

Identification of 3',5'-Cyclic Adenosine Monophosphate Response Element and Other *Cis*-Acting Elements in the Human Androgen Receptor Gene Promoter

Atsushi Mizokami*, Shu-Yuan Yeh*, and Chawnsiang Chang

Department of Human Oncology, Comprehensive Cancer Center, and Endocrinology-Reproductive Physiology Program University of Wisconsin Madison Wisconsin 53792

Androgen and androgen receptor (AR) play an important role in sexual differentiation and prostate proliferation. To investigate AR gene transcriptional regulation, a 2.3-kilobase AR gene promoter region was isolated, sequenced, and characterized. Chloramphenicol acetyltransferase (CAT) assay and sequence homology search of AR gene promoter among human, rat, and mouse revealed some potential *cis*-acting elements, including a GC box, a suppressor region, and a purine-rich element. Deletion analysis and gel retardation assay using a 50-base pair (bp) double-strand purine-rich element showed that this purine-rich element can bind to specific proteins in nuclear extract of LNCaP and HeLa cells and may be essential for AR gene transcription. Furthermore, to investigate the effect of cAMP on AR gene transcription, we treated LNCaP and HeLa cells with 10 mM (Bu)₂cAMP after transfection with CAT gene reporter plasmids linked to the AR gene promoter. This treatment induced several folds of CAT activity in LNCaP cells only, and the induction was further confirmed at AR mRNA level by Northern blot analysis and reverse transcription-polymerase chain reaction assay. Deletion analysis of the AR gene promoter showed that a region between 530 bp and 380 bp upstream of AR gene transcription initiation site, which includes one potential cAMP response element (CRE), is responsible for cAMP induction. Gel retardation analysis using this CRE (AR/CRE1) showed that AR/CRE1 can bind to specific proteins in nuclear extract of LNCaP cells, which appears to form a different binding complex compared to somatostatin/CRE. (Molecular Endocrinology 8: 77-88, 1994)

INTRODUCTION

Androgens play an important role in male sexual differentiation and development via an androgen receptor

(AR) (1). Structural analysis of AR cDNA indicated AR is a member of the steroid receptor superfamily which includes thyroid hormone receptor, vitamin D receptor, and retinoid receptors (2, 3). Recently, it has been demonstrated that AR may regulate androgen target genes by binding to a DNA consensus sequence androgen response element (ARE; 5'-GG[A/T]ACAN₃TGTTCT) which is similar to glucocorticoid response element (GRE) (3, 4). So far, AREs have been identified in mouse mammary tumor virus long terminal repeat (MMTV-LTR) (5, 6), tyrosine aminotransferase gene promoter (7), C3 first intron (8, 9), and prostatic-specific antigen gene promoter (10, 11).

Several hormones and growth factors can modulate AR gene expression. For example: FSH can stimulate the AR mRNA level in Sertoli cells (12), GH and PRL can increase the AR mRNA level in prostatic cells (13), and epidermal growth factor may decrease the AR mRNA level (14). To further understand the regulation of the AR gene at the transcriptional level, the promoter region of the human AR gene (hAR) was isolated and sequenced. The results showed the transcription initiation site was located at both 1127 base pairs (bp) (AR-TIS I), and 1116 bp (AR-TIS II) upstream of AR translation initiation site (15, 16). The promoter region of hAR does not have TATA box or CCAAT box but contains a GC box which may play a role on transcript started from AR-TIS II (16) and match the previous reports that, by binding to the SP-1, the GC box may play an important function for the gene transcription. However, it remains unclear if other important *cis*-acting elements in the promoter region of the AR gene may also be involved in the AR gene transcriptional regulation.

The regulation of gene expression by intracellular protein phosphorylation has been a key phenomenon in the physiology of multicellular organisms. Many genes appear to be mediated through protein phosphorylation, which includes unmasking of a protein kinase activity of the receptor molecule itself or activa-

tion of a ubiquitous protein kinase system of the cell. Two of the major protein kinase systems in control of gene transcription are the pathways leading to the activation cAMP-dependent protein kinase A (18) and diacylglycerol (DAG)-dependent protein kinase C (19). The nuclear effects could then be mediated by phosphorylation or dephosphorylation of *trans*-acting proteins (20). It will be very interesting to know if AR, a nuclear steroid receptor, is one of such candidates.

In the present study, we isolated and sequenced the hAR gene promoter region up to 2.3 kilobases (kb) upstream of the AR transcription initiation site. Using deletion analysis and chloramphenicol acetyltransferase (CAT) assay, we were able to locate several potential *cis*-acting regions important for AR gene transcriptional regulation. We found that a 50-bp purine-rich region at 70 bp upstream of the AR transcription initiation site may be the most important *cis*-acting element for AR gene transcription. In addition, a functional cAMP response element (AR/CRE1), which may be responsible for cAMP induction of AR gene expression, was identified within the hAR gene promoter region. The sequence and character of this AR/CRE1 was different from published somatostatin CRE (21).

RESULTS

Cloning and Sequencing the Promoter Region of the hAR Gene

Three positive λEMBL3 plaques were isolated by screening a λEMBL3 human genomic library with a ³²P-labeled 640-bp *Eco*RI-*Sma*I fragment of AR 5'-untranslated region (2) as a probe. One of the positive clones (ARg3) was found to contain approximately a 13-kb insert which includes 3.6 kb upstream of the published AR transcription initiation site by restriction enzyme mapping (Fig. 1). A 2.9-kb 5'-flanking fragment from *Hind*III at the position -2.3 kb to *Pst*I at the position +0.57 kb was subcloned into pBluescript sk(-) and sequenced by the dideoxy chain termination method (Fig. 2).

We then searched for the known potential *cis*-acting elements from the translation initiation site to 2.3 kb upstream of the transcription initiation site in the AR gene (Fig. 2 and Table 1). The region from -2327 to +1 contained neither putative TATA box (22) nor CCAAT box (23) but contained a GC box (17) that is known as a putative Sp1 binding site (5'-GGCGGG-3') at -45 to -40, an inverted sequence of NF-1 binding motif (5'-TGGN₇CCA-3') (24) at -317 to -313, and a purine-rich region at -118 to -75 (four tandem repeats of GGGGA). The promoter region of the AR gene also contained five potential AP-1 binding motifs (5'-TGAGTCA-3') (25, 26) with a 1-bp mismatch at -2319 to -2313, -2307 to -2300, -1955 to -1949, -1382 to -1376, and -1233 to -1227, and six potential CREs (5'-TGACGTCA-3') (6) with a 2-bp mismatch at -2056 to -2049, -1895 to -1888, -1807 to -1800, -1060

