Structural and functional analysis of the promoter of a mouse gene encoding an androgen-regulated protein (MSVSP99)

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ABSTRACT

MSVSP99 (mouse seminal vesicle secretory protein of 99 amino acids) is a member of the rat and mouse seminal vesicle secretory protein family. The gene encoding MSVSP99 is under androgenic control and we demonstrate here that this regulation involves a complex interplay of positive and negative regions. First, we show that the promoter region (−387/+16) sufficient to mediate a full androgen induction is a complex enhancer organized in two regulatory regions. These two regions are inactive individually and must act together to confer a 40-fold androgen induction to the MSVSP99 gene and androgen responsiveness is not only dependent on the presence of functional androgen response element (ARE) sequences but results from complex cooperations between ARE and non-ARE sequences forming an androgen response unit. Secondly, we characterized a new regulatory region (−824/−632) that decreases androgen-dependent transcriptional activity of the MSVSP99 promoter. This region, also able to repress the transcriptional activity of the heterologous thymidine kinase promoter, contains a functional promoter on the inverted strand (−826 to −387) and we identified a transcription initiation site located at position −639 with respect to the cap site of the MSVSP99 promoter. Sequence analysis of the flanking DNA also revealed that the MSVSP99 gene is surrounded by long interspersed repeated sequences called LINEs.

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INTRODUCTION

Transcriptional control is a primary mechanism underlying cell-specific gene expression in mammalian cells. It is now established that steroid hormone receptors are ligand-activated transcriptional modulators that control gene expression in either a positive or negative direction. Several types of DNA elements are involved in the control of steroid responsive genes: canonical promoter elements such as the TATA box, hormone responsive elements which are consensus sequences binding receptor–steroid complexes and binding sites for ubiquitous and/or tissue-specific factors (Evans 1988, Mangelsdorf et al. 1995, Beato & Sanchez-Pacheco 1996). The majority of these binding proteins are known to play a positive role in transcription. Further, in a smaller number of cases, DNA regions have been identified that appear to suppress transcriptional activity (reviewed by Drouin 1993).

The seminal vesicles secrete a set of proteins involved in semen coagulation in rodents and in humans (Aumüller & Seitz 1990). In rodents, the seminal vesicle secretory (SVS) proteins share a common pattern of tissue-specific regulation by androgens and have a common developmental profile (Higgins et al. 1976, Fawell et al. 1986, Normand et al. 1989). They are encoded by a multigene family from which we have isolated the gene encoding MSVSP99 (mouse SVS protein of 99 amino acids), which is the murine equivalent of the rat SVS VI gene (Simon et al. 1995). Moreover, some regions were found to be conserved between the MSVSP99 gene and the human semenogelin I and II genes (Simon et al. 1995). Semenogelins I and II are the predominant seminal vesicle-secreted proteins (Lilja et al. 1989). We have studied the MSVSP99 gene, which is under hormonal, developmental and tissue-specific control (Guilbaud et al. 1993, Simon et al. 1997). The MSVSP99 gene is...
regulated at the transcriptional level by the androgen receptor (AR), and a functional androgen response element (ARE), located at position −208 upstream from the cap site, has been identified (Brochard et al. 1997, Simon et al. 1997). However, little is known about the DNA sequences and nuclear factors that mediate the response of the SVS genes to androgens. As an ongoing effort to understand the factors that control MSVSP99 gene expression, we report here the characterization of DNA regions that appear to enhance or repress androgen-dependent transcriptional activity.

MATERIALS AND METHODS

Animals

Mice of Swiss strain (CD-1; Charles River, St Aubin lès Elbeuf, France) were used for total RNA isolation.

