Tissue-Specific Expression and Androgen Regulation of Different Genes Encoding Rat Prostatic 22-Kilodalton Glycoproteins Homologous to Human and Rat Cystatin

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22-Kilodalton (kDa) protein cDNA clones were isolated from a rat prostatic library. Nucleotide sequence analysis revealed three different cDNA sequences encoding two somewhat different open reading frames of 176 amino acids. The N-terminal 24 amino acids of these sequences show the typical characteristics of signal peptides of secretory proteins. The C-terminal end of the derived protein sequences displays sequence similarity to a number of cysteine proteinase inhibitors, called cystatins, suggesting a common physiological function. Upon Northern blotting with a labeled cDNA fragment, three different 22-kDa protein mRNAs, *i.e.* 950 nucleotides (nt), 920 nt and 860 nt, could be detected in the rat ventral prostate and the lacrymal gland. In both tissues these messengers were regulated by androgens showing the most rapid androgen response for the 950 nt mRNA form. Administration of cycloheximide nearly completely abolished the observed androgen effect suggesting that a short-living protein is required for the full induction of the 22-kDa protein genes. Hybridization experiments with specific oligonucleotides which distinguish between the mRNAs encoding both 22-kDa protein variants indicate that one protein form is less androgen dependent in the ventral prostate and not expressed in the lacrymal gland. (Molecular Endocrinology 4: 657–667, 1990)

INTRODUCTION

The rat ventral prostate is commonly used as a model system for the study of the mechanism of action of androgens. It synthesizes and secretes a number of androgen-regulated proteins including prostatic binding protein (PBP) (1, 2), a kallikrein-related protease (3, 4), a spermine-binding protein (5), the proline-rich polypeptides (6, 7), and a 22-kilodalton (kDa) glycoprotein (8, 9).

The 22-kDa glycoprotein was first described by Parker *et al.* (8) as androgen-regulated prostatic protein-α and further characterized as an oligomeric glycoprotein (9, 10). Recent immunochemical studies indicate that most of the protein is located in the intraluminal secretion from the epithelial cells, although the observation of some staining within the stroma suggests that the protein may be transported to the latter (11). Studies with cloned cDNA have shown that the synthesis of this protein is regulated by androgens at the mRNA level (8, 9), but no data were available on the primary structure of the encoded protein or its mRNA. In the present communication we report on the isolation and sequence determination of different cDNAs corresponding to full-size mRNA encoding the 22-kDa glycoprotein. Examination of the encoded amino acid sequence reveals the existence of two variants of this protein and a striking homology with the amino acid sequence of the human and rat cysteine proteinase inhibitor (12–14).

In addition, androgen-regulated expression of the 22-kDa protein could be demonstrated in the rat lacrymal gland using immunoblotting and RNA hybridization.

RESULTS

Purification of 22-kDa Glycoprotein and Preparation of an Antiserum

The 22-kDa protein was purified on a small scale from rat prostatic cytosol by successive gel filtration on

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Sephadex G-100 and ion exchange chromatography on diethylaminoethyl (DEAE)-cellulose and carboxymethyl (CM)-cellulose, monitoring the purification by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. On the initial gel filtration column the 22-kDa protein (probably as dimer or oligomer) comigrates with a large peak consisting mainly of PBP (M_r 50 kDa). After passage through a DEAE-cellulose column, on which PBP is retained, the 22-kDa protein is the only major protein in the flowthrough of this column. For final purification these flowthrough fractions were applied on a CM-cellulose column, from which the 22-kDa protein was eluted by means of a 0 to 0.5 N NaCl gradient.

The purified 22-kDa glycoprotein was used for the immunization of rabbits. As shown by the Ouchterlony technique both immunized animals reacted with the formation of antibodies. The specificity of these polyclonal antibodies was further demonstrated by immunoblots after SDS gel electrophoresis (see further).

Isolation of cDNAs Encoding Different Forms of a 22-KDa Glycoprotein

In a first experiment, the polyclonal antibody raised against the 22-kDa glycoprotein was used to screen a λ gt11 expression library of rat ventral prostate cDNA. Seven phages producing recombinant protein (designated 22K1imm, 22K2imm *etc.*) were isolated after screening 10^6 independent plaques. The positive phages contained relatively small inserts ranging between 150–260 base pairs (bp), as estimated by agarose gel electrophoresis of an *Eco*RI digest. Furthermore, these inserts were found to overlap, since they all hybridized with the labeled insert from clone 22K4imm upon Southern blotting. Northern blot analysis of total ventral prostate RNA revealed specific hybridization to messages of approximately 950 nucleotides (nt), 920 nt, and 860 nt. Therefore, it was concluded that our clones carried an incomplete cDNA fragment possibly derived from internal *Eco*RI cleavage occurring during construction of the library. This was confirmed by sequence analysis. Indeed, all clones ended at the internal *Eco*RI endonuclease site (see Fig. 1, position 368) and contained sequences located upstream from this site. Interestingly, two distinct nucleotide sequences were obtained differing at a few randomly dispersed positions. In order to isolate full-length cDNAs, a commercial prostatic cDNA library, constructed into λ gt10, was screened with the labeled 22K4imm cDNA insert. Twenty positive phages were isolated and further purified. Unfortunately, all phages contained cDNA inserts comparable in size to the ones isolated from the λ gt11 library. Therefore, a third library was constructed into an oligo(dT)-tailed pUC9 plasmid as described by Heidecker and Messing (15). Since this construction does not require *Eco*RI digestion, the problem of internal cDNA cleavage could be overcome. After screening 10^3 recombinant clones, 21 positive clones were isolated containing inserts of about 1 kilobase (kb), except for clone p22K16 which contains a somewhat smaller insert. Plasmid sequencing was performed on four different clones. Three different cDNA sequences were found encoding two different open reading frames of 176 amino acids (Fig. 1). Clones p22K16 and p22K20 encode an identical open reading frame, but differ in the 3′-untranslated region. Indeed, the 3′-untranslated region of clone p22K16 contains only 239 nt instead of 244 nt and its poly(A) tail is markedly shorter. Clone p22K15, on the other hand, encodes a somewhat different open reading frame of similar length and its 5′-untranslated region contains only 51 nt instead of 53 nt. Moreover, the 3′-untranslated region of this clone contains two polyadenylation consensus sequences located 69 and 25 nt upstream from the poly(A) tail. In contrast, only the latter signal is found in clones p22K16 and p22K20.

