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Expression of Crisp-1 mRNA Splice Variants in the Rat Epididymis, and Comparative Analysis of the Rat and Mouse Crisp-1 Gene Regulatory Regions

KENNETH P. ROBERTS,* LAURA B. HOFFMAN,† KATHY M. ENSRUD,† AND DAVID W. HAMILTON†

*From the Departments of *Urologic Surgery and †Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, Minnesota.*

ABSTRACT: The rat Crisp-1 gene encodes Protein DE (acidic epididymal glycoprotein; AEG), a glycoprotein secreted by the epididymal epithelium that associates with maturing sperm and has been implicated in the process of sperm-egg fusion. Previous characterization of the Crisp-1 messenger RNA in the rat epididymis has demonstrated the presence of 3 splice variants (Klemme et al, 1999). This study was undertaken to determine if expression of the Crisp-1 splice variants in the rat epididymis is region-specific and correlates with the region-specific pattern of synthesis of the D and E forms of the Crisp-1 protein. Expression of each of the splice variants was shown by RNase protection assays to be under the control of androgens, but they are not differentially regulated either within the epididymal segments or along the length of the organ. The reported structure of the mouse Crisp-1 gene does not include an exon that is equivalent to the rat exon 1, suggesting that the rat splice variants cannot exist in the mouse and may be specific to the rat. Furthermore, the mouse transcription start site is situated in a different region of the gene than in the rat. In this study, a comparison of the mouse and rat genes in the region flanking the mouse exon 1 and the rat exon 2 (within the rat intron 1) shows greater than 80% sequence identity, including the conservation of several putative androgen receptor binding sites. In addition, the rat gene is shown to have a corrupted TATA box in intron 1 that corresponds to the TATA box located in the mouse gene. These observations explain the preferential transcription for the mouse gene in this region, while the predominant start site for the rat gene is 5′ of the upstream exon 1. Although an exon corresponding to the rat exon 1 has not been found in the mouse gene, reverse transcription–polymerase chain reaction experiments using mouse epididymal RNA suggest that such an exon exists in the mouse gene and is transcribed at low frequency.

Key words: Acidic epididymal glycoprotein, AEG, Protein DE, androgen regulation.

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Spermatozoa released from the testis are immature in that they are immotile and unable to fertilize an oocyte. As sperm move through the epididymis they undergo a series of maturational changes that render them motile and capable of fertilization. These changes include modifications of the lipid composition of the sperm plasma membrane, processing of proteins and sugars on the sperm surface, and addition of proteins to the sperm plasma membrane (for review see Jones, 1998; Tulsiani et al, 1998).

Among the proteins secreted by the epididymal epithelium that interact with the maturing sperm is acidic epididymal glycoprotein (AEG), also known as Protein DE because it runs as 2 forms that differ in molecular weight by approximately 2 kd when analyzed by polyacrylamide gel electrophoresis (Cameo and Blaquier, 1976; Xu and Hamilton, 1996). In the rat, the D form interacts with the sperm head and the E form binds to the sperm tail (Rochwerger and Cuasnicu, 1992; Moore et al, 1994). Although Proteins D and E can be distinguished by a unique epitope on the amino terminus of Protein E, the exact nature of the difference or differences between the 2 forms of AEG remains unknown (Xu and Hamilton, 1996). Protein DE has been shown by direct amino acid sequencing and deduced amino acid sequence to be rich in cysteine residues in the carboxyl half of the molecule (Xu and Hamilton, 1996; Xu et al, 1997). Based on this observation, Protein DE and the gene encoding it have been renamed Crisp-1 for *c*ysteine-*ri*ch *s*ecreted *p*rotein 1 (Eberspaecher et al, 1995; Klemme et al, 1999). In several species Crisp-1 is known to be a member of a 3-gene family of proteins that have high sequence similarity and conservation of their 16 cysteine amino acid residues (Kratzschmar et al, 1996; Haendler et al, 1997). Although its function remains unknown, Crisp-1 has been implicated in the process of sperm-egg plasma membrane fusion (Cohen et al, 1996).