Plasmid constructs

The pSV-ARo contains the full-length human AR cDNA directed by the SV40 promoter (Brinkmann et al. 1989). The p2-4-chloramphenicol acetyltransferase (p2-4CAT), p0-8CAT, p0-4CAT, and p0-3CAT constructs contain sequences extending respectively from −2365 to +16, −824 to +16, −387 to +16 and −261 to +16 (Brochard et al. 1997). The p0-632CAT and p0-329CAT constructs were generated by inserting a PCR fragment covering the MSVSP99 promoter, obtained using respectively the HindIII (−632/−612) 5′-AGA TAAGCTTCTTGGAGCTGAC-3′ and the XbaI (−329/−303) 5′-AAGTCTAGACTGCTTGA GGACATTTG-3′ forward primers with the reverse SaII (+24/+4) 5′-CATCAGTGAGGAAGCTTGGT-3′, into the HindIII/XbaI restriction sites in the pBLCAT3 vector. The AREp-thymidine kinase (Tk)-CAT was obtained by inserting the double-stranded MSVSP99 AREp 5′-GAAGAAGCTTCTCAGTTGAAAGAC TTG-3′ primer, into the HindIII/XbaI and the XbaI/XhoI restriction sites in the pBLCAT3 vector. The AREp-thymidine kinase (Tk)-CAT was obtained by inserting the double-stranded sequences extending from −2365 to +16 and deleted for the −823 to −388 fragment. This construct was made by inserting a −2365/−823 HindIII/blunted XhoI restriction fragment of the p2-4CAT construct into the HindIII/XhoI restriction sites in front of the −387/+16 promoter fragment of the p0-4CAT construct (Brochard et al. 1997). The p0-43iCAT construct was obtained by inserting a PCR fragment containing the −819 to −389 genomic sequence in reverse orientation with respect to the MSVSP99 promoter in the XbaI/XhoI sites present in the polylinker of the pBLCAT3 vector (Luckow & Schütz 1987). This PCR fragment was generated using the forward primer SaII (−829/−806) 5′-AGCAGTCGACTGAGGGAAGCTTGGAAAGAC TTG-3′ and the reverse primer XbaI (−397/−379) 5′-CCTCATCAGTTGAAAGAC TTG-3′.

Cell culture and transfection

Monkey kidney CV-1 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with glutamine (2 mM), insulin (4 µg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% calf serum. For each transfection experiment, cells were seeded at a density of 1 × 10^5 cells/dish (diameter 10 cm) and transfected 8 h later using the calcium phosphate/DNA precipitation method (Chen & Okayama 1987). The DNA quantities used were: 2.5 µg for pSV-ARo, 10 µg for the different TkCAT constructs and 20 µg for the MSVSP99CAT constructs. The cells were incubated in fresh DMEM supplemented with 2.5% steroid-free calf serum supplemented or not with 10^{-6} M dihydrotestosterone (DHT), 10^{-6} M dexamethasone (DEX) or 10^{-6} M progesterone (PROG). Cells were harvested after 60 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). Average inductions and standard deviations were calculated from at least four independent transfections.

Localization of transcription start point

Total RNAs were extracted with RNAzol (Bioprobe-Quantum, Montreuil sous bois, France), from CV1 cells transfected with the p0-43iCAT
A 33-mer oligonucleotide 5′-GTTCTT TACGATGCCATTTGGGATATATCAACGC-3′ complementary to nucleotides 29–61 of the CAT cDNA in the pBLCAT3 vector (Luckow & Schütz 1987) was used as primer to initiate both a reverse transcription (RT) reaction on CV1 total RNA as described by Simon et al. (1995) and a sequencing reaction using the p0-43iCAT construct as template. The sequencing reaction was carried out using the T7 Sequencing kit (Pharmacia, Bois d’Arcy, France) following the manufacturer’s instructions. Electrophoresis was performed at 60 W in a 89 mM Tris-borate, 2 mM EDTA buffer, pH 8.3, on a 6% (w/v) polyacrylamide gel under denaturing conditions (7 M urea). When fixed and dried, gels were autoradiographed using Amersham Life Science Hyperfilm MP (RPN7H; Amersham, Les Ulis, France).