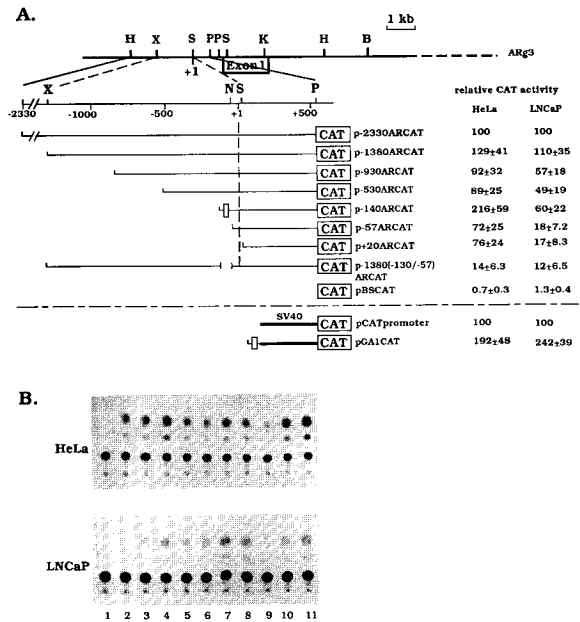


Fig. 1. Structure of the hAR Gene and CAT Activity of Deletion Mutant of the AR Gene Promoter

A, Scheme of AR gene structure and CAT reporter plasmids linked to the AR gene promoter. Positions of *Hind*III (H), *Xba*I (X), *Sma*I (S), *Pst*I (P), *Kpn*I (K), and *Bam*H (B) sites in ARg3 are indicated. The open box in the deletion plasmid represents the purine-rich element. CAT activity in HeLa and LNCaP cells transfected with various CAT reporter plasmids was assayed as described in *Materials and Methods* and normalized by β-galactosidase activity. Relative CAT activity of all deletion plasmids was calculated with CAT activity of each of HeLa and LNCaP transfected with p-2330ARCAT as 100%. Relative CAT activity of pGA1CAT was calculated with CAT activity of the parent CAT vector, pCAT-promoter, as 100%. The data show the mean ± SD of at least four experiments. B, CAT assay data of deletion mutants of the AR gene promoter. LNCaP and HeLa cells were transfected with 8 μg CAT reporter plasmid. Lane 1, pBSCAT; lane 2, p+20ARCAT; lane 3, p-57ARCAT; lane 4, p-140ARCAT; lane 5, p-530ARCAT; lane 6, p-930ARCAT; lane 7, p-1380ARCAT; lane 8, p-2330ARCAT; lane 9, p-1380(-130/-57)ARCAT; lane 10, pCAT-promoter; and lane 11, pGA1CAT.

to -1053, -1016 to -1009, and -508 to -501. Two potential retinoic acid response elements (RAREs; repeat of 5'-PuG[G/T]TCA-3') (27, 28) were located at -2147 to -2126 and -1295 to -1281. Three potential complete interleukin-6 (IL-6) response elements (29) were located at -1704 to -1695, -1405 to -1397, and -785 to -777. One potential 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) response element (30) was located at +967 to +973. We also compared hAR gene promoter region with published rat and mouse AR genes up to approximately 500 bp upstream of the AR transcription initiation site (31, 32, 15). We found that the region from -120 to +1, including a GC box and a purine-rich region, was the most conserved among human, rat, and mouse AR gene promoter regions. The highly conserved sequence in this GC box and purine-

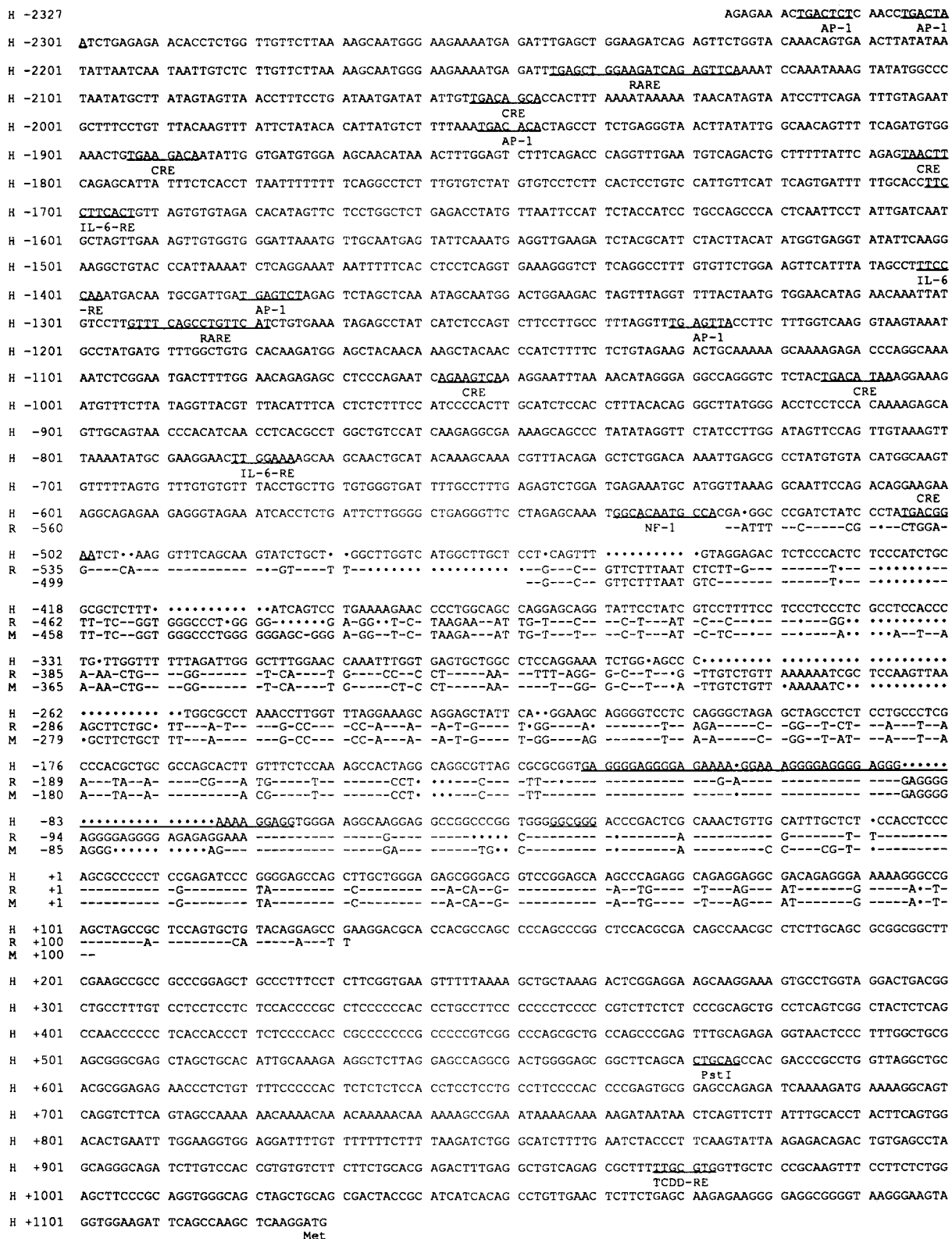


Fig. 2. Nucleotide Sequence of hAR Gene Promoter Region and Comparison with the Partial Rat and Mouse AR Gene Promoter Region
 The sequence from *Hind*III site at 2.3 kb upstream of the transcription initiation site to translation initiation site of the hAR gene (H) is shown. Nucleotides are numbered relative to the transcription initiation site (designated +1) which was published by Tilley *et al.* (15). Potential *cis*-acting elements are *underlined*. The hAR gene promoter region was compared with the published rat (R) and mouse (M) AR gene promoter regions.

