primer Extension

Primer extension was performed with a 23 bp primer complimentary to the sequence of the hamster aFGF cDNA, most 3′ in the 5′ non-coding exon (5′ CTGCTGCACCTGGCTGCCTTTC 3′) [31]. The oligonucleotide primer was labelled at the 5′ ends with ^{32}P by treatment with T4 polynucleotide kinase and γ-ATP-^{32}P (3,000 Ci/mmole) [34]. Approximately 2×10^5 CPM of primer was used per reaction. The primers were annealed with 50 μg total RNA in 40 mM PIPES pH 6.5, 0.4 M NaCI, 2 mM EDTA, 50% formamide at 45°C for 6–16 h and extension was carried out by diluting the annealing mixture fourfold with reverse transcriptase buffer and incubation at 42°C for 60 min with 200 U MuLV reverse transcriptase (Superscript, BRL) [35]. The resultant cDNA fragments were analyzed on an 8% denaturing-urea polyacrylamide gels.

Mammalian Cell Cultures and Transfections

DDT1 and NIH 3T3 cells were grown in monolayer in humidified, 5% CO_2 95% air atmosphere at 37°C. The DDT1 cells were first grown in DFITS media (DME/F12 1:1) + 5 μg/ml insulin, 5 μg/ml transferrin, 3×10^{-8} M H_2SeO_4, and antibiotics) and 10% fetal calf serum (FCS). The NIH 3T3 cells were grown in DMEM and 10% FCS. Two days before electroporation, the media was changed and the concentration of FCS reduced to 2%. The cells were harvested (the DDT1 cells by scraping and the 3T3 cells by trypsin digestion) and washed with PBS prior to electroporation. Approximately 1–3 $\times 10^7$ cells were resuspended in 0.8 ml of PBS and placed into an electroporation cuvette with 50 μg of CAT plasmid and 5 μg RSV-β galactosidase plasmid. The constructs were transfected using the Gene Pulser (BioRad, Gathisberg, MD) electroporation apparatus at a setting of 320 volts and 960 μFD. After the shock, the cells were divided into aliquots, replated in media with 10% FCS and allowed to recover for 24 h. The media was then changed again and half the cells were placed in 10% FCS and the other half in 0.2% FCS for an additional 48 h.

DDT1 cells for the testosterone (T, 10^{-7}, M) and aFGF (15 ng/ml) studies were placed in

TABLE I. DNA Elements in the 5′ Flanking Region of the aFGF Gene That May Be Involved in Androgen and aFGF Transcriptional Responsiveness*

Element	Sequence	Location	
1. ARE/PRE/GRE	AGAACA	−767	−384
1/2 sites	AGGACA		
2. Nur77/COUP-like RE	TGACCTTTGA		−3419
	CTGACCTGTCAG		−992
3. Pu-Py Tract	$[TG]_{34}$	−187	
4. SRE-like	$TGTAGTGG(A/T)_6GCCTTACA$		−1795
5. Zif 268/Egr-1 RE	GCGTGGAAG		−1380
	GTGGGGAGG		−63
	GAGGGAAG		−70
6. AP-1 RE	2 × TGAGTCA		−3479
	GAGTCA		−710
	GAGTCAC		−22
7. CRE	GACGTCA		−1362

*ARE/PRE/GRE, androgen, progesterone, glucocorticoid receptor DNA 1/2 site of the palindromic response element; Nur 77, orphan receptor transcription factor response element involved in early growth and differentiation signals and transcriptionally activated by androgens [21,22]; COUP, chick ovalbumin upstream binding protein transcription factor; Pu-Py, purine-pyrimidine alternating; SRE-like, serum response-like DNA element; zif 268/Egr-1, early response transcription factor 1 with three Cys-His Zn+ fingers and involved in both growth and differentiation; AP-1, the fos-jun binding site. CRE; cyclic AMP response element (cAMP can replace androgens in stimulating growth of DDT1 [24]). RE = DNA response element.