RT-PCR analysis

Total RNAs were extracted from CV1 cells transfected with the genomic clone containing the whole MSVSP99 gene plus the 5′-flanking region (Simon et al. 1995), and from tissues of 2-day and 60-day-old male and female mice (muscle, liver, adrenals, kidney, lung, submaxillary gland, intestine, brain, lacrimal gland and spleen for both sexes, with testis, epididymis, vas deferens and prostate for males, and ovary and uterus for females) using RNAzol. Two micrograms total RNA were de-

characterized in the presence of 100 pmol random hexanucleotides at 65 °C for 5 min, then reverse transcribed with 200 U of MMLV-RT (Promega, Charbonnieres, France) at 37 °C according to the manufacturer’s instructions. All first-strand cDNAs were amplified by PCR using 100 pmol specific primers and Vent exo-DNA polymerase (Biolabs Ozyme, Saint Quentin en Yvelines, France) following the manufacturer’s instructions. Amplification was carried out using MSVSP99-flanking region-specific primer (5′-AGCAGTCGACTGG GGACTGGAAGG-3′: position −829/−806 with respect to the MSVSP99 start site and 5′-ACT ATATATACATACACAAC-3′: position −651/−670), or β-actin-specific primer (5′-CGTGGG CGGCCCTAGGCACCA-3′: position +102/+122 with respect to the ATG initiator (Inr) codon, and 5′-TTGGCCCTTAGGTTCCAGAAGG-3′: position +344/+323). PCR products were then analyzed by agarose gel electrophoresis.

Statistics

Values are means ± s.d. The significance of differences between means was calculated using Student’s t-test. Differences were considered to be significant at P<0.05.

RESULTS

Characterization of MSVSP99 sequences responsible for androgen-stimulated expression

A 403 bp fragment (−387 to +16) functions as an androgen-responsive unit in four different cell lines

Previously, we have shown that a minimal region (−387 to +16) was able to direct androgen-regulated expression in CV1 cells (Brochard et al. 1997). Possible effects of cell-specific factors on androgen responsiveness were investigated by transient transfections again in CV1 cells and in three additional cell lines that do not normally produce MSVSP99: T47D, SC115 and Y1 cells.

Cells were cotransfected with the p0-4CAT construct (the −387 to +16 fragment of the MSVSP99 5′-flanking region fused to the CAT reporter gene) and pSV-ARo in the absence and the presence of 10−6 M DHT. As shown in Fig. 1, good induction of CAT activity was seen with DHT in all cell lines, indicating that sequence elements located between −387 and +16 are sufficient to confer androgen-stimulated expression. Since the strongest CAT activity was observed in CV1 cells they were chosen as host cells for further experiments.

FIGURE 1. Comparison of androgen-dependent enhancer activities of the minimal MSVSP99 promoter in CV1, T47D, SC115 and Y1 cells. Autoradiogram of CAT gene expression assays measured in homogenates of cells cotransfected with 20 µg of 4 kb MSVSP99 fragment and 2.5 µg human AR. Cells were grown (60 h) in the absence or presence of 10−6 M DHT.

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Specificity of the hormonal response

The ability of AR, glucocorticoid receptor (GR) and PROG receptor (PR) to activate the p0·4CAT construct was tested in CV1 cells. To explore how specific to androgen was the response of the MSVSP99 upstream region, the AR, GR and PR were cotransfected with the MSVSP99-CAT reporter and subsequently the cells were treated with 10⁻⁶ M DHT (for the AR), 10⁻⁶ M DEX (for the GR) or 10⁻⁶ M PROG (for the PR). Under the same conditions, the activity of the pSV2CAT plasmid control is not stimulated (not shown). The addition of 10⁻⁶ M PROG or DEX to the growth medium of cells transfected with androgen responsive 0·4 kb fragment resulted in approximately a 4- and a 5-fold increase in CAT activity in the cell homogenates (Fig. 2A). The CAT activity induced by the same concentration of DHT was 3- to 4-fold greater than that with PROG and DEX (P<0·05).

