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Direct Activation of TGF-β1 Transcription by Androgen and Androgen Receptor Complex in Huh7 Human Hepatoma Cells and its Tumor in Nude Mice

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Abstract Importance of androgen for promotion of hepatocelullar carcinoma (HCC) has long been supported by clinical and experimental evidences. However, mechanisms involved in the carcinogenesis have not yet been fully elucidated. Moreover, unbalanced expression of TGF-β1 during tumor progression results in prooncogenic rather than growth inhibition. To investigate the effect of androgen on transcriptional regulation of TGF-β1, we isolated rat TGF-β1 promoter, based on our previous report (GenBank AF249327), and examined regulation of its promoter activity by dihydrotestosterone in Huh7, LNCaP, and PC3 cells. Several putative transcription factor-binding sites were found, but no TATA box. When the full-length (−4784 to +68) and variously deleted promoter DNAs were evaluated, the promoter region spanning from −2732 to −1203 showed the highest activity towards dihydrotestosterone in a dose-dependent manner in both Huh7 and PC3 cells with androgen receptor (AR) expression. Putative androgen response sequence half site (5′-TGTCCT-3′) was identified to be located within −1932 to −1927, proved by mutant (5′-*AGA*CCT-3′) analysis and chromatin immunoprecipitation (ChIP) assay. AR mediated upregulation of TGF-β1 expression was confirmed by HCC developed in nude mice with AR-overexpressed Huh7-cells. This work presents in vivo and in vitro evidences of activation of TGF-β1 expression by androgen and AR, and implicates the modulation of hepatocarcinogenesis by AR through the regulation of TGF-β1 expression. J. Cell. Biochem. 97: 393–411, 2006.

Key words: androgen; androgen receptor; TGF-β; hepatocellular carcinoma; Huh7 cells; Huh7-AR hepatoma

Transforming growth factor β1 (TGF-β1) is a multifunctional cytokine to influence homeostatic processes of various tissues [Massague, 1998]. In liver, its increased expression has been found during progression of HCC [Romieu et al., 1997; Tsai et al., 1997; Abou-Shady et al., 1999; Matsuzaki et al., 2000], cirrhosis [Qi et al., 1999; Roulot et al., 1999; Nakamura et al., 2000], and

Abbreviations used: TGF-β1, transforming growth factor-β1; HCC, hepatocellular carcinoma; DEN, diethylnitrosamine; AR, androgen receptor; ARE, androgen response element; PSA, prostate specific antigen; KLK-2, glandular kallikrein-1; Slp, sex-limited protein; MVDP, mouse vas deferens protein; TFIIF, transcription factor IIF; cFBS, dextran-treated charcoal-stripped FBS; DHT, dihydrotestosterone; RLTK, thymidine kinase promoter(TK)-driven Renilla luciferase plasmid; pCMV-hAR, cytomegalovirus-human androgen receptor plasmid; MMTV-Luc, mammary tumor virus promoter-luciferase; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; USF, upstream stimulatory factor; NF-κB, nuclear factor κB; HRE, hormone response element.

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liver damage and regeneration [Braun et al., 1988; Jakowlew et al., 1991; Davis and Chen, 1996], implying its critical roles involved. These effects are orchestrated outcomes of various cellular processes, including cell proliferation [Bissell et al., 1995] and growth inhibition [Lim et al., 1997; Choi et al., 1998], and result from cellular signaling mediated by TGF-β1, its receptors and intracellular Smad proteins [Schuster and Krieglstein, 2002]. However, unbalanced expression of TGF-β1 during tumor progression may result in pro-oncogenic rather than growth inhibition [Roberts and Wakefield, 2003].

In our earlier study with diethylnitrosamine (DEN)-nodularin-induced hepatocarcinogenesis model in rat [Song et al., 1999], the reciprocal expression of TGF-β1 and its receptor was found to be one of the important maneuvers for selective proliferation of altered hepatocytes, suggesting a critical role of TGF-β1 regulation in carcinogenesis [Lim et al., 1999]. Although TGF-β1 expression and a few intracellular factors, including H-ras and E2F-1, in response to external stimuli have amply been elucidated [Geiser et al., 1991; Kim et al., 1998], the regulation of TGF-β1 expression in the process of hepatocarcinogenesis remains poorly understood.

The importance of steroidal hormone, androgen, in the etiology of HCC has been implicated and supported by several clinical or experimental evidences [Nagasue et al., 1985; Ostrowski et al., 1988; Yu et al., 1995; Matsumoto et al., 2000]. Androgenic hormones exert powerful effects on liver by direct transcriptional regulation through androgen receptor (AR) or indirect influence through hypothalamus and pituitary. The presence of an AR within the liver has increasingly attracted attention, especially with respect to its relationship with HCC. The maintenance of AR, as opposed to decreased estrogen receptor, has been observed in the progression from hyperplasia to HCC in rat [Eagon et al., 1996], which implies a role of AR in regulation of HCC development. Moreover, it warrants the use of antihormonal therapy for the treatment of HCC, particularly in patients who express high AR levels [Nagasue et al., 1990; Eagon et al., 1994, 1996]. Androgen plays its regulatory role in gene expression by two different mechanisms. One is through binding its receptor, AR, and interacting with *cis*-acting DNA sequences, termed androgen response elements (ARE), located in the promoter region of its target genes [Karvonen et al., 1997]. There are many androgen-regulated genes, encoding proteins such as prostate specific antigen (PSA) [Riegman et al., 1991], glandular kallikrein-1 (KLK-2) [Murtha et al., 1993], prostatein C3 protein [Tan et al., 1992], tyrosine aminotransferase [Denison et al., 1989], sex-limited protein (Slp) [Adler et al., 1991], probasin [Rennie et al., 1993], $p21^{WAF1}$ [Lu et al., 1999], and mouse vas deferens protein (MVDP) [Fabre et al., 1994]. The second mechanism is through interaction of AR with other transcription factors such as c-Jun or transcription factor IIF (TFIIF) [Kallio et al., 1995; McEwan and Gustafsson, 1997]. The possibility that androgen may regulate the expression of TGF-β1 was indicated by Northern blot analyses after androgen treatment in a few cell lines such as prostate cancer cells, adrenocortical cells, and osteoblasts [Kim et al., 1996; Gill et al., 1998; Zatelli et al., 1998, 2000]. However, it remains still unclear whether the regulation is through direct interaction of AR through ARE of *TGF-β1* gene in liver.

Although 1,232 bases of 5′ flanking promoter region of rat TGF-β1 has earlier been isolated and sequenced [Yang et al., 1998], no putative ARE consensus sequence was found within the region. In the present study, in order to investigate whether androgen can regulate TGF-β1 expression through direct interaction of AR and ARE within *TGF-β1* gene, we cloned and sequenced 4,852 bases, spanning the 5′-flanking region of rat *TGF-β1* gene.

Fig. 1. Nucleotide sequence of the cloned rat TGF-β1 promoter and 5′ untranslated region. Numbered horizontal arrows indicate the primer sequences used for cloning: four sets of primer 1 and 3, 2 and 5, 4 and 6, and 4 and 7 were used for cloning four independent PCR fragments, and full size clone was generated by sequential rejoining of digested fragments by BamHI, AvrII (open box) and other available restriction enzymes on the muticloning site of the TA cloning vector, pCR3.1. Sequence from −4784 to −200 is the region newly cloned in the present study (GenBank AF249327), and the sequence in bold letter was derived from the sequence reported by Dr. L. M. Wakefield (GenBank AF105069). The translation start site is indicated as thick arrow. The dotted underlined sequences indicate HRE-like consensus sequences, and the double underlined sequences indicate putative ARE sequences.


