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DNA-binding of Androgen Receptor Overexpressed in Mammalian Cells

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In order to investigate the DNA-binding properties of the rat androgen receptor (rAR) in mammalian cells after addition of androgens and antiandrogens, we established a gel-shift assay with extract from COS-1 cells (CV-1 cells transformed with the DNA-tumour virus SV40) overexpressing the rAR. First, the rAR was overexpressed in COS-1 cells. Therefore the full-length AR cDNA was inserted immediately downstream from the SV40 early promoter of pECE to generate pECE–AR. Expression of the rAR driven by the SV40 early promoter yields constant and high levels of rAR protein. In addition, the vector contains the SV40 origin of replication for obtaining high copy vector numbers in COS-1 cells. The rAR-containing expression vector was transiently transfected into COS-1 cells using Transfectam Reagent, in order to achieve high transfection efficiency. Expression of biologically active receptor was tested by analyzing the effect of the synthetic androgen R1881 on induction of transiently transfected pMMTV-CAT. Steroid binding assays were carried out to confirm overexpression of biologically active AR and to determine the binding of different hormones and antihormones to AR in COS-1 cells transiently transfected with pECE–AR. Gel-shift experiments performed with whole cell extract of those cells, containing ~700 fmol AR/mg protein, and labeled AR-binding GRE (glucocorticoid responsive element) showed that R1881 induced the formation of a protein–GRE complex. Furthermore, the R1881-induced formation of the protein–GRE complex could be competed by addition of unlabeled excess of GRE but not of unspecific oligonucleotides, confirming sequence-specific binding of the R1881-induced protein–GRE complex.

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INTRODUCTION

The androgen receptor (AR) belongs to the family of steroid receptor proteins that act as ligand-regulated transcriptional activators [1, 2]. After binding of androgen the AR interacts with androgen responsive elements (AREs) of androgen-regulated genes, thereby inducing a cascade of transcriptional events [2]. Antiandrogens are synthetic antagonists of natural androgens and are used in the clinical treatment of different androgen-dependent diseases [3]. Antiandrogens bind to the hormone-binding site of the androgen receptor [4], thereby competing with the natural ligand, and are thought to inhibit the agonistic effect of androgens on induction of transcription [5].

Since we were interested in getting more precise knowledge of the molecular level of androgen and antiandrogen action, we set out to study the DNA-binding properties of the AR. A commonly used method to investigate the binding properties of a DNA-binding protein like the AR is the "gel retardation assay". In this assay different binding conditions for these proteins can be tested. However, studies of DNA-binding and other biochemical properties of the AR have been hampered by the scarcity and the instability of this steroid receptor in tissues [6]. Thus expression of the AR in suitable host systems like in *E. coli* [7] or in the baculovirus system [8] were performed in order to investigate DNA-binding parameters, while expression of the AR cDNA in mammalian cells did not yield quantities of the receptor sufficient for such functional studies [9].

Therefore the major aim of the present work was to get sufficient overexpression of AR in mammalian cells to investigate the DNA-binding properties of authentic AR. To achieve this, we established a gel retardation assay with AR overexpressed in COS-1 cells. This gel-shift assay carried out with AR from transiently transfected COS-1 cells provides a powerful tool to study the effects of androgens and antiandrogens on

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the DNA-binding properties of the AR produced in an environment close to the *in vivo* situation.

MATERIALS AND METHODS

Hormones and antihormones

R1881 (17β-hydroxy-17-methylestra-4,9,11-triene-3-one) and radioactive [17α-methyl-^{3}H]R1881 (sp. act.: 87 Ci/nmol) were purchased from New England Nuclear (Boston, MA). Dihydrotestosterone, testosterone, estradiol, cyproterone acetate (17-acetoxy-6-chlor-1,2α-methylene-4,6-pregnadiene-3,20-dione), promegestone (17α,21-dimethyl-19-norpregna-4,9-dione-3,-20-dione), casodex [ICI 176334; (2RS)-4′-cyano-3-(4-fluorophenyl-sulfonyl)-2-hydroxy-2-methyl-3′-(trifluoromethyl)propionanilide], hydroxyflutamide [2-hydroxy-2-methyl-*N*(4-nitro-3(trifluoromethyl)-phenyl)propanamide] and dexamethasone (9-fluoro-11β,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione) were synthesized by Schering AG (Berlin).