Previous studies have demonstrated the presence of a functional ARE at position −208 (AREp) in the MSVSP99 gene promoter (Brochard et al. 1997). To determine whether steroids exert an effect through this ARE independently of other enhancer sequences, it was cloned in front of the heterologous Tk promoter-CAT construct (AREpTkCAT). The addition of 10⁻⁶ M DHT, DEX or PROG to the growth medium of cells transfected with the AREpTkCAT construct resulted in a stimulation of the Tk promoter activity. CAT activity increased in a similar manner with the three hormones used (Fig. 2B). This effect was not observed with the control TkCAT vector (pBLCAT2). In comparable transfection experiments, the MSVSP99 hormone-responsive elements showed a greater induction by androgens than did AREpTkCAT elements.

Androgen induction of the MSVSP99 gene requires the presence of both ARE and non-ARE elements

Previous studies have shown that three different cis-acting DNA elements bind the AR: two putative AREs occurring at positions −361 (AREd) and −208 (AREp) and an AR-binding region (ARBR) located between positions −317 and −293 (Brochard et al. 1997) (Fig. 3A). Point-directed mutations of either AREd or ARBR did not affect the androgenic induction of the p0·4CAT construct, whereas mutations of AREp dramatically decreased the capacity of p0·4CAT to respond to androgens (Brochard et al. 1997). In order to delineate the minimum promoter fragment able to mediate an androgenic induction, the p0·3CAT construct deleted for both AREd and ARBR was obtained (Fig. 3A). The resulting fragment lost androgen

Figure 2. Comparison of androgen, glucocorticoid and PROG-dependent enhancer activities of the minimal MSVSP99 promoter (A) and AREpTkCAT constructs (B). The CV1 cells were cotransfected with 20 µg 0·4 kb MSVSP99 fragment or 10 µg AREpTkCAT construct and 2·5 µg human AR, or 2·5 µg human GR or 2·5 µg rabbit PR. Cells were grown (60 h) in the absence or presence of 10⁻⁶ M DHT, DEX or PROG. CAT activity is expressed as fold increase over basal level. Values are means ± s.d. (n=5).

Figure 3. (A) Schematic representation of the structure of the MSVSP99 promoter. (B) Androgen-dependent transcriptional activities of the MSVSP99 promoter. The CV1 cells were cotransfected with wild-type MSVSP99 promoter (−387/+16), or deleted MSVSP99 promoter (−329/+13: p0·329 and −260/+16: p0·3) or mutated promoter containing a substitution in the AREp sequence (p0·4AREpm: Brochard et al. 1997) and human AR. Cells were grown (60 h) in the absence or presence of 10⁻⁶ M DHT. CAT activity is expressed as fold increase over basal level. Values are means ± s.d. (n=6).
sensitivity suggesting that cis DNA sequences located in this region are necessary for hormonal response (Fig. 3B) in cooperation with AREp. In an attempt to localize these cis DNA elements, a subfragment of p0-4CAT extending from nucleotide −329 to +16 was generated (p0-329CAT construct). The absence of the sequence −387/−329 abolished the androgenic responsiveness of the MSVSP99 promoter, indicating that sequences distinct from AREd are involved in the promoter transactivation in cooperation with AREp.

Characterization of far upstream MSVSP99 sequences that contain negative regulatory elements

A 435 bp fragment (−823 to −388) contains a putative negative regulator

Previously, we found that maximal androgen induction of the MSVSP99 gene occurs after deletion of the sequences upstream from the −387 position (Brochard et al. 1997). Transfections of plasmids which contain the MSVSP99 region extending from nucleotides −2365 to +16 fused to the CAT reporter gene (p2-4CAT) showed detectable androgen-dependent transcriptional activity. In the presence of 10^{-6} M DHT, CAT activity increased about 4-fold over the level of basal activity (Brochard et al. 1997). In order to identify an extinguisher region, subfragments were analyzed. As shown in Fig. 4, deletion of the 5′-flanking region between nucleotides −824 and −387 (p2-4ACAT) resulted in a strong increase in the transcriptional activation by androgen. After 60 h exposure of cells to 10^{-6} M DHT, CAT activity was increased about 7-fold compared with that measured with p2-4CAT. As the basal transcription activity of the −2365/+16 promoter fragment remains weak even when deleted for the −823/−388 region, we could not determine whether the silencing effect could result from interactions involving the AR or from a decrease of the basal transcription activity. With that aim, the transcriptional extinguisher activity of the −823 to −388 fragment was assessed on the heterologous Tk promoter placed under the control of the ARE sequence of the proximal MVDP promoter (Fabre et al. 1994) either in the presence or absence of 10^{-8} M DHT. The MVDP AREp sequence was chosen because of its strong androgen-dependent enhancer activity. As shown on Fig. 5A, the Tk promoter activity is strongly reduced (5-fold) in the p-800/-400AREmpwdfTkCAT construct compared with that of the AREmpwdfTkCAT construct in the absence of androgens. Interestingly, addition of DHT 10^{-8} M to transfected cells shows a similar fold induction (5-fold) of the transcriptional activity of both constructs (Fig. 5B), thus suggesting that the silencing effect exerted by the −823/−388 fragment on the promoter activity is independent of the presence of AR but rather involves interactions with the basal transcription machinery.

