

# Identification of an Androgen Response Element in Intron 8 of the Sterol Regulatory Element-binding Protein Cleavage-activating Protein Gene Allowing Direct Regulation by the Androgen Receptor\*

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Sterol regulatory element-binding proteins (SREBPs) are transcription regulators that play a pivotal role in intracellular lipid homeostasis. They are synthesized as inactive precursor proteins in the endoplasmic reticulum, where they are retained by SREBP cleavage-activating protein (SCAP), a sterol sensing protein that in turn is linked to a retention protein complex. Low intracellular sterol concentrations weaken the interaction of SCAP with its retention proteins and allow translocation of the SREBP-SCAP complex to the Golgi compartment where SREBP is proteolytically cleaved and activated. Previous studies on the mechanisms by which androgens provoke a coordinated activation of lipogenic pathways in prostate cancer cells have suggested an alternative pathway of activation in which androgens increase the expression of SCAP and favor translocation of the SREBP-SCAP complex to the Golgi apparatus by disturbing the balance between SCAP and its retention proteins. Here we show that the SCAP gene contains an androgen-responsive region located in intron 8. This region interacts directly with the androgen receptor and confers androgen responsiveness to promoter-reporter constructs transfected in LNCaP cells. It contains a noncanonical androgen response element GGAAGAAAATGTACC that interacts not only with the androgen receptor but also with the glucocorticoid receptor and that also confers glucocorticoid responsiveness. The identification of a steroid response element in intron 8 of the SCAP gene further supports the contention that SCAP is a direct target for steroid hormone action.

Sterol regulatory element-binding proteins (SREBPs)<sup>1</sup> represent an important family of transcription regulators (SREBP-

1a, -1c, and -2) controlling intracellular lipid homeostasis (1). SREBPs are synthesized as 125-kDa inactive precursor proteins, and immediately upon their synthesis they are inserted into the membranes of the endoplasmic reticulum where they form tight complexes with an escort protein known as the SREBP cleavage-activating protein (SCAP). SCAP plays a pivotal role in the control of SREBP signaling. In fact, SCAP does not only bind SREBP but, through its amino-terminal sterol-sensing domain, it also interacts with a retention protein complex consisting of at least two endoplasmic reticulum proteins (designated insulin-induced gene 1 and 2; Insig 1 and 2) that serve to retain the SREBP-SCAP complex into the membranes of the endoplasmic reticulum. In the "classical" SREBP activation pathway, a decrease in the intracellular concentration of sterols changes the conformation of the sterol-sensing part of SCAP, weakens its interaction with the retention proteins, and allows translocation of the SREBP-SCAP complex to the Golgi apparatus where SREBP is proteolytically cleaved and activated. The active 68-kDa SREBP fragment migrates to the nucleus where it increases the transcription of a large set of sterol-responsive element (SRE) containing genes encoding lipogenic enzymes belonging to the pathways of fatty acid and cholesterol synthesis (1–7).

Our studies on the mechanisms by which androgens provoke a coordinated activation of lipogenic pathways in androgen-responsive prostate tumor lines have suggested an alternative pathway of SREBP activation in which androgens change the expression rather than the conformation of SCAP (8, 9). In this pathway, increased expression of SCAP shifts the balance between SCAP and its retention proteins and favors translocation of the SREBP-SCAP complex to the Golgi apparatus. Evidence for the existence of this pathway was derived from several observations (9). 1) In two independent prostate tumor lines (LNCaP and MDA-PCa-2a) androgens cause major changes in the expression of SCAP both at the mRNA and at the protein level, whereas no or only minor changes were observed for other critical components of the SREBP pathway (the SREBP precursor proteins SREBP-1a, -1c, and -2; the site 1 and site 2 proteases responsible for SREBP cleavage). 2) The observed increase in SCAP expression was shown to be a cause rather than a consequence of SREBP activation. Induction of SCAP expression coincided with nuclear translocation of SREBP but

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<sup>1</sup> The abbreviations used are: SREBP, sterol regulatory element-

binding protein; SCAP, SREBP cleavage-activating protein; SRE, sterol-responsive element; ARE, androgen response element; FCS, fetal calf serum; GR, glucocorticoid receptor; AR, androgen receptor; DBD, DNA-binding domain; EMSA, electrophoretic mobility shift assay.

TABLE I  
Primers used to screen a human PAC genomic library for the genomic sequence of SCAP

DNA amplified	Primer pair	Primer sequence	PCR products bp
SCAP promoter	5' 3'	gagacggagggtctcctacgttg catgcgtagtcgaaccacattc	279
SCAP exon 22	5' 3'	tggggatgtcacctcccttac tgctgaatggagtagaacttgatgc	113

preceded the increased expression of lipogenic genes involved in cholesterol and triglyceride synthesis. Moreover, activation of SREBP signaling by an alternative route (sterol depletion) did not result in SCAP induction. 3) Overexpression of SCAP could be shown to result in activation of the SREBP pathway.

Because experiments with actinomycin D indicated that the stimulatory effects of androgens on SCAP require transcriptional activity (9), we wanted to answer the question of whether SCAP is a direct target for androgen action. To this end we performed a systematic search for *cis*-acting regulatory sequences in the SCAP gene that might mediate the effects of androgens. Here we describe the identification and characterization of an androgen response element (ARE) located within intron 8 of the SCAP gene.

#### EXPERIMENTAL PROCEDURES

**Cell Culture**—The human prostate carcinoma cell line LNCaP was obtained from the American Type Culture Collection (Manassas, VA) and was routinely maintained in phenol red-free RPMI 1640 medium supplemented with 10% FCS (Invitrogen), 3 mM L-glutamine (Invitrogen), 100  $\mu$ g/ml streptomycin, and 100 units/ml penicillin (Invitrogen). The cells were cultured at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> in air. In experiments assessing the effects of androgens, FCS was pretreated with dextran-coated charcoal (CT-FCS) (10) to reduce the background levels of steroids. The synthetic androgen R1881 (methyltrienolone) was purchased from PerkinElmer Life Sciences, dissolved in absolute ethanol, and added to the cultures from a 1000-fold concentrated stock. Control cultures received similar amounts of ethanol only. Final ethanol concentrations did not exceed 0.1% (v/v).

