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Characterization of the Hamster DDT-1 Cell aFGF/HGBF-I Gene and cDNA and Its Modulation by Steroids

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Syrian hamster DDT-1 cells are derived from smooth muscle of the ductus deferens. DDT-1 cell growth is increased by the addition of testosterone (T). Acidic fibroblast growth factor (aFGF) or basic fibroblast growth factor (bFGF) also known as heparin binding growth factor I and II (HBGF-I and HBGF-II) can replace T in the stimulation of growth in these cells. This phenomenon is correlated with testosterone's ability to elevate aFGF/HBGF-I mRNA. The increase steady-state levels of aFGF/HBGF-I mRNA were documented by northern blots and by in situ hybridization. Using a 520 bp human aFGF/HBGF-I cDNA probe, a genomic clone with a 38 kb DNA insert was isolated from a cosmid library. By restriction enzyme analysis and southern hybridization, it was determined that there are three coding exons. DNA sequence analysis showed all of the coding region and 3′ noncoding sequences were on this clone. A 5′ noncoding exon not in the 38 kb insert is indicated, based on the cDNA sequences and genomic sequences of aFGF/HBGF-I's from hamster DDT-1 cells and several other species. The cDNA for hamster aFGF/HBGF-I was isolated from a DDT-1 lambda gt11 library and sequenced. Comparison of the coding region of aFGF/HBGF-I from four species shows a >90% conservation of amino acid sequence.

Key words: DDT-1 cells, acidic FGF, HBGF-I, gene and cDNA, androgen, in situ hybridization

We have been investigating the role that androgens and glucocorticoids play in the gene expression of fibroblast growth factors (FGF also known as heparin binding growth factors or HBGF) and the effect of these steroids on the FGF/HBGF receptor(s) in DDT-1 cells. The FGF/HBGF family contains several species such as acidic fibroblast growth factor (aFGF), which belongs to HBGF class I, and basic FGF (bFGF), which belongs to HBGF class II [1,2]. More recently, the oncogenes int-2, hst/KS3, FGF-5, and KGF have been shown to encode proteins related to the FGF/HBGF family

Abbreviations used: FGF, fibroblast growth factor; HBGF, heparin binding growth factor; DDT-1, ductus deferens tumor cell clone MF-2; T, testosterone.

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[3–5,27]. We are employing the Syrian hamster DDT-1-MF2 tumor cell line in these studies, since these cells are growth responsive to and have steroid receptors for both androgens and glucocorticoids [6–9].

Either aFGF/HBGF-I or bFGF/HBGF-II can replace androgen (testosterone) in stimulating growth of DDT-1 cells in serum-free media [10]. The aFGF/HBGF-I gene is amplified in the genome of DDT-1 cells [11]. These results encouraged us to undertake an investigation into the mechanism of the increase of aFGF/HBGF-I messenger RNA and the regulation of aFGF/HBGF-I mRNA by steroids and other factors. This report deals with (1) isolation and sequence analysis of the aFGF/HBGF-I gene from a DDT-1 cosmid genomic library, (2) isolation of the aFGF/HBGF-I cDNA from a DDT-1 cDNA library, and (3) a demonstration of the increase in aFGF/HBGF-I mRNA levels caused by androgens using in situ hybridization and northern analysis of total RNA using ^{32}P-cRNA probes to aFGF/HBGF-I.

MATERIALS AND METHODS

Northern Analysis

Culture. DDT-1 cells were grown in a monolayer on 150 mm petri dishes in a humidified 5% CO_2-95% air atmosphere at 37°C. The 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 0.1% fetal calf serum. When the cells were 30–60% confluent, the media was removed and replaced with DFITS ± 10 nM testosterone. After 3 days, the cells were harvested.

RNA preparation. The cells were scraped from the dishes and pelleted at 1,500g for 10 min at 4°C. The cell pellet was washed 3× with PBS (10 mM $NaPO_4$ [pH 7.4], 130 mM NaCl, and 5 mM KCl) and resuspended in 1 ml ice cold sterile NTM (10 mM NaCl, 10 mM Tris [pH 7.4], and 1 mM $MgCl_2$). Then 9 ml of cold sterile lysis buffer (NTM + 5% NP-40) was added to the 1 ml cell suspension. After a 10 min incubation on ice, the lysed cells were spun at 3,000g for 15 min at 4°C. The supernatant was removed and 0.2 volumes of RNA extraction solution (500 mM NaCl, 5% SDS, and 50 mM Tris pH [7.4]) was added. This solution was extracted 5× with an equal volume of phenol-chloroform and 1× with chloroform, then ethanol precipitated for 1 h at −20°C.

