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The rat androgen receptor gene promoter

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Summary

The androgen receptor (AR) is activated upon binding of testosterone or dihydrotestosterone and exerts regulatory effects on gene expression in androgen target cells. To study transcriptional regulation of the rat AR gene itself, the 5′ genomic region of this gene was cloned from a genomic library and the promoter was identified. S1-nuclease protection analysis showed two major transcription start sites, located between 1010 and 1023 bp upstream from the translation initiation codon. The area surrounding these start sites was cloned in both orientations in a CAT reporter plasmid. Upon transfection of the constructs into COS cells, part of the promoter stimulated transcription in an orientation-independent manner, but the full promoter showed a higher and unidirectional activity. In the promoter/reporter gene constructs, transcription initiated from the same positions as in the native gene. Sequence analysis showed that the promoter of the rat AR gene lacks typical TATA and CCAAT box elements, but one SP1 site is located at about 60 bp upstream from the major start site of transcription. Other possible promoter elements are TGTYCT sequences at positions −174 to −179, −434 to −439, −466 to −471, and −500 to −505, resembling half-sites of the glucocorticoid-responsive element (GRE). Furthermore, a homopurine stretch containing a total of 8 GGGGA elements and similar to sequences that are present in several other GC-rich promoters, is located between −89 and −146 bp upstream from the major start site of transcription.

Introduction

Following differentiation of the testis, androgen action is essential for development of the male reproductive tract, virilization and full initiation and maintenance of spermatogenesis (Griffin and Wilson, 1989). The androgenic hormones testosterone and dihydrotestosterone exert their effects on gene expression through binding to the androgen receptor (AR). The AR belongs to the family of ligand-activated transcription factors that includes the other steroid hormone receptors as well as the thyroid hormone, the retinoic acid and the vitamin D_3 receptors. The progesterone and glucocorticoid receptor are structurally most closely related to the AR (Tilley et al., 1989).

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Recently, the cDNAs encoding the human and rat AR have been cloned (Chang et al., 1988; Lubahn et al., 1988; Trapman et al., 1988). The rat AR mRNA is approximately 10 kb, containing large 5′ and 3′ untranslated regions (UTRs) of approximately 1 and 7 kb respectively. The open reading frame encodes a protein with 902 amino acid residues. The rat and human AR mRNAs show a high degree of sequence similarity in the coding regions and the 5′ UTRs.

The regulation of AR protein levels may constitute an important level of control at which the physiological effects of testosterone can be modulated. In the testis, AR protein is expressed in Sertoli cells, Leydig cells and peritubular cells, but not in the developing germ cells (Grootegoed et al., 1977; Buzek and Sanborn, 1988). Recently, it has been shown that both follicle-stimulating hormone (FSH) and testosterone can stimulate AR protein levels in cultured Sertoli cells from immature rats (Verhoeven and Cailleau, 1988; Blok et al., 1989). However, only FSH stimulated the amount of AR mRNA, whereas testosterone had no effect on mRNA expression (Blok et al., 1989). In other tissues, including prostate, kidney, brain and epididymis, the AR mRNA content is down-regulated by testosterone (Quarmby et al., 1990).

The cellular amount of AR mRNA may be regulated at the level of transcription, but also through alteration of its stability. To study a possible regulatory effect of testosterone and FSH on the rate of AR gene transcription, the AR gene promoter should be defined. Here we report the identification and characterization of the rat AR gene promoter.

Materials and methods

Isolation of 5′ genomic rat AR clones

A rat genomic library was constructed by cloning rat DNA that had been partially digested with *Mbo*I into the *Bam*HI site of λEMBL3. This library (1.7×10^6 independent plaques) was screened using human AR probes corresponding to the 5′ region of the human AR first exon (Faber et al., 1989). Hybridization was carried out using standard methods (Maniatis et al., 1982) under conditions of low stringency. Positive clones were selected and rescreened to obtain purified single plaques. Five independent clones were isolated: GrAR2, 3, 4, 6, 7.