-4784 TGAGACCCAC ATTGTAGCCC ATGCTGACTT CACACTCATC AGGCAGCCCA GGAAAGCCCT CCACACACTC CCACATGCTG AGATTTCAGG TGTAGACTGC
(1)
-4684 GGGTGCCAAG ATCTGAATCC AGATATTTGG TTCTAGTGTT TACACTGTGT AACTCCCCAC TCCCAAATAC CAGGGGAAAA CAAACCAGGT CTCCTTCCTG
-4584 AAGGGTGCTA CTCACCCCCA ACCCTGTGTC CTGTTCTCAT TACAGGGGCC GCATGGAAGC TCCTGTCAGG CGTCCGGGAA GGCCAGACGC AGGTAGACAC
-4484 ACCCCAGACA GGGGACATGG CTTACTGGTC CCACCCCATT GACCTGCACT TTGCTACAAA AGGTCTCCAA GGTACGTACA GGATGACAGG AAGGGAAGGG
-4384 GCAGTCAAAC TAGGAAGAGC TGGGCTTGGA ACACCCTGGC CGTCTCTGCT CCCCATACTT TGTGGGTCCT TTTTCTGGTG CATTTGAGGC TTCACTCGAG
-4284 CTATGCAAAA CCCTACCTAG GCCGTGGAAT GTGCCAGAGG TCGGGGTAGA GGAGATAGCA GGGGGAGGGG GGTCAAGTGG CAGAGGAGAA GGCAGTGAGG
-4184 AGGCCGGGTA GGGTGAAGAG AGCTGGATGG GAAGGCGAGG GACAGGAAAT GGGCCAGTTG CCGCTGCTCA CACACGGACA GAGGTCCTGC TCAAAGGGGA
-4084 AGTGAGTGTA GGAGGGAGGA GGTAGCATGG CAGTGCTGCT TTGCCGGGAA GCTGCCCGGG TATTTCAGCA ATGTCAGTCA GTTGTCAGTT TGCTGAGTCT
-3984 GTGCCTGCCC AGCTTGAGAA GGGATGTCCC CAACTAAGAC AGGACCCTCC AAGCCCTGTC TTCAGGGCCT CTATTCTAGG TAGAAGATGG ACCCGTTTCT
-3884 AGATGGGGCC AGCCCAGAGG CCGTGGAGAC CCAGAAGTCA TCTCTGGTTG GTCCCATGGC CTTATATGGC AGAGGGGTTA GAGCTCTGCT GTAAAGGCCA
-3784 GGAGAAGCTA ACTAGTCCCA AGGACAAGGG CAGAGGGTGG AGGCAGAGAG CCGGAGACGT GGGTATTAGA CGACGTGGGT ATTAGACGTC TGCGGGAGAG
-3684 AACAGCAGGG GATGGGGAGG TAGGGACAGA ATGTAGGGCC TTGGCTGCAA AGGTGCTAAG ACTCTCTCCT GAAGGCTCTG GGGAGCCCAG GAAGAAATGG
-3584 ATGGGTCAGA TTTTCCCCTT GGACAGCATA CTAGTTTCTG TGTGAGCACT GAGTCCTTGT TGCCTCAGTT CTGGCCTTTG GTAGCTTCCT CCCTGCTTGC
-3484 TTGCTTCTTA GTCTCTCTCA CTTAGCTGTT CACCAGCAAA CCCTAGGGGC TCTGAAACCC ACTTCTGCTT CTCACCCCAT TCCTTCTGCT TCTCACCCCA
-3384 GCCACCCCAC TGAGCCCTCT CTGCCCTTCT TCTCTCTTTT TTTTTTTTTT TGTTTTTTGT TTTTTGGGTT TTTTTTTTTT TTTGGAGTTG GGGACCAAAC
-3284 CCAGGGCCTT GTGCTTGCTA GGCAAGCGCT CTACCACTGA GCTAAATCCT GCCCTTCTTC TCTTACCTGG ACCATTCTTC CTTCCCGTCT TCACTGTGCT
-3184 CCCAGACCCA GGCATCAGGG TCTGGGACTG CACCGGACCC TGGGAGGACA CAAATACGAC CATGTTCTTC CTCTGCACAG AACCTTCCTG CAGATCTCCT
-3084 GTCTCTCAGA GCAAACGCTG GAGTCTTCTC TAGCATCACA TGGCCCCATG TAATCTGGAG GTCACCTCTG TCCTTTGTCC CCCATGACTC CCTGTTTTAG
-2984 TCCCGTTGGG CCCACCAGGC CAGCTCCTAC TTCACAACTG TTGCACTGAC GATCTCGTCC CCCTAGGTGC CTAGCTAGCC CACTCCTTTA AGCATTTCTA
-2884 TACCGGTTGC ATCCTGGTTG CTTTGTTTGC TTTTGAGAAC ATAGTGCAGG CCAGCCTGGA ACCTCAGCAG CCCAAGTGTG CACCCTTCCA GGTGAGGTCT
-2784 TCCTGTATAG CCTAGAACTC ATGTGTAGCC CAGGCTAGCC TTGAACTTGC AGGGATCCTC TTGCCTCTAC CTCCCAAGAG CTGACAGTCC AGCTGTACAC
(2)
(3)
-2684 TGCAGTGCTC AGCCCAAATG TCCCCATCTT TAGTAAAGCT TCCTTTAGCT GCTCCCCACT TTTCCAAGCT ACATCTCCAC CCCTCCCTTT CTGGCTGTTG
-2584 TTTTTCCCCA GAGCGGTTAT CACCATCTGA CATACCCTAT AATTTACTTA TTTACTATGT TGGTTGTCTA GGATGTCAGC TTCACAGGGC AGGAATTTTT
-2484 GTCTGCTGTG TTCATTGCTG TGTCCCCAGC CTGGAGAACA GGGCCTGCCA GGGAGGAGCT CAGGGTAGCG GGGGACCTGG GCTGCACGTC CAAGCATTGG

Fig. 1.

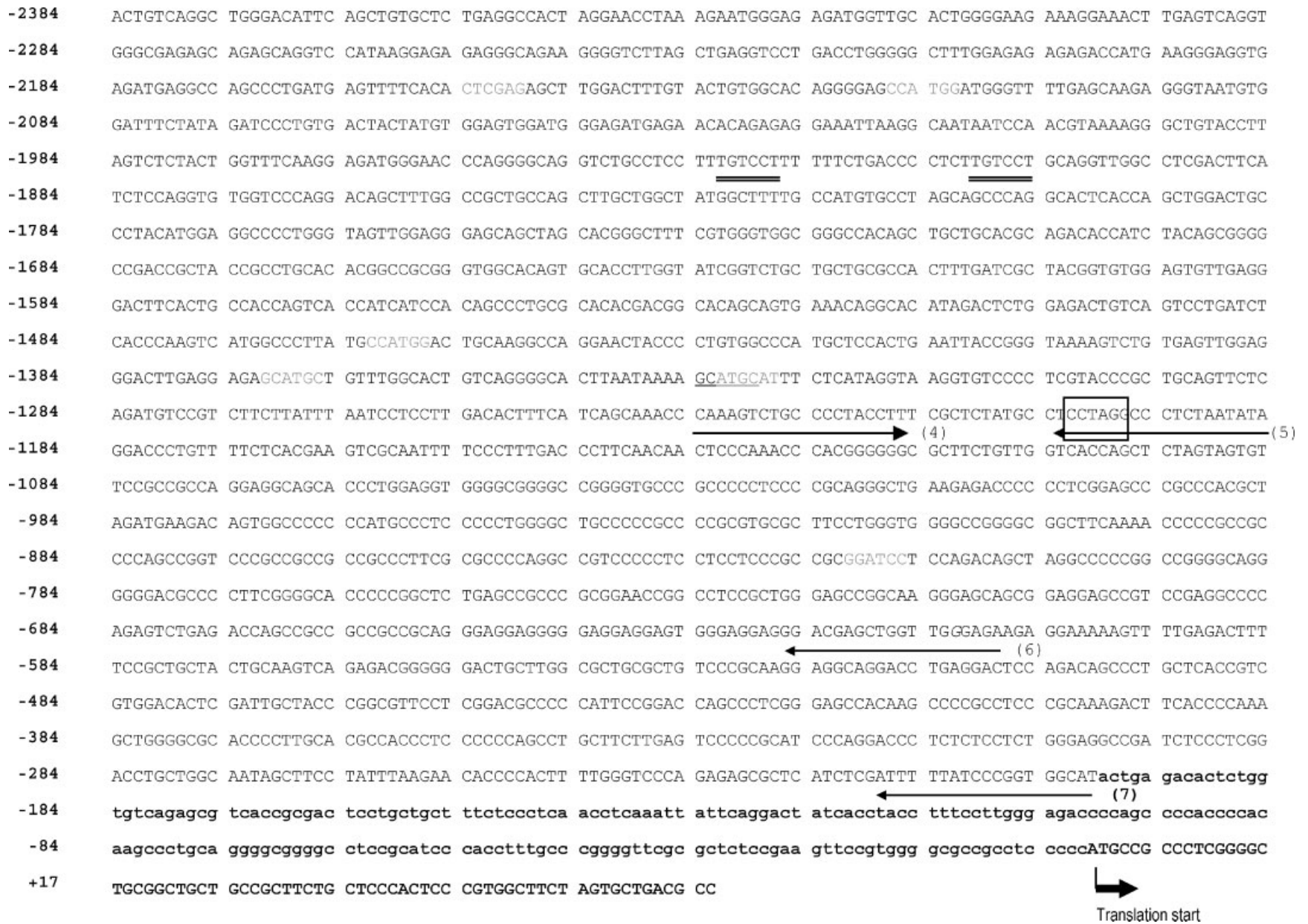


Fig. 1. (*Continued*)

Furthermore, to evaluate the effect of androgen on transcriptional activities of TGF-β1 promoter, human prostate cancer LNCaP cell-line was selected, because of its high expression of AR, high sensitivity to androgen, and well-characterized androgen responsiveness. Human hepatoma Huh7 cells and prostate cancer PC3 cells were also used, since these cells lack of AR expression. Using a series of TGF-β1 promoter-luciferase deletion constructs, we identified one discrete positive regulatory region at −(2732 to −1203 and analyzed its characteristics. Moreover, the sequence from −1932 to −1927 (5′-TGTCCT-3′) was found to be the potential ARE half site within the promoter. We present here in vivo and in vitro evidences that androgen can directly regulate TGF-β1 expression through binding of AR to ARE in TGF-β1 promoter, and suggest that such activation might regulate the progression of HCC both in human and animal model systems.

MATERIALS AND METHODS

Cloning and Sequencing of the Promoter Region of TGF-β1

In order to isolate promoter sequences of rat *TGF-β1* gene (−4784 to +68) containing translation start site (Fig. 1), we used our previously cloned construct, which was isolated, sequenced, and reported to GenBank (AF249327). PCR products were obtained sequentially by using Ex-Taq (Takara, Inc., Shiga, Japan), various sets of primers, and genomic DNA isolated from F344 rat as a template. Reverse primers were designed from the known sequences of rat TGF-β1 promoter, which has earlier been cloned. For forward primers, two or three primers were designed against the mouse TGF-β1 promoter sequence (GenBank, L42456). Four PCR products (−4784/−2694, −2732/−1184, −1234/−608, −687/−200 nt) with more than 30 base-pairs overlapping were isolated and cloned into pCR3.1 (Invitrogen, Groningen, Netherland). Sequencing was carried out by the dideoxynucleotide chain termination method, using automatic sequencer (ABI 377). Sequencing primers were generated by using the canonical T3 or T7 sites flanking the multiple cloning sites of the vector or complementary to the experimentally established internal sequences, according to primer-walking strategy.

Construction of TGF-β1 Promoter-Luciferase Reporter Genes

All plasmid constructs were prepared according to the standard method [Sambrook and Russell, 2000]. Luciferase reporter plasmid containing full-length promoter of rat TGF-β1 and spanning from −4784 to +68 was generated, using the promoterless basic pGL3 (Promega, Madison, WI), by sequential subcloning of each restriction fragments from four independently cloned plasmids. Briefly, after subcloning AvrII/EcoRV fragment (−1202 to −608) into pGL3-basic plasmid, BamHI/AvrII fragment (−2732 to −1202) and KpnI/BamHI fragment (−4784 to −2732) were sequentially cloned, and finally BamHI/SmaI fragment (−821 to +68), digested from the plasmid containing TGF-β1 promoter region (GenBank, AF105069) was introduced. The genomic promoter region investigated comprised 4852 bp.