The Protein D and E forms of Crisp-1 appear to be

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Correspondence to: Dr Ken Roberts, Department of Urologic Surgery, University of Minnesota Medical School, D595 Mayo Memorial Building, 420 Delaware Street, SE, Minneapolis, MN 55455 (e-mail: roberts@med.umn.edu).

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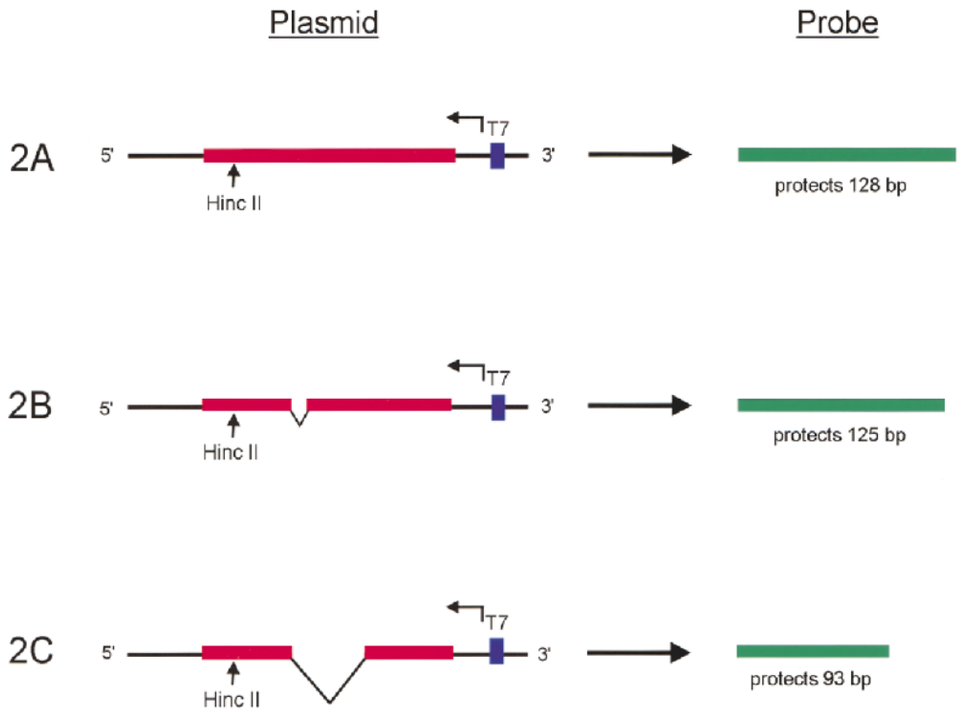


Figure 1. Diagrammatic representations of the 3 pGEM7Z plasmid constructs containing cDNAs encoding each splice variant (red). Nucleic acid, which is spliced out, is represented by the bent solid line. The plasmids were linearized with *Hinc* II and T7 RNA polymerase was used to generate probes. Each probe will hybridize to all forms of the Crisp-1 mRNA but the full-length probe will only be protected by the corresponding form of the mRNA. The predicted size of the largest protected RNA fragment is depicted in green.

