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Direct Progesterone Receptor and Indirect Androgen Receptor Interactions with the Kallikrein-Related Peptidase 4 Gene Promoter in Breast and Prostate Cancer

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Abstract

Kallikrein 4 (*KLK4*) is a member of the human *KLK* gene family of serine proteases, many of which are implicated in hormone-dependent cancers. Like other *KLKs*, such as *KLK3/PSA* and *KLK2*, *KLK4* gene expression is also regulated by steroid hormones in hormone-dependent cancers, although the transcriptional mechanisms are ill defined. Here, we have investigated the mechanisms mediating the hormonal regulation of *KLK4* in breast (T47D) and prostate (LNCaP and 22Rv1) cancer cells. We have shown that *KLK4* is only expressed in breast and prostate cancers that express the progesterone receptor (PR) and androgen receptor (AR), respectively. Expression analysis in PR- and AR-positive cells showed that the two predominant *KLK4* variants that use either TIS1 or TIS2a/b are both up-regulated by progesterone in T47D cells and androgens in LNCaP cells. Two putative hormone response elements, K4.pPRE and K4.pARE at -2419 bp and -1005 bp, respectively, were identified *in silico*. Electrophoretic mobility shift assays and luciferase reporter experiments suggest that neither K4.pARE nor ~2.8 kb of the *KLK4* promoter interacts directly with the AR to mediate *KLK4* expression in LNCaP and 22Rv1 cells. However, we have shown that K4.pPRE interacts directly with the PR to up-regulate *KLK4* gene expression in T47D cells. Further, chromatin immunoprecipitation experiments showed a time-

dependent recruitment of the PR to the *KLK4* promoter (-2496 to -2283), which harbors K4.pPRE. This is the first study to show that progesterone-regulated *KLK4* expression in T47D cells is mediated partly by a hormone response element (K4.pPRE) at -2419 bp. (Mol Cancer Res 2009;7(1):129-41)

Introduction

Breast cancer and prostate cancer are significant diseases in Western countries, accounting for ~30% of all newly diagnosed cancers for 2006 (1). Although the etiology of both diseases is not fully understood, there are some similarities in the epidemiology and pathogenesis between breast and prostate cancers. For example, the incidence of breast and prostate cancers are reported to be higher in Caucasians and African Americans when compared with women and men with Asian ancestry (2, 3). However, the most significant association is that in a majority of cases, both breast and prostate cancers are regulated by hormones in the initial stages of disease and current therapeutic options involve targeting the estrogen receptor (ER) and androgen receptor (AR) signaling axes, respectively (4, 5). Consequently, much of the research into breast and prostate cancers has focused on genes that are involved in the estrogen and androgen signaling pathways. However, despite extensive research efforts, the precise molecular events leading to the initiation and progression of these diseases are still largely unknown.

Steroid hormones, such as estrogen, progesterone, and androgens, mediate their cellular effects through their cognate receptors. Upon ligand binding, steroid receptors participate in a sequence of events that ultimately result in the translocation of the receptor into the cell nucleus whereupon it binds to hormone response elements (HRE) in the promoters of target genes to initiate transcription. One such family of hormone target genes that are regulated by steroid hormones in prostatic, breast, and endometrial cells, and which are thought to play a role in the progression to neoplasia, are the tissue kallikrein-related (*KLK*) peptidases (6, 7). The *KLKs* are a multigene family of serine proteases that are involved in the posttranslational processing of polypeptide precursors to their biologically active forms (6, 7), a function that is central to a number of biological events. Additionally, *KLKs* can activate proteases such as urokinase-type plasminogen activator and cleave structural proteins of the extracellular matrix (6, 7), which are critical events in tumorigenesis, invasion, and metastasis.

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KLK4 is a relatively recently described *KLK* family member (8-11). Although the porcine and mouse orthologue of *KLK4*, enamel matrix serine protease-1 (EMSP-1), plays a role in tooth development by degrading amelogenin, which is the predominant protein in the enamel matrix of developing teeth (12), the physiologic role of *KLK4* remains to be established. By analogy, *KLK4* may have similar extracellular matrix remodeling properties and potentially facilitate in local invasion and/or metastatic progression of carcinomas. In support of this notion, *KLK4* can activate pro-PSA and single-chain urokinase-type plasminogen activator (13) and recombinant *KLK4* also degrades prostatic acid phosphatase (13) and members of the insulin-like growth factor binding protein family (14). Collectively, this suggests that *KLK4* may have an important role in prostate tumorigenesis.

At the mRNA level, *KLK4* has been shown to be up-regulated by androgens, estradiol, progesterone, and glucocorticoids in prostate cancer cells (10, 15) and by the synthetic progestin (norgestrel) in BT-474 breast cancer cells (16). Further, *KLK4* protein levels are increased by as much as 4-fold in response to estradiol in ovarian cancer cells (17) and by 40-fold in response to estradiol and progesterone in endometrial cancer cells (18). However, functional HREs have yet to be characterized for the *KLK4* promoter, although putative AREs have been predicted using *in silico* modeling (11). In fact, functional HREs mediating hormone-induced gene expression of the *KLKs* have only been identified in the promoter and enhancers for the *KLK2* and *KLK3* (prostate-specific antigen, *PSA*) genes (19-27).

To date, the promoter of the *KLK4* gene has not been well defined with two groups mapping alternative transcription initiation sites (TIS) using 5'-random amplification of complementary ends (5'-RACE; refs. 9, 15). Initially, the full-length *KLK4* transcript was reported to be derived from the classic five exons (10). However, subsequent studies have identified a variant transcript that begins in exon 2 (15).

