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Identification of a Functional Androgen Response Element in the Promoter of the Gene for the Androgen-regulated Aldose Reductase-like Protein Specific to the Mouse Vas Deferens*

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Mouse vas deferens protein (MVDP), a member of the aldo-keto reductase superfamily, is exclusively produced in the epithelial cells of the deferent duct under androgenic regulation. To better understand androgen-regulated MVDP gene expression, the location and sequences of androgen response elements (AREs) in the 5′-flanking DNA were determined. Sequence analysis revealed two putative AREs as follows: one between positions −1186 and −1171 (distal ARE) and the other between −111 and −97 (proximal ARE). To study hormonal regulation, fragments of the MVDP promoter region, extending from residue −1804 to +41, were linked to the chloramphenicol acetyltransferase (CAT) reporter gene and cotransfected with a human androgen receptor expression vector into T47D cells in a transient expression assay. A minimal region (−121 to +41) was identified as being sufficient for androgen-regulated gene expression. A mutation in proximal ARE almost completely abolished androgen induction of CAT. One copy of the sequence TGAAGT tcc TGTTCT, cloned in the opposite orientation in front of the thymidine kinase promoter, confers androgen responsiveness to the CAT reporter gene. Androgen transcriptional activity was not detected with the distal ARE.

The data provide strong evidence that transcriptional regulation of the MVDP gene occurs via the sequence TGAAGT tcc TGTTCT.

Transcription of eukaryotic genes by RNA polymerase II is mediated by two major cis-acting sequences as follows: promoter elements, which bind general transcription factors and define a basal transcriptional activity of the gene, and regulatable elements, which mediate either a positive or negative effect on the basal promoter activity (Maniatis *et al.*, 1987). These regulatory sequences may act as hormone-dependent enhancers and confer hormone responsiveness to homologous and heterologous promoters irrespective of distance or orientation (Beato, 1989). *In vivo*, the expression of steroid-responsive genes is more complex, due to the action of tissue-specific factors, which also may act via enhancer-like sequences (Adler *et al.*, 1991). All of these cis-acting sequences serve as binding sites for sequence-specific DNA-binding proteins that are required in trans to mediate the transcriptional response. The classical steroid receptor proteins have been identified as the trans-acting factors required for function of the specific cis-acting steroid response elements (Beato, 1989).

The androgen receptor belongs to the superfamily of ligand-responsive transcription factors, which includes steroids, thyroid hormones, and retinoic acid receptors (Carson-Jurica *et al.*, 1990). Specific cis-acting sequences with high affinity for glucocorticoids, progesterone, and estrogen receptors have been thoroughly characterized (for review, see Beato (1989)). The DNA-binding domains of the glucocorticoid, progesterone, mineralocorticoid, and androgen receptors (AR)[1] are highly conserved sequences (homology of 80–90%), suggesting that they recognize similar cis-acting sequences (Evans, 1988; Lubahn *et al.*, 1988). It has been found that in transfection experiments, glucocorticoids, progesterone, and androgens can all induce a reporter gene via a canonical glucocorticoid response element (GRE) (Ham *et al.*, 1988). The DNA sequences to which steroid receptors bind are composed of two hexanucleotides arranged as an imperfect palindrome separated by three nonconserved base pairs (Beato, 1989; Nordeen *et al.*, 1990). The consensus sequence of the responsive element for the glucocorticoid receptor and progesterone receptor is GGTACA nnn TGTT/CCT (Beato, 1989; Nordeen *et al.*, 1990). It has been found that GREs from the mouse mammary tumor virus (MMTV) long terminal repeat and from the tyrosine aminotransferase gene also mediate transcriptional activation by androgens (Ham *et al.*, 1988; Denison *et al.*, 1989).

Although a large number of genes have been demonstrated to respond to androgens, there is little information regarding interactions between androgen receptors and specific sequences of androgen-responsive genes. Among the cellular genes that are transcriptionally regulated by androgens, the rat C3 (1) gene, which codes for one of the constituent peptides of prostatic binding protein, has been studied in more detail. Two restriction fragments located in the promoter region and in the first intron that are able to bind partially purified AR contain imperfect palindromic sequences resembling the GRE/PRE consensus sequence (Rushmere *et al.*, 1987; Claessens *et al.*, 1990). The intron fragment is shown to confer androgen responsiveness to the thymidine kinase promoter, and mutations within the GRE-like sequence suppress the response to androgens, suggesting that this sequence functions as an ARE (Claessens *et al.*, 1989; Tan *et al.*, 1992).

We have studied the mouse vas deferens protein (MVDP) gene, which is under hormonal, developmental, and tissue-specific control (Taragnat *et al.*, 1988, 1990; Martinez *et al.*, 1990,

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U00149.

[1] The abbreviations used are: AR, androgen receptors; MVDP, mouse vas deferens protein; ARE, androgen response element; CAT, chloramphenicol acetyltransferase; kb, kilobase(s); GRE, glucocorticoid response element; MMTV, mouse mammary tumor virus; bp, base pair(s); DHT, dihydrotestosterone; PCR, polymerase chain reaction; PRE, progesterone response element.

1991). The amino acid sequence of MVDP, deduced from its cDNA nucleotide sequence, exhibits a high degree of homology with members of the aldo-keto reductase superfamily (Pailhoux *et al.*, 1990), and the structure of the MVDP gene is very similar to that described for the human aldose reductase gene (Pailhoux *et al.*, 1992). Although the physiological role of aldose reductase is still unclear, numerous studies suggest that this enzyme may play a key role in the etiology of some complications of diabetes mellitus (Kinoshita *et al.*, 1981). Aldose reductase is a ubiquitous "housekeeping" enzyme, probably functional in all cells (Jeffery and Jörnvall, 1983; Iwata *et al.*, 1990). It has been shown that in lens and renal inner medulla, expression of the aldose reductase gene is highly regulated by hyperglycemia and hyperosmolarity (Bondy *et al.*, 1989a, 1989b; Petrash *et al.*, 1992). However, to our knowledge, there is no example of an androgen-regulated aldose reductase.

MVDP is under androgen control, and testosterone is the signal that triggers MVDP gene expression at the protein and mRNA levels (Taragnat *et al.*, 1988; Martinez *et al.*, 1989). Hormonal induction of the MVDP gene is achieved mainly at the transcriptional level (Martinez *et al.*, 1990). In addition, *in vitro* studies have shown that pure vas deferens epithelial cell cultures are able to produce MVDP in response to androgens in a medium devoid of other hormones, growth factors, and cAMP, suggesting that none of these factors exert a permissive influence on androgen action (Manin *et al.*, 1992). The promoter of the MVDP gene contains a TATA (CATAA) box, a CAAT box, a GC-rich motif, and a 5′-TGTTCT-3′ element that closely resembles the GRE/PRE consensus sequence (Pailhoux *et al.*, 1992).