DFITS + 0.2% charcoal stripped FCS (CSS) plus and minus factors, after only overnight recovery period in 10% whole serum. After 48 h, the cells were collected and subjected to CAT assay [36,39].

The β-galactosidase gene, pRSV-β-galactosidase (gift of W. Rutter, UCSF, San Francisco) co-transfected as an internal control, showed about a fivefold lower activity in the +T and aFGF experiments. This suggests the CSS reduced uptake and/or expression of plasmid DNA in general about 5×. The CAT activity using whole serum vs. CSS for the 2 experiments is, however, in the same range, when corrected for plasmid uptake. In the design of the initial deletion experiments, DDT1 cells were allowed to recover after transfection for 2 days in whole serum. As stated in the +T and aFGF experiments, DDT1 cells were incubated only overnight in 10% whole serum before being switched to CSS, plus and minus factors.

CAT Analysis

After 48 h transfected cells were harvested and subjected to the CAT assay, as previously described [36], with the following modifications. Samples were sonicated for 10 seconds, after 3 cycles of freeze-thawing. For each sample a total of 0.150 $O.D._{280}$ of cell extract was processed at 37°C for 1 h, using 0.5 μCi-^{14}C-chloramphenicol. Approximately 0.02 $O.D._{280}$ was used for β-galactosidase assay as described [39]. Less than 10% variability was found between $O.D._{280}$ and β-galactosidase activity under any given condition.

RESULTS

Screening and Sequencing

Screening 2 × 10^6 plaques from the genomic hamster DDT1 EMBL3 library detected 15 signals using the 69 bp Ban I-EcoRI probe. Six unique clones were isolated from these signals. Using restriction enzyme analysis, the overlaps of the clones were determined and it was calculated that the clones span approximately 30 kb (Fig. 1). Southern and sequence analysis located the 69 bp 5′ non-coding exon sequence in each of the clones. Approximately 4.5 kb of the genomic DNA was completely sequenced from 300 bp 3′ of the 69 bp fragment to 4.0 kb in the 5′ direction. A consensus 5′ splice site was found at the 3′ end of the 69 bp sequence in the genomic clones at the position predicted by the cDNA [31]. Several candidate regulatory sequences were found in the 5′ region (Table I).

RNase Protection

RNase protection assays were first carried out with riboprobe RP1. This probe contains the ATG containing 155 bp of coding sequence and 76 bp of the 5′ non-coding region (231 bp). Using total DDT1 RNA, a 230 bp fragment was pro-

tected. Total RNA from normal Syrian hamster kidney cells protected a fragment that migrates at 170 bp, indicating the absence of the DDT1 5′ non-coding region (Fig. 3). Hamster kidney cells may have an alternately spliced exon 1 or use an alternate promoter for the aFGF gene.

Riboprobe RP2, which contains sequence for the DDT1 5′ non-coding exon and 5′ upstream sequences, protects fragments at 114 bp and 116 bp (Fig. 4). Lane E in Figure 4 shows results from RPA using 15 μg of total RNA from DDT1 cells transfected with pCAT-1.7. One might expect a higher amount of the 114/116 bp fragments in RNA from transfected cells due to the addition of the construct. This lane shows similar intensity in the RPA to 30 μg of total RNA from non-transfected DDT1 cells (lane B). Thus the amount of protection is increased using total RNA from DDT1 cells that have been transfected with the CAT construct, pCAT-1.7. This suggests transcription initiation on the pCAT-1.7 construct is similar as the endogenous gene. No fragments are protected with NIH 3T3 RNA or probe alone. These protected fragments map to within 16 bp of Stu I site as marked (Fig. 2). Since the Ban I site in the BSS M1/Ban 1 fragment used for RNA probe is 11 bp from the splice site, the predicted size of this DDT1 5′ non-coding exon is 125–127 bps, by this RNase protection assay.