The studies reported here were designed to further characterize the hormonal gene regulation of *KLK4* in breast cancer (T47D) and prostate (LNCaP and 22RV1) cancer cells. We confirm that the predominantly used TIS for *KLK4* gene expression in breast and prostate cancer cells is similar to that previously reported in exon 2 (15). We have shown that the *KLK4* promoter region encompassing 2.8 kb from this TIS is not responsible for the observed androgen regulation of the *KLK4* gene expression in LNCaP and 22RV1 prostate cancer cells. However, our analysis suggests that a progesterone-responsive region in this 2.8 kb of the *KLK4* promoter regulates progesterone-induced *KLK4* gene expression in the T47D breast cancer cells.

Results

Hormonal Regulation of KLK4 Gene Expression in Breast and Prostate Cancer Cells

The association of *KLK4* gene expression with progesterone receptor (*PR*) and *AR* status was assessed for four breast and seven prostate cell lines using reverse transcription-PCR (RT-PCR). Figure 1A shows that *KLK4* is expressed in one breast (T47D) and three prostate (LNCaP, 22RV1, and MDA-

PCa-2b) cell lines. Importantly, *KLK4* was found to be only expressed in *PR*-positive breast and *AR*-positive prostate cells (Fig. 1A). Consequently, the progesterone and androgen regulation of *KLK4* was further investigated using quantitative RT-PCR in T47D and LNCaP cells, respectively.

Treatment of T47D cells with 10 nmol/L of progesterone over 24 hours resulted in a 1.38-fold ($P < 0.01$) increase in *KLK4* mRNA and a 1.65-fold ($P < 0.01$) response for *HSD11B2*, a prototypical progesterone-regulated gene (Fig. 1B). Further, the attenuation of *KLK4* and *HSD11B2* response by the *PR* antagonist RU486 shows that regulation of *KLK4* and *HSD11B2* by progesterone was mediated through the *PR* (Fig. 1B).

In LNCaP cells, *KLK4* expression was up-regulated by 3.2-fold ($P = 0.03$) after the cells were treated for 24 hours with 1 nmol/L of the synthetic androgen, R1881, when compared with the vehicle control (Fig. 1C). Androgen-induced up-regulation of *KLK4* expression was attenuated when cells were treated with 1 μ mol/L bicalutamide, an *AR* antagonist, which suggests that the observed regulation is mediated through the *AR* (Fig. 1B). The *KLK4* response to androgen \pm antagonist was similar to the *PSA/KLK3* gene (positive control for androgen regulation), although *PSA* had a much higher response to androgen treatment (~ 8 -fold, $P < 0.05$; Fig. 1C).

To further validate the hormonal regulation of *KLK4* gene expression, quantitative RT-PCR (RT-qPCR) was also carried out on T47D and LNCaP cells that were cultured in hormone-depleted medium. After maintaining both T47D and LNCaP cells in medium containing 10% charcoal-stripped FCS for 4 days, *KLK4* expression was found to be down-regulated by 4.2-fold ($P = 0.01$) and 2.4-fold ($P < 0.01$), respectively, compared with cells grown in regular FCS (Fig. 1D and E).

Identification of the TIS for KLK4 in Breast Cancer and Prostate Cancer Cells

To identify potential HREs in the *KLK4* promoter, it was essential to first map the location of the *KLK4* TIS for breast cancer (T47D) and prostate cancer (LNCaP) cells. Using 5'-RLM-RACE, a *KLK4* TIS in LNCaP cells (TIS2b) was found downstream of the originally reported TIS1 (9) and 43 bp upstream of the *KLK4* exon 2 ATG. In T47D cells, the TIS (TIS2a) was 35 bp longer and situated 78 bp upstream of the exon 2 ATG (Fig. 2A-C). Transcripts generated from these TISs would be derived from just four exons and thus are shorter than the published full-length transcript (Fig. 2D) but similar to that reported in LNCaP cells (15). The relative expression of transcripts arising from each TIS, as well as the hormonal regulation of these variants in T47D and LNCaP cells, was assessed using RT-qPCR. Figure 2E shows that transcripts using TIS1 have markedly lower expression when compared with transcripts that use TIS2a or TIS2b for T47D or LNCaP cells. Yet, despite their differing abundance, similar fold increases of both variants were observed with androgen and progesterone treatment.

Identification of Putative HREs in the Promoter Region of the KLK4 Gene

Given our observations that *KLK4* gene expression is regulated by progesterone and androgens in breast and prostate