Since only a limited amount of sequence information has been published for the 5′-upstream part of the MVDP gene (500 bp), we completed sequencing, including 1805 bp of the 5′-flanking region. To identify functional androgen response elements, transient expression experiments were performed using an androgen receptor vector and a reporter gene. As recipient cells, we have used the heterologous human breast cancer cell line T47D. By these means, we have identified a functional ARE that is essential for androgen-regulated expression of the MVDP gene.

EXPERIMENTAL PROCEDURES

DNA Sequencing—The mouse genomic clones employed in these studies were isolated from an amplified library (Clontech Laboratory) obtained by partial *Sau*3AI digestion of BALB/c liver DNA and insertion into the λEMBL 3 vector (Pailhoux *et al.*, 1992). MVDP 5′-flanking DNA was digested with restriction enzymes and the resulting fragments cloned into the pUC19 vector for DNA sequencing. DNA was sequenced by the dideoxy termination method (Sanger *et al.*, 1977) by using the T7 sequencing kit (Pharmacia LKB Biotechnology Inc.). Each subclone extremity was sequenced using universal and reverse pUC19 primers. Restriction maps of these clones were obtained by using the λmap system (Promega).

Construction of CAT Fusion Plasmids—All plasmid constructs were prepared using standard methods (Sambrook *et al.*, 1989). The promoterless basic plasmid pBLCAT3 (Luckow and Schütz, 1987) was used for cloning promoter restriction fragments from the MVDP gene upstream of the bacterial CAT gene. The genomic region investigated comprises 1845 bp of the 5′-flanking sequence. The vector pBLCAT2 contains a herpes simplex viral thymidine kinase promoter directing transcription of the CAT gene. The MMTV-CAT vector contains the upstream sequence of the long terminal repeat promoter of the mouse mammary tumor virus in front of a CAT reporter gene. The pSV-AR_0 vector (Brinkmann *et al.*, 1989) contains an SV40 promoter directing the transcription of the full-length human androgen receptor cDNA. Recombinant plasmids were purified from clear lysates on cesium chloride gradients. Fragments from restriction enzyme-cleaved plasmids were analyzed by electrophoresis on 1% (w/v) agarose gels and identified in relation to molecular size markers by staining with ethidium bromide. Extensive restriction mapping was used to verify all constructs.

Cell Culture and Transfection—No androgenic induction was seen when fragments of the C3 (1) and prostate-specific antigen genes were cloned directly in front of the CAT gene or in front of a thymidine kinase-CAT construct and transfected in homologous prostate cell lines containing androgen receptors (Parker *et al.*, 1987; Riegman *et al.*, 1991). We have chosen heterologous T47D cells in which testosterone stimulated MMTV promoter activity (Parker *et al.*, 1987). T47D cells were grown in Dulbecco's modified Eagle's medium supplemented with glutamine (2 mM), insulin (4 μg/ml), penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% fetal calf serum. For each transfection experiment, cells from a subconfluent flask were seeded at a density of 1.6×10^6 cells/dish and transfected 24 h later with plasmid DNAs using the DEAE-dextran method (Cato *et al.*, 1986). Each transfection included 2.5 μg of pSV-AR_0 vector (receptor expression) and 15 μg of MVDP DNA (regulation of the MVDP promoter) or 10 μg of MMTV-CAT (positive control of androgen induction) or 5 μg of pBLCAT2 vectors. Following a 1-h incubation with the DEAE-dextran/DNA precipitate and dimethyl sulfoxide shock, the cells were incubated in fresh Dulbecco's modified Eagle's medium supplemented with 10% steroid-free donor calf serum containing either 10^{-6} M dihydrotestosterone (DHT) or no additives. Cells were harvested after 48 h of hormone exposure for CAT assays.

CAT Assays—CAT activity of cell extracts was assayed according to the method of Neumann *et al.* (1987). Protein concentration was determined by the Bradford method (Bio-Rad). Samples containing 50–100 μg of protein were incubated in 100 μl of 0.25 M Tris (pH 7.5), 0.4 μM acetyl coenzyme A, 0.2 μCi of [^{14}C]chloramphenicol for 3 h at 37 °C. After thin-layer chromatography and autoradiography, acetylated and nonacetylated forms were cut out and quantitated by scintillation counting. CAT activity corresponds to the ratio of acetylated form radioactivity to total radioactivity (both forms). Average inductions and standard deviations were calculated from at least four independent transfections.

PCR-mediated Mutation of the ARE—For the mutation of the ARE (−111/−97) contained in the MVDP promoter, the fragment −125 to +75 was amplified by the PCR. The forward PCR primer (−125/−86) carrying three mutations (*underlined*) for *Hin*dIII site production (position −119) and for ARE inactivation (positions −102 and −99) 5′-CACAAGCTTGACATGAAGTTCCTTTTTTCATGCCCCAACC-3′ and the reverse PCR primer (+51/+75) placed just downstream of a *Pst*I site 5′-TGGTACTGAGTTCCACGAAGGTGGC-3′ were used for p0.16 mCAT MVDP construct. A second forward PCR primer (−125/−105) carrying one mutation (*underlined*) for *Hin*dIII site production (position −119) 5′-CACCAAGCTTGACATGAAGTTC-3′ and the reverse PCR primer (shown previously) were used for p0.16 CAT MVDP construct.

The PCR products were cut with *Hin*dIII and *Pst*I and isolated from a 2% agarose gel. The isolated fragment was ligated in *Hin*dIII-*Pst*I digested plasmid pBLCAT3. The new plasmids p0.16 mCAT and p0.16 CAT were checked by sequencing.

RESULTS

Sequence of the 5′-Flanking DNA Adjacent to the MVDP Gene—The complete sequence of the MVDP gene (11 kb) and 0.5 kb of the 5′-flanking region have been previously determined (Pailhoux *et al.*, 1992). We completed sequencing the gene and report here a sequence of 1.8 kb upstream from the transcription start site (Fig. 1). A CAAT box, a GC box, and a TATA box sequence are respectively located 65, 51, and 31 bp upstream from the transcription start site. A 94-nucleotide long stretch of purines, interrupted by only 10 pyrimidine residues, is located at position −691. Five potential recognition sequences AGGAAG/A for the PEA3/Ets1 factor are located in this stretch of purines. Numerous potential regulatory sites are present that bind proteins not known to affect MVDP gene expression (Table I). Other potential recognition elements for ATF, TCF-1, eF-1, F-ACT 1, USF, PCF, SIF, and NFkB (Faisst and Meyer, 1992) are also present in the 5′-flanking region of the MVDP gene. A poly(T) sequence of 14 nucleotides and a poly(A) sequence of 14 nucleotides are observed flanking the homopurine stretch at positions −746 and −472, respectively.