Primer Extension

Using a labeled oligonucleotide which hybridizes to the most 3′ sequence of the 5′ non-coding exon, an extended fragment, dependent on RNA from testosterone treated DDT cells, migrates at 136 bp. By comparison with the genomic sequence, the start site of transcription in this assay will be at the G, as indicated in Figure 5 and 136 bp from the splice site. Therefore, the G at bp 136 from the 3′ end of the non-coding exon is the approximate transcription start site and the non-coding exon is defined as 136 bp long by this assay. The differences in the predicted size between RNase protection and primer extension are small and may be due differences in mobility of RNA and DNA. Other bands in the RNase protection are artifacts due to non-specific protection or read through transcripts in probe preparations, or partially degraded mRNA and probe. There are also smaller sized bands that appear in the primer extension gel, which may indicate alternate start sites or incomplete extensions. They do not, however, correspond to the

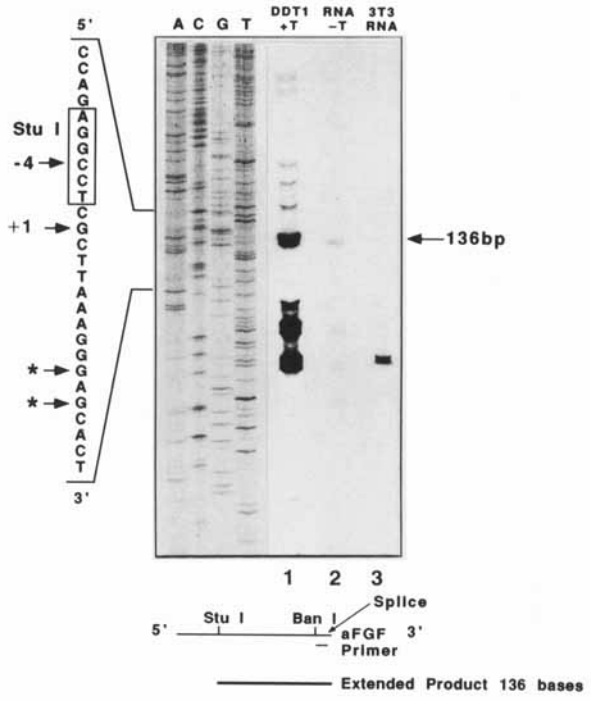


Fig. 5. The primer extension assay. **Lane 1:** Total RNA (50 μg) from DDT1 cells treated with 10^{-7} M testosterone (+T) for 3 days. **Lane 2:** DDT1 total RNA (50 μg) from cell withdrawn 4 days from testosterone (−T). **Lane 3:** NIH 3T3 total RNA (50 μg). The sequence ladder is from genomic DNA using the same primer as in the extension reaction (5′CTGCTGGCACCTGGCTGCCTTTC 3′). The sequence shown on the left represents the sense strand and is the complement of the ladder (anti-sense) sequence. The (→) indicates the G designated as the start site as determined by the band that migrates at that point. The (*→) indicate the bases where the RNase protection assay predicted the start sites to be. The Stu I site indicated is the one used in the aFGF-CAT construct, pCAT + 1 (−4/+131)

major bands in the RNase protection assay. The *arrows indicates the positions protected by the RP2 in the RNAse protection assay.

In summary, the transcription start site for DDT1 aFGF is near the Stu I site. As indicated in Figure 2 and Figure 5 we designated +1, 4 bp 3′ of the Stu I cut site as the major transcription start site.

Promoter Analysis

Cell specificity and deletion analysis. To test the functional properties of the 5′ flanking region of the aFGF gene, the CAT assay was employed [36,39]. Two aFGF 5′ flanking CAT constructs, pCAT-3.0 (−3067/+131) and pCAT-1.7 (−1645/+131) were tested in DDT1 cells and NIH 3T3 cells. These constructs include both the non-coding exon plus approximately 3 kb or 1.7 kb of 5′ flanking sequences, respec-