Subcloning and sequencing

Several restriction fragments derived from GrAR2 were subcloned into pGEM7 (Promega, Madison, WI, U.S.A.) using standard techniques (Maniatis et al., 1982). Partial overlapping clones were sequenced by dideoxy-chain termination (Sanger et al., 1977) in two orientations using T7 polymerase (Pharmacia, Uppsala, Sweden). Double-stranded plasmid DNA was used as a template. The promoter/reporter gene constructs were prepared as follows: The genomic *Hin*dIII fragment (−296/+120) was cloned in the *Hin*dIII site of the polylinker from the pCATENH vector (Promega) in the antisense direction (HASCAT). pGEM7H3.1, containing the *Hin*dIII fragment (−296/+120), was partially cut with *Nhe*I, treated with Klenow enzyme and dNTPs to prepare blunt ends, followed by a complete *Hin*dIII digestion. Subsequently, the appropriate *Hin*dIII-*Nhe*I fragment (−296/+97) was isolated from low-melting-point agarose. The fragment was ligated into pCATENH which had been cut with *Xba*I and blunt-ended with Klenow enzyme followed by a second digestion with *Hin*dIII (HSCAT). The *Pst*I fragment (−507/+912) was cloned into the *Pst*I site from pCATENH in the antisense direction (PASCAT). pGEM7E3, containing the *Eco*RI fragment (−570/+1005), was cut with *Kpn*I, treated with T4 polymerase and dNTPs to create blunt ends, followed by digestion with *Pst*I. The *Kpn*I-*Pst*I fragment was isolated from low-melting-point agarose and cloned into pCATENH (PSCAT), which had been cut with *Hin*dIII, blunt ended with Klenow and digested with *Pst*I. The insertion of the correct fragment in the desired orientation was checked by restriction enzyme digestion.

RNA isolation

Frozen tissues and cultured cells were lysed and homogenized in 3 M LiCl/6 M ureum, and left on ice for several hours. The RNA was pelleted through ultracentrifugation (25×10^3 rpm, 25 min, SW40 rotor). The pellet was dissolved in 0.1% (w/v) sodium dodecyl sulfate (SDS) and proteins were removed by repeated phenol extraction and a

single proteinase K treatment in 0.1% (w/v) SDS. After precipitation the RNA was dissolved in a small volume of water and the amount of RNA was determined by spectrophotometric optical density (OD) measurement at 260 nm. RNA was stored in 0.3 M NaAc/70% ethanol at −20°C.

S1-nuclease protection assay

pGEMH3.1 was cut with *Hin*dIII, dephosphorylated and end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP according to Maniatis et al. (1982). The *Hin*dIII fragment was subsequently isolated from low-melting-point agarose and dissolved in 0.3 M NaAc (pH 5.2). Approximately 0.5–1 × 10^5 cpm were precipitated with the appropriate amount of total RNA. After centrifugation, the pellet was dissolved in hybridization buffer containing 80% (v/v) deionized formamide, 40 mM Pipes (pH 6.3), 0.4 M NaCl and 1 mM EDTA according to Favaloro et al. (1980). The samples were denatured for 3 min at 90°C followed by an overnight hybridization at 55°C. S1-nuclease (Boehringer Mannheim, Mannheim, F.R.G.) digestions (Favaloro et al., 1980) were carried out for 1 h at 37°C or for 3 h at 20°C, with similar results. The protected DNA fragments were analyzed on a 6% or 8% polyacrylamide sequencing gel.

Cell culture and transient transfection assays

COS cells were grown in Dulbecco's modification of Eagle's medium (Gibco BRL, Grand Island, NY, U.S.A.), supplemented with 10^5 IU/l of penicillin, 100 mg/l streptomycin and 5% fetal calf serum. All cultures were maintained at 3°C under an atmosphere of 5% CO_2 in air. Before transfection, cells were plated in 6 cm diameter culture dishes (Nunclon, Roskilde, Denmark) at approximately 10% confluence and cultured for 24 h. Transfections were carried out according to Graham and van der Eb (1973) using 10 μg of plasmid DNA per dish. The cells were shocked 24