A total of three potential cis-acting steroid response elements were identified in the 5′-flanking region of the MVDP gene by selecting for the right half-site of the consensus described. All are imperfect palindromes. A consensus sequence similar to that reported to function as an estrogen-responsive element is

A

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-1804 GATCTTCTCT TTCCAGTGTG TCAGTGGGAT TTCAGAGAGG GGAACATGTA GAGACGTCAC
-1744 AGTGTGAGAT GGAGCTTGGC TATGATTTCT ACCTGATCTT TAGCTTTCTT GCCTAGTTAT
-1684 CTGGTCTTTG ATGGGGTGAG CCTGTGTGTA GGCTTCAGAG ATGTCCATAT GACTATCCAG
-1624 TCTCTAGCCA CAGAGCCTAC TCCCTGAGTC CAATTTACCT GACTGTGACA AGTGTATGCC
-1564 AGTACATGAC ATTGTTTTAC AATTCTAACT ACGTGGTGGC TAGCTGGTGC TCTTCTTCAG
-1504 CTAGATTCTA CAGCACATGT AGACTATCCC ATGTGCGAAG ACTCAAGAAA TACAGAGGGA
-1444 ACCCTCTGCA CATACTGGGG CAGTAATTGT CAGTGTCCTG GGATATGGAA CTTCTATGAA
-1384 ATACCTACAA TGGTACACTT AGGGATATTA CTCATTCCTA TTATAAACCA ATCGAAAACA
-1324 AACTAAATAT TTAAGAATAG TGGAAATGAC CAAATAAATG TTGACAAAAT AAAATCTTTT
-1264 TGCAACCATT AAAATGTATG TGGTAAAAGT AATTTTAAAA TGGTGTTTTA GGGCTGGAAA
-1204 GAAGGCTTAG CGGTTAAAAG AACATGCTGC TCTAACCGAG GACCCATGTT CAATTCCCAC
-1144 ACCCAGTGGC TCACAACCAC CTATAGCCTC CAGGGGATCT GATGTCCTGG GATGGCCTCC
-1084 ACAGGGCTGC ACACGCGTGC ATGGGGCACA TGCAAAAGTA AGAAACAAAA CCAAAACATG
-1024 AAGTCAAAGT GACTTGTTGT AGATGTTTAG AAAGTTTGTT TTTATGAAGT TAGCTTCCAA
 -964 AAATATATGT ATAGTATGTA AGAACTAAGA CATATATACT CAGAGACATT ATTAATAAAC
 -904 ACATATGCAA AGACAGAAGA AAACAGCACT GCGAATGGCA GTTTAGGTGA TGGGGATATT
 -844 CTGCTTCAGG TTCTCACCCT TATAAACCAT CCTTGCGTTT CACAGTTGAG TACATGACAT
 -784 TTCATAGTCA CAAAAGATTG TTTTCAAATC AGGCACAGTT TTTTTTTTTT TTTAATGTCG
 -724 TTATAAAACA CAATTGGTCA AATCCCTAAA TCTGAAAGAA AAGAAAAGGA AGAAGAAGAA
 -664 GAAAAGTAGG CAACGAGGAA GAAAGGAAAA ACAAAGGAAG GAGAAAGAGA ATTTCATTAG
 -604 GAAAGAACCT GTCCCAGTGG CTAGCAGGTC ATTTTCATCC TCAGGGAATC TTCTTGCCTT
 -544 GCAATCTGAC AGAAAACGCA CAGATACAGG CATTTCTAGA AAGGTGTCCA TGACCTACAT
 -484 TTAAAAAAAA AAAAAATCTT TCCACAAGGT CACCTGCCAG TTTCCTCTGT AACAGACATC
 -424 CCTTAGTTTT GTGGCCAAGG ACAGTTATTT CAAAGTAGTT ACTGTAAAAG ATGTCTCATT
 -364 TTTCCTCGAC ATTTTTATTT GCCTCAAACT GCATGAGCAC CCACGCAGTT CACTAGCGTT
 -304 ATATCACTCC CAGGGCAATG ACCCTTCCCA AATAAACTTA CCAGTTCATC TTGGATGACC
 -244 TTTCAACCAT GTCACTTAGG TTCACAAACC TCTGATCCGA TACCCAATAA AGCATCGATT
 -184 CTGCCCAGAT AGCCTTTGCA CTGTGCTGAC TTTAATCTTT GTTTACGATG ATCAGTGTGG
 -124 CACAAGATTG ACATGAAGTT CCTGTTCTCA TGCCCCAACC CTTGGCTGTG GCTGCTTGCC
  -64 AATGTGGTAA GAGCCCGCCT CCTTTATCCA GGACATAAAA ATGTCACCAG CCTCCTTGTA
   -4 GAGAAAGCAG GCATTTCATC TGCTCACTCA GAGAACTCTC TGCAG +41
      +1
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B

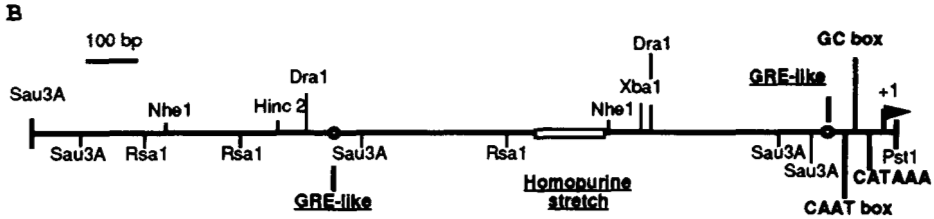

FIG. 1. **Sequence features of the MVDP gene promoter and 5′-flanking region.** *A*, about 1800 residues of the sequence extending from nucleotides −1804 to +41 are shown. All negative numbering is relative to the start site of transcription (+1). The TATA box, GC box, CAAT box, and 94-bp homopurine stretch are *underlined*. GRE-like sequences are *double underlined*. *B*, restriction enzyme map of the MVDP gene promoter extending from nucleotides −1804 (*Sau*3AI) to +41 (*Pst*I).

located at position −503. Two GRE-like sequences were identified at positions −1186 and −111 (Table I). The two AREs show a complete homology with the right half-site of the GRE/PRE consensus (5′-TGTTCT-3′). For the distal ARE, this homology is situated on the noncoding strand.

Analysis of MVDP Promoter Activity—As a control of the transfection efficiency and androgen induction, MMTV promoter activity was initially examined in T47D cells. Such constructs were described to be androgen-responsive in T47D cells that contain receptors for androgen, glucocorticoid, progestin, and estrogen (Cato *et al.*, 1987; Parker *et al.*, 1987). In our experiments, MMTV promoter activity can be stimulated by progesterone but not by DHT (not shown). Cotransfection of T47D cells with MMTV-CAT and the androgen receptor expression vector (pSV-AR_0) gave positive results, suggesting that low levels of androgen receptor are responsible for the failure of DHT to induce MMTV promoter activity.