Construction of Deletion or Mutant Plasmids and Expression Plasmids

All deletion mutant plasmid constructs were prepared according to the standard methods by using digestion with restriction enzymes and ligation; −1202/−608 plasmid was made by inserting AvrII/EcoRV fragment into pGL3-basic plasmid; −2732/−608 plasmid was generated by inserting the BamHI/AvrII fragment (−2732 to −1202) into −1202/−608 plasmid; −4784/−608 plasmid was obtained by introducing the KpnI/BamHI fragment (−4784 to −2732) into the −2732/−608 plasmid; −4784/−41 plasmid was made by inserting the BamHI/SmaI fragment (−821/−41) into −4784/−608 plasmid; −2429/−41 plasmid was produced by deleting the SacI fragment (−4784 to −2429) from −4784/−41 plasmid; All constructs were confirmed by mapping restriction enzymes sites and partially sequencing with available primers which were used for cloning. A mutant plasmid, ARE_m, was generated against the −2732/−608 plasmid by QuickChange™ Site-Directed Mutagenesis (Stratagene), using the primer sets of 5′-GGTCTGCCTCCTT**AGACCT**TTT-TTCTGAC-3′ and 5′-GTCAGAAAAA**AGGTC-T**AAGGAGGCAGACC-3′.

Cell Culture and Application of Steroids

PC3 and LNCaP cells were maintained in RPMI 1640 (Gibco BRL, Gaitherburg, MD) supplemented with 10% (v/v) fetal bovine serum

(FBS; Gibco BRL), and Huh7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10% FBS. All cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. Dextran-treated charcoal stripped FBS (cFBS) was used in the place of FBS, when steroids such as dihydrotestosterone (DHT), dexamethasone, progesterone, hydroxyflutamide, and testosterone were applied. Briefly, DHT was dissolved in ethanol before addition to culture medium. The final alcohol concentration was 0.001%, and the final concentration of each steroid is indicated under the legend.

Transient Transfection and Luciferase Assay

Cells were seeded into six-well plates at 50% confluent, and were subjected to transfection by $CaPO_4$ precipitation method with two of reporter plasmid and 0.5 μg of thymidine kinase promoter(TK)-driven Renilla luciferase plasmid (RLTK) (Promega, Madison, WI) as an internal control in the presence of 1 μg of cytomegalovirus-human androgen receptor plasmid (pCMV-hAR) plasmid or pcDNA3 plasmid. After washing with PBS, the cells were then incubated for an additional 48 h in 10% cFBS containing media in the absence or presence of 10 nM DHT. pGL3-control (Promega) and mammary tumor virus promoter-luciferase (MMTV-Luc) plasmid were used as control. The cells were lysed, and luciferase activity of cell extracts was measured by TD20/20 luminometer (Turner, BioSystems, CA) as relative light units, according to the manual provided with the Dual-Luciferase Reporter Assay System (Promega). The luciferase activities were normalized on the basis of the Renilla luciferase activity. All transfection experiments and luciferase assays were carried out in triplicate and also repeated at least twice.

Western Blot Analysis

For measuring the level of AR expression, cells were washed twice with PBS and lysed with lysis buffer [50 mM Tris/HCl (pH 7.5), 0.1 M NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 10 μg/ml each of aprotinin and leupeptin, and 1 mM PMSF], and a portion (5–50 μg) of the cell lysate was subjected to 10% SDS–polyacrylamide gel electrophoresis. The proteins on the gel were subsequently transferred to nitrocellulose membrane, and the intensity of the protein bands was monitored by the enhanced chemiluminescence method (Amersham Pharmacia Biotech, Buckinghamshire, England) after reacting with anti-AR antibodies and peroxidase-conjugated anti-rabbit IgG (Amersham Pharmacia) as the secondary antibody.

Nude Mice Hepatoma Formation

To evaluate in vivo effect of androgen-AR complex on TGF-β1 expression, HCC tumors were produced in nude mice (CBy. Cg-Foxn1^{nu}) obtained from Jackson Laboratory (Bar Harbor, ME) and maintained in Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea) in the specific pathogen free condition. Huh7-AR_m and Huh7-V_m cells were subcutaneously injected at left thigh of mice (5×10^6 cells per mouse, and 10 and 11 mice per group). Tumors were excised from all the mice at around 40 days after the injection, when the tumor volume became over 1,000 mm^3, and used for RNA preparation to evaluate TGF-β1 expression. Huh7-AR_m and Huh7-V_m cells were prepared by stable transfection with pCMV-hAR plasmid and its vector pcDNA3 in Huh7 cells by using LipofectAMINE in DMEM. After transfection for 6 h, the cells were further incubated for 18 h without LipofecAMINE, and then selected with G418 (1 mg/ml) for 2 weeks. G418-resistant cells were pooled and named as Huh7-AR_m and Huh7-V_m, and maintained in G418 (0.5 mg/ml) containing media. Before pooling the cells, several single clones were selected under a microscope.

RNA Isolation and RT-PCR

Total RNA was isolated from cultured cells by extraction with guanidinium isothiocyanate/phenol/chloroform. Total cDNAs for RT-PCR were obtained by consecutively incubating 0.5 μg of total RNA, oligo (dT) primer, and AMV RT XL at 30°C for 10 min, 56°C for 30 min, and then 90°C for 5 min, according to the protocol provided with PCR kit (Version 2.1, Takara Inc., Shiga, Japan). PCR conditions were: denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and elongation at 72°C for 30 s. Hundred nanograms each of TGF-β1 primers set and 50 ng each of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer sets were mixed with Ex Taq polymerase for a single reaction (total volume, 100 μl), and serial PCR products from 25 to 32 cycles were screened. Oligonucleotide primers designed for

human TGF-β1 were 5′-GCCCTGGACACCAA-CTATT-3′ and 5′-TCAGCTGCACTTGCAGG-AG-3′ and the PCR product spanned from exon 5 to exon 7. Primers for GAPDH were 5′-CCATGGAGAAGGCTGGGG-3′ and 5′-CA-AAGTTGTCATGGATGACC-3′. Primers for human AR expression in Huh7 cells and HCC tissues were 5′-CCTTTGCTGCCT TGTTATCT-3′ and 5′-GCATGCAATGATGCGATCAA-3′. The final RT-PCR products were electrophoresed on 1.2% agarose gel, stained with 0.5 μg/ml of ethidium bromide solution, and visualized on UV trans-illuminator.

ChIP Assay

Huh7-AR_m cells were seeded into 100 mm culture dish with 40% confluent and transfected with pT−2732/−608 using LipofectAMINE for 5 h in a serum free medium, and the medium was changed with complete medium for 20 h. The cells were further maintained in DMEM with 10% cFBS for 22 h before stimulating with 10 nM DHT for 12 h. To cross-link protein to DNA, the cells were then treated with 1% formaldehyde and washed twice with ice-cold PBS. Nuclei were isolated according to the published method [LiKe et al., 2004], and incubated for 10 min on ice in 300 μl of lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS). The isolated chromatin was sonicated (550 Sonic dismembrator, Fisher Scientific, Pittsburgh, PA) for 10 s three times to shear DNA, and centrifuged at 13,000 rpm for 10 min. Immunoprecipitation was carried out in 2 ml of ChIP buffer (12.5 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1.25% Triton X-100, 1 mM EDTA) with 250 μl of the supernatant obtained above, protease inhibitors and anti-AR antibody (3 μg), after pre-clearing the reaction mixture with protein-G agarose for 1 h at 4°C without anti-AR antibody. The mixture was incubated overnight at 4°C, and then 40 μl of protein-G agarose was added for 1 h to the mixture (Invitrogen, Carlsbad, CA.). Agarose beads were sequentially washed three times with the wash buffer (20 mM Tris-HCl, pH 8.1, 2 mM EDTA, 1% Triton X-100, 0.1% SDS) containing 150 mM NaCl, 500 mM NaCl, and the buffer (10 mM Tris-HCl, pH 8.1, 1 mM EDTA, 1% deoxycholic acid, 1% NP40) with 0.25M LiCl, followed by washing twice with 10 mM Tris, pH 8.0, and 1 mM EDTA. The immunoprecipitates were eluted from the beads with 250 μl of ChIP extraction buffer (1% SDS, 0.1 M $NaHCO_3$) by rotating for 15 min at room temperature and spinning down the beads. To reverse the protein-DNA crosslink, 12.5 μl of 4 M NaCl was added and the mixture was incubated at 65°C for 4 h. DNA was purified by incubation at 45°C for 1 h with 5 μl of a solution containing 0.5 M EDTA, 10 μl of 1 M Tris-HCl, pH 6.5 and 1 μl of 10 mg/ml proteinase K, followed by phenol/chloroform extraction. PCR amplification was performed on recovered DNA products with the potential ARE specific primers; sense 5′-GATGAGAACACAGAGAGGAA-3′ (−2041/−2022) and antisense 5′-TGCTGGCTATGGCT-TTTG-3′ (−1842/−1825) through denaturing at 95°C for 5 min and 29 cycles of the 95°C (30 s), 53°C (30 s), 72°C (30 s), and the final synthesis at 72°C for 5 min. To quantify the PCR products over a broad range of DNA concentration, ARE irrelevant internal region (−1329/−1183) was also amplified through the initial denaturation at 95°C for 5 min and 30 cycles of 95°C (30 s), 51.5°C (30 s) and 72°C (30 s), and the final synthesis at 72°C for 5 min with the sense 5′-CATTTCTCATAGGTAAGG-3′ and antisense 5′-CTATATTAGAGGGCCTAGG-3′ primers.

