4. MATERIALS AND METHODS

4.1. General materials and methods

4.1.1. Chemicals and cell culture media

R1881 (methyltrienolone) was from Dupont NEN, dexamethasone from Sigma and R5020 was synthesized in-house at Schering. RPMI 1640, MEM, OPTI-MEM, streptomycin, penicillin, geneticin and L-glutamine were obtained from Gibco BRL Life Technologies. Fetal calf serum (FCS) was from PAA. The oligonucleotides were purchased from Roth. Trypsin, chymotrypsin, Pefabloc SC, the complete protease inhibitor mix and FuGene 6 were from Roche Molecular Biochemicals. Media for bacterial cultures were prepared according to Sambrook et al. (1989).

4.1.2. Bacterial strains and cell lines

***E.coli* host strain**	**Genotype**	**Company**
Top 10	endA1, hsdR1, lacZ∆M15, ∆lacZX74, recA1	Invitrogen
XL1-Blue	recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac	Stratagene
BL21	BF^-, dcm, ompT, hsdS($r_B^-m_B^-$), gal	Stratagene

Cell line	**Specifications**	**Origin**
CV-1	African green monkey (*Cerciopithecus aethiops*) kidney cell line which lacks all steroid receptors	American type culture collection
PC-3	Prostate carcinoma cell line not expressing the AR	American type culture collection
PC-3/AR	Prostate carcinoma cell line stably transfected with the AR	Dr. A. Cato Forschungs-zentrum Karlsruhe

4.1.3. Plasmids

Name	Host cell	Other features	Company
pCRII-TOPO	*E.coli*		Invitrogen
pCR2.1-TOPO	*E.coli*		Invitrogen
pSG5	Eukaryotic cell lines	early SV40 promoter	Stratagene
pcDNA3.1+/myc-his	Eukaryotic cell lines	CMV promoter	Invitrogen
pGL3	Eukaryotic cell lines	Luciferase gene	Promega
pCMV-AD	Eukaryotic cell lines	Contains the NF-κB-transactivation domain	Stratagene
pCMV-BD	Eukaryotic cell lines	Contains GAL4 DNA binding domain	Stratagene
pFR-Luc	Eukaryotic cell lines	Luciferase gene under control of GAL4 response elements	Stratagene

4.1.4. DNA and protein size standards

Name	Company
DNA 1 kb ladder	Gibco BRL
DNA 100 bp ladder	Gibco BRL
Protein kaleidoscope prestained standard	BIO-RAD

4.1.5. DNA preparation

DNA was prepared with the Quiaprep Spin Miniprep kit, Plasmid Maxi kit or Quiaquick gel extraction kit, all from Quiagen.

4.1.6. Commonly used buffers

Standard buffers for basic molecular biology techniques were prepared according to Sambrook et al. (1989). H_2O was deionised and ultrafiltrated using a millipore apparatus.

10x Towbin buffer for protein transfer (blotting)	25 mM 192 mM 0,1% 20%	Tris Glycine SDS Methanol
Cell lysis buffer	20 mM 10% 1.5 mM 10 mM 0.1% 2.5 mM 0.2 μM	HEPES pH 7.6 Glycerol $MgCl_2$ NaCl Triton X-100 DTT R1881 Protease inhibitory mix
4x SDS loading buffer	4% 20% 120 mM 100 mM 0.02% 150 mM	SDS Glycerin Tris base DTT Bromophenol blue Pefablock SC

4.1.7. Coomassie staining

SDS gels were fixed in 10% (v/v) acetic acid and stained with Coomassie solution. They were subsequently destained with a 20% (v/v) MetOH, 10% (v/v) acetic acid, 5% Glycerol solution.

4.2. Materials and methods used in Results section 2.1.

4.2.1. Plasmid construction for receptor studies

For the Pem promoter constructs, the -1139 to -36 or -444 to -36 fragment was PCR-amplified using the *Taq* polymerase (Perkin Elmer Cetus) while adding the appropriate restriction sites. The resulting fragments were introduced between the *Nhe*I and *Hin*dIII sites of the pGL3-Basic plasmid (Promega).

The luciferase reporter plasmids were based on the pGL3-Basic plasmid (Promega) and harbored two copies of the following response elements: Pem ARE-1 5´-AGATCTcattcTGTTCC-3´ (Barbulescu et al, 2001), Pem ARE-2 5´-AGCACAtcgTGCTCA-3´ (Barbulescu et al., 2001), 2.43 SRE 5´-GGAACAaaaTGTCCC-3´ (Roche, et al., 1992), the –1253 DNA element of the *CRISP-1* promoter hereafter named CRISP-1 SRE 5´-GGTACAtctTGTTCA-3´ (Schwidetzky et al., 1997) or the consensus (cons.) high-binder element 5´-GGTACAttgTGTTCT-3´ (Liu et al., 1996). For multicopy constructs a spacing of twelve base pairs between the elements was chosen.