A series of MVDP putative promoter fragments were cloned in front of the CAT reporter gene. Since the transcription start site was found 40 nucleotides upstream of a *Pst*I site, we ligated the *Sau*3AI-*Pst*I (p1.8 CAT), *Hin*cII-*Pst*I (p1.3 CAT), *Rsa*I-*Pst*I (p0.8 CAT), *Xba*I-*Pst*I (p0.5 CAT), and *Hin*dIII-*Pst*I (p0.16 CAT)

TABLE I

Potential regulatory sequences in MVDP gene 5′-flanking region

Nucleotide numbering is according to the transcription start site (+1) and refers to the 5′ nucleotide. Regulatory proteins are abbreviated. Consensus DNA sequences for regulatory factor binding are according to Faisst and Meyer (1992).

Nucleotide number	Sequence	Regulatory protein	Homology
			%
−1788	TGTGTCA	AP 1	85.7
−1635	TGACTAT	AP 1	85.7
−1600	TGAGTCC	AP 1	85.7
−1529	GTGGCTAG	C/EBP	87.5
−1477	CCCATGTG	AP 2	87.5
−1466	AGACTCA	AP 1	85.7
−1357	TTACTCA	AP 1	85.7
−1357	TTACTCA	AP 1	100[b]
−1305	GTGGAAAT	C/EBP	87.5
−1297	TGACCAA	AP 1	85.7
−1283	TGACAAA	AP 1	85.7
−1245	GTGGTAAA	C/EBP	87.5
−1186	AGAACATGCTGCTCT	PR/GR/AR	93.3
−1162	CCCATGTT	AP 2	75
−1150	TCCCACACCCA	CACCC[a]	81.8[b]
−1149	CCCACACC	AP 2	87.5
−1142	CCAGTGG	AP 2	87.5
−1138	TGGCTCA	AP 1	85.7
−798	TGAGTAC	AP 1	85.7
−592	CCCAGTGG	AP 2	87.5
−588	GTGGCTAG	C/EBP	75
−574	ATTTTCAT	OCT 1	87.5[b]
−561	GGGAATCTTC	NFKB	90
−503	AGGTGTCCATGACCT	ER	86.6
−500	TFTCCATGACCT	RAR	75
−467	CTTTCCAC	C/EBP	100[b]
−373	TGTCTCA	AP 1	85.7
−348	ATTTGCCT	OCT 1	87.5[b]
−345	TGCCTCA	AP 1	85.7
−325	CCCACGCA	AP 2	87.5
−296	CCCAGGGC	AP 2	100
−111	TGAAGTTCCTGTTCT	PR/GR/AR	86.6
−102	CCCCAACC	AP 2	87.5
−101	CCCAACCC	AP 2	87.5
−76	TGGCTGCTTGCCAA	NF1	100
−61	GTGGTAAG	C/EBP	100
−52	GCCCGCCTC	SP1	88.9[b]

[a] Myers *et al.*, 1986.

[b] With sequence in the antisense strand.

fragments containing this start point to the CAT reporter gene (Fig. 2). To delineate the responsive region, MVDP constructs were cotransfected with pSV-AR_0 in T47D cells in the absence or in the presence of 10^{-6} M DHT (T47D cells do not produce MVDP).

Cotransfection of p1.8 CAT (−1804, +41) with pSV-AR_0 in the absence of DHT resulted in a basal expression of 3% acetylation. Constructs containing subfragments of 1.3, 0.8, and 0.5 kb of the MVDP upstream region showed similar basal activity. However, basal expression for the minimal construct containing the −121 to +41 fragment was significantly higher (about 3-fold) than that of the largest segments of the MVDP 5′-flanking DNA.

The 1.8-kb fragment, which contains two GRE-like sequences, showed detectable androgen-dependent transcriptional enhancer activity. In the presence of 10^{-6} M DHT, CAT activity increased about 4-fold over the level of basal activity in the absence of androgen (Fig. 2). In order to identify the active androgen-responsive element, subfragments were analyzed. As shown in Fig. 2, deletion of the MVDP 5′-flanking DNA between nucleotides at positions −1804 and −121 resulted in a progressive increase in the absolute level of androgen-induced CAT activity. A similar observation was made when CAT activity was expressed as -fold increase over basal level, except for the p0.16 CAT construct. The lesser -fold increase observed for this fragment is probably related to its high basal activity (see above). On the basis of this data, it is clear that androgen-dependent transcriptional activity is not decreased by deletion of the distal GRE-like sequence. Similarly, deletion of the homopurine stretch does not significantly affect the activity of the resulting fragments (p0.5 CAT compared with p0.8 CAT, Fig. 2).

Although a minimal region located −121 to +41 is sufficient for androgen-dependent transcriptional activity, the region −510 to +41 gave maximal androgen induction (about 12-fold). Cotransfection with pSV-AR_0 in the presence of DHT at various concentrations showed that DHT stimulated p0.5 CAT construct in a dose-dependent manner (Fig. 3). To determine whether the 0.5-kb fragment contains a functional ARE responsible for androgen responsiveness, it was cloned in opposite orientation in front of the heterologous thymidine kinase promoter-CAT construct. Various concentrations of DHT showed stimulation of the thymidine kinase promoter activity (1.6–2.6-fold increase) in a dose-dependent fashion, irrespective of the 0.5-kb fragment orientation (Fig. 3). This effect was not observed with the control pTk-CAT vector (pBLCAT2). Thus, within the −510 to +41 fragment, a DNA motif is able to function as an androgen-dependent enhancer.

Identification of Functional AREs—The GRE-like sequence at position −111 is the most likely candidate to be involved in the androgen responsiveness. To determine the role of this TGAAGT tcc TGTTCT sequence present in p0.16 CAT, it was mutated to TGAAGT tcc TTTTTT in p0.16 mCAT (see "Experimental Procedures"). The mutated construct was cotransfected with the AR vector (pSV-AR_0) into T47D cells. As shown in Fig.