RESULTS

Cloning and Sequence Analysis of Promoter Region of Rat *TGF-β1* Gene

In order to investigate the possibility of direct transcriptional regulation of TGF-β1 expression by androgen, we previously isolated and sequenced 4,595 base pairs, spanning the 5′-flanking region of rat *TGF-β1* gene (GenBank, AF249327). Using PCR-based cloning strategy, three separate genomic clones which contained part of rat TGF-β1 promoter region were isolated by the method described in Materials and Methods (Fig. 1). It should be noted here that Yang et al. [1998] has earlier reported the sequences of 1,232 bases of rat TGF-β1 promoter, including 5′-untranslated region, however, the program search failed to find any putative consensus sequence for hormone responsive element (5′-TGTTCT-3′) within the region, such as glucocorticoid responsive element, progesterone responsive element or ARE.

Comparison of the rat TGF-β1 promoter sequence with published sequences for human (GenBank, J04431) and mouse (GenBank, L42456) genes showed generally high homology of 86% and 87%, respectively. The proximal region contained two highly conserved regions

among the three species from −1890 to −1314 (Fig. 2A, 577 bases, 95% homology) and from −1175 to −708 (Fig. 2B, 468 bases, 97% homology). To identify potential *cis*-regulatory elements for androgen in the promoter regions, we computationally analyzed possible transcription factor binding sites of the sequences by using the TFSEARCH or TRANSFAC program, and found them for upstream stimulatory factor (USF), nuclear factor kB NF-κB, E2F, Sp1, and p300, but no TATA and CAAT box (Fig. 2).

In order to screen the possible responsiveness of the cloned rat TGF-β1 promoter toward DHT, we attempted to find putative hormone responsive sequences to which ARE belonged with high homology. Hormone response elements (HRE) are known to typically consist of two six-nucleotide palindromic sequences with a 3-bp spacer having a well-demonstrated consensus sequence 5′-GGA/TACAnnnTGTT/CCT-3′. The palindromic sequence implies its action as a dimer through AR binding on the sequences [Nordeen et al., 1990]. Two half-site sequence (5′-TGTTCT-3′) for potential steroid response elements are situated at −4548 and −3117 in the 5′-distal promoter region (Fig. 2C). Both are imperfect palindromes with only the right half of the consensus sequences, implying its incomplete action. However, it is not clear whether TGF-β1 can be regulated by androgen through androgen-AR-ARE complex formation on its promoter region, because several non-consensus ARE sequences have also been reported [Le and Burnstein, 1996].

Determination of Androgen Responsiveness of TGF-β1 Promoter, Using Androgen Sensitive Prostate Cancer Cells, LNCaP

To investigate whether the rat TGF-β1 promoter could be regulated by androgen, we first subcloned the major TGF-β1 transcription start site [Yang et al., 1998], containing segment of the 4,177 bases (from −4784 to −608, pT−4784/−608) genomic clone, into plasmid pGL3-basic which contained a firefly luciferase reporter gene cassette. The murine mammary tumor virus (MMTV) promoter sequence was compared as a positive control of androgen responsiveness. The pT−4784/−608 showed significant androgen-dependent promoter activity in LNCaP cells. In the presence of 10 nM DHT, the Luc activity increased about sevenfold over the basal level observed in the absence of DHT (Fig. 3A). To prove direct involvement of AR in the activity, the activity was examined in an androgen-independent PC-3, which does not express AR. In the PC3 cells, the pT−4784/−608 did not show any activity, whereas co-transfection of pCMV-hAR expressing plasmid regained the activation by DHT, implying that the activation by DHT was mediated through AR (Fig. 3B). The activation might be due to the above mentioned two putative HRE-like half-consensus sequences (5′-TGTTCT-3′) located at −4548 and −3117.

To further define the active androgen-responsive region, two shorter subfragments (pT−2732/−608 and pT−1202/−608) were analyzed. As shown in Figure 3B, deletion of the TGF-β1 5′-distal region between nucleotides at positions −4784 and −2732 resulted in an increased activation (35-fold) only in PC3 cells with AR expression. A lesser degree of induction was observed with the fragment pT−1202/−608. On the basis of this data, it was clear that ARE existed between nucleotides −2732 and −1202 and the two HRE-like half-sites (5′-TGTTCT-3′) were not active enough, probably due to the absence of proper palindromic partner sequences or presence of any strong repressive element at the −2732 upstream base. The activity of pT−2732/−608 in the PC3/AR expression cells increased dose-dependently in the presence of DHT up to 10 nM (Fig. 3C).

Response of TGF-β1 Promoter to DHT in Huh7 Hepatoma Cells

Although the possibility that TGF-β1 can be induced by androgen has been suggested by several groups [Kim et al., 1996; Gill et al., 1998; Zatelli et al., 1998, 2000], it is not clear whether TGF-β1 in hepatocytes can be transcriptionally induced by androgen and also through the direct action of AR. To address this possibility, we tested the regulation of TGF-β1 promoter activity by DHT in Huh7 hepatoma cells. As shown in Figure 4A, the basal activity of the pT−4784/608 in Huh7 cells was stronger than that of MMTV promoter in the media supplemented with 10% cFBS, instead of FBS. Even with DHT added, none of the promoter constructs showed increased activity. However, when AR was overexpressed by transient co-transfection, the activity of pT−4784/−608 was increased 2-fold and that of pT−2732/−608 was increased more (3.75-fold) in Huh7 cells (Fig. 4B), thus showing similar pattern

observed in PC3 cells with AR expression. The AR expression of Huh7 cells was clearly detectable, only when pCMV-hAR expressing plasmid was transfected (0.5 and 1.5 μg/lane). AR-levels in the AR-transfected Huh7 cell lysates (50 μg/lane) were almost comparable to those of LNCaP cell lysates loaded 5 μg and 10 μg per lane, respectively (Fig. 4C). These results indicate that TGF-β1 can be induced by androgen even in HCC cells if the cellular AR level becomes high for any reason.

Specificity of Androgen Analogs and Hormones for the TGF-β1 Promoter Activity

Next, we have examined the specificity of androgen analogs for the TGF-β1 promoter activity. Among various androgen analogues, including DHT, testosterone, and R1881 (synthetic analog), DHT showed the highest activity as expected, and the activity was suppressed by co-treatment with cyproterone acetate that has an anti-androgen effect, thus further confirming androgen-specific activation of the promoter (Fig. 5A). We also tested the response to other steroid hormones such as glucocorticoid and progesterone, because no putative HRE was found within the sequences from −2429 to −1203. The promoter activity was activated not only by androgen, but also by progesterone, although activation by progesterone required exogenous expression of progesterone receptor. However, the activity with TGF-β1 promoter pT−2732/−608 was not increased by dexamethasone (Fig. 5B).

Identification of a Potential ARE Sequence within Rat TGF-β1 Promoter

Recently, a novel ARE, 5′-TGTCCT-3′, was found in the coding region of AR, and it was still active as the half-site [Le and Burnstein, 1996]. As seen in Figure 6B, at nucleotides −1927 and −1905 of the rat TGF-β1 promoter, two 5′-TGTCCT-3′ sequences were found, however, only the −1927 sequence was conserved in the mouse promoter region. To confirm whether the conserved ARE half site at −1927 might possibly be involved in the TGF-β1 promoter activation by DHT, we prepared a mutated reporter plasmid (pT-AREm) by substituting the sequence of −1932 to −1927 with 5′-AGACCT-3′. The activation of pT-AREm plasmid by DHT in Huh7/AR cells was only one third of that of the pT−2732/−608. Moreover, it was dramatically decreased in the PC3/AR cells, indicating that the 5′-TGTCCT-3′ in −1932 to −1927 might function as one of the important ARE sequences on rat TGF-β1 promoter (Fig. 6A).