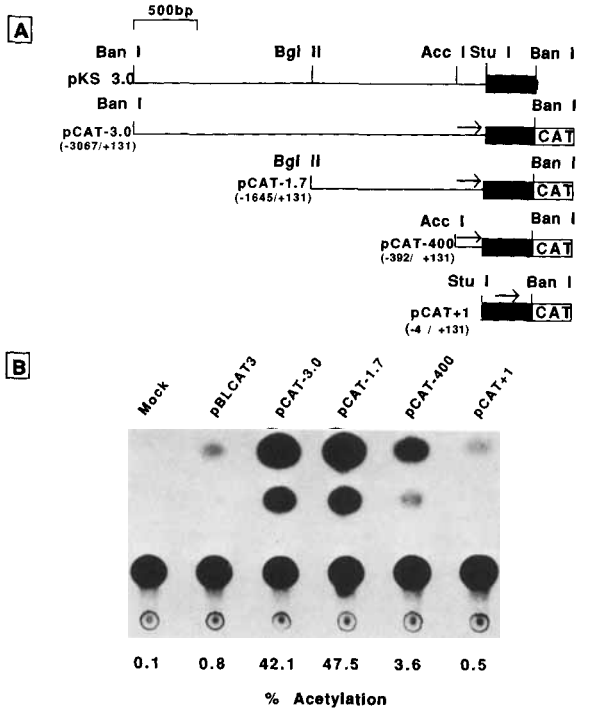
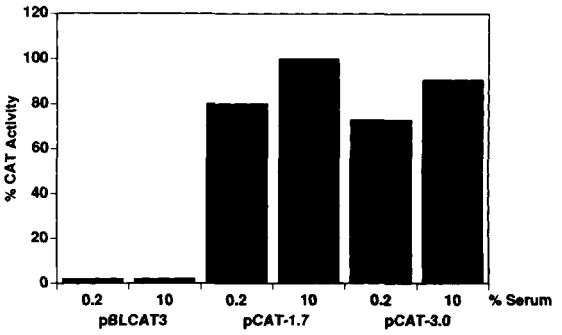


Fig. 6. **A:** Diagram of the hamster DDT1 aFGF-CAT constructs. A 3.0 kb Ban I fragment was subclone into the Bluescript vector, pKS. This fragment was then either placed in pBLCAT3 [32] or cut with Ban I, Bgl II, Acc I, or Stu I, then inserted into pBLCAT3 (see Methods and Materials for details). The black box indicates the 5′ non-coding exon 1 (Exon 1.C). The numbers under the CAT constructs correspond to the numbers utilized in Fig. 2. The CAT box represents the chloramphenicol acetyl transferase reporter gene. **B:** The autoradiogram of the CAT assays using the extracts of DDT1 cells transfected with the CAT clones diagrammed in A. The numbers below each lane indicate CAT activity as percent acetylation. β-galactosidase activity was equivalent in the different extracts and correlated with $O.D._{280}$ of the extracts. Therefore $O.D._{280}$ measurements were used to normalize the amount of extract to use in the CAT assays.



Vector	Percent Serum	DDT1 %Acetylation*	NIH 3T3 %Acetylation*
pBLCAT3	0.2	1	< 1
	10	1	< 1
pCAT 1.7	0.2	42	< 1
	10	53	< 1
pCAT 3.0	0.2	38	< 1
	10	51	< 1

* Values have been normalized for variation in transfection efficiency using β galactosidase activity

Fig. 7. Effect of serum on CAT activity of transfected pCAT-1.7 and pCAT-3.0 constructs into DDT1 cells and NIH 3T3 cells. The highest % acetylation is set to 100% CAT activity. Both constructs produce about 20% greater CAT activity in 10% serum than in extracts from DDT1 cells treated with 0.2% serum. These DDT1 aFGF-CAT constructs showed no activity in NIH 3T3 cells. Also shown is the data used to normalize the presentation in the graph.