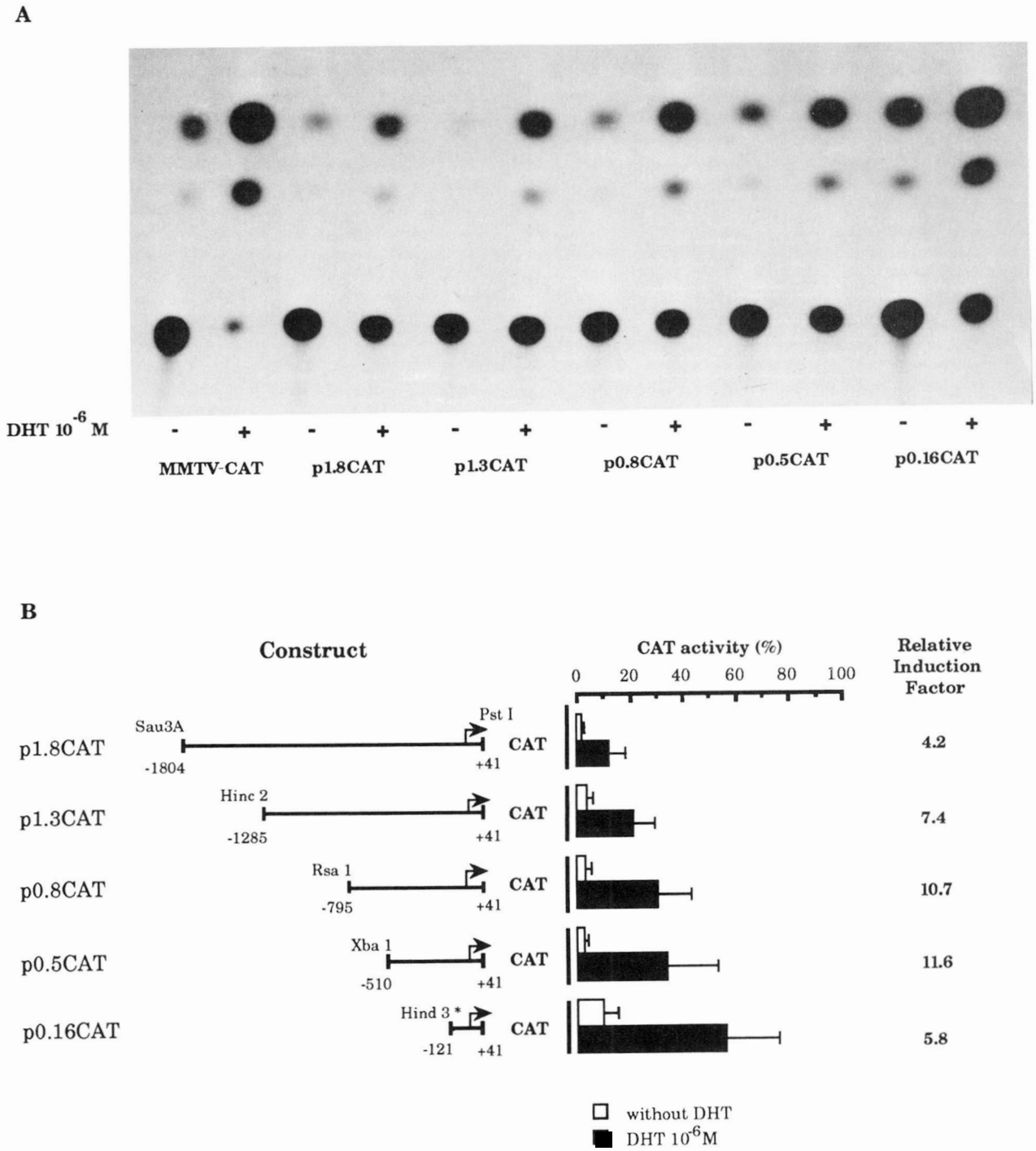

FIG. 2. **Androgen-dependent transcriptional enhancer activities of the MVDP promoter and 5′-flanking region.** *A*, autoradiogram of CAT gene expression assays measured on homogenates of cells cotransfected with different plasmid constructs and pSV-AR_0. *B*, induction of CAT activity by MVDP-CAT constructs. Restriction fragments of MVDP 5′-flanking region were cloned upstream of the CAT reporter gene in pBLCAT3. Nucleotide numbering is according to Fig. 1. (The *arrow* represents the transcription start site.) Constructs were cotransfected with pSV-AR_0 into T47D cells. Cells were subsequently grown in the absence or presence of 10^{-6} M DHT. *Asterisk*, restriction site produced by PCR (experimental details are as described under "Experimental Procedures").

4, the mutation resulted in an almost total loss of the transcription activation by DHT compared with p0.16 CAT. The results strongly suggest the importance of this motif in the androgen responsiveness of the MVDP gene.

As shown above, deletion of the distal GRE-like sequence does not decrease activity of the resulting fragments. However, to determine whether this sequence can act as a functional ARE, it was taken out of the MVDP promoter and linked to the thymidine kinase-CAT construct. We ligated the *Hinc*II-*Sau*3AI (about 180 nucleotides) and *Sau*3AI-*Xba*I (about 1300 nucleotides) to the pBLCAT2 plasmid, and these constructs were cotransfected with the AR vector in the presence of 10^{-6} M DHT. The results indicated that the distal GRE-like sequence, either alone (180 nucleotides) or associated with the homopurine stretch (1300 nucleotides), failed to enhance the activity of the thymidine kinase promoter (data not shown).

DISCUSSION

Sequence analysis of the upstream region from the androgen-regulated MVDP gene was carried out to locate conserved

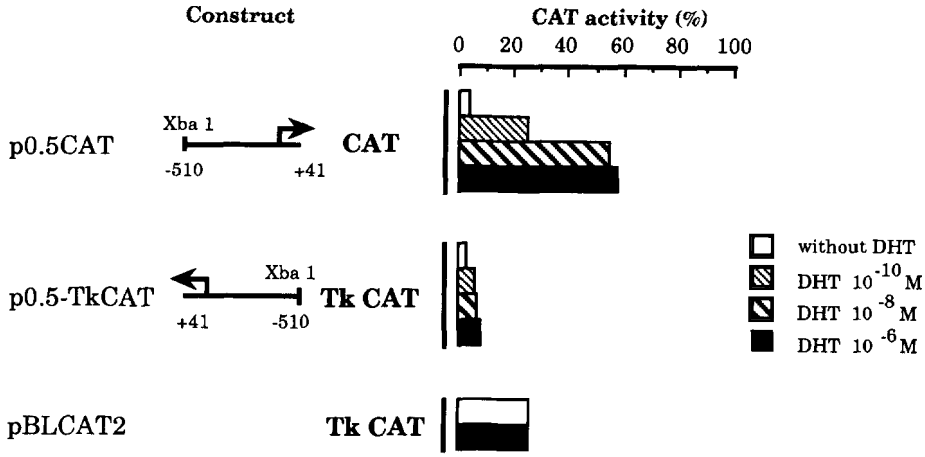

FIG. 3. **Trancription-stimulating activity of the 0.5-kb fragment transfected into T47D cells in the presence of various concentrations of DHT.** In the p0.5-thymidine kinase-CAT construct, the genomic fragment (-510 to +41) of the MVDP promoter was linked to the heterologous thymidine kinase promoter in opposite orientation and cotransfected with pSV-AR_o. Nucleotide numbering is according to Fig. 2. *Tk*, thymidine kinase.

