Transcriptional Regulation of Prostate Kallikrein-Like Genes by Androgen

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Using gene-specific synthetic oligonucleotides the expression and regulation of kallikrein-like genes in the human prostatic cancer cell line LNCaP were studied. Prostate-specific antigen (PSA) and human glandular kallikrein (hGK-1) together constitute a subfamily of serine proteases exclusively produced in the human prostate. RNA analysis revealed that both genes are expressed in LNCaP cells with PSA basal levels being 2-fold higher than hGK-1 levels. Both mRNAs are induced over a period of 24 h in the presence of 3.3 nM of the synthetic androgen mibolerone. Stimulation of PSA RNA is about 5-fold,whereas hGK-1 stimulation is less pronounced. Nuclear run-on analysis revealed that androgen induction of kallikrein-like genes in LNCaP cells is a rapid event (<3 h) occurring at the level of transcription initiation. Treatment of cells with cycloheximide demonstrates that, while PSA/hGK-1 basal transcription strictly depends on continuous protein synthesis, transcriptional induction by androgen does not. This suggests the direct involvement of the androgen receptor in the induction process independent of additional labile protein factors necessary for kallikrein basal transcription. A binding motif is present in the PSA and hGK-1 promoters, closely resembling the consensus sequence for steroid-responsive elements. The androgen antagonist cyproterone acetate was also able to stimulate transcription of kallikrein-like genes in LNCaP cells. In contrast, androgen-dependent transcriptional suppression of the protooncogene c-*myc* was strongly counteracted by cyproterone acetate. Thus, antiandrogens act differentially on androgen-regulated prostate-specific (PSA, hGK-1) and growth-related (c-*myc*) gene expression in LNCaP cells. (Molecular Endocrinology 6: 753–762, 1992)

INTRODUCTION

Activation of gene expression by extracellular stimuli often requires a multistep signal transduction pathway. Typically, an external signal molecule binds specifically to a receptor which releases a second messenger into the cytosol, leading to signal transformation and transduction into the nucleus, ultimately resulting in the activation of responsive genes. Ligand-inducible transcription factors of the steroid and thyroid hormone receptor superfamily are unique among transcription activators in that they combine each of these steps in a single molecule. The steroid hormone-receptor complex formed in the cytosol undergoes a conformational change induced by hormone binding and dissociation from heat shock protein 90 and translocates into the nucleus where it binds to specific recognition sequences near the promoter of the gene to be activated (for review see Refs. 1–3). The present study characterizes some aspects of steroid hormone regulation of two androgen-dependent genes from the human prostate.

Prostate-specific antigen (PSA) and human glandular kallikrein (hGK-1) belong, together with kidney/pancreas kallikrein (4, 5), to the subfamily of kallikrein-like serine proteases (for review see Ref. 6). The 34-kilodalton PSA glycoprotein (7, 8) which has a chymotrypsin-like specificity (9) was reported to cleave seminogelin, a major seminal vesicle protein responsible for the gel-like structure of semen (10, 11). The appearance of PSA in male serum is diagnostic of destruction of tissue boundaries by prostatic neoplasms and correlates well with tumor progression and the patients' prognosis (12, 13). hGK-1 may be a typical kininogen-processing enzyme (6). Its precise function in prostate physiology remains to be elucidated.

PSA and hGK-1 expression has been demonstrated in human prostate only, and consequently PSA cDNA was cloned from benign and malignant prostatic tissue by several groups (14–16). The hGK-1 gene was isolated by screening a human genomic library with a mouse kallikrein gene fragment (17). Chromosome mapping showed that both genes are tandemly located in the same orientation on chromosome 19 (18). As the gene for kidney/pancreas kallikrein has also been assigned to chromosome 19 (19), this suggests a common ancestor of kallikrein-like genes in evolution. Sequence comparison revealed a close relationship between PSA and hGK-1 in exon-intron architecture with an overall homology of 82% (20, 21). The promoter

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regions also exhibit a high degree of homology and contain, within 200 base pairs (bp), several well known *cis*-regulatory elements which have been defined by sequence homology only (20, 21). These include a variant TATA box, a transcription factor Sp1 binding site, and a CACCC motif. A 15-bp imperfect palindrome closely resembling the consensus sequence for nuclear steroid receptor binding sites (1) resides upstream of the transcription start site at positions −155 to −170 of the published genomic sequences of both PSA and hGK-1 (21–23).

In recent publications we and others (24–27) have demonstrated androgen-dependent induction of PSA-related steady state mRNA in the androgen receptor-containing human prostatic cancer cell line LNCaP. In the present study we have used this cell line, which is the best suited *in vitro* model of prostate cancer available (28, 29), to study the androgen dependence of kallikrein-like genes in greater detail. By specific oligonucleotide hybridization and nuclear run-on analysis we show that both genes, PSA and hGK-1, are induced by androgen at the level of transcription initiation. This is the first demonstration of direct androgen induction of kallikrein genes.