Table 1. Potential *cis*-Acting Elements in AR 5'-Flanking Region

<i>cis</i> -Acting element	Potential sequence and position	Consensus sequence
GC box	5'-GGCGGG-3' (-45/-40)	5'-GGCGGG-3'
NF-1	5'-TGGCACAATGCCA-3' (-541/-529)	5'-TGGN ₇ CCA-3'
Purine-rich region	5'-AGGGGAGGGGAGAAAAGGAAAGGGGAG- GGGAGGGAAAAGGAGG-3' (-118/-75)	5'-GGGGA-3' repeat
AP-1	5'-TGA CT CT-3' (-2319/-2312) 5'-TGA CT AA-3' (-2306/-2301) 5'-TGA CT ACA-3' (-1955/-1948) 5'-TGA CT TCT-3' (-1382/-1376) 5'-TGA CT TTA-3' (-1233/-1227)	5'-TGAGTCA-3'
CRE	5'-TGACAGCA-3' (-2056/-2049) 5'-TGAAGACA-3' (-1895/-1888) 5'-AGAAGTCA-3' (-1060/-1053) 5'-TGACATAA-3' (-1016/-1009) 5'-TGACGGAA-3' (-508/-500)	5'-TGACGTCA-3'
RARE	5'-TGAGCTGGAAGATCAGAGTTCA-3' (-2147/-2126) 5'-GTTTCAGCCTGTTC-3' (-1295/-1281)	5'-PuG[G/T]TCA-3' repeat
IL-6-RE	5'-TTCCCAA-3' (-1405/-1399) 5'-TTGAAA-3' (-783/-777)	5'-PyTGGPuAA-3'
TCDD-RE	5'-TTCCTCACT-3' (-1704/-1695) 5'-TTGCGTG-3' (+967/+973)	5'-AGTGANGNAA-3' 5'-T[A/T]GCGTG-3'

rich region suggests that this region may contain very important *cis*-acting elements required for AR gene transcription (Fig. 2).

Identification of Several *cis*-Acting Elements in the hAR Gene Promoter Region

To see whether a 2.3-kb AR gene promoter region has enough promoter activity, a CAT expression plasmid containing the 2.9-kb fragment from *Hind*III to *Pst*I (-2330 to +573 of the published AR gene transcription initiation site) was constructed (Fig. 1, p-2330ARCAT) by ligating to a parent reporter plasmid, pBSCAT, which could not induce CAT activity in LNCaP prostate cancer cells and HeLa cells (Fig. 1B, lane 1). When p-2330ARCAT was transfected into LNCaP and HeLa cells, the strong CAT activity was observed (Fig. 1B, lane 8). To investigate which regions contain important *cis*-acting elements that are related to AR gene transcription, we constructed CAT expression plasmids containing a progressive 5' to 3' deletion mutant of AR gene promoter (Fig. 1, p-2330ARCAT, p-1380ARCAT, p-930ARCAT, p-530ARCAT, p-140ARCAT, p-57ARCAT, and p+20ARCAT). Only p+20ARCAT did not contain the AR transcription initiation site. These plasmids were transfected into LNCaP and HeLa cells. Although almost the same CAT activity was observed by transfection with either p-2330 ARCAT or p-1380ARCAT (Fig. 1, A and B, lanes 7 and 8), transfection into LNCaP and HeLa cells with p-930ARCAT resulted in 2-fold and 1.4-fold decreases in CAT activity, respectively, as compared with p-1380ARCAT (Fig. 1B, lane 6). These results indicate the existence of *cis*-acting elements between -1380 to -930 that may be important for AR gene transcription. As compared with p-530ARCAT, transfection into HeLa and LNCaP cells

with p-140ARCAT resulted in a 2.4-fold and a 1.3-fold increase in CAT activity, respectively. In HeLa cells the strongest induced CAT activity was found by transfection with p-140ARCAT (a 2-fold increase when compared with p-2330ARCAT). These results indicate the existence of a negative *cis*-acting element(s) between -530 to -140 that represses AR gene transcription and also suggest that HeLa cells may contain some specific proteins that are interacted with the element(s) more than LNCaP cells. When p-57ARCAT was transfected into LNCaP and HeLa cells, a 3-fold decrease in CAT activity was observed when compared with p-140ARCAT. This region (from -140 to -57) contains four tandem repeats of the GGGGA sequence. Interestingly, we also found that there was no significant difference in CAT activity between p-57ARCAT and p+20ARCAT, a plasmid without the transcription initiation site of the AR gene. These results suggest that the region located from -140 to -57, which may play some important roles in AR gene transcription, the GC box from -45 to -40 in the AR gene promoter region may not play a significant role in AR gene transcription.

The GGGGA Purine-Rich Region May Be an Essential *cis*-Acting Element for AR Gene Transcription

Since the biggest difference in CAT activity between progressive 5' to 3' deletions of the AR gene promoter was observed in the purine-rich region between -140 and -57, we started to focus on this region and constructed an internal deletion between -130 to -57 [Fig. 1A, p-1380(-130/-57)ARCAT]. This internal deletion resulted in a decrease in CAT activity to the same level as p-57ARCAT in LNCaP cells (Fig. 1, A and B). Interestingly, in HeLa cells p-1380(-130/-57)ARCAT re-

sulted in the decrease in CAT activity to five times lower than p-57ARCAT, although both plasmids included -57 to +570 of the AR gene promoter region. (Fig. 1, lanes 3, 7, and 9). A negative *cis*-acting region (between -1380 and -140, especially between -530 and -140) of the AR gene promoter region may affect the CAT activity of p-57ARCAT due to deletion of a purine-rich region. The reason for this finding, however, remains unclear. These observations indicate that the purine-rich region of the AR gene promoter may be essential for the transcriptional activity of AR gene.

To examine if the purine-rich region can also enhance other promoter activity, the region between -140 to -57 was ligated to the upstream of the SV40 promoter in the parent pCAT promoter plasmid (Fig. 1, pGA1CAT) and transfected into HeLa and LNCaP cells. The CAT activity of pGA1CAT was increased 2-fold higher than that of the parent vector (Fig. 1, A and B, lanes 10 and 11). We also constructed a plasmid (pGA3CAT) in which three tandem repeats of the purine-rich region were ligated to upstream of the SV40 promoter and found the CAT activity was the same as pGA1CAT (data not shown).