Primer Extension Assay

In order to determine whether our clones carried full-length cDNA, we performed primer extension with a 20-mer oligonucleotide complementary to the N-terminal part of the leader sequence (5′-double underlined sequence in Fig. 1). As shown in Fig. 2, two prominent extension products differing in length by only one nt are found for clones p22K16 and p22K20. The longest extension product, 62 nt, is equal in length to the number of base pairs from the 5′-end of the primer to the 5′-end of the cDNA clones, confirming that our cDNAs correspond to full-length mRNA.

Primary Structure of the Encoded Protein

The amino-terminal part of both open reading frames contains many hydrophobic residues characteristic for the signal peptide of most secretory proteins. Although the N-terminus of the mature proteins encoded by our clones has not been directly determined, a computer prediction based on the (−3,−1) rule (16) and weight matrix analysis (17) places the tentative cleavage site between Ala^{24} and Thr^{25} (Fig. 3). Another possibility is cleavage between Ala^{26} and Lys^{27} which results in a slightly lower score. As shown in Table 1, there is good agreement between the cDNA-derived amino acid composition of both mature protein variants and that published by Chamberlin *et al.* (9). The same authors have demonstrated that the 22-kDa protein is a glycoprotein. A potential N-glycosylation site (Asn-X-Ser/Thr) is located at positions +47 and at position −5 (Asn-Phe-Ser) in each clone. Computer epitope scanning performed using the method of Hopp and Woods (18) identified a single possible epitope site between amino acid residues +64 and +74, a region which is highly homologous among our clones. Since this region is located upstream from the *Eco*RI endonuclease site, this would explain the failure to isolate cDNA clones containing sequences downstream from this site upon screening the λ gt11 expression library.

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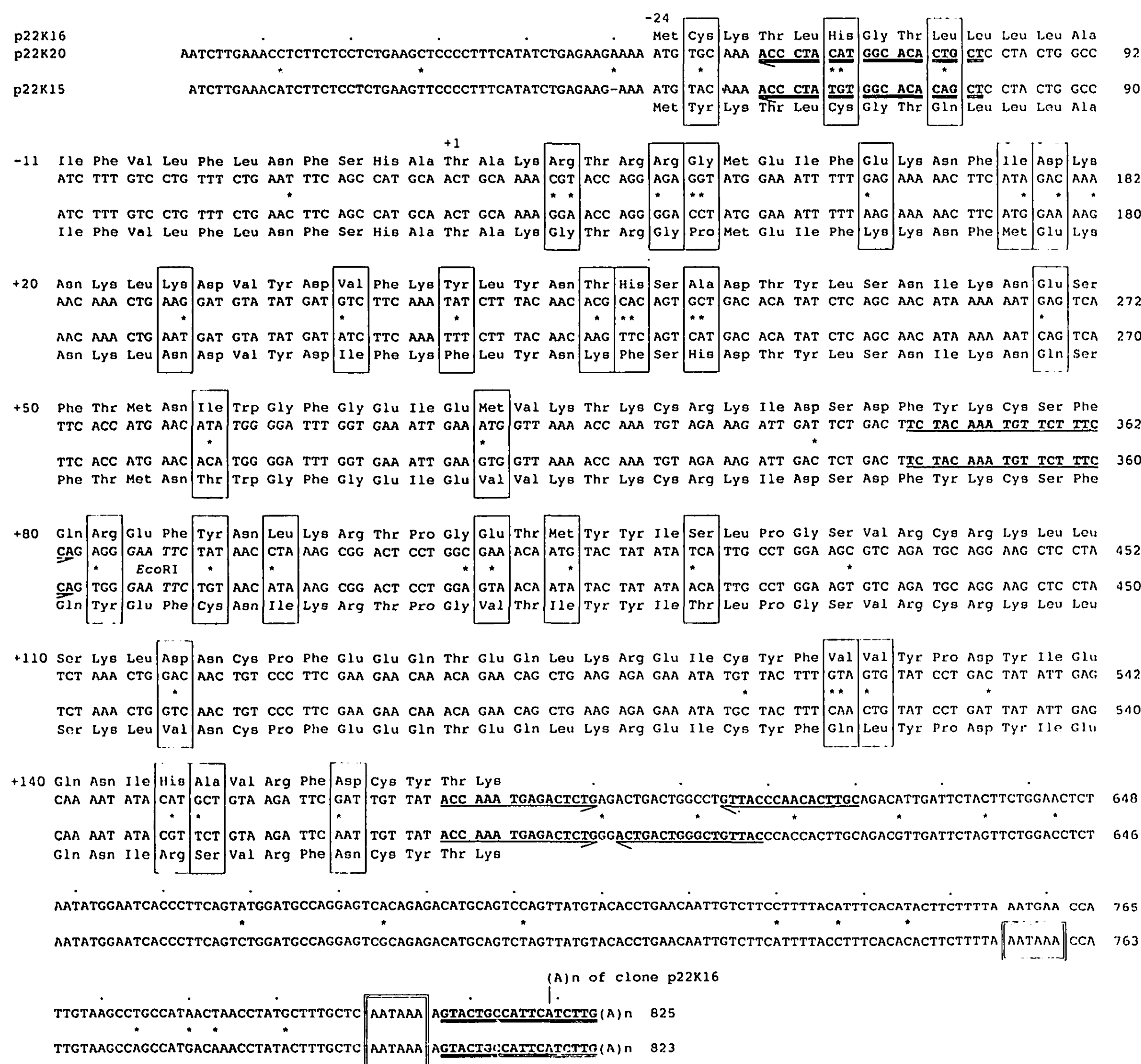

Fig. 1. Comparison of the Nucleotide Sequences and Derived Amino Acid Sequences of the Different 22-kDa Proteins
Differences between the two cDNA sequences are marked by an *asterisk* and the resulting amino acid substitutions are shown *boxed*. The internal *Eco*RI endonuclease cleavage site is indicated and printed in *italic mode*. The oligonucleotides used for primer extension and hybridization assays are *double underlined*. The oligonucleotides used for sequencing analysis are shown *underlined* and the direction of sequencing is indicated. The polyadenylation consensus sequences are *double boxed*.