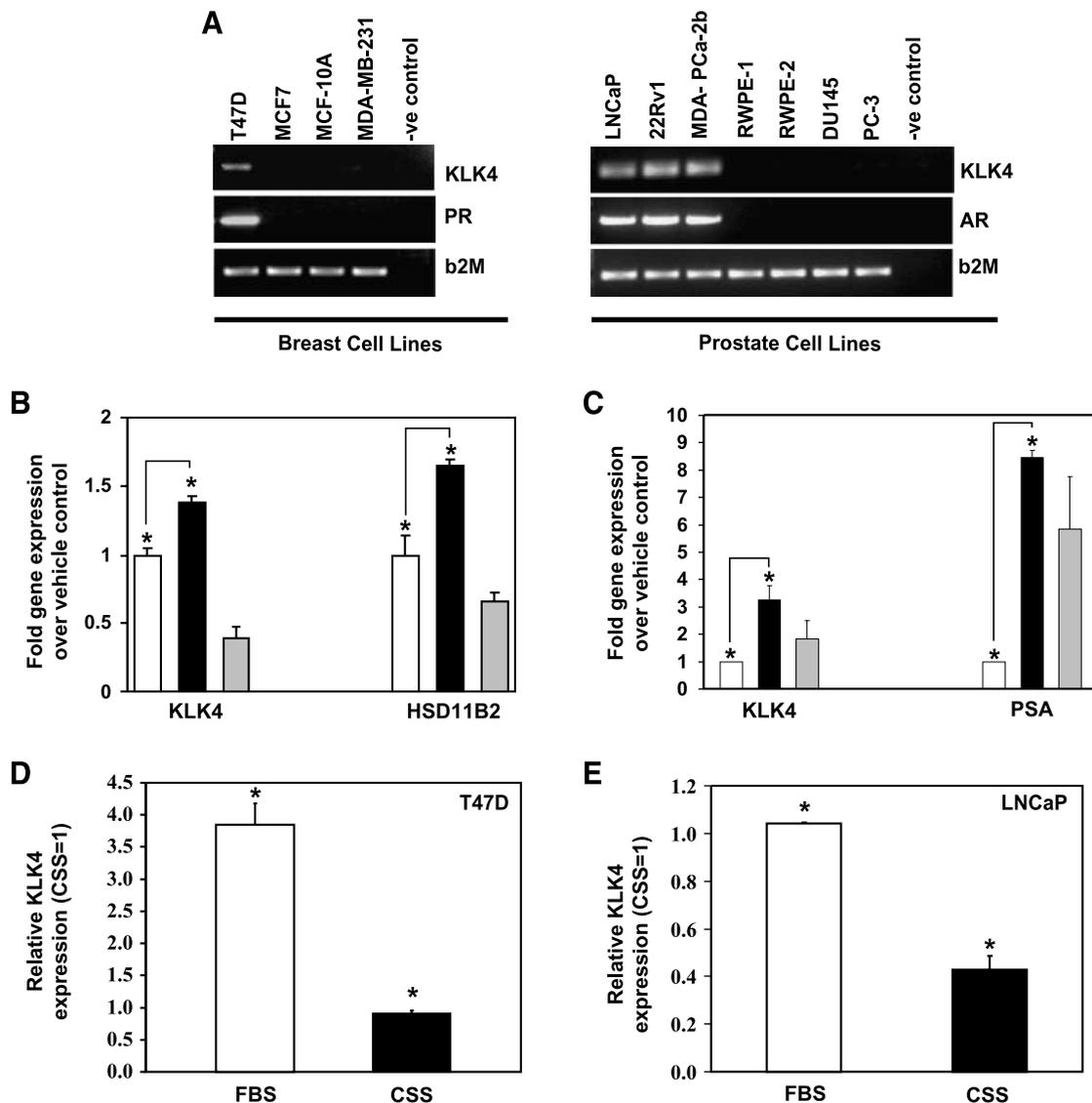


FIGURE 1. Hormonal regulation of *KLK4* in breast and prostate cancer cells. **A.** Expression of total *KLK4*, *PR*, *AR*, and β 2M was assessed in four breast and seven prostate cell lines using RT-PCR. **B.** Progesterone regulation of *KLK4* in breast cancer (T47D) cells. Cells were treated for 24 h with ethanol (white columns) or 10 nmol/L progesterone (black columns) + 1 μ mol/L of the PR antagonist RU486 (gray columns). The expression of total *KLK4* and *HSD11B2*, a positive control for progesterone responsiveness, was determined using RT-qPCR. **C.** Androgen regulation of *KLK4* in prostate cancer (LNCaP) cells. Cells were treated for 24 h with ethanol (white columns) or 1 nmol/L of the synthetic androgen, R1881 (black columns) + 1 μ mol/L of the AR antagonist, bicalutamide (gray columns). The *KLK3/PSA* gene was used as a positive control. Relative *KLK4* gene expression was also assessed in T47D (**D**) and LNCaP (**E**) cells that were maintained for 4 d in hormone-depleted medium (CSS) versus cells that were maintained in basal medium with hormones (FBS). All data are represented as the fold changes in gene expression over vehicle control (0.1% ethanol) or relative to 18S. Columns, mean from three independent experiments that were each carried out in duplicate or triplicate; bars, SE. *, $P < 0.05$, significant difference between treatments.

cancer cells, we carried out *in silico* analyses to identify HREs within a region encompassing ~ 3 kb of the *KLK4* promoter from TIS2b that may mediate these responses. Using four different gene analysis programs, we identified two putative HRES (K4.pPRE and K4.pARE) that have consensus-like half sites, which could interact directly with class I steroid hormone receptors (androgen, progesterone, mineralocorticoid, and glucocorticoid receptors; Fig. 3A). The K4.pPRE sequence (5'-AGAACAAtgagagAGAACA-3') consists of a direct repeat motif that is separated by six nucleotides and is located 2,419 bp upstream of the TIS2b identified here in LNCaP cells and 397 bp upstream from the previously reported TIS1. The

K4.pARE sequence (5'-GGTGCAGgaGATTGT-3'), located at -1005 bp from TIS2b (1017 bp downstream from TIS1), more closely resembles the classic HRE motif that is traditionally characterized by a palindromic hexameric repeat sequence that is separated by three nucleotides.