Constructs

The expression plasmid p6RAR containing the full-length coding sequence of the rat AR (rAR) was kindly provided by Dr R. Miesfeld. The expression plasmid pECE [10] was kindly provided by Dr G. Langer. pECE contains the SV40 early and late promoters, the SV40 origin of replication, the SV40 polyadenylation site and a polylinker site. Plasmid p6RAR was cleaved with restriction enzymes *Kpn*I and *Xba*I to generate a 3 kb *Kpn*I–*Xba*I fragment, containing the complete protein coding part of the rAR. The *Kpn*I–*Xba*I–rAR fragment was inserted into the *Kpn*I and *Xba*I site of the polylinker of the expression plasmid pECE to generate pECE–AR.

The pMMTV-CAT plasmid [11] contains the MMTV promoter linked to a CAT gene and was kindly provided by Dr A. C. B. Cato.

Cell culture and transfection

COS-1 cells [12] were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS, Gibco, Berlin), 4 mmol/l L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

Transient transfection was performed using "Transfectam Reagent" (Promega, Heidelberg) according to a procedure recommended by the manufacturer [13]. The transfection cells were seeded onto 100 mm dishes at a density of 1×10^6 cells/dish. Cells were typically about 40–50% confluent after 24 h, prior to transfection. Before transfection, cells were washed twice with 3 ml phosphate-buffered saline (PBS). For each dish 5 μg of AR-containing DNA was diluted with 0.5 ml DMEM without FCS; for the CAT assay, 5 μg of the pMMTV-CAT plasmid were cotransfected. In addition, 7.5 μl "Transfectam Reagent" was diluted with 0.5 ml DMEM without FCS. Next, the DNA and "Transfectam Reagent" were combined in a polystyrene snap cap tube to obtain 1 ml of transfection solution per dish, gently mixed, added dropwise to the washed cells and filled up with DMEM without FCS to a volume of 3 ml/dish. After 5 h the transfection solution was replaced by 10 ml DMEM supplemented with 10% FCS. After 24 h cells were cultured for another 24 h in medium supplemented with 3% charcoal stripped FCS [14]. Before harvesting, cells were incubated for 3 h in the presence or absence of 10 nmol/l of the synthetic androgen R1881 (NEN).

Preparation of whole cell extract

Transiently transfected cells ($10–15 \times 10^6$) were washed twice with 3 ml PBS per dish and harvested using a "rubber policeman". Next, cells were disrupted on ice in 0.2 ml TEGM-buffer (10 mmol/l Tris–HCl, 1 mmol/l EDTA, 10% glycerin, 2 mmol/l mercaptoethanol, 10 mmol/l sodium molybdate, pH 7.2) [9] containing protease inhibitors according to Klein-Hitpass *et al.* [15] [5 mmol/l leupeptin, 0,1 mmol/l benzamidin, 1 μg/ml pepstatin A, 0,5 μg/ml aprotinin (all Merck, Darmstadt) and 0.2 mmol/l PMSF (Sigma, Deisenhofen)] with 30 strokes in a homogenizer (B. Braun, Melsungen) with a strong pestle. For gel retardation experiments a high salt buffer [10 mmol/l sodium phosphate, pH 7.4, 1 mmol/l EDTA, 0,5 mmol/l dithiothreitol (DTT), 10% Glycerin, 400 mmol/l KCl] instead of TEGM-buffer was used for disruption. The homogenate was centrifuged at 100,000 *g* for 1 h at 0–2°C, the supernatant was collected and protein concentration was determined according to Bradford [16].

Preparation of rat prostate extract

Ventral prostates of 10 castrated adult male rats (Wistar rats, 350 g, Schering AG) were washed in ice-cold buffer (0.25 mol/l sucrose, 0.01 mol/l Tris, pH 7.5) cleaned and dried on tissue paper. 1.3 ml preparation buffer (10 mmol/l Tris, 10% glycerin, 2 mmol/l DTT, 20 mmol/l sodium molybdate, pH 7.4) was added per g prostate and prostate was homogenized using an ultraturrax. The homogenate was centrifuged at 100,000 *g* for 1 h at 0–2°C, the supernatant was stored in aliquots at −80°C.