RESULTS

Stimulation of PSA-Related mRNA by Androgen

To monitor the time course of PSA steady state RNA induction, LNCaP cells were treated for increasing periods of time with 3.3 nM of the nonmetabolizable synthetic androgen mibolerone (MIB; 7α,17α-dimethyl-19-nortestosterone) (30) to provide a constant androgenic stimulus to the cells. This concentration of synthetic androgen has proven to be maximally effective in stimulating the LNCaP androgen receptor (31). Isolated RNAs were subjected to Northern analysis by hybridization with a PSA cDNA probe (15, 24). As shown in Fig. 1A, the level of PSA mRNA, with an expected size of 1.5 kilobases (kb), rises continuously within 4–72 h after hormone addition. Maximal induction is 10-fold after 72 h as compared to control levels simultaneously determined in the absence of MIB (Fig. 1B). Note that the time scale in Fig. 1B is not linear. The additional bands seen in Fig. 1A are likely to represent alternatively processed kallikrein transcripts, as has been described (16). The housekeeping enzyme glyceraldehyde-phosphate-dehydrogenase (GAPDH) is not significantly influenced by MIB (32), and the corresponding mRNA can thus be used to correct for changes in hybridization signal due to differences in the amounts of RNA loaded on the gel.

Comparison of Androgen Effects on PSA and hGK-1 Expression

Due to the high degree of homology in the coding regions of the PSA and hGK-1 genes, hGK-1 mRNA might contribute considerably to the Northern blot signal obtained with a PSA cDNA probe (6, 33). To explore whether both prostatic kallikrein genes are regulated by androgen, we synthesized oligonucleotides which were recently shown to be able to discriminate between PSA and hGK-1 in primer extension experiments (33). The slot blot hybridization in Fig. 2A shows that both genes, PSA and hGK-1, are expressed in LNCaP cells. In unstimulated control cells (time points 0, 24, 48, and 72 h in the absence of MIB) the level of PSA messenger exceeds the level of hGK-1 mRNA by a factor of 2. Greater abundance of PSA RNA compared to hGK-1 RNA has also been noticed in benign prostate hyperplasia (33). Both mRNAs increase within 72 h after hormone addition (for a quantitative assessment see densitometer scan in Fig. 2B). However, hGK-1 induction by MIB is less pronounced compared to PSA induction in LNCaP cells. The signal obtained by hybridization against PSA cDNA reveals the specificity of the oligonucleotides used (Fig. 2A, *top*). These results show that, in LNCaP cells, both prostatic kallikrein genes are positively regulated by androgen.

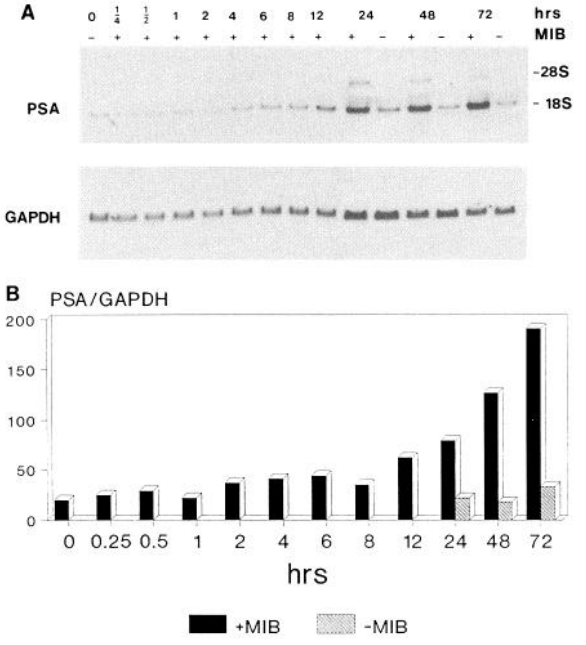

Fig. 1. Northern Blot Analysis of Total RNA of LNCaP Cells after Treatment with the Synthetic Androgen MIB

A, LNCaP cells were cultivated in the presence (+) or absence (−) of 3.3 nM MIB for various periods from 0–72 h. RNA was extracted and analyzed on Northern blots (20 μg/lane) and hybridized with a PSA *Eco*RI/*Bam*H1 cDNA probe labeled by random priming. Subsequently the probe was washed off, and the filter was rehybridized with a probe for the housekeeping enzyme GAPDH (72). B, The autoradiograms were scanned densitometrically, and the results of PSA hybridization relative to GAPDH are shown schematically in a block diagram.

Androgen Induction of PSA-related Genes Takes Place at the Level of Transcription Initiation

PSA-related mRNAs progressively increase from 4–72 h in LNCaP cells upon androgen administration. The

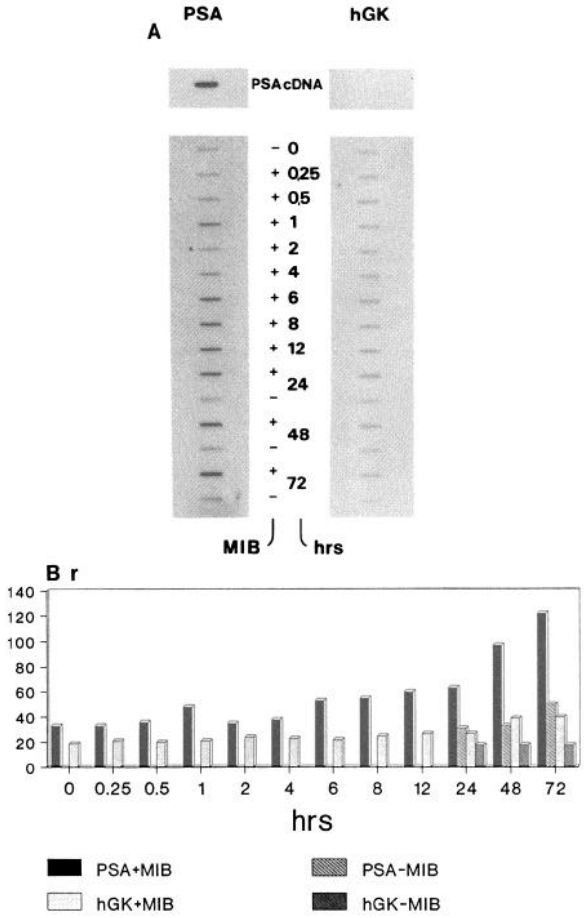

Fig. 2. Slot Blot Analysis of LNCaP RNA Showing the Androgen Dependence of PSA and hGK-1

A, Five micrograms of the same RNAs as described in Fig. 1 were transferred to nylon membranes using a commercial manifold and probed with equal amounts of PSA- and hGK-1-specific oligonucleotide probes labeled by polynucleotide kinase. B, Densitometer scan of PSA and hGK-1 hybridization signals normalized to the GAPDH signal (r = PSA/GAPDH or hGK-1/GAPDH) obtained by rehydridization with a GAPDH-specific probe (autoradiograms not shown).

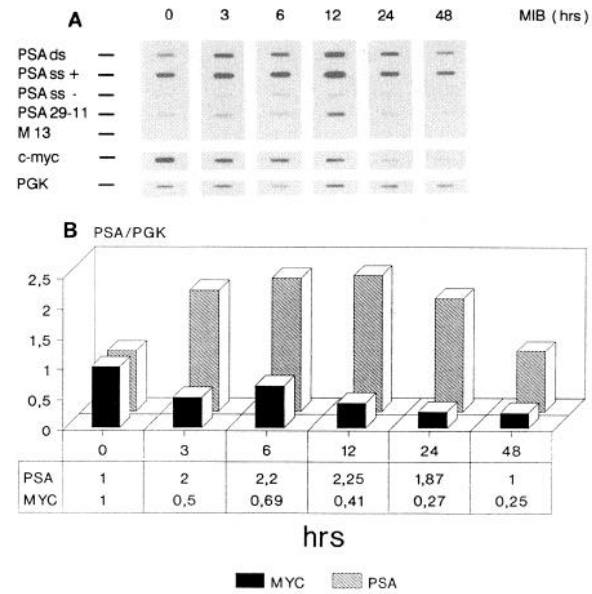

Fig. 3. Transcriptional Run-On Activity of PSA-Related Genes and c-*myc* in MIB-Treated LNCaP Cells

A, Plasmids containing double-stranded and single-stranded DNA probes were transferred to nylon membranes by slot blot and hybridized with nuclear run-on RNA (10^7 cpm/3 ml hybridization buffer) from LNCaP cells treated without (0 h) or with MIB for 3–48 h. The PSA probe cloned in both orientations in M13 phages was a 1.5-kb *Eco*RI cDNA fragment encompassing the complete coding sequence for the mature PSA protein. The PSA probe 29/11 was an approximately 780-bp *Pst*I/*Sac*I fragment, spanning 580 bp of the second intron and 200 bp of the third exon (23). The *c-myc* insert was a 1.4-kb *Eco*RI/*Cla*I cDNA fragment cloned in pBR 328 containing parts of the third exon (32). The PGK plasmid contains a 1.8-kb *Eco*RI cDNA fragment cloned in pBR 322 (24). B, Autoradiograms were scanned densitometrically, normalized to the PGK signal, and results are shown relative to basal transcription levels in untreated control cells.

early onset of RNA induction could reflect a direct effect of MIB on gene transcription. To test this hypothesis we performed nuclear run-on analyses to measure the androgen dependence of the density of actively transcribing polymerase II molecules on PSA-related genes. Double-stranded and single-stranded PSA cDNA fragments presumably recognizing both PSA and hGK-1 RNA were immobilized on nylon filters and hybridized with ^{32}P-labeled transcripts isolated from nuclei of LNCaP cells treated for different periods with MIB. Probes for the phospho-glycerate kinase gene (PGK) and the c-*myc* gene which is negatively regulated by androgen (32) were used as internal controls. As shown in Fig. 3, PSA/hGK-1 transcription is 2-fold induced by MIB. Significant transcription is only detectable on the coding strand of the PSA/hGK-1 genes. Peak induction is observed between 3–12 h after hormone addition. From 24–48 h PSA/hKG-1 transcription slowly returns to pretreatment levels (Fig. 3B). This may be due to autologous down-regulation of the androgen receptor after hormone stimulation (25, 34). While PGK transcription remains almost stable during the course of the experiment, c-*myc* gene transcription slowly decreases concomitantly to androgen-induced reduction of cell proliferation as has been reported earlier (24, 32).

Contribution of Posttranscriptional Mechanisms to Hormonal PSA/hGK-1 Regulation

The kinetics of PSA/hGK-1 induction in Northern blots and run-on assays are somewhat different in that maximal induction of steady state RNA is achieved at time points when transcription already decreases again. We therefore attempted to evaluate the possible participation of posttranscriptional regulation. In the absence of MIB, PSA/hGK-1 RNA half-life was approximately 24 h (Fig. 4). In the presence of MIB (72 h), PSA/hGK-1 RNA levels showed some variability in response to treatment

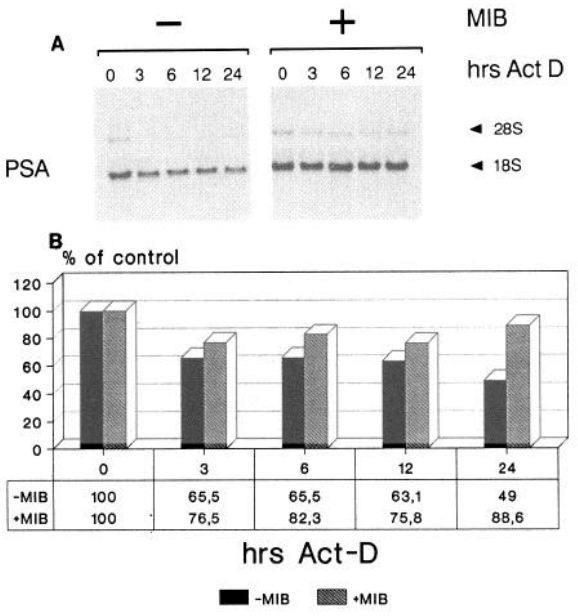

Fig. 4. Half-Lives of PSA/hGK-1 RNA in the Absence and Presence of MIB

A, Untreated LNCaP cells and cells treated with MIB for 72 h were incubated for various periods from 0–24 h with actinomycin D (5 μg/ml), and RNA was isolated and subjected to Northern analysis by hybridization with a PSA cDNA probe. B, Autoradiograms were scanned, and results are shown relative to PSA/hGK-1 levels obtained at the time point 0 h after actinomycin D treatment.

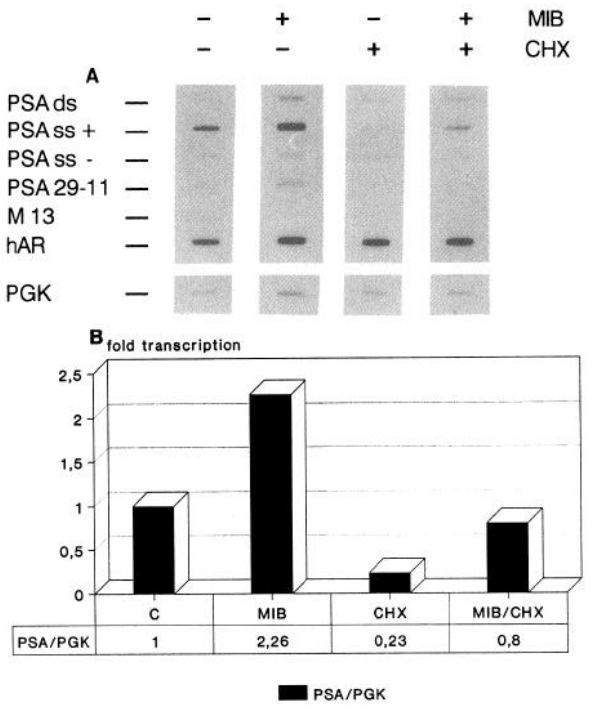

Fig. 5. Effect of Inhibition of Protein Synthesis on PSA/hGK-1 Transcription

A, Filters prepared as described in Fig. 3 were hybridized with nuclear run-on RNA of LNCaP cells treated with MIB (+/− MIB) and with 10 μg/ml of the protein synthesis inhibitor CHX (+/− CHX) for 6 h. The hAR cDNA probe contained within a eukaryotic expression plasmid (74) spans the complete coding region for the human androgen receptor protein (kindly provided by Dr. J. Trapman). B, Autoradiograms were scanned, and results are shown as in Fig. 3.

with actinomycin D for different periods but were still 90% of untreated control levels 24 h after actinomycin D administration (Fig. 4B). The high stability of PSA/hGK-1 RNA in MIB-induced cells precluded the exact determination of its half-life by conventional treatment of cells with actinomycin D. Nevertheless, the low degradation rate of PSA/hGK-1 RNA explains quite well the ongoing accumulation of transcripts several hours after transcriptional induction has occurred. These results indicate an involvement of posttranscriptional mechanisms in the hormonal PSA/hGK-1 induction process.

Androgen Induction of PSA/hGK-1 Transcription is Independent of Protein Synthesis

To establish whether protein synthesis is required for the immediate induction of kallikrein gene expression, we carried out run-on transcription in nuclei from cells treated with the protein synthesis inhibitor cycloheximide (CHX) for 6 h. Figure 5 again shows PSA/hGK-1 induction by MIB. However, PSA/hGK-1 basal transcription strictly depends on newly synthesized proteins. Only a few elongated transcripts are detectable after CHX treatment for 6 h. Note that the transcription rates for the human androgen receptor (hAR) gene and the PGK gene are not significantly affected by CHX. Simultaneous administration of CHX + MIB restores transcriptional activity nearly to the level observed in untreated control cells (Fig. 5B). The magnitude of the induction effect of MIB in the presence of CHX (= 2.5×) compared to cells treated with CHX alone is equal to the induction observed by MIB alone compared to untreated control cells (= 2.2×). These results demonstrate that PSA/hGK-1 basal transcription but not induction by MIB is dependent on the ongoing synthesis of a labile protein factor.

Effect of Antiandrogen on PSA and hGK-1 Expression

The time course of the run-on analysis strongly suggests that the early androgen-dependent induction of PSA-related mRNA is a direct hormone effect on gene expression, presumably involving the androgen receptor (AR). In order to explore the possible participation of the AR we tried to antagonize the effect of MIB by the AR antagonist cyproterone acetate (CA). Due to the relatively low affinity of CA to the AR a more than 500-fold molar excess (1.8 μM) over the androgen is required for efficient blockage of androgen action (35). The Northern blots in Fig. 6 hybridized with PSA- and hGK-1-specific oligonucleotides show the effect of MIB and CA on kallikrein-like mRNAs in LNCaP cells. Again an

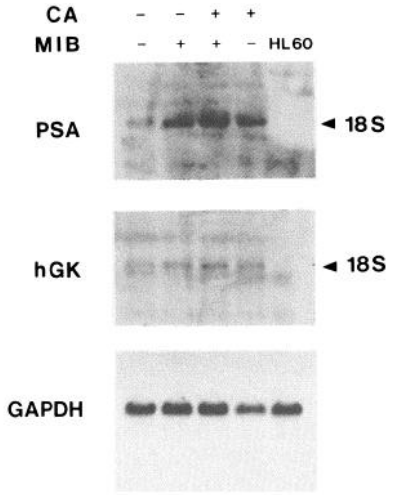

Fig. 6. Northern Blot Analysis of RNA of LNCaP Cells Treated with the Synthetic Androgen MIB and the Antiandrogen CA

LNCaP cells were maintained in the presence (+) or absence (−) of 3.3 nM MIB and 1.8 μM CA for 48 h. RNA was isolated and analyzed on Northern blots (20 μg/lane) with PSA- and hGK-1-specific oligonucleotides as in Fig. 2. RNA of the human promyelocytic cell line HL 60 was used as a control for hybridization specificity. Filters were reprobed with GAPDH to document equal loading of RNA per lane.

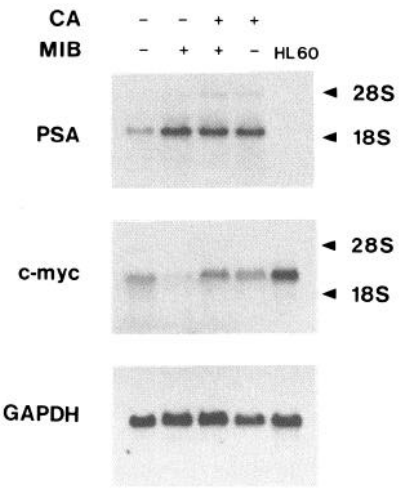

Fig. 7. Differential Effect of the Antiandrogen CA on PSA/hGK-1 and c-*myc* Expression

The Northern blots shown in Fig. 6 were reprobed with a PSA cDNA and a c-*myc* cDNA probe specific for the third exon labeled by random priming.

increase in signals corresponding to the mol wt of PSA and hGK-1 mRNA (both 1.5 kb) is distinctly detectable in LNCaP cells 48 h after MIB treatment, but not in the promyelocytic cell line HL 60. Unexpectedly, CA alone also stimulates PSA and hGK-1 expression. The stimulatory potency of 1.8 μM CA is equivalent to 3.3 nM MIB. However, combined administration of MIB and CA reveals no antagonistic activity.

Differential Effect of Antiandrogen on PSA/hGK-1 and c-*myc* Expression

In previous work (24) we have already demonstrated that 1.8 μM CA potently antagonizes the effect of 3.3 nM MIB on the proliferation of LNCaP cells, while not having any effect when administered alone. To resolve further the unexpected agonism of antiandrogen on androgen-dependent gene expression, we compared the effect of CA on kallikreins with its effect on c-*myc*, a gene known to be involved in growth regulation. The same filters as in Fig. 6 were reprobed with PSA cDNA and a third exon probe of the human c-*myc* gene. As shown in Fig. 7, steady state RNA of c-*myc* is suppressed by MIB, while PSA/hGK-1 RNA is increased. However, whereas in the case of PSA/hGK-1, antiandrogen has a considerable androgen agonistic effect, in the case of c-*myc* the androgen-induced repression of steady state RNA is fully overcome by the simultaneous addition of CA (Fig. 7, lanes +MIB and +CA). CA alone has, in contrast to PSA/hGK-1, no effect on c-*myc* expression and therefore behaves as a true androgen antagonist.

To investigate whether agonistic action of antiandrogen on PSA/hGK-1 expression also takes place at the level of transcription, we performed nuclear run-on experiments in the presence of CA and CA + MIB, respectively. As shown in Fig. 8A, CA induces PSA/hGK-1 transcription, nearly to the same extent as MIB.

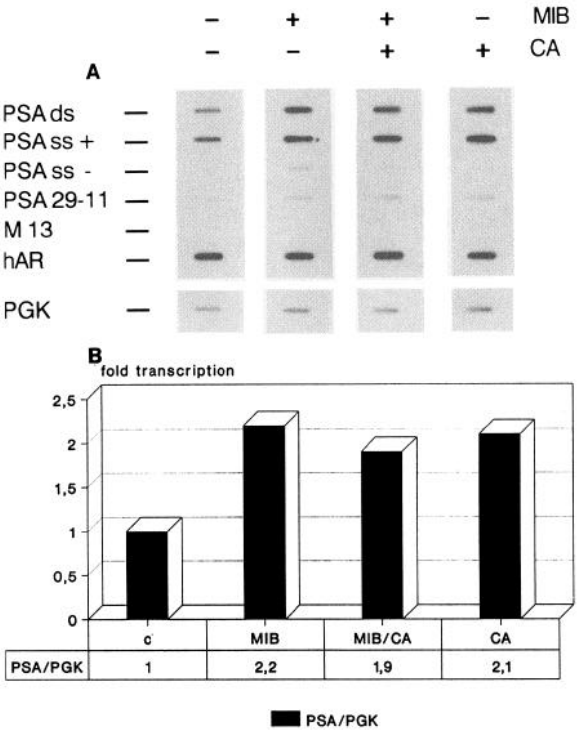

Fig. 8. Effect of CA on PSA/hGK-1 Transcription

A, Identical filters as described in Fig. 5. were hybridized with run-on RNA of LNCaP cells treated with (+) or without (−) 3.3 nM MIB and/or 1.8 μM CA for 6 h. B, Autoradiograms were scanned, and results are shown as described in Fig. 3.

Simultaneous administration of CA plus MIB also increases PSA/hGK-1 transcription but does not result in overstimulation (Fig. 8B). These results establish that the androgen agonism of CA on kallikrein gene expression in LNCaP cells is mediated by the same transcriptional induction pathway as the effect of MIB.

DISCUSSION

Thus far, expression and regulation of kallikrein genes have been extensively studied only in rodents. The kallikrein gene families of rat and mouse are probably much larger than that of man (6), consisting of 24 members in the mouse (36, 37) and 8–17 members in the rat (38, 39). Rodent kallikrein genes have been shown to be subject to complex tissue-specific (39, 40) and hormonal (41–43) regulatory mechanisms. Regulation of kallikrein protein synthesis and steady state RNA levels by androgens has also been observed (44–46). Expression of mouse kallikrein genes mGK-3, 4, and 5 was shown to be induced by testosterone in the salivary gland (45). In the rat prostate, kallikrein S3 and P1 mRNAs disappeared 8 days after castration and were restored to control levels by dihydrotestosterone administration for 8 days (46). However, both responses are slow and thus imply that androgens are not necessarily acting directly on rodent kallikrein genes (45, 46). Immediate transcriptional regulation of kallikrein expression has thus far only been noticed in the rat pituitary by dopamine (47). In contrast, the results shown in the present study demonstrate that, unlike in rodents, regulation of the human kallikrein-related PSA and hGK-1 genes by androgen in LNCaP cells occurs primarily at the level of transcription initiation, presumably involving the androgen receptor. Note however that these results do not rule out the possibility that still unidentified members of the presumably larger human kallikrein gene family contribute to the hybridization signal obtained with the full length PSA cDNA probe used in the run-on transcription assays.

Several gene fragments have definitely been shown to act as androgen-dependent enhancers of transcription. These are the long terminal repeat of mouse mammary tumor virus (48), an upstream element of the mouse sex-limited protein gene (49), and a part of the first intron of the rat prostatic binding protein gene, C3[1] (50). The sequence 5′-*AGTACGTGATGTTCT*-3′ contained within the C3[1] gene was shown to confer androgen inducibility onto a heterologous promoter (50) and to specifically bind a bacterially expressed chimeric androgen receptor (51). The PSA promoter also harbors a partially palindromic 15-bp stretch closely resembling the above androgen response element of the rat (21, 23). The sequence 5′-*AGCACTTGCTGTTCT*-3′ occurring in the PSA promoter at positions −155 to −170 of the noncoding strand differs in the hGK-1 promoter in only one base at position −170 (TG). Although preliminary studies have revealed the binding of a nuclear protein to the PSA sequence in gel shift experiments (Wolf, D. A., unpublished observation), it is at present unknown whether this element mediates the transcriptional regulation of PSA/hGK-1 by androgen. Further investigations are required to verify this speculation and the possibility that additional sequences present in the PSA/hGK-1 promoters, namely the Sp1 binding site and the CACCC motif, contribute to PSA regulation, since these elements have recently been shown to cooperate with steroid hormone response elements (52).

The finding that protein synthesis is dispensible for the induction of PSA/hGK-1 transcription in LNCaP cells confirms Northern blot data provided by Young *et al.* (27). However, these workers have found superinduction of PSA steady state RNA by MIB in the simultaneous presence of CHX. Thus CHX appears to drastically stabilize PSA RNA while decreasing PSA/hGK-1 basal level transcription (Fig. 5). Unfortunately the effect of CHX alone on PSA steady state RNA expression was not investigated. Taking into account that CHX might further increase the stability of PSA RNA, which is exceptionally stable anyway, it appears questionable whether the inhibiting effect of CHX on PSA/hGK-1 basal level transcription could be detected in Northern blot analyses. Regulation by steroid hormones independent of protein synthesis has been observed for several genes in various cell types (53–56). In most cases this has been interpreted to reflect direct gene induction via the homologous hormone receptor. Thus our CHX data lend further support to the concept that androgen induction of prostate kallikrein gene transcription is mediated by the AR.

Our results demonstrating differential effects of the antiandrogen CA on prostate-specific and growth-related gene expression in LNCaP cells raise some interesting questions on gene regulation by androgens. While CA antagonizes androgen suppression of c-*myc* transcription, it does not antagonize androgen induction of PSA/hGK-1 and even has androgen agonistic activity on kallikrein transcription (Figs. 7 and 8).

An important aspect of antiandrogen action in LNCaP cells is given by the recent publication of a point mutation in the androgen receptor gene in LNCaP cells, which results in a single amino acid substitution (ThrAla) in the steroid binding domain (31). By amplification with the polymerase chain reaction and sequence analysis of this region we have recently confirmed this mutation in our stock of LNCaP cells (data not shown). This alteration in receptor structure was shown to cause both increased affinity for progestins (57) and receptor-mediated inducibility of a glucocorticoid/progestin response element by CA (31). Since the wild type receptor was unable to respond to CA (31), the androgen agonism of CA on PSA/hGK-1 transcription observed in LNCaP cells could be a special feature of the mutated receptor in these cells. However, this interpretation alone is not sufficient to explain the differential effect of CA on PSA/hGK-1 *vs.* c-*myc*.

In general, the mechanisms of antihormone action on

hormone-responsive genes are not completely understood, although several have been proposed (58, 59). For, example the effects of the antiestrogen 4-hydroxytamoxifen have been shown to vary greatly with the species, tissues, cells, and response being studied (for review see Refs. 60–62). 4-Hydroxy-tamoxifen has variable activity, ranging from full estrogenic to totally antiestrogenic activity, for the induction of a number of RNA species in cultured breast cancer cells (63–65). These results emphasize the marked cell-type and promoter-context dependence of antihormone action.

The action of antiandrogens on gene expression is much less well documented. Recent studies have shown that the antiandrogen CA can have considerable androgen agonistic activity on the expression of androgen-regulated genes (34, 66). Androgen antagonism of CA has also been demonstrated on the androgen-induced suppression of the nerve growth factor receptor gene in rat testis (67). Our results from LNCaP cells show that CA can have androgen-agonistic as well as -antagonistic activity even within one cell type. At present the reasons for this discrepancy are not understood. As the receptor mutation clearly cannot be responsible for both effects, alternative mechanisms may be operative.

Although both PSA-related genes and the c-*myc* gene are regulated by androgen at the level of transcription they differ in several notable aspects: 1) c-*myc* RNA has an exceptionally short half-life of 20 min (68) and encodes a nuclear protein which has been implicated in growth regulation (for review see Refs. 69, 70), while, at least, authentic PSA encodes a secretory protein, the corresponding RNA of which has a half-life of approximately 24 h (Fig. 4); 2) PSA-related genes are positively regulated by androgen, whereas c-*myc* is negatively regulated (32); and 3) whereas regulation of PSA transcription is a rapid event taking less than 3 h until maximal induction has occurred, maximal c-*myc* down-regulation is slow (>48 h; Ref. 32 and Fig. 3). It is therefore reasonable to assume that the differential effect of CA reflects differential mechanisms of androgen action on gene regulation, possibly depending on additional regulatory steps and the particular position of the regulated gene in the functional context of the whole cell.

Although the detailed mechanism of the differential effects of CA on tissue-specific and growth-related gene expression in LNCaP cells cannot be fully assessed at present, their consideration may offer future directions to a more complete understanding of gene regulation by androgens.

MATERIALS AND METHODS

Cell Culture and Hormones

The human prostate cancer cell line LNCaP (28) was from the Human Cancer Cell Laboratory, Sloan Kettering Institute for Cancer Research (Rye, NY). LNCaP cells between passages 70 and 80 were used for the experiments described. Cells were maintained in RPMI 1640 as monolayers in the presence of 10% fetal calf serum and 2 mM glutamine as described (24). For the preparation of seed stocks, cells were grown to 50–75% confluency before use. Hormones were added 48 h after seeding as ethanol solutions. Final concentrations of the hormones were 3.3 nM for the synthetic androgen MIB (Upjohn, Kalamazoo, MI) and 1.8 μM for the antiandrogen CA (Schering AG, Berlin, Germany). The protein synthesis inhibitor CHX (Sigma, St. Louis, MO) was used at a concentration of 10 μg/ml and actinomycin D (Boehringer Mannheim, Mannheim, Germany) at a concentration of 5 μg/ml.

RNA Extraction and Northern Blot Analysis

Total cellular RNA was isolated by the LiCl/urea method described in detail elsewhere (24). RNA samples (20 μg/lane) were fractionated on 1.2% agarose gels containing 6% formaldehyde. Integrity and relative amounts of RNA per lane were checked by staining with ethidium bromide. 28S and 18S rRNAs were used as internal size markers. Gels were blotted onto Biodyne nylon membranes (PALL, Glen Cove, NY) and baked for 2 h in a vacuum oven. Filters for hybrization with cDNA probes were prehybridized in 5-fold concentrated standard saline citrate (SSC; 1× SSC = 150 mM NaCl, 15 mM Na_3-citrate, pH 7.0), 0.5% sodium dodecyl sulfate (SDS), 1× Denhardt's solution, and hybridization was carried out for 20 h at 68 C in 5 ml of the same solution containing 1.5×10^6 cpm/ml of the respective cDNA probe. Filters were washed at 68 C once in 4× SSC, 0.5% SDS, 1× Denhardt's solution for 30 min, twice for 15 min in 2× SSC, 0.5% SDS, 30 min in 2× SSC, and subsequently exposed overnight at −80 C to Fuji (Tokyo, Japan) x-ray films between intensifier screens. Autoradiograms were scanned in an LKB UltraScan XL Laser Densitometer (LKB, Bromma, Sweden).

Hybridization Probes

^{32}P labeling was performed with the random-primed labeling kit (Boehringer Mannheim) according to the recommendations of the supplier. The 1.4-kb *Eco*RI/*Bam*HI cDNA fragment used as a PSA probe (15) spans the coding region of the complete mature PSA protein. The c-*myc* probe was a 1.4-kb *Eco*RI/*Cla*I fragment covering the third exon (71), and the probe for the housekeeping enzyme glyceraldehyde-phosphate-dehydrogenase was a synthetic single-stranded oligonucleotide (100 bases) derived from the published sequence (72). The oligonucleotides specific for PSA (5′-TTCTGAGGGTGA-ACTTGCGCA-3′) and hGK-1 (5′-CTTCTCAGAGTA-AGCTCTAGC-3′) were derived from sequences coding for amino acids 161–167. These oligonucleotides have recently been shown to be able to discriminate between PSA and hGK-1 in primer extension assays (33). The hybridization temperature was determined according the specific oligonucleotide content by the formula: $T_H(^0C) = 4(G + C) + 2(A + T) - 3$. Filters were prehybridized in 6× SSC, 0,1% SDS, 1× Denhardt's solution, and 50 μg/ml denatured salmon sperm DNA for 2 h at 57 C and hybridized overnight in the same solution containing equal amounts (1.5×10^6 cpm/ml) of PSA or hGK-1 oligonucleotide probe labeled to a specific activity of 6×10^7 cpm/μg with polynucleotide kinase. Filters were washed in 4× SSC, 0.1% SDS for 30 min at 57 C and twice for 15 min in 2× SSC at 40 C. Autoradiography was carried out for 48 h as described above.

Nuclear Run-On Analysis

Preparation of nuclei and hybridization were performed as described (32). Cells (2×10^8) were scraped from the culture dishes and washed twice in PBS. Cells were resuspended in 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% (vol/vol) NP40, and incubated on ice for 5 min. The nuclear pellets were spun down at 500 × *g* and washed by resuspen-

sion in 10 ml of the same buffer. The pelleted nuclei were resuspended in storage buffer (50 mM Tris-HCl, pH 8.3, 40% (vol/vol) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA) and frozen in liquid nitrogen in portions of 100 μl corresponding to 2×10^7 nuclei. For nuclear run-on assays the nuclei were mixed with 100 μl reaction buffer [10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 300 mM KCl, 0.5 mM ATP, CTP, and GTP, and 100 μCi [α-^{32}P]UTP (800 Ci/mmol; Du Pont, Wilmington, DE)] and incubated for 20 min at 28 C. DNAse I was added to a final concentration of 10 μg/ml, and the incubation was continued for 5 min at 28 C. After addition of 200 μl STE buffer (100 mM Tris-HCl, pH 7.5, 50 mM EDTA, and 0.5% SDS) and 200 μl proteinase K (10 mg/ml, preincubated at 37 C for 1 h) the samples were incubated for 1 h at 40 C. Nuclear transcripts were separated from unincorporated nucleotides on a Sephadex G-50 column equilibrated with 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1% SDS. The labeled RNA was boiled for 10 min, chilled on ice, and hybridized to DNA probes immobilized on Biodyne nylon membranes in Church buffer (0.5 M sodium phosphate, pH 7.1, 7% SDS, and 0.1 mM EDTA) (73) after preincubation of the filters in the same buffer. After hybridization the filters were washed twice at 50 C in 0.1× SSC, 1% SDS, once in 2× SSC containing 10 μg/ml RNase A at 25 C, and finally twice again in 0.1× SSC, 1% SDS at 50 C. The filters were exposed for 24–48 h to Fuji X-ray films using a DuPont Lightning Plus intensifying screen.

All run-on assays were repeated at least twice with independent preparations of cell nuclei in order to document reproducibility.

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