tively. DDT1 cells and NIH 3T3 cells, transfected with these constructs, were placed in high (10%) and low (0.2%) serum conditions. The NIH 3T3 cell extracts showed no CAT activity under either serum conditions. Transfection efficiency into NIH 3T3 cells was similar to DDT1 cells, as assayed by β-galactosidase activity. The extracts from DDT1 cells, however, transfected with pCAT-3.0 and pCAT-1.7 showed a forty- to fiftyfold increase in CAT activity over those transfected with control pBLCAT3 plasmid (Fig. 6). Cells treated under high serum had reproducibly 20% more CAT activity than those in low serum for both pCAT-3.0 and pCAT-1.7 (Fig. 7). Figure 6 also demonstrates that extracts from DDT1 cells transfected with pCAT-400 (−392/+131) had CAT activity that was sevenfold that of pCAT + 1 (−4/+131). pCAT + 1 (−4/+131) resulted in CAT activity in DDT1 cells two- to threefold lower than parent pBLCAT 3 vector.

Testosterone and aFGF stimulate aFGF promoter. DDT1 cells transfected with pCAT-1.7 (−1645/+131), treated with 0.2% charcoal stripped serum (CSS) and either testosterone or aFGF or both, exhibited threefold more CAT activity than those treated with 0.2% CSS alone (Figs. 8, 9). There was 25-fold more CAT activity in extracts from DDT1 cells treated with testosterone (T) or aFGF1 as compared to DDT1 cells transfected with pCAT+1 (−4/+131). Thus, DDT1 cells transfected with pCAT+1 (−4/+131) or pBL3 CAT showed no stimulation of CAT activity when treated with testosterone (T) or aFGF itself. Again, however, pCAT+1 was consistently two- to threefold lower in CAT activity than the parent pBL3 CAT vector, indicating a possible negative transcription or translation element in the 5′ non-coding exon. RNA elements in the 5′ non-coding region of PDGFB gene and other mRNAs have been identified that inhibit translation of their respective mRNA [40].

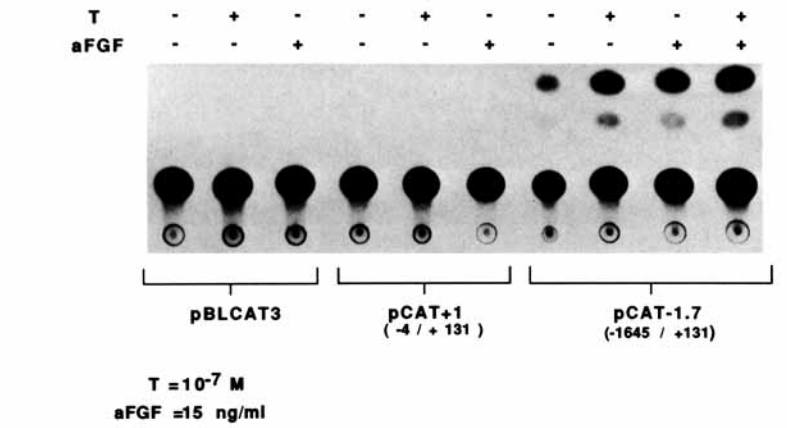
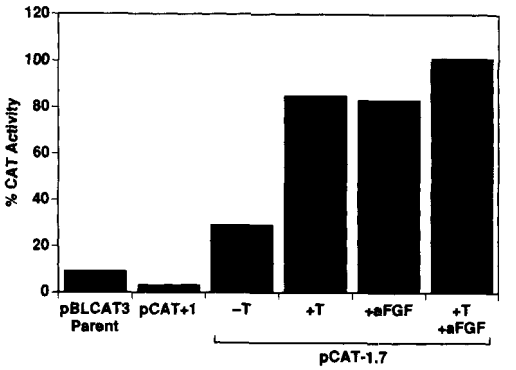


Fig. 8. The CAT assays demonstrating that the DDT1 5′ flanking region of the aFGF gene is responsive to testosterone (T) and aFGF protein growth factor itself. The conditions for each lane are noted above and pBLCAT3 and pCAT + 1 (−4/+131) are used as controls. They demonstrate that the induction of CAT activity is dependent upon the presence of the DDT1 −1645/−4 DNA 5′ flanking region of the aFGF gene. The pCAT +1 (−4/+131) showed no response to either testosterone or aFGF protein, at the indicated concentrations.