K4.pARE Binds Indirectly with the Androgen Receptor

Electrophoretic mobility shift assays (EMSA) were carried out with the K4.pARE to determine if the element bound to endogenous AR or recombinant AR-DBD. Incubation of K4.pARE with LNCaP nuclear extracts from cells that were

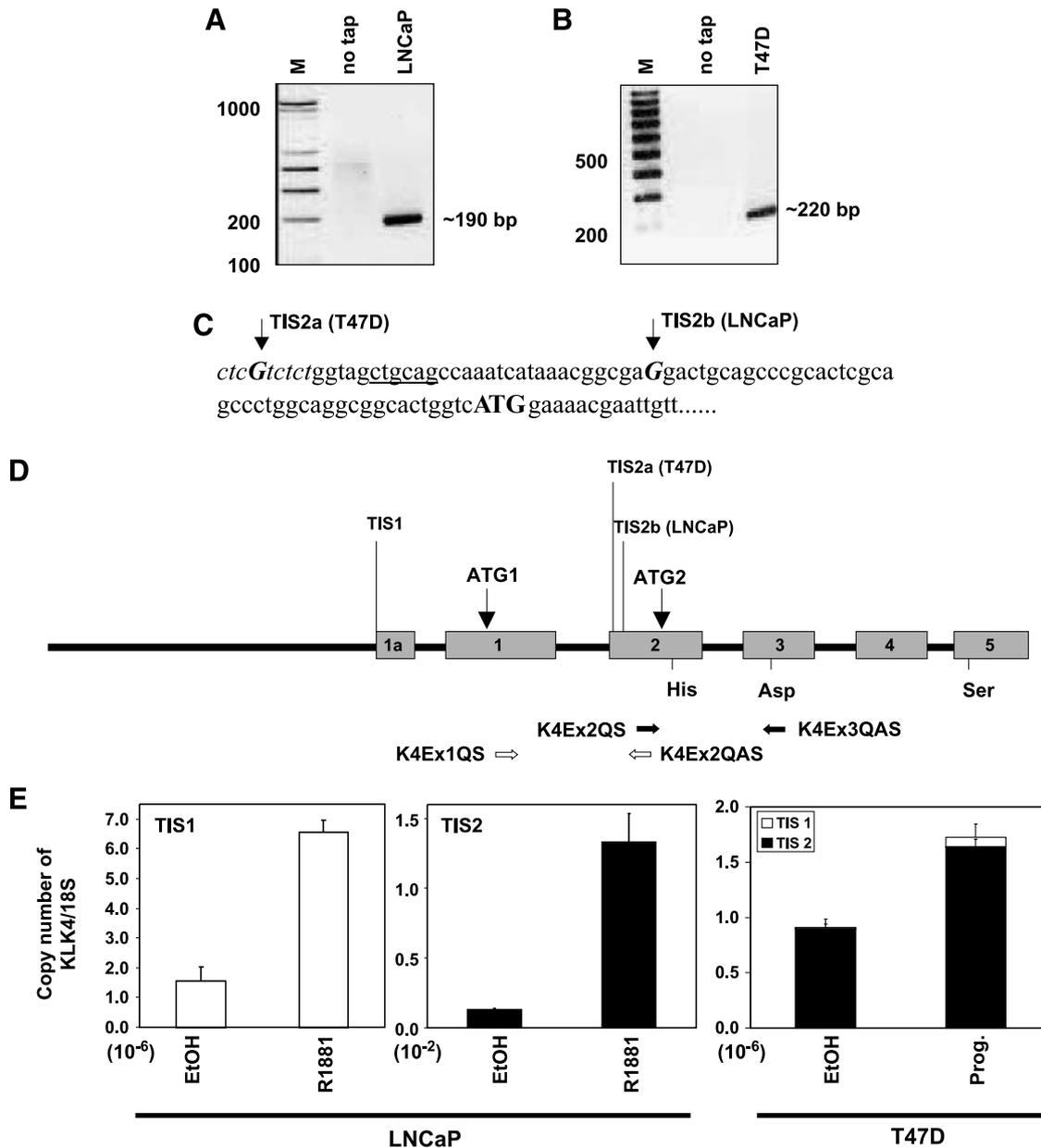


FIGURE 2. Characterization of the *KLK4* promoter. 5'-RLM-RACE was carried out to identify the predominant *KLK4* TIS in (A) prostate (*LNCaP*) and (B) breast (*T47D*) cancer cells. M, DNA marker; no tap, negative TAP control. 5'-RLM-RACE products were ~190 and 220 bp for *LNCaP* and *T47D*, respectively. C. *KLK4* 5'-untranslated region sequence data showing the TIS (**bold, capitalized, and italicized G**) in *T47D* (*TIS2a*) and *LNCaP* (*TIS2b*) cells. The start of the coding region in exon 2 is shown as ATG (**capitalized**). A palindromic sequence 5'-ctgcag-3' (underlined) represents a region that is statistically overrepresented in 5'-untranslated regions. The italicized sequence *ctcgtctct* represents a putative initiator element. D. Diagram of the genomic structure of the *KLK4* gene showing the two potential TISs (*TIS1* and *TIS2a/b*). Putative ATG start sites for translation (*ATG1* and *ATG2*) and the coding structure of the *KLK4* copy numbers relative to 18S from three independent experiments that were each performed in duplicate; bars, SE. Quantitative RT-PCR was carried out on RNA extracted from cells maintained in either 1 nmol/L R1881, 10 nmol/L progesterone, or vehicle control (*EtOH*) for 24 h using TIS-specific primers. The relative expression of the larger *TIS1* variants was subtracted from the total expression of *TIS2a/b* variants to calculate the absolute relative expression of *TIS2a/b* variants.