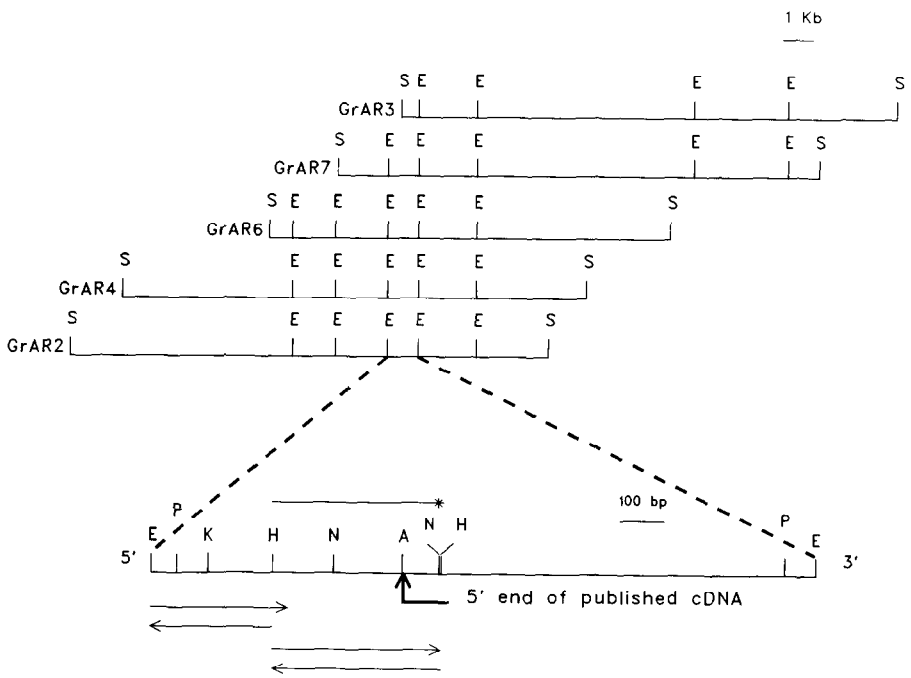

Fig. 1. Schematic representation of isolated lambda clones, containing genomic rat AR sequences (GrAR2, 3, 4, 6, 7). Part of GrAR2 is shown in more detail. The 5′ end of the published rat AR cDNA (Lubahn et al., 1988) is indicated. The horizontal arrows show the sequence strategy. The horizontal line above the restriction map of GrAR2 represents the probe that was used for the S1-nuclease protection assays. The asterisk indicates the site used for end-labeling by [γ-^{32}P] ATP. A = *Sau*3A, E = *Eco*RI, H = *Hin*dIII, K = *Kpn*I, N = *Nhe*I, P = *Pst*I, S = *Sal*I.

h later for 2 min with 15% glycerol in phosphate-buffered saline (PBS), and 48 h after transfection the cells were harvested and broken by three cycles of freezing and thawing. After centrifugation, supernatants were assayed for CAT activity as described by Gorman et al. (1982). A transfection with pSV2CAT was included in each experiment as a control to compare the transfection efficiencies of the different experiments.

Results

Isolation and characterization of 5′ genomic rat AR clones

A rat liver genomic library was screened with human AR cDNA probes representing the 5′ end of the AR mRNA. Five independent overlapping clones were isolated as presented in Fig. 1. GrAR2 contained the farthest 5′ extending sequences and this clone has been used for subsequent studies.

Based upon hybridization results, we have subcloned and sequenced several restriction fragments derived from GrAR2. The sequencing strategy is also shown in Fig. 1 and the obtained sequence is presented in Fig. 2. The 3′ end of this sequence (117 bp) is identical to the published 5′ end of the rat AR cDNA (Lubahn et al., 1988).