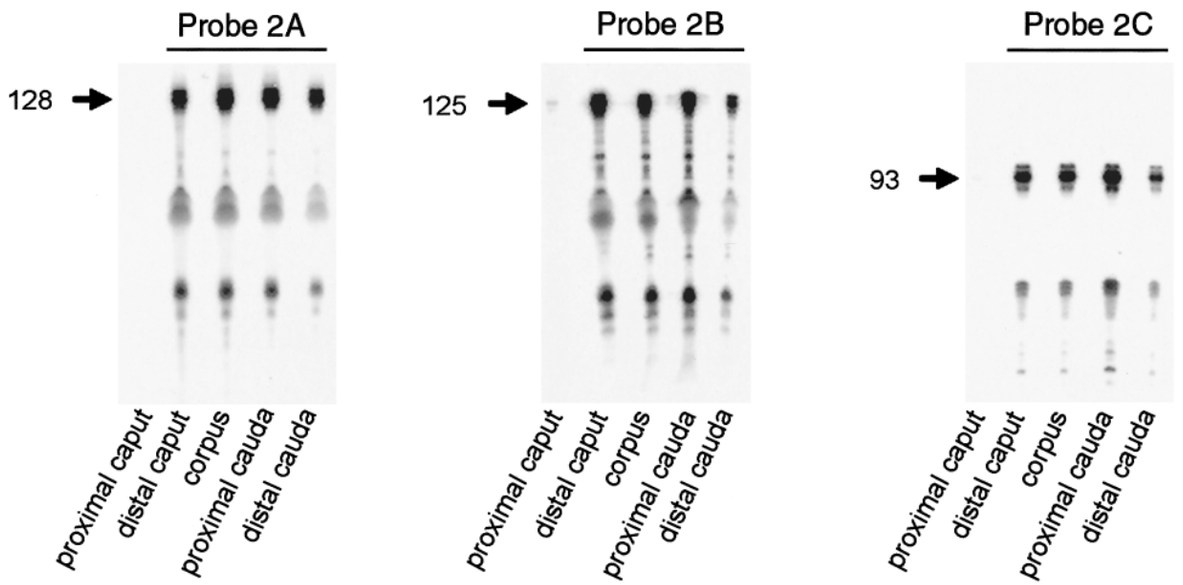


Figure 2. Regional expression of the Crisp-1 mRNAs determined by RNase protection assays of RNA isolated from 5 regions of the epididymis. The protected products were separated on 6% urea denaturing polyacrylamide gels. The largest protected fragment from each probe is labeled by its length in base pairs. The results show that the pattern of expression is similar for all forms of the mRNA. Expression is lowest in the proximal caput and uniformly high in the distal caput, corpus, and proximal cauda. There is a slight decrease of the level of each form of the message in the distal cauda.

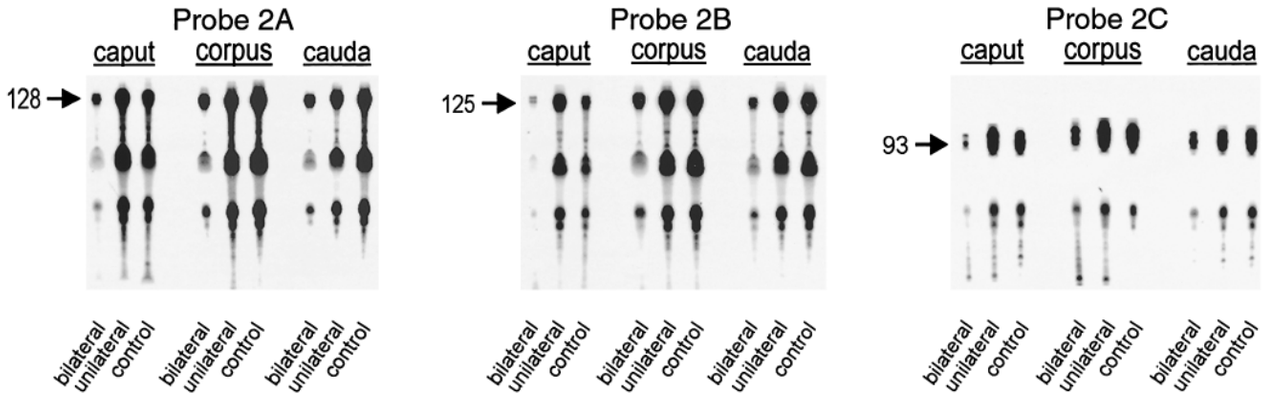


Figure 3. Androgen-regulated expression of the Crisp-1 mRNAs determined by RNase protection assays of RNA isolated from 3 regions of the epididymis. Animals were either bilaterally castrated to remove androgens (bilateral), unilaterally castrated to remove luminal androgens and other luminal factors (unilateral), or sham-operated (control). The protected products were separated on 6% urea denaturing polyacrylamide gels. The largest protected fragment from each probe is labeled by its length in base pairs. The results show that the levels of all 3 forms of the mRNA are decreased in all 3 segments of the epididymis when circulating androgens are removed by castration. Unilateral castration did not result in a decrease in Crisp-1 mRNA compared with control.