The inverted complementary strand contains a functional promoter

Sequences analysis of the −823 to −388 region shows two potential binding sites for transcription factor IID (TFIID) located at positions −745 and −792. To determine if the minus strand contains elements that could initiate transcription, the XhoI/XbaI fragment (−825 to −388) was cloned in the opposite orientation into a promoterless pBLCAT3 vector (p0-43iCAT). As shown in Fig. 6, the p0-43iCAT construct transfected into CVI cells resulted in strong induction of CAT enzyme activity (54 ± 3.5% acetylation) whereas cells transfected with the pBLCAT3 vector did not show enzymatic activity (data not shown), thus demonstrating the presence of a promoting activity carried by the −823/−388 fragment.

Transcription start site and sequence of the reverse promoter

The pattern of transcriptional initiation sites for the reverse promoter was determined by primer extension experiments. The p0-43iCAT construct was transfected into CVI cells and total cytoplasmic RNA was isolated and analyzed by primer extension assay using a synthetic oligonucleotide complementary to positions 29–61 of the CAT DNA sequence. The products of the reaction were analyzed on
polyacrylamide gel electrophoresis (Fig. 7). A major site of transcription initiation was observed on the T residue located at position −639 with respect to the cap site for the MSVSP99 promoter. This start nucleotide corresponds to an A on the transcribed strand (Fig. 7). Sequences upstream from the cap site of the reverse promoter were examined for the presence of elements specific to gene promoters. Neither TATA nor CCAAT box elements are located in close proximity to the site of transcription initiation, but an Inr-like element 5′-ATATCTT-3′ is found at position -637. Inr elements showing the consensus sequence 5′-YYANWYY-3′ have been described as characteristic of TATA-less promoters (Roeder 1996).

To examine whether the fragment carrying the reverse promoter acts as the cis repressor of the MSVSP99 gene promoter activity, we generated the p0·362CAT construct deleted for nucleotide −824/−633. It was cotransfected in CV1 cells with pSV-ARo in the presence of 10⁻⁸ M DHT. As shown in Fig. 8, deletion of the inverted promoter is sufficient to induce a strong increase in androgen responsiveness.

![Figure 5](image_url) Figure 5. Analysis of the silencing effect of the MSVSP99 −823/−388 fragment on the heterologous Tk promoter placed under the control of the MVDP gene proximal to ARE (Fabre et al. 1994). The CV1 cells were cotransfected with 10 µg AREpmvdptkCAT construct or 10 µg −823/−388AREpmvdptkCAT vector, and 2·5 µg human AR. (A) Effect on the basal transcription activity. Cells were grown 60 h in absence or presence of 10⁻⁸ M DHT. CAT activity is expressed relative to the AREpmvdptkCAT construct basal activity used taken as 100%. (B) Fold induction. Values are mean ± s.d. (n=5).