Identification of the 5′ end of the rat AR mRNA

In order to locate the cap site of the rat AR mRNA we have performed S1-nuclease protection assays. A genomic *Hin*dIII fragment that sur-

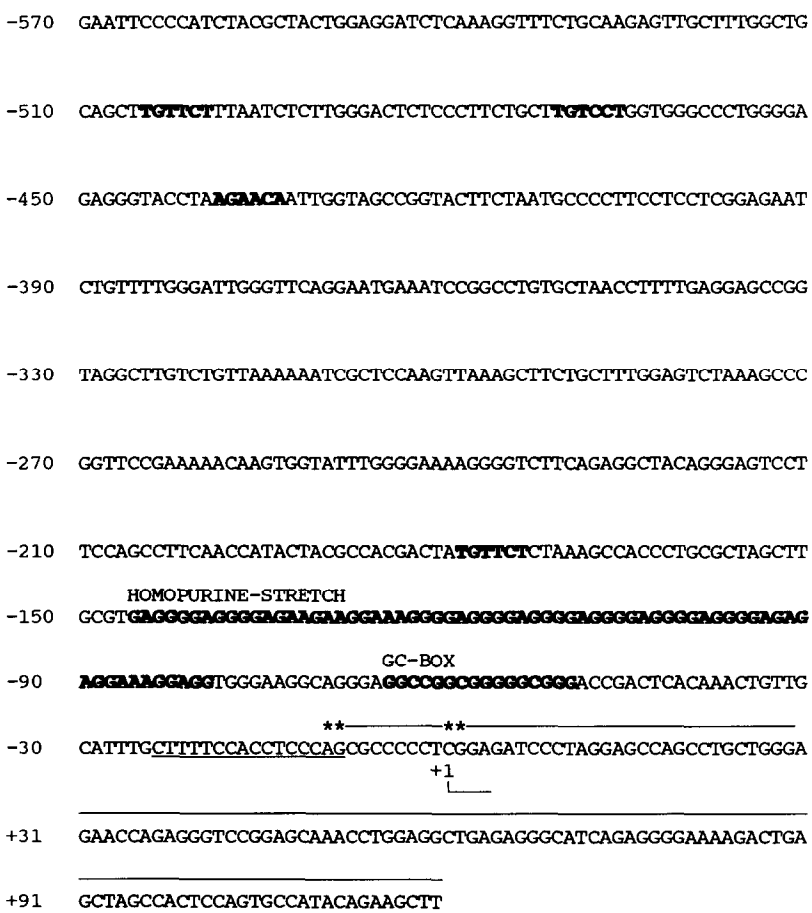

Fig. 2. Sequence of the 5′ genomic region of the rat AR gene containing putative promoter elements. The +1 position is defined at the major transcriptional start site. The asterisks above the sequence indicate the other start sites. Putative promoter elements are bold-faced: TGTYCT and AGAACA, the homopurine stretch and the GC box. The possible splice acceptor site is underlined and the transcribed sequence is overlined.

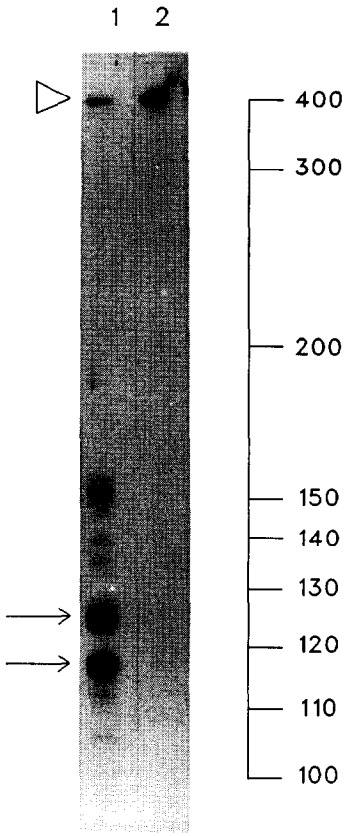

Fig. 3. S1-nuclease protection assay of the 5′ genomic *Hin*dIII fragment. Protected fragments obtained with 50 μg of total testis RNA (lane 1), and 50 μg of total spleen RNA (lane 2) are shown. The triangle indicates intact probe, and arrows indicate the major bands. The numbers on the right represent the lengths of the marker DNA in bp.

rounds the 5′ end of the cDNA was used as a probe (Fig. 1). Total RNA preparations from 21-day-old rat testis or spleen were hybridized to the end-labeled DNA probe, and subsequently the S1-nuclease digestion was carried out; Fig. 3 shows the resulting protected DNA fragments after analysis on a denaturing polyacrylamide gel.