**Luciferase Reporter Plasmids**—The pGL3-SCAP construct was generated by cloning an approximately 1.1-kb promoter fragment of the SCAP gene (bp −1018 to +42) (11) into the pGL3 basic vector (Promega, Madison, WI). The pGL3-SCAPmutSRE construct was made by performing site-directed mutagenesis using the primer pair 5'-ctgtggcctcgacctccgggagactgtgcactctacccagctcctgag-3' (forward primer) and 5'-ctcaggagctgaggtaggagtcacacagctccgggaggtcgaggccacag-3' (reverse primer).

To obtain the entire genomic sequence of SCAP, a human PAC genomic library was screened by PCR (Incyte Genomics, Palo Alto, CA) using both a primer pair targeted at the promoter region of the SCAP gene (designated SCAP promoter) (bp −452 to −173) (11) and a primer pair amplifying a genomic region encompassing exon 22 (designated SCAP exon 22) (bp 3475–3588; GenBank™ accession number D83782) (Table I). A positive clone, designated PAC-SCAP, was grown according to the supplier's recommendations. PAC-SCAP DNA was purified using a Qiagen plasmid kit (very low copy plasmid purification protocol). KpnI restriction fragments derived from PAC-SCAP and subfragments thereof were cloned into the pGL3 promoter vector or into a vector driven by a minimal promoter (pE1bLUC) (12). Following a NCBI human genomic BLAST search, the location and orientation of the genomic DNA fragments were determined in relation to the sequence of the SCAP gene. The oligonucleotides used in band shift assays (see below) were hybridized and cloned into a NheI-digested pES vector (a pGL3-derived plasmid driven by a thymidine kinase-TATA minimal promoter), resulting in the insertion of two copies of the oligonucleotide with alternating orientation upstream of the minimal TATA box. The genomic inserts of all constructs were subjected to nucleotide sequencing using an automated laser fluorescence sequencer (Amersham Biosciences).

**Transient Transfections**—LNCaP cells were seeded in 60-mm dishes at a density of  $1 \times 10^6$  cells/dish in RPMI 1640 medium containing 5% CT-FCS. The next day, transfection mixtures were prepared. For each dish, 2 ml of serum-free Dulbecco's modified Eagle's medium (Invitrogen) was supplemented with 3  $\mu$ g of promoter-reporter construct. In case of cotransfection experiments, 100 ng of an expression construct encoding the human glucocorticoid receptor (GR) was added. After the

addition of 9  $\mu$ l of Transfast (Promega), the transfection mixture was incubated for 15 min at room temperature. The cells were washed with serum-free medium, the transfection mixture was added, and the cell cultures were incubated for 30 min at 37 °C. Four ml of RPMI medium with 5% CT-FCS was added. The next day, the medium was replaced (RPMI with 5% CT-FCS), and the cells were treated with  $10^{-8}$  M R1881,  $10^{-8}$  M dexamethasone, or ethanol vehicle for 2 days. The cells were washed with phosphate-buffered saline and lysed in 500  $\mu$ l of passive lysis buffer (Promega). Aliquots of 10  $\mu$ l of cleared lysate were assayed for luciferase activity by using a luciferase reporter assay kit from Promega and a Berthold Microumat LB96P luminometer.

**Electrophoretic Mobility Shift Assay**—Recombinant glutathione S-transferase-tagged AR-DBD or GR-DBD was expressed in *Escherichia coli* BL21(DE3)pLysS from pGEX-2TK plasmid as described (13) and was purified on immobilized glutathione (Pierce) according to the manufacturer's instructions. All oligonucleotides used in the band shift assays contain XhoI and NheI overhanging ends. Oligonucleotides were hybridized and 5' overhanging ends were filled in using the Klenow fragment of DNA polymerase I in the presence of [ $\alpha$ -<sup>32</sup>P]dCTP (PerkinElmer Life Sciences). Labeled oligonucleotide dimer (20,000 cpm, ~5–10 fmol) was incubated with recombinant AR-DBD (or GR-DBD) in a solution containing 10 mM Hepes, 2.5 mM MgCl<sub>2</sub>, 0.05 mM EDTA, 8.5% (v/v) glycerol, 50 mM NaCl, 1 mM dithiothreitol, 2.5 ng/ $\mu$ l poly(dI-dC) (Roche Applied Science) for 20 min on ice. The DNA-protein complexes were resolved on a 5% nondenaturing polyacrylamide gel in 22 mM Tris, 22 mM borate, 0.5 mM EDTA and 0.05% Triton X-100 at room temperature. The gels were vacuum-dried and exposed to Amersham Biosciences Hyperfilm-MP.

In binding assays, a constant amount of radiolabeled double-stranded oligonucleotide (20,000 cpm, corresponding to 5–10 fmol) was incubated with increasing amounts of receptor DBD. The percentage of radioactivity that was retarded by dimeric receptor-DBD in each lane of the gel, relative to the total amount of radioactivity in that lane was plotted against the AR-DBD or GR-DBD concentration that was used. The radioactive signals were quantitated using PhosphorImager screens (Amersham Biosciences). To calculate the apparent dissociation constant ( $K_d$ ), the Sigma Plot software package (SPSS Inc., Chicago, IL) was used. The best fits were calculated to four parameter curves with allosteric Hill kinetics.