treated with R1881 for 12 hours resulted in the formation of two specific shifts (Fig. 3B). These shifts were also observed in experiments where cells were incubated with *PSA* AREI. A decrease in shift intensity, particularly for the lower shift, in experiments where cells were incubated with 200- and 400-fold

excess unlabeled K4.pARE suggests that these complexes are specific (Fig. 3B). To determine whether these shifts may represent AR/ARE complexes, blocked shift analysis using 1 and 2 μ g of AR antibody was carried out. Incubation of both the *PSA* ARE I and the K4.pARE, with increasing amounts of

AR antibody, decreased the shift intensity for both shifts (Fig. 3B). The blocking of these shifts by the AR antibody suggests these complexes may be formed by interaction of the AR with K4.pARE. The specificity of K4.pARE was further shown by abrogating the binding of the lower receptor complex with the AR antibody but not by the nonspecific rabbit IgG antibody in experiments in which LNCaP cells were treated for 24 hours with 1 nmol/L R1881 (Fig. 3C). Both shifts for K4.pARE were identical to the *PSA* ARE I control and further supports the hypothesis that K4.pARE is forming similar DNA/receptor complexes to those observed for *PSA* ARE I. The differences in shift intensity and blocking patterns between the two blocking shift experiments likely represent the dynamic receptor/DNA complexes formed from the different treatment times and the consequent accessibility of the AR antibody to the AR. These blocked shifts were also observed using the N-20 AR antibody (Supplementary Fig. S1). However, EMSA analysis of K4.pARE with purified AR-DBD did not result in the formation of any high molecular complexes that are indicative of receptor/DNA complexes, although there was a clear *PSA* ARE I/AR-DBD complex formed (Fig. 3D). Further, competition of the *PSA* ARE I reactions with increasing amounts (5- to 40-fold molar excess) of unlabeled K4.pARE did not result in any observed abrogation of *PSA* ARE I binding with the AR-DBD.

K4.pARE Is Not Responsive to Androgens in LNCaP and 22Rv1 Cells

Reporter assays for androgen responsiveness of K4.pARE were carried out in both LNCaP and 22Rv1 cells as the blocked shift assays suggest that K4.pARE may be interacting with the AR in some way, although not directly with the AR-DBD. Three tandem repeats of the putative K4.pARE and its flanking sequences were cloned into the pGL3-Promoter vector (K4.pAREX3-Luc) to enhance the hormonal responsiveness for this element (Fig. 4A). In this assay, the pGL3-Promoter was not significantly regulated by R1881 in either LNCaP ($P = 0.074$) or 22Rv1 ($P = 0.12$) cells. For *PSA* AREX3-A-Luc, treatment with R1881 resulted in a 3.9- and 4.1-fold up-regulation of promoter activity over the vehicle control in 22Rv1 ($P = 0.011$) and LNCaP cells ($P = 0.037$), respectively, confirming that the cells were appropriately sensitive to androgens (Fig. 4B). For K4.pAREX3-Luc, there was no difference in promoter activity in LNCaP or 22Rv1 cells (Fig. 4B).

The Proximal *KLK4* Promoter Region Is Not Responsive to Androgens

Seven deletion luciferase promoter constructs that encompass up to 2.8 kb of the *KLK4* promoter from TIS2b were