differentially expressed in the epididymis. Protein D is expressed throughout the epididymis while Protein E is produced in the distal caput, corpus, and proximal caudal portions of the organ (Moore et al, 1994). The complementary DNA (cDNA) for rat Crisp-1 has been cloned and an allelic variant has been demonstrated (Charest et al, 1988). Both of these cDNA forms are predicted to encode the same protein. Recent characterization of the rat Crisp-1 gene, which encodes the messenger RNA (mRNA) for Protein DE, has revealed the presence of 3 splice variants of the mRNA involving exon 2 in the 5′ untranslated region of the mRNA (Klemme et al, 1999). Because it is possible that the splice variants are responsible for the D and E forms of rat Crisp-1, the experiments reported herein were designed to assess the region-specific expression of these variants and to determine if the expression pattern coincided with the expression pattern of the Protein D and E forms. Because it is known that Protein D is androgen-regulated, regional regulation of the individual splice variants by androgen was also investigated. An additional question that was asked in these experiments was whether the mouse and rat forms of the Crisp-1 gene are the same because comparison of reported sequences in the 2 species suggests that there is no sequence equivalent to the rat exon 1 in the mouse. The apparent lack of the first exon in the mouse gene is surprising because the remaining gene structure is nearly identical to the rat (Klemme et al, 1999). However, comparison of the reported mouse 5′ regulatory region (Schwidetzky et al, 1997) to the rat genomic sequence flanking exon 2 (reported herein) shows greater than 80% sequence identity. Reverse transcription–polymerase chain reaction (RT-PCR) studies of the mouse suggest that there is likely a form of the mouse Crisp-1 mRNA that contains a 5′ sequence similar to the rat exon 1.