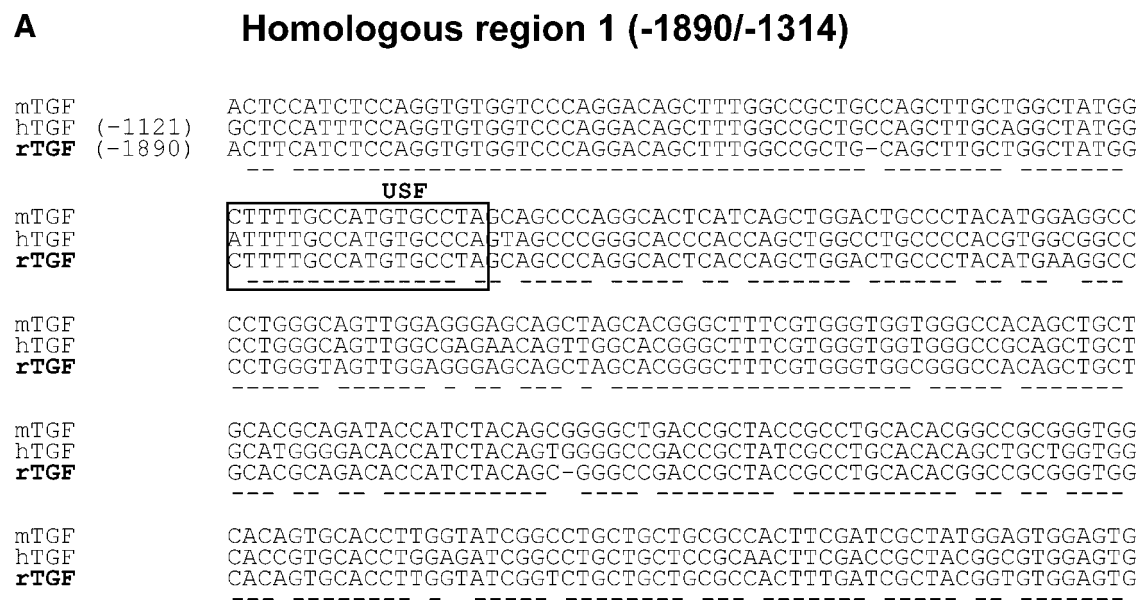
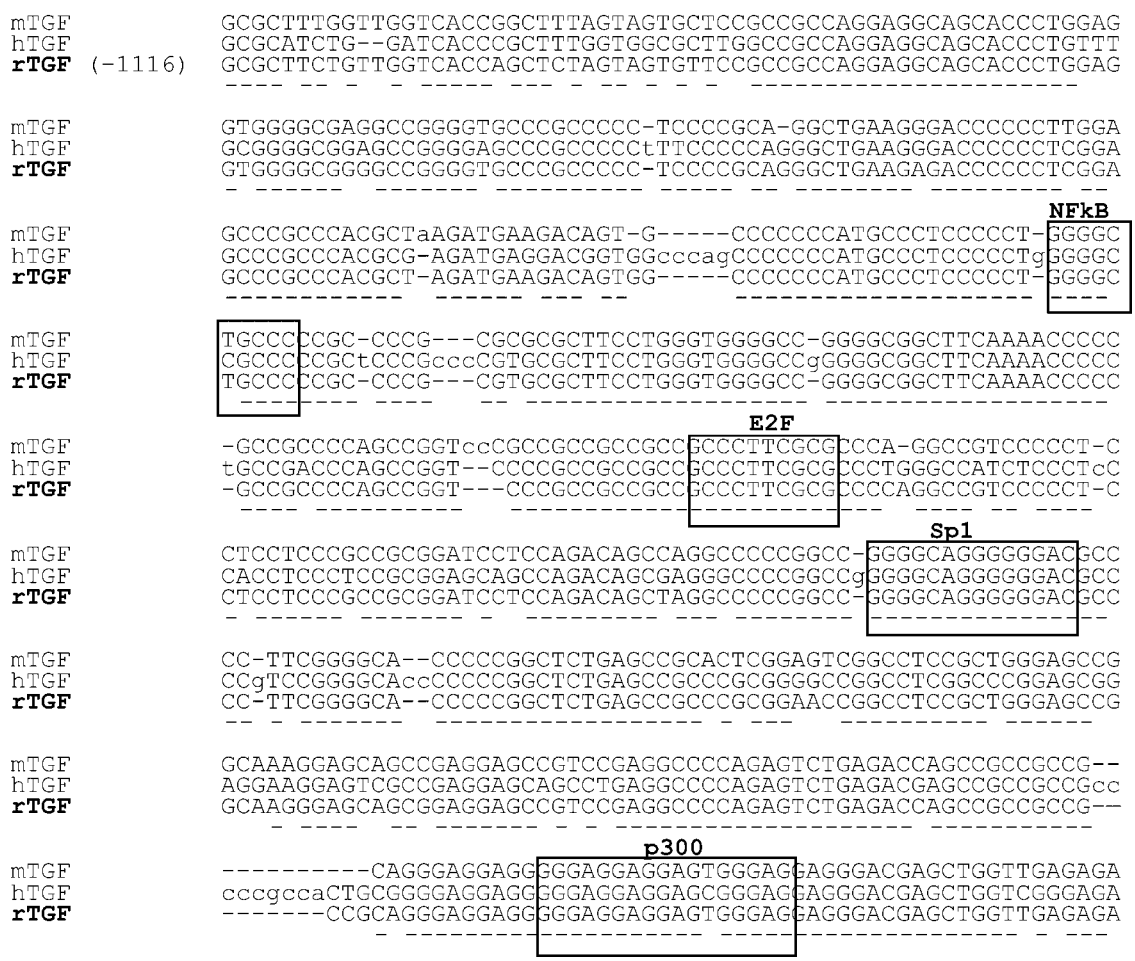


Fig. 2. Sequence comparison of the highly homologous regions of the *TGF-β1* promoter gene in mouse, human and rat. **A**: Homologous region 1 at −1890 to −1314. **B**: Homologous region 2 at −1 175 to −708. The open boxes indicate conserved putative transcription factor binding sites, and the underlines show regions of sequence identity between the three species. **C**: Sequence comparison of the ARE in the *TGF-β1* gene with the well characterized ARE sequences.

B Homologous region 2 (-1175/-708)



C HRE consensus sequence and ARE sequences reported

		Sequence reported	Ref.
	HRE(GRE, PRE, MRE) consensus:	**AGAACAnnnTGTTCT**	
HRE-like ARE sequence			
	by random sequence selection:	**AAC**GG**A**aca**TGTTCT**	Zhou et al. 1997
	p21(waf1) ARE:	**AG**C**AC**GcgaG**GTTC**C	Lu et al. 1999
	hKLK2 ARE:	G**GAACA**gcaA**GT**G**CT**	Murtha et al. 1993
	C(3) ARE:	**AGTAC**GtgaT**GTTCT**	Tan et al. 1992
	PSA ARE:	**AGAACA**gcaA**GT**G**CT**	Riegman et al. 1991
		G**GAACA**tat**TGT**ATC	
	Slp ARE3:	**AGAACA**ggc**TGTT**TC	Adler et al. 1991
	MVDP:	T**GAA**GTtcc**TGTTCT**	Fabre et al. 1994
	Probasin:	G**GTTCT**tcc***AGTACT***	Rennie et al. 1993
Non-HRE-like ARE sequence			
	AR ARE2:	*AGTACT*CC CAGGGATCAGGGAGTCTCAC	LeDai & Burnstein 1996
Putative HRE sequence in the TGF-β1 promoter region			
	-4563/ -4548 :	CCTGTGtcc**TGTTCT**	
	-3130/ -3117:	ATACGAcca**TGTTCT**	

Fig. 2. (*Continued*)