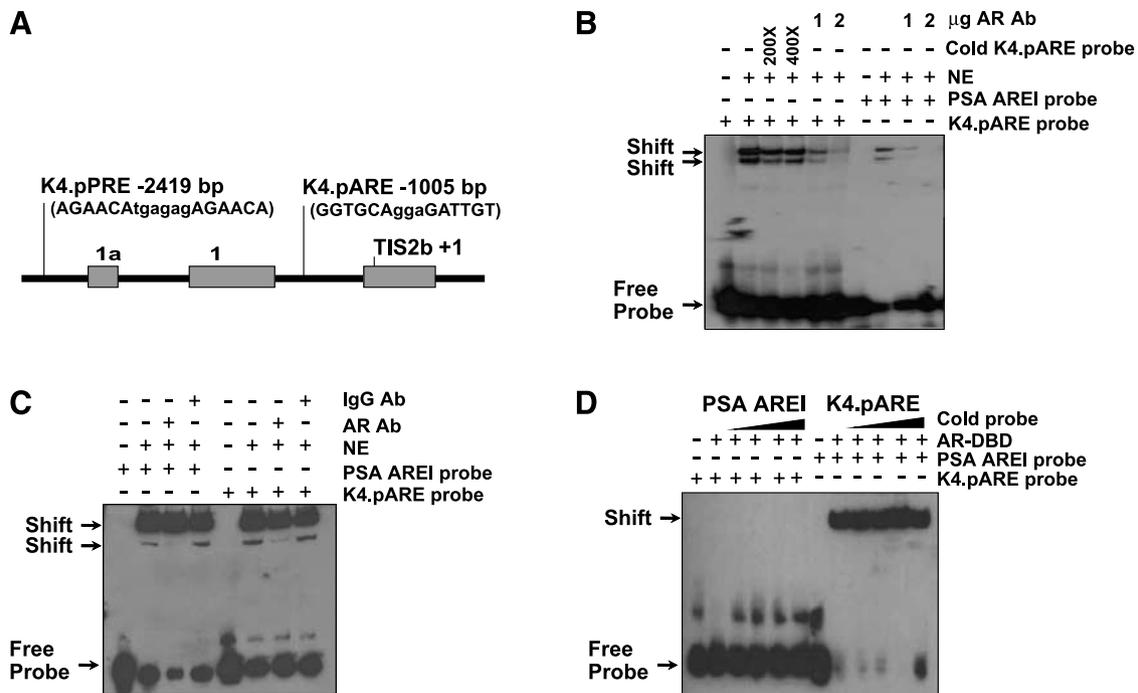


FIGURE 3. EMSA and blocked shift analyses of K4.pARE. **A.** Two putative HRE (*K4.pPRE* and *K4.pARE*) sequences that were predicted by *in silico* modeling are shown with their positions, -2419 bp and -1005 bp, respectively, relative to TIS2b. K4.pARE was incubated with nuclear proteins that were extracted from LNCaP cells treated with 1 nmol/L R1881 for 12 h. **B.** Incubation of K4.pARE with nuclear extracts (NE) resulted in the formation of two specific shifts that were also present in lanes that were probed with *PSA* ARE I. The intensity of these specific shifts (for both the *KLK4* and *PSA* probes) was partially or completely diminished when molar excess (200×, 400×) unlabeled (Cold) K4.pARE or 1 and 2 μg of androgen receptor antibody (AR Ab) was added to the reactions. **C.** The specificity of K4.pARE and *PSA* ARE I shifts are shown by blocking of the lower shift by the AR antibody but not by the nonspecific IgG antibody (IgG Ab) in experiments carried out on nuclear proteins from LNCaP cells treated with 1 nmol/L R1881 for 24 h. **D.** EMSA was carried out with 0.2 μg of purified AR-DBD. A specific shift was only observed in lanes from *PSA* ARE I/AR-DBD reactions. Competition with increasing amounts of molar excess (5×–40×) unlabeled K4.pARE did not outcompete binding of *PSA* ARE I with the AR-DBD. Labeled K4.pARE was also incubated with increasing amounts (5×–40×) of unlabeled *PSA* ARE I probes.