![Figure 6](image_url) Figure 6. Transcriptional activity of the −824/−388 genomic fragment. The −824/−388 fragment was cloned in reverse orientation in the pBLCAT3 vector (p0·43iCAT construct). Twenty micrograms of each construct were transfected in CV1 cells and grown for 60 h in the absence of DHT. CAT activity is shown as percent conversion of chloramphenicol to acetylated chloramphenicol. Values are means ± s.d. (n=5).
Localization of the transcription start site of the inverted promoter. The experiment was carried out as described in Materials and Methods. A 33-mer oligonucleotide, complementary to nucleotides 29–61 of the CAT cDNA in the pBLCAT3 vector, was used as primer to initiate both an RT reaction on CV1 cell total RNA and a sequencing reaction using the p0-43iCAT construct as template. Ext: primer extension reaction. Lad. sequence: ladder sequence corresponding to the p0-43iCAT sequence. The arrow on the right indicates the transcription start site that corresponds to the T (A on the transcribed strand) located at −639 upstream from the transcription start site of the MSVSP99 gene.

Influence of the inverted promoter on the androgen-dependent transcriptional activity of the MSVSP99 gene. The p0-632CAT construct is deleted for the inverted promoter. The CV1 cells were cotransfected with 20 µg MSVSP99 promoter constructs and 2·5 µg human AR. Cells were grown 60 h in the absence or presence of 10⁻⁶ M DHT. CAT activity is expressed as fold increase over basal level. Values are means ± S.D. (n=5). *Significantly different (P<0·05) from basal activity.
To determine the presence of a gene on the complementary strand, all 5'-flanking region available upstream from nucleotide -824 present in a genomic clone (Simon et al. 1995) was sequenced (Genbank accession number: AF 055466). Examination of the sequence downstream from the cap site did not reveal any significant homologies with cDNA sequences referenced in the EMBL databank. Nevertheless, we looked for the presence of mRNA transcribed from the reverse promoter in the mouse seminal vesicle. First we investigated the target sequence to amplify by using as a template mRNA obtained from CV1 cells transfected with the 7·4 kb genomic fragment containing the whole MSVSP99 gene and 5'-flanking sequences. As shown in Fig. 9, forward primers 5'-AGTATATA
TACATACACAAC-3' (position -651/-670 with respect to the MSVSP99 gene cap site) and reverse primers 5'-AGCAGTCGACTGGGGACTGGA
AGG-3' (position -829/-806) yielded the predicted product of 178 bp which was always faint in several independent experiments. PCR performed without RT yielded no product, indicating no plasmid DNA contamination. We examined whether the reverse transcripts detected in transiently transfected CV1 cells are present in mouse tissues. In all tissues examined, including seminal vesicles from immature (data not shown) and mature animals (Fig. 8), reverse transcripts were undetected.

DNA sequence analysis revealed that the reverse promoter is located near the 3'-untranslated region of a highly repetitive L1 element. The sequence between positions -1300 to -2699 exhibits a 93% homology with the L1MdA13 line (Shehee et al. 1987). Although greater than 90% of them are truncated, full-length long interspersed element-1 (LINE-1) elements are approximately 6–8 kb in length and contain 5'-untranslated regions with internal transcription regulatory elements, two open reading frames and a 3'-untranslated region that terminates in a polyA tail (Rikke et al. 1995). The sequenced region contains the end of ORF2 and the 3'-untranslated region of the LINE until the polyadenylation signal (nucleotide 6083 to 7482 of the full L1 element). The untranslated region of the MSVSP99 gene (+2232 to +2837) is 73% identical to a LINE 1 subfamily element called L1MA2.

**DISCUSSION**

In the absence of an available seminal vesicle-derived cell line, analysis of androgen regulation of MSVSP99 promoter is performed in CV1 cells. The results described here extend our own previous work (Brochard et al. 1997). In the present study we characterized more precisely the proximal enhancer found in the -387 to +16 region and described a
new regulatory element, spanning position −824 to −632, that strongly represses enhancer activity.