Steroid-binding assay

The steroid hormone receptor levels in COS-1 cells transiently transfected with rAR and rat prostate tissue were determined in duplicates by the dextran-coated charcoal procedure [17] using saturating concentrations of ligand (5 nmol/l [^{3}H]R1881, 87 Ci/mmol, NEN) competing with a 200-fold excess of non-radioactive R1881 (NEN).

Binding affinities of hormones and antihormones were determined in cytosol preparations of COS-1 cells and rat prostate tissue. 10 μl of [^{3}H]R1881 (final concentration 5×10^{-9} mol/l) and 10 μl unlabeled R1881 for the standard curve or 10 μl of the test substance in appropriate concentrations were added to 40 μl of cytosol. Each sample was incubated for 2 h at 4°C. After the incubation the unbound steroids were

adsorbed using the dextran-coated charcoal procedure [17].

CAT assay

Transiently transfected cells were disrupted by freezing–thawing in 37°C water bath/dry ice-acetone three times each. Protein concentration of the cell extracts was determined according to the procedure of Bradford [16]. CAT assay was performed as described by Gorman *et al.* [18].

Gel retardation assay

The gel retardation assay was performed according to Barberis *et al.* [19]. 15 μg protein of whole cell extract were incubated with 1 μg poly (dI·dC) (Pharmacia, Berlin) at 4°C for 15 min. For the binding reaction, a "glucocorticoid responsive element" (GRE [20]) with the sequence 5′ AGCTTAGAACACAGTGTTCTCTAGAG 3′ (GRE underlined) was used. 10–50 ng of the sense oligonucleotide was annealed to the overlapping antisense oligonucleotide to obtain a double-strand DNA, which was fill-in labeled with [^{32}P] dATP (3000 Ci/mmol, NEN) using Klenow polymerase (Boehringer, Mannheim). Binding reaction was initiated by adding 40,000–50,000 dpm (10 pg) of the purified double-strand oligonucleotide and was performed at 4°C for 20 min. The final concentration of buffer components in a 30 μl binding reaction was as follows: 10 mmol/l Tris–HCl (pH 7.5), 1 mmol/l DTT, 10% glycerol and 75 mmol/l KCl. A pre-electrophoresed (4 mA for 30 min) 5% polyacrylamide gel (acrylamide to bisacrylamide ratio 30:0.8) containing 0.5 × TBE buffer (0.89 mol/l Tris, 0.89 mol/l borate, 20 mmol/l EDTA, pH 8.3) was used to separate the receptor–DNA complex at 150 V and 8 mA at 4°C with buffer recirculation. After 3 h the gel was vacuum-dried for autoradiography. For the gel retardation competition assay different concentrations of unlabeled competition oligonucleotide were added to the binding reaction.

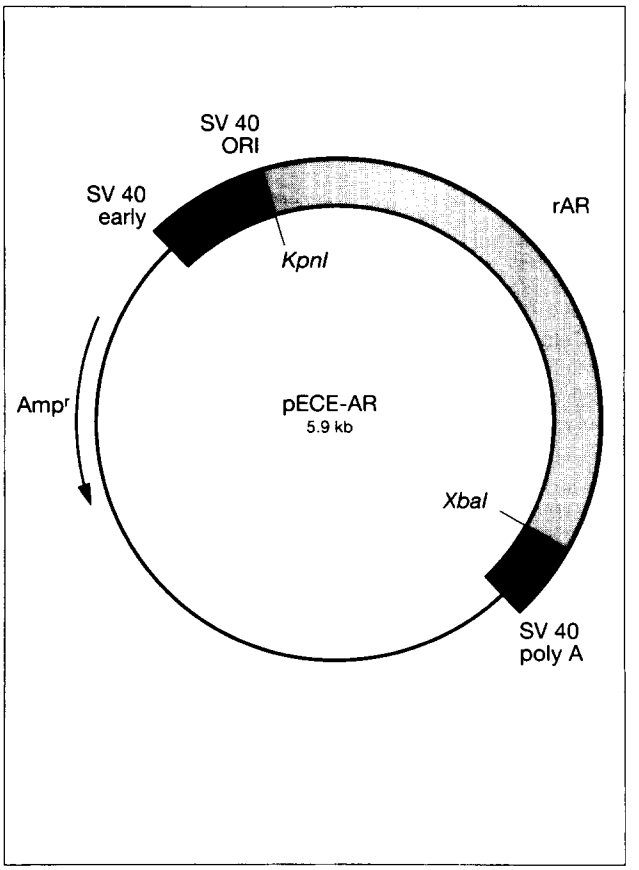

Fig. 1. Structure of the AR-expression vector pECE–AR. The 3 kb full-length coding sequence of rAR [21] was inserted into the *Kpn*I and *Xba*I sites of the expression vector pECE [10].