Expression vectors containing cDNAs for full-length AR, PR, GR, TIF2, ARA55, Ubc9 and PIASxα inserted in the pSG5 plasmid (Stratagene) were generated and used for transfections. For the mammalian two-hybrid assays, individual domains of AR and PR were amplified by PCR and inserted into the pCMV-AD and pCMV-BD vectors (Stratagene). Site-directed mutagenesis of AR, PR and GR constructs was carried out using the QuickChange kit (Stratagene) and the heat-stable polymerase mix from the Advantage kit (Clontech). DNA sequencing was performed using standard procedures.

4.2.2. Cell culture and transactivation assays

The CV-1 cells were grown at 37°C in a 5% CO_2 atmosphere in MEM, 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 4 mM L-glutamine. For the transactivation assays, the cells were seeded in 96-well plates at a concentration of 10,000 cells/100 µl per well in MEM supplemented as above except that 5% charcoal-stripped FCS was used. The PC-3/AR cells were routinely cultured at 37°C in a 4.5% CO_2 atmosphere in RPMI 1640, 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 4 mM L-glutamine, 600 µg/ml geneticin. For the transactivation assays, the cells were seeded at a concentration of 15,000 cells/100 µl per well in RPMI 1640

supplemented as above except that 5% charcoal-stripped FCS was used. For both cell lines, the transfection was carried out 18 h later using FuGene 6 in OPTI-MEM and 100 ng of reporter plasmid. Expression plasmids for human AR, GR or PR (100 ng each) were cotransfected into CV-1 cells when indicated. Induction was performed 5 h later by adding 1 nM R1881, 1 nM dexamethasone or 1 nM R5020. Measurement of luciferase activity was carried out 24 h later after adding 100 µl of LucLite or LucLite Plus reagent (Packard) in a Lumicount luminometer (Packard). The activity of pGL3 promoter vector (Promega) was determined on parallel samples to assess transfection efficiency. For all points the average value of six wells treated in parallel was taken. The experiments were repeated at least three times independently.
For the steroid selectivity experiments, 100 ng of pSG5-AR, pSG5-PR or pSG5-GR were transfected into CV-1 cells. For the cofactor assays, 25 ng of pSG5-AR and 70 ng of cofactor-expressing plasmid or of β-galactosidase control plasmid were used.

4.2.3. In vitro translation

Human AR cDNA was cloned into the pCRII-TOPO plasmid. *In vitro* translation was carried out with 1 µg plasmid using the TNT T7/SP6 Coupled Reticulocyte Lysate system and the SP6 RNA polymerase, following the manufacturer´s instructions (Promega). The level of AR synthesized was assessed by SDS-polyacrylamide gel electrophoresis and subsequent Western blot analysis using the anti-AR (441) antibody (Santa Cruz Biotechnology). The reaction products were stored at -70°C in small aliquots.

4.2.4. Preparation of nuclear extracts

PC-3/AR cells were grown as described above and induced with 0.1 µM of R1881 for 24 h before harvesting. The nuclear extracts were prepared essentially as described (Liu et al., 1996). Approximately 100 million cells were treated with lysis buffer (20 mM HEPES pH 7.6, 10% glycerol, 1.5 mM $MgCl_2$, 10 mM NaCl, 0.1% Triton X-100, 2.5 mM DTT, 0.2 µM R1881 and protease inhibitor). They were then centrifuged for 4 min at 13,000 g, and the pelleted nuclei were resuspended in lysis buffer containing 600 mM NaCl and 10 mM $MgCl_2$ and gently vortexed for 30 min at 4°C. Undissolved material was removed by centrifugation. The buffer was exchanged on a NAP25 column (Pharmacia) using binding buffer (20 mM HEPES pH 7.5, 0.5 mM EDTA, 2.5

mM Mg acetate, 130 mM NaCl, 0.1% NP-40, 10% glycerin, 2 mM DTT and a proteinase inhibitor mix (Roche)).

4.2.5. DNA binding and protease digestion assays

The following biotinylated oligonucleotides containing two copies of each element (underlined) and their complementary sequence were annealed in annealing buffer (40 mM Tris-HCl pH 7,5; 20 mM $MgCl_2$; 50 mM NaCl) and incubated with Streptavidin Sepharose beads (Sigma) overnight in binding buffer.