Using total testis RNA, two pronounced bands were observed (Fig. 3; lane 1). The 5′ ends of the two major bands map just upstream from the 5′ end of the published rat AR cDNA which is located in Fig. 2 at position +5. Using total RNA derived from spleen, an AR-negative tissue (Lubahn et al., 1988), no labeled DNA fragments protected from S1-nuclease digestion were detected. The S1-nuclease digestion has been carried out at 37°C as well as at 20°C, and similar results were obtained at both temperatures (not shown).

We have also performed primer extension analysis as described by Krug and Berger (1987), to identify the transcription start sites using another method. However, this method did not yield results, most likely because the RNA was present in a conformation which interfered with AMV reverse transcriptase activity.

Structure of the 5′ genomic region of the rat AR gene

The finding that different bands were obtained with the S1-nuclease protection assay strongly implies the presence of multiple start sites of transcription, a feature that is common to most promoters that lack a TATA box. In concordance with this, sequence analysis of the 5′ genomic region shows that no typical TATA or CCAAT box is present (Fig. 2). However, several structural elements indicative of a promoter can be indicated. The genomic region from −1 to −300 has an overall G/C content of 58%, and one SP1 site (GGCGGG) is located around position −60 within an uninterrupted stretch of G/C residues. Furthermore, a homopurine stretch containing a total of eight GGGGA elements is located between −89 and −146 bp upstream from the major start site of transcription. These GGGGA sequences might represent promoter elements (see Discussion).

Other possible promoter elements in this region are the TGTYCT consensus elements that are present at positions −174 to −179, −434 to −439, −466 to −471, and −500 to −505, and which can be considered as glucocorticoid-responsive element (GRE) 'half sites'. The GRE consists of a palindromic pair of hexameric TGTYCT sequences separated by three nucleotides (Klock et al., 1987). It should be noted that the 5′ genomic region also contains a possible splice acceptor site (Fig. 2).

Functional promoter activity in the 5′ genomic region of the rat AR gene

From the above described results, it is not clear whether the protected bands are derived from different start sites of transcription, or might re-

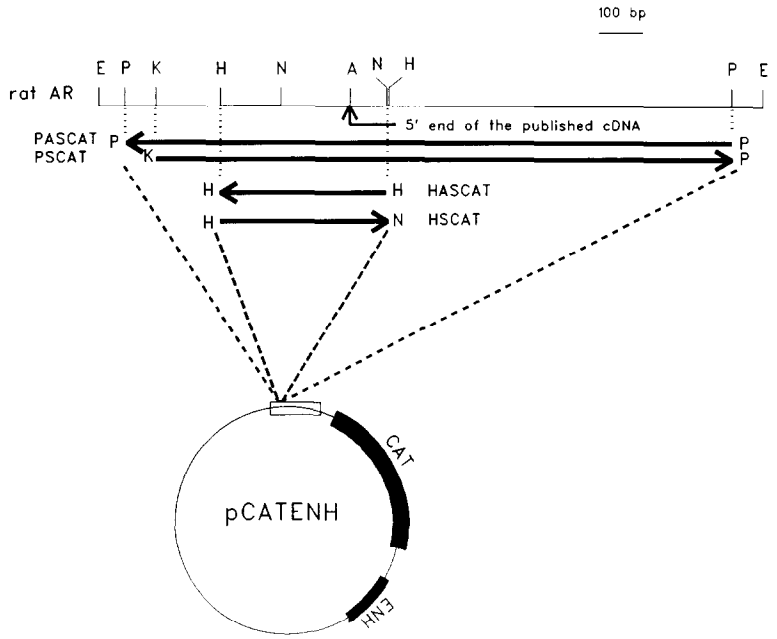

Fig. 4. Promoter constructs cloned into the pCATENH vector. The promoter fragments and the genomic region from which they were derived are shown. Arrows pointing to the right indicate the sense orientation. A schematic drawing of the pCATENH vector shows the relative positions of the polylinker (open box), the CAT gene, and the SV40 enhancer (closed boxes). Restriction enzyme abbreviations are as in Fig. 1.

sult from the use of a splice acceptor site: The sequence 5'TTTCCACCTCCAG3' is located in the area that contains the supposed start sites of transcription (Fig. 2) and is in accordance with a possible intron/exon splice acceptor site. Consequently, further experimental evidence was needed to determine that the region shown in Fig. 2 indeed represents the rat AR gene promoter.