To investigate whether in the LNCaP and HeLa cells some proteins may exist which can interact directly with the purine-rich element, we synthesized a 50-bp double-strand oligonucleotide of purine-rich element and carried out a gel retardation assay using an end-labeled oligonucleotide as a probe with LNCaP and HeLa cell nuclear extract. When this probe (0.25 ng/lane) was reacted with both cells' nuclear extracts, two major shift bands were detected (Fig. 3, lanes 2 and 8). These

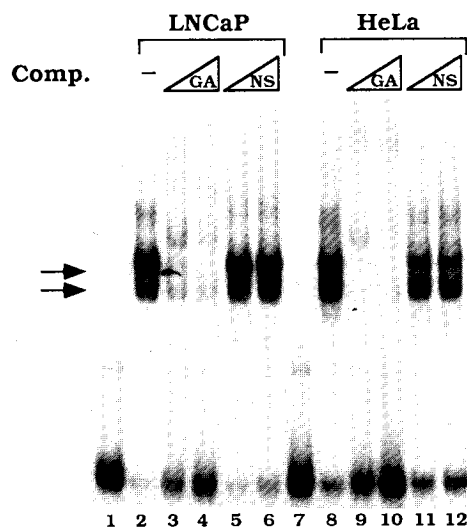


Fig. 3. Gel Retardation Assay with the Purine-Rich Element
Gel retardation assays were performed by using ^{32}P -labeled 50-bp double-strand oligonucleotide (0.25 ng/lane) including the purine-rich element and LNCaP and HeLa nuclear extract. As a specific competitor (GA) and nonspecific competitor (NS), 50-fold or 200-fold unlabeled probe and 100-fold or 400-fold pBluescript/*Hpa*I fragments were used, respectively. Lanes 1 and 7 represent the absence of nuclear extract. Arrows represent retardation bands.

two bindings were abolished by 50 times or 200 times unlabeled oligonucleotide probe as a specific competitor, but not by 400 times nonspecific DNA (Fig. 3). These results indicate that nuclei of LNCaP and HeLa cells contain some protein(s) that can specifically bind to this purine-rich element and play some important roles in AR gene transcription.

cAMP Induction of hAR Gene Expression

To examine the molecular basis of cAMP regulation of hAR gene, we transfected LNCaP cells with p-2330ARCAT. In the presence of 5–10 mM cAMP analog $(\text{Bu})_2\text{cAMP}$ for 20 h, we observed a 4- to 6-fold increase in CAT activity (Fig. 4A, lanes 4 and 5). Cholera toxin (data not shown) and forskolin, which can stimulate adenylate cyclase, also greatly stimulated CAT activity, but no induction could be observed when we used 1,9-dideoxy forskolin, a nonfunctional analog of forskolin (Fig. 4A, lane 2).

cAMP induction of AR gene transcription was further confirmed at the AR mRNA level in LNCaP cells by the Northern blot analysis (Fig. 4B) and reverse transcription-polymerase chain reaction (RT-PCR) assay (data not shown). The LNCaP cells in Dulbecco's modified Eagle's medium (DMEM)/F-12 containing 5% charcoal-stripped fetal calf serum (CCS) was cultured for 20 h in the presence of 10 mM $(\text{Bu})_2\text{cAMP}$. Total RNA was then isolated for the quantification of the AR mRNA level by Northern blot analysis (Fig. 4B) and RT-PCR (data not shown). The results all showed that cAMP can increase AR mRNA level up to the 2-fold but not to the 4- to 6-fold levels seen by induction of cAMP to the AR gene promoter-CAT gene expression. This discrepancy may be due to additional regulatory mechanisms, such as attenuation, and the stability of AR mRNA. Nevertheless, these data clearly indicated that AR gene expression can be induced by cAMP.

The Specific CRE within the AR Gene Promoter Region

To further characterize the CRE identified in Fig. 4, a serial of progressive 5' to 3' deletion mutants of AR gene promoter linked to the CAT gene (p-2330ARCAT, p-1380ARCAT, p-1030ARCAT, p-800ARCAT, p-530ARCAT, p-390ARCAT, and p-200ARCAT) were transfected into LNCaP cells. Relative CAT activity for each deletion mutant in the presence or absence of 5 mM $(\text{Bu})_2\text{cAMP}$ for 20 h were determined as shown in Fig. 5. Although $(\text{Bu})_2\text{cAMP}$ induced CAT activity severalfold in the cells transfected with p-2330ARCAT, p-1380ARCAT, p-1030ARCAT, p-800ARCAT, and p-530ARCAT, no induction by $(\text{Bu})_2\text{cAMP}$ was observed in cells transfected with p-390ARCAT and p-200ARCAT. These results indicate that the region from 530–390 bp upstream of the AR gene transcription initiation site may contain CREs. To further confirm whether the DNA fragment around this region contains CREs, we inserted the DNA fragment from -738 to