Using in vitro protein–DNA interaction assays and transient transfection experiments, at least two separate active regions (region I: −387/−260 and region II: −260/+16) were identified within the androgen-dependent enhancer. In each of the two regions, at least one AR-binding site which resembles the core consensus ARE (Roche et al. 1992) was identified (Brochard et al. 1997). The observation of different AR-binding sites suggested that multiple domains might be required for function. Mutation in the AREp abolished induced expression, indicating that region I is individually functionally inactive. However, 5′-deletion of the MSVSP99-flanking DNA, which eliminates the first regulatory region, results in a loss in hormone inducibility. This indicates that AREp must cooperate with region I for androgen induction of the MSVSP99 gene. Such functional synergism involving multiple ARE-like sequences has already been described in the complex enhancer elements of the Slp, 20 kDa protein and prostate-specific antigen (PSA) genes (Adler et al. 1992, Ho et al. 1993, Cleutjens et al. 1996, Schuur et al. 1996). However, AREd or ARBR alone do not influence the induced expression of the MSVSP99 promoter (Brochard et al. 1997), and the lack of response to androgenic stimulation of the 0·329 fragment (Fig. 3) suggests that cis DNA element(s) distinct from AREd might act in synergy with AREp to potentiate the transcriptional activity of the MSVSP99 promoter in response to androgen. Then, the study demonstrates that the two regulatory regions in the MSVSP99 gene act together as components of a complex androgen response unit involving both AREp and non-ARE sequences.

In transient transfection assays, site-directed mutation in AREp of the MSVSP99 gene abolished androgen inducibility (Brochard et al. 1997). When cloned in front of the Tk promoter, AREp responds to AR, GR and PR in a similar manner, indicating that this sequence does not dictate hormonal specificity. In the same conditions, MSVSP99 gene (p0-4CAT) expression was more effectively induced by androgens than by glucocorticoids and PROG. Moreover, the AREp by itself exerts only a weak activation of the Tk promoter (about 3- to 4-fold) compared with that seen with the MSVSP99 promoter (12-fold). Thus, the preferential androgen regulation of the MSVSP99 gene involves the participation of different cis-acting DNA elements. A similar situation has been described for the androgen-regulated probasin gene: AR, GR and PR are able to stimulate probasin promoter activity, although AR is markedly more potent than GR and PR in this respect (Rennie et al. 1993). There is no evidence that sequence differences distinguish AREs from glucocorticoid response elements or progestin response elements in vivo. The only example of a simple ARE specifically recognized by the AR is the probasin ARE2 (Claessens et al. 1996). The complex promoters of the Slp and 20 kDa protein genes show an AR but no GR response in transfection assays. However, this stringent hormonal control does not reside in the AR-binding site itself but in adjacent sequences that include multiple steroid response elements together with recognition sequences for other transcription factors (Adler et al. 1991, 1992, Ho et al. 1993). This is in contrast to results on the PSA promoter showing that the apparent AR specificity of this promoter in LNCaP cells is due to the absence of other members of the steroid receptor family in this cell line (Cleutjens et al. 1996). Thus, it appears that the specific transcriptional response to androgens may result from combined actions of different elements including intrinsic properties of AREs, and cell type-dependent interaction with other factors via regions distinct from the AR-binding domain. Our data suggest that, in addition to AR, other factors are involved in specific AR-regulated MSVSP99 promoter activation. To determine which accessory elements are required for androgen-dependent transcription, we have examined binding of CV1 crude cellular extracts to the enhancer. In vitro footprinting assays failed to indicate significant protein binding (L Morel, unpublished data). Each of the AR-binding sites seen in the MSVSP99 promoter binds to the AR in vitro, but with apparent weak affinity. Low receptor-binding activity has also been described for the complex androgen-dependent enhancers of the Slp and 20 kDa protein genes (Adler et al. 1991, Ho et al. 1993). It has been suggested that the absence of strong individual response elements within a complex enhancer may ensure specificity of the hormonal response.

The transient expression studies reported here demonstrate that sequences spanning nucleotide position −824 to −388 contain elements that appear to repress androgen-dependent transcriptional activity of the MSVSP99 gene. Functional analysis revealed that this region contains a second promoter which is capable of specifying transcription on the opposite strand with respect to that of the MSVSP99 promoter itself. Analysis of RNA isolated from the CV1 cells has shown that there is a major site for initiation located at position −639 with respect to the cap site for the MSVSP99 promoter. However, reverse promoter activity was...
not detected in mouse tissues including seminal vesicles. It is possible that the reverse promoter is active only in certain developmental or physiological conditions. Alternatively, a neutralizing element, present in the native MSVSP99 gene, can inactivate the reverse promoter so that the MSVSP99 gene can be activated by androgens in its natural context. It is also possible that this activity is of no biological significance and due to spurious transcription. The reverse promoter lacks TATA and CCAAT boxes but contains an Inr-like element at position −637. All Inrs so far described always overlap the cap site which is located on the third nucleotide of the Inr element (Roeder 1996). The transcription start site of the inverted promoter is in agreement with the positioning of the Inr-like element.

The mechanisms underlying the repression of gene transcription are less well understood than those governing activation. At least two distinct mechanisms have been recognized in causing transcriptional suppression. Negative regulation of gene transcription appears to be carried out either by mechanisms involving protein–protein interaction or alternatively by mechanisms dependent on protein–DNA interaction (Drouin 1993). In the first case, transcriptional repression involves a physical interaction between two proteins in a DNA-independent manner and probably the formation of a transcriptionally inactive complex. An example of this mechanism is the mutual inhibition of transcriptional activity by the GR and c-jun (Jonat et al. 1990, Yang-Yeng et al. 1990). For the latter case, the negative regulatory region appears to block the activity of constitutive or inducible, adjacent or overlapping DNA-binding sites (Akerblom et al. 1988, Biggin & Tjian 1989). How does the MSVSP99 repressor work? The −824/−388 fragment (437 bp) displays no sequence homology with the G-rich elements of the catalase and AR genes that have been described as acting as inhibitors of expression (Sato et al. 1992, Kumar et al. 1994). The data presented here show that the 437 bp fragment is able to repress the androgen-stimulated activity of the MSVSP99 promoter. However, the weak constitutive activity of this promoter prevented us from determining whether this repression results from interference with the AR or the basic transcription machinery. The important repressive effect of the −824/−388 fragment on the basal transcription of an ARE-driven Tk promoter and the capacity of the Tk promoter to be stimulated by androgens suggest that the −824/−388 fragment interferes with the basic transcription machinery.

MSVSP99 belongs to the SVS protein family (rat SVS II, IV, V, VI). Members of this family present a similar organization: the first exon encodes the signal peptide and N-terminal part of the secreted protein; the C-terminal part is encoded by the second exon, and the 3′-untranslated region is mainly represented by the last exon (Lundwall & Lazure 1995, Simon et al. 1995, Lundwall 1996a). A similar organization has been described for the human semenogelin genes, the mouse SVS II gene and the guinea-pig gene that yields SVP1, SVP3 and SVP4 (Lundwall & Lazure 1995, Hagstrom et al. 1996, Lundwall 1996a). Recently it was suggested that the semenogelin and SVS genes constitute the REST (rapidly evolving seminal vesicle transcribed) gene family, as most members seem to encode substrates for transglutaminase (Lundwall & Lazure 1995, Lundwall 1996a). A comparison of the semenogelin I and semenogelin II genes suggests that they evolved by duplication, probably by a mechanism involving recombination between L1 elements (Lundwall 1996b). The semenogelin locus contains several types of repetitive DNA sequences. Most abundant are the LINEs located in the intergenic region and in the DNA flanking the semenogelin genes (Lundwall 1996b). LINE-1 elements are interspersed repetitive DNA present in mammalian genomes (100 000 copies per haploid genome in mice). Similarly, the 5′-flanking region of the mouse semenoclotin, the murine equivalent of rat SVS II, contains L1 elements (Lundwall 1995; Genbank accession number: X91270). Results reported here showed that the MSVSP99 gene is surrounded by LINE-1 elements. Repeated elements, such as Alu sequences, have been involved in the formation and evolution of multigene families including alpha-1 acid glycoprotein (Merritt et al. 1990), glycoporphin (Onda et al. 1993) and haptoglobin (Erickson et al. 1992). Data from MSVSP99 and semenoclotin genes suggest that the members of the SVS family have arisen from a common ancestor by a gene duplication mechanism involving homologous recombination between LINE elements.

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