Name of oligonucleotides	***Sequence in 5'-3' orientation (response elements are underlined)***
Pem ARE-1	GATAATTCTTAAAGATCTCATTCTGTTCCATTGATTTTTAGAGATCTCATTCTGTTCCAATTCATTGATT
Pem ARE-2	GATAATTCTTAAAGCACATCGTGCTCAATTGATTTTTAGAGCACATCGTGCTCAAATTCATTGATT
2.43 SRE	GATAATTCTTAAGGAACAAAATGTCCCATTGATTTTTAGGGAACAAAATGTCCCAATTCATTGATT
Cons. SRE	GATAATTCTTAAGGTACATTGTGTTCTATTGATTTTTAGGGTACATTGTGTTCTAATTCATTGATT
CRISP-1 SRE	GATAATTCTTAAGGTACATTGTGTTCAATTGATTTTTAGGGTACATTGTGTTCAAATTCATTGATT

Excess oligonucleotides were removed by washing with binding buffer. Then, 70 µl of the 1:1 oligonucleotide-loaded Sepharose solution were mixed with 100 µl of 1 mg/ml nuclear extract supplemented with 2.6 µg of poly(dI-dC) (Amersham Pharmacia Biotech) and 2.6 µg of poly(dA-dT) (Amersham Pharmacia Biotech) for 20 min at room temperature followed by an additional 2 h incubation at 4°C under constant shaking. The beads were washed twice with 1 ml of binding buffer without proteinase inhibitor.

Determination of bound AR was carried out following elution with binding buffer containing 1.2 M NaCl, TCA precipitation and gel separation (4-12% PAA Gel, Invitrogen). The proteins were transferred onto a polyvinylidene fluoride membrane (PVDF; Invitrogen). For immunoblot analysis the anti-AR (441) was used. Processing was with the Enhanced Chemiluminescensce Detection Kit (Perkin Elmer).

For the protease digestions, 75 µl of trypsin or chymotrypsin dilutions in binding buffer were added to the beads-bound protein/DNA complexes. The total protein concentration was identical in all samples as checked following SDS-PAA gel electrophoresis and Coomassie blue staining. After 10 min at 25°C the reaction was stopped by adding 35 µl of sample buffer (4% SDS, 20% glycerin, 120 mM Tris base, 100 mM DTT, 0.02% bromophenol blue, 150 mM Pefablock SC) and heating to 95°C for 5 min. Aliquots of supernatant were loaded on parallel polyacrylamide gels for Coomassie blue and Western blot analyses. Coomassie blue staining was performed to check that the total protein amount was identical. Western blot anaylsis of the AR digestion products was carried out after transfer to a PVDF membrane by incubating with the anti AR C-19 antibody (C-19) and processing with a detection kit, as above.

4.3. Materials and methods used in Results section 2.2.

4.3.1. Identification and cloning of OTEX

A BLAST search of public databases allowed the identification of an EST (AI631510) sharing significant sequence identity with *PEPP* family members. This led to the identification of a genomic region (AC005023) containing the sequence information for an open reading frame putatively encoding a novel PEPP protein. This information was used to design the primers 5´-ATGGCGCGTTCGCTCGTCCACGAC-3´ (OT1) and 5´-TAGTCCACGACGATGTAGACACAG-3´ (OT2) which were used for PCR amplification of full-length *OTEX*, starting from human ovary first-strand cDNA. PCR conditions were: 30 s at 94°C, 30 s at 58°C, 2 min at 68°C for 40 cycles using the Advantage 2 PCR-kit (Clontech, Heidelberg, Germany). Additional PCR amplifications were carried out for a precise determination of the extremities, using ovary and testis Marathon-Ready cDNA (Clontech) as templates. For analysis of the 5´ end we used the *OTEX*-specific reverse primer 5´-TTGAGTGTGTCGGAAAACACTTTCCAG-3´ (OT4) and the universal primer 5´-CCATCCTAATACGACTCACTATAGGGC-3´. For the 3´ end we used the *OTEX*-specific forward primer 5´-CTGGGGGCAGCATCAAGCGCAGAAGG-3´ (OT3) and the universal primer 5´-CCATCCTAATACGACTCACTATAGGGC-3´. The generated products were separated on agarose gel (Gibco BRL Life Technologies, Karlsruhe, Germany), and subcloned into the pCRII-TOPO vector (Invitrogen, Karlsruhe, Germany). Sequencing was performed with the *Taq* polymerase using the BigDye

Terminator Cycle Sequencing kit (Roche Applied Biosystems, Foster City, CA, USA). The amplified products were purified from the dye terminators using Centriflex gel filtration cartridges (MoBiTec, Göttingen, Germany) and their sequence was determined on both strands (Agowa, Berlin, Germany).

4.3.2. RT-PCR analysis

RNAs from different human tissues were obtained from Invitrogen, Clontech (Heidelberg, Germany) and Ambion (Huntingdon, UK). Total RNA from PC-3 and PC-3/ARwt cells was purified using the RNAeasy kit (Qiagen, Hilden, Germany). The RNA was reverse-transcribed using the ProSTAR kit (Stratagene, La Jolla, CA, USA). Primer pairs for amplification of full-length *OTEX* (OT1 and OT2) or of a 300-bp internal fragment (OT3 and OT4) were used in PCR. Primers specific for the gene coding for the housekeeping 23kDa highly basic protein or for β-actin were used for amplification of an internal control. PCR conditions were 30 s at 95°C, 30 s at 62°C and 1 min at 72°C for 35 cycles, using Ready-To-Go PCR beads (Amersham Pharmacia Biotech, Freiburg, Germany).

4.3.3. Construction of OTEX expression plasmids

Two constructs were engineered for use in the one- and two-hybrid assays. Full-length *OTEX* cDNA was PCR-amplified while adding compatible flanking restriction sites and inserted between the *Bam*HI and *Xba*I sites of the pCMV-AD and pCMV-BD plasmids (Stratagene). The resulting constructs were checked by DNA sequencing using the BigDye Terminator Cycle Sequencing kit (Roche), as above.

For the immunofluorescence experiments, full-length *OTEX* cDNA was amplified by PCR while adding compatible flanking restriction sites and inserted between the *Bam*HI and *Eco*RI sites of the pcDNA3.1/myc-his(+)A plasmid (Invitrogen) giving the plasmid pOTEXwt. For mutation of the NLS, we used the QuickChange kit (Stratagene) to insert compensatory frameshift mutations before and after the candidate region in two steps. First we inserted a base pair at the 3´ end using the 5´-CATCAGAGAGAATTTAATGCTCGCC-3´ primer and its complementary sequence. In the following step we deleted a basepair 18 codons upstream using the 5´-GGTGTGACTGAAGACAAGTGCGGG-3´ primer and its complementary sequence (pOTEX mut1). A second mutant was generated by deleting a base pair 10 codons upstream of the initial insertion using the 5´-GGTTTAAGAATAAAGGGCCAGATG-3´

primer and its complementary sequence (pOTEX mut2). The presence of the mutations was confirmed by DNA sequencing.

4.3.4. Cell culture and transfections for studies of OTEX

The CV-1 cells were grown at 37°C in a 5% CO_2 atmosphere in MEM, 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 4mM L-glutamine. The PC-3 cells were grown at 37°C in 5% CO_2 atmosphere using Kaighn´s modification of Nutrient Mixture F-12K, 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine. The PC-3/ARwt cells were cultured at 37°C in a 4.5% CO_2 atmosphere in RPMI 1640, 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 4 mM L-glutamine, 600 µg/ml Geneticin.

For the hormone treatment, the cells were seeded in medium supplemented with charcoal-stripped FCS for 24 h before adding 1 nM R1881. The cells were harvested 24 h later for RNA purification.

For the one-hybrid and two-hybrid assays, the cells were seeded at a concentration of 10,000/100 µl per well in 96-well plates. Transfection was carried out 18 h later using FuGene6 (Roche Molecular Biochemicals, Mannheim, Germany) in OPTI-MEM and the pCMV-based plasmids. Measurement of luciferase reporter activity was carried out 24 h later in a Lumicount luminometer (Packard, Dreieich, Germany) after adding 100 µl of LucLite Plus reagent (Packard). The activity of the pGL3 promoter vector (Promega, Mannheim, Germany) was determined on parallel samples to assess transfection efficiency. For all points the average value of six wells treated in parallel was taken. The experiments were repeated at least three times independently.

4.3.5. Proliferation assays

CV-1 cells were seeded at a concentration of 3000/100 ul per well in 96-well plates and transfected with pOTEX wild type, pOTEX mutant 1, pOTEX mutant 2, and a LacZ expressing construct as described above. After 0 h, 24 h, 48 h and 72 h the viable cells were quantitated using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) which determines the ATP content. Each sample was measured as sextuplicate values and the experiment repeated at least three times independently.