RESULTS AND DISCUSSION

Construction of the rAR expression plasmid

The first step to overexpress the AR in mammalian cells was the construction of a suitable expression vector. Therefore, the full-length AR cDNA of the rAR [21] was inserted immediately downstream from the SV40 early promoter of pECE [10] to generate pECE–AR (Fig. 1). Expression of the rAR driven by the SV40 early promoter yields constant and high levels of rAR protein. In addition, the vector contains the SV40 origin of replication for obtaining high copy vector numbers in COS-1 cells.

Overexpression of AR in COS-1 cells

In order to achieve high transfection efficiency, the AR-containing expression vector pECE–AR was transiently transfected into COS-1 cells using "Transfectam Reagent" (Stratagene). Steroid binding assays were carried out to confirm overexpression of the AR in those cells. As shown in Fig. 2, transfection of the AR expression vector pECE–AR using Transfectam Reagent yielded a receptor content of about 650 fmol/mg protein. In contrast, transfection carried out with other methods (lipofection, calcium phosphate, DEAE-dextran) yielded much lower quantities of expressed AR (data not shown). Unexpectedly, untransfected COS-1 cells used as a negative control contained about 50 fmol receptor /mg protein (Fig. 2). This amount of androgen-binding protein did not correspond to biologically active AR, because no transactivation of the CAT reporter gene could be found in untransfected COS-1 cells cultured in the presence of 10 nmol R1881, as shown in Fig. 3. Extract of rat prostate tissue used as a positive control showed an amount of about 40 fmol/mg AR (Fig. 2) as described previously [22].

Furthermore, binding experiments were performed with AR overexpressed in COS-1 cells to test the ability of various hormones and antihormones to compete with [^{3}H]R1881 for the hormone binding site of the receptor. To get a broader perspective, AR from rat prostate tissue was used for comparison. The results of these comparative binding studies are summarized in Table 1. The binding affinities of AR overexpressed in

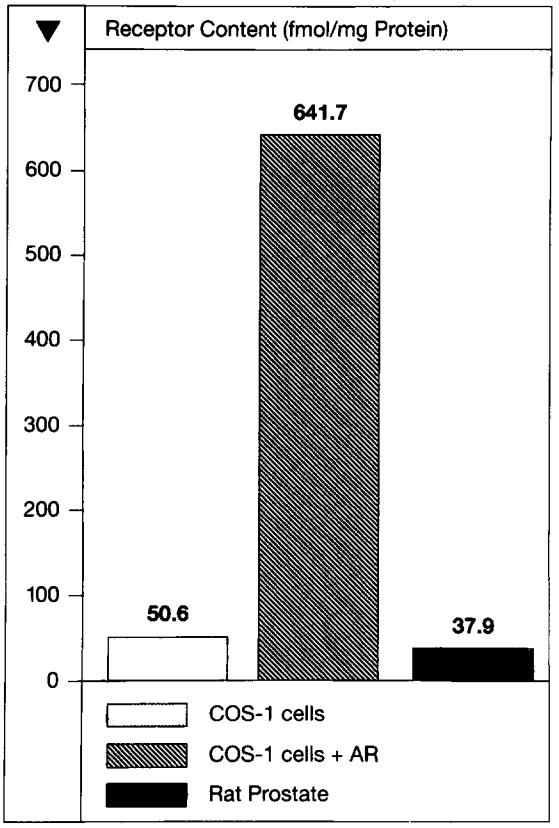

Fig. 2. Receptor content in COS-1 cells after Transfectam-mediated transfection of the AR-expression vector pECE–AR. Steroid binding assays were carried out with the ^{3}H-labeled synthetic androgen R1881 (NEN) as described previously [17]. Untransfected COS-1 cells were used as negative control, extract of rat prostate tissue as positive control.