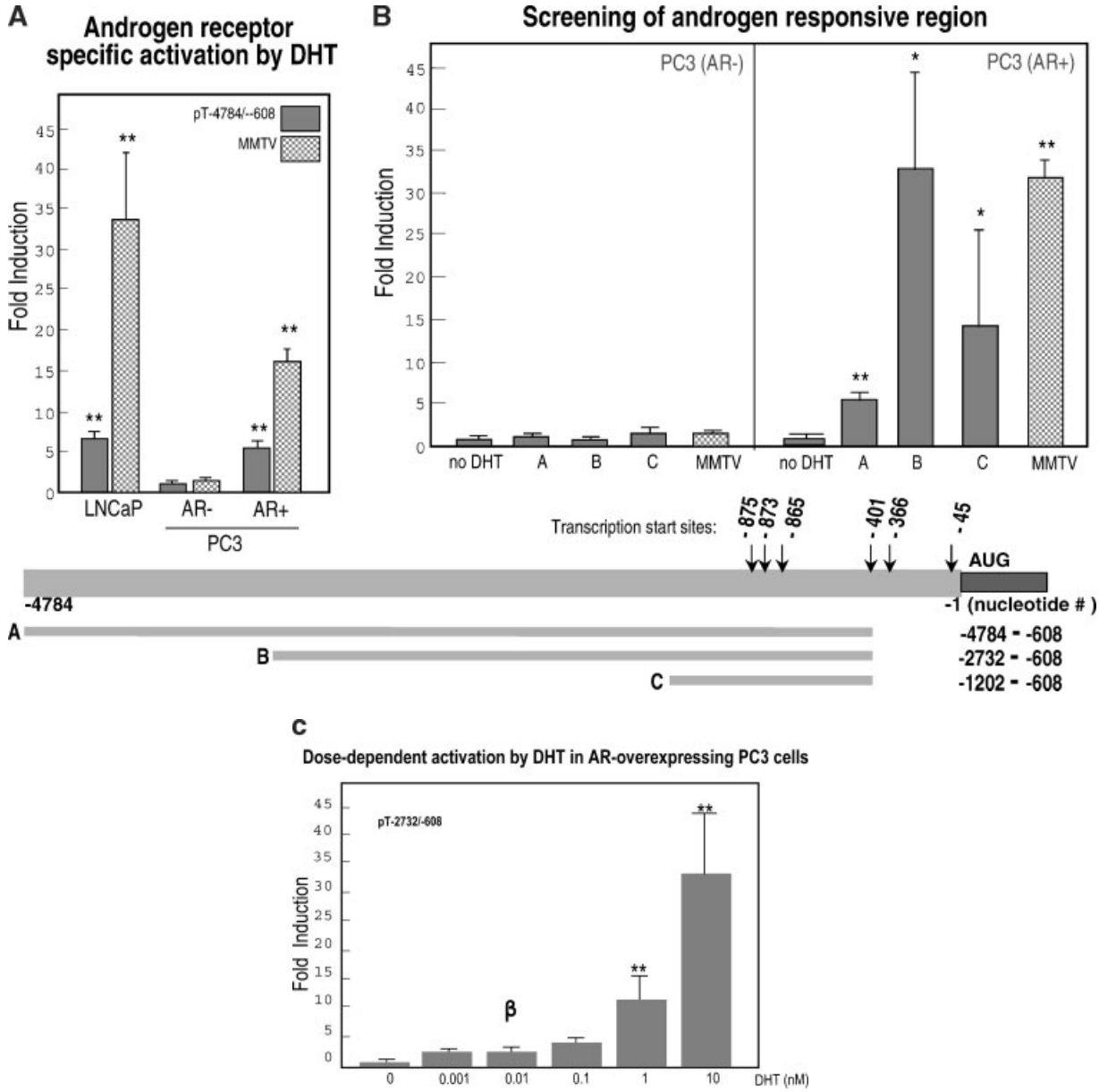


Fig. 3. Transcriptional activation of TGF-β1 promoter by DHT in human prostate cancer cells. LNCaP and PC3 cells were subjected to transfection with 2 μg of reporter plasmids and 0.5 μg of an internal control RLTK plasmid. When applied to PC3 cells, pCMV-hAR expression plasmid (1.5 μg) was cotransfected. After 12 h of incubation, the cells were washed with PBS and refreshed with RPMI 1640 media containing 10% charcoal-stripped FBS in the presence or absence of 10 nM DHT for 48 h. The cells were lysed, and a portion of the lysates was applied to dual luciferase assay system. pMMTV-Luc plasmid was used as the positive control for responsiveness to androgen. Fold induction of luciferase activity in DHT-treated against that in non-treated cells was obtained. Each bar represents mean ± SD. **, $P < 0.001$; *, $P<0.05$ versus non-treated cells analyzed by Student's *t*-test. **A**: AR-dependent activation of TGF-β1 promoter. The reporter construct containing the region from −4784 to −608 of TGF-β1 promoter (solid bar) and control pMMTV plasmid (hatched bar) was applied (fold inductions of TGF-β1 promoter and MMTV promoter in LNCaP were 6.99 ± 3.37 and 33.54 ± 11.85, 0.97 ± 0.38, and 1.47 ± 0.50 in PC3, 5.46 ± 2.91 and 18.02 ± 4.85 in PC3/AR+ cells, respectively). **B**: Screening of androgen responsive region of TGF-β1 promoter in the PC3 and PC3/AR+ cells. Upper panel*:* Mapping of androgen responsive region. Transcriptional activation of the reporter plasmids (pT−4784/−608, pT−2732/−608, and pT−1202/−608) by DHT were examined in the absence (AR−) or presence (AR+) of over-expressed AR by cotransfection. The values observed were pT−4784/−608; 0.97 ± 0.39 (−AR) and 5.46 ± 2.92 (+AR), pT−2732/−608; 0.46 ± 0.02 (−AR) and 34.49 ± 13.42 (+AR)], and pT−1202/−608; 1.47 ± 0.50 (AR−) and 16.10 ± 3.88(AR+). Lower panel: the promoter regions used. **C**: DHT-dependent activation of TGF-β1 promoter activity in PC3/AR+ cells. pT−2732/−608 reporter plasmid and 0–10 nM DHT in ethanol were employed as indicated. The fold inductions were 2.54 ± 0.83 (0.001 nM), 2.71 ± 1.27 (0.01 nM), 4.35 ± 1.02 (0.1 nM), 11.58 ± 3.66 (1 nM), and 33.76 + 7.94 (10 nM).

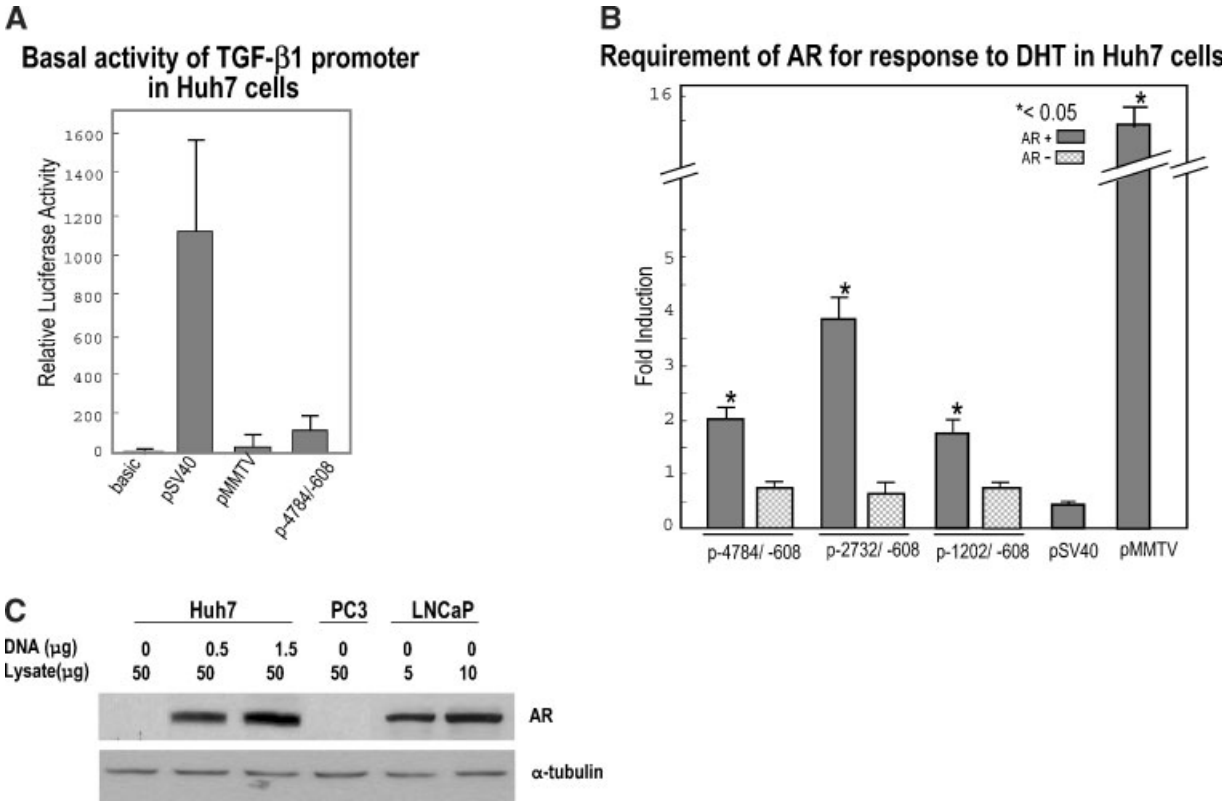


Fig. 4. Enhancement of TGF-β1 promoter activity by DHT in Huh7/AR+ cells. Huh7 cells were subjected to transfection with 2 μg of reporter plasmids and 0.5 μg of an internal control RLTK plasmid in the absence or presence (1.5 μg) of pCMV-hAR expression plasmid. After 12 h of incubation, the cells were washed with PBS and refreshed with DMEM media containing 10% cFBS in the presence or absence of 10 nM DHT for 48 h. The cells were then lysed, and a portion of the lysates was applied to dual luciferase assay. pMMTV-Luc plasmid was used as positive control for responsiveness to androgen. Fold induction of luciferase activity in DHT-treated versus that in non-treated cells was obtained. Each bar represents mean ± SD. *<0.05 versus non-treated cells analyzed by Student's *t*-test. **A**: Basal activity of TGF-β1 promoter. Basic (promoterless), pSV40, pMMTV, and pT−4784/−608 reporter plasmids were tested for their basal activities in Huh7 cells with DMEM media containing 10% cFBS. The luciferase activities were compared to that of basic plasmid, and the values were 1,224 ± 356 (pSV40), 13 ± 5 (pMMTV), 67 ± 13 (pT−4784/−608). **B**: Requirement of AR for response to DHT in Huh7 cells. Activation of TGF-β1 promoter by DHT in Huh7 cells was measured in the absence (AR−) or presence (AR+) of androgen receptor. The values were pT−4784/−608, 2.14 ± 0.69 (AR+), and 0.73 ± 0.24 (AR−); pT−2732/−608, 3.75 ± 0.42 (AR+), and 0.67 ± 0.32 (AR−); pT−1202/−608, 1.65 ± 0.41 (AR+), and 0.88 ± 0.34 (AR−); pSV40, 0.64 ± 0.04 (AR+), pMMTV, 15.08 + 2.26 (AR+). **C**: Comparison of AR level of AR overexpressing Huh7 cells to that of LNCaP cells. Western blot analyses revealing AR expression in the Huh7 cell lysates co-transfected with 0.5 or 1.5 μg of pCMV-hAR plasmid. Lysates of PC3 and LNCaP cells were used as negative or positive control for AR expression, respectively. The cell lysates loaded per lane were 50 μg in the Huh-cells and PC3 cells, as opposed to 5 and 10 μg per lane in the LNCaP cells.

Specific Binding of Androgen-AR Complex to the Potential ARE in TGF-β1 Gene Increases TGF-β1 Expression in Huh7-AR Cells and HCC in Nude Mice

To further elucidate in vivo activation of TGF-β1 transcription by AR, Huh7-AR_m cells were employed. When AR expression was measured by RT-PCR (Fig. 7A) and immunoblot (Fig. 7B) analyses, the vector transfected Huh7 cells (V_m) and its single clones (1, 2, 3, and 6) failed to express AR at both the mRNA and protein levels, however, the expression was variable in the AR expressing single clones (3, 4, 12, 20, and 21) and Huh7-AR mixture (AR_m). The discrepancy between the transcription and translational efficiencies shown in the single clones C4 and C20 can be explained by a mechanism, such as the insertional mutagenesis in the 5′ non-coding region of the gene and excessive secondary structure at the 5′ end of eukaryotic mRNA [Svensson and Akusjarvi, 1985; Simoes and Sarnow, 1991], the *cis*-acting elements on the 5′ and 3′ non-coding regions in eukaryotic cells