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Sequence Homology between the 22-kDa Glycoproteins and the Human and Rat Cysteine Proteinase Inhibitor

Protein and nucleotide databases were searched for homologies to the 22-kDa protein cDNAs and the encoded proteins. At the amino acid level a striking homology was found at the carboxy-terminal end of both 22-kDa proteins and a number of cysteine proteinase inhibitors, called cystatins. Figure 4 illustrates the sequence similarity of the 22-kDa protein p22K16 and p22K20 to human and rat salivary cystatin S (12, 13) and to human placenta cystatin C (14). A number of amino acid residues have been conserved. Most strikingly this is the case for all cysteine residues within this region which align completely and for an LDNCPF sequence which is present at an identical position in both the 22-kDa protein and the human placenta cystatin C. The observed homology will extend further when a number of conservative amino acid exchanges are taken into consideration.

The 22-kDa Glycoprotein is Expressed in the Rat Ventral Prostate and in the Lacrymal Gland

We next analyzed the tissue-specific distribution of the 22-kDa protein mRNA. Total RNA was prepared from a number of rat tissues including spleen, brain, thymus,

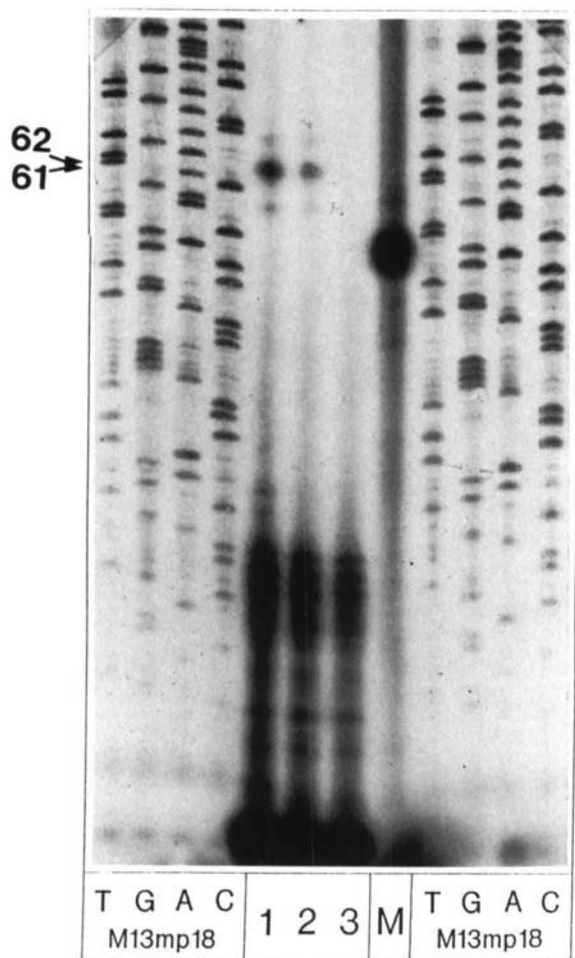

Fig. 2. Primer Extension Assay

The primer extension assay was performed as described in *Materials and Methods*. The sequence ladders of M13mp18 and labeled restriction fragments (lane M) were used to provide molecular weight markers. Different amounts of poly(A)$^+$ RNA were added to the reaction mixture: lane 1, 500 ng; lane 2, 10 ng. Lane 3 represents the labeled primer only. The two major extension products are indicated.

intestine, lung, ovaria, testis, preputial gland, submandibular gland, lacrymal gland, ventral prostate, and liver. Whereas the 22-kDa protein mRNA remained undetectable in most tissues, specific hybridization to different mRNA sizes, *i.e.* 950 nt, 920 nt and 860 nt, was obtained in the ventral prostate and in the lacrymal gland (Fig. 5).

Influence of Castration and Androgen Treatment on the Expression of the 22-kDa Glycoprotein

The effect of castration on the 22-kDa protein mRNA levels in the ventral prostate was studied in 3-month-old rats which had been castrated for 2, 3, or 7 days. Changes in mRNA concentrations were evaluated by dot-blot analysis using a labeled *Eco*RI fragment of clone p22K20 and subsequent densitometric scanning of the autoradiograms. Castration resulted in a marked and rapid decrease of the 22-kDa protein mRNA level. Indeed, the amount of 22-kDa protein mRNA (expressed per milligram RNA) dropped to approximately 13% and 3.4% of the level found in intact males at 2 and 3 days after castration, respectively. One week after castration 22-kDa protein mRNA was hardly detectable (results not shown).

Rapid androgen-mediated effects on the expression of 22-kDa protein mRNA were studied in 2-day castrated animals, since changes in tissue composition (*i.e.* the stroma cell *vs.* epithelial cell ratio) are still limited at that time. Total RNA was extracted from the ventral prostate and the lacrymal gland of 2-day castrated rats and similar animals which had been injected sc with androgens 3 or 6 h before killing. As shown in Fig. 6, castration decreased the relative level of 22-kDa protein mRNA to 13% in the ventral prostate and 20% in the lacrymal gland. Administration of androgen to these rats resulted in a gradual increase of the concentration of 22-kDa protein mRNA in both tissues to 38% (ventral prostate) and 45% (lacrymal gland) of the level measured in intact animals. On Northern blots (Figs. 5 and 7), the different mRNA forms reacted differently to castration and androgen treatment. Indeed, unlike the 920 nt and 860 nt mRNA forms, the largest mRNA (950 nt) could no longer be detected in 2-day castrated animals but reappeared after 3 and 6 h of androgen supplementation in the ventral prostate and after 6 h of androgen treatment in the lacrymal gland.

The influence of castration could also be demonstrated at the protein level. As shown in Fig. 8, castration resulted in a gradual decrease of the 22-kDa protein content. This phenomenon is much slower in the prostate gland, probably because considerable amounts of the secreted proteins are stored in the prostatic acini. Figure 8 also shows that several protein forms are recognized by the polyclonal antiserum in both tissues. Probably this reflects partial proteolytic degradation of the 22-kDa proteins; although differences in protein processing or tissue-specific expression of certain 22-kDa protein genes cannot be ruled out.

Effect of Cycloheximide (CHX)on the Androgen-Mediated Elevation of 22-kDa Glycoprotein mRNA Concentrations

To establish whether protein synthesis was required for the androgen stimulation of 22-kDa protein mRNA, the effect of androgens was also studied after administration of the protein synthesis inhibitor CHX. A single ip injection of the inhibitor (3 mg) was given to 2-day castrated animals 1 h before killing (control) or 1 h before 3 or 6 h of androgen treatment. In the ventral prostate CHX alone appeared to produce a slight transient increase of 22-kDa protein mRNA, but the androgen-induced stimulation of the latter was almost completely abolished (Figs. 6 and 7). A similar pattern was observed for the lacrymal gland; although a minor effect of androgens was still apparent in the presence of CHX.

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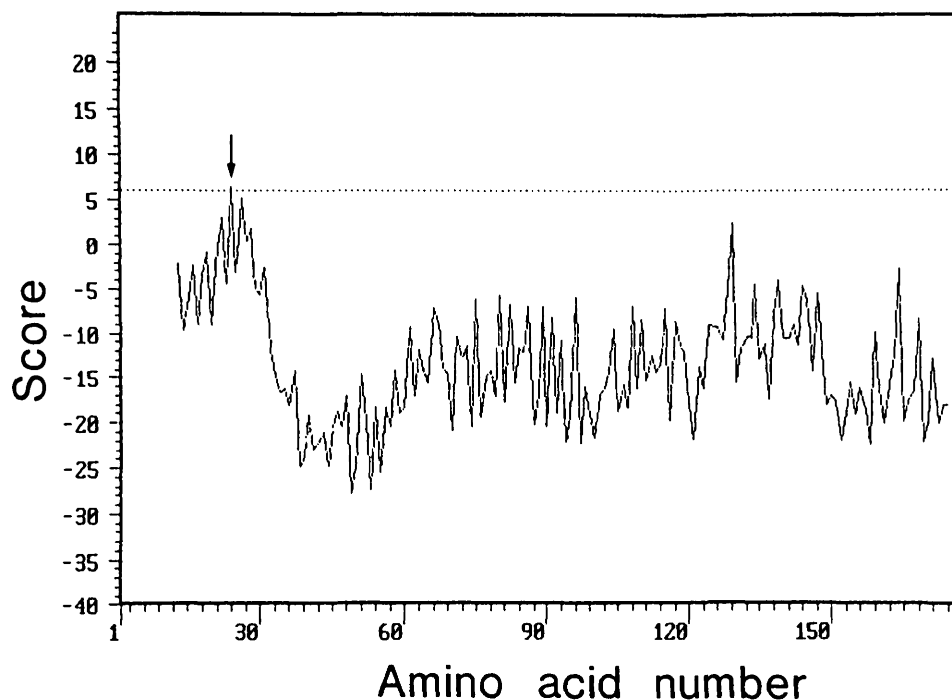

Fig. 3. Detection Curve for Eucaryotic Secretory Signal Sequences

The amino acid sequence deduced from p22K20 was analyzed in order to determine the cleavage site between the signal sequence and the mature protein making use of the algorithm described previously by von Heijne (17). The *arrow* indicates the most probable cleavage site.

Table 1. Comparison of the Amino Acid Content of 22-kDa Proteins

	(mol amino acid/mol protein)		
	22-kDa Protein[a]	p22K16 p22K20	p22K15
Asp	19.35	18	17
Thr	8.85	10	11
Ser	8.19	8	8
Glu	18.04	16	16
Pro	4.92	4	5
Cys	4.81	6	7
Gly	6.40	5	6
Ala	3.60	3	1
Val	7.22	7	7
Met	3.60	4	3
Ile	9.18	10	11
Leu	11.16	9	9
Tyr	11.16	12	10
Phe	12.13	14	13
His	1.96	2	1
Lys	14.42	16	17
Arg	7.54	10	8
Trp	n.d.	1	1

[a] The amino acid content of the mature proteins derived from the cloned mRNAs (p22K16/20, p22K15) was compared to the amino acid composition determined for a 22-kDa protein from the rat ventral prostate previously reported by Chamberlin *et al.* (9) and recalculated for a 152 amino acid protein.

When Northern blot analysis was performed on the same RNA preparations, the effect of androgens was most marked in both tissues for the largest mRNA form (950 nt), which did not reappear during the combined treatment. These results indicated that ongoing protein synthesis is essential for full induction of 22-kDa protein mRNAs by androgens.

Selective Hybridization Assays

As shown above two clearly different cDNA sequences (p22K15 and p22K20) and a third slightly different variant (p22K16) of the latter were isolated from the rat ventral prostate mRNA. By selective hybridization with specific oligonucleotide probes (Fig. 7) we tried to establish in how far these mRNA sequences contributed to the different forms of 22-kDa protein mRNA, observed on Northern blots of prostatic and lacrymal gland mRNA.

Northern blots of prostatic RNA hybridized with an *Eco*RI p22K20 cDNA fragment revealed the existence of at least three sizes of 22-kDa protein mRNA, corresponding to approximately 950 nt, 920 nt, and 860 nt (Figs. 5 and 7; lane A). The same three bands were detected in a similar relative proportion with the N-terminal oligo 16/20 (Fig. 7; lane B), which is common to clones p22K16 and p22K20. Consequently, this oligo does not recognize specifically one of these mRNA bands. For that reason, it could not be established whether the rapid androgen induction of the 950 nt

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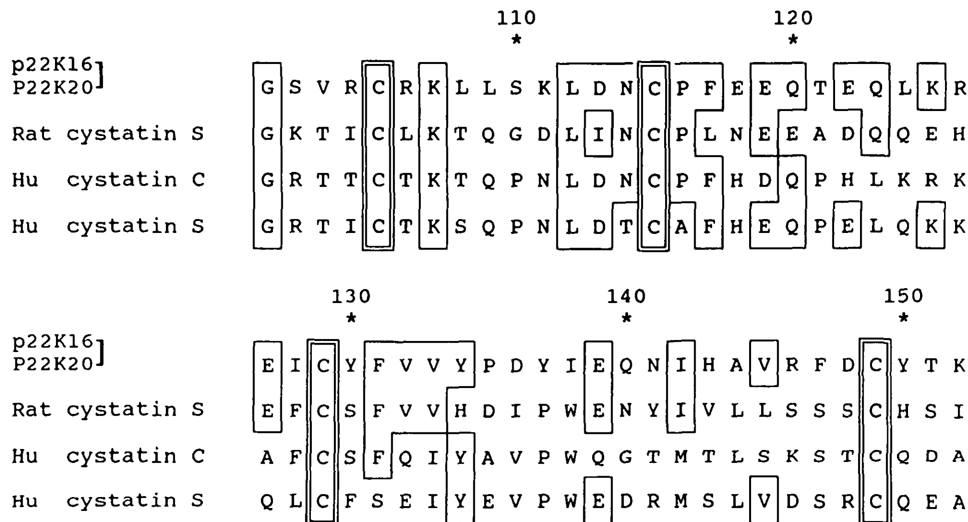

Fig. 4. Comparison of the Primary Amino Acid Sequences of 22-kDa Protein and Different Human and Rat Cystatins
The homologous regions between the human and rat salivary cystatin S, the human placenta cystatin C, and the predicted 22-kDa protein are shown. Matching amino acids are *boxed*. The cysteine amino acid residues are *double boxed*.

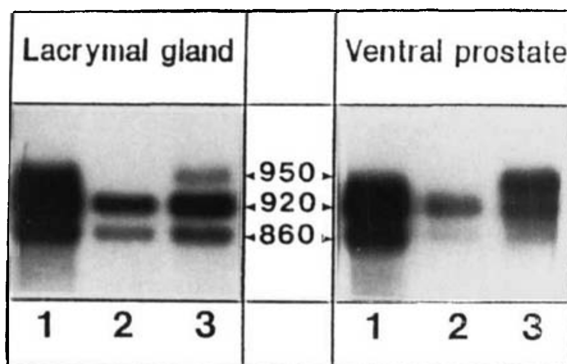

Fig. 5. Northern Blot Analysis of 22-kDa Protein mRNA in the Ventral Prostate and Lacrymal Gland
Total RNA (10 μg) was extracted from the ventral prostate and lacrymal gland of intact male rats (1), 2-day castrates (2), and 2-day castrates which were subsequently treated with androgens for 6 h. The RNA was separated by electrophoresis on a 1.7% agarose gel, blotted onto nylon membranes, and subsequently hybridized with a labeled *Eco*RI fragment of clone p22K20. The sizes of the 22-kDA protein mRNAs were estimated on the basis of the migration distances of both 18S and 28S ribosomal RNAs.

form represents the transcription of a specific androgen-dependent 22-kDa protein gene or whether it results from androgen-mediated changes in RNA processing. The N-terminal oligo 15, on the other hand, which is specific for clone p22K15 (Fig. 7; lane c), only hybridized to a 920 nt mRNA form. Moreover, far less pronounced changes in mRNA levels were observed after castration and androgen treatment when this oligo was used for hybridization. In the lacrymal gland, the cDNA fragment and oligo 16/20 again hybridized with the three mRNA forms and a clear pattern of androgen regulation was observed. Surprisingly however, no hybridization signal was detected with oligo 15 under the same hybridization conditions as used for ventral prostate RNA. Consequently, the corresponding gene is probably silent in the lacrymal gland.

Since the p22K16 cDNA clone has a much shorter poly(A) tail in addition to a deletion of five nucleotides at its 3′-end, we were interested to know whether it could represent the smallest mRNA form of 860 nt detected upon hybridization with the p22K20 cDNA fragment or oligo 16/20 in both tissues. Therefore, a third oligo (oligo 15/20) was synthesized which corresponds to the 3′-end of clones p22K15 and p22K20 (3′-double underlined sequences in Fig. 1) and which would not hybridize under the chosen conditions to messengers with the p22K16 sequence. The 860 nt mRNA form, however, was still detected with this oligo, indicating that at least part of the 860 nt mRNA is not identical to clone p22K16 (data not shown).

DISCUSSION

In the present communication we describe the isolation and sequencing of different cDNAs encoding the 22-kDa protein, one of the major androgen-regulated proteins from the rat ventral prostate. Using antibodies raised against the purified proteins incomplete cDNA inserts were isolated from a λ gtll expression library which could be used to isolate full-size cDNAs from a library constructed into an oligo(dt) tailed pUC9 plasmid. The corresponding mRNAs encode two different but largely homologous precursor proteins consisting of 152 amino acids starting with typical signal peptide sequences of 24 amino acids at their N-terminal end. The presence of a signal peptide, as we found in both the encoded protein forms, is not surprising. Indeed, recent immunohistological studies have provided good

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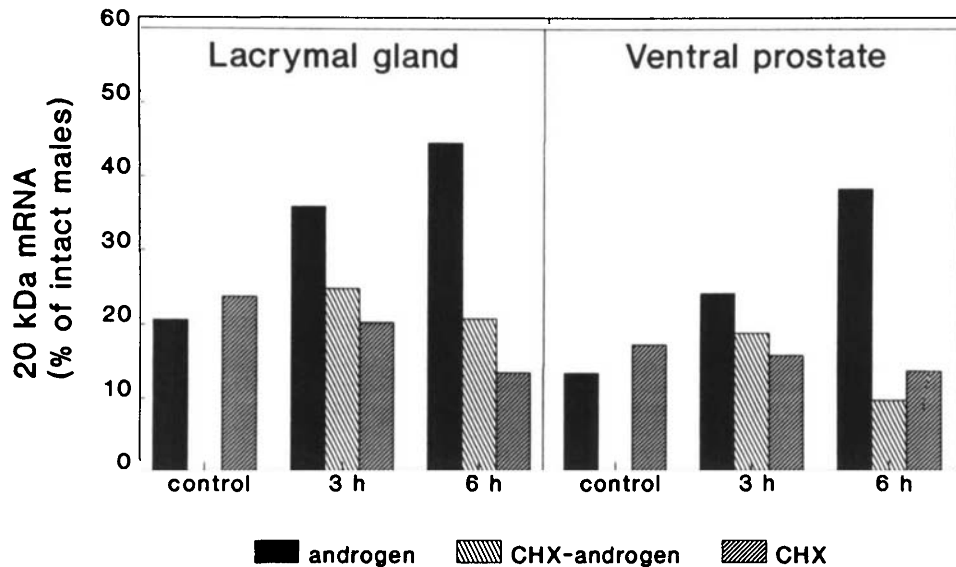

Fig. 6. Hormonal Regulation of mRNA Levels for the 22-kDa Protein in Both Ventral Prostate and Lacrymal Gland

Total RNA was prepared from ventral prostates and lacrymal glands of intact rats (five rats per sample) and from 2-day castrated animals which had been treated with androgens, CHX, or both. Changes in mRNA concentrations were evaluated by dot-blot analysis using a labeled *Eco*RI cDNA fragment of clone p22K20 and subsequent densitometric scanning of the autoradiograms. The mRNA levels (estimated per milligram RNA) are expressed as percentages of the level measured in intact male rats. The control lanes show the 22-kDa protein mRNA level found in 2-day castrates and in 2-day castrates treated with CHX for 1 h. The 3 h lanes show the 22-kDa protein mRNA levels in 2-day castrates treated for 3 h with androgens, with androgens (3 h) and CHX (4 h) or with CHX alone (4 h). The 6 h lanes show the 22-kDa protein mRNA concentrations measured in 2-day castrates treated with androgens (6 h), with androgens (6 h) and CHX (7 h), or with CHX alone (7 h).

evidence for the secretory nature of the 22-kDa protein and suggest that the previously reported cytoplasmatic and nuclear forms of this protein may be cell fractionation artefacts (11). After subtraction of the contribution of the signal peptide, there is a good agreement between the amino acid composition derived from the cDNA sequence and that reported for the purified protein by Chamberlin *et al.* (9), confirming the immunological identification of the cDNAs.

An interesting observation is the homology of the C-terminal end of the encoded proteins and that of cystatin, a cysteine proteinase inhibitor (12–14). Most strikingly in this respect is the conservation of four cysteine residues at identical positions from the C-terminal end of the protein. In cystatin, these cysteine residues are involved in the formation of two functionally important disulfide bridges (19). The N-terminal end of the 22-kDa proteins, on the other hand, shows little or no homology with that of cystatin. Although there is suggestive evidence that this N-terminal end is involved in the protease inhibitory activity of cystatin (20), a similar function could make sense for the 22-kDa proteins in view of the description of various proteolytic enzymes and protease inhibitor activities in the prostate (21). It should be noted, however, that the protease inhibitor described by Mills *et al.* (22) in the mouse ventral prostate and other sex accessory glands is a member of the Kazal family of secretory protease inhibitors and is not related to cystatin or the 22-kDa proteins.

The data in the present study clearly indicate that the 22-kDa protein is not expressed exclusively by the rat ventral prostate, but also by the lacrymal gland. In this organ, however, only one of both sequence variants of the protein seems to be expressed, as indicated by hybridization with specific oligonucleotides. In the ventral prostate, on the other hand, both 22-kDa variants are expressed at a considerable level, although their relative contribution is difficult to evaluate. With respect to androgen regulation, our data confirm previous studies which have shown such regulation in the ventral prostate at the protein and mRNA levels (9, 23) and extend these observations to the lacrymal gland. Furthermore, there appears to be specific androgen regulation of different mRNA forms. This can be concluded from Northern blots, showing the most rapid androgen response for the largest mRNA form, but it is not clear, whether this is due to specific transcriptional regulation of a particular 22-kDa protein gene or to androgen-mediated changes in mRNA processing. On the other hand, within the time course of the experiment, very little androgen response was observed for the 22-kDa variant mRNA, which is expressed exclusively in the prostate as shown by hybridization with a specific oligonucleotide probe (oligo 15).

Another interesting aspect is the observation that administration of CHX, a protein synthesis inhibitor, 1 h before androgen treatment nearly completely abolishes the stimulation of 22-kDa protein mRNA at 3 or 6 h of androgen treatment. This may indicate that the androgen effect on the 22-kDa protein mRNA is me-

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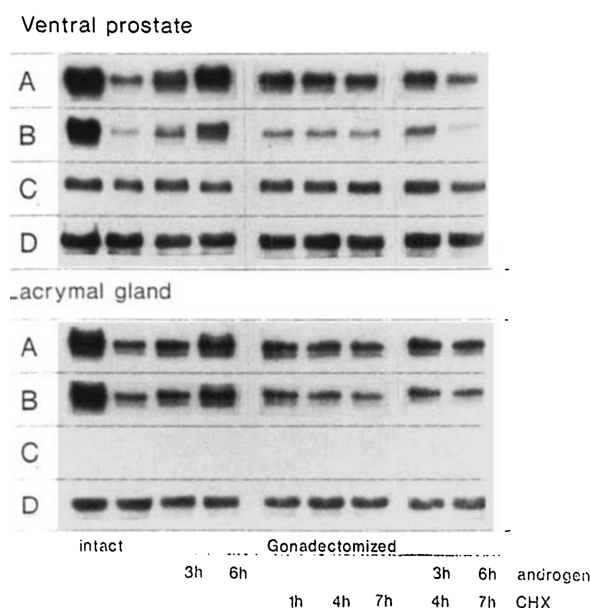

Fig. 7. Effect of Androgen, CHX, and Androgen-CHX Treatment on the Levels of 22-kDa Protein as Shown by Northern Blotting

Total RNA was prepared from the ventral prostates and lacrymal gland of intact rats (five rats per sample), 2-day castrates, or 2-day castrates treated with androgens, with CHX, or with androgens and CHX as indicated. RNA was loaded separated through a 1.2% agarose gel, blotted onto nylon membranes, and hybridized with different probes: the labeled *Eco*RI restriction fragment of clone p22K20 (A), oligo 16/20 (B), oligo 15 (C), and 185 (D).

diated indirectly by another androgen-regulated protein. Alternatively androgen-receptor complexes may interact directly with the 22-kDa protein genes, but the presence of an additional short-living transcription factor may be required for the full induction of 22-kDa protein mRNA synthesis (24).

During preparation of this manuscript Ho *et al.* (25) reported on the primary structure and androgen regulation of a 20-kDa protein specific to the rat ventral prostate. The published cDNA sequence and derived protein structure is essentially the same as that of the p22K20 form in the present study. There can thus hardly remain any doubt on the identity of the encoded proteins. Moreover, Ho *et al.* (25) supported the relation to the 22-kDa glycoprotein with partial amino acid sequences from the purified protein. In general our sequence data show an excellent agreement. However, a small but significant discrepancy exists for the region encoding the N-terminal end of the protein. Indeed, in the sequence of Ho *et al.* (25) an additional A at position 123 results in a frame shift of the upstream-located nt triplets. As a consequence, their open reading frame does not include the sequences of the 24 N-terminal amino acids, which have the typical characteristics of signal peptides of secretory proteins. It should be noted, on the other hand, that the amino-terminal end sequence of the isolated protein does not correspond to that derived from our cDNA sequences after cleavage of the proposed signal peptide or that derived from the cDNA sequence of Ho *et al.* (25). As proposed by the latter authors some further proteolytic cleavage must have occurred after Arg + ^{9} or after Lys + ^{16} (Arg + ^{7} and Lys + ^{14} in our sequences). Such proteolytic breakdown is not surprising in view of the high concentration of a kallikrein-related protease in rat prostatic secretion (3). With regard to tissue specificity Ho *et al.* (25) concluded that the 22-kDa protein is expressed exclusively in the rat ventral prostate, but their study did not include the lacrymal gland, wherein we also demonstrated androgen-regulated synthesis of this protein. Finally, the same authors observed no effect of cycloheximide on the androgen induction of 22-kDa protein mRNA in the prostate. In their study, however, the inhibitor was added only during the last 3 h of a 15-h period of androgen treatment. Under these conditions this would reflect a lack of effect of cycloheximide on the stability of the 22-kDa protein mRNA rather than on its induction by androgens.

MATERIALS AND METHODS

Materials

All reagents used for the isolation of mRNA and for the hybridization assays were of the highest purity available. Oligo(dT) cellulose, Sephadex G-100, DEAE-cellulose and CM-cellulose were obtained from Pharmacia (Uppsala, Sweden), nitrocellulose membranes from Schleicher and Schuell (Dassel, West Germany), [α-32P]dcTP, the cDNA cloning kit, and the Hybond-N transfer membrane from Amersham International (Buckinghamshire, England). The random primed labeling kit was from Boehringer Mannheim Gmbh (Mannheim, West Germany). The λ gt11 expression vector and the anti-rabbit second antibodies conjugated with alkaline phosphatase were obtained from Promega (Madison, WI); the peroxidase-labeled goat anti-rabbit immunoglobulin G (IgG) from Nordic (Leuven, Belgium). CHX was obtained from Serva (Heidelberg, West Germany). Restriction endonucleases and DNA modifying enzymes were purchased from GIBCO-BRL (Ghent, Belgium), Boehringer Mannheim Gmbh (Mannheim, West Germany), and Pharmacia (Uppsala Sweden). A λ gt10 cDNA library from rat prostatic RNA was obtained from Clonetech (Palo Alto, CcA). Testosterone was purchased from Sigma (St. Louis, MO), testosterone propionate from Roussel Uclaf (Paris, France). The PC/gene software used for computer analysis was obtained from Genofit (Geneva, Switzerland).

Oligonucleotides

Different oligonucleotides, prepared using a Cyclone DNA synthesizer (New Brunswick, Canada), were used throughout this study. Oligo 16/20 corresponds to the N-terminal sequence of clones p22K16 and p22K20 and is complementary to the cDNA sequence ACCCTACATGGCACACTGCT. Likewise, oligo 15 corresponds to the homologous sequence found in clone p22K15. Oligo 15/20 contains a sequence complementary to the last 18 nucleotides of clones p22K15 and p22K20. In addition, a 20-mer oligo(dT) and an oligonucleotide complementary to sequences specifically found in 18S RNA (26) were used as controls for quantitative dot-blot assays.

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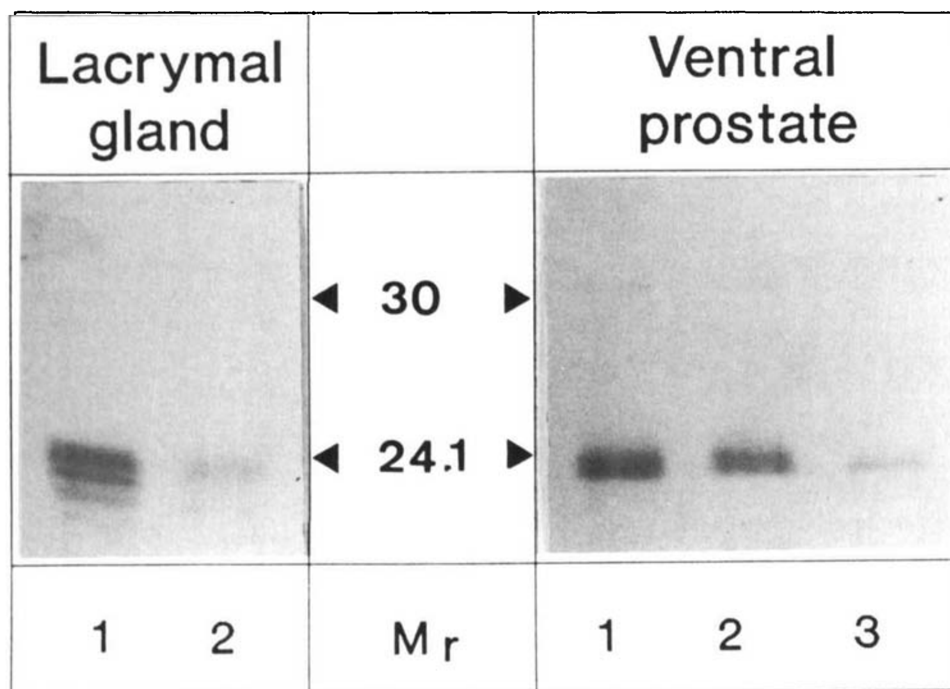

Fig. 8. Effect of Castration at the Protein Level

Cytosols were prepared from the ventral prostate and lacrymal gland of intact rats (lane 1), 2-day castrates (lane 2), and from 7-day castrates (ventral prostate; lane 3). Equal amounts of cytosolic proteins were separated by SDS-PAGE on 15% gels. Immunoblotting was performed using the purified polyclonal antibody. Carbonic anhydrase (M_r 30 kDa) and trypsin inhibitor (M_r 21.4 kDa) were used as molecular weight markers.

Animals

Male Wistar albino rats (3 months old) were used for this study. Castration was performed under ether anesthesia. Some castrated animals received sc injections of androgens (0.5 mg testosterone propionate and 0.5 mg testosterone in 0.2 ml olive oil). Before removal of organs, rats were anesthetized with ether and bled to death via the carotid artery. Immediately after killing, organs were removed and frozen in liquid nitrogen.

Purification of the 22-kDa Glycoprotein

For small scale purification of this protein cytosol was prepared from the ventral prostates of three rats in 50 mM Tris-HCl, pH 7.4, 0.2 N NaCl. The cytosol was applied onto a Sephadex G-100 column (1.5 × 89 cm), equilibrated with the same buffer. Fractions containing the 22-kDa protein, as shown by SDS gel polyacrylamide gel electrophoresis (27), were equilibrated against 50 mM Tris-HCl buffer on PD 10 columns and applied on a DEAE-cellulose column (1.5 × 5 cm) equilibrated with this buffer. Finally the flowthrough of this column was applied on a CM-cellulose column, from which the 22-kDa protein was eluted by means of a O-0.5 N NaCl gradient.

Immunization of Rabbits

Two rabbits were immunized with approximately 0.5 mg purified protein by means of multiple intradermal injections for primary immunization (28) followed by two im booster injections. The specificity of the antisera was monitored using the Ouchterlony technique (29) and immunoblotting (30).

Polyacrylamide Gel Electrophoresis and Immunoblotting

SDS-polyacrylamide gel electrophoresis was performed on 15% separating gels as described by Laemmli (27). Immunoblotting was performed according to Towbin *et al.* (30) using the rabbit anti-22K antiserum as primary antibody at a 1:50 dilution and peroxidase labeled goat anti-rabbit IgG at a 1:2000 dilution as secondary antibody.

Preparation of RNA and Hybridization Assays

Total cellular RNA was extracted as described by Chirwing *et al.* (31) with minor modification. Poly(A)$^+$ RNA was prepared by chromatography on oligo(dT)-cellulose according to Maniatis *et al.* (32). Northern blotting and dot-blot hybridization with labeled 22-kDa protein cDNA fragments were done as described previously (3). Oligo-hybridization was performed according to Geliebter *et al.* (33) at a temperature 5 C below the melting point temperature except for the 18S oligo which was hybridized at 72 C.

Primer Extension Assay

Primer extension was performed as described by Williams and Mason (34). Briefly, 2.5 fmol labeled primer were hybridized to different concentrations of poly(A)$^+$ RNA for 6 h at 55 C in 10 μl 10 mM Pipes, pH 6.4, 400 mM NaCl. Samples were diluted with 90 μl extension reaction buffer [50 mM Tris, pH 8.2, 10 mM dithiothreitol, 6 mM MgCl, 0.5 mM dNTP], and 10 U reverse transcriptase and incubated for 1 h at 42 C. The nucleic acids were subsequently precipitated by ethanol rinsed with 90% ethanol and dried under vacuum. The pellet was resuspended in 5 μl formamide dye mix (0.3% xylene cyanol, 0.3% bromophenol blue in formamide), heated for 5 min at 95 C, and analyzed in a 6% polyacrylamide/50% ureum sequencing gel.

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Construction of the cDNA Libraries

The construction of the prostatic λ gt11 cDNA expression library has been described previously (3). The cDNA library inserted into pUC9 was constructed as described by Heidecker and Messing (15).

Sequence Analysis

Recombinant phage DNA of the λ gt11 and λ gt10 phages was digested with *Eco*RI and subcloned either in pUC18 or pGEM7. The clones obtained from the pUC9 library were directly used for sequence determination without further subcloning. Dideoxy sequence analysis was performed on both strands of denaturated plasmids according to Chen and Seeburg (35).

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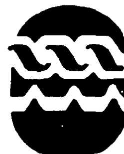

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