Isolation and characterization of the androgen-dependent mouse cysteine-rich secretory protein-3 (*CRISP-3*) gene

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The mRNA for cysteine-rich secretory protein-3 (CRISP-3) was originally identified in the mouse salivary gland as an androgen-dependent transcript, and is closely related to CRISP-1 and CRISP-2 which are abundantly expressed in the epididymis and testis respectively. Overlapping phage clones encompassing the entire length of the *CRISP-3* gene were isolated from a λEMBL3 genomic library and analysed. DNA sequencing revealed that the gene consisted of eight exons ranging between 55 and 740 bp in size, and seven introns. All exon–intron junctions conformed to the GT/AG rule established for eukaryotic genes. The length of the introns was determined by PCR and was found to vary between 1.0 and 3.7 kb, indicating that the gene spans over 20 kb of the mouse genome. Primer extension allowed the mapping of the major transcription initiation site to an adenine located at the appropriate position downstream of a *bona fide* TATA box, in a region corresponding well to the eukaryotic consensus sequence. Over 800 bp of *CRISP-3* promoter region were determined and two regions almost exactly matching the androgen-responsive element consensus RGWACANNNTGTWCY detected. In addition, sequences described in the *Drosophila melanogaster Sgs-3* gene as being involved in its salivary gland-specific expression as well as two putative OTF- and GATA-binding elements were also found.

INTRODUCTION

Members of the cysteine-rich secretory protein (CRISP) family or the corresponding transcripts have been detected in several mouse tissues that contain androgen receptors. CRISP-1 is mainly expressed in the epididymis and, to a lesser extent, in the salivary gland [1–3]. CRISP-2 (also named Tpx-1) is found in the testis [4] and CRISP-3 is an abundant salivary gland product [1,3]. CRISP-1 and CRISP-3 share 78% amino acid sequence identity, whereas CRISP-2 is more distantly related with 55 and 48.5% identity to the other forms. However, all 16 cysteine residues, of which ten are concentrated in the C-terminal part, are conserved between the CRISPs, suggesting an identical structure for the members of the family. Based on the localization of internal disulphide bridges, we have proposed the existence of a two-domain structure for CRISP-1 [2] which is probably also shared by CRISP-2 and CRISP-3.

Closely related members of the CRISP family have also been identified in the rat (DE/AEG [5,6] which corresponds to mouse CRISP-1) and in human (TPX1 [4] which corresponds to mouse CRISP-2/Tpx-1). Very recently we have been able to identify a new member of the family, helothermine, in the venom of the Mexican lizard *Heloderma horridum horridum* [7]. This protein shares between 43 and 47% sequence identity with the CRISPs and was found to have a lowering effect on the body temperature of mice after intraperitoneal or intravenous injection [8].

Mouse CRISP-1 and rat DE/AEG are expressed in abundance in the epididymis [2,9–11]) where spermatozoa undergo late stages of maturation including the acquisition of motility and of zona pellucida binding ability. Immunization studies have pointed to the importance of DE/AEG in the fertilization process [12,13], and an association of the protein with the heads of spermatozoa, possibly via a specific receptor, has been proposed [11,14]. Furthermore, a redistribution of DE/AEG has been observed on spermatozoa after their capacitation, in line with a possible involvement in a specific stage of fertilization [15]. In addition a recent study reports the existence of complementary sites for DE/AEG on the rat egg plasma membrane [16]. Altogether these data point to a fundamental role in gamete interaction but the precise role of DE/AEG has so far not been elucidated.

Little is known about the function of CRISP-3 which has only been identified in the mouse. RNA analysis and *in situ* hybridization showed that *CRISP-3* transcripts are only found in the submandibular salivary gland where they are synthesized by the cells of granular convoluted tubules [3]. Castration and hormone substitution experiments have established that the expression of the *CRISP-3* gene in the salivary gland is controlled by androgens, albeit to a lesser extent than the *CRISP-1* gene [1,3]. As a first step towards deciphering the elements responsible for the tissue-specific expression and androgen dependence of the *CRISP* genes, we have now isolated and characterized the complete *CRISP-3* gene, including over 800 bp of 5′ upstream region.