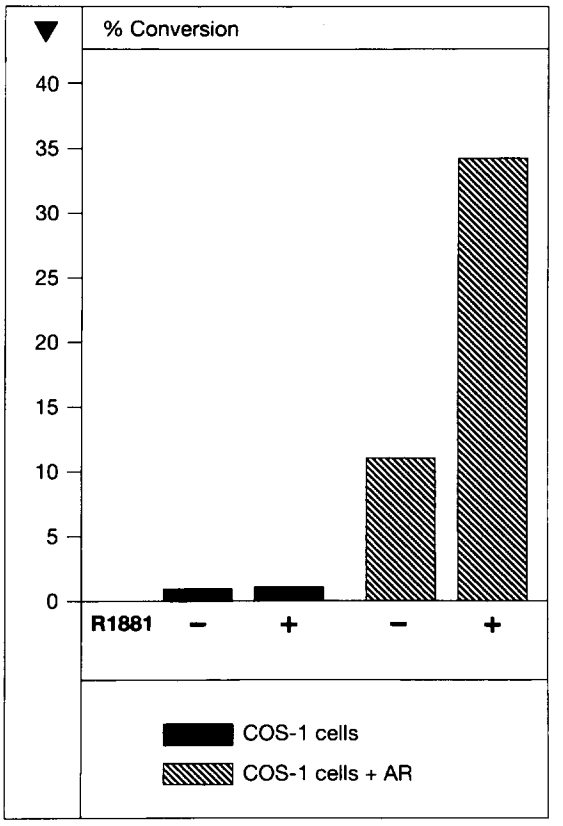

Fig. 3. Specific induction of CAT activity by R1881 in the transient transfected COS-1 cells. COS-1 cells were transiently cotransfected with 5 μg of the AR-expression vector pECE–AR and the reporter plasmid 5 μg of pMMTV-CAT [11], respectively. COS-1 cells transfected with pMMTV-CAT alone were used as a negative control. Transfections were carried out with "Transfectam Reagent". After transfection cells were grown 24 h in charcoal-treated FCS and then incubated for 3 h either in the presence or absence of 10 nmol/l R1881 (NEN). CAT activity was determined in 20 μg protein extract as described by Gorman *et al.* [18] and is shown as percent conversion from chloramphenicol to acetylated chloramphenicol.

COS-1 cells were in agreement with receptor binding affinities obtained with AR from rat prostate (Table 1). Therefore AR overexpressed in COS-1 cells shows the typical binding profile of a functional AR.

Expression of biologically active AR in COS-1 cells transiently transfected with rAR was tested by analyzing the effect of the synthetic androgen R1881 on induction of transiently cotransfected reporter-plasmid pMMTV-CAT [11]. Transient transfection of the parental cell line COS-1 was used as a negative control. As shown in Fig 3, a low background CAT activity in COS-1 cells transiently transfected with pMMTV-CAT only could be observed in the absence or presence of 10 nmol/l R1881. In contrast, CAT-expression could be induced in the pECE–AR transfected COS-1 cells (Fig. 3). Surprisingly, CAT activity was also found in AR-expressing COS-1 cells in the absence of R1881. One possible explanation for this observation would be the activation of overexpressed AR by endogenous testosterone (10^{-11} mol/l) in the culture medium.

Taken together, these results show the overexpression of a biologically active AR after transient transfection of the AR-gene into COS-1 cells using "Transfectam Reagent".

Gel retardation experiments with AR overexpressed in COS-1 cells

Next gel retardation experiments were carried out to investigate the binding of biologically active AR

Table 1. *Relative binding affinities (RBA, expressed in percent) of different steroids to the AR*

Test substances	RBA rat prostate	RBA COS-1 cells
Dihydrotestosterone	78	83.3
Testosterone	50	47.6
Cyproterone acetate	8.3	6.1
Promegestone	1.0	3.6
Casodex	0.83	2.3
Estradiol	2.0	1.6
Hydroxyflutamide	0.4	1.6
Dexamethasone	NC	NC

The reference compound was R1881. The RBA value of the reference compound R1881 was arbitrarily designated 100%. NC, no competition.

overexpressed in mammalian cells to an AR-binding "hormone responsive element" (HRE). As AR-binding HRE a GRE [20] was used, since GREs not only mediate glucocorticoid and progestin but also androgen activation of transcription [23].