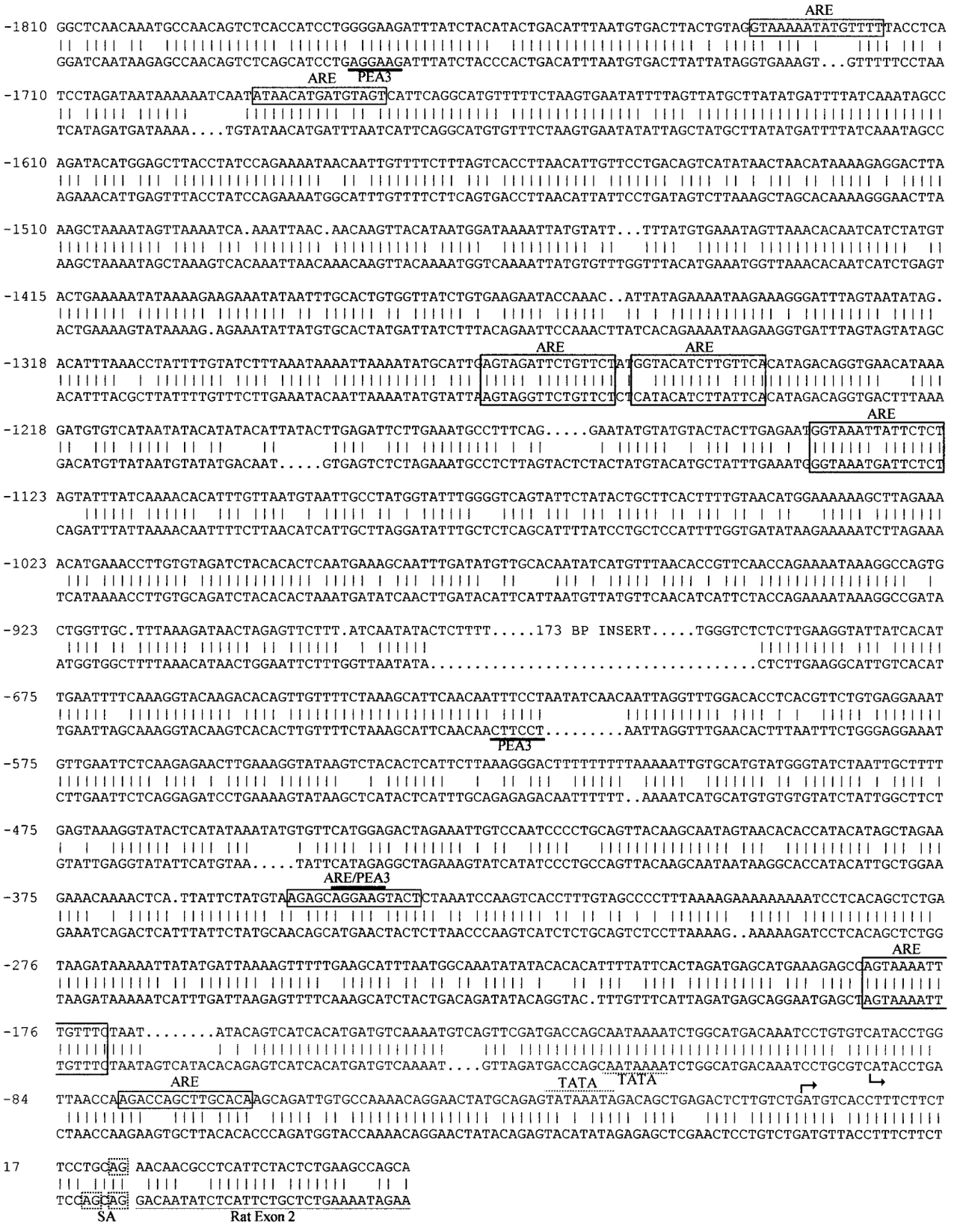
Methods

Materials

Sprague-Dawley rats and CD1 mice were purchased from Harlan Sprague-Dawley (Indianapolis, Ind). TOPO-TA cloning vector (*pC*RII) was purchased from Invitrogen (Carlsbad, Calif) and pGEM 7Z cloning vector was purchased from Promega (Madison, Wis). All restriction enzymes were from Promega. SP6/T7 RNA polymerase kits and RNase protection kits were purchased from Roche (Indianapolis, Ind). Protected RNA fragments were analyzed by polyacrylamide gel elecrophoresis using the NOVEX precast gels and the NOVEX electrophoresis system (Invitrogen). ^{35}S-UTP was purchased from Amersham (Arlington Heights, Ill). All other reagents and chemicals were purchased from Sigma Chemical Company (St Louis, Mo).

Cloning of Rat Splice Variant cDNA Probes

An upstream oligonucleotide primer complementary to rat exon 1 sequence (5′-GAGAGCAGATAAGCTTCAGGG-3′) and a downstream oligonucleotide primer complementary to exon 3 (5′-AATACAGCAGCCAGGAACAA-3′) were synthesized (Microchemical Facility, University of Minnesota, Minneapolis, Minn). PCR was performed with cDNA synthesized from rat corpus epididymidis mRNA as a template using oligo dT as primer. PCR products were cloned into the TOPO TA *pC*RII vector according to the manufacturer's instructions (Invitrogen). Several clones were isolated, plasmid DNA was prepared, and inserts were sequenced. Complementary DNA clones representing each of 3 splice variants (Klemme et al, 1999) were subcloned into pGEM7Zf (Promega) using *Eco*RI (a site in the *pC*RII vector) and *Hind*III (an endogenous site in exon 1 of rat Crisp-1 cDNA). These vectors were linearized with *Hinc*II, an endogenous site in exon 1 of rat Crisp-1 cDNA, and ^{35}S-labeled cRNA synthesized using T7 RNA polymerase according to the manufacturer's protocol (Roche). These cRNA probes protect the corresponding splice variant of the Crisp-1 mRNA (Figure 1).