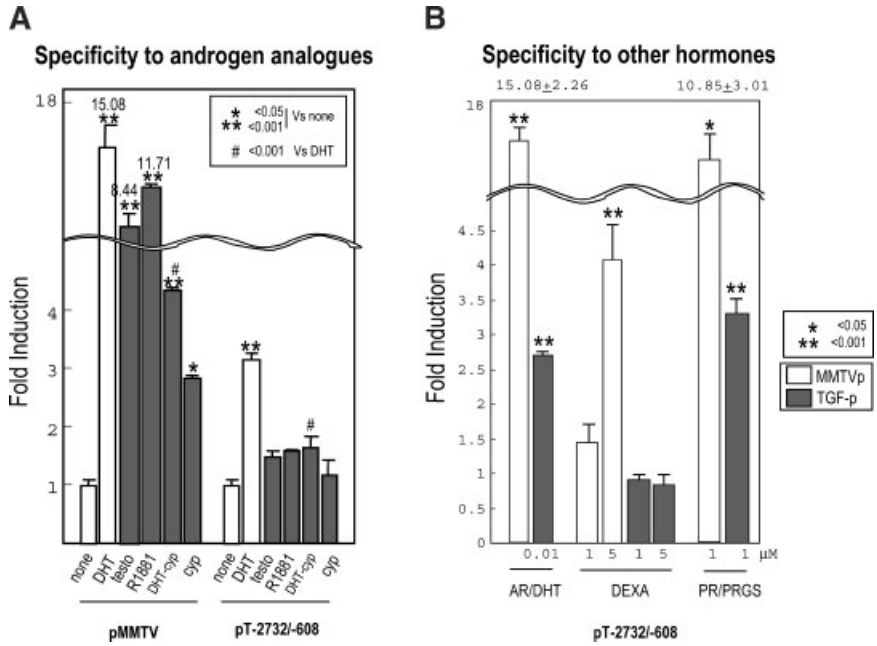


Fig. 5. Specificity of androgen-responsive region of TGF-β1 promoter in Huh7 cells. pT−2732/−608 reporter plasmid (2 μg), RLTK plasmid (0.5 μg), and pCMV-hAR expression plasmid (1.5 μg) were transfected for 12 h, and the cells were washed with PBS and refreshed with DMEM media containing 10% cFBS in the presence of androgen analogues or steroid hormones for 48 h. The cells were lysed, and a portion of the lysates was applied to dual luciferase assay system. pMMTV-Luc was used as positive control for responsiveness to hormones. Fold induction of luciferase activity versus that of non-treated cells was obtained. Each bar represents mean ± SD, ** (or #) $P < 0.001$; *$P < 0.05$ versus non-treated (or vs. DHT-treated) cells analyzed by one-way ANOVA test. **A**: Specificity test of DHT-induced activation of TGF-β1 promoter to androgen analogues. The results were pMMTV, 15.08 ± 2.26 (DHT, 10 nM), 8.44 ± 1.65 (testosterone) 11.71 ± 0.62 (R1881), 4.32 ± 0.52 (DHT + cyproterone), 2.82 ± 0.18 (cyproterone); pT−2732/−608, 3.2 ± 0.17 (DHT), 1.47 ± 0.05 (testosterone) 1.57 ± 0.13 (R1881), 1.62 ± 0.09 (DHT + cyproterone), 1.17 ± 0.11 (cyproterone). **B**: Specificity of the TGF-β1 promoter for steroid hormones. To test the responsiveness to progesterone, progesterone receptors (PR_A and PR_B) were overexpressed by cotransfection of pCMV-PRA and pCMV-PRB, respectively. The results were DHT treatment (10 nM) with AR over-expression, 15.08 + 2.26 (pMMTV), 2.75 + 0.07 (pT−2732/−608); dexamethasone treatment, 1.45 + 0.33 (1 μM DEXA with pMMTV), 4.09 + 0.64 (5 μM DEXA with pMMTV), 0.92 + 0.03 (1 μM DEXA with pT−2732/−608), 0.83 + 0.12 (5 μM DEXA with pT−2732/−608); progesterone treatment (1 μM), 10.58 + 3.00 (pMMTV), 3.30 + 0.30 (pT−2732/−608).

[Parkin et al., 1988; Grens and Scheffler, 1990], the incorrect RNA splicing due to mutation of intronic sequences after DNA transfection [Kang et al., 1998] and silencing of IRES [Creancier et al., 2001]. On the other hand, 5′ cap and 3′ polyA tail enhance translation initiation [Wiklund et al., 2001]. When TGF-β1 expression was measured by RT-PCR, it was much higher in the Huh7-AR_m cells than that in the Huh7-V_m cells (Fig. 7C). Similar finding was also obtained in HCC developed in nude mice; all HCC tissues developed from the Huh7-AR_m cells (lanes 5–8, Fig. 7D) expressed higher level of TGF-β1 than the HCC of Huh7-V_m cells (lanes 1–4, Fig. 7D), however, there was no statistical significance between the two groups. That might be due to the endogenous and heterogeneous expressions of TGF-β1 in response to various stimulations, as well as different concentrations of androgen in mice. To confirm the activity of ARE in rat TGF-β1 promoter, ChIP assay was performed with anti-AR antibody in Huh7-AR_m cells after transfection with pT−2732/−608. As shown in Figure 7E, binding of the AR to the potential ARE in rat TGF-β1 promoter significantly increased in 4 h of 10 nM DHT treatment, whereas endogenous binding without DHT treatment was much less even in the presence of anti-AR antibody (compare lanes 1 and 2, Fig. 7E). AR-ARE complex was maintained until 12 h, and the anti-AR antibody was specific (compare lanes 3 and 4, Fig. 7E). Persistent amplification of the ARE irrelevant promoter region verified equal amount of DNA templates added to the reaction mixtures. These data strongly support the possibility that androgen can directly induce TGF-β1 expression through the action of androgen-AR complex in AR-overexpressed hepatoma cells both in vitro and in vivo.

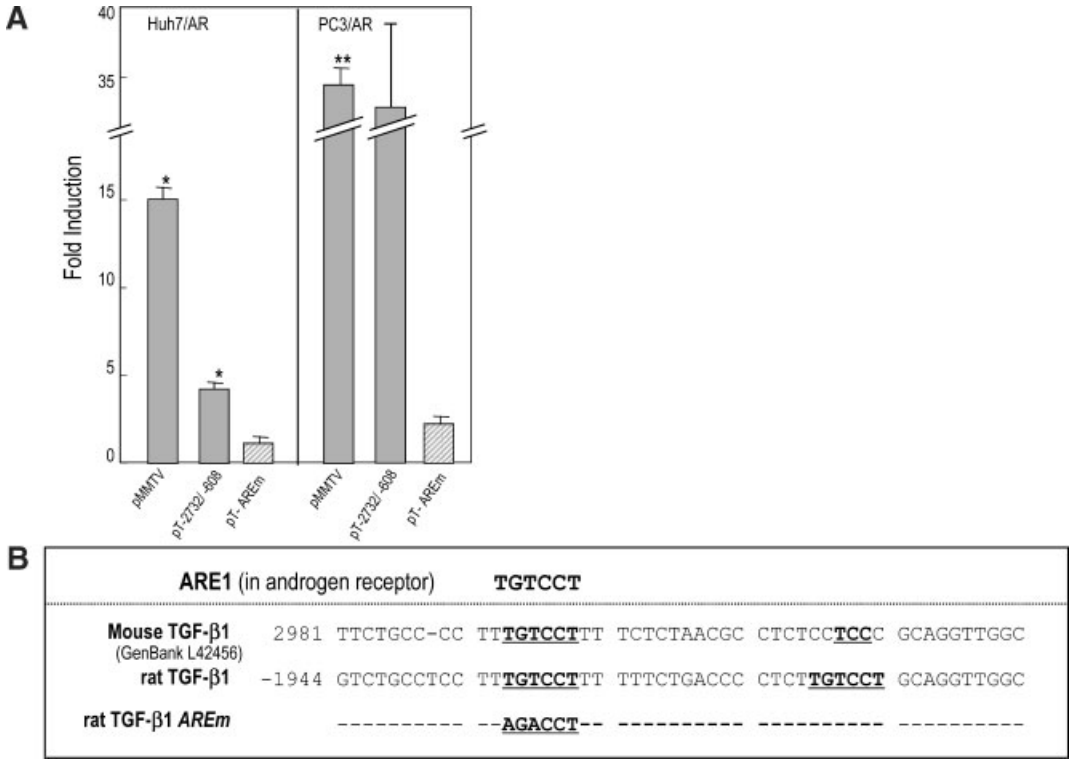


Fig. 6. Identification of putative ARE within TGF-β1 promoter. **A**: Promoter activity of pT−2732/−608 and its mutant *AREm* was measured in Huh7/AR+ and PC3/AR+ cells with 10 nM DHT. Activity of the *AREm* was significantly reduced, compared with that of the pT−2732/−608 both in the Huh7/AR+ and PC3/AR+ cells. **B**: Comparison of putative ARE sequence of TGF-β1 promoter in mouse and rat. To analyze activity of the conserved putative ARE sequence located at −1932/−1927 (5′-TGTCCT-3′), mutated sequence (5′-AGACCT-3′) was generated within the reporter plasmid, pT−2732/−608, as described in Materials and Methods.

DISCUSSION

We have earlier reported that TGF-β1 expression was transiently increased in the hyperplastic nodule induced by DEN and nodularin with concomitant loss of its receptor expression [Lim et al., 1999], indicating a tricky maneuver of proliferating hepatocytes to escape from TGF-β1 induced apoptosis. At the same time, serum testosterone level becomes significantly reduced by treatment of male rats with DEN or nodularin due to the damage of Leydig cells [Park et al., 2002], which strongly suggest a possibility of decreased TGF-β1 transcription, therefore, which might help abnormal liver cells escape from apoptosis during the early stage of carcinogenesis. In the present study, we demonstrated that androgen regulated transcription of TGF-β1 through direct binding of DHT-AR complex to the potential ARE within TGF-β1 promoter region (Fig. 4). We also isolated rat TGF-β1 promoter region up to −4784, including translation start site (Fig. 1), and mapped ARE to the sequence of 5′-TGTCCT-3′ at −1932/−1927 of rat TGF-β1 promoter DNA by mutation analysis (Fig. 6) and ChIP assay (Fig. 7E). These findings were confirmed in vitro and in vivo with the Huh7-AR_m cells after transfection with rat TGF-β1 promoter (pT−2732/−608) and the HCC tissues developed from Huh7-AR_m cells in nude mice (Fig. 7C and D).

During carcinogenesis in liver induced by either chemical carcinogens or by viruses, the consistent finding associated with HCC development is almost always cirrhotic changes: Liver cirrhosis is the final output of repeated damage and repair, that is, hepatocyte death and regeneration during the promotion stage of carcinogenesis. It has been reported that TGF-β induces cell death in hepatocytes [Fausto, 1993; Benedetti et al., 1995]. On the other hand, TGF-β can activate survival signals in fetal rat hepatocytes [Valdés et al., 2004] as well as various cancer cells [Roberts and Wakefield, 2003], indicating two faces of TGF-β1 and DHT interaction during the carcinogenic process in liver.