Northern hybridization. The RNA was dissolved in RNase free water. The RNA concentration was determined by OD_{260} reading. Five micrograms of RNA from each experimental condition were denatured in 2.2 M formaldehyde/50% (vol/vol) formamide and run on a 1% agarose gel containing 2.2 M formaldehyde (100 volts for 3.5 h). The gel was stained with ethidium bromide (5 μg/ml) and photographed. The gel was then destained in 50 mM NaCl overnight and transferred to a nitrocellulose filter by capillary blotting with 10× SSPE buffer [12]. After transfer, the filter was dried and baked in a vacuum oven for 2 h at 80°C. The Riboprobe protocol (Promega, Madison, WI) was followed to make a specific RNA probe from a plasmid containing the T7 promoter and 520 bp human aFGF/HBGF-I cDNA. Prehybridization was carried out at 52°C in 5× Denhardt's solution + 6× SSPE, 50% formamide, 0.5% SDS, 100 μg/ml denatured salmon sperm DNA, and 50 mM Na_3PO_4 (pH 6.5). After 1 h prehybridization, the RNA probe was added to the solution at a concentration of 5×10^5 cpm/ml. Hybridization was for 12–16 h at 52°C. Filters were washed at 62°C, then 64°C for 20 min, each in 0.1× SSPE + 1% SDS. The filters were dried at room temperature and exposed at −20°C overnight with an intensifying screen.

Libraries

The cDNA for the lambda gt11 library was made from DDT-1 poly A RNA by the method of Gubler and Hoffman [13]. The cDNA was packaged using a Gigapack lambda gt11 kit from Stratagene (La Jolla, CA). The cosmid library was generated by previously described methods [14,15]. The DDT-1 DNA was partially digested by Mbo I into 30–50 kb pieces, size fractionated on sucrose gradients, and then inserted into the vector pCV108 [14]. The cosmid constructs were packaged with Gold Gigapack phage extracts and transduced into ED8767 cells [14].

Screening

Genomic and cDNA clones were detected using a 520 bp Bgl II-Eco RI probe from the human aFGF/HBGF-I cDNA [16]. The probes were labeled using a random priming kit from Pharmacia (Piscataway, NJ). A total of 5×10^5 cosmid clones and 3×10^6 gt11 plaques were screened to isolate the genomic and the cDNA fragment, respectively.

Sequencing

The genomic clone was partially sequenced by subcloning restriction enzyme fragments into the Bluescript SK-vector (Stratagene). Some of the larger (>3 kb) clones were prepared for sequencing by using an Exo III/S1 nuclease double-stranded nested deletion kit from Pharmacia. Sequence information was produced by the ^{35}S dideoxy chain termination method described by United States Biochemical (Cleveland, OH) using Sequenase version 2. Also, automated sequencing on the Applied Biosystem (ABI) 370A DNA sequencer (Foster City, CA) was employed following ABI's method for sequencing double stranded template with Sequenase version 2. The 700 bp hamster aFGF/HBGF-I cDNA clone was cut from the phage DNA by Eco RI and subcloned into the Bluescript plasmid. The sequence of the cDNA was obtained by automated sequencing with this ABI 370A.

In Situ Hybridization

Culture. DDT-1 cells were plated at a density of $<2{,}000/cm^2$ on Lab-Tek Tissue Culture Chamber/Slides (Naperville, IL) using DFITS media with 0.1% fetal calf serum. After 24 h, the media was removed and DFITS ± 10 nM testosterone was added to the chambers. The chambers were incubated at 37°C for 72 h. The media was then removed and the cells washed 1× with PBS and fixed in fresh 2% paraformaldehyde in PBS + 5 mM $MgCl_2$ for 15 min. The cells were washed with 70% ETOH, and the slides were then stored at −20°C in 70% ETOH. After air drying, the cells were rehydrated in PBS + 10 mM $MgCl_2$ for 10 min at room temperature followed by a 10 min incubation at room temperature in 0.2 M Tris (pH 7.4) + 0.1 M glycine. The slides were then transferred to 50% formamide in 2× SSC at 65°C for 10 min.