ARE
PEA3
ARE/PEA3
TATA
TATA
SA
Rat Exon 2

Regional and Androgen-Regulated Expression of Rat Crisp-1 Splice Variants

For analysis of regional rat Crisp-1 mRNA levels, epididymides from 4 rats were isolated and divided into proximal caput, distal caput, corpus, proximal cauda, and distal cauda regions. For analysis of androgen-regulated expression of the rat Crisp-1 mRNA, epididymides were collected from groups of 4 adult Sprague-Dawley rats that were bilaterally and unilaterally castrated. Castrations were performed by exposing the testes and epididymides via an abdominal incision, ligating the efferent ducts with a 4.0 silk suture, and removing the testes by cutting proximal to the suture. Control rats were sham-operated by exposing the testes via an identical abdominal incision. Seven days after unilateral or bilateral castration or sham operation, epididymides were collected, cut into caput, corpus, and cauda regions, and homogenized in TRIzol homogenization buffer (Life Technologies, Grand Island, NY). In the unilateral castrate animals the ipsilateral epididymis was used for RNA isolation using the TRIzol system. In all RNase protection experiments, homogenates from 4 epididymides were pooled by region, total RNA was isolated using the TRIzol reagent, and RNase protection assays were performed (see following discussion). The analysis of mRNA levels by RNase protection was repeated at least twice.

RNase Protection Assay

RNase protection assays were carried out according to the manufacturer's protocol (Roche). Briefly, in each reaction, 5 μg of total RNA was hybridized with 3×10^5 cpm of ^{35}S-UTP labeled cRNA probe in 30 μL of hybridization solution (40 mM PIPES, 400 mM NaCl, 1 mM EDTA, 80% formamide, pH 6.4) and allowed to hybridize at 45°C overnight. The reactions were then subjected to digestion by RNase A/RNase T1 for 30 minutes at 30°C followed by Proteinase K digestions for 15 minutes at 37°C. The protected products were extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with yeast transfer RNA carrier. The protected RNA precipitate was analyzed by 6% urea denaturing polyacrylamide gel electrophoresis (NOVEX System, Invitrogen). The gels were dried and bands visualized by exposure to Kodak XOmat x-ray film (Eastman Kodak, Rochester, NY).

RT-PCR of Mouse Crisp-1

An upstream oligonucleotide primer complementary to rat exon 1 sequence (5′-ACTCCTCAGGAAGACCAGCAG-3′) and a downstream oligonucleotide primer complementary to a region in mouse exon 6 (5′-GGCATTCAGCAACTCCACATG-3′) were synthesized (Microchemical Facility, University of Minnesota). PCR was performed with cDNA synthesized from total mouse epididymis RNA as template using oligo dT as primer. PCR products were cloned into the TOPO TA *pC*RII vector according to the manufacturer's instructions (Invitrogen). Several clones were isolated, plasmid DNA was prepared, and inserts were sequenced.

Sequence Comparisons

Sequence alignments were performed using the University of Wisconsin Genetics Computer Group (1991) software program and identification of intron/exon splice acceptor sites was performed using the BCM Search Launcher programs (Baylor College of Medicine, [dot.imgen.bcm.tmc.edu:9331/seq-search/gene-search.html]).

Results

Total RNA isolated from 5 regions of the epididymis (proximal caput, distal caput, corpus, proximal cauda, and distal cauda) were subjected to RNase protection analysis using cRNA probes specific for the 3 splice variants described in ''Materials.'' The results show that the pattern of expression is similar for all 3 forms of the message (Figure 2). The levels of each form are very low in the proximal caput epididymides; uniformly high in the distal caput, corpus, and proximal cauda; and reduced in the distal cauda epididymides (although higher in the distal cauda than in the proximal caput). The absolute level of expression is lowest for the splice variant missing all of exon 2 (complementary to probe 2C).

The maintenance of the rat Crisp-1 mRNA and protein is known to be dependent on androgens (Cameo et al, 1976; Brooks et al, 1986; Charest et al, 1988). To determine if expression of the Crisp-1 mRNA splice variants are differentially regulated by androgens, the regional expression of the 3 forms was compared in the caput, corpus, and cauda epididymides of castrate and control rats, and in the ipsilateral epididymides in hemicastrate rats. The results show that all 3 forms of the message are equally suppressed by the withdrawal of androgens and that circulating androgen levels in the hemicastrate rats are sufficient to maintain levels of the Crisp-1 mRNAs (Figure 3).