DISCUSSION

DDT1-MF2 (DDT1) cells treated with testosterone (T) or aFGF exhibit changes in growth rates [21,24]. It has been shown by Northern analysis and in situ hybridization that the level of aFGF is increased in these cells when stimulated by testosterone (T) [21,31]. In our efforts to study the mechanisms of transcriptional regulation of growth factors involved in steroid regulation of growth and alterations of that pathway in the development of hormone independence, DNA sequencing and characterization was carried out on the DDT1 5′ non-coding exon (5′ NC) and the 5′ flanking region of the aFGF gene. Approximately 4 kb of DNA was sequenced in the 5′ flanking region. By RNase protection and primer extension assays, we estimate that in DDT1 cells, the 5′ NC exon is about 130 bp. This 5′ NC exon was not found in kidney aFGF mRNA, suggesting tissue specifically for this promoter. In both the primer extension and RNase protection, some larger and smaller protected fragments and extension products are noted. They are not the major bands nor do they correlate with the two assays. We cannot exclude minor transcription start sites upstream or downstream of where we have presently indicated, the major transcription start site (Fig. 5), near the Stu I site.

Several 5′ flanking aFGF-CAT constructs were prepared and transfected into DDT1 cells and NIH 3T3 cells. Some of those constructs were then tested for responsiveness to testosterone and aFGF in DDT1 cells. Briefly, the −392/



Clone	Conditions	% Acetylation*
pBLCAT3	Control	0 5
	+T	0 4
	+aFGF	0 5
pCAT+1	Control	0 2
	+T	0 1
	+aFGF	0 2
pCAT 1 7	Control	1 6
	+T	4 9
	+aFGF	4 8
	+T +aFGF	5 8

* Values represent duplicate determination and within the experiment have been normalized for variation in transfection efficiency using β-galactosidase activity measurements Similar results were obtained in two independent transfection experiments

Fig. 9. Comparison of CAT activity between +/− testosterone (T) and aFGF. The highest % acetylation is set to 100% CAT activity. pBLCAT3 represents the vector alone in any condition (i.e., +/− T). pCAT+1 contains 131 bp 5′ non-coding sequence and 4 bp of 5′ flanking DNA (−4/+131). pCAT-1.7 contains −1645/+131 of 5′ flanking region of aFGF gene. T = testosterone 10^{-7} M. aFGF = acidic fibroblast growth factor, 15 ng/ml. Also shown is the data used to normalize the presentation in the graph. These are the results of duplicate determinations within one experiment that varied less than 20%. Similar results were also obtained in a totally independent transfection experiment.

+131 had sevenfold more promoter activity than −4/+131 DNA in DDT1 cells. The −1645/+131 aFGF promoter region showed forty- to fiftyfold more promoter activity than the −4/+131 DNA. Thus two regions of the aFGF gene in DDT1 cells with promoter activity were identified. None of these constructions showed activity in NIH 3T3 cells. This indicates a degree of cell or tissue specificity for the this promoter region of the aFGF gene. This is consistent with the results obtained with hamster kidney RNA, where the aFGF gene may use another promoter, since we presume hamster kidney aFGF mRNA has a different 5′ NC exon than DDT1 cells (Fig. 3).

The −1645/+131 region also responds to aFGF protein itself or testosterone (T). There was a highly reproducible threefold stimulation, with aFGF or testosterone (T). The −4/+131 region did not respond to either factor. Thus between −1645 and −4, there are DNA elements directly or indirectly responsive to testosterone (T) and aFGF and which may or may not be overlapping.