Promoter trap constructs were used to test the functional capacity of the putative promoter. The cloning vector pCATENH (described in Materials and Methods) contains the CAT reporter gene and

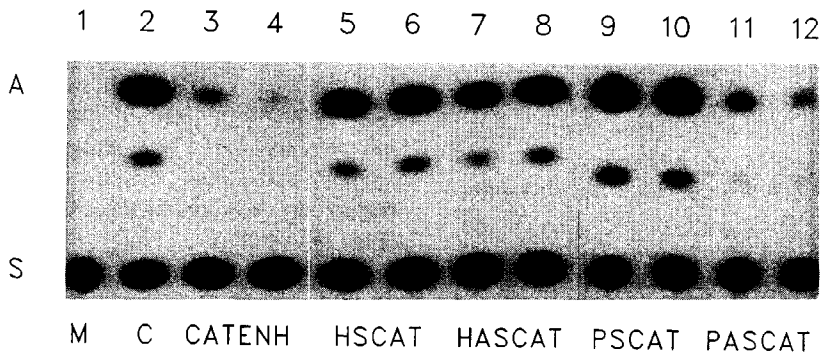

Fig. 5. Rat AR promoter activity in COS cells. CAT activity in COS cells which were transfected with: no DNA (M; lane 1), PSV2CAT (C; lane 2), pCATENH (lanes 3 and 4), HSCAT (lanes 5 and 6), HASCAT (lanes 7 and 8), PSCAT (lane 9 and 10), PASCAT (lanes 11 and 12). The S denotes the position of the substrates in the chromatogram, and the A is located at the position of the acetylated products of the enzymatic reaction.

a SV40 enhancer sequence, but no promoter. Two types of constructs were made, which contained the putative promoter sequences and flanking regions in either the sense or antisense orientation with respect to the CAT reporter gene. A schematic representation of the different constructs is shown in Fig. 4. After transfection into COS cells, the stimulatory effect of the different 5′ genomic fragments on the rate of CAT gene transcription was determined by performing CAT assays (Fig. 5). pSV2CAT, the positive control plasmid that contains a SV40 enhancer and promoter, showed a high CAT activity as compared with pCATENH. The construct HSCAT as well as the antisense analogue HASCAT stimulated the transcription of the CAT gene several fold, as compared to the background activity of the parent vector pCATENH. These constructs contain about 300 bp of possible promoter sequences and approximately 100 bp of the 5′ UTR. The larger construct, PSCAT, that extends 150 bp further upstream and 850 bp further downstream than HSCAT and HASCAT, resulted in a somewhat higher CAT expression relative to HSCAT and HASCAT. In contrast, PASCAT, identical to PSCAT but containing the promoter in the antisense orientation, did not show stimulation of CAT activity.

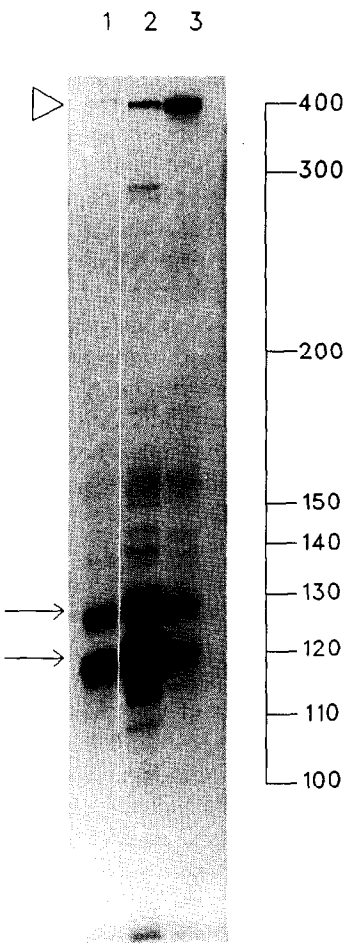

Fig. 6. Analysis of transcriptional start sites in promoter/reporter gene constructs. The probe shown in Fig. 1 was hybridized to RNA extracted from: COS cells transfected with PSCAT (lane 1; 20 μg), COS cells transfected with PASCAT (lane 2; 20 μg), and total testis (lane 3; 60 μg). The triangle points to the intact probe and the arrows indicate the major protected fragments. The numbers on the right indicate the lengths of the marker DNA in bp.