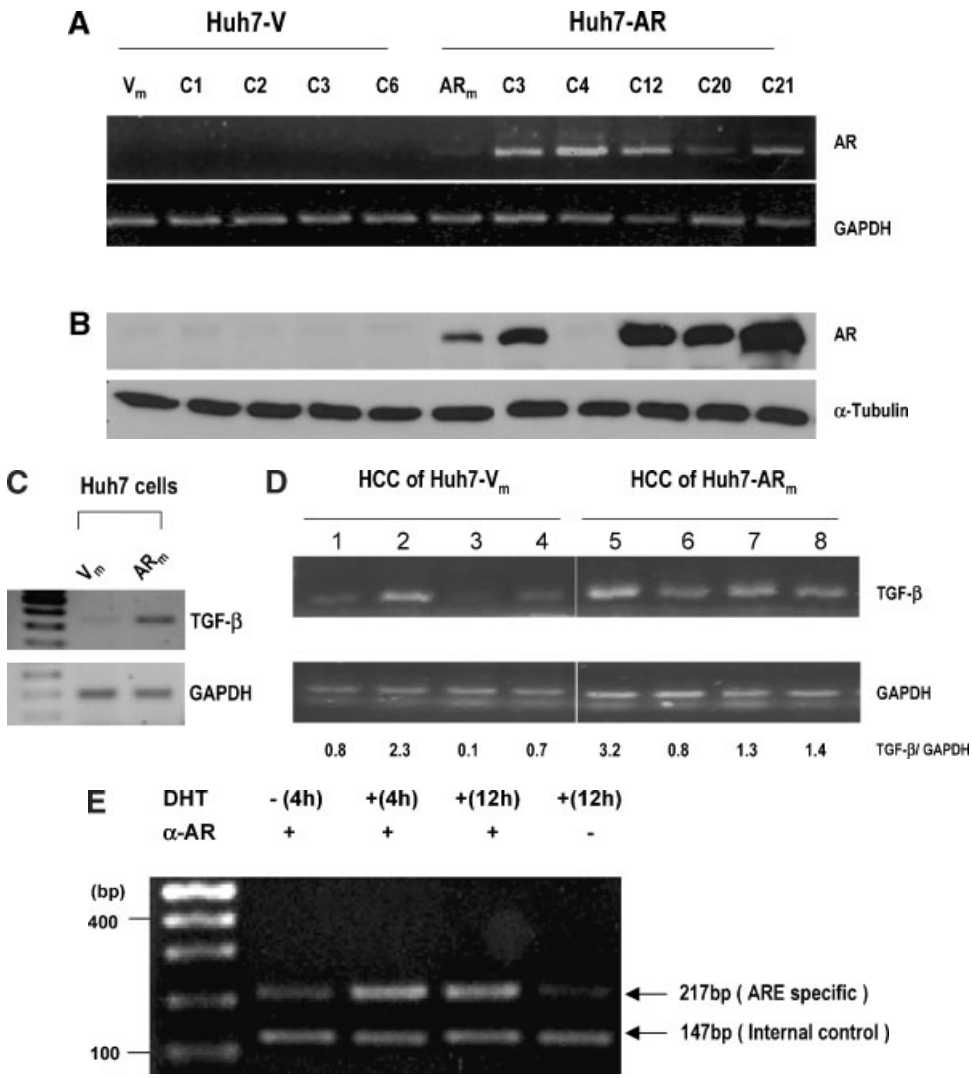


Fig. 7. TGF-β1 expression in vivo and in vitro is specifically regulated by DHT, when AR is overexpressed. Huh7-V and Huh7-AR cells were established by stable transfection of Huh7 cells with vector alone and pCMV-hAR plasmid, respectively. Numbers indicate single clone cells, whereas V_m and AR_m indicate the pooled cells which are resistant to G418 treatment. Overexpression of AR in the transfected cells was examined by RT-PCR (**A**) and immunoblot analyses (**B**); Huh7-AR single clones revealed much stronger expression of AR than Huh7-AR_m cells and Huh7-V cells. **C**: RT-PCR analysis showing increased expression of the TGF-β1 mRNA in the Huh7-AR_m cells than the Huh7-V_m. **D**: Increased TGF-β1 expression was confirmed by RT-PCR analyses in the HCC developed by Huh7-AR_m cells in nude mice. Each lane represents individual animal variations observed in 10–11 mice per each group. Numbers of the bottom panel indicate the fold induction of TGF- β1, compared with the expression of GAPDH. **E**: ChIP assay; DHT induced direct binding of AR to potential ARE in the rat TGF-β1 promoter. Huh7-AR cells were transfected with pT−2732/−608 promoter DNA for 5 h, and the cells were then incubated for 20 h with 10% FBS before culture with 10% cFBS in DMEM for 22 h and stimulated with 10 nM DHT for 12 h. When the chromatins prepared from the DHT treated cells were incubated with anti-AR antibody, AR-ARE binding was found to be significantly increased by DHT treatment (lane 2), compared to its control (lane 1). The AR-potential ARE binding was specific, when anti-AR antibody was added to the reaction mixture (compare lanes 3 and 4), and their binding was persistent for 12 h after DHT treatment.

Although the increased expression of both AR and TGF-β1 has often been observed during the development of HCC, no clear correlation and/or mechanism involved have been proposed, yet. Therefore, in the present study, we investigated whether androgen could directly regulate TGF-β1 expression by transcriptional activation through androgen-AR complex. Comparison of the cloned promoter sequence showed strong homology with those of human and mouse genes, implying possibly similar transcriptional regulation involved. These sequences included USF, NF-κB, E2F, SP1, and p300 sites, but not TATA box (Fig. 2).

Several *cis*-acting sequences responsible for androgenic regulation have been identified in various AR target genes [Denison et al., 1989; Adler et al., 1991; Rennie et al., 1993; Lu et al., 1999]. Typically, the palindromic hormone responsive element-like consensus sequence (5′-AGAACAnnnTGTTCT-3′) as ARE is involved in androgen regulation, however, non-HRE-like sequence has rarely been reported [Le and Burnstein, 1996]. This sequence was initially termed as glucocorticoid response element, later as HRE to indicate recognition by multiple steroid receptors [Tan et al., 1992]. Recently, a novel ARE-1 (5′-TGTCCT-3′) and ARE-2 (5′-AGTACTCC-3′) have been shown in the hormone-binding domain of AR, and ARE-1 was found to be necessary, but not sufficient for maximum induction of AR, indicating the ARE-2 as a synergistic element [Le and Burnstein, 1996]. In the present study, there was no proper palindromic HRE-like consensus sequence in our cloned promoter region, however, two half-site sequences (5′-TGTTCT-3′) were found far-upstream of the promoter (−4563/−4548 and −3130/−3117). Nevertheless, the promoter region containing −4784 to −608 showed its activation in response to DHT in LNCaP cells, suggesting possible direct transcriptional regulation by androgen (Fig. 3A). As confirmed in PC3 cells, this response was dependent on DHT concentration and AR expression (Fig. 3C). Activation of the promoter was not only due to ligand binding to AR, but also autologous upregulation of AR expression by DHT, in agreement with the previous report [Mora and Mahesh, 1999]. These data suggest that transcription of TGF-β1 can be upregulated by DHT through the action of AR, and that the upregulation is partly due to the auto-activation of AR expression by DHT.

Next, we tested whether TGF-β1 expression could be regulated by DHT even in liver cells. TGF-β1 promoter activity was about 3.75-fold enhanced by DHT, when AR was over-expressed. Therefore, we screened the possible androgen responsive region with randomly deleted genes with luciferase reporter. The promoter region, spanning from −2732 to −1203, displayed the highest response to DHT (Fig. 4). Among various androgenic analogues tested, DHT was the most active ligand for transcriptional activation by AR and the activity was inhibited by cyproterone, an anti-androgen, implying that any androgen could upregulate TGF-β1 expression in AR expressing hepatocytes (Fig. 5). Further detailed sequence analysis of the region showed a putative ARE sequences, 5′-TGTCCT-3′ from −1932 to −1927, which was also conserved in mouse promoter DNAs. By mutating the sequence to 5′-AGACCT-3′, we confirmed that the sequence was one of the ARE within the TGF-β1 promoter (Fig. 6). The ARE mutant pT−2732/−608 promoter revealed less than fivefold induction, not ∼15-fold, in response to DHT treatment (Fig. 6A), in spite of presence of the intact −1202/−608 sequences in it (Fig. 3B). The finding indicates that the potential ARE site at the −1932/−1927 may be critical for upregulation of pT−2732/−608 activity, and suggests the possible presence of a repressive element between −2732 and −1202 in rat TGF-β1 promoter. Not only rat and mice, the complete sets of (-TGTCCT-) sequence are conserved in the 5′ end of human *TGF-β1* gene isolated from a human leukocyte genomic DNA library, and they are located at −685/−680 and −503/−498 (GenBank J04431) [Kim et al., 1989] downstream of the human promoter DNA (−1121/−822) shown in Figure 2A with alignment of mouse and rat sequences. From these result, we became more convinced that the expression of TGF-β1 in liver can be induced by androgen, whenever the cellular AR level becomes high for any reason. This contention could be proved in vivo and in vitro in the HCC developed in nude mice (Fig. 7); TGF-β1 expression was significantly higher in the Huh7-AR_m cells and its tumor tissues as compared with those of the Huh7-V_m. In conclusion, these data demonstrate that androgen might regulate hepatocarcinogenesis by increasing transcription of TGF-β1 through direct interactions with AR and ARE in the *TGF-β1* gene, even though abnormal hepatocytes escape from TGF-β1 induced cell death and growth arrest through the concomitant loss of TGF-β receptor expression [Lim et al., 1999] and the reduced testosterone biosynthesis in liver [Park et al., 2002].

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