Gel retardation experiments performed with whole cell extract of AR-transfected COS-1 cells (700 fmol AR/mg protein) and labeled AR-binding GRE showed that R1881 induced the formation of a protein–GRE complex (Fig. 4, lane 5). No retarded complex could be seen with whole cell extract of untransfected COS-1 cells in the absence (Fig. 4, lane 2) or presence (Fig. 4, lane 3) of R1881 and with extract of AR-transfected COS-1 cells in the absence of the synthetic hormone (Fig. 4, lane 4). Furthermore, the R1881-induced formation of the protein–GRE complex could be competed for by addition of an excess of unlabeled GRE (Fig. 4, lanes 6–8), whereas 100-fold excess of the unspecific oligonucleotide with the binding region of the "octamer binding factor" (Oct-1) (Fig. 4, lane 9) could not compete for the formation of the GRE–protein complex. These results confirmed sequence-specific binding of the R1881-induced protein–GRE complex.

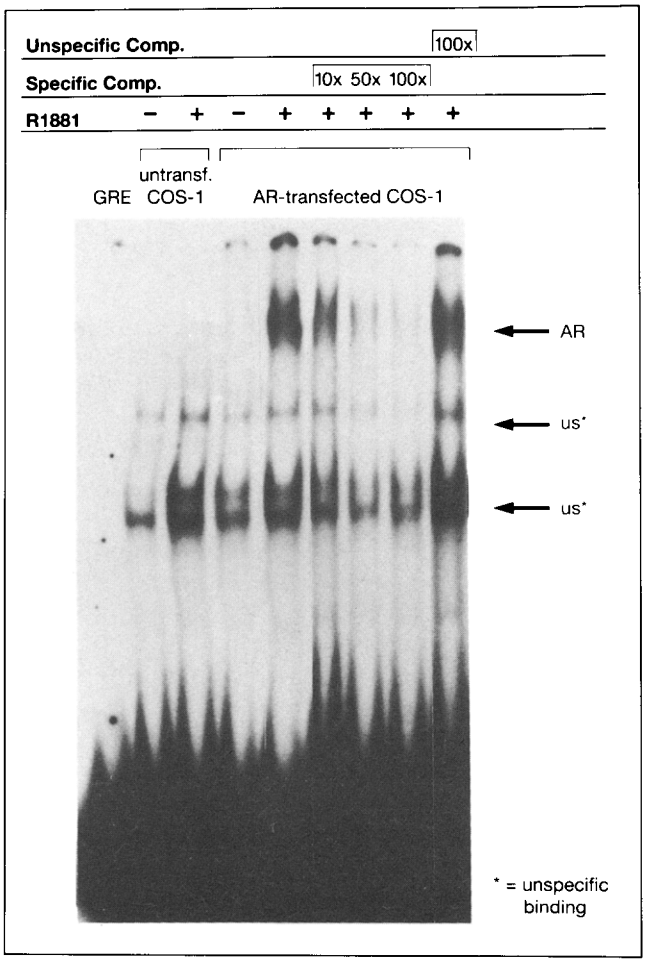

Fig. 4. Gel retardation assay with the overexpressed AR. COS-1 cells overexpressing the rAR were grown for 3 h in the presence or absence of 10 nmol/l R1881. For binding reactions extracts of these cells were incubated with 10 pg ^{32}P-labeled GRE-oligonucleotide for 20 min at 4°C. After binding reaction a PA-gelelectrophoresis was performed according to Barbaris *et al.* [19] to separate protein-bound from unbound oligonucleotide.

In order to investigate the DNA-binding properties of the AR, we established a gel retardation assay for the AR overexpressed in mammalian cells. In comparison with AR overexpressed in *E. coli* [7] or in the baculovirus system [8], AR overexpressed in our mammalian system offers the significant advantage of being a biologically active receptor produced in an environment close to the *in vivo* situation. Furthermore, DNA-binding properties and transactivation of the AR can now be investigated in the same cell type. In conclusion, this gel retardation assay with AR overexpressed in mammalian cells provides a powerful tool to study the effects of androgens and antiandrogens on the DNA-binding properties of the AR.

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