To investigate whether transcription of the promoter/CAT reporter gene constructs starts at the same sites as the transcription of the wild type AR promoter, we performed S1-nuclease protection assays with RNA extracted from the transfected COS cells, using as a probe the genomic *Hin*dIII fragment that had also been used to locate the cap site in our previous experiment. The assay was performed on total cellular RNA derived from COS cells transfected with either the PSCAT (sense) or PASCAT (antisense) construct (Fig. 6).

Using the sense and anti-sense rat AR genomic sequence constructs, stable transcripts were obtained in both orientations, although CAT-gene derived sequences are only included in the PSCAT transcripts. This experiment was performed to show that the 5′ ends of the protected fragments in RNA from the transfected cells mapped at exactly the same positions as those observed using RNA from rat testis (Fig. 6; lane C). It can be concluded, therefore, that the promoter region in PASCAT as well as PSCAT directs transcription from the correct initiation sites and on the correct DNA strand.

Discussion

Structural features of the rat AR gene promoter

From the present results it is concluded that the minimal promoter of the rat AR gene is located within the area from position +100 to −300 in the 5′ genomic region of the rat AR gene. The sequence upstream of position +1 is not effi-

ciently transcribed into mRNA as shown by S1-nuclease protection analysis. A regulatory function of the largely untranscribed sequence −1 to −300 is reflected in the presence of known sequences in this area that have been proven to be functional elements of other promoters.

First, a GC box (GGCGGG) is located around position −60. This sequence is identical to a recognition site for the transcription factor SP1 that has been shown to activate the transcription of several genes (Saffer and Singer, 1984).

Second, a homopurine stretch of 67 bp, located between positions −79 and −146. Within this region a total of eight repeats of the sequence GGGGA is present. Several other genes also contain purine-rich sequences in the promoter (Christophe et al., 1989; Claessens et al., 1989; Watson and Milbrandt, 1989; Young et al., 1989), some including the GGGGA element (Watson and Milbrandt, 1989; Young et al., 1989). No functional role has yet been assigned to this sequence motive, although homopurine stretches are known to contain sites that are sensitive to S1-nuclease digestion indicating an irregular DNA structure (Claessens et al., 1989; Young et al., 1989). The GGGGA element could very well represent a regulatory protein binding site. For example, the transcription factor ETF can bind to sequences that contain CCCC or GGGG repeats separated by one nucleotide. Through binding to these consensus repeats, ETF specifically enhances transcription from promoters which do not contain a TATA box (Kageyama et al., 1989). The P2 promoter of the c-*myc* gene also contains a GGGGA element which is involved in protein binding and promoter function. The sequence element GGGGAGGGA, located 48 bp upstream of the start site of transcription in the c-*myc* P2 promoter, can be specifically bound by protein in a gel retardation assay and is capable of increasing the promoter activity (Hall, 1990).

Finally, TGTYCT elements are present at four different positions between −174 and −505 in the promoter region. A glucocorticoid responsive element (GRE) consists of a palindromic pair of the hexameric TGTYCT sequence, separated by three nucleotides (Klock et al., 1987). In a random distribution, the TGTYCT sequence would be expected to occur approximately once in every 10^3 bp. It might be suggested that this non-palindromic sequence, either as repeat, or together with flanking sequences, might also have some function in steroid hormone responsive promoters in general. In this respect, it is of interest that TGTYCT sequences are present in the promoter of the androgen-dependent prostatic binding protein genes C1, C2 and C3 (Claessens et al., 1989a). Furthermore, fragments from the promoter and first intron of the C3 gene that contain this consensus sequence have been shown to bind AR-steroid complex (Rushmere et al., 1987). Only the intron-derived sequence could confer androgen responsiveness to a heterologous promoter and this effect could be annulled by a single point mutation in one of the two TGTYCT motives that were located in this area (Claessens et al., 1989b).