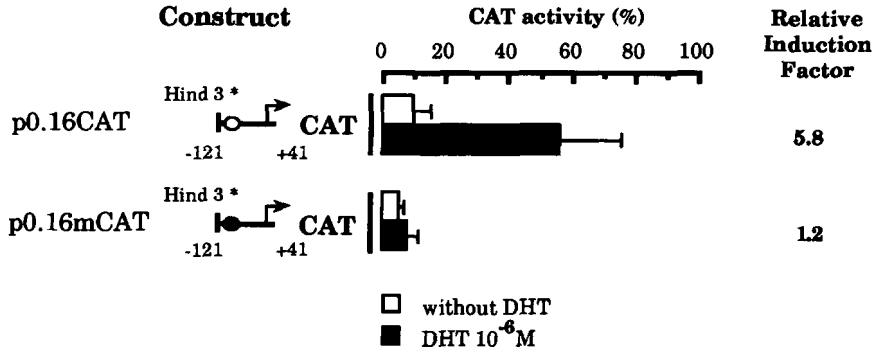

FIG. 4. **Effect of point mutations on ARE activity.** The effect of DHT on CAT activity was determined for constructs in which the ARE sequence (-111/-97) TGAAGT tcc TGTTCT (p0.16 CAT, ○) was mutated to TGAAGT tcc TTTTTT (p0.16 mCAT, ●). Constructs were cotransfected with pSV-AR_o into T47D cells. *Asterisk*, restriction site produced by PCR. Experiments were carried out as described under "Experimental Procedures." Nucleotide numbering is according to Fig. 2.

sequence motifs that could possibly be involved in the androgen regulation or tissue specificity of transcription. Sequences with proven GRE/PRE activity have been well characterized (Cato *et al.*, 1986; Beato, 1989), and elements that mediate androgen-dependent regulation of gene transcription might be expected to be similar, since they share sequence similarity with the DNA-binding domain of glucocorticoid and progesterone receptors (Evans, 1988; Lubahn *et al.*, 1988).

Two GRE-like sequences were identified within the 1.8 kb upstream of the MVDP gene, and we determined their activities in transcriptional enhancer assays. Gene fusion experiments with deleted fragments in the upstream region showed that a -121- to +41-bp fragment was sufficient for androgen-responsive CAT activity. We have identified a canonical ARE at position -111 to -97 that can function both in the context of the MVDP promoter or as a separate element when cloned in front of the heterologous thymidine kinase promoter. This sequence plays a critical role in androgen responsiveness, as its mutation causes a loss of induction. Then, we may conclude that the minimal promoter of the MVDP gene contains a functional ARE that does not depend on tissue-specific factors from the vas deferens epithelial cells. The sequence 5′-TGAAGT tcc TGTTCT-3′ of the functional ARE in the MVDP gene closely resembles one of the most effective synthetic oligonucleotides, 5′-GGTACA nnn TGTTCT-3′, showing GRE and ARE activity (Ham *et al.*, 1988; Roche *et al.*, 1992). However, poor homology was observed with the left half-site of the GRE/PRE consensus. Studies on the regulation of specific gene expression by androgen receptors have not been as successful as similar studies for other steroid hormones, owing to the fact that good model systems to study androgen-induced gene expression are in short supply. Most studies of androgen response elements have used the MMTV long terminal repeat, which responds to glucocorticoid, progesterone, and androgen via GRE/PRE consensus (Cato *et al.*, 1987; Beato, 1989). Although the expression of a large number of genes is known to be androgen-dependent, relatively little is known about the interaction of the androgen receptor with the corresponding AREs. At present, among cellular genes, eight gene fragments have been shown to act as ARE. In the rat C3 (1) gene, a strong androgen binding region located in the first intron confers androgen responsiveness of the thymidine kinase promoter and contains a GRE half-site, which plays a critical role, as its mutation causes loss of induction (Rushmere *et al.*, 1987; Claessens *et al.*, 1989, 1990; Tan *et al.*, 1992). A 0.75-kb fragment, which was found to reside within the 5′ long terminal repeat of an ancient endogenous provirus in the upstream region of the mouse sex-limited protein gene, was sufficient, in either orientation, for androgen-inducible expression (Loreni *et al.*, 1988; Stavenhagen and Robins, 1988). The promoter of the prostate-specific antigen gene contains a functional ARE, the mutation of which almost completely abolishes androgen-inducible expression (Riegman *et al.*, 1991). The mouse ornithine decarboxylase gene contains an ARE-like DNA motif starting at about -910 from the cap site, which was shown to be able to bind recombinant androgen receptor protein *in vitro* and to confer androgen responsiveness upon a reporter gene (Eisenberg and Jänne, 1989; Crozat *et al.*, 1992). The plasma factor IX promoter contains a sequence resembling a consensus ARE that can bind androgen receptor *in vitro* but functions well only in CAT assays when multimerized (Crossley *et al.*, 1992). The mouse β-glucuronidase gene contains an ARE-like sequence, located in the ninth intron, that mediates an-

TABLE II
Alignment of functional ARE sequences characterized in 9 androgen-regulated genes

Genes	Sequences	References
C3 (CORE II)	5′-AGTACG tga TGTTCT-3′	Claessens *et al.*, 1989
SLP (HRE 3)	5′-GAAACA gcc TGTTCT-3′	Adler *et al.*, 1991
PSA	5′-AGCACT tgc TGTTCT-3′	Riegman *et al.*, 1991
Probasin (ARE 1)	5′-ATAGCA tct TGTTCT-3′	Rennie *et al.*, 1993
GUS (intron 9)	5′-AGTACT tgt TGTTCT-3′	Lund *et al.*, 1991
ODC	5′-AGTCCC act TGTTCT-3′	Crozat *et al.*, 1992
Factor IX	5′-AGCTCA gct TGTACT-3′	Crossley *et al.*, 1992
MEP 24	5′-AGAACA acc TGTTGA-3′	Ghyselinck *et al.*, 1993
MVDP (proximal ARE)	5′-TGAAGT tcc TGTTCT-3′	Present results
Prevalence	5′-AGAACA nnn TGTTCT-3′ 774684 999888	

drogen regulation (Lund *et al.*, 1991). The promoter of the probasin gene contains two functional AREs acting together for androgen regulation (Rennie *et al.*, 1993). The gene for the glutathione peroxidase-like androgen-regulated murine epididymal protein (MEP24) contains an imperfect palindromic sequence at position −896 to −882, which confers androgen regulation (Ghyselinck *et al.*, 1993). Thus, androgen regulation of these genes, and probably others, appears to be exerted through sequences that resemble a consensus GRE and that function at a distance upstream and downstream of the promoter. A comparison of the sequences of these functional AREs is shown in Table II. The right half-site TGTTCT is well conserved and present in seven of the nine AREs characterized. A possible consensus sequence of the ARE is AGAACA nnn TGTTCT, which is a perfect palindrome. This sequence is almost identical to the ARE sequence GGA/TACA nnn TGTTCT determined by a DNA-binding site selection assay (Roche *et al.*, 1992). The sequence conservation of the left half-site is lower than that of the right half-site (Table II). This asymmetry is consistent with studies, indicating that steroid receptors bind to their responsive element in the form of homodimers, one component of the dimer interacting with one half-site of the pseudo-palindromic sequence (Kumar and Chambon, 1988; Dahlman-Wright *et al.*, 1991). For the glucocorticoid receptor, only one subunit seems to interact specifically with the consensus target and the other nonspecifically with a noncognate element (Luisi *et al.*, 1991). Although a perfect palindrome was not required for maximal androgen induction, the presence of ACA in the GREs of the MMTV long terminal repeat conferred the highest androgen inducibility (Ham *et al.*, 1988). The presence of ACA is also observed in the AREs of cellular androgen-regulated genes (Table II). The ornithine decarboxylase gene-ARE exhibits a low affinity for androgen receptor; when the C at position 4 was converted to an A, its competitive ability increased over 10-fold (Crozat *et al.*, 1992). These observations suggest that an A at position 4 in the left half ARE motif plays a critical role in androgen responsiveness.