#### RESULTS

**A 1.1-kb Proximal SCAP Promoter Fragment Does Not Harbor Androgen-responsive Region(s)**—The human SCAP gene is located on chromosome 3 (3p21.3) and consists of 23 exons and 22 introns (11). At the start of our investigations, only the coding sequence of the SCAP gene and the nucleotide sequence of a DNA region of ~1.1 kb located in the 5' region flanking the start of transcription of the SCAP gene were available (11). In a search for potential androgen-responsive domains, the 1.1-kb promoter region was cloned into the pGL3 basic vector. LNCaP cells transiently transfected with the resulting promoter-reporter construct were treated with R1881 ( $10^{-8}$  M) or ethanol vehicle for 2 days. No significant effects of androgens on reporter gene activity were observed. Neither expansion of this 1.1-kb region to ~6 kb 5'-upstream nor shortening altered androgen responsiveness. Mutation of a putative SRE described in this region (11) markedly reduced overall expression but did not affect androgen regulation (data not shown).

**Isolation of a 122-bp Androgen-responsive Region within Intron 8 of the SCAP Gene**—Because no androgen-regulatory regions were found in the 1.1-kb promoter region, we decided to explore the entire genomic sequence of the gene encoding SCAP for regions mediating the stimulatory effects of androgens. To

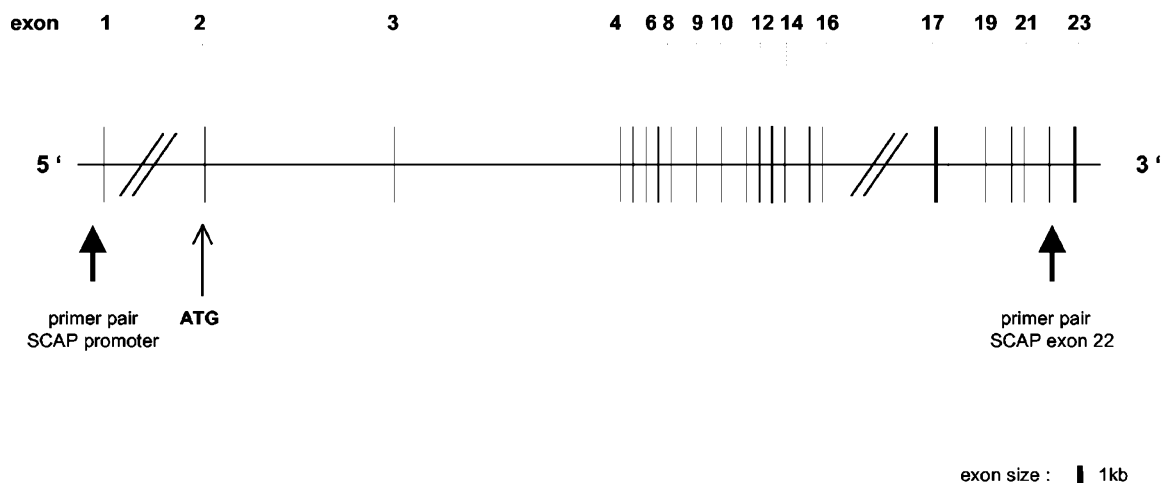


FIG. 1. **Genomic structure of the gene encoding SCAP.** Schematic representation of the exon/intron organization of the human gene encoding SCAP (11). The sizes of the exon regions are depicted in relative proportion. The start of translation (ATG), located within the second exon, and the locations of the target sites for the primer pairs (SCAP promoter and SCAP exon 22) used to screen a human PAC library for the gene encoding SCAP are indicated.

this end, a human PAC genomic library was screened for the genomic sequence of SCAP by a PCR screening using both a primer set targeted at the promoter region of the SCAP gene and a primer pair amplifying a genomic fragment corresponding to exon 22 (Fig. 1). Positive clones thus isolated (designated PAC-SCAP) contain most of the genomic sequence of the SCAP gene.

Genomic fragments obtained by restriction enzyme digestion of DNA isolated from the resulting PAC-SCAP clone and covering ~70% of the SCAP gene were individually cloned into the enhancer site of the pGL3 promoter vector, which is driven by an SV40 promoter. Following automated laser fluorescence sequencing of the genomic inserts and using the NCBI human genomic blast search program, the locations of the genomic DNA fragments in the SCAP gene were determined. Upon transient transfection into LNCaP cells, several of these constructs showed a slight and often poorly reproducible increase in reporter activity after androgen exposure. In most cases, the extent to which reporter gene activity was induced barely exceeded the induction observed with the empty pGL3 promoter-reporter vector (~1.2-fold; data not shown). One promoter-reporter construct (designated SCAP-X), however, harboring a 4.5-kb genomic insert located in a region of the SCAP gene encompassing exons 8–10, consistently displayed a 2–3-fold induction of reporter activity after androgen exposure (Fig. 2). This construct was selected for further analysis. By restriction enzyme digestion the genomic DNA insert of SCAP-X was divided into three independent fragments that were individually cloned into pGL3. As shown in Fig. 2B, only one of the three resulting promoter-reporter constructs (designated SCAP-X3) responded to androgen exposure with a major (~3-fold) increase in reporter activity, suggesting the presence of (an) androgen-responsive region(s) within this DNA segment (Fig. 2B). To more precisely locate the androgen-responsive region(s) within this 2.3-kb DNA fragment located in a genomic region encompassing exon 9, the DNA insert of SCAP-X3 was subjected to deletions. Transient transfection experiments using the deletion constructs indicated the presence of an androgen-responsive domain within a 122-bp DNA fragment located in intron 8 of the gene encoding SCAP (construct SCAP-122bp). Androgen treatment of transiently transfected LNCaP cells gave rise to an ~2.3-fold increase in SCAP-122bp reporter gene activity (Fig. 2B). Interestingly, introduction of this 122-bp

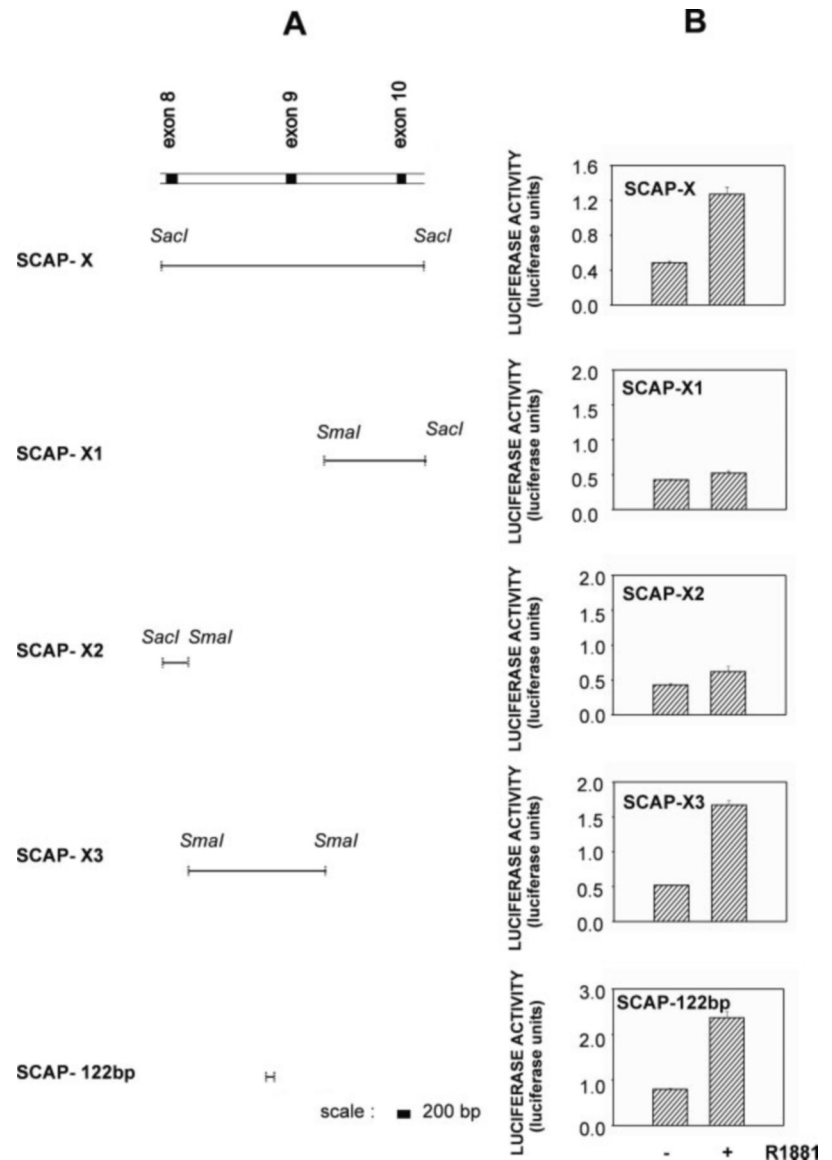
fragment into the enhancer site of a reporter construct driven by a minimal promoter containing only a TATA box (pE1bluc-SCAP-122bp) (12) yielded a  $9.3 \pm 3.1$ -fold ( $n = 3$ ) induction of reporter gene activity, confirming the presence of an androgen response domain within the tested DNA fragment.

**Identification of an AR-binding Site within Intron 8 of the SCAP Gene**—To explore whether the androgen stimulation of SCAP-122bp reporter gene activity is mediated by a direct interaction between the 122-bp genomic DNA fragment and the AR, electrophoretic mobility shift assays (EMSAs) were set up using recombinant AR-DBD protein and radiolabeled probes corresponding to DNA fragments encompassing approximately one-third of the original 122-bp fragment (designated oligonucleotide SCAP 1, 2, and 3; see Fig. 3A). To avoid inadvertent disruption of any region(s) mediating androgen effects, these oligonucleotides were designed to contain a 8-bp overlap with their neighboring DNA fragments. As shown in Fig. 3B, a shifted DNA-protein complex was observed when AR-DBD was added to oligonucleotide SCAP 2. In contrast, the AR-DBD displayed no or very weak interaction with either oligonucleotide SCAP 1 or oligonucleotide SCAP 3. To evaluate whether the observed binding of AR-DBD to oligonucleotide SCAP 2 can indeed give rise to transcriptional activation of the SCAP gene, two copies of each oligonucleotide (oligonucleotides 1–3) were cloned in a luciferase vector upstream of the heterologous thymidine kinase minimal promoter (pES vector), and the androgen responsiveness of the resulting promoter-reporter constructs was evaluated in transient transfection assays in LNCaP cells. A marked increase in the reporter activity of pES-oligoSCAP2 was observed following androgen treatment, indicating that the interaction between AR-DBD and oligonucleotide SCAP 2 is indeed functionally relevant (Fig. 3C). Consistent with the findings described in Fig. 3B, the androgen induction of reporter activity of pES-oligoSCAP 1 and 3 constructs was marginal.

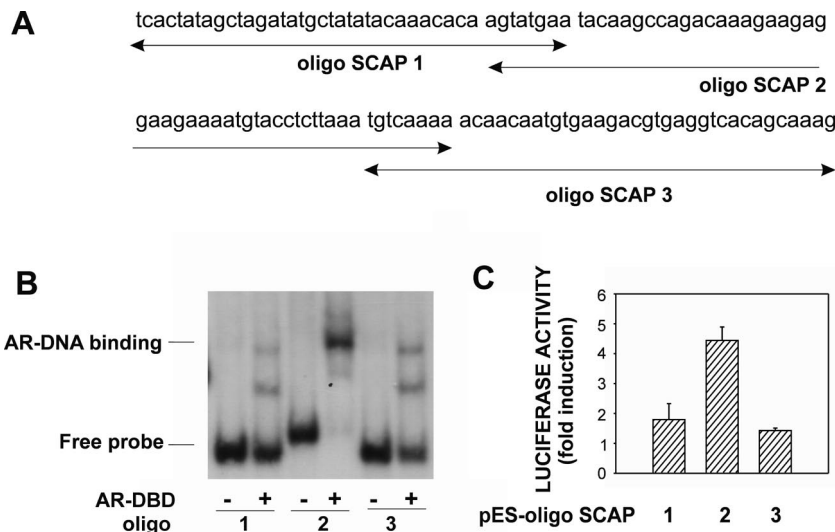
**Characterization of the AR-binding Site within Intron 8 of the SCAP Gene**—To determine the exact site of AR-DNA interaction within oligonucleotide SCAP 2, the nucleotide sequence of oligonucleotide SCAP 2 (Fig. 3A) was analyzed in more detail. This analysis revealed the presence of a TGTACC sequence that bares resemblance to the consensus ARE core-binding motif TGTCT. Because an ARE typically consists of a three-nucleotide-spaced inverted repeat of a 5'-TGTCT-3'-



**FIG. 2. Identification of a 122-bp intronic DNA region capable of conveying androgen responsiveness to reporter genes.** A, schematic representation of the location of the genomic DNA fragments under investigation in relation to the structure of the gene encoding SCAP. Cleavage sites for the restriction enzymes *Sma*I and *Sac*I, used to generate the SCAP-X1, SCAP-X2, and SCAP-X3 subfragments, are indicated. B, LNCaP cells were transiently transfected with a promoter-reporter construct harboring a 4–5 kb genomic DNA region encompassing exons 8–10 of the gene encoding SCAP (SCAP-X), or with subfragments thereof (SCAP-X1, SCAP-X2, SCAP-X3, and SCAP-122bp). After 2 days of exposure to R1881 ( $10^{-8}$  M), the cells were harvested, and luciferase activity was measured. Columns, means of incubations performed in triplicate; bars, S.E. The results shown are representative of at least two independent experiments.

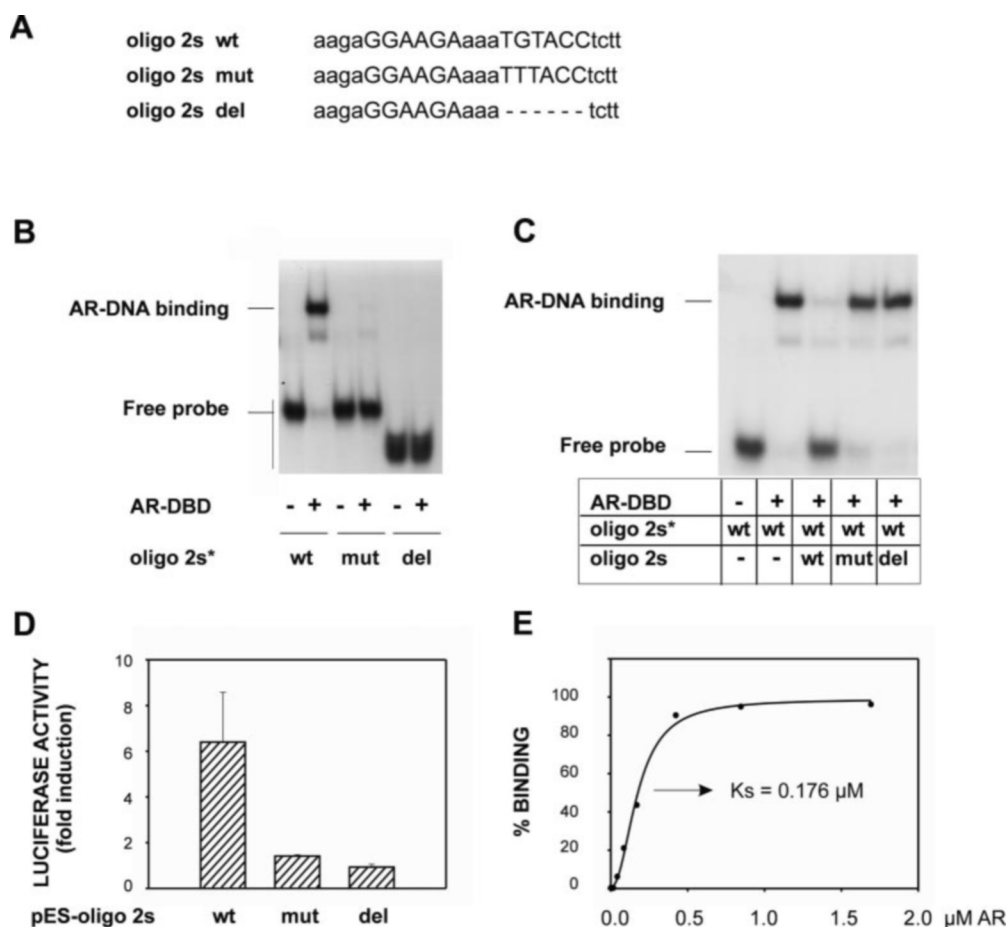


**FIG. 3. Binding of the AR-DBD to the genomic insert of SCAP-122bp correlates with androgen-responsive transcription of a luciferase reporter gene.** A, nucleotide sequence of the genomic insert of SCAP-122bp and delineation of the oligonucleotides SCAP 1, 2, and 3, used in DNA binding studies described below. B, 10 fmol of oligonucleotide SCAP 1, 2, and 3 were radiolabeled and incubated in the presence (+) or absence (-) of 423 nM recombinant AR-DBD protein. After incubation on ice, the reaction mixtures were subjected to EMSA as described under "Experimental Procedures." C, two copies of oligonucleotide SCAP 1, 2, or 3 were cloned into the pES vector. LNCaP cells were transiently transfected with the resulting constructs and exposed to R1881 ( $10^{-8}$  M) or ethanol vehicle. After 2 days, the cells were lysed, and luciferase activity was measured. Luciferase activity was expressed as fold induction following androgen exposure. Columns, means of three independent experiments; bars, S.E.



like monomer-binding motif (14), we hypothesized that the GGAAGAAAATGTACC sequence within oligonucleotide SCAP 2 might represent a novel ARE. To test this hypothesis, an

oligonucleotide corresponding to this particular sequence (aa-gaGGAAGAAAATGTACCttt, designated oligonucleotide SCAP 2 short) was generated and used in gel



**FIG. 4. Delineation and further characterization of the site of AR binding within oligonucleotide SCAP 2.** *A*, nucleotide sequence of wild type (*wt*), mutated (*mut*), and deleted (*del*) oligonucleotide SCAP 2 short (*oligo 2s*). *B*, 10 fmol of oligonucleotide 2s *wt* (*wt*), oligonucleotide 2s *mut* (*mut*), or oligonucleotide 2s *del* (*del*) were radiolabeled (\*) and incubated in the presence (+) or absence (–) of 423 nM recombinant AR-DBD protein. After incubation on ice, binding reactions were subjected to EMSA. *C*, radiolabeled (\*) SCAP 2s *wt* oligonucleotides and recombinant AR-DBD were used in gel shift assays in the absence (–) or in the presence (+) of a 400-fold molar excess of unlabeled competitor oligo. Wild type oligonucleotides (*wt*) and oligonucleotides in which the ARE sequence was mutated (*mut*) or deleted (*del*) were used as competitors. *D*, two copies of wild type, mutated, or deleted oligonucleotide 2s were cloned into the pES vector. LNCaP cells were transiently transfected with the resulting constructs and exposed to R1881 ( $10^{-8}$  M) or ethanol vehicle. After 2 days, the cells were lysed, and the luciferase activity was measured. Luciferase activity was expressed as fold induction following androgen exposure. Columns, means of three independent experiments; bars, S.E. *E*, to generate binding curves of AR-DBD to oligonucleotide 2s, a constant amount (10 fmol) of radiolabeled oligonucleotide 2s was incubated with increasing amounts of AR-DBD (ranging from 1 to 400 ng, corresponding 4.23–1692 nM). The amount of radioactivity present in bound DNA was calculated relative to the total amount of radioactivity in each lane and plotted against the concentration of protein that was used. Fits were calculated to a curve with four parameter allosteric Hill kinetics, and the apparent dissociation constant ( $K_s$ ) was calculated. The data shown are representative of two independent experiments using two different preparations of AR-DBD.

shift assays (Fig. 4A). As shown in Fig. 4B, a shifted AR-DBD-DNA complex was observed when oligonucleotide 2s was used in EMSA studies, indicating that the oligonucleotide 2s sequence is indeed sufficient for AR dimer binding. Substitution of the guanine residue within the TGTACC motif, which is a unique and consistent feature of AREs (14), by a thymine (TGTACC → TTTACC) (oligonucleotide 2s *mut*) almost completely prevented binding of AR-DBD. Moreover, deletion of the entire TGTACC right half-site (oligonucleotide 2s *del*) abolished AR-DBD binding (Fig. 4B). Conversely, deleting the GGAAGA left half-site or mutation of this site (GGAAGA → CGAAGA) gave rise to an altered AR-DBD binding pattern, suggesting a shift from dimer to monomer binding (data not shown). In addition, an excess of unlabeled homologous competitor fragment displaced AR-DBD binding to labeled oligonucleotide 2s DNA. Oligonucleotides in which the guanine residue within the TGTACC sequence was mutated into a thymine as well as oligonucleotides lacking the entire TGTACC site were unable to compete for AR-DBD binding (Fig. 4C). In contrast, inclusion of an excess of DNA fragments encompassing a well described ARE (rTAT-GRE) (15) displaced binding of the AR-

DBD to oligonucleotide 2s (data not shown). The addition of oligonucleotides harboring no androgen-responsive regions did not affect AR-DBD binding (data not shown).

Interestingly, the binding patterns of AR-DBD to wild type or mutated 2s oligonucleotides reflected the ability of the tested sequences to confer transcriptional activation to reporter genes; reporter gene activity of a construct containing two copies of intact oligonucleotide 2s markedly (7-fold) increased following androgen treatment, whereas mutation or deletion of the TGTACC sequence (almost) completely prevented such an increase in reporter gene activity (Fig. 4D). In addition, the deranged AR-DBD binding to mutated oligonucleotide 2s left half-site sequences reflected the diminished ability of these sequences to confer androgen responsiveness to reporter genes (data not shown).

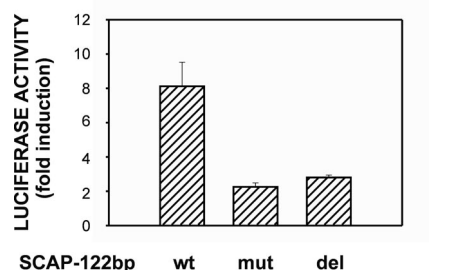
To further characterize binding of the AR-DBD to the newly identified SCAP ARE, binding studies were set up, and an apparent dissociation constant ( $K_s$ ) was deduced. To this end, radiolabeled 2s oligonucleotides were incubated with increasing concentrations of recombinant AR-DBD (4.23–1692 nM). A binding curve was generated, and a best fit was calculated to a plot

with four-parameter Hill kinetics. Binding of AR-DBD to oligonucleotide 2s is sigmoidal, the plateau of 95% of total binding is reached at  $\approx 630$  nM of protein and a  $K_s$  of 151 nM (127 and 176 nM, respectively, in two independent experiments using two different preparations of AR-DBD) was deduced (Fig. 4E).

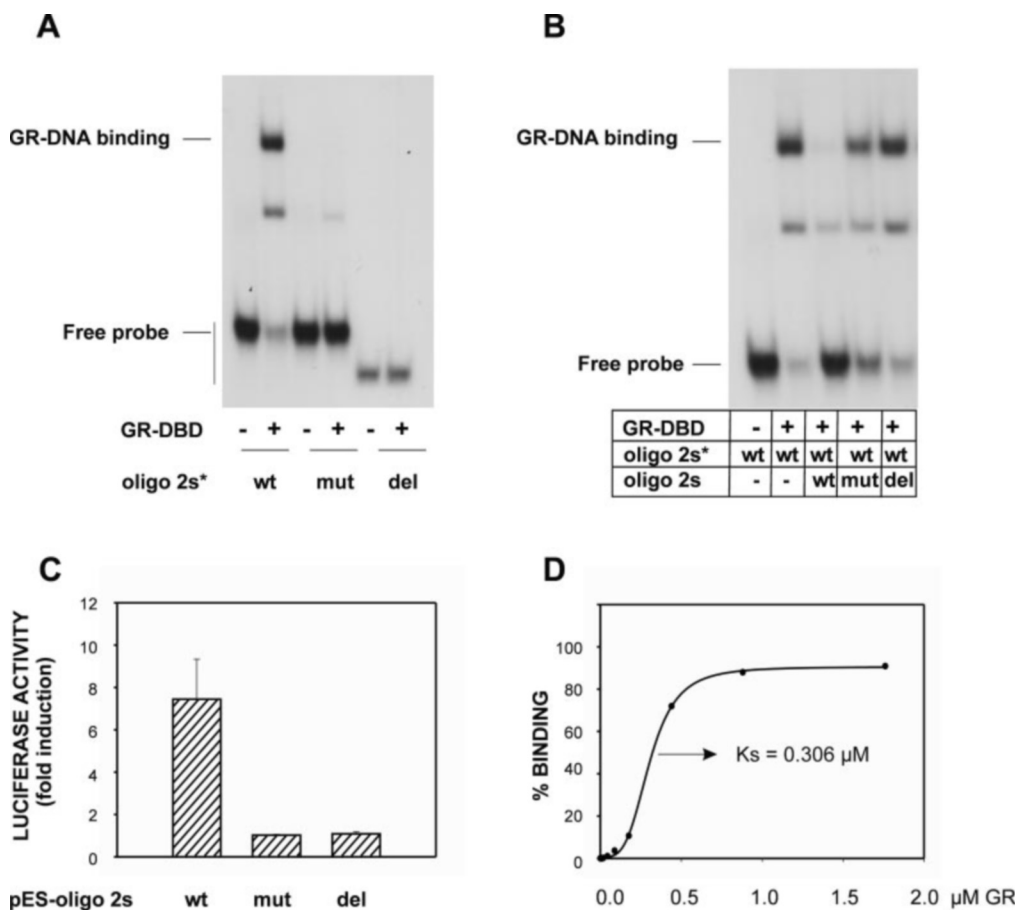
Taken together, the data presented in Fig. 4 provide evidence for the existence of a novel ARE located within intron 8 of the SCAP gene (SCAP ARE). To further explore the importance of this particular sequence for the androgen induction of SCAP gene expression, we evaluated the impact of the presence of

this sequence on androgen stimulation of SCAP-122bp reporter gene activity. As shown in Fig. 5, androgen stimulation of LNCaP cells transiently transfected with the wild type pE1bluc-SCAP-122bp construct gave rise to an  $\sim 8$ -fold induction of reporter gene activity. Mutation (TGTACC  $\rightarrow$  TTTACC) or deletion (TGTACC sequence) of the SCAP ARE markedly decreased the response, indicating the importance of the presence of the intact SCAP ARE for the androgen-induction of gene transcription.

**SCAP ARE Represents a Non-AR-specific Regulatory DNA Region**—As a final part of our studies, we investigated whether the SCAP gene ARE can be activated also by other class I steroid hormone receptors or belongs to a subset of AREs that selectively bind the AR (14). Thus, we tested whether the GR, known to bind several AREs (Ref. 14 and references therein), is able to interact with the SCAP gene ARE. EMSA studies using recombinant GR-DBD and the oligonucleotides described in Fig. 4 demonstrated GR binding to oligonucleotide 2s. As was the case for the AR-DBD, mutation or deletion of the TGTACC sequence within this sequence abolished GR-DBD binding (Fig. 6A). In addition, the presence of an intact GGAAGA left half-site was required to obtain normal binding of GR-DBD to oligonucleotide 2s (data not shown). Moreover, an excess of wild type oligonucleotide was able to compete for GR-DBD binding, whereas addition of oligonucleotide 2s mut or oligonucleotide 2s del did not affect binding (Fig. 6B). To examine the functional significance of GR-DNA binding, LNCaP cells, which do not express an endogenous GR, were cotransfected with an expression construct encoding the human GR and promoter-reporter constructs containing two copies of wild type or mutated oligonucleotide 2s oligonucleotides. As shown in Fig. 6C,



**FIG. 5. Presence of the newly identified SCAP ARE is critical for androgen responsiveness mediated by the 122-bp intronic SCAP DNA fragment.** The genomic insert of SCAP-122bp (see Fig. 2) was cloned into the enhancer site of pE1bluc, driven by a minimal promoter. LNCaP cells were transiently transfected with pE1bluc-SCAP-122bp or with a similar construct in which the ARE was mutated (*mut*) or deleted (*del*). The next day, medium was replaced, and the cells were treated with R1881 ( $10^{-8}$  M) or ethanol vehicle for 2 days. The cells were lysed, and luciferase activity was measured. Luciferase activity was expressed as fold induction following androgen treatment. Columns, means of three independent experiments; bars, S.E. *wt*, wild type.



**FIG. 6. The SCAP ARE interacts with glucocorticoid receptor as well as androgen receptor.** Binding of GR-DBD to oligonucleotide SCAP 2 short and functional significance of this binding was evaluated as described for the AR-DBD (see legend to Fig. 4).

exposure to dexamethasone stimulated reporter gene activity of the intact construct. Consistent with the binding pattern observed in Fig. 6A, mutation or deletion of the SCAP ARE right half-site within this construct resulted in a loss of glucocorticoid responsiveness. Similar results were obtained by disruption of the GGAAGA sequence within oligonucleotide 2s (data not shown). Binding studies revealed that binding of GR to oligonucleotide 2s reaches a plateau (~90% of total binding) at a GR-DBD concentration of ~880 nM. However, GR-DBD interacted with the SCAP ARE at a somewhat lower affinity than AR-DBD; a  $K_s$  of 294 nM was calculated (306 and 282 nM, respectively, in two independent experiments using two different preparations of GR-DBD (Fig. 6D).

#### DISCUSSION

In previous studies we demonstrated that the marked and coordinated up-regulation of lipogenic genes provoked by androgens in androgen-responsive prostate tumor cells is the result of activation of the SREBP pathway (8). Moreover, using two independent tumor lines we presented evidence that this activation was caused by an androgen-induced increase in the expression of SCAP resulting in an imbalance between SREBP and its retention proteins, translocation of the SREBP-SCAP complex to the Golgi apparatus and proteolytic activation of SREBP (9). In the present paper we searched for regions in the SCAP gene that might mediate androgen-induced transcription either by direct interaction with the AR or through a more indirect mechanism.

Original attempts to identify androgen-responsive regions in the SCAP promoter using promoter-reporter constructs containing either 1.1 or 6 kb of the 5'-upstream region of the SCAP gene failed. Like many other components of the SREBP signaling cascade, the SCAP promoter contains a (putative) SRE (11). Destruction of this SRE strongly reduced basal activity, but no evidence was found for its involvement in the effects of androgens on SCAP induction (data not shown).

In view of these findings a PAC clone harboring the complete human SCAP gene sequence was isolated, and a more systematic search for regions able to mediate androgen responsiveness was performed. Genomic fragments of the SCAP-PAC clone were individually subcloned into the enhancer site of the pGL3 promoter vector, driven by a SV40 promoter. In this way ~70% of the entire gene could be investigated. For the remainder 30% subcloning in the pGL3 vector failed. Transient transfection studies in LNCaP cells pointed to a 4.5-kb region encompassing exons 8–10 as an androgen-responsive region consistently yielding a 3-fold stimulation in reporter expression. By further digestion, the active region could be pinned down to a 122-bp fragment yielding a 9.3-fold stimulation when subcloned in the enhancer of a reporter construct driven by a minimal promoter containing only a TATA box (pE1bluc).

This responsive region displays a number of interesting characteristics. 1) It is located in an intron (intron 8) of the SCAP gene. Intronic localization of androgen-responsive regions has also been reported for the genes encoding the C1 and C3 components of prostatic binding protein (16, 17) and for the gene encoding  $\beta$ -glucuronidase (18). 2) Gel retardation studies reveal that the relevant 122-bp fragment interacts directly with the AR, suggesting that it may contain an ARE. 3) Further analysis reveals the presence of a sequence aagaGGAA-GAaaTGTACctt that bears resemblance to the 5'-TGT-TCT-3' consensus core-binding motif of canonical AREs. This short sequence (SCAP 2s) binds the AR-DBD and is able to confer androgen responsiveness to promoter reporter constructs when transfected in LNCaP. Moreover, mutations of bases considered critical for the function of an ARE or deletions of the GGAAGA left half of the proposed ARE reduce both

binding and activity. The same mutations and deletions also destroyed the ability of the 122-bp fragment to act as an ARE. 4) Because AREs can be divided in at least two categories, the classical AREs, which apart from the AR also bind other class 1 steroid hormone receptors and which are organized as (imperfect) inverted repeats, and the "specific AREs" which selectively recognize the AR and are structured as partial direct repeats (14), we also studied the ability of SCAP 2s to act as a GRE. The data summarized in Fig. 6 indicate that SCAP 2s behaves as a classical ARE and binds the GR-DBD with an affinity that is only slightly lower than that observed for the AR-DBD ( $K_s$  = ~300 nM versus ~150 nM for AR binding).

Taken together, these data support the contention that SCAP is a direct target for androgen action and that androgen-induced increases in SCAP expression are at least in part the result of a direct interaction of the AR with an ARE located in intron 8 of the SCAP gene. At this stage, it cannot be excluded that additional elements may contribute to the androgen-induced lipogenesis in prostate tumor cells. Although SCAP is a pivotal element in the control of SREBP signaling, many elements in this signaling pathway remain incompletely understood and may turn out to be additional targets for androgen action. Similarly, because we could study only 70% of the sequences of the SCAP gene, the existence of additional AREs in some of the uninvestigated domains of the gene cannot be excluded. Another intriguing observation is that stable introduction of the AR in the otherwise androgen-unresponsive context of PC3 prostate cancer cells failed to evoke a full androgen response of the SCAP gene (data not shown). This may point to the requirement of other (*cis*-acting, epigenetic, etc.) factors in addition to the AR to mediate the SCAP response to androgens.

The finding of an ARE in the SCAP gene may not only be relevant to androgen-induced lipogenesis in prostate tumor cells. In fact, it has recently been demonstrated that androgens also provoke a coordinated activation of lipogenic pathways involved in cholesterol and triglyceride synthesis in noncancerous target tissues such as the rat prostate and lacrimal gland (19) and in hamster sebaceous glands (20). In the rat prostate this increase in lipogenesis was shown to be accompanied by activation of the SREBP pathway (19), and SCAP was demonstrated to be an androgen-inducible gene (21). Moreover, steroid induced lipogenesis has also been observed in mammary tumor cells (22, 23). In this case progestagens are the most active inducers, and at least in some cell lines induction seems to be mediated by SREBPs (24). Given the fact that the ARE identified in the SCAP gene belongs to the family of nonselective response elements for steroid receptors of class 1, it is tempting to speculate that here too SCAP may turn out to be a direct target for steroid action.

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