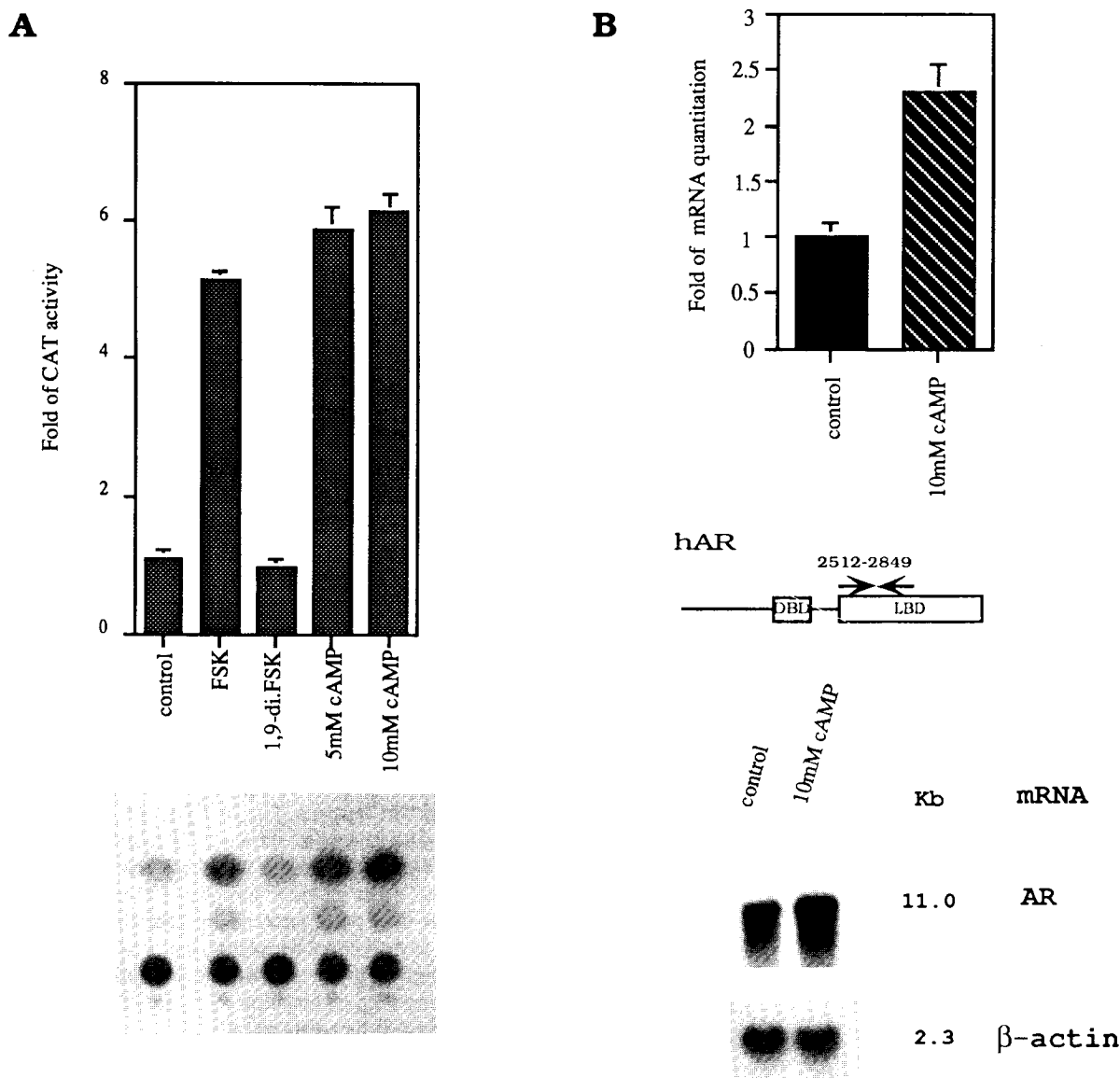


Fig. 4. The Effect of cAMP on AR Gene Expression

A, LNCaP cells transfected with 6 μ g p-2330ARCAT were incubated in the absence or presence of 10 μ M forskolin, 10 μ M 1,9-dideoxyforskolin, and 5 mM and 10 mM (Bu)₂cAMP. After 24 h the cells were harvested and CAT assay was carried out. B, 1 \times 10⁶ LNCaP cells were plated on a 6-cm dish. One day later medium was changed to DMEM/F-12 containing 5% CCS and cultured for 20 h, and 10 mM (Bu)₂cAMP were then added to the medium. After 24 h the total RNA of LNCaP cells was purified, and the Northern blot analysis was performed to quantitate the amount of AR mRNA. DBD and LBD, DNA-binding domain and ligand-binding domain of AR, respectively. The arrows represent the DNA fragment used in Northern blot analysis, and the numbers indicate the sites of AR DNA published by Chang *et al.* (2).

–332 of the AR gene promoter in front of the pCAT promoter [Fig. 6A, pCATpro(–738/–332)]. The CAT activity of this construct could be induced by (Bu)₂cAMP to 4.1-fold (Fig. 6B). As mentioned above, we have identified several potential CREs in the AR gene promoter region by comparing with the published CREs (33). One of these candidate CREs was found at –508 to –501 bp upstream of the AR transcription initiation site, named AR/CRE1 (5'-TGACGGAA-3') which has two mismatches compared to the consensus CRE (5'-

TGACGTCA-3') (34). Other reports have shown that a CRE with two mismatches could still be a functional CRE (21, 33). We then synthesized AR/CRE1, 5'-TCCCTATGACGGAATCTAAG-3', and somatostatin/CRE (SOMA/CRE), 5'-CTTGGCTGACGTCAGAGAGA-3', for further characterization. We ligated three tandem repeats of AR/CRE1 and SOMA/CRE in front of an SV40-promoter of pCAT promoter and transfected these two constructs, pCATpro(AR/CRE1)₃ and pCATpro(SOMA/CRE)₃ into LNCaP cells, respectively.

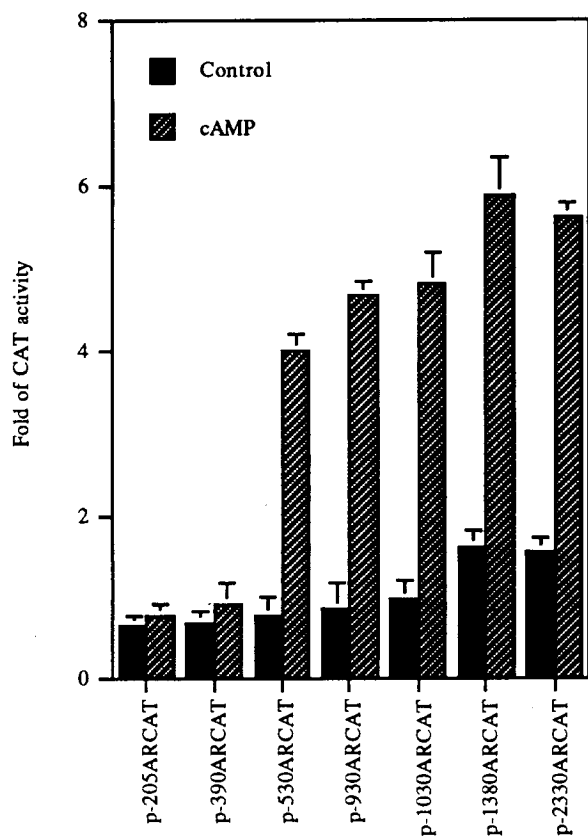


Fig. 5. Effect of cAMP on CAT Activity of Serial Deletions of AR Gene Promoter Fused to the CAT Gene

LNCaP cells transfected with 8 μ g of the different reporter plasmids were incubated in the absence or presence of 10 mM (Bu)₂cAMP. After 20 h cells were harvested and CAT activity was determined as described in *Materials and Methods*. All transfections were carried out in triplicate, and the data were expressed as the mean \pm SD. The results presented are typical of seven separate experiments. The CAT activity was normalized by the β -galactosidase activity.

The addition of (Bu)₂cAMP resulted in a 4- to 6-fold induction of CAT activity in these two plasmid (Fig. 6C). No induction of CAT activity by (Bu)₂cAMP was observed with control parent pCAT promoter. These results suggested that the cAMP effect is relatively specific to the AR/CRE1 and SOMA/CRE.

The Novel Mechanism of cAMP Induction of AR Gene Expression

Sequence analysis demonstrated that the AR/CRE1 has two bases mismatched to the classic palindrome CRE, 5'-TGACGTCA-3'. Gel retardation assay using synthesized double-strand AR/CRE1 and SOMA/CRE as probes also proved the different gel retardation binding pattern between these two CREs; the retardation pattern formed by AR/CRE1 is different from that formed by SOMA/CRE. (Fig. 7). The complexes derived from each of the probes were sequence specific, since these complexes were abolished by each of the unlabeled probes (Fig. 7, lanes 1, 2, 3, 6, 9, and 10).

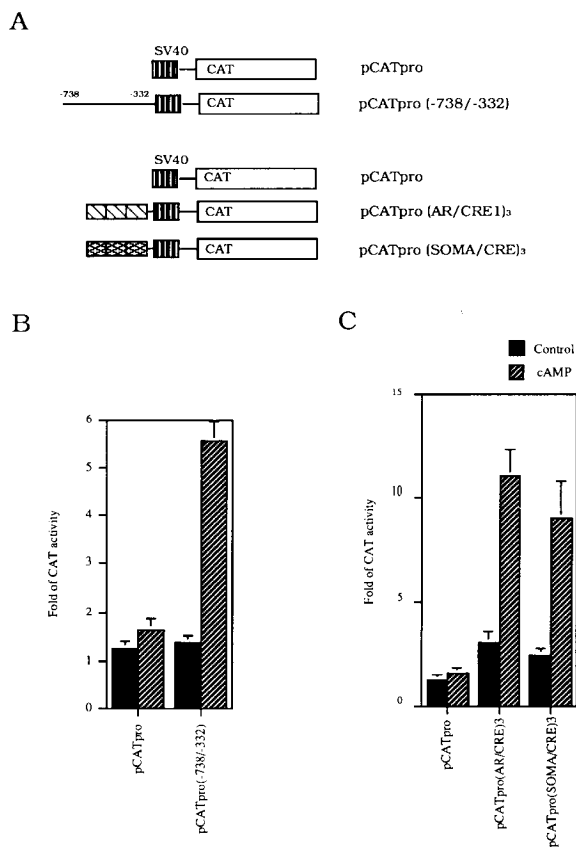


Fig. 6. The cAMP Induction of AR/CRE1

A, The map of different constructs used for transfection. B, The CAT activity of pCATpro(-738/-332) by the induction of cAMP. C, The CAT activity of pCATpro(AR/CRE1)₃ and pCAT(SOMA/CRE)₃ by the induction of cAMP. For performing B and C, 6 μ g pCAT-promoter, pCATpro(-738/-332), pCATpro(AR/CRE1)₃ or pCATpro(SOMA/CRE)₃ were transfected into LNCaP cells for 24 h. Cells were then changed to medium with 5 mM (Bu)₂cAMP and cultured for another 24 h before harvest for the CAT assay.

Furthermore, unlabeled AR/CRE1 could compete with labeled SOMA/CRE on complexes I and II, and SOMA/CRE could only compete with AR/CRE1 on complex II (Fig. 7, lanes 4, 5, 7, and 8). These results indicate that the transcription factors binding to SOMA/CRE may also be able to bind to AR/CRE1. When increasing amounts of unlabeled competitor were added, SOMA/CRE exhibited a higher affinity than AR/CRE1 for competing complexes I and II. One of the explanations of this phenomenon may be due to an imperfect CRE and different flanking sequences of AR/CRE1 (35). Yamamoto *et al.* (36) compared half-site CRE, 5'-TGACGCTG-3', with perfect CRE, 5'-TGACGTCA-3', in a gel retardation assay and found that the binding affinity of the half-site CRE is less than the perfect palindrome CRE which is similar to our present data.

Interestingly, our gel retardation competition assay showed that the AR/CRE1 but not SOMA/CRE can form another specific complex, complex III (Fig. 7, lane

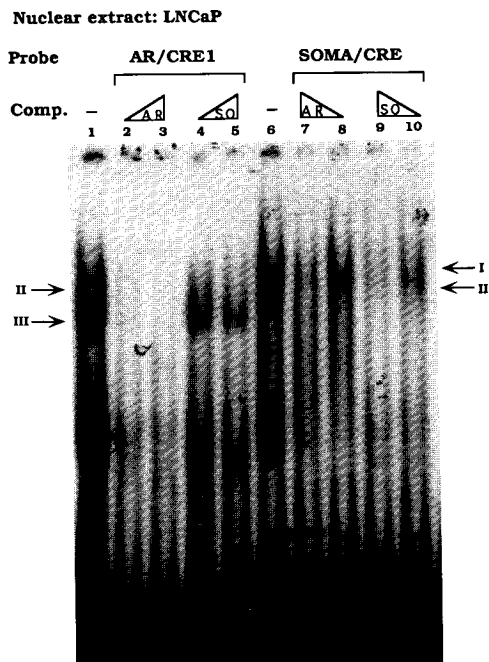


Fig. 7. Characterization of DNA-Protein Complexes Formed by Oligonucleotides Containing the AR/CRE1 and SOMA/CRE Sequences

Four micrograms of LNCaP nuclear extract were incubated with ³²P-labeled double-stranded oligonucleotide corresponding to the region from -55 to -34 of the somatostatin promoter (SOMA/CRE) or the region from -514 to -495 of the AR gene promoter containing AR/CRE1. Competition experiments were performed in the presence of 50-fold or 100-fold molar excess of unlabeled oligonucleotides (AR, AR/CRE1; SO, SOMA/CRE) as indicated. Arrows represent the positions of complexes I, II, and III.

1 vs. 6). The complex III was not abolished by 50 times or 100 times unlabeled SOMA/CRE (Fig. 7, lanes 1, 4, and 5). This competition assay showed the different characteristics between these two CREs and suggested that some different proteins and regulatory mechanism may be involved in the cAMP induction of AR and somatostatin transcriptions.

DISCUSSION

Since androgens control their target gene expression via AR, a study including the regulation of AR gene transcription in AR target cells may provide some crucial clues to understand the molecular mechanism of androgen action on sexual differentiation and prostate proliferation. We have cloned, sequenced, and characterized the AR gene promoter region to 2.3 kb upstream of the transcription initiation site and found that there were several potential *cis*-acting elements such as GC box, NF-1 binding motif, AP-1 binding motif (with one base mismatch), and CRE (with two base mismatches). The AR gene promoter region also contained two potential

RAREs, three IL-6 response elements, and one TCDD response element. The roles of these potential *cis*-acting elements on AR gene transcription are still unclear. The treatment of 12-O-tetradecanoylphorbol-13-acetate (TPA), which induces AP-1 via activation of protein kinase C (19), did not change the expression level of AR mRNA in LNCaP cells (our unpublished data). AP-1, however, may activate AR gene transcription in other cells. RARE, as retinoids, like androgens, may also play a major role in embryogenesis, differentiation, and homeostasis (37, 38). It is possible that retinoids may indirectly affect sexual differentiation by affecting AR gene transcription via these RAREs. In breast cancer cell lines, retinoids have been demonstrated to affect the AR mRNA level (39). We are in the process of further characterizing the effects of retinoic acid on AR gene transcription and prostate proliferation/differentiation.

Based on deletion analysis and CAT assay, we found that the genomic DNA from -1380 to -930 of the AR gene promoter contained *cis*-acting region(s) that may activate AR gene transcription, and the region from -530 to -140 may contain suppressor element(s). The differences of CAT activity between p-530ARCAT and p-140ARCAT were more remarkable in HeLa cells than in LNCaP cells. One of the basic differences between these two cell lines is that LNCaP cells, but not HeLa cells, can express endogenous AR mRNA (our unpublished data). The differential expression of CAT activity between LNCaP and HeLa cells also suggested that different cells may have their unique transcription factors associated with AR gene transcription.

With respect to *cis*-acting elements necessary for essential AR gene transcription, the AR gene promoter did not contain a TATA box or CCAAT box but contained a GC box at -45 to -40. Faber *et al.* (16) demonstrated that the GC box can influence the transcript initiated from AR-TIS II but does not influence the transcript initiated from AR-TIS I. Although we compared the CAT activity of p-57ARCAT plasmid that contains the GC box with that of p+20ARCAT, which does not contain a GC box, no significant difference in CAT activity between these two plasmids was observed. Since the main transcript of AR is the one initiated from AR-TIS I (16), our CAT assay might not detect the significant difference.

The AR gene promoter contained a purine-rich region (four tandem repeats of GGGGA) at the position of -119 to -75, which was well conserved among human, rat, and mouse (31, 32, 15). A purine-rich region also exists in the promoter region of several other genes, such as osteonectin, *c-myc*, progesterone receptor, *c-Ki-ras*, insulin receptor, and epidermal growth factor receptor (40-45). A purine-rich element in the *c-myc* and *c-Ki-ras* gene promoter region appears to play an important role in their transcription (41, 43). Our results using progressive 5' to 3' deletions of this region and an internal deletion of this region also suggest that the purine-rich region may play an important role in AR gene transcription. And this purine-rich region may in-

fluence the major transcript initiated from AR-TIS 1. Furthermore, since this purine-rich element can also enhance other promoters activity, such as SV40 promoter, it is possible that the purine-rich element in the promoter region may act as an important *cis*-acting element in many other genes. Our gel-retardation assay data showed that LNCaP and HeLa cells may have some nuclear protein(s) that can bind to the purine-rich element. These proteins may be general transcriptional factors associated with gene transcription. Further experiments will be necessary to make it clear if this purine-rich region itself can initiate transcription like TATA box.

The AR gene may also contain other essential *cis*-acting elements. When we transfected p-1380/+20ARCAT, which was deleted between +20 to +574 of the AR 5'-untranslated region but still included the published transcription initiation site and purine-rich element, into LNCaP and HeLa cells, CAT activity was almost abolished (our unpublished data). AR mRNA contains a very long 5'-untranslated region (1127 bp in length). The purine-rich element binding protein may regulate AR gene transcription by interacting with AR 5'-untranslated region.

Our Northern blot analysis and RT-PCR assay as well as CAT assay demonstrated that AR gene expression can be induced by the cAMP in LNCaP cells. Gel retardation assay and competition study (Figs. 4 and 5) also clearly indicated that AR/CRE1, located at -508 to -501 of the AR transcription initiation site, can form one special complex different from SOMA/CRE. The complexes I and II formed by SOMA/CRE could be abolished by AR/CRE1, and the complex II formed by AR/CRE1 could be also abolished by SOMA/CRE. However, the complex III formed by AR/CRE1 was not abolished by SOMA/CRE. These results suggest that there may exist some specific factors which can modulate AR gene expression via cAMP. It is well known that the classic CRE, 5'-TGACGTCA-3', can bind with CREBs which are related to the activating transcription factors (ATFs). ATFs can bind to CRE-like elements in the adenovirus gene promoter and can be activated by the E1A proteins (46, 47). At least 10 structurally distinct CREB/ATF cDNAs have been cloned and studied. The amino acid sequences of these proteins have extensive similarity within the DNA binding domain and have been characterized as a class of transcription factors of the leucine zipper proteins (ZIP). All ZIP proteins have the potential capacity to form homodimer or heterodimer with other ZIP proteins, and the protein-protein interaction can modify the DNA binding specificity (48-50). We are in the process of further investigating whether the complex III specifically formed by AR/CRE1 contains a new heterodimer in the CREB/ATF family.

Many genes important for physiological functions are regulated by cAMP (protein kinase A pathway) and steroid hormones as well as their receptors. Ree *et al.* (51) have described that cAMP can biphasically modulate the estrogen receptor at the mRNA level, but the

mechanism remains unknown. Although they have not been able to characterize the CRE in estrogen receptor gene promoter (51), they did suggest that cAMP could result in growth arrest of the human adenocarcinoma cell line MCF-7. Other reports also suggested that cAMP could result in growth arrest in the human androgen-independent prostate carcinoma PC-3 cell line (52). Our preliminary data, however, show that cAMP can stimulate the proliferation of LNCaP cells. Since AR plays an important role in prostate growth and differentiation, we speculate that up-regulation of AR gene expression by cAMP may be a critical modulating mechanism for androgen actions and may present the complexity of growth regulation in prostate cells.

It has been reported that cAMP, like FSH, can stimulate AR gene expression at the mRNA level in rat Sertoli cells (12). Other reports also indicated that FSH could stimulate the proliferation of some specific cells by increasing the intracellular cAMP level (18, 53). GH-releasing hormone and vasoactive intestinal peptide can also stimulate cAMP production in cultured Leydig cells (54). Prostaglandin E and IL-1 α or IL-2 may also have their function via cAMP (55, 56). As our data indicates that cAMP may induce hAR gene expression via AR/CRE1, it will be very interesting to further investigate what kinds of hormones or inducers can affect the prostate cAMP level.

By comparing the AR gene promoter among mouse, rat, and human (Fig. 2), we found that AR/CRE1 only exists in the hAR gene. The species-specific CRE sequence has also been reported in other genes such as human P450-c17 gene (57) and suggests that the cAMP/PKA pathway could be mediated by species-specific proteins.

In sum, CRE and other *cis*-acting elements were identified in the hAR gene promoter. These *cis*-acting elements may play very important roles in androgen actions.

MATERIALS AND METHODS

Isolation of the hAR 5'-Flanking Genomic DNA Clone

A human genomic λ EMBL3 library was screened with a 32 P-labeled 640-bp *EcoRI-SmaI* fragment of pGEM-3Z hAR plasmid (2) containing a part of the AR 5'-untranslated region as a probe. DNA probe was hybridized on membranes overnight at 42 C in hybridization buffer [50% formamide, 5 \times SSPE (1 \times SSPE = 150 mM NaCl, 10 mM NaH₂PO₄, 1 mM EDTA), 5 \times Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), and 50 μ g/ml heat denatured salmon sperm DNA]. The membranes were then washed twice for 30 min with 2 \times SSC (1 \times SSC = 0.15 M NaCl, 0.015 M Na citrate, pH 7.0)-0.2% SDS at 37 C and twice for 30 min with 0.1 \times SSC-0.2% SDS at 65 C. The insert in one of three positive clones isolated (ARg3) was mapped (Fig. 2), and a 2.9-kb *HindIII-PstI* fragment was subcloned into the *EcoRV* site of pBSCAT (p-2330ARCAT) according to Sambrook *et al.* (58). The 2.9-kb fragment was sequenced using the dideoxy chain termination method.

Constructions of Different AR Gene Promoter-CAT Plasmids

A 1.7-kb CAT gene fragment of pSV0CAT was ligated into the *Sma*I site of pBluescript sk(-) (Stratagene, La Jolla, CA) (pBSCAT). The 5'-flanking region of the CAT gene was adjacent to the *Kpn*I site of pBluescript. A 2.9-kb DNA fragment extending from the *Pst*I site (+574) to the *Hind*III site located at 2330 bp upstream of the AR transcription initiation site published by Tilley *et al.* (15) was blunt-ended and subcloned into the *Eco*RV site of pBSCAT (p-2330ARCAT). p-1380ARCAT was constructed by deletion of a 900-bp *Sal*I-*Xba*I fragment of p-2330ARCAT. p-57ARCAT was constructed by inserting a 630-bp *Nae*I-*Pst*I fragment into the *Eco*RI-*Pst*I sites of pBSCAT. p+20ARCAT was constructed by deletion of a 2.35-kb *Sal*I-*Sma*I fragment of p-2330ARCAT. To produce other progressive 5' to 3' deletion mutants of the AR gene promoter-CAT plasmid (p-1030ARCAT, p-930ARCAT, p-800ARCAT, p-530ARCAT, p-390ARCAT, p-200ARCAT, and p-140ARCAT), Exonuclease III (Promega, Madison, WI) was used according to Sambrook *et al.* (58) after p-1380ARCAT was digested with *Ap*I and *Xho*I. To produce p-1380(-130/-57)ARCAT, p-1380ARCAT was digested with *Sma*I, followed by digestion with Exonuclease III. The digested p-1380ARCAT was then digested with *Ap*I, and approximately 1.2-kb fragments were cut out. These 1.2-kb fragments were inserted into the *Cl*I and *Ap*I sites of p-57ARCAT. p-1380/+20ARCAT was produced by self-ligation after p-1380ARCAT was digested with *Sma*I and *Pst*I. The ends of deletion mutant promoters produced using Exonuclease III were confirmed by sequencing using the dideoxy chain termination method. To produce pGA1CAT, an 85-bp *Bss*HII-*Hpa*II fragment including the purine-rich region was cut out from p-1380ARCAT. This 85-bp fragment was inserted into the *Bgl*II site in front of the SV40 promoter of parent pCAT-promoter. To construct pCATpro(-738/-332), a 407-bp DNA fragment including the AR/CRE1 was cut out from p-1380ARCAT with *Hha*I and *Bst*XI. This 407-bp fragment was inserted into the *Bgl*II site in front of the SV40 promoter in parent pCAT-promoter. To construct pCATpro(AR/CRE1)₃ and pCATpro(SOMA/CRE)₃, we used the DNA fragments made by annealing the following oligonucleotide pairs (CRE and CRE-like sequences are underlined): AR/CRE1 (5'-GATCTCCCTATGACGGAATCTAAG-3' and 5'-GATCCTTAGATTCCGTCATAGGGA-3'); SOMA/CRE (5'-GATCCTTGGC-TGACGTCAGAGAGA-3' and 5'-GATCTCTCTGACGTCAGCCAAG-3'). The annealed DNA fragments were inserted into the *Bgl*II site in front of SV40 promoter in the parent pCAT-promoter. We performed DNA sequencing to check the inserted DNA fragment.

Cell Culture, Transfection and CAT Assay

LNCaP and HeLa cells were cultured in DMEM/F-12 medium containing 5% fetal calf serum. Twenty-four hours after plating on 6-cm dishes, cells were transfected with 8 μ g AR gene promoter-CAT plasmids using the calcium phosphate method (59); 0.4 μ g β -galactosidase expression plasmid was cotransfected as an internal control to normalize transfection efficiencies. After normalization by β -galactosidase activity, whole cell extracts were used for CAT assays. The CAT activity was quantitated by Phosphor Imager (Molecular Dynamics, Sunnyvale, CA).

For investigating the effect of cAMP on the AR gene promoter region, 1×10^6 LNCaP cells were plated on 6-cm dishes. One day later the culture medium was changed to DMEM/F-12 medium containing 5% CCS (charcoal-stripped calf serum) and 1% antibiotics. Twenty hours after changed medium, the transfection was performed as mentioned above. Twenty hours after transfection, the medium also was replaced to DMEM/F-12 containing 5% CCS with either 5 mM or 10 mM (Bu)₂cAMP, 10 μ M forskolin, or 10 μ M 1,9-dideoxyforskolin, and the cells was grown for another 20 h. The procedures for harvesting cells and CAT assay were the same as above.

Preparation of Nuclear Extract

Nuclear extract from LNCaP cells was prepared after nuclei were isolated according to Groudine *et al.* (60). Briefly, after LNCaP cells were harvested, cells were washed with PBS twice and swollen in hypotonic buffer (10 mM HEPES, pH 7.8, 10 mM NaCl, 3 mM MgCl₂, and 1 mM dithiothreitol). Cell membrane was then destroyed with hypotonic buffer containing 0.5% NP-40, and nuclei were isolated. Nuclei were washed with hypotonic buffer three times and were lysed with hypertonic buffer (10 mM HEPES, pH 7.8, 0.5 M NaCl, 3 mM MgCl₂, 1 mM dithiothreitol, and 20% glycerol). After centrifugation, supernatant was stored at -70 C.

Gel Retardation Assay

For the gel retardation assay, two 50-bp oligonucleotides containing the purine-rich region (PR1, 5'-GATCTGAGGGGAGGGGAGAAAAGGAAAGGGGAGGGGAGGGAAAA - GGAGGA-3'; PR2, 5'-GATCTCCTCCTTTTCCCTCCCCTCCCTCCCTTTTCTTTTCTCCCCTCCCCTCA-3') were annealed, end-labeled with [³²P], and used as a specific probe. Gel retardation assay was carried out mainly according to Carthew *et al.* (61). Briefly, this probe (5000 cpm) was incubated with 6 μ g LNCaP nuclear extract or with 4 μ g HeLa nuclear extract in the presence of 2.0 μ g poly(dI-dC)-poly(dI-dC) (Pharmacia, Piscataway, NJ) in a final vol of 12 μ l at room temperature for 20 min. The samples were then electrophoresed on a low-ionic strength 5% polyacrylamide gel. As a specific competitor and a nonspecific competitor, cold probe and pBluescript/*Hpa*II fragment were used, respectively. The gel was then transferred to Whatman (Clifton, NJ) 3MM, dried, and autoradiographed.

The probes used for the CRE-specific complexes were made by annealing of the oligonucleotide pairs (CRE and CRE-like sequences are underlined): AR/CRE1 (5'-GATCTCCCTATGACGGAATCTAAG-3' and 5'-GATCCTTAGATTCCGTCATAGGGA-3'); SOMA/CRE (5'-GATCCTTGGC-TGACGTCAGAGAGA-3' and 5'-GATCTCTCTGACGTCAGCCAAG-3'). The annealed oligonucleotides were end-labeled with ³²P. Unlabeled double-strand DNAs were used for competition studies. The reactions were the same as mentioned above.

The Quantification of AR mRNA

Sixty-per cent confluent LNCaP cells were treated with 10 mM (Bu)₂cAMP in DMEM/F-12 containing 5% CCS for 48 h, and total mRNA was isolated by using single-step acid guanidinium isothiocyanate/phenol/chloroform extraction (62). The amount of total RNA in aqueous solution was quantitated by absorbance at 260 nm. Finally, 20 μ g each LNCaP total RNA were electrophoresed in a 0.8% agarose-formaldehyde gel and transferred to the Hybond-N membrane (Amersham, Arlington Heights, IL). Human AR and β -actin mRNA were detected by using a 337-bp hAR cDNA (Fig. 4) and β -actin cDNA. Purified cDNA inserts were labeled by an Amersham (Arlington Heights, IL) multiprime labeling kit with [α -³²P]dCTP. The membrane was washed twice with 2 \times SSC-0.1% SDS at 4 C for 30 min and twice with 0.1 \times SSC-0.1% SDS at 60 C for 30 min. The intensities of the signals were calculated using Phosphor Imager.

Note

The sequence of the 2.3-kb hAR gene promoter region was accepted in GenBank. The accession number is L14435.

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Address requests for reprints to: Dr. Chawnsang Chang, Department of Human Oncology, University of Wisconsin-Madison, 600 Highland Avenue, K4-632, Madison Wisconsin 53792

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* Both authors contributed equally to this work and should be viewed as first author in this paper.

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