Mutation of the TGTTCT sequence of the MVDP gene proximal ARE to TTTTTT resulted in an almost total loss of androgen responsiveness, indicating that the presence of G and/or C nucleotides in this half-site is essential to maintain the ARE-mediated induction of the MVDP gene transcription. Similarly, in the C3 (1) gene, the substitution G to T in the core II hexanucleotide sequence TGTTCT abolished androgen responsiveness (Claessens *et al.*, 1989). In the factor IX promoter, a unique G to C mutation in the ARE sequence TGTACT prevents any expression of the protein after puberty (Crossley *et al.*, 1992). It appears that *in vitro*, as well as *in vivo*, a G nucleotide at position 2 of the right half-site of ARE is essential for AR/DNA interactions.

Based on sequence similarity, another potential ARE is present in the 5′-flanking region of the MVDP gene at position −1172. Although androgen transcriptional activity was not detected with this sequence and although androgen responsiveness was not affected by its deletion, the possibility exists that in homologous vas deferens epithelial cells, this distal ARE functions cooperatively with the proximal one. Cooperativity among AREs has been described in several genes regulated by androgens (Adler *et al.*, 1991; Rennie *et al.*, 1993). Similarly, some androgen-regulated genes require the presence of additional DNA sequences (the nature of which remains to be determined) for full androgen induction (Riegman *et al.*, 1991; Lund *et al.*, 1991; Crossley *et al.*, 1992).

In addition to the potential cooperativity among the two AREs within the region upstream of the MVDP gene, androgen receptors may also interact cooperatively with other transcription factors to modulate MVDP gene expression. Several sequences, homologous with known recognition sites for gene regulatory proteins, were present in the 5′-flanking region of the MVDP gene (Table I). It has been previously shown that GREs can undergo synergistic interactions with a wide variety of heterologous binding sites, such as those for SP1, NF1, and CCAAT box binding factors (Schüle *et al.*, 1988; Strähle *et al.*, 1988; Cato *et al.*, 1988). Interestingly, consensus sequences for ubiquitous SP1, NF1, and CCAAT box binding factors were closely associated with the proximal ARE of the MVDP gene. Other consensus sequences for NFkB (Adler *et al.*, 1991) and OCT-1 (Brüggemeier *et al.*, 1991), present in the MVDP 5′ region, have been described as potential cooperating factors with AR and glucocorticoid receptor. The five potential recognition sequences for the PEA3/Ets1 factor, located in the homopurine stretch, are of particular interest, since it has been shown that the PEA3 protein is expressed in epididymis (Xin *et al.*, 1992) and testis (Ito *et al.*, 1993) of mouse. Numerous putative AP1- and AP2-binding sites are present in the 5′-flanking DNA of the MVDP gene. These transcription factors mediate induction by two different signal transduction pathways as follows: protein kinase C and cAMP (Imagawa *et al.*, 1987). As the expression of the MVDP gene is confined to the vas deferens epithelial cells (Martinez *et al.*, 1990), some transcription factors required for activation of the MVDP promoter should be cell-specific. Evidence was obtained that there are tissue-specific differences in the proteins that bind to the C3 (1) promoter region and that CAAT box/enhancer binding protein itself may be one of the transcription factors involved (Zhang and Young, 1991). Whether these motifs and proteins are functional in the MVDP gene remains to be determined. The implications of some factors mentioned above in the full specific responsiveness of the transcription of the MVDP gene to androgens is suggested by cycloheximide experiments, showing that the induction of the MVDP gene by androgens depends on continuous protein synthesis (Dassouli *et al.*, 1994).

Male accessory sexual organs of most mammals contain both aldose reductase and sorbitol dehydrogenase and provide the fructose and sorbitol of seminal plasma (Mann, 1964). Sperm contains sorbitol dehydrogenase, and it has been recently shown that a fructose transporter is expressed in human spermatozoa (Burant *et al.*, 1992). Whether the expression of the human (Graham *et al.*, 1991a) and rat (Graham *et al.*, 1991b) aldose reductase and MVDP genes, which have a common evolutionary origin (Pailhoux *et al.*, 1990), is subject to the same regulation remains to be determined.