Recently, we have also cloned the human AR gene promoter (Faber et al., manuscript in preparation). Comparison of the rat and human sequences indicates that the AR promoter shows a similar structural organization in these two species. The major start sites of transcription are located at the same positions within a region that shows a high degree of sequence similarity. The human AR gene promoter also contains one SP1 site and a homopurine stretch, but no other conserved elements are located within 570 bp upstream from the major transcription start site.

Two major start sites of transcription and a putative splice acceptor site are located within the same region

The results of the S1-nuclease protection assay indicated the presence of two major start sites of transcription between 1010 and 1023 bp upstream from the translation initiation codon. However, it is important to exclude that the results reflect the presence of an intron/exon boundary (splice acceptor site), or that the results are derived from non-specific hybridization. This is indicated by the following. First, the two major bands were not found when spleen derived RNA was used. Second, RNA from COS cells expressing the promoter-trap constructs PSCAT and PASCAT, resulted in the same S1-nuclease protection pattern as that obtained using total testis RNA. In PSCAT and PASCAT, the possible splice acceptor site is separated from any possible splice donor site in the

genome. It can be concluded therefore, that the two double bands observed in the S1-nuclease assay are derived from hybridization of the probe to AR mRNAs, and represent two start sites of transcription.

The minimal promoter of the rat AR gene is located within the 5′ genomic region between positions +100 and −300

Promoter trap constructs were used to test the functional capacity of the DNA sequences surrounding the start sites of transcription to act as a promoter. By cloning 5′ genomic sequences in either the sense or antisense orientation, additional information about the direction of transcription initiation should be obtained. The fragment that spans the region from −296 to +120 (constructs HSCAT and HASCAT) activates transcription in an orientation-independent manner. This is not surprising from what is currently known about the characteristics of promoters lacking typical TATA or CCAAT box sequences, also called housekeeping or GC-rich promoters. The transcription factors SP1 and ETF can activate transcription on both DNA strands (Saffer and Singer, 1984; Kageyama et al., 1989) and the upstream half of the calcium-dependent protease (CANP) gene promoter shows activity in either orientation (Hata et al., 1989).

However, a larger construct of the rat AR 5′ genomic region appears to function in one direction only. This can be concluded from the observation that the transcriptional activity is stimulated by PSCAT that contains the promoter fragment in the sense orientation, but not by the PASCAT antisense construct. PSCAT and PASCAT contain a promoter fragment which includes additional regulatory up- and downstream sequences as compared to the H(A)SCAT constructs.

The start sites of transcription that were functional in the promoter/reporter gene constructs in the transfected COS cells were found to map at exactly the same positions as the start sites that were identified using testis RNA. Hence, the protected DNA fragments that were obtained with RNA transcribed from both the sense and antisense constructs can only be the result of transcription initiation. Intron/exon splicing could not have resulted in the same protected fragments from either orientation of the promoter fragment, unless the splice donor site would be present in the genomic fragment. However, this is very unlikely not only because we have shown that the different constructs resulted in transcription starting at the rat AR gene promoter, but also because there is no splice donor consensus sequence present in the tested promoter reporter gene constructs.

No consensus cyclic AMP responsive element (CRE) is located within or near the AR gene promoter region. This may point to a complex and indirect regulatory mechanism for the effect of FSH on AR mRNA expression in Sertoli cells; the transcription of several CRE-containing genes in Sertoli cells is stimulated by FSH and/or dbcAMP (Hall et al., 1988; Klaij et al., 1990). By transfection of AR promoter/reporter gene constructs into Sertoli cells, the upstream regions and possible regulatory elements of the rat AR promoter will be functionally analyzed.

In conclusion, the rat AR gene promoter lacks TATA and CCAAT box elements, but it contains one SP1 site and several other possible binding sites for transcription factors. Part of the promoter can function in an orientation-independent manner, but the full promoter shows a higher and unidirectional activity.

Acknowledgement

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Note added in proof

The findings described in the present paper are in agreement with recently published data concerning the promoter of the human androgen receptor gene (Tilley et al., J. Biol. Chem. 265 (1990) 13776–13781).

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