EXPERIMENTAL

Materials

Fresh salivary glands were obtained from NMRI mice kept at the in-house facility. The mouse (C57 black) genomic library was from Clontech. Restriction endonucleases, the reverse transcriptase, RNase A and the 5′ DNA end-labelling kit were purchased from Boehringer Mannheim. *Taq* polymerase and the PCR kit were from Perkin Elmer Cetus. T7 gene 6 exonuclease

Abbreviations used: ARE, androgen-responsive element; dATP[αS], deoxyadenosine 5′-[α-thio]triphosphate; CRISP, cysteine-rich secretory protein.

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and the Sequenase kit version 2.0 were obtained from USB. RNAsin was from Promega and the Fast Track mRNA Isolation kit from InVitrogen. The pBluescript plasmid was from Stratagene. The 1 kb DNA molecular mass standard was from GIBCO BRL. Oligodeoxynucleotides were made on an Applied Biosystems 381A DNA synthesizer. The labelled nucleotides [α-^{32}P]dCTP (~ 3000 Ci/mmol), [γ-^{32}P]ATP (~ 3000 Ci/mmol) and [^{35}S]deoxyadenosine 5′-[α-thio]triphosphate (dATP[αS]) (> 1000 Ci/mmol) were purchased from Amersham. Denhardt's solution and the salmon sperm DNA were from Sigma. Agarose and low-melting agarose (SeaPlaque) were from Life Technologies and FMC, respectively. The Biodyne A (1.2 μm) hybridization membranes were obtained from Pall and the films for autoradiography from Kodak. The University of Wisconsin GCG package [17] was used for sequence analysis.

Isolation of phage clones

A mouse genomic library established in the λEMBL3 vector was plated as advised by the manufacturer and two replicas made on nylon membranes. Between 0.5×10^6 and 1×10^6 plaques were analysed for each screening. Prehybridization and hybridization were carried out in 5 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.1 % SDS, 5 × Denhardt's, 100 μg/ml denatured salmon sperm DNA for 4 and 20 h respectively. Washing steps were in 5 ×, 3.5 × and 2 × SSC/0.2 % SDS for 15 min in each case. A probe was generated from the approximately 300-bp-long internal *Nco*I fragment of the *CRISP-3* cDNA (positions 25 to 347; [1]) using the primer 5′-CAGCACCATGGCATTAATGCTTGTGC-3′ (positions 19 to 44; [1]) and [α-^{32}P]dCTP, in four cycles of 2 min at 94 °C, 2 min at 42 °C and 3 min at 72 °C and in the presence of *Taq* polymerase. Alternatively, oligonucleotides were labelled with T4 polynucleotide kinase and [γ-^{32}P]ATP: 5′-AAGTTGCATGTGGAGTTG-3′, 5′-GTGCCAGTTGTCCTGATC-3′ and 5′-TGTCAATATAAGGATATG-3′ (positions 471–488, 606–623 and 653–670 in the *CRISP-3* cDNA [1]). Plaques scoring positive were rescreened at least twice and DNA was prepared from the phages using a large-scale procedure [18]. The DNA was then digested with *Xho*II and subcloned into a *Bam*HI-cut pBluescript plasmid as described previously [19]. Bacterial colonies transformed with the subclones were screened for the presence of *CRISP-3* gene fragments using the above-mentioned probes.

DNA sequencing

The dideoxy chain-termination method [20] was used to sequence a double-stranded plasmid DNA with a Sequenase kit and [^{35}S]dATP[αS]. Phage DNA was sequenced after preparing single-stranded template by T7 gene 6 exonuclease digestion or by asymetric PCR. For exonuclease digestion, 24 μg of phage DNA was incubated for 30 min at 37 °C in the presence of 75 units of enzyme. The reaction was stopped by incubation at 78 °C for 10 min before sequencing. For asymetric PCR, the target DNA was first amplified conventionally with *CRISP-3*-specific primers and purified on a low-melting agarose gel, and then amplified again using a 1:50 molar ratio of the same primers to generate single-stranded DNA which was directly used for sequencing with internally priming oligonucleotides.