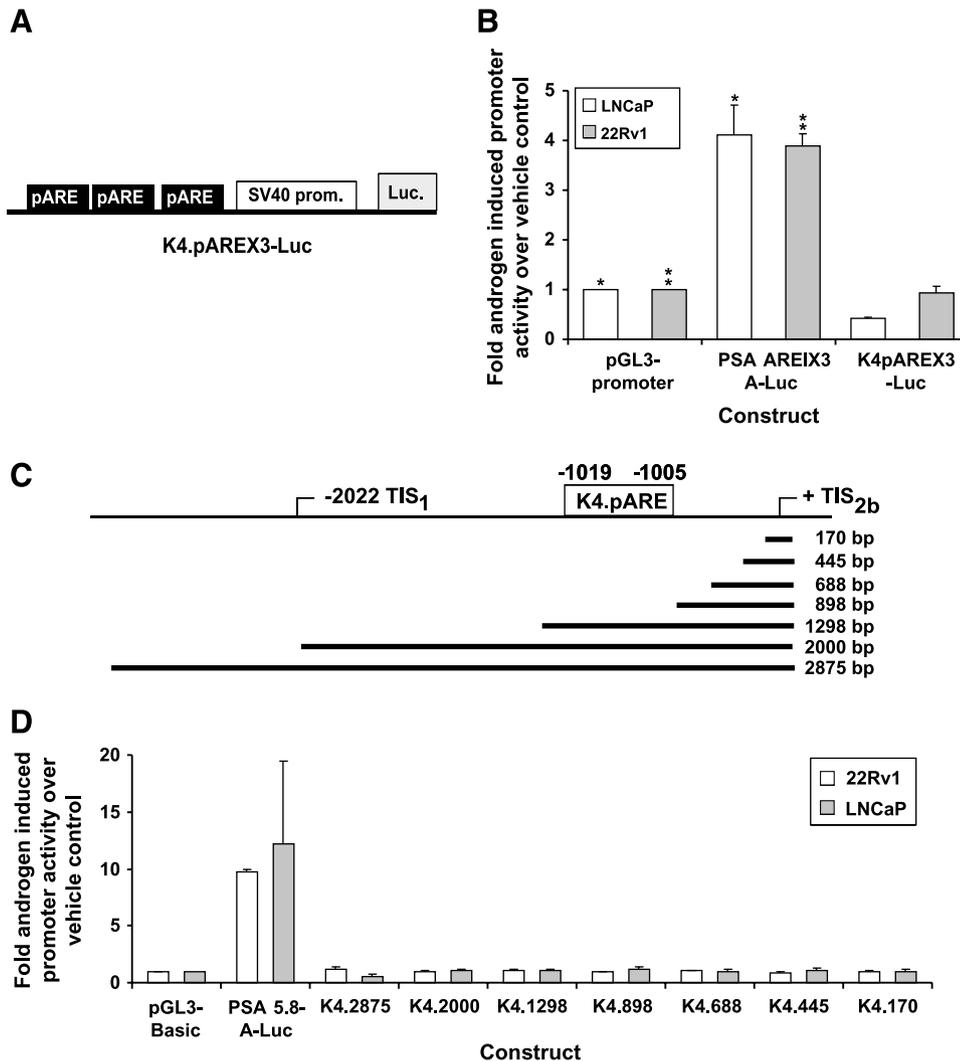


FIGURE 4. Androgen responsiveness of the *KLK4* promoter in prostate cancer cells. Androgen (1 nmol/L R1881) regulation of the *KLK4* putative ARE was carried out by luciferase reporter assays using (A) a construct comprising three tandem copies of the putative *KLK4* ARE (*K4.pARE-Luc*) in (B) both LNCaP and 22Rv1 cells. pARE, putative ARE; SV40 prom., SV40 mammalian viral promoter; Luc, luciferase gene. C. Diagram of the seven *KLK4* promoter constructs used in luciferase reporter assays relative to the two *KLK4* TISs. Construct sizes are relative to TIS2b. D. Analysis of the androgen (1 nmol/L R1881) responsiveness of the *KLK4* promoter was carried in both LNCaP and 22Rv1 cells. All luciferase data are expressed as fold androgen-induced promoter activity over vehicle control (0.1% ethanol) and normalized to pGL3-Promoter or pGL3-Basic activity. Columns, mean from two independent experiments (each carried out in triplicate); bars, SE. *, $P < 0.05$, significant difference between constructs.

generated (Fig. 4C) to identify if other hormone-responsive regions could be identified and to determine whether K4.pARE needs to act synergistically with other *cis*-elements to mediate the *KLK4* gene expression seen at the mRNA level. Androgen regulation of the *KLK4* promoter was assessed in both LNCaP and 22Rv1 cells. R1881 (1 nmol/L) stimulated promoter activity for pGL3-Basic was 0.85- and 0.75-fold increased over vehicle control (0.1% ethanol) in 22Rv1 and LNCaP cells, respectively (Fig. 4D). Promoter activity for PSA 5.8-A-Luc was 9.7-fold increased in 22Rv1 cells and 12.2-fold increased in LNCaP cells after treatment with R1881. However, none of the seven *KLK4* promoter constructs transfected in either LNCaP or 22Rv1 cells (transfected for 6 hours and either for 24 hours) were significantly regulated ($P < 0.05$) by R1881 when compared with cells that were treated with vehicle control.

The Proximal *KLK4* Promoter Is Responsive to Progesterone

As there was no androgen regulation of the *KLK4* promoter in prostate cancer cells, the focus of the hormonal studies was shifted to the progesterone responsiveness observed in

T47D cells. Initial analysis was carried out using the K4.898, K4.2000, and K4.2875 promoter constructs (Fig. 5A). Significant basal promoter activity was observed for all three constructs (K4.2875, ~4-fold, $P < 0.05$; K4.2000, ~8-fold, $P < 0.05$; K4.898, ~3-fold, $P < 0.05$), when compared with the insert-less pGL3-Basic control (Fig. 5B). There was no significant increase in promoter activity for the K4.2000 and K4.898 constructs after cells were treated with 10 nmol/L progesterone for 24 hours. However, a modest but significant (~1.2-fold, $P < 0.05$) increase in K4.2875 promoter activity, which was comparable with that seen at the mRNA level, was observed after cells were treated with 10 nmol/L progesterone (Fig. 5B). Further, a trend of increased transcriptional activity of K4.2875 was observed in T47D cells that were treated with increasing amounts of progesterone (Fig. 5C).

The *KLK4* Progesterone Response Element Complexes with the Progesterone Receptor

As the progesterone-induced luciferase data support the *in silico* analysis, suggesting that the K4.pPRE may be functional, EMSA analysis was then carried out to assess the direct

interaction of K4.pPRE with the PR. Two shifts were observed with a time-specific shift of the upper band seen when the K4.pPRE was incubated with nuclear extracts from T47D cells that were treated with 10 nmol/L progesterone over 24 hours (Fig. 6A). This shift is likely to represent progesterone response element (PRE)/PR complexes as no shifts were observed for experiments that used nuclear proteins from the PR-negative breast cancer cell line MDA-MB-231 (Fig. 6B). Further, addition of PR but not AR antibodies in blocked shift experiments were able to abrogate binding of the higher molecular DNA/protein complexes, although no supershift was observed (Fig. 6C).

The PR Is Recruited to the *KLK4* PRE Region

Chromatin immunoprecipitation (ChIP) assays were carried out to assess the active recruitment of the PR to the *KLK4* PRE promoter region in a more cellular context. Thus, T47D cell lines were grown in the absence of progesterone for 72 hours followed by treatment with or without saturating levels (100 nmol/L) of progesterone for 0 to 24 hours. The PR

antibody effectively immunoprecipitated the progesterone-responsive region (−2496 to −2283) of the *KLK4* promoter in a manner that was dependent on progesterone treatment and time (Fig. 7, I). In contrast, the PR antibody failed to immunoprecipitate a region of the *KLK4* promoter that did not contain K4.pPRE (−2012 to −1839) or the chromatin DNA from a non-progesterone-responsive gene, *β2-microglobulin* (Fig. 7, II and V, respectively). The respective input DNA controls (Fig. 7, II, IV, and VI) amplified the appropriate product, indicating the specificity of the PCR. As expected, the genomic DNA PCR controls were positive for all panels, indicating the specificity of the primer pairs and validating the PCR.