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REFERENCES

Adler, A. J., Scheller, A., Hoffman, Y., and Robins, D. M. (1991) *Mol. Endocrinol.* **5,** 1587–1596
Beato, M. (1989) *Cell* **56,** 335–344
Bondy, C. A., and Lightman, S. L. (1989a) *Mol. Endocrinol.* **3,** 1417–1425
Bondy, C. A., Lightman, S. L., and Lightman, S. L. (1989b) *Mol. Endocrinol.* **3,** 1409–1416
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) *J. Steroid Biochem.* **34,** 307–310
Brüggemeier, U., Kalff, M., Franke, S., Scheidereit, C., and Beato, M. (1991) *Cell* **64,** 565–572
Burant, C. F., Takeda, J., Brot-Laroche, E., Bell, G. I., and Davidson, N. O. (1992) *J. Biol. Chem.* **267,** 14523–14526
Carson-Jurica, M. A., Schrader, W. T., and O'Malley, B. W. (1990) *Endocr. Rev.* **11,** 201–220
Cato, A. C. B., Miksicek, R., Schütz, G., Arnemann, J., and Beato, M. (1986) *EMBO J.* **5,** 2237–2240
Cato, A. C. B., Henderson, D., and Ponta, H. (1987) *EMBO J.* **6,** 363–368
Cato, A. C. B., Skroch, P., Weinmann, J., Butkeraitis, P., and Ponta, H. (1988) *EMBO J.* **7,** 1403–1410
Claessens, F., Celis, L., Peeters, B., Heyns, W., Verhoeven, G., and Rombauts, W. (1989) *Biochem. Biophys. Res. Commun.* **164,** 833–840
Claessens, F., Rushmere, N. K., Davies, P., Celis, L., Peeters, B., and Rombauts, W. A. (1990) *Mol. Cell. Endocrinol.* **74,** 203–212
Crossley, M., Ludwig, M., Stowell, K. M., De Vos, P., Olek, K., and Brownlee, G. G. (1992) *Science* **257,** 377–379
Crozat, A., Palvimo, J. J., Julkunen, M., and Jänne, O. A. (1992) *Endocrinology* **130,** 1131–1144
Dahlman-Wright, K., Wright, A., Gustafsson, J. A., and Caristedt-Duke, J. (1991) *J. Biol. Chem.* **266,** 3107–3112
Dassouli, A., Manin, M., Veyssière, G., and Jean, Cl. (1994) *J. Steroid Biochem. Mol. Biol.*, in press
Denison, S. H., Sands, A., and Tindall, D. J. (1989) *Endocrinology* **124,** 1091–1093
Eisenberg, L. M., and Jänne, O. A. (1989) *Nucleic Acids Res.* **17,** 2359
Evans, R. M. (1988) *Science* **240,** 889–895
Faisst, S., and Meyer, S. (1992) *Nucleic Acids Res.* **20,** 3–26
Ghyselinck, N. B., Dufaure, I., Lareyre, J. J., Rigaudière, N., Mattéi, M. G., and Dufaure, J. P. (1993) *Mol. Endocrinol.* **7,** 258–272
Graham, A., Brown, L., Hedge, P. J., Gammack, A. J., and Markham, A. F. (1991a) *J. Biol. Chem.* **266,** 6872–6877
Graham, C., Szpirer, C., Levan, G., and Carper, D. (1991b) *Gene (Amst.)* **107,** 259–267
Ham, J., Thompson, A., Needham, M., Webb, P., and Parker, M. (1988) *Nucleic Acids Res.* **16,** 5263–5276
Imagawa, M., Chiu, R., and Karin, M. (1987) *Cell* **51,** 251–260
Ito, E., Toki, T., Ishihara, H., Ohtani, H., Gu, L., Yokoyama, M., Engel, J. D., and Yamamoto, M. (1993) *Nature* **362,** 466–468
Iwata, N., Inazu, N., and Satoh, T. (1990) *Arch. Biochem. Biophys.* **282,** 70–77
Jeffery, J., and Jörnvall, H. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 901–905
Kinoshita, J. H., Kador, P., and Catiles, M. (1981) *JAMA* **246,** 257–261
Kumar, V., and Chambon, P. (1988) *Cell* **55,** 145–156
Loreni, F., Stavenhagen, J., Kalff, M., and Robins, D. M. (1988) *Mol. Cell. Biol.* **8,** 2350–2360
Lubahn, D. B., Joseph, D. R., Sar, M., Tan, J., Higgs, H. N., Larson, R. E., French, F. S., and Wilson, E. M. (1988) *Mol. Endocrinol.* **2,** 1265–1275
Luckow, B., and Schütz, G. (1987) *Nucleic Acids Res.* **15,** 5490
Luisi, B. F., Xu, W. X., Otwinowski, Z., Freedman, L. P., Yamamoto, K. R., and Sigler, P. B. (1991) *Nature* **352,** 497–505
Lund, S. D., Gallagher, P. M., Wang, B., Porter, S. C., and Ganschow, R. E. (1991) *Mol. Cell. Biol.* **11,** 5426–5434
Maniatis, T., Goodbourn, S., and Fischer, J. A. (1987) *Science* **236,** 1237–1245
Manin, M., Veyssière, G., Cheyvialle, D., Chevalier, M., Lecher, P., and Jean, Cl. (1992) *Endocrinology* **131,** 2378–2386
Mann, T. (1964) in *Biochemistry of Semen and of the Male Reproductive Tract*, pp. 237–303, Methuen, London
Martinez, A., Berger, M., and Jean, Cl. (1989) *J. Endocr.* **120,** 67–74
Martinez, A., Pailhoux, E., Berger, M., and Jean, Cl. (1990) *Mol. Cell. Endocrinol.* **72,** 201–211
Martinez, A., Pailhoux, E., and Jean, Cl. (1991) *Mol. Cell. Endocrinol.* **76,** 191–200
Myers, R. M., Tilly, K., and Maniatis, T. (1986) *Science* **232,** 613–618
Neumann, J. R., Morency, C. A., and Russian, K. O. (1987) *Biotechniques* **5,** 444–447
Nordeen, S. K., Such, B. J., Kuhnel, B., and Hutchinson, C. A. (1990) *Mol. Endocrinol.* **4,** 1966–1973
Pailhoux, E. A., Martinez, A., Veyssière, G. M., and Jean, C. G. (1990) *J. Biol. Chem.* **265,** 19932–19936
Pailhoux, E., Veyssière, G., Fabre, S., Tournaire, C., and Jean, Cl. (1992) *J. Steriod Biochem. Mol. Biol.* **42,** 561–568
Parker, M. G., Webb, P., Needham, M., White, R., and Ham, J. (1987) *J. Cell. Biochem.* **35,** 285–292
Petrash, J. M., Flath, M., Sens, D., and Bylander, J. (1992) *Biochem. Biophys. Res. Commun.* **187,** 201–208
Rennie, P. S., Bruchovsky, N., Leco, K. J., Sheppard, P. C., McQueen, S. A., Cheng, H., Snoek, R., Hamel, A., Bock, M. E., McDonald, B. S., Nickel, B. E., Chang, C., Liao, S., Cattini, P. A., and Matusik, R. J. (1993) *Mol. Endocrinol.* **7,** 23–36
Riegman, P. H. J., Vlietstra, R. J., van der Korput, J. A. G. M., Brinkmann, A. O., and Trapman, J. (1991) *Mol. Endocrinol.* **5,** 1921–1930
Roche, P. J., Hoare, S. A., and Parker, M. G. (1992) *Mol. Endocrinol.* **6,** 2229–2235
Rushmere, N. K., Parker, M. G., and Davies, P. (1987) *Mol. Cell. Endocrinol.* **51,** 259–265
Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 5463–5467
Schüle, R., Muller, M., Otsuka-Murakami, H., and Renkawitz, R. (1988) *Nature* **332,** 87–90
Stavenhagen, J. B., and Robins, D. M. (1988) *Cell* **55,** 247–254
Strähle, U., Schmid, W., and Schütz, G. (1988) *EMBO J.* **7,** 3389–3395
Tan, J-A., Marschke, K. B., Ho, K.-C., Perry, S. T., Wilson, E. M., and French, F. S. (1992) *J. Biol. Chem.* **267,** 4456–4466
Taragnat, C., Berger, M., and Jean, Cl. (1988) *J. Reprod. Fertil.* **83,** 835–842
Taragnat, C., Berger, M., and Jean, Cl. (1990) *J. Androl.* **11,** 279–286
Xin, J. H., Cowie, A., Lachance, P., and Hassell, J. A. (1992) *Genes Dev.* **6,** 481–486
Zhang, H., and Young, A. P. (1991) *J. Biol. Chem.* **266,** 24332–24338