The mouse gene is reported to be structurally different from the rat gene in that no sequence homologous to the rat exon 1 has been reported in the mouse gene. The primary form of the mouse mRNA is produced by tran-

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Figure 4. Comparison of the mouse gene in the 5′ flanking region and the rat gene in the 5′ region of exon 2. The mouse sequence is listed above and is numbered relative to the published transcription start site (Schwidetzky et al, 1997). The sequences are 82% identical over 1865 base pairs, not including an unmatched segment of 187 base pairs beginning at nucleotide −883. Of the 8 AREs identified in this region of the mouse gene, 4 are conserved in the rat gene (boxed with solid line). The consensus PEA3 site in the mouse gene (−345) is not present in the rat gene; however, the rat gene sequence contains 2 distinct PEA3 sites (mouse positions −626 and −1777). The mouse TATA box is labeled (dotted line) above the mouse sequence and the functional rat TATA box (dotted line) is labeled below the rat sequence (Klemme et al, 1999). The demonstrated transcription start sites for the mouse and rat genes are labeled with bent arrows. The sequence corresponding to the rat exon 2 is underlined and the consensus splice acceptor sites in the mouse and rat sequences are boxed (dotted line) and labeled (SA).

Figure 5. Sequence of cloned products obtained by RT-PCR using mouse epididymal RNA as template, a rat exon 1 primer upstream (underlined) and a mouse exon 6 primer downstream (not shown) compared with the rat mRNA sequence. Sequence corresponding to the rat exon 2 is boxed. New mouse sequence obtained downstream of the exon 1 primer is shown in bold and compared with exon 1 sequence from the rat, also in bold. The mouse and rat sequences are 78% identical over this 50-base-pair sequence.

scription from a start site upstream of the mouse-assigned exon 1 (Schwidetzky et al, 1997). A weak transcription start site in the corresponding region of the rat gene (5′ of exon 2) has also been reported (Klemme et al, 1999). Figure 4 compares the rat genomic sequence 5′ to exon 2 with the mouse genomic sequence flanking the mouse exon 1. These 2 regions have 82% sequence identity, including several putative androgen response elements (AREs) and PEA3 recognition sites. The rat gene does not have a strong consensus TATA box in this region, whereas the mouse gene does possess a TATA box, likely explaining the difference in transcription efficiency from this site between the rat and the mouse.

When analyzed by the FGENES software (BCM Search Launcher; dot.imgen.bcm.tmc.edu:9331/seq-search/gene-search.html), a consensus splice acceptor site was predicted in the mouse gene just downstream of the transcription start site (Figure 4), suggesting that an upstream exon in the mouse gene could splice in this region. To determine if the mouse gene has a sequence equivalent to the rat exon 1 that is spliced at low frequency at this site, RT-PCR was performed on mouse epididymal RNA using a 5′ primer against the rat exon 1 and a 3′ primer identical to the mouse exon 6. The reaction yielded a single band when analyzed by agarose gel electrophoresis (data not shown). The PCR product was cloned and several colonies were isolated and sequenced. All the clones had identical sequence and were identical to the published mouse Crisp-1 cDNA except that they possessed an extended 5′ sequence that was 78% identical to the rat exon 1 sequence, excluding the primer sequence (Figure 5). These data suggest that the mouse gene also contains an exon that is analogous to the rat exon 1. Several attempts to demonstrate this form of the message by 5′ RACE failed, suggesting that it is a rare form of the mouse Crisp-1 mRNA.