Discussion

It has long been established that hormones play an important role in the pathogenesis of breast and prostate cancer. Consequently, the identification of downstream target genes with functions that may contribute to neoplasia is an important area of research to better understand the etiology of these two cancers. One such family of hormone-target genes are the tissue

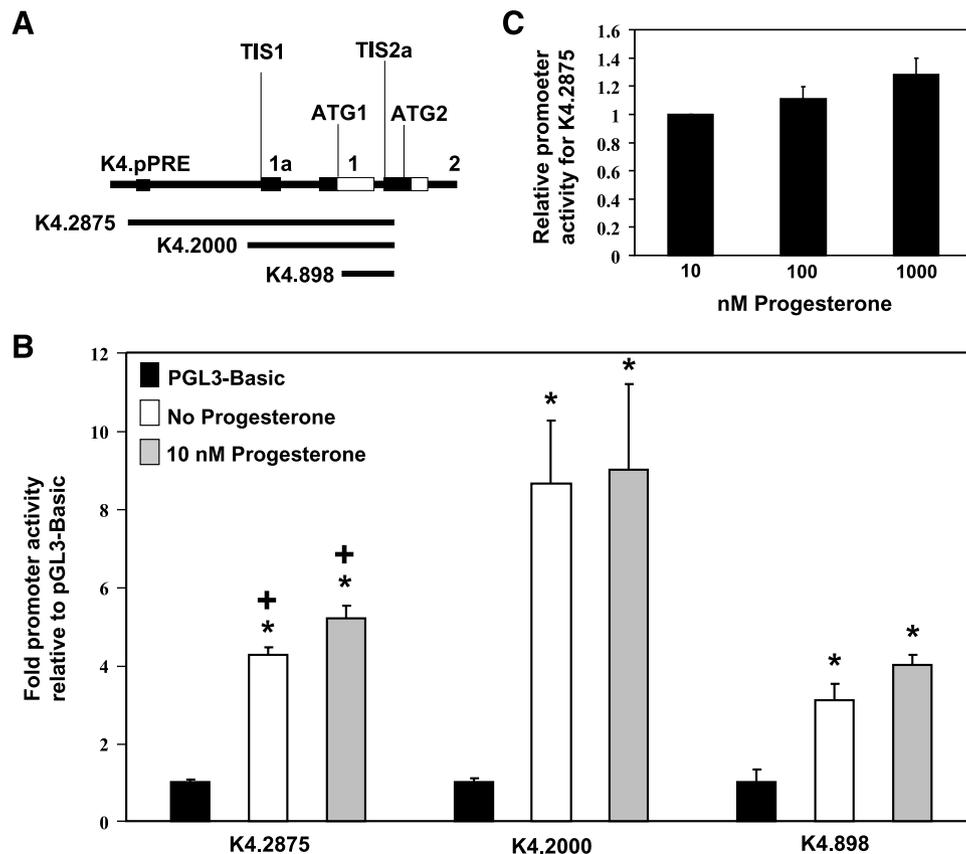


FIGURE 5. Progesterone responsiveness of the *KLK4* promoter in breast cancer cells. **A.** Diagram of the *KLK4* putative PRE (K4.pPRE) and luciferase promoter constructs (K4.2875, K4.2000, and K4.898) relative to the coding (white boxes) and noncoding (black boxes) regions of the *KLK4* gene. K4.pPRE is indicated at −2400 bp from TIS2b. **B.** Progesterone-induced luciferase reporter assays were carried out in breast cancer cells (T47D). Luciferase activity was normalized to the Renilla transfection control. There was no significant difference ($P < 0.05$) in pGL3-Basic activity in response to progesterone so data is represented as luciferase activity normalized to pGL3-Basic activity (black columns). The three *KLK4* promoter constructs had significantly higher luciferase activity in cells that were treated with (gray columns) and without (white columns) 10 nmol/L progesterone when compared with pGL3-Basic (*, $P < 0.05$). Columns, mean from at least three independent wells; bars, SE. +, $P < 0.05$, significant difference in promoter activity over vehicle control (0.01% ethanol). **C.** Luciferase reporter assay demonstrating that K4.2875 promoter activity increases in T47D cells treated with increasing amounts of progesterone. Columns, mean from two independent experiments, each carried out in triplicate; bars, SE.

kallikrein-related proteases (*KLK*), which are a multigene family of serine proteases that are highly expressed in many hormone-dependent cancers (6, 7, 28). The kallikrein 4 gene (*KLK4*), a more recently cloned member of the *KLK* gene family, has been reported to be regulated by progestins and androgens in breast and prostate cancer cells, respectively (10, 15, 16). Although the (patho)physiologic role of *KLK4* is yet to be established, it has been proposed that this gene may be important in the pathogenesis and progression of hormone-dependent cancers as it is aberrantly expressed in cancer cells when compared with benign cells (17, 29-31), and has known or putative functions that are important in cancer biology (12-14, 32-34). In this study, we have further characterized the hormonal regulation of the *KLK4* gene in breast and prostate cancer by interrogating the *KLK4* promoter to identify putative HREs that may mediate this response.

Initial profiling on a panel of breast and prostate cells showed that *KLK4* is only present in breast cells expressing *PR* and prostate cells expressing *AR*, suggesting that progestins and androgens play an active role in mediating *KLK4* gene expression. Consequently, we further assessed the hormonal regulation of *KLK4* gene expression using quantitative RT-PCR

in breast and prostate cancer cells, particularly as previous studies have only taken semiquantitative or qualitative approaches (10, 15, 16). The results from these experiments show that *KLK4* is moderately up-regulated in T47D cells that were treated with progesterone for 24 hours (~1.38-fold). In LNCaP cells, *KLK4* was also moderately up-regulated (3.2-fold) in response to androgens. The observed up-regulation of *KLK4* by progesterone and androgens are likely to be mediated through their cognate receptors as these responses were attenuated in cