# Androgen-receptor-specific DNA binding to an element in the first exon of the human secretory component gene

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Androgens and glucocorticoids are steroid hormones, which exert their effects *in vivo* by binding and activating their cognate receptors. These intracellular receptors are transcription factors that can bind specific DNA sequences, called hormone response elements, located near the target genes. Although the androgen receptor (AR) and the glucocorticoid receptor (GR) bind the same consensus DNA sequence, androgen-specific responses can be achieved by non-conventional androgen response elements (AREs). Here we determine the specificity mechanism of such a selective element recently identified in the first exon of the human gene for secretory component (*sc* ARE). This *sc* ARE consists of two receptor-binding hexamers separated by three nucleotides. The DNA-binding domains of the AR and GR both bind the *sc* ARE, but, although the AR fragment dimerizes on the element, the GR fragment does not. Comparing the affinities of the DNA-binding domains for mutant forms of the *sc* ARE revealed that

dimeric GR binding is actively excluded by the left hexamer and more precisely by the presence of a G residue at position −3, relative to the central spacer nucleotide. Inserting a G at this position changed a non-selective element into an androgen-selective one. We postulate that the AR recognizes the *sc* ARE as a direct repeat of two 5′-TGTTCT-3′-like core sequences instead of the classical inverted repeat. Direct repeat binding is not possible for the GR, thus explaining the selectivity of the *sc* ARE. This alternative dimerization by the AR on the *sc* ARE is also indicated by the DNA-binding characteristics of receptor fragments in which the dimerization interfaces were swapped. In addition, the flanking and spacer sequences seem to affect the functionality of the *sc* ARE.

Key words: androgen response element, androgen specificity, dimerization, direct repeat, glucocorticoid receptor.

## INTRODUCTION

Steroid hormones are important endocrine messengers with a broad range of physiological functions. They are small, hydrophobic, cholesterol-derived molecules that enter the cell by passive diffusion through the cell membrane and bind to their cognate steroid receptors. The ligand-bound receptors become activated and translocate to specific DNA motifs called hormone response elements (HREs) in the regulatory regions of responsive genes [1]. On binding such motifs, the receptors are thought to interact with the transcription initiation complex, along with co-activators and chromatin factors, resulting in the activation or repression of neighbouring promoters [2]. The steroid receptors belong to the nuclear receptor superfamily [3,4], which can be divided into four classes on the basis of their differences in DNA-binding specificity and binding configuration, which can be homodimeric, heterodimeric or monomeric [5,6]. The androgen receptor (AR) and the glucocorticoid receptor (GR) belong to the same class I receptors that interact with their response elements as homodimers. Their DNA-binding domains (DBDs) are highly conserved and recognize an identical DNA motif [7–9], the consensus of which has been determined as 5′-GGTACAnnnTGTTCT-3′, a partial palindromic repeat of the core sequence 5′-TGTTCT-3′ separated by a three-nucleotide spacer [4,10–12], called androgen/glucocorticoid response element (ARE/GRE).

Because the expression of several genes is normally controlled by only one steroid hormone *in vivo*, this raises the question of

how the specificity of transcriptional responses to the different steroid hormones is established [1]. One possible mechanism for hormonal selectivity is a difference in availability of the receptors and their ligands by cell-type-specific steroid metabolism and/or the tissue-specific expression of receptors [13,14]. Another factor that might control the receptor specificity is the local chromatin structure [15]. This has been shown for the murine-mammary-tumour virus long-terminal-repeat promoter, which is inducible by all class I receptors in transient transfections, whereas the activation from a chromosomally integrated template is limited to the GR [16]. In addition, non-receptor transcription factors that are structural components of androgen-responsive enhancers can bind next to receptor-binding sites or can make receptor-specific interactions potentiating the responsiveness to one specific steroid hormone. This possibility has been proposed for the mouse sex-limited protein (SLP) and the rat 20 kDa protein [17–19]. The existence of non-conventional response elements that are specifically recognized by the AR is a more recently described mechanism for androgen specificity [20–22]. Non-specific binding sites are believed to be high-affinity palindromic AREs matching or nearly matching the consensus, whereas more selective elements, which are bound preferentially by a particular receptor type, can deviate considerably from this consensus [10,20,23].

We previously identified an androgen-specific enhancer approx. 3.5 kb upstream of the transcription initiation site of the human secretory component (SC) gene. This enhancer contains four 5′-TGTTCT-3′-like ARE half-sites (cores 1–4) and a binding

site for nuclear factor I (NF-I) [22]. The major AR-binding site is composed of cores 1 and 2 (*sc* ARE1.2) and is not recognized by the GR *in vitro*. This *sc* ARE1.2 is arranged as an unusual partial direct repeat of two core binding sites with a three-nucleotide spacer instead of the conventional palindromic repeat. Point mutations in the left half-site that increase the inverted repeat character at the expense of the direct repeat character allow GR-DBD binding and increase the responsiveness to glucocorticoids, thus abrogating the androgen specificity. A similar effect has been described for the androgen-specific *slp*-HRE-2 of the SLP enhancer [24]. In-depth analysis of the differences between AR-DBD and GR-DBD provided evidence for a new mode of binding for the AR. Indeed, the AR can bind to direct repeat elements; this results in androgen selectivity [21,25].

Recently, we identified an additional motif in the first exon of the human SC gene (*sc* ARE) conferring an androgen-selective transcriptional response to a heterologous simian virus 40 (SV40) promoter [26]. Here we unravel the underlying mechanism responsible for this selectivity.

## EXPERIMENTAL

### Materials

Restriction and modifying enzymes were obtained from Gibco-BRL Life Technologies (Grand Island, NY, U.S.A.), Pharmacia Biotech (Uppsala, Sweden), Promega (Madison, WI, U.S.A.), Takara Shuzo Co. Ltd. (Shiga, Japan) or Roche (Mannheim, Germany). Oligonucleotides were purchased from Eurogentec (Seraing, Belgium). The sequences of all oligonucleotides used in this study are shown in Figure 1. The SC, C3(1)(SC), SC/C3(SC), C3/SC(SC), mut1(SC) and mut2(SC) oligonucleotides have flanking sequences of the *sc* ARE (5′-ggcNNNNNNctg-NNNNNNtgaag-3′) as indicated by the (SC) annotation. The SC(C3), C3(1), SC/C3(C3), C3/SC(C3), mut1(C3) and mut2(C3) elements have the same core sequences, transferred into the C3(1) flanking sequences (5′-acatNNNNNNtgaNNNNNNcaag-3′) as indicated by the (C3) annotation.

### Plasmid constructs

The SC wild-type, mut88 and mut90 oligonucleotide constructs (see Figure 2), were created by cloning phosphorylated double-stranded oligonucleotides with *Nhe*I and *Eco*RI sticky ends into the *Nhe*I site of a pTK-TATA luciferase reporter vector [21], resulting in a tandem of two oligonucleotides in a head-to-head configuration upstream of the TATA box. The wild-type *sc* ARE consists of the ARE identified in the first exon of the human SC gene from position +77 to position +99 [26]. Mut88 and mut90 have a G → T point mutation at positions +88 and +90 respectively. The SC, C3(1), SC(C3), C3(1)(SC), SC/C3(SC), C3/SC(SC), mut1(SC) and mut2(SC) oligonucleotide constructs, used in transient transfection experiments in T-47D cells (see Figures 5A and 6B) were created by ligating phosphorylated double-stranded synthetic oligonucleotides with *Mlu*I and *Eco*RI sticky ends into the *Mlu*I site of the pGL3 promoter vector (Promega), resulting in the cloning of two oligonucleotides in a head-to-head configuration upstream of the SV40 promoter. All sequences of the sense strands, except for the C3(1)(SC) and SC(C3) constructs, are indicated in Figure 3. The C3(1)(SC) oligonucleotide consisted of the core II sequence of the first intron of the rat C3(1) gene [27], which is indicated in capital letters, placed in the context of the SC sequence, which is indicated in lower-case letters (5′-gccAGTACGctgTGTTCTt-gaa-3′). The SC(C3) oligonucleotide consisted of the SC core

### A. OLIGONUCLEOTIDES IN SC CONTEXT : mutants of the SC wild type

| SC wild type (*Nhe*I/*Eco*RI) | 5′-*ctagc*gccAGCAGGctgTGTCCCtgaag*g*-3′<br>3′-*gcg*gTCGTCCgacACAGGGacttc*cttaa*-5′ |
|---|---|
| mut88 (*Nhe*I/*Eco*RI) | 5′-*ctagc*gccAGCAGGct**t**TGTCCCtgaag*g*-3′<br>3′-*gcg*gTCGTCCga**a**ACAGGGacttc*cttaa*-5′ |
| mut90 (*Nhe*I/*Eco*RI) | 5′-*ctagc*gccAGCAGGctgT**T**TCCCtgaag*g*-3′<br>3′-*gcg*gTCGTCCgacA**A**AGGGacttc*cttaa*-5′ |

### B. OLIGONUCLEOTIDES IN SC CONTEXT : mutants of SC

| SC (*Mlu*I/*Eco*RI) | 5′-*cgcgt*gccAGCAGGctgTGTCCCtgaag*g*-3′<br>3′-*acg*gTCGTCCgacACAGGGacttc*cttaa*-5′ |
|---|---|
| C3(1)(SC) (*Mlu*I/*Eco*RI) | 5′-*cgcgt*gcc**AGTACG**ctg**TGTTCT**tgaag*g*-3′<br>3′-*acg*g**TCATGC**gac**ACAAGA**acttc*cttaa*-5′ |
| C3/SC(SC) (*Mlu*I/*Eco*RI) | 5′-*cgcgt*gcc**AGTACG**ctgTGTCCCtgaag*g*-3′<br>3′-*acg*g**TCATGC**gacACAGGGacttc*cttaa*-5′ |
| SC/C3(SC) (*Mlu*I/*Eco*RI) | 5′-*cgcgt*gccAGCAGGctg**TGTTCT**tgaag*g*-3′<br>3′-*acg*gTCGTCCgac**ACAAGA**acttc*cttaa*-5′ |
| mut1(SC) (*Mlu*I/*Eco*RI) | 5′-*cgcgt*gccAG**T**AGGctgTGTCCCtgaag*g*-3′<br>3′-*acg*gTC**A**TCCgacACAGGGacttc*cttaa*-5′ |
| mut2(SC) (*Mlu*I/*Eco*RI) | 5′-*cgcgt*gccAGCA**C**GctgTGTCCCtgaag*g*-3′<br>3′-*acg*gTCGT**G**CgacACAGGGacttc*cttaa*-5′ |

### C. OLIGONUCLEOTIDES IN C3(1) CONTEXT : mutants of C3(1) ARE

| C3(1) (*Mlu*I/*Eco*RI) | 5′-*cgcg*ttacatAGTACGtgaTGTTCTcaagg*g*-3′<br>3′-aatgtaTCATGCactACAAGAgttcc*cttaa*-5′ |
|---|---|
| C3(1)mut1 (*Mlu*I/*Eco*RI) | 5′-*cgcg*ttacatAGTA**G**GtgaTGTTCTcaagg*g*-3′<br>3′-aatgtaTCAT**CC**actACAAGAgttcc*cttaa*-5′ |

### D. OLIGONUCLEOTIDES IN C3(1) CONTEXT : mutants of SC(C3)

| SC(C3) (*Mlu*I/*Eco*RI) | 5′-*cgcg*ttacatAGCAGGtgaTGTCCCcaagg*g*-3′<br>3′-aatgtaTCGTCCactACAGGGgttcc*cttaa*-5′ |
|---|---|
| C3/SC(C3) (*Mlu*I/*Eco*RI) | 5′-*cgcg*ttacat**AGTACG**tgaTGTCCCcaagg*g*-3′<br>3′-aatgta**TCATGC**actACAGGGgttcc*cttaa*-5′ |
| SC/C3(C3) (*Mlu*I/*Eco*RI) | 5′-*cgcg*ttacatAGCAGGtga**TGTTCT**caagg*g*-3′<br>3′-aatgtaTCGTCCact**ACAAGA**gttcc*cttaa*-5′ |
| mut1(C3) (*Mlu*I/*Eco*RI) | 5′-*cgcg*ttacatAG**T**AGGtgaTGTCCCcaagg*g*-3′<br>3′-aatgtaTC**A**TCCactACAGGGgttcc*cttaa*-5′ |
| mut2(C3) (*Mlu*I/*Eco*RI) | 5′-*cgcg*ttacatAGCA**C**GtgaTGTCCCcaagg*g*-3′<br>3′-aatgtaTCGT**G**CactACAGGGgttcc*cttaa*-5′ |

**Figure 1    Sequences of the oligonucleotides used in this study**

The core sequences, which are indicated in capital letters, are either placed in the SC context (**A**, **B**) or in the C3(1) context (**C**, **D**), as indicated in lower-case letters. Mutated nucleotides are indicated in bold. The oligonucleotides have either *Nhe*I/*Eco*RI (**A**) or *Mlu*I/*Eco*RI (**B–D**) sticky ends, which are indicated in italics.

placed in the C3(1) context (5′-acatAGCAGGtgaTGTCCCcaag-3′). Both oligonucleotides had the same *Mlu*I and *Eco*RI sticky ends. All oligonucleotides are also summarized in Figure 1. The SC, C3(1)(SC), SC/C3(SC), C3/SC(SC), mut1(SC) and mut2(SC) oligonucleotide constructs, used in transient trans-fection experiments in COS-7 cells (see Figure 5B), were created by recloning a *Not*I/*Nhe*I fragment of the corresponding pGL3promoter constructs containing the AREs into the *Not*I and *Nhe*I site of the pTK-TATA vector upstream of the TATA box.

### Cell culture and transient transfection assays

The human breast cancer cell line T-47D and the COS-7 African green monkey kidney cells were obtained from the American Type Culture Collection (A.T.C.C., Manassas, VA, U.S.A.). The cells were grown in Dulbecco's modified Eagle's medium (Gibco-BRL Life Technologies) containing 1000 mg/l glucose, supplemented with 100 i.u./ml penicillin, 0.1 mg/ml streptomycin, 4 $\mu$g/ml insulin and 10 % (v/v) heat-inactivated foetal calf serum

(Gibco-BRL Life Technologies). For the transfection experiments, cells were plated in 24-well dishes (Nunc, Roskilde, Denmark) at a density of $10^5$ cells per well in the same culture medium but with 5% (v/v) foetal calf serum that had been stripped of steroids by treatment with dextran-coated charcoal. At 24 h after plating, the cells were transfected with the FuGENE6 transfection reagent (Roche) as described by the manufacturer, with 500 ng of luciferase reporter plasmid and 50 ng of an expression vector for either the AR or the GR. To correct for transfection efficiency, 50 ng of a $\beta$-galactosidase expression plasmid (pCMV-$\beta$-gal; Stratagene, La Jolla, CA, U.S.A.) was used as an internal control. In transfection experiments in T-47D cells, either the pSV-AR$_0$ human AR expression plasmid [28] or the pRSV-hGR$\alpha$ human GR expression plasmid [29] was co-transfected. In COS-7 cells, the human AR expression plasmid pSG5-hAR [30] or the pSG8-rGR rat GR expression plasmid [31] was used.

After incubation overnight, the medium was replaced and cells were stimulated for 48 h with either the synthetic androgen methyltrienolone (1 nM) (R1881; DuPont–New England Nuclear, Boston, MA, U.S.A.) or the synthetic glucocorticoid dexamethasone (10 nM) (Sigma-Aldrich, Bornem, Belgium), or equal volumes of vehicle (ethanol) alone. After 48 h of stimulation, cells were harvested; protein concentration, luciferase activity and $\beta$-galactosidase activity were then determined as described [26]. Luciferase activity was determined as the amount of chemiluminescence measured per $\mu$g of protein, corrected for transfection efficiency by normalizing against $\beta$-galactosidase activity. The results of the transient transfection experiments are expressed as fold induction, which corresponds to the luciferase activity of extracts of cells stimulated with hormones divided by the activity of non-stimulated cells. All transfection experiments were performed in duplicate at least three times independently ($n = 3$).

### Expression of the AR-DBD, GR-DBD, AG-DBD and GA-DBD

Recombinant DBDs were prepared as glutathione S-transferase fusion proteins with pGEX expression plasmids [21]. In the AG-DBD and GA-DBD constructs, the first Zn-fingers of the AR-DBD (Asp$^{533}$ to Ala$^{569}$) and the GR-DBD (Ala$^{432}$ to Ala$^{467}$) were swapped. AG-DBD contains the first Zn-finger of the AR and the second Zn-finger and the first part of the hinge region of the GR. GA-DBD contained the first Zn-finger of the GR and the second Zn-finger and the first part of the hinge region of the AR. The fusion proteins were expressed and purified as described previously [21]. The purified proteins were aliquoted and stored at $-80$ °C in PBS [140 mM NaCl/2.7 mM KCl/10.1 mM Na$_2$HPO$_4$/1.8 mM KH$_2$PO$_4$ (pH 7.3)] containing 15% (v/v) glycerol. The protein concentration was measured with the Coomassie protein assay (Pierce, Rockford, CA, U.S.A.), and the purity and size of the products obtained were determined by SDS/PAGE.

### Preparation of COS-7 nuclear extracts containing full-size AR and GR

COS-7 cells were transiently transfected with the FuGENE6 transfection reagent (Roche) as described by the manufacturer, with 10 $\mu$g of either the pSG5-hAR human AR expression plasmid [30] or the pSG8-rGR rat GR expression plasmid [31]. At 48 h after transfection, cells were stimulated for 1 h with either the synthetic androgen methyltrienolone (1 nM) (R1881; DuPont-New England Nuclear) or the synthetic glucocorticoid
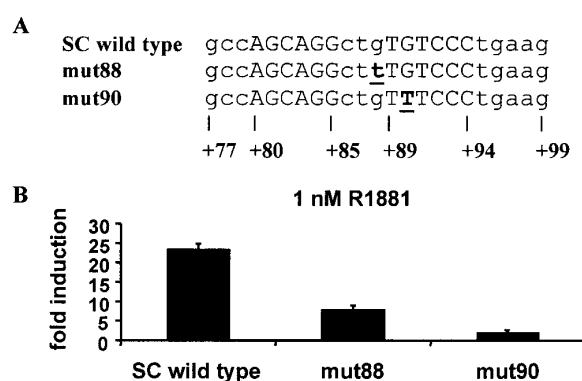


**Figure 2    Sequences (A) and functional analysis in transient transfection experiments in COS-7 cells (B) of the wild-type *sc* ARE and the two mutated elements mut88 and mut90**

COS-7 cells were transiently transfected with the oligonucleotide reporter constructs and were subsequently stimulated for 48 h with 1 nM R1881 as described in the Experimental section. Luciferase activity was determined and the results are represented as fold induction of the luciferase activity of androgen-stimulated cells relative to the activity of cells stimulated with vehicle only. The error bars indicate S.E.M. ($n = 3$).

dexamethasone (10 nM) (Sigma-Aldrich), or equal volumes of vehicle (ethanol) alone. After 1 h of stimulation with hormone, the medium was removed and nuclear extracts of the cells were prepared as described [25]. The nuclear extracts were aliquoted, frozen in liquid nitrogen and stored at $-80$ °C. The protein concentration was measured with the Coomassie protein assay kit (Pierce). The yield was approx. 100 $\mu$g of protein from $10^6$ cells.

### Band-shift assays, determination of the apparent dissociation constant ($K_s$) and supershift assays

Synthetic complementary oligonucleotides were hybridized and labelled; band-shift assays were performed as described previously [26]. For determination of the $K_s$, the radioactivity was measured by scanning dried gels with a PhosphorImager (Molecular Dynamics). The percentage of retarded probe contained in the DNA–protein dimer complexes was plotted against the protein concentration used. The data were analysed with the Fig. P software package (Fig. P Software Corporation, Durham, NC, U.S.A.). Because the DBDs bind as dimers to the DNA and this involves a co-operative protein–protein interaction between monomers, the formula for Hill kinetics resulted in the best fit to the curves and was used for the determination of the $K_s$ values. For an accurate comparison of the DNA-binding affinities of the AR-DBD and GR-DBD for the different probes, the $K_s$ values were determined for data from at least three independent protein preparations. The corresponding binding curves represent the percentages of the total amount of probe that was retarded in a dimeric complex as a function of an increasing amount of protein.

For the supershift assays, 10 $\mu$g of nuclear extracts of COS-7 cells containing overexpressed full-size AR or GR was incubated with the same buffer as for the recombinant DBDs [26], but a larger amount (1 $\mu$g) of poly(dI:dC) was added. To obtain supershifts, a rabbit antiserum against human AR [32] or a monoclonal mouse antibody against rat GR [33] was added. As a negative control for the anti-AR, preimmune serum was added.
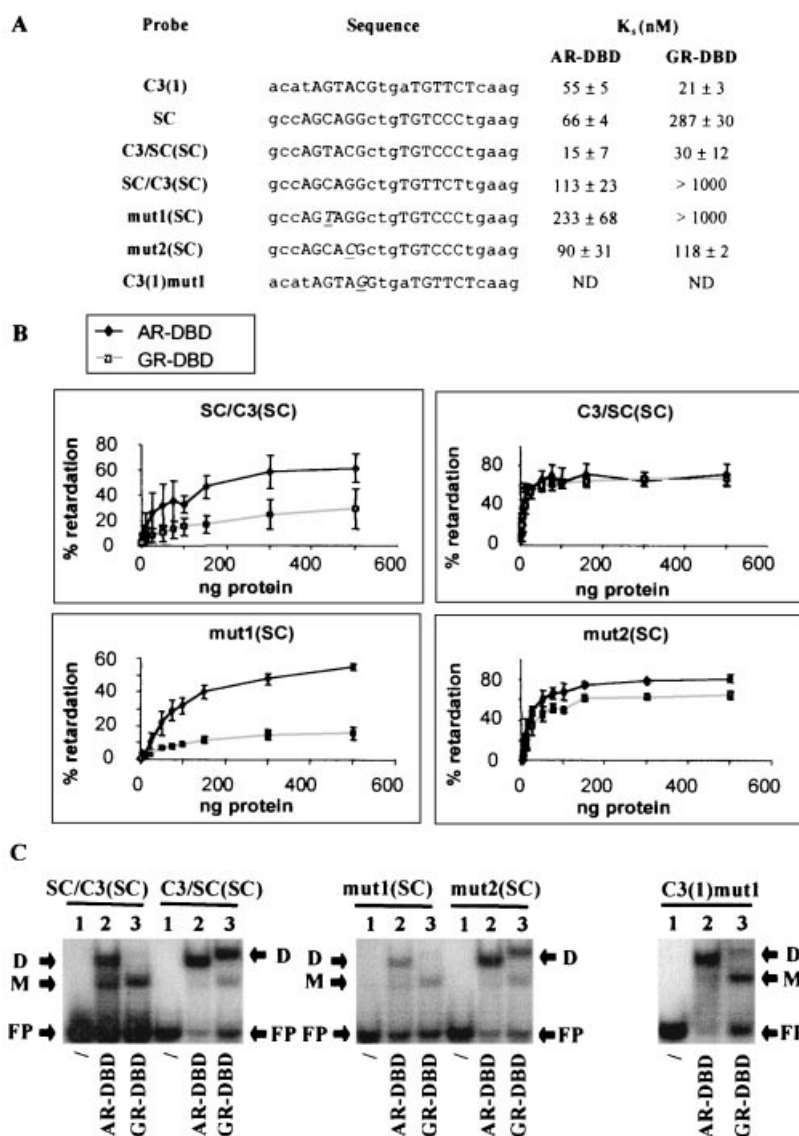
**Figure 3    Substitution analysis of the human *sc* ARE in the SC context, and analysis of the mutant C3(1)mut1 ARE**

(**A**) Names and sequences of the oligonucleotides are given at the left. The core sequences are represented in capital letters, the flanking and spacer sequences in lower-case letters. The binding affinity of the different elements for the AR-DBD and the GR-DBD were analysed by determining the $K_s$ values as described in the Experimental section. $K_s$ values (means ± S.E.M.) were determined on data from three independent protein preparations ($n = 3$). (**B**) Binding curves of the C3/SC(SC), SC/C3(SC), mut1(SC) and mut2(SC) oligonucleotides, representing the percentages of the total amount of probe that was retarded in a dimeric complex as a function of an increasing amount of protein. The error bars indicate S.E.M. ($n = 3$). (**C**) The binding characteristics of the different elements were determined by band-shift assays. Labelled probes were incubated with 100 ng of AR-DBD or GR-DBD. Free and protein-bound DNA were separated on a non-denaturing polyacrylamide gel and revealed by autoradiography. The dimeric complexes (D), monomeric complexes (M) and the free probe (FP) are each indicated with an arrow. ND, not determined.

## RESULTS

### Mapping the core sequence of the *sc* ARE

Recently, we identified by DNase I footprinting a receptor-binding site within the first exon of the human SC gene. It displayed a selective androgen-specific functionality that was correlated with a higher DNA-binding affinity for the AR-DBD than for the GR-DBD [26]. In the present study we investigated how this receptor selectivity is achieved. In the right half of the footprinted sequence, called *sc* ARE, two TGT motifs are present that are known to be recognized by the first Zn-finger of the AR and GR. We mutated both G bases to T, resulting in mut88 and

mut90 respectively (Figure 2A). The wild-type *sc* ARE and both mutants were cloned in duplicate into the pTK-TATA vector upstream of the TATA box; the androgen responsiveness of these constructs was analysed by transient transfection in COS-7 cells (Figure 2B). The wild-type construct was inducible up to 23-fold by 1 nM R1881. Whereas the mutation at position +88 lowered the androgen inducibility to 8-fold, the mutation at position +90 resulted in a complete loss of androgen responsiveness. This indicates that the core sequence of the right half-site of the *sc* ARE can be mapped between positions +89 and +94 and the left half-site is therefore between positions +80 and +85.
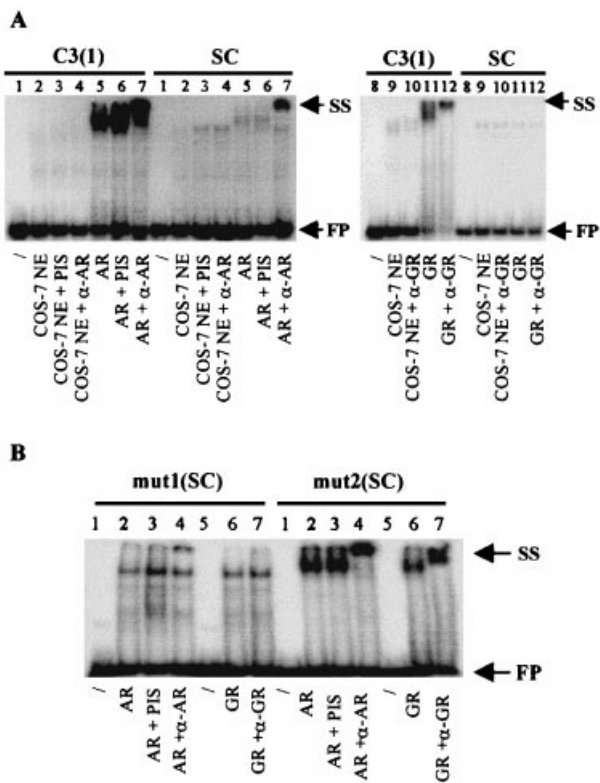
**Figure 4   Supershift experiments of wild-type and mutant AREs with full-size AR and GR**

(**A**) Supershift experiments of the C3(1) and wild-type *sc* AREs with nuclear extracts of COS-7 cells. Band-shift assays were performed as for Figure 3(C). Labelled probes were incubated with either nuclear extracts of untransfected COS-7 cells (COS-7 NE, lanes 2, 3, 4, 9 and 10), cells transfected with an expression plasmid for full-size AR (lanes 5–7) or cells transfected with an expression plasmid for full-size GR (lanes 11 and 12). No protein was added to lanes 1 and 8. The supershifts were obtained with specific antibodies against AR ($\alpha$-AR, lanes 4 and 7) and GR ($\alpha$-GR, lanes 10 and 12). As a negative control for the polyclonal anti-AR, preimmune serum (PIS, lanes 3 and 6) was used. The positions of the free probe (FP) and the supershifted complexes (SS) are each indicated with an arrow. (**B**) Supershift experiments of the mutant mut1(SC) and mut2(SC) AREs in the SC context with nuclear extracts of COS-7 cells transfected with full-size AR or GR. The experiments were performed by incubating the labelled probes shown in Figure 3(A) with nuclear extracts of COS-7 cells transiently transfected with full-size AR (lanes 2–4) or GR (lanes 6 and 7). No protein was added to lanes 1 and 5. The supershifts were obtained with specific antibodies against AR ($\alpha$-AR, lane 4) or GR ($\alpha$-GR, lane 7). As a negative control for the polyclonal anti-AR, preimmune serum (PIS, lane 3) was used. The positions of the free probe (FP) and the supershifted complexes (SS) are each indicated with an arrow.

## AR specificity is due to the upstream half-site of the *sc* ARE

To determine which part of the *sc* ARE is responsible for the androgen specificity, we analysed the binding of the AR-DBD and the GR-DBD to chimaeric elements containing parts of the specific *sc* ARE [26] and the non-specific ARE of the first intron of the rat C3(1) gene [27]. The chimaeric elements were created by replacing the left or right half-site of the *sc* ARE by the corresponding half-site of the C3(1) element, resulting in C3/SC(SC) and SC/C3(SC) respectively (Figure 3A). The $K_s$ values of the AR-DBD and the GR-DBD for the different sequences were determined from allosteric Hill kinetics (Figure 3A). The AR-DBD and the GR-DBD showed equal affinities for the C3/SC(SC) element, the $K_s$ values being $15 \pm 7$ and $30 \pm 12$ nM (means $\pm$ S.E.M.) respectively, whereas AR-DBD had a much higher affinity for the SC/C3(SC) element than the GR-DBD,

the $K_s$ values being $113 \pm 23$ and more than 1000 nM respectively. The corresponding binding curves are shown in Figure 3(B). In band-shift assays with 100 ng of either recombinant AR-DBD or GR-DBD, the retarded complexes were either predominantly dimeric or monomeric (Figure 3C). Because the chimaeric element C3/SC(SC) did not show any difference in DNA-binding characteristics between the AR-DBD and GR-DBD, it can be considered a non-specific element, like the C3(1) ARE. In contrast, the SC/C3(SC) element behaved like the SC wild-type element, because it bound the AR-DBD preferentially as a dimer and the GR-DBD as a monomer. It can therefore be considered an androgen-specific element. The non-specificity of the C3/SC(SC) element and the androgen specificity of the SC/C3(SC) element was also confirmed by the $K_s$ values of AR-DBD and GR-DBD binding.

## GR-DBD dimerization is prevented by a G at position $-3$

Because the specificity of the *sc* ARE was due to the left half-site (5′-AGCAGG-3′) and because this sequence differed in only two nucleotides from the corresponding half-site of the non-selective C3(1) ARE (5′-AG<u>TAC</u>G-3′), two mutants of the *sc* ARE, mut1(SC) and mut2(SC), were synthesized in which one of these nucleotides was converted to the corresponding nucleotide of the C3(1) ARE. The binding characteristics of both probes showed that mut1(SC) bound AR-DBD preferentially as a dimer and the GR-DBD as a monomer, whereas mut2(SC) bound both DBDs as a dimer (Figures 3A and 3C). $K_s$ values were determined as for the chimaeric C3/SC(SC) and SC/C3(SC) elements. Mut2(SC) showed similar DNA-binding affinities for the AR-DBD and the GR-DBD because the corresponding $K_s$ values were $90 \pm 31$ and $118 \pm 2$ nM respectively. Mut1(SC), in contrast, had a higher affinity for the AR-DBD ($K_s = 233 \pm 68$ nM) than for the GR-DBD ($K_s > 1000$ nM). The corresponding binding curves are shown in Figure 3(B). These data indicate that the G at position $-3$ in the left half-site of the *sc* ARE (5′-AGCA<u>G</u>G-3′) has a key role in its specificity. To test whether this would be a general mechanism, we introduced a G at the same position in the non-specific C3(1) ARE, resulting in C3(1)mut1 (Figure 3A). In gel-shift assays with 100 ng of recombinant DBDs, this mutated C3(1) ARE was bound by dimeric AR-DBD and monomeric GR-DBD (Figure 3C). This confirms the destructive role of a G at position $-3$ for the binding of dimeric GR-DBD.

## Binding specificity of the full-size receptors

To analyse the influence of the other receptor domains on the binding of DNA to the non-specific and specific C3(1) and *sc* AREs, band shifts with full-size AR and GR expressed in COS-7 cells were performed (Figure 4A). A Western blot analysis of COS-7 nuclear extracts confirmed the high level of expression of the full-size AR and GR (results not shown). Band-shift experiments with nuclear extracts of untransfected cells showed only very weak or almost no interaction with either the C3(1) or the *sc* AREs. In addition, no supershift was seen when preimmune serum, anti-AR or anti-GR was added. This was in contrast with the band shifts with the nuclear extracts of COS-7 cells transfected with expression plasmids for AR or GR, which showed a clear interaction between the C3(1) ARE and the AR and GR. Specific supershifts were observed with the C3(1) ARE when anti-AR and anti-GR were added, which was not seen with the preimmune serum. This indicates that the overexpressed receptors could interact specifically with the C3(1) ARE. Although under the same conditions the SC probe showed only a weak interaction with the overexpressed AR, the complex could clearly be
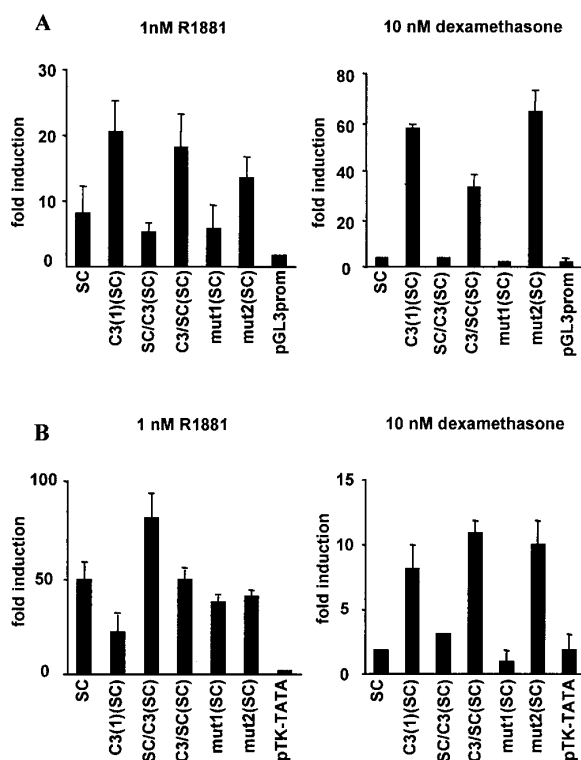
**Figure 5 Functional analysis of the wild-type, chimaeric and mutant AREs in the SC context by transient transfection experiments of oligonucleotide constructs in T-47D cells (A) and COS-7 cells (B)**

All elements were cloned in duplicate into the pGL3 promoter vector upstream of the SV40 promoter (T-47D cells) or into the pTK-TATA vector upstream of the TATA box (COS-7 cells). The cells were transiently transfected with those constructs and were subsequently stimulated for 48 h with 1 nM R1881 or 10 nM dexamethasone as described in the Experimental section. The results are represented as fold inductions of the luciferase activity in hormone-stimulated cells relative to the activity in cells stimulated with vehicle only. Error bars indicate S.E.M. ($n = 3$).

supershifted with the anti-AR but not with the preimmune serum, indicating that the *sc* ARE did indeed interact specifically with the full-size AR. However, under the same conditions no interaction was observed between the *sc* ARE and full-size GR.

Similar supershift experiments were performed with the mutant mut1(SC) and mut2(SC) probes (Figure 4B). The mut2(SC) showed specific supershifted complexes with both full-size AR and GR, in contrast with the mut1(SC) probe, which showed a supershift only with AR and not with GR. This indicates that the interactions of the *sc* ARE and the other specific elements with the DBDs were reproducible when using full-size receptor, and that the DBD and the first part of the hinge region were sufficient for the specificity.

## Transcriptional functionality is correlated with the binding characteristics

To analyse whether the differences in receptor binding *in vitro* were correlated with differences in responsiveness to androgens and glucocorticoids, the functionality of the SC, C3(1)(SC), SC/C3(SC), C3/SC(SC), mut1(SC) and mut2(SC) AREs was tested. Oligonucleotide constructs were transiently transfected into either T-47D or COS-7 cells, which were co-transfected with an expression plasmid for either AR or GR (Figure 5). The cells
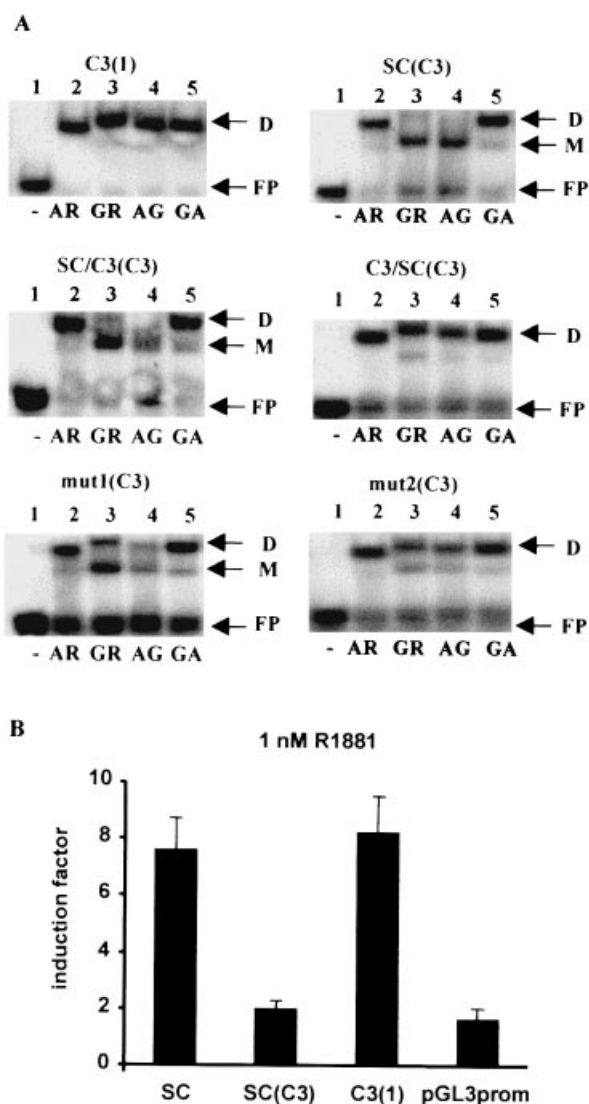
**Figure 6 Analysis of the influence of the context of an ARE on the binding characteristics of the AR-DBD and the GR-DBD *in vitro* and on transcriptional functionality**

(**A**) Band-shift assays of the C3(1), SC(C3), SC/C3(C3), C3/SC(C3), mut1(C3) and mut2(C3) AREs in the C3(1) context with the AR-DBD and the GR-DBD and the mutant AG-DBD and GA-DBD. The sequence of the C3(1) ARE is shown in Figure 3(A). The core sequences of the SC, SC/C3(SC), C3/SC(SC), mut1(SC) and mut2(SC) elements, indicated in capital letters in Figure 3(A), were transferred into the C3(1) flanking sequences (5′-acatNNNNNNtgaNNNNNNcaag-3′), resulting in the SC(C3), SC/C3(C3), C3/SC(C3), mut1(C3) and mut2(C3) elements respectively. Band-shift assays were performed by incubating labelled probes with 100 ng of AR-DBD, GR-DBD, AG-DBD or GA-DBD (lanes 2, 3, 4 and 5 respectively). Protein–DNA complexes were separated from the free probe by PAGE. The dimeric (D) and monomeric (M) complexes and the free probe (FP) are each indicated with an arrow. (**B**) Comparison of the responsiveness of the *sc* ARE in the SC and C3(1) context. T-47D cells were transiently transfected with the indicated oligonucleotide constructs and then stimulated for 48 h with 1 nM R1881 as described in the Experimental section. The fold induction of the luciferase activity of stimulated cells relative to cells stimulated with vehicle only is given. Error bars indicate S.E.M. ($n = 3$). pGL3prom, pGL3 promoter vector.

were subsequently stimulated with either the synthetic androgen methyltrienolone (R1881) or the synthetic glucocorticoid dexamethasone. Although in both cell lines all constructs were responsive to androgens, with induction factors of 5–21 in T-47D and 22–82 in COS-7 cells, only the non-specific C3(1)(SC),

C3/SC(SC) and mut2(SC) constructs were inducible by gluco-corticoids, with induction factors of 34–64 in T-47D and 8–10 in COS-7 cells. The androgen inducibility of the specific SC, SC/C3(SC) and mut1(SC) elements was somewhat lower than that of the non-specific elements in the T-47D cell line but not in the COS-7 cell line.

### Influence of surrounding sequences on the functionality of AREs

All core sequences of the SC, SC/C3(SC), C3/SC(SC), mut1(SC) and mut2(SC) oligonucleotides were transferred into the C3(1) flanking sequence, resulting in the SC(C3), SC/C3(C3), C3/SC(C3), mut1(C3) and mut2(C3) elements respectively. The binding characteristics of these elements were determined by band-shift assays with the AR-DBD and the GR-DBD (Figure 6A, lanes 2 and 3). All elements that were specific in the SC context retained this property in the C3(1) context because the SC(C3), SC/C3(C3) and mut1(C3) probes bound AR-DBD preferentially as a dimer, in contrast with the GR-DBD, which was bound as a monomer. The non-specific elements C3(1), C3/SC(C3) and mut2(C3) bound both receptor DBDs as a dimer. Supershift experiments of the elements in the C3(1) context with full-size receptors confirmed that the SC(C3), SC/C3(C3) and mut1(C3) elements were androgen-specific in binding studies *in vitro*, and that the C3(1), C3/SC(C3) and mut2(C3) elements were non-specific (results not shown). The elements in the C3(1) context had similar $K_s$ values and thus similar affinities for the AR-DBD and the GR-DBD to those of the corresponding elements in the SC context (results not shown). The flanking sequences therefore do not seem to influence markedly the binding characteristics of an ARE *in vitro*.

To investigate to what extent the surrounding sequences affect the transcriptional activation potential of an ARE, the androgen responsiveness of the SC and SC(C3) elements were compared in transient transfection experiments in T-47D cells, which were co-transfected with an expression plasmid for AR. Surprisingly, in contrast with the SC and C3(1) constructs, which were clearly androgen responsive, the SC(C3) construct was not induced at all (Figure 6B).

### Determination of the binding characteristics by the second Zn-finger

To determine which part of the DBD has a role in the specific recognition of the *sc* ARE, band-shift assays were performed with mutant AR-DBD and GR-DBD in which the first Zn-fingers had been swapped, resulting in the mutant AG-DBD and GA-DBD respectively [21]. Both constructs are known to bind DNA. The binding characteristics of the C3(1), SC(C3), SC/C3(C3), C3/SC(C3), mut1(C3) and mut2(C3) elements were compared (Figure 6A). The GA-DBD and the AR-DBD bound all probes as dimers. However, although the first Zn-finger is known to contain all the sequence-discriminating residues [21], the AG construct did not bind the AR-specific elements SC(C3), SC/C3(C3) and mut1(C3) as a dimer.

### DISCUSSION

### Exact mapping of the *sc* ARE

The AR and GR recognize very similar DNA motifs [7,8]. However, the existence of AR-specific response elements was demonstrated first for the rat probasin gene [20] and also for the upstream enhancers of the mouse gene encoding SLP [17,24] and the human SC gene [22]. Here we have studied in more detail the

underlying mechanism responsible for the specificity of another element, the *sc* ARE, which we previously identified in the first exon of the human SC gene [26]. We situated the exact position of the core sequence of the right half-site between positions +89 and +94 (5′-TGTCCC-3′) and not between positions +87 and +92 (5′-TGTGTC-3′) (Figure 2). This is not surprising because, although the latter core sequence has the first three bases (position +2 to +4, relative to the central spacer nucleotide) identical with those of the consensus, the last three nucleotides (positions +5 to +7), indicated in bold, diverge greatly. The base at position +5 does not make contact with the receptor, although a pyrimidine is preferred in this position [10,34]. The sixth position in the consensus is occupied by a C. The complementary G makes specific contacts with the GR-DBD in crystal structures [35] and is always present in natural GREs [36].

### Androgen selectivity is due to the presence of a G at position −3

An analysis of chimaeric elements by band-shift assays revealed that the specificity of the *sc* ARE can be contributed by its left half-site (Figure 3). This was also seen for the PB-ARE-2 element of the rat probasin gene [20] and the upstream *sc* ARE1.2 of the human SC gene [22]. A more detailed point mutation analysis of the *sc* ARE revealed that the G at position −3 is critical for the specificity of the ARE. Moreover, replacing the C by a G at position −3 in the C3(1) ARE switches the non-specific element into a specific one. In the classical inverted repeat recognition by the GR-DBD, the G in the opposite strand at position −3 is contacted by the receptor and is essential for high-affinity binding [12]. Although a C at position −3 is highly conserved in naturally occurring AR binding sites, some elements do have a G at position −3, as in ARBSd/crp2 [37], MVDP AREp [38] and MSVSP99 AREp [39]. It would be interesting to test whether these elements are responsive to glucocorticoids. The mechanism of specificity of the *sc* ARE seems different from that described previously for PB-ARE-2, *slp*-HRE-2 and *sc*-ARE1.2 because they have no G at position −3 [24,25].

### Importance of the dimerization interface in androgen specificity

All non-specific elements used in the band-shift assays in this study bound both the AR-DBD and the GR-DBD as a dimer, whereas the specific elements bound dimeric AR-DBD, in contrast with the GR-DBD, which bound only as a monomer (Figure 3C). Dimeric binding suggests a high degree of co-operativity for the AR-DBD, which is not allowed for the GR-DBD. For the GR, the presence of a G at position −3 in the *sc* ARE is most probably responsible for a repulsive force on the second monomer that is stronger than the co-operativity driving towards dimeric binding. Band-shift assays with mutant AR-DBD and GR-DBD in which the first Zn-fingers had been swapped showed that the second Zn-finger and the first part of the hinge region of the AR are necessary for dimeric DBD binding to selective AREs (Figure 6A). Because the second Zn-finger is known to be involved in the DNA-dependent dimerization, we propose that the AR specificity of the *sc* ARE is indeed due to the inability of the GR-DBD to dimerize on it co-operatively. Because full-size GR is also unable to bind the AR-selective sequences (Figure 4), the dimerization function in the ligand-binding domain [4] does not seem to have a role in androgen selectivity.

Class I nuclear receptors are known to recognize response elements that consist of a partial palindrome of two core sequences separated by three spacer nucleotides and to bind such sequence as homodimers in a head-to-head way [5,6]. However,
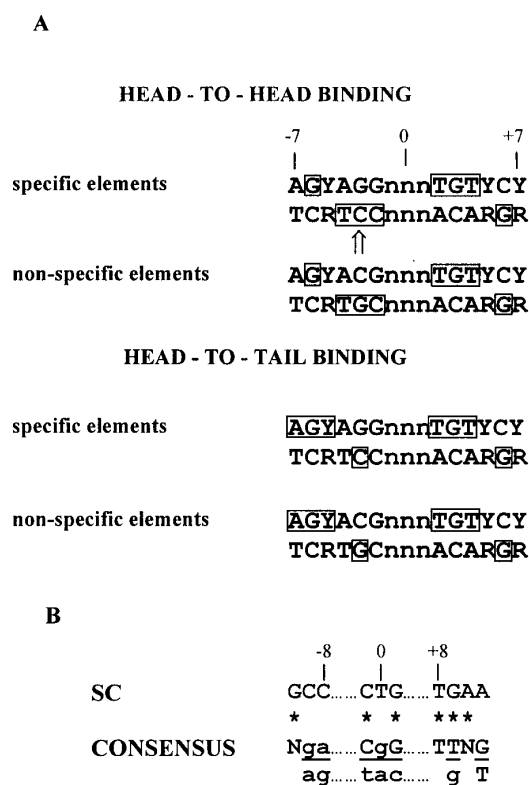
**A**

HEAD - TO - HEAD BINDING

```
            -7          0         +7
             |          |          |
specific elements    AGYAGGnnnTGTYCY
                     TCRTCCnnnACARGR
                            ⇑
non-specific elements AGYACGnnnTGTYCY
                     TCRTGCnnnACARGR
```

HEAD - TO - TAIL BINDING

```
specific elements    AGYAGGnnnTGTYCY
                     TCRTCCnnnACARGR


non-specific elements AGYACGnnnTGTYCY
                     TCRTGCnnnACARGR
```

**B**

```
                 -8      0      +8
                  |      |       |
     SC          GCC....CTG....TGAA
                 *      * *     ***
 CONSENSUS       Nga....CgG....TTNG
                 ag.....tac....  g  T
```

**Figure 7   Comparison of different AR-selective and non-selective consensus sequences with the *sc* ARE**

(**A**) The consensus sequences of the AR-specific and non-specific elements were determined. Nucleotides within the core half-sites that made specific contacts with the AR-DBD and the GR-DBD either in the conventional head-to-head or in the alternative head-to-tail binding are boxed. An arrow indicates the G at position $-3$ that we believe to exclude the GR from head-to-head binding. The positions in the binding sites are numbered relative to the central spacer nucleotide, which is designated as position 0. (**B**) Comparisons of the context of the *sc* ARE and the consensus of the AR-specific flanking sequences [40]. The flanking and spacer sequences of the *sc* and C3(1) AREs are compared and aligned with the consensus for AR-specific binding sites, as determined by a competitive amplification and binding assay in which AR-binding sites were selectively enriched in the presence of competing GR [40]. Strongly selected nucleotides are denoted in capital letters; nucleotides selected to a smaller degree are shown in lower-case letters. The nucleotides of the SC sequence that fit the consensus are each indicated with an asterisk. The positions of the nucleotides are numbered relative to the position of the central spacer nucleotide, which is designated as position 0.

the AR can have a different mode of binding because it can recognize direct repeats of the same 5′-TGTTCT-3′ core sequence [22,24,25]. Indeed, the probasin promoter, the SC upstream enhancer and the mouse gene encoding SLP contain such AR-selective direct repeats [22,24]. The results that we obtained here from a mutational analysis of the *sc* ARE are in agreement with our hypothesis of an AR-selective binding to direct repeats.

Consensus sequences derived from all specific, as opposed to non-specific, elements from this report are shown in Figure 7(A). In a classical head-to-head binding, major contacts between the receptor and the DNA are at positions $+2$, $+3$ and $+4$ in the sense strand and at $+6$ in the anti-sense strand for the right half-site and at positions $-2$, $-3$ and $-4$ in the anti-sense strand and at $-6$ in the sense strand for the left half-site [12]. In the consensus of the AR-specific elements, the G at position $-3$ in the sense strand, corresponding to a C in the other strand (indicated with an arrow), probably excludes the head-to-head binding. All bases of the non-specific consensus that contact the

receptor permit DNA–protein contacts in classical head-to-head binding. In the alternative head-to-tail binding, the receptor molecule is supposed to contact the DNA at positions $-7$, $-6$ and $-5$ in the upper strand and at position $-3$ in the lower strand. The G at position $-6$, which is essential for DNA binding in the GR-DBD–GRE co-crystal, is present. Thus we postulate that the key mechanism for specificity is the difference in dimerization possibilities between the AR and GR. The presence of a G at position $-3$ prevents the receptor from binding in the classical head-to-head manner and leaves only the alternative head-to-tail configuration as a possible dimeric mode of binding. This latter configuration is only allowed for the AR and not for the GR.

When comparing the PB-ARE-2, *sc*-ARE1.2 and *slp*-HRE-2, another AR-specific consensus was determined (5′-KGNTCWn-nnAGTWCT-3′) [24]. In this sequence there is a C at position $-3$. However, the T at position $-4$ was proved to be important for the specificity. This T probably makes the AR-specific head-to-tail configuration as the preferred option for the receptor to interact with the motif, as does the G at position $-3$ in the *sc* ARE.

Functional analysis of the wild-type, chimaeric and mutant *sc* AREs in transient transfection assays revealed that all elements were responsive to androgens, whereas only the elements that could bind dimeric GR-DBDs were responsive to glucocorticoids (Figure 5).

There are clear differences in androgen responsiveness of the different constructs in the two cell lines used. However, most of these differences are probably attributable to the overexpression of the receptors in the COS-7 cells after co-transfection of expression plasmids of the AR and GR. Although the T-47D cell line has the advantage of low receptor levels when expression plasmids are co-transfected, this cell line has the disadvantage of the presence of other steroid receptors, which is not true of the COS-7 cells.

## Influence of surrounding sequences on functionality

Roche et al. [11] have shown the impact of spacer and flanking sequences of AR-binding sites on their functionality, but AR selectivity was not examined. However, AR-specific sequences have also been amplified in a selection assay that included competing GR-DBD [40]. When the SC sequence is compared with the consensus of the sequences thus obtained (Figure 7B), the first two nucleotides (TG) immediately downstream of the right half-site at positions $+8$ and $+9$, relative to the central spacer nucleotide, and the C and G in the spacer sequence at positions $-1$ and $+1$ respectively are present in both elements.

We therefore examined the influence of the surrounding nucleotides of the *sc* ARE. Transferring the core sequences from the SC context to the C3(1) context did not have any effect on the AR-DBD and GR-DBD binding characteristics *in vitro* (Figure 6A, and results not shown). Surprisingly, moving the *sc* ARE from its own context into the C3(1) context greatly impaired its functionality in the transfection assays (Figure 6B). The same was true for the other AR-specific elements SC/C3(C3) and mut1(C3), which showed AR-selectivity in transfection assays only in the SC context, but not in the C3(1) context (Figure 5, and results not shown). These results indicate that the observation of a dimeric complex *in vitro* is not strictly correlated with transactivation by the receptors. Surrounding sequences could provide additional stabilizing protein–DNA contacts necessary for the formation of a surface for the recruitment of co-activator complexes or for interaction with the basal transcription ma-

chinery. These additional stabilizing forces could be crucial for low-affinity binding sites; sequences surrounding the core could thereby contribute to functionality. This is probably true of the specific low-affinity binding site *sc* ARE, whose functionality depends on the presence of its own context.

The classical DNA-binding site selection assay revealed a small preference for G at position +1 in the spacer and for A and T in the 3′ flanking region [11]. Mutational analysis of these preferred sites led to the conclusion that sequences surrounding the two 6 bp half-sites influence both the binding affinity for the AR and the functional activity of the response element. These results fit our data well, because the *sc* ARE does indeed have a G at position +1 in the spacer. In addition, three of the four bases downstream of the right half-site of the *sc* ARE are T or A. The role of the G in the spacer is also indicated by the effect of its mutation in the *sc* ARE (mut88 in Figure 2B). However, whereas the mut88 construct is still responsive to androgens, the SC(C3) construct is not, which again indicates the contribution of the spacer sequence and other flanking sequences to functionality.

In conclusion, we have shown that a single nucleotide in the left half-site excludes the GR from binding the *sc* ARE. The presence of a G at position −3 seems to impair the classical head-to-head binding. Because the AR is known to bind some DNA elements in an alternative head-to-tail configuration, we think that this explains the specificity of the *sc* ARE. In addition, it is clear that on low-affinity binding sites the surrounding nucleotides can contribute to the functionality of the AREs. It remains to be elucidated whether this involves secondary protein–DNA interactions or whether the surrounding sequences impose structural constraints when the elements are cloned in a circular plasmid and transferred to the nucleus of transfected cells.

## REFERENCES

1   Beato, M. (1989) Gene regulation by steroid hormones. Cell **56**, 335–344
2   Xu, L., Glass, C. K. and Rosenfeld, M. G. (1999) Coactivator and corepressor complexes in nuclear receptor function. Curr. Opin. Genet. Dev. **9**, 140–147
3   Evans, R. M. (1988) The steroid and thyroid hormone receptor superfamily. Science **240**, 889–894
4   Tsai, M.-J. and O'Malley, B. W. (1994) Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu. Rev. Biochem. **63**, 451–486
5   Forman, B. M. and Samuels, H. H. (1990) Interactions among a subfamily of nuclear hormone receptors: the regulatory zipper model. Mol. Endocrinol. **4**, 1293–1301
6   Glass, C. K. (1994) Differential recognition of target genes by nuclear receptor monomers, dimers, and heterodimers. Endocr. Rev. **15**, 391–407
7   Cato, A. C. B., Henderson, D. and Ponta, H. (1987) The hormone response element of the mouse mammary tumour virus DNA mediates the progestin and androgen induction of transcription in the proviral long terminal repeat region. EMBO J. **6**, 363–368
8   Ham, J., Thomson, A., Needham, M., Webb, P. and Parker, M. (1988) Characterization of response elements for androgens, glucocorticoids and progestins in mouse mammary tumour virus. Nucleic Acids Res. **16**, 5263–5276
9   Umesono, K. and Evans, R. M. (1989) Determinants of target gene specificity for steroid/thyroid hormone receptors. Cell **57**, 1139–1146
10  Nordeen, S. K., Suh, B. J., Kühnel, B. and Hutchison III, C. A. (1990) Structural determinants of a glucocorticoid receptor recognition element. Mol. Endocrinol. **4**, 1866–1873
11  Roche, P. J., Hoare, S. A. and Parker, M. G. (1992) A consensus DNA-binding site for the androgen receptor. Mol. Endocrinol. **6**, 2229–2235
12  Truss, M. and Beato, M. (1993) Steroid hormone receptors: interaction with deoxyribonucleic acid and transcription factors. Endocrine Rev. **14**, 459–479
13  Strähle, U., Boshart, M., Klock, G., Stewart, F. and Schütz, G. (1989) Glucocorticoid- and progesterone-specific effects are determined by differential expression of the respective hormone receptors. Nature (London) **339**, 629–632
14  Funder, J. W. (1993) Mineralocorticoids, glucocorticoids, receptors and response elements. Science **259**, 1132–1133
15  Li, Q. and Wrange, Ö. (1995) Accessibility of a glucocorticoid response element in a nucleosome depends on its rotational positioning. Mol. Cell. Biol. **15**, 4375–4384
16  Lambert, R. J. and Nordeen, S. K. (1998) Steroid-selective initiation of chromatin remodeling and transcriptional activation of the mouse mammary tumor virus promoter is controlled by the site of promoter integration. J. Biol. Chem. **273**, 32708–32714
17  Adler, A. J., Scheller, A. and Robins, D. M. (1993) The stringency and magnitude of androgen-specific gene activation are combinatorial functions of receptor and nonreceptor binding site sequences. Mol. Cell. Biol. **13**, 6326–6335
18  Ho, K.-C., Marschke, K. B., Tan, J., Power, S. G. A., Wilson, E. M. and French, F. S. (1993) A complex response element in intron 1 of the androgen-regulated 20-kDa protein gene displays cell type-dependent androgen receptor specificity. J. Biol. Chem. **268**, 27226–27235
19  Ning, Y.-M. and Robins, D. M. (1999) AML3/CBFalpha1 is required for androgen-specific activation of the enhancer of the mouse sex-limited protein (Slp) gene. J. Biol. Chem. **274**, 30624–30630
20  Claessens, F., Alen, P., Devos, A., Peeters, B., Verhoeven, G. and Rombauts, W. (1996) The androgen-specific probasin response element 2 interacts differentially with androgen and glucocorticoid receptors. J. Biol. Chem. **271**, 19013–19016
21  Schoenmakers, E., Alen, P., Verrijdt, G., Peeters, B., Verhoeven, G., Rombauts, W. and Claessens, F. (1999) Differential DNA binding by the androgen and glucocorticoid receptors involves the second Zn-finger and a C-terminal extension of the DNA-binding domains. Biochem. J. **341**, 515–521
22  Verrijdt, G., Schoenmakers, E., Alen, P., Haelens, A., Rombauts, W. and Claessens, F. (1999) Androgen specificity of a response unit upstream of the human secretory component gene is mediated by differential receptor binding to an essential androgen response element. Mol. Endocrinol. **13**, 1558–1570
23  Rundlett, S. E. and Miesfeld, R. L. (1995) Quantitative differences in androgen and glucocorticoid receptor DNA binding properties contribute to receptor-selective transcriptional regulation. Mol. Cell. Endocrinol. **109**, 1–10
24  Verrijdt, G., Schoenmakers, E., Haelens, A., Peeters, B., Verhoeven, G., Rombauts, W. and Claessens, F. (2000) Change of specificity mutations in androgen-selective enhancers. J. Biol. Chem. **275**, 12298–12305
25  Schoenmakers, E., Verrijdt, G., Peeters, B., Verhoeven, G., Rombauts, W. and Claessens, F. (2000) Differences in DNA binding characteristics of the androgen and glucocorticoid receptors can determine hormone-specific responses. J. Biol. Chem. **275**, 12290–12297
26  Haelens, A., Verrijdt, G., Schoenmakers, E., Alen, P., Peeters, B., Rombauts, W. and Claessens, F. (1999) The first exon of the human *sc* gene contains an androgen responsive unit and an interferon regulatory factor element. Mol. Cell. Endocrinol. **153**, 91–102
27  Claessens, F., Celis, L., Peeters, B., Heyns, W., Verhoeven, G. and Rombauts, W. (1989) Functional characterization of an androgen response element in the first intron of the C3(1) gene of prostatic binding protein. Biochem. Biophys. Res. Commun. **164**, 833–840
28  Brinkmann, A. O., Faber, P. W., van Rooij, H. C. J., Kuiper, G. G. J. M., Ris, C., Klaassen, P., van der Korput, J. A. G. M., Voorhorst, M. M., van Laar, J. H., Mulder, E. and Trapman, J. (1989) The human androgen receptor: domain structure, genomic organization and regulation of expression. J. Steroid Biochem. **34**, 307–310
29  Giguère, V., Hollenberg, S. M., Rosenfeld, M. G. and Evans, R. M. (1986) Functional domains of the human glucocorticoid receptor. Cell **46**, 645–652
30  Alen, P., Claessens, F., Schoenmakers, E., Swinnen, J. V., Verhoeven, G., Rombauts, W. and Peeters, B. (1999) Interaction of the putative androgen receptor-specific coactivator ARA70/ELE1$\alpha$ with multiple steroid receptors and identification of an internally deleted ELE1$\beta$ isoform. Mol. Endocrinol. **13**, 117–128
31  Schmitt, J. and Stunnenberg, H. G. (1993) The glucocorticoid receptor hormone binding domain mediates transcriptional activation *in vitro* in the absence of ligand. Nucleic Acids Res. **21**, 2673–2681

32   Marivoet, S., Hertogen, M., Verhoeven, G. and Heyns, W. (1990) Antibodies against synthetic peptides recognize the human and rat androgen receptor. J. Steroid Biochem. Mol. Biol. **37**, 39–45

33   Okret, S., Wikström, A.-C., Wrange, Ö., Andersson, B. and Gustafsson, J.-Å. (1984) Monoclonal antibodies against the rat liver glucocorticoid receptor. Proc. Natl. Acad. Sci. U.S.A. **81**, 1609–1613

34   Zilliacus, J., Wright, A. P. H., Carlstedt-Duke, J. and Gustafsson, J.-Å. (1995) Structural determinants of DNA-binding specificity by steroid receptors. Mol. Endocrinol. **9**, 389–400

35   Luisi, B. F., Xu, W. X., Otwinowski, Z., Freedman, L. P., Yamamoto, K. R. and Sigler, P. B. (1991) Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. Nature (London) **352**, 497–505

36   Beato, M., Chalepakis, G., Schauer, M. and Slater, E. P. (1989) DNA regulatory elements for steroid hormones. J. Steroid Biochem. **32**, 737–748

37   Devos, A., Claessens, F., Alen, P., Winderickx, J., Heyns, W., Rombauts, W. and Peeters, B. (1997) Identification of a functional androgen-response element in the exon 1-coding sequence of the cystatin-related protein gene *crp2*. Mol. Endocrinol. **11**, 1033–1043

38   Fabre, S., Manin, M., Pailhoux, E., Veyssière, G. and Jean, C. (1994) Identification of a functional androgen response element in the promoter for the gene of the androgen-regulated aldose reductase-like protein specific to the mouse vas deferens. J. Biol. Chem. **269**, 5857–5864

39   Brochard, D., Morel, L., Cheyvialle, D., Veyssiere, G. and Jean, C. (1997) Androgen induction of the SVS family related protein MSVSP99: identification of a functional androgen response element. Mol. Cell. Endocrinol. **136**, 91–99

40   Nelson, C. C., Hendy, S. C., Shukin, R. J., Cheng, H., Bruchovsky, N., Koop, B. F. and Rennie, P. S. (1999) Determinants of DNA sequence specificity of the androgen, progesterone, and glucocorticoid receptors: evidence for differential steroid receptor response elements. Mol. Endocrinol. **13**, 2090–2107