Determination of the intron size

PCR was carried out on 1 ng of phage DNA for 22 to 28 cycles of 1 min 30 s at 94 °C, 30 s at 41–51 °C and 3 min 30 s to 4 min 30 s at 72 °C, depending on the primers and expected size of product. The amplified fragments were separated on a 1 % agarose gel and stained with ethidium bromide [18]. The size was determined by comparison with a DNA molecular mass standard.

Determination of the transcription initiation site

Poly(A)$^+$ RNA was isolated from mouse salivary gland using the FastTrack mRNA Isolation kit. A labelled antisense oligonucleotide (5′-TTACTGGTTGAAAGTTTCTCAAGACTGTTC-3′, positions 100–129 in the *CRISP-3* cDNA [1]) was mixed with 2 or 10 μg of poly(A)$^+$ RNA and precipitated in ethanol. The sample was dissolved in 30 μl of 3 M NaCl/0.5 M Hepes (pH 7.5)/1 mM EDTA (pH 8.0), heated at 85 °C for 10 min and left to anneal at 30 °C for 14 h. After ethanol precipitation, the RNA was copied into cDNA for 90 min at 42 °C using the Moloney murine leukaemia virus reverse transcriptase (40 units) in the presence of 4 mM dNTPs and 1.25 μl of RNAsin. The reaction was stopped by treatment with 0.5 M EDTA and 1 μg/μl RNase A for 30 min at 37 °C followed by phenol/chloroform extraction and ethanol precipitation. The sample was analysed on a 6 % acrylamide/bisacrylamide (30:1)/7.5 M urea gel and compared to a sequencing reaction loaded at the same time.

RESULTS

Isolation of phage clones covering the *CRISP-3* gene

A mouse genomic library established in λEMBL3 was screened with a probe corresponding to a 300 bp sequence of the 5′ end of the *CRISP-3* cDNA. The positive clones were further analysed with oligonucleotide probes to discriminate between *CRISP-3* and the closely related *CRISP-1*. One of them, λ30, was then selected for further characterization and *Xho*II restriction fragments subcloned into a plasmid vector. DNA sequencing revealed the presence of five *CRISP-3* exons and 817 bp of 5′ upstream region in the λ30 insert (Figure 1). Additional screenings of the library were carried out with oligonucleotide probes derived from the 3′ part of *CRISP-3* (see the Experimental section), and

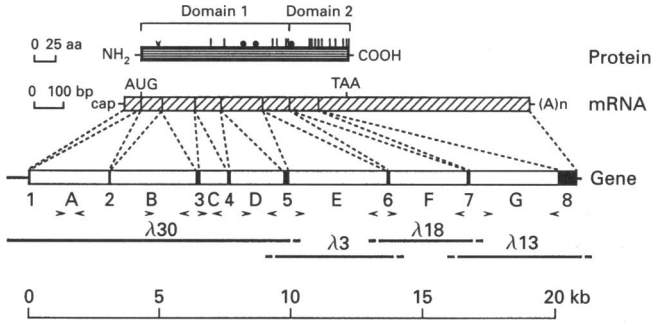

Figure 1 Organization of the *CRISP-3* gene

The *CRISP-3* gene is schematically represented in the centre as a bar with the exons shown in black and marked 1 to 8, and the introns marked A to G. The converging arrowheads below indicate the location of the PCR primers used to determine the size of the different introns. The four overlapping phage clones from which the sequence information was derived are drawn under the gene structure, with the corresponding scale. The top of the Figure shows a schematic representation of the CRISP-3 protein (bar with horizontal lines) and the corresponding scale. The vertical arrowhead shows the limit between the signal sequence and the mature protein, small vertical lines point to cysteine residues and filled circles to potential glycosylation sites. The boundaries of the two putative domains are shown. *CRISP-3* mRNA is represented immediately below (bar with diagonal lines), with the corresponding scale. The positions of the AUG and TAA translation initiation and stop codons are indicated.

Intron	Exon		Intron	Phase
ATGTC..	1	(55 bp)	..CAGCAgtaagtag (~2.95 kb)	/
aaacatcataatgcatgtttttgttctcttctttgttctctagCCATG.. M1	2	(74 bp)	..CTCAGgtatgggt (~3.25 kb) Q24	0
tcatacactaatgtgactttcattttatttccctttagGAGAA.. E25	3	(117 bp)	..ATATGgtgagggc (~1.0 kb) M63	0
attattatttatagctatctccttttttttctctctctagGAATG.. E64	4	(88 bp)	..AACTAgtatgtga (~2.0 kb) N93	1
tgctaccttaatgattggtctatttctttcccatagATTTA..	5	(146 bp)	..CTCAGgtaagaaa (~3.7 kb) Q141	0
tttttctctgacacatgctccatattcattcctgggtatgcagGTTGT.. V142	6	(95 bp)	..CCTGTgtaagtat (~2.9 kb) V173	2
aaatcatctgatgattttgcagATTGA..	7	(101 bp)	..GTGCAgtaagttt (~3.25 kb) T207	1
gttgacattaacagaggaaaataaaaataaagtaatgaatatttttttttctttatttcagCCAAG..	8	(740 bp)	..TTATT	/

Figure 2 Exon–intron junctions of the *CRISP-3* gene

Exon and intron sequences are given in upper-case and lower-case letters respectively. The phase refers to the boundary relative to the last codon of an exon. The amino acids affected by the intron interruption are indicated with their respective location in the protein [1]. The branchpoint consensus motif at the 3′ end of the introns [25,26] is underlined.

three phage clones carrying exons 5 and 6 (λ3), 6 and 7 (λ18) and 7 and 8 (λ13) of the *CRISP-3* gene were isolated (Figure 1). The four clones were overlapping and together contained the entire transcription unit of the *CRISP-3* gene.

Sequence and organization of the *CRISP-3* gene

The exon parts and their neighbouring regions were sequenced. Comparison with the cDNA sequence [1] did not show any discrepancy and revealed that the *CRISP-3* gene was composed of eight exons and seven introns (Figure 1). The internal exons 2 to 7 are of similar sizes (between 74 and 101 bp long; Figure 2) thereby falling into the main category described [21]. The first exon is the shortest (see below) and codes for almost all of the 5′ untranslated part. Even though the precise limits between leader sequence and mature protein have not been determined for CRISP-3, they are probably similar to those found for CRISP-1 [2], so the second exon codes for the entire 20-amino-acid-long signal peptide and the four N-terminal amino acids of mature CRISP-3. The equivalents of the six cysteines which are pairwise linked and delineate a discrete N-terminal domain in CRISP-1 [2] are encoded by exons 4, 5 and 6 of *CRISP-3* whereas exons 7 and 8 code for the cysteine-rich C-terminal domain of the protein. Exon 8 is much longer than the others but its protein coding part is similar in size to that of exons 2 to 7, in line with previous observations [21]. It covers in addition the entire 3′ untranslated part, including the polyadenylation signal [22].

Seven introns interrupt the *CRISP-3* gene (Figure 1). Introns B to G split the protein coding regions in the three possible phases [23] with a preponderance of phase 0 introns (Figure 2). In all cases the exon/intron boundaries conformed to the GT/AG splice rule [24] (Figure 2). A putative branchpoint for the lariat formed during splicing [25,26] was found in the appropriate position upstream of the 3′ extremity of each intron (Figure 2). The size of the introns was determined by PCR amplification of phage DNA, employing primer pairs hybridizing to two consecutive exons or their intervening region (Figure 1). The length of the amplification product was determined on an agarose gel by comparison with a standard (Figure 3) and the value rectified with respect to the exact location of the primers. For each intron,

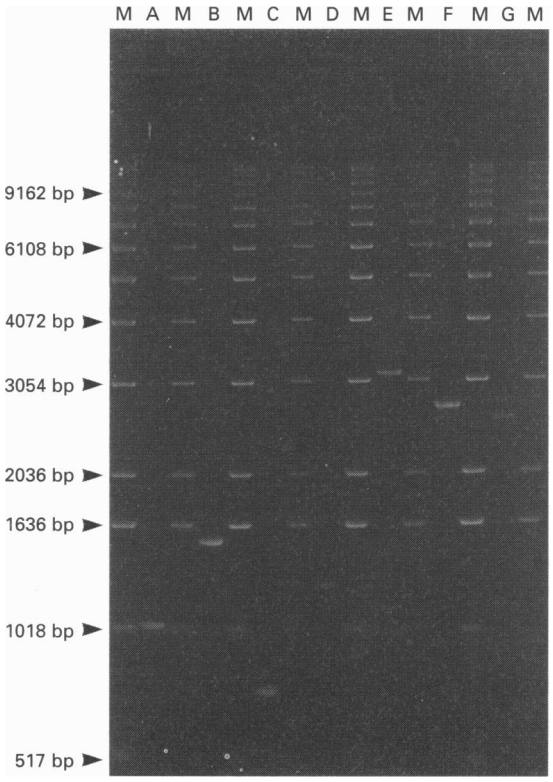

Figure 3 Determination of intron sizes

PCR products amplified from introns A to G were separated on an agarose gel and stained with ethidium bromide. The 1 kb DNA ladder (GIBCO BRL) was used as a size marker (M).

a second pair of primers was additionally used for an independent confirmation and a more precise determination of the size (results not shown). The length of the introns varied between 1.0 and 3.7 kb such that the entire *CRISP-3* gene spanned approximately 20 kb of the mouse genome.

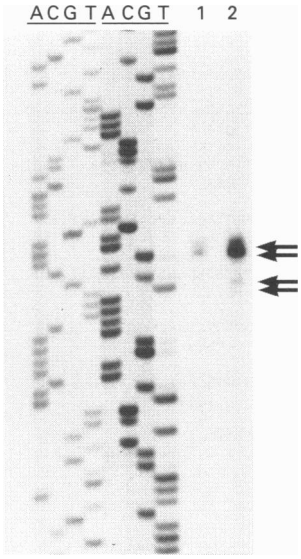

Figure 4 Primer extension mapping of the transcription initiation point

Mouse salivary gland poly(A)$^+$ RNA (lane 1, 2 μg; lane 2, 10 μg) was used as a template for reverse transcription. The arrows point to the extension products. Two different sequencing reactions (lanes ACGT) served as markers for a precise determination of the length. Exposure was for 48 h using an X-OMAT film.

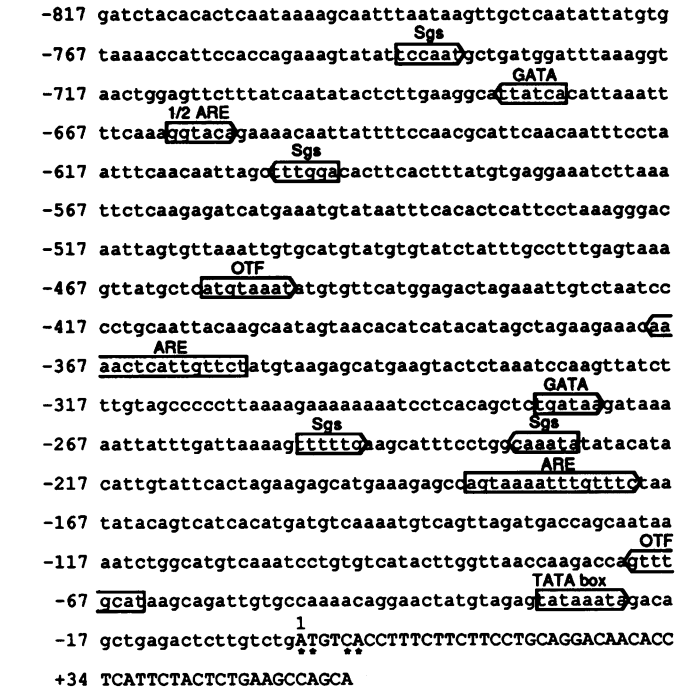

Figure 5 Nucleotide sequence of the *CRISP-3* promoter region

The DNA sequence of the 5′ upstream region (lower-case letters) and of the first exon (upper-case letters) are shown. Asterisks indicate the transcription initiation sites as determined by primer extension. Putative *cis*-acting elements are highlighted with arrows. The GeneBank/EMBL accession number for this sequence is X85321.

Determination of the transcription initiation site

Primer extension was used for the mapping of the transcription initiation site. A 30-nucleotide-long antisense oligonucleotide priming near the 5′ end of the *CRISP-3* cDNA was used for reverse transcription of salivary gland poly(A)$^+$RNA. Two major bands differing by one nucleotide in length and two shorter, much fainter bands with also one nucleotide difference were generated (Figure 4). The longest product finished 57 bp upstream of the AUG translation initiation codon and 32 bp upstream of the 5′ extremity of the published *CRISP-3* cDNA [1]. This position is occupied by an adenine residue embedded in a region, in good agreement with the consensus cap site sequence [27] and at a correct distance downstream of the TATA box (see below). It was taken as nucleotide 1 in the genomic sequence (Figure 5). These results are compatible with the previously determined size of the *CRISP-3* mRNA [1] and indicate that the first exon is made of 55 bp of 5′ untranslated sequence.

Characterization of the promoter region

An 817-bp-long stretch of 5′ upstream region was determined (Figure 5). A TATA box was detected at the appropriate position with regard to the transcription initiation site (positions −29 to −22) whereas no sequence closely resembling a CAAT box (CCAAT; [28,29]) was identified. Two stretches closely resembling the androgen-responsive element (ARE) consensus sequence RGWACANNNTGTWCY [30] (only one mismatch) were found at positions −369 to −355 and −185 to −171, along with half an ARE (−661 to −656). In addition, sequences matching the consensus TNTTTG, TCCANW or their complements which are present in the *Drosophila melanogaster* salivary gland-expressed *Sgs-3* genes [31] were detected at positions −741 to −736, −602 to −597, −250 to −245 and −231 to −226. A sequence reminiscent of the general enhancer element ATTTGCAT (seven out of eight possible matches) recognized by the octamer-binding transcription factors (OTFs) [32–34] was observed twice, at positions −458 to −451 and −71 to −64 and a motif with 100% identity to the GATA transcription factor recognition site WGATAR [35] twice also, at positions −682 to −677 and −279 to −274.

DISCUSSION

The genomic organization of the mouse *CRISP-3* gene was elucidated from four overlapping phage clones and found to consist of eight exons and seven introns. Comparison with the structure of the *CRISP-2* (*Tpx-1*; [36]) gene reveals that in both cases the introns interrupt the protein coding sequence at equivalent positions and in the same phases. Consequently the cysteine-rich C-terminal part of CRISP-2 and CRISP-3, which is characteristic of the family and might play a specific functional role due to its compact structure [2], is encoded by the last two exons. Interestingly, this domain is absent from proteins which are more distantly, though still significantly, related to the CRISPs, namely members of the pathogenesis-related protein family of plants (e.g. tobacco PR-1 [37,38]), insect allergens (e.g. hornet Dol m V [39,40] and ant Sol i II [41]) and Sc7 and Sc14 from the basidiomycete *Schizophyllum commune* [42] (for an alignment of these proteins see [7]). The structural organization of the genes for Sc7 and Sc14 is known and comparison with *CRISP-3* shows that the position of only one intron, between exons 5 and 6, is conserved, probably reflecting the oldness of the diverging event for the ancestors of these genes. This and the conspicuous absence of the cysteine-rich domain suggest that different functions are served by the vertebrate and non-vertebrate proteins of the superfamily.

Two differences between the *CRISP-3* and *CRISP-2* genes are

worth mentioning. First, *CRISP-2* contains two additional exons coding for 5′ untranslated sequence, which are not present in *CRISP-3* (nor in *CRISP-1*; U. Schwidetzky, B. Haendler and W.-D. Schleuning, unpublished work). This fits with the published size of the 5′ untranslated part of the cDNAs, which is much longer in CRISP-2 (213 bp) than in CRISP-3 or CRISP-1 (25 bp) [1,4]. Secondly, an unusually large intron upstream of the region coding for the cysteine-rich domain was found in *CRISP-2* and hypothesized to be a vestige of the recruitment event that led to the incorporation of this domain into the encoded protein [36]. This particularity was not found in *CRISP-3* (nor in *CRISP-1*; U. Schwidetzky, B. Haendler and W.-D. Schleuning, unpublished work) and can be explained by a deletion or insertion event that took place after the divergence of the ancestor genes for *CRISP-2* and *CRISP-1*/*CRISP-3*. Altogether these differences are in line with the higher similarity existing between CRISP-3 and CRISP-1 than between CRISP-3 (or CRISP-1) and CRISP-2.

The promoters of the *CRISP-3* and *CRISP-2* genes display no significant sequence similarity. Contrarily to *CRISP-2*, a classical TATA box and two motifs closely matching AREs are present in the *CRISP-3* upstream region. Previous studies have shown that the expression of the *CRISP-3* gene is under androgen control [1,3] but further experiments have now to be carried out to prove that the *cis* elements we identified are indeed responsible for this regulation. Several half AREs are also found in the *CRISP-2* promoter but the androgen-dependency of the gene has so far not been studied.

Motifs common to several salivary gland-expressed *Drosophila* genes [31] were found in the *CRISP-3* promoter. In *Drosophila*, this motif is made of two 6-bp-long sequences in one or the other orientation and separated by non-conserved spacer sequences. In the *CRISP-3* promoter, this spacer region is much longer and might therefore contain additional elements, which will complicate the analysis of the contribution of these sequences to the tissue specificity. None of the sequences previously identified as implicated in salivary gland-specific expression of mammalian genes like the macaque *MnP4* gene [43], the rat *GRP-Ca* gene [44] or the human *amylase* gene [45] was found in the *CRISP-3* gene promoter.

A putative OTF-binding octanucleotide [32–34] was detected at two locations of the *CRISP-3* gene upstream region. Interestingly it has also been reported in another androgen-responsive, salivary gland-expressed gene, namely that for the prolactin-inducible protein PIP/GCDFP-15 [46], and is thought to represent a general modular enhancer element recognized by several transcription factors. The presence of two GATA-binding motifs is at first intriguing, since they were originally described as erythroid gene regulatory regions [47] but later studies have, however, shown that the range of genes controlled by the GATA factors is wider [35]. Interestingly, interactions of GATA-1 with the glucocorticoid receptor have recently been reported in erythroleukaemia cells [48] and a similar interaction might be envisaged with the androgen receptor. In line with this, putative GATA-binding sites have also been described in the promoter of the gene coding for the epididymal, androgen-dependent glutathione peroxidase-like protein arMEP24 [49].

Altogether these results suggest complex interactions between factors involved in the androgen dependence and tissue specificity of the *CRISP-3* gene. Reports on the few other androgen-dependent genes that have so far been analysed have already pointed to the importance of the cooperation between receptor and non-receptor binding sites and only additional studies using appropriate reporter constructs and cell lines will clarify if the *cis* elements detected are functional.

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