Probe preparation. A 520 bp human aFGF/HBGF-I cDNA was cloned into the Bam HI site of PTZ-19R plasmid (Promega). Both the sense and anti-sense probes were linearized with Eco RV. The plasmids were phenol-chloroform extracted and ethanol precipitated. The RNA probes were then prepared using ^{32}P-UTP (800 Ci/mmole; 20 μM) and a Stratagene transcription kit. The probe specific activity was 1.4×10^9 cpm/μg RNA.

Hybridization. To each well (4.84 cm^2) was added 10 μl of RNA probe mix containing 10 μg yeast t-RNA, 10 μg sheared salmon sperm DNA, 20 μg BSA, and 10^6 cpm of the RNA probe, all in 2× SSC and 50% formamide, that had been previously heated at 90°C for 10 min. Coverslips were then applied, and the slides were incubated in a humidified chamber at 50° for 3 h. The slides were washed 3× at 52° with agitation in 50% formamide-2× SSC, 10 min each, then rinsed 4× in 2× SSC at room temperature. Next the slides were treated with 0.4 μg/ml RNase A for 30 min in 2× SSC at 37°C, then washed 2×in 2× SSC for 1 min each. The slides were then transferred to 50% formamide in 2× SSC at 52°C for 5 min with agitation, then dehydrated in 70%, 80%, and 95% ethanol for 1 min each. The slides were air dried before dipping in Kodak NTB2 emulsion (1:1 dilution with H_2O, prewarmed to 40°C). The coated slides were then air dried vertically overnight and stored at 4°C for 5 days. The exposed slides were developed in D19 for 4 min, fixed, rinsed, and air dried. They were then stained with hematoxylin and eosin Y. Photographs were then taken with TMAX 400 at 630x under oil using a phase-contrast objective.

RESULTS

Northern Analysis

The northern blot (Fig. 1A) shows an increase of 2× in hybridization of the human ^{32}P-cRNA to DDT-1 RNA from cells treated with 10^{-8} M testosterone for 3 days. There was no detectable hybridization in the control HepG2 RNA lane. The increase was quantitated by scanning densitometry. Tracings of the −T and +T lanes are represented in Figure 1B. Each lane contained 5 μg of total RNA as determined by OD_{260} readings. There were equal amounts of ethidium bromide staining both on the gel and after transfer to nitrocellulose.

Isolation of the aFGF/HBGF-I Gene

After screening 5 $\times 10^5$ cosmid clones, one clone (cos 7) was positive for aFGF/HBGF-I. Restriction enzyme analysis of various subcloned EcoR1, Hind III, and Kpn I subclones of cos 7 showed that three coding exons of the transcription unit were on three different Eco RI fragments (two 7 kb and one 3 kb). Figure 2 shows the relative positions of the exons in cos 7. The DNA sequence of the exons and flanking region is also presented in Figure 2. Table I lists the exon/intron boundaries of aFGF/HBGF-I in cos 7. Exon 2 is defined as the first coding exon, containing the ATG initiation codon and designated Exon 2 because most of the 5′ noncoding region of the aFGF/HBGF-I mRNA is not present in the cos 7 clone. At the present time we assume that the 5′ noncoding sequence is contained in one exon. It is labeled "Exon 1." Exon 2 and 3 are separated by 12 kb and Exon 3 and 4 by 5 kb.

Isolation of the aFGF/HBGF-I cDNA

Several clones containing a hybridizing 700 bp insert were isolated from the λgt 11 DDT-1 cDNA library. After subcloning the cDNA insert into a Bluescript plasmid, it was analyzed. The sequence (Fig. 3) contains the entire coding region (465 bp) and 115 bp of 5′ and 120 bp of 3′ noncoding sequence. The presumed protein coded for by the cDNA (155 amino acids) was then compared to human, bovine, and rat (Fig. 4).

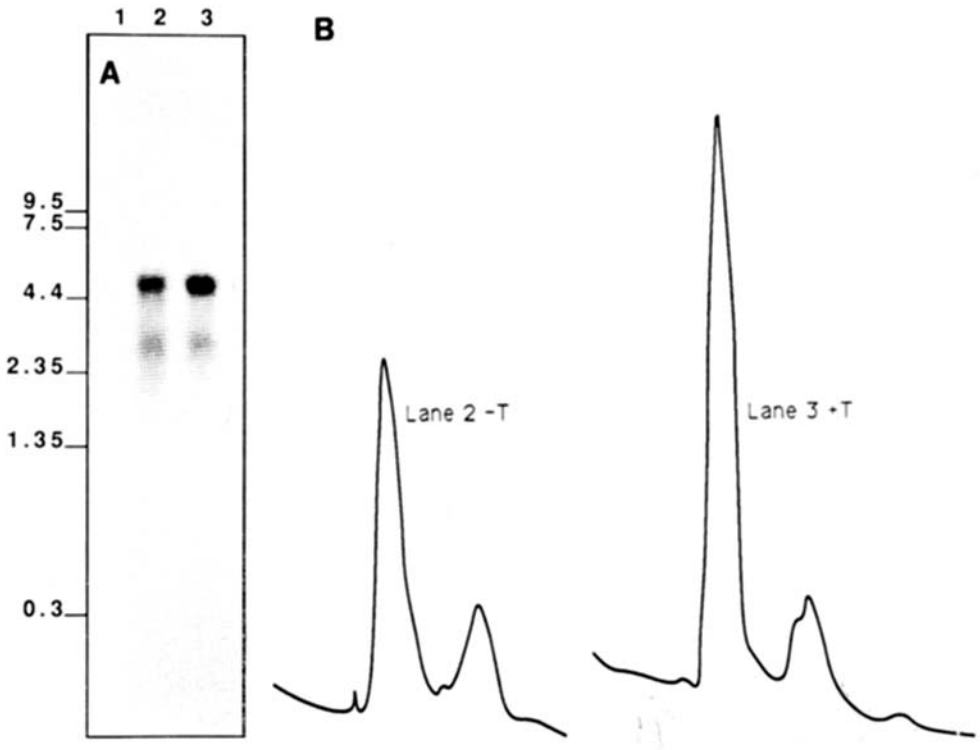

Fig. 1. **A:** A northern analysis of total DDT-1 RNA probed with human aFGF/HBGF-I ^{32}P-cRNA. The aFGF/HBGF-I mRNA steady-state levels are elevated by testosterone (10 nM) added to serum free DFITS media. **Lane 1:** 5 μg of HepG2 RNA. **Lane 2:** 5 μg DDT-1 RNA −T. **Lane 3.** 5 μg DDT-1 RNA +T (10 nM). The numbers on the left indicate size in base pairs. **B:** Densitomer scans of radioautogram from the northern analysis in **A.** The area under each peak was determined. The area under the peak obtained from RNA sample of DDT-1 cells treated with androgens was 2.2×that obtained from RNA samples of similar untreated (−T) cells grown under these conditions.

In Situ Hybridization

DDT-1 cells treated with T show significantly higher hybridization with the ^{32}P-cRNA (antisense) than those not treated with androgens (Fig. 5). This was determined by counting grains/cell. Here 5 to >20× more grains per cell appear in the cultures treated with T for 72 h. Over 50% of the cells treated with 10^{-7} M testosterone were scored positive for hybridization (>5×over-T control). The background was determined by hybridization with a probe identical (sense) to the aFGF/HBGF-I mRNA. The sense probe showed approximately twofold less hybridization than that of the −T control with the antisense probe (data not shown). Therefore, DDT-1 cells not treated with androgen under these in situ low density conditions are expressing a basal level aFGF/HBGF-I mRNA. In summary, androgen treatment for 72 h results in an overall 2–20× increase in the steady-state aFGF/HBGF-I mRNA levels, as assayed by in situ hybridization or Northern analysis.

DISCUSSION

DDT-1 cells treated with androgens (T) exhibit increased growth rates. By northern analysis and in situ hybridization, the level of aFGF/HBGF-I mRNA is also

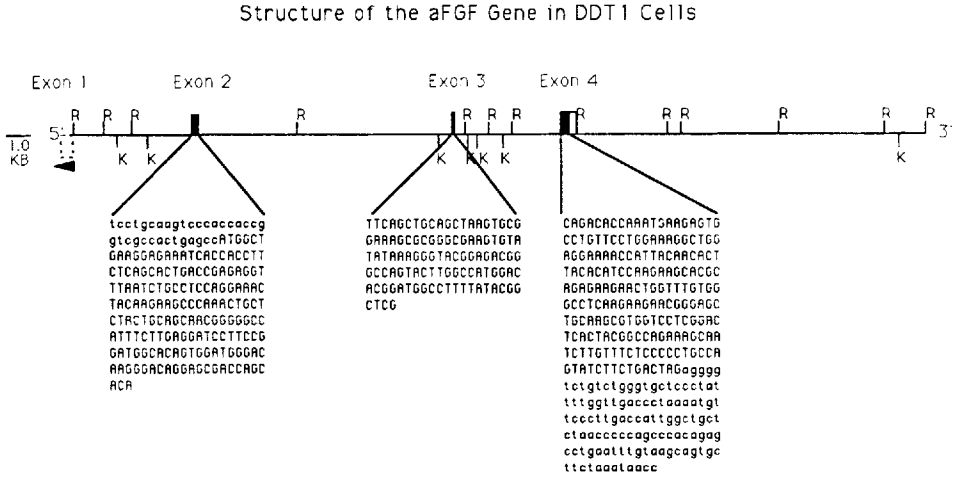

Fig. 2. An Eco RI (R) and Kpn I (K) map of aFGF/HBGF-I gene in DDT-1 cells. The filled boxes indicate the fully characterized exons. The open-dashed box indicates that exon 1 is 5′ of this cos 7 hamster DNA fragment. Below each filled box is the DNA sequence in cos 7, which is identical to the DNA sequence found in the 700 bp cDNA to hamster DDT-1 aFGF/HBGF-I mRNA. The open box in exon 4 represents sequence with high (>80%) similarity to the 3′ noncoding region of rat, human, and bovine aFGF/HBGF-I mRNA.

TABLE I. Size and Sequence of Intron Boundaries*

Intron	Approximate size (kb)	Sequence		
		Exon	Intron	Exon
A	?	?	?...TTCATTTTTTTTCAG	TCTGC
B	12	CAG CAC A	GTNNGCT...TTATTTTCATTCCAG	TT CAG CTG
		Gln His I		Ie Gln Leu
C	5	TAC GGC TCG	GTNNGTN...TTTTTATCCGTTTAG	CAG ACA CCA
		Tyr Gly Ser		Gln Thr Pro

*The exon/intron junctions of the hamster aFGF/HBGF-I gene. The intron sizes and splice site composition are presented. Introns A, B, and C reside between exons 1 and 2, 2 and 3, 3 and 4, respectively. The size of intron A has not yet been determined.

increased in these cells when stimulated by T. From genomic and cDNA DDT-1 libraries we have isolated part of the aFGF/HBGF-I gene and the cDNA coding for hamster DDT-1 aFGF/HBGF-I peptide. It has been reported that the aFGF/HBGF-I protein is very highly conserved across species [17]. Our findings extend this homology to the hamster. The protein coded by the DDT-1 cDNA has 98% homology with rat aFGF/HBGF-I, 97% with human, and 90% with bovine [16–18] (Fig. 4). Based on these homologies, the 700 bp cDNA clone contains the entire coding sequence and ~100 bp each of the 5′ and 3′ noncoding regions. From the northern analysis we estimate the full-length cDNA to be 4.8 kb. This is similar to the rat and human.

In the cos 7 DNA fragment, the coding sequence is divided into three exons (#2, 3, 4) separated by introns of 12 kb and 5 kb (B and C), respectively. The exon/intron boundaries are presented in Table I. Exon 2 is 203 bp long. Exon 3 contains 124 bp. The hamster DDT-1 aFGF/HBGF-I mRNA sequence so far characterized comprises only ~15% of the full-length aFGF/HBGF-I mRNA. Therefore, the size and sequences of

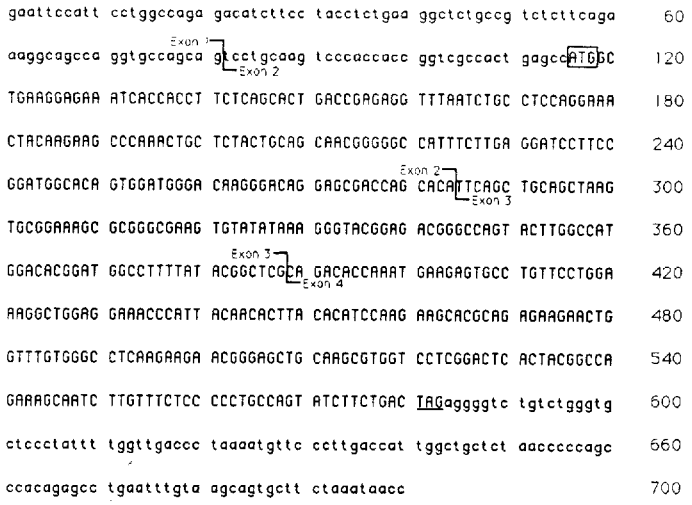

Fig. 3. DNA sequence of the 700 bp hamster DDT-1 cDNA insert that hybridizes to human aFGF/HBGF-I cDNA probe. The presumed start codon and stop codon are indicated, as well as the splice sites as determined from the genomic cos 7 clone.

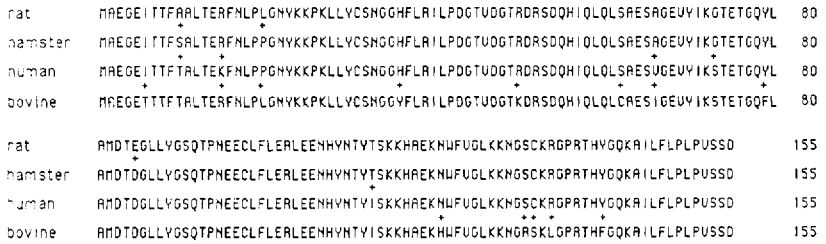

Fig. 4. Comparison of aFGF/HBGF-I amino acid sequences. The + sign denotes differences in the sequences between species. The rat sequence is from Dunning tumor 3327 AT-3 cells [17], the hamster from DDT-1 cells, the human from brain stem cells [16], and the bovine from bovine retinal cells [18]. The protein coded for by the DDT1 mRNA is presumably a normal cell associated aFGF/HBGF-I and has 98% homology with rat aFGF/HBGF-I, 97% with human, and 90% with bovine aFGF/HBGF-I.

exon 1 and 4 are estimated from other species. Using the rat aFGF/HBGF-I cDNA as a model, cos 7 contains all of the protein coding region (465 bp) and 3′ noncoding region (~3 kb, based on partial hybridization and the assumption it is contained on one exon). The bovine aFGF/HBGF-I cDNA so far characterized contains ~916 bp of 5′ noncoding region and 2619 bp of 3′ noncoding region and may be close to full length [18]. Only 25 bases of the 5′ noncoding region are in exon 2 of the hamster cos 7 genomic clone. This is based on the fact that the 25 bp 5′ of the ATG in cos 7-exon 2 is identical to the 25 bp 5′ of the ATG codon in the hamster aFGF/HBGF-I cDNA. A consensus splice site is found in cos 7 at this position. The cDNA contains a further area of 85 bp, which presumably belongs to a 5′ noncoding exon (~1,000 bp based on the bovine aFGF/HBGF-I mRNA structure), which is not in cos 7. From the 85 bp of 5′ noncoding sequence in the hamster cDNA aFGF/HBGF-I cDNA, a 72 bp probe is now being used to rescreen DDT-1 genomic libraries for the 5′ noncoding exon(s) and flanking regions.