As noted in Table I, there are several candidate response elements that may be involved in the testosterone (T) and/or aFGF responses. Testosterone (T) is known to activate the PKA pathway in DDT1 cells. Thus the candidate CRE element at −1362 may play a role. Both testosterone (T) and aFGF activate a variety early response genes in DDT1 cells, such a c-fos, egr-1/zif 268, and nur 77 [21,22]. These are obvious candidates for involvement in regulation of aFGF gene promoter. At this point in time we cannot rule out direct effects of the ligand activated androgen receptor on transcriptional regulation of the aFGF gene, although no standard palindromic androgen response elements (ARE) were noted in the area sequenced.

Overall, our findings are in general agreement with previous observations that addition of testosterone (T) to DDT1 cells cause two- to fivefold increase of aFGF mRNA and that aFGF added to cell culture media can replace testosterone in stimulating DDT1 cells to grow [21,24]. Adding both testosterone (T) and aFGF shows no additive or synergistic effect on the aFGF promoter region tested in these experiments. This suggests that testosterone (T) through the androgen receptors and aFGF through the aFGF receptor(s) ultimately may affect similar DNA elements of the gene, and both stimuli may affect the production and/or activation of common DNA binding proteins and transcription factors. Several candidate factors involved in this cascade maybe some of the early growth response genes, such as c-fos, c-jun, Nur 77, and zif 268/egr-1 as indicated earlier [21].

Both acidic FGF and basic FGF lack leader peptides and are not secreted by the usual mechanisms, but are found in the extracellular matrix [41]. Recent experiments indicate that both acidic FGF and basic FGF immunostaining can be found in the nucleus after 10–24 h after addition of the growth factor to the media in several different systems. It has been shown that aFGF (HBGF1) contains a nuclear localization peptide at position 21–27 which, if removed, prevents translocation of aFGF to the nucleus, and more importantly, a failure for aFGF to promote growth [41]. These results suggest a direct nuclear role for at least part of the aFGF molecule in regulation of gene expression and possibly the aFGF gene itself.

Recent work on the 5′ non-coding regions for HBGF1 or acidic FGF cDNAs and gene fragments in human, brain, and kidney have identified at least 3 different 5′ non-coding exons [3,42]. The aFGF gene in human heart tissue appears to use another alternate 5′ non-coding exon [43]. We have recently identified a new aFGF 5′ non-coding exon expressed in the human prostate carcinoma cell line, LNCAP [14,44]. Thus there are at least 4 different 5′ non-coding exons of the aFGF gene utilized in different human tissues.

The human prostate cancer cell line, LNCAP, aFGF 5′ non-coding exon was found to have a region with high identity to the DDT1 5′ non-coding exon [44]. Moreover, using hamster DDT1 5′ non-coding and flanking sequence probes, a region in the human aFGF gene locus with extensive sequence similarity was recently found [45]. By sequence comparison there is over 70% similarity in the first 1,000 base pairs of hamster DDT1 aFGF 5′ flanking region and human Exon 1.C containing aFGF 5′ flanking region [45]. This human aFGF 5′ non-coding exon (Exon 1.C) turns out to be identical to the prostate LNCAP 5′ non-coding exon [45]. These results again raise the intriguing possibility of tissue specific promoters for the aFGF gene, and that human prostate cancer LNCAP cells and DDT1 cells utilize homologous aFGF gene promoters.

Our observations strongly suggest a coordination of growth and elevation of the aFGF mRNA. Also they indicate that testosterone and aFGF

are involved in the modulation of aFGF transcription. We have now defined the approximate start site of one of the transcription units for Syrian hamster aFGF gene. Additionally the promoter is responsive to testosterone and the growth factor, aFGF or HBGF1. Analysis of 5′ flanking sequences indicates the presence of several possible regulatory elements. The above information will now be used to develop a model in understanding the mechanisms for steroid regulation of growth in hormone dependent prostate tumors. We can now identify specific response elements and their transcription factors and how they may be regulated by steroid receptors. Alteration in this steroid dependent pathway in the loss of hormone dependence may lead to new insight into understanding the development of hormone independent cancers.

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