Discussion

The rat epididymal Crisp-1 mRNA is produced as 3 splice variants. These 3 forms of the message are variations involving exon 2, which is an exon encoding the 5′ untranslated sequence in the mRNA (Klemme et al, 1999). Thus, all 3 of the splice variants reported here are predicted to encode the same amino acid sequence. This is in contrast to other epididymal genes, such as the PEM homeobox gene and the relaxin-like factor gene, which encode splice variants that in turn encode protein variants (Maiti et al, 1996; Zarreh-Hoshyari-Khah et al, 1999). It is possible, however, that the rat Crisp-1 splice variants encode Crisp-1 proteins that are differentially processed to yield the D and E forms. For this hypothesis to be correct, differential processing of the variants would have to be region-specific because the expression pattern of the D and E forms of the protein differ while the relative amounts of each splice variant remains constant in all regions of the epididymis. The direct testing of this hypothesis awaits further experiments.

Each splice variant form of the rat Crisp-1 mRNA was reduced when androgens were reduced by castration. However, the ratio of splice variant forms was unaffected by this decrease in androgen levels. These results taken together show that splicing is not differentially regulated by androgens and that all forms of the mRNA are under androgen control, similar to what has been shown previously for the rat DE/AEG mRNA (Brooks et al, 1986; Charest et al, 1988). It is known that luminal factors from the testis are required for the synthesis of many epididymal proteins (Hinton et al, 1998). The levels of all Crisp-1 splice variants were maintained at control levels in the ipsilateral epididymis of the hemicastrate rats, indicating that luminal androgens or other luminal factors are not required for the maintenance of Crisp-1 mRNA levels. Taken together, the results of this study suggest that the splice variants involving exon 2 in the rat Crisp-1 gene are the result of splicing efficiency and not a mechanism of differential gene regulation.

This study shows that the mouse and rat Crisp-1 genes are highly similar (82% identical) in the sequence that flanks the rat exon 2 and mouse exon 1, suggesting that the rat and mouse genes are structurally very similar. The intron/exon structure of the mouse and rat genes are identical except that the mouse has no reported exon equivalent to the rat exon 1 (Klemme et al, 1999). Comparison

of the rat exon 2 sequence to the mouse exon 1 sequence shows that both these regions contain numerous transcription factor binding sites, including several consensus steroid response elements. Several of these AREs are likely functional because they contain 3 or fewer mismatches with the ARE consensus (Schwidetzky et al, 1997). The AREs at position −1253 and −1270 (AREs 3 and 4, Figure 4), which are conserved in the rat sequence, have been shown in the mouse to interact with the androgen receptor protein and are thus likely to be functional (Schwidetzky et al, 1997). In addition, both the mouse and rat genes encode binding sites for PEA3, one of the few transcription factors known to be expressed in the epididymis (Xin et al, 1992).

One striking difference between the rat and mouse sequences is in the lack of a consensus TATA box in the rat sequence corresponding to the TATA box in the mouse gene, which likely results in greatly reduced expression of the rat gene from this site flanking exon 2. We have previously shown that the rat gene is in fact transcribed from this region of exon 1 but the transcription start site is 92 base pairs upstream of the mouse start site (Klemme et al, 1999).

RT-PCR using a rat exon 1 primer with a mouse epididymal RNA-derived cDNA template demonstrates that there is a form of the mouse Crisp-1 mRNA with a 5′ sequence similar to the rat exon 1 sequence. The 3′ end of this new sequence begins 24 base pairs downstream of the published transcription start at a site that would be predicted, based on the rat mRNA sequence, to be the boundary between exon 1 and exon 2. Analysis of this sequence shows that it contains a predicted splice acceptor site, suggesting that RNA splicing could occur at this site. The additional mouse mRNA sequence is 78% identical to the rat exon 1 sequence, excluding the rat-derived 5′ primer sequence used in the analysis. These data taken together suggest that mouse and rat Crisp-1 genes have similar exons encoding the 5′ untranslated regions of the mRNAs. The mouse and rat differ in that the mouse gene is preferentially transcribed from the start site in the sequence corresponding to the rat intron 1 and that the rat gene is preferentially transcribed from a start site in the 5′ flanking region of exon 1. The preferred upstream start site in the rat gene results in splice variants of the rat mRNA that are not present in measurable amounts in the mouse gene due to its preferential downstream start site. The low transcription efficiency from the intron 1 start site in the rat is likely due to the less than optimal TATA box in this region of the gene.

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