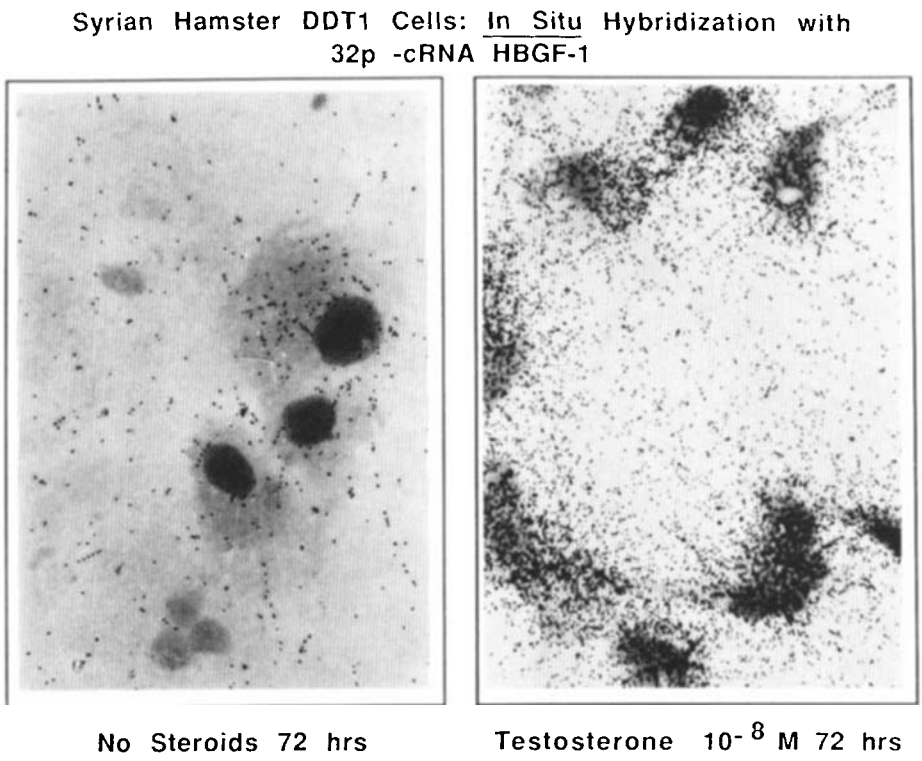

Fig. 5. In situ hybridizations of DDT-1 cells probed with a human aFGF/HBGF-I ^{32}P-cRNA. Most cells treated with T show a greater than fivefold increase (determined by grain count/cell) of aFGF/HBGF-I message over the nontreated cells (see methods for growth conditions). A sense control strand probe shows approximately twofold less binding than the level seen in DDT-1 cells not treated with androgens and hybridized with the antisense strand probe (no steroids, 72 h).

Among different aFGF, bFGF, FGF-5, int-2, or hst (KS) species, there is a uniform homology in the peptides of different exonic domains [3–5,19]. No particular area in the peptides seems to be more conserved than other areas. However, two cysteine residues and the middle coding exons' size are two features conserved throughout this FGF/HBGF family of genes. It does appear that aFGF/HBGF-I in the hamster, human [2], and rat (S. Goodrich, personal communication) has a large intron 5′ of the ATG initiation codon exon and other possible exons not yet defined. It is presently not clear if the 5′ noncoding region is on one or several exons. It is assumed to be contained on one exon ("Exon 1"). Among the coding exons, the position of the splice sites is very close between the different FGF/HBGF's. All FGF/HBGF genes, including the hamster, have three exons containing protein coding sequence. The introns are in the same positions with respect to the coding sequences, but their sizes vary. Computer analysis indicates exon 2 and 4 of aFGF/HBGF-I have a net negative charge while exon 3 has a positive one.

In this tumor cell line we have found only a cDNA for normal presumed matrix or cell-associated aFGF/HBGF-I and not a secreted oncogenic form such as hst (KS) or FGF-5, which have leader peptides [4,5]. The presence of oncogene type FGF/HBGF molecules in these cells is presently unknown. However, since the aFGF/HBGF-I gene is

amplified in DDT-1 cells, the normal aFGF/HBGF-I may play an autocrine role in growth of these cells [20,21].

It has been shown that v-sis, or a PDGF like gene, is also regulated by androgens in DDT-1 cells [9,22,23]. Recent results indicate that porcine platelet PDGF added at 40 ng/ml to low density cultures can stimulate aFGF/HBGF-I mRNA accumulation (unpublished observations). Thus androgens may increase v-sis expression, which in turn could modulate the expression of the amplified aFGF/HBGF-I gene. This model is now being tested.

The larger difference seen in steady-state aFGF/HBGF-I mRNA levels between −T and +T with the in situ hybridization results is likely due to the lower cell density (<2,000 cells/cm^2) and greater syncrony at which the cells were grown. The DDT-1 cells utilized in the in situ hybridization experiments were allowed to incubate for 24 h in media without T before the experiment was initiated with fresh media plus and minus androgens. For the northern analysis, and to obtain workable amounts of RNA, it was first necessary to let the cells grow to 50% confluence in media without androgens. The media was then removed and add fresh media plus and minus androgen was added. Under these conditions, DDT-1 cells may become "conditioned" with factors associated with the extracellular matrix, which are not easily removed by simply changing the media. Recent evidence suggests that the FGF/HBGF family and v-sis and c-sis gene products are not found in the conditioned media but are associated with the matrix and/or other cellular components [24].

Also DDT1 cells grown under these conditions are an asyncronous population with respect to stage in the cell cycle. Therefore, in the cells prepared for northern analysis, the baseline level of the aFGF/HBGF-I mRNA is higher as compared with the in situ hybridization results. This suggests that cell-cell contacts or matrix associated aFGF/HBGF-I may have a modulating effect on androgen-stimulated aFGF/HBGF-I mRNA accumulation under high density asynchronous culture conditions. Similar results of cell density effects on bFGF/HBGF-II mRNA expression have been noted in other cell culture systems [25].

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