Androgen Induction of a Human Prostate-Specific Kallikrein, *hKLK2*: Characterization of an Androgen Response Element in the 5′ Promoter Region of the Gene†

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ABSTRACT: The human prostate-specific kallikreins, human glandular kallikrein-1 (*hKLK2*) and prostate-specific antigen (*hKLK3*), have been shown to be regulated by androgens. To determine whether the androgen induction of these genes is transcriptionally regulated via an androgen response element, an *hKLK2* promoter DNA fragment was linked to a promoterless chloramphenicol acetyltransferase (CAT) reporter gene and cotransfected with an androgen receptor expression vector in an androgen receptor-less human prostate cell line, PC-3. Dose response and steroid specificity experiments showed that the *hKLK2* promoter confers androgen receptor-mediated gene induction in a ligand-specific manner. Moreover, 5′ deletion constructs of the *hKLK2* promoter DNA and internal deletion constructs devoid of the 5′ half-site of the putative androgen responsive element (ARE) were used to show that the putative ARE is indeed acting as a functional ARE in prostate cells. In addition, multiple AREs from both *hKLK2* and *hKLK3* were able to reconstitute androgenic induction, further strengthening the argument that the AREs are functional. Although previous studies have shown that *hKLK3* mRNA is expressed at a higher level than that of *hKLK2*, our results suggest that the *hKLK2* ARE may have higher androgenic inducibility than the *hKLK3* ARE. These results suggest that other cis-acting elements may be involved in coordinating *in vivo* androgenic induction of *hKLK2* and *hKLK3* genes.

Human glandular kallikrein-1 (hGK-1), prostate-specific antigen[1] (PSA), and human pancreatic/renal kallikrein are three members of a subgroup of serine proteases (Watt et al., 1986; Evans et al., 1988; Lundwall, 1989; Schedlich et al., 1987) that are potentially involved in the activation of specific polypeptides through post-translational processing (Clements, 1990). Recently, these three genes have been redesignated as *hKLK1* for human pancreatic/renal kallikrein, *hKLK2* for human glandular kallikrein-1, and *hKLK3* for prostate-specific antigen, according to a new nomenclature system (Berg et al., 1992). Since the name PSA has been used classically in clinical studies, we use this term interchangeably with *hKLK3*. The *hKLK2* and PSA mRNAs exhibit a high degree of sequence homology (Schedlich et al., 1987; Digby et al., 1989) and are expressed exclusively in the prostate (Morris, 1989; Qui et al., 1990; Young et al., 1992). There is approximately 80% homology between PSA and *hKLK2* in the 5′ flanking region from -300 to -1 with respect to the cap site. Each promoter contains a putative androgen responsive element (ARE) at about -160, which differs by one nucleotide (Young et al., 1992). PSA protein is well characterized and is an important marker for prostate cancer (Wang et al., 1981). The *hKLK2* protein has yet to be isolated and its function is uncertain, although its amino acid sequence has been deduced from the complementary DNA that is translatable in *in vitro* translation systems (Schedlich et al., 1987; Young et al., 1992).

Androgens play an important role in the development and secretory properties of the prostate. Androgens may also be crucial for initial development of prostate neoplasia (Carter & Coffey, 1990; Carter et al., 1990). Although many genes are known to be regulated by androgens (Berger & Watson, 1989), the molecular mechanism by which the androgen receptor complex regulates gene expression is not fully understood. The recent cloning of the androgen receptor gene (Chang et al., 1988; He et al., 1990; Luban et al., 1988; Tilley et al., 1989; Young et al., 1990) has provided a valuable tool for studying androgen-regulated gene expression. Also, recent studies have led to the identification and characterization of androgen response elements in several androgen responsive genes (Riegman et al., 1991; Tan et al., 1992). Such elements appear to be important regulators of transcription by their interaction with the androgen receptor complex.

The expression of PSA and *hKLK2* mRNAs has been demonstrated to be regulated by androgens (Young et al., 1991, 1992). A functional ARE (Riegman et al., 1991) has been described in the PSA gene. Our efforts were to determine whether androgenic regulation of *hKLK2* is transcriptional and to provide evidence that the putative ARE identified in the promoter region of *hKLK2* is in fact a functional ARE within human prostate cells.

MATERIALS AND METHODS

Plasmid Constructs. The 5′ flanking DNA fragments of the PSA and *hKLK2* genes were obtained by PCR amplification of 200 ng of human genomic DNA (Clontech, Palo Alto, CA), using oligonucleotide primers corresponding to the PSA and *hKLK2* genes. The sequences used for the synthesis of primers in this report were described elsewhere (Lundwall, 1989; Schedlich et al., 1987). The sequences for

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[1] Abbreviations: *hKLK2*, human glandular kallikrein; PSA or *hKLK3*, prostate-specific antigen; ARE, androgen responsive element; MIB, mibolerone; CAT, chloramphenicol acetyltransferase.

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these primers are 5′-CATTGTTTGCTGCTGCACGTTG-GAT-3′ and 5′-TCCGGGTGCAGGTGGTAAGCTTGG-3′ for the PSA gene and 5′-CTGTAATCTATCACTTT-GGGCA-3′ and 5′-TCCACGGCCAGGTGGTGA-3′ for the *hKLK2* gene. The PCR fragments were purified by gel electrophoresis, phosphorylated with T4 polynucleotide kinase, and ligated with *Sma*I-digested, dephosphorylated pGEM 7Zf-(+). pGEM7Zf(+) DNA containing a PSA or *hKLK2* 5′ flanking fragment was purified from transformed *Escherichia coli* HB101 and sequenced by a double-stranded DNA–dideoxynucleotide chain termination method. PSA and *hKLK2* 5′ flanking fragments were excised from pGEM7Zf-(+) by digestion with *Sph*I plus *Bam*HI and *Xba*I plus *Bam*HI endonucleases, respectively. The PSA and *hKLK2* DNA fragments were ligated with pBLCAT3 vector, which had been digested with *Sph*I plus *Bam*HI and *Xba*I plus *Bam*HI, respectively. The resulting PSA–pBLCAT3 and *hKLK2*–pBLCAT3 are designated as PSA-624 and *hKLK2*-597, respectively. The pBLCAT3 and pBLCAT2 described below were constructed originally by Luckow and Schutz (1987).

A series of 5′ deletion fragments of *hKLK2* 5′ flanking DNA was generated, and the fragments were designated as *hKLK2*-468, *hKLK2*-323, *hKLK2*-171, and *hKLK2*-158, indicating their 5′-most ends relative to the cap site of the *hKLK2* transcript. Except for *hKLK2*-468, the rest of the constructs were produced by a PCR reaction with 5′ end sense primers 5′-CGACTCTAGAAAGAATCAGTGATCATC-CCAAC-3′, 5′-CGACTCTAGAGGAACAGCAAGTGCTG-GCTCT-3′, and 5′-CGACTCTAGATGCTGGCTCTCCCC-TTCCACA-3′, 3′ end anti-sense primer 5′-CGGATCCGT-GTCCACGGCCAGGTGGTC-3′, and *hKLK2*-597 as a template. As noted, the sense primer contains an *Xba*I sequence at the 5′ end, and the anti-sense primers contain a *Bam*HI sequence at the 3′ end. The PCR products generated with the above primers were treated with *Xba*I plus *Bam*HI enzymes, gel-purified, and ligated with *Xba*I plus *Bam*I digested pBLCAT3. For the *hKLK2*-468 construct, *hKLK2*-597 was digested with *Sph*I enzyme to remove the 5′ portion of the *hKLK*-613 DNA. The resulting plasmid was ligated with T4 ligase to form *hKLK2*-468.

The 5′ half-site of the putative *hKLK2* ARE was deleted from *hKLK2*-597 to produce *hKLK2*-597Δ as follows: Primers 5′-CGACTCTAGACTGTAATCTCATCACTTTGGA-3′ and 5′-ACATACTAGATCAGTCTGGAG-3′ as well as primers 5′-CAAGTGCTGGCTCTCCCTCCC-3′ and 5′-CGGATCCGTGTCCACGGCCAGGTGGTC-3′ were used to generate two respective DNA fragments in a PCR with *hKLK2*-597 as template. These two DNA fragments correspond to the 5′ and 3′ portions of the *hKLK2*-597 fragment relative to the position of the putative ARE. The 5′ fragment contains an *Xba*I sequence at its 5′ end. The 3′ fragment contains a *Bam*HI sequence. These two fragments were digested with *Xba*I and *Bam*HI, respectively, phosphorylated with T4 polynucleotide kinase, and ligated with *Xba*I plus *Bam*HI digested pBLCAT3. To generate the *hKLK2*-323Δ construct, primers 5′-CGACTCTAGAAAGAATCAGT-GATCATCCCAAC-3′ and 5′-CGGATCCGTGTCCACG-GCCAGGTGGTC-3′ were used in a PCR with *hKLK2*-597Δ as the template. The PCR product was digested with *Xba*I plus *Bam*HI enzymes and ligated with *Xba*I plus *Bam*HI digested pBLCAT3.

To generate ARE–tk–CAT constructs, the sense strands of the oligonucleotides, containing two or three tandem repeats of putative *hKLK2* ARE (5′-GGAACAGCAAGTGCT-3′) flanked with a *Bam*HI sequence at both ends, were synthesized and annealed with complementary oligonucleotides to form a double-stranded DNA. A construct with three tandem repeats of the putative PSA ARE (5′-AGAACAGCAAGT-GCT-3′) flanked by *Bam*HI sequences at both ends was also synthesized. The *hKLK2* and PSA AREs differ by only one base pair. The double-stranded oligonucleotides containing the AREs were ligated with pBLCAT2 (tK promoter plus CAT gene). A double-stranded DNA–dideoxynucleotide chain termination technique was used to confirm the sequence in the above constructs.

Transfections. PC-3 cells were grown in RPMI 1640 medium with 5% fetal calf serum at 37 °C until the cells reached approximately 50% confluency. Cells were washed with TBS (10 mM Tris-HCl (pH 7.4) and 0.15 M NaCl) and transfected with plasmids containing *hKLK2* or PSA construct at concentrations ranging from 0 to 15 μg/plate, RSV β-galactosidase (β-gal) (5 μg/plate), and mouse AR (mAR) expression vector (1 μg/plate), using DEAE–dextran (0.05 mg/mL) in TBS for 30 min. The DEAE–dextran solution was removed, and 100 μM chloroquine diphosphate in RPMI 1640 was added to each plate. After 5 h, the chloroquine solution was removed and replaced with 1% charcoal–striped fetal calf serum RPMI 1640 either with or without 3.2 nM mibolerone (MIB). After 48 h, cells were collected and extracted for use in β-gal and CAT assays, following published methods (Sambrook et al., 1989). All treatments were prepared in duplicate or triplicate. Parental vector pBLCAT3 was used as a control in the transfection assays. For steroid specificity, PC-3 cells transfected with *hKLK2*-597 (10 μg/plate) were treated with the following steroids: 100 nM diethylstilbestrol (DES), 100 nM dexamethasone (DEX), 10 nM dihydrotestosterone (DHT), 3.2 nM MIB, 100 nM R5020, 50 nM testosterone (T), or 100 nM triamcinolone acetonide (TA).

The above procedure was also used to transfect PC-3 cells with *hKLK2*-597, pBLCAT2 containing two or three copies of *hKLK2* ARE, pBLCAT3 containing PSA promoter DNA (PSA-624), or pBLCAT2 containing three copies of PSA ARE and control (pBLCAT2 or pBLCAT3), β-gal, and mAR. Transfected cells were treated with either MIB (3.2 nM) or DHT at concentrations ranging from 0 to 100 nM.

RESULTS

In order to study the androgenic regulation of *hKLK2* and PSA gene expression in prostatic cells at the transcriptional level, the 5′ flanking regions of both genes (approximately 627 and 655 bp, respectively) were cloned from human genomic DNA by PCR and linked to a CAT reporter gene. A human prostatic adenocarcinoma cell line PC-3 was used in the gene-transfer experiment to investigate the androgenic inducibility of *hKLK2* and PSA promoters. Since PC-3 cells do not express the androgen receptor (Brolin et al., 1992), cells were cotransfected with an androgen receptor expression vector.

First, we determined optimum conditions for transfection. Dose response experiments were performed with varying concentrations of mibolerone (a synthetic androgen) and constant amounts of *hKLK2* or varying concentrations of *hKLK2* and constant mibolerone. The results in Figure 1a show that mibolerone at concentrations as low as 0.1 nM induced expression of CAT activity from the *hKLK2* promoter (*hKLK2*-597). The concentrations of mibolerone required to induce CAT activity are coincident with the affinity of the androgen receptor for its cognate ligand, which is less than 1 nM. Moreover, Figure 1b shows that 5-10 μg of plasmid DNA *hKLK2*-597 was required to produce optimal CAT

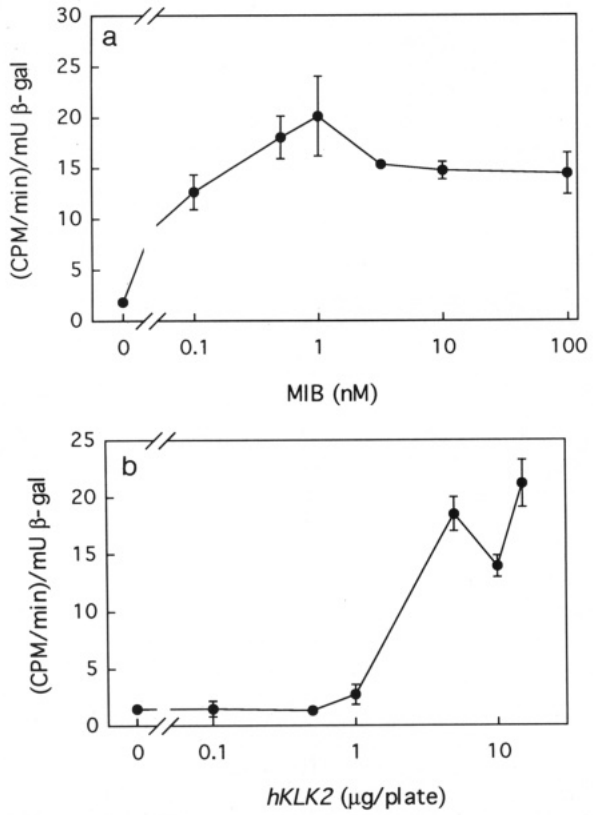

FIGURE 1: Concentration effects of *hKLK2*-597 and mibolerone in transfection assays. (a) PC-3 cells were transfected with *hKLK2*-597 at 5 μg/plate and treated with MIB at concentrations from 0 to 100 nM. Cell extracts for both experiments were assayed for β-galactosidase (β-gal) and CAT activity. CAT activity was normalized by β-gal and expressed as cpm/min/munits β-gal. Error bars indicate +1 standard error for three replicates in one experiment. (b) PC-3 cells were transfected with *hKLK2*-597 at concentrations ranging from 0 to 15 μg/plate and then treated with 3.2 nM MIB.

activity in the presence of androgen. Therefore, 5–10 μg of *hKLK2* promoter-linked CAT constructs with 3.2 nM mibolerone was used in subsequent experiments.

To demonstrate the androgen specificity of the *hKLK2* promoter, cells cotransfected with *hKLK2*-597 and androgen receptor vectors were treated with various steroid hormones. All of the androgens tested, including the naturally occurring androgens, testosterone and dihydrotestosterone, as well as the synthetic androgen, mibolerone, caused marked increases in CAT activity when compared to cells with no hormone treatment (Figure 2). Nonandrogenic compounds, including DES, a synthetic estrogen, DEX and TA, synthetic glucocorticoids, and R5020, a synthetic progestin, showed little or no inducibility. The above studies studies strongly suggest that *hKLK2* promoter confers androgen receptor-mediated gene transcription ability.

To identify regions of the *hKLK2* promoter that may have regulatory effects on androgen-induced gene expression, transfection experiments were performed with various 5′ deletion constructs of *hKLK2*-597. For each construct, CAT activity from cells treated with MIB were compared with that from untreated cells (Figure 3). The -597, -468, and -323 constructs all showed significant increases in CAT activity with MIB treatment compared to no hormone treatment (t-test, $p < 0.05$). Interestingly, removal of sequences between -468 and -323 resulted in levels of androgen-induced CAT activity more than 3-fold greater than that of *hKLK2*-597. The deletion construct *hKLK2*-171 still contained the putative ARE at its 5′-most end; however, this construct showed a

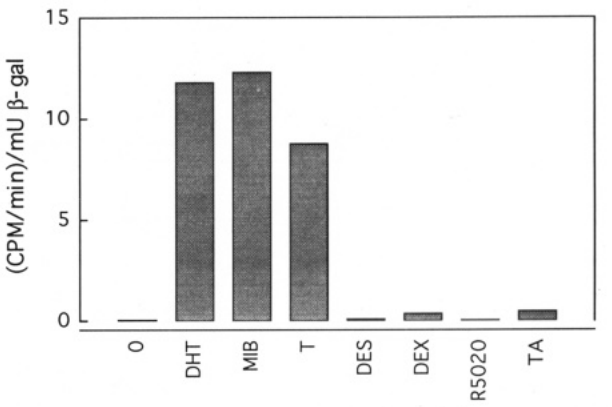

FIGURE 2: Steroid specificity of *hKLK2* gene induction. PC-3 cells were transfected with *hKLK2*-597 (10 μg/plate) and treated with one of the following steroids: 100 nM DES, 100 nM DEX, 10 nM DHT, 3.2 nM MIB, 100 nM R5020, 50 nM T, or 100 nM TA. Cell extracts were assayed for β-gal and CAT activity. CAT activity was normalized by β-gal activity and expressed as cpm/min/munits β-gal.

slight, but statistically insignificant, increase with mibolerone treatment over no treatment. The deletion construct *hKLK2*-158, with complete removal of the putative ARE, showed complete loss of androgenic inducibility. To verify that the putative ARE is functional in cells, the 5′ half-site (six base pairs) of the putative ARE was deleted from constructs *hKLK2*-597 and -323, designated as *hKLK2*-597Δ and -323Δ, respectively. As shown in Figure 3, deletion of the half-site of the ARE completely knocked out the androgenic induction of CAT activity.

Additional evidence that the putative ARE being studied is functional was provided by experiments using multiple ARE constructs. Two or three copies of *hKLK2* or PSA ARE were linked with the herpes simplex viral thymidine kinase (tk) promoter–CAT gene (Figure 4). Transfection results showed that the inclusion of two copies of *hKLK2* ARE created androgen inducibility in the tk promoter (Figure 4). The inclusion of a third ARE nearly doubled the activity compared to just two AREs. This indicates that the putative ARE site identified in *hKLK2* is in fact a true ARE. The construct containing three PSA AREs also exhibited increased activity compared to the vector alone, but only about one-half that of the *hKLK2* ARE3. In addition, comparison of the hKLK2–597 and PSA-624 constructs shows *hKLK2* producing about 2-fold higher activity than PSA. These results indicate that the *hKLK2* ARE may be more potent than the PSA ARE, possibly due to the single nucleotide variation between the two ARE sequences. Moreover, treatment of *hKLK2* ARE3 with varying DHT concentrations showed that the ARE was inducible by androgen in a dose response manner, further strengthening the argument that the ARE is functional (Figure 5).

DISCUSSION

Previous studies have shown *hKLK2* mRNA expression to be under androgenic regulation (Young et al. 1992). In this article, we have described the utilization of the androgen receptor-less human prostatic cell line PC-3 and an androgen receptor expression vector to further characterize the androgen regulatory properties of the *hKLK2* promoter. As demonstrated by both dose response and steroid specificity experiments, the *hKLK2* promoter confers the ability for androgen receptor-mediated gene induction in a ligand-specific manner. Among the three androgens tested, mibolerone seems to be the most effective, whereas testosterone is the least effective,

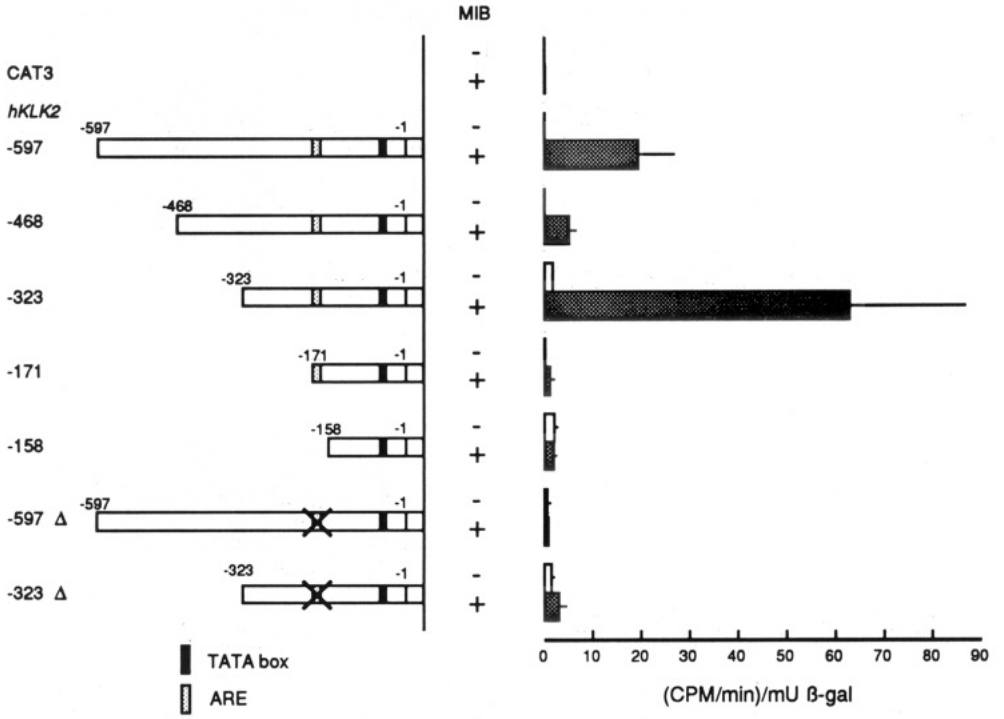

FIGURE 3: Androgen induction of *hKLK2* deletion constructs. Cells were transfected with either *hKLK2*-597 or one of its deletion constructs (8–10 μg/plate). For each construct, plates were treated in duplicate with (+) or without (–) 3.2 nM MIB. Cell extracts were assayed for β-gal and CAT activity, and data are expressed in cpm/min/munits β-gal. The left panel of the diagram is a schematic representation of the *hKLK2* promoter segment of each construct; the CAT3 vector structure is not shown. Numbers describing constructs denote base pair positions relative to the cap site. The X through the ARE site in constructs -597Δ and -323Δ indicates that the 5′ half-site of the ARE sequence was deleted from that construct. The right panel of the diagram shows the results of the CAT assay. Error bars indicate the standard error of its mean for three assays.

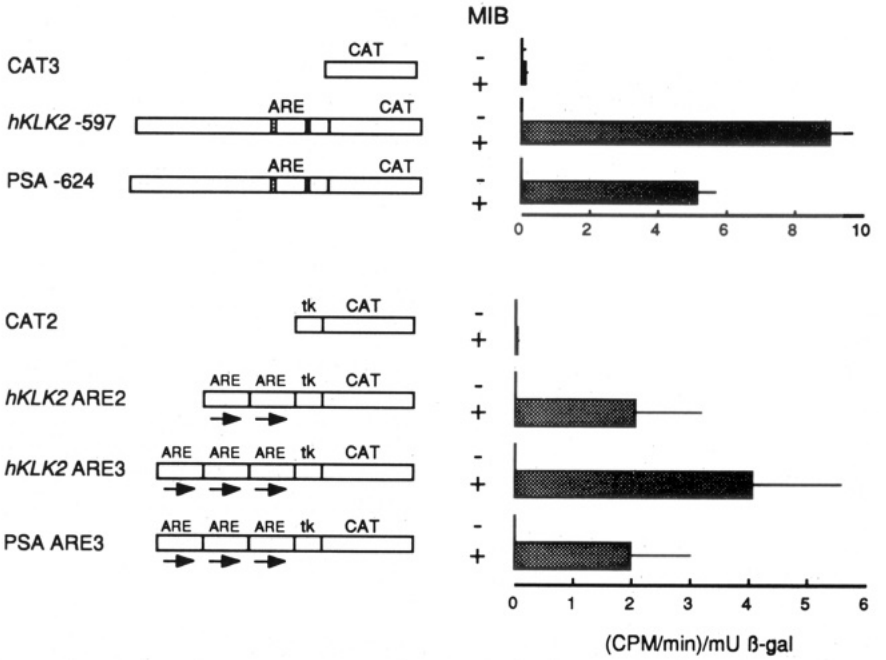

FIGURE 4: Androgen induction of multiple ARE constructs. PC3 cells were transfected with one of the following constructs (5-10 μg/plate): *hKLK2*-597 or PSA-624 promoter construct, *hKLK2* ARE2, *hKLK2* ARE3, PSA ARE3, pBLCAT2, or pBLCAT3. Cultures were treated, in duplicate, with or without 3.2 nM MIB. Cell extracts were assayed for β-gal and CAT activity, and the results are expressed in cpm/min/munits β-gal. The left side of the diagram represents each construct. The ARE sequences in the multiple ARE constructs were cloned into the CAT2 vector in the same orientation as in the *hKLK2* or PSA promoter construct, indicated by the arrows. The right side of the diagram shows the results of the CAT assay. Error bars represent the standard error of the mean for four repetitions of the experiment.

which correlates with their affinities for the androgen receptor (Grino et al., 1990). It has also been shown (Murthy et al., 1986) that prostate cells contain steroid-metabolizing enzymes that can convert active androgens to nonandrogenic steroids (e.g., estrogen) or inactive androgens. Moreover, mibolerone is a nonmetabolizable androgen (Murthy et al., 1986). Therefore, both the affinity and metabolic properties of these three androgens may contribute to their efficiency of CAT induction in this study.

Many of the mouse kallikrein genes in the salivary gland are up-regulated by androgens *in vivo*. The slow rate of androgenic induction of the mouse kallikrein genes (van

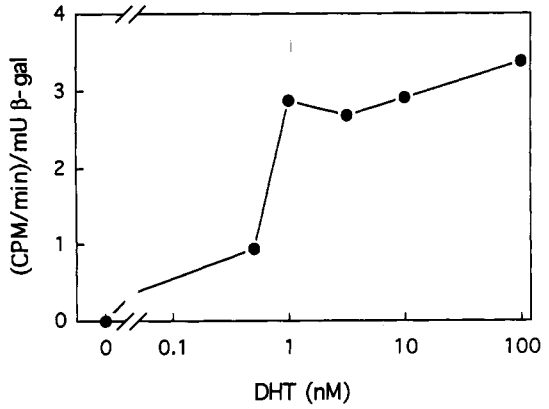

FIGURE 5: Dose response of DHT inducibility of the *hKLK2* ARE3 construct. Cells were transfected with ARE3 (8 μg/plate) and treated with DHT at concentrations ranging from 0 to 100 nM. Cell extracts were assayed for β-gal and CAT activity, and the results are expressed in cpm/min/munits β-gal.

Leekwen et al., 1987) suggests that these effects may be mediated at the post-transcriptional level, but the mechanism remains to be elucidated. *In situ* hybridization has revealed that these murine genes are expressed at different levels (van Leekwen et al., 1987). *In situ* hybridization studies from our laboratories demonstrated that both *hKLK2* and *hKLK3* (PSA) are expressed at different levels in prostate epithelial cells.

Sequence analysis of *hKLK2* reveals an ARE-like element approximately 160 base pairs upstream of the cap site (Schedlich et al., 1987). Deletion of the 5′ half-site of this ARE from the *hKLK2*-597 promoter region and from the -323 segment virtually eliminated androgen-induced gene expression. The removal of the 5′ region, including the ARE in construct -158, also eliminates androgen inducibility. Furthermore, the insertion of this putative ARE into a thymidine kinase promoter–CAT construct resulted in androgen receptor-mediated induction of CAT expression when stimulated with androgen. These results clearly demonstrate that the putative site is indeed acting as a functional ARE.

Comparison of the putative ARE sequences in *hKLK2* or PSA with the consensus glucocorticoid responsive element (GRE; GGTACAnnnTGTTCT) (Beato, 1989) indicates that the *hKLK2* ARE is less homologous with the consensus GRE than the PSA ARE. Moreover, the *hKLK2* ARE is a less perfect palindrome than the PSA ARE. It has been reported in this (Young et al., 1992) and other (Wolf et al., 1992) laboratories that PSA mRNA levels are higher than that for *hKLK2* in prostate tissues, as determined by *in situ* hybridization and Northern blot analysis. Moreover, androgenic stimulation of LNCaP cells results in greater expression of PSA mRNA than that for *hKLK2*. These results have led us to predict that PSA ARE might be more potent than *hKLK2* ARE. Surprisingly, the results shown in Figure 4 suggest that *hKLK2* ARE has higher androgenic inducibility when compared to PSA ARE under the conditions used. The discrepancy between the results produced in this study and mRNA levels for *hKLK2* and PSA in previous studies could be due to the involvement of other regulatory components in the androgenic regulation of PSA and *hKLK2* genes. Other cis-acting elements may reside in or nearby the *hKLK2* and PSA genes, but not in the promoter regions used in this study.

The deletion of the sequence upstream of the -323 position in *hKLK2* revealed a putative negative regulatory element. Comparison with the *hKLK2*-597 construct shows this regulatory element to reduce, but not completely eliminate, androgen-induced activity. Sequence analysis revealed that the promoter region of *hKLK2* contains an Alu sequence from -499 to -320. A negative regulatory element (termed the "reducer" element) has also been found within a member of the African green monkey Alu family of interspersed sequences (Saffer & Thurston, 1989). Thus, the *hKLK2* Alu sequence may also contain a reducer-like negative regulatory element.

Interestingly, although the deletion construct *hKLK2*-171 still retains the putative ARE at its 5′-most end, androgen-dependent induction of CAT activity was largely reduced. This observation suggests that ARE interaction with sequences upstream of the ARE may be required for optimal androgenic induction. It raises the possibility that the androgen receptor may interact with a transcription factor(s) bound to the upstream sequence, thereby opimizing androgen-regulated gene expression.

In summary, we have identified a functional ARE in the *hKLK2* 5′ flanking promoter region by gene-transfer assays. It appears that the androgen receptor and the ARE complex are the major factors required for *hKLK2* gene expression in the region examined. Deletion of the 5′ half-site of the ARE was sufficient to abolish the androgen-inducted transcription. Multiple AREs were able to reconstitute the androgen induction of a heterologous gene. On the other hand, a single ARE appears to be insufficient to drive the androgenic induction unless upstream sequences participate in the induction process. It will be important to identify and fully characterize those potential cis elements and transactivators to obtain more insight into the mechanism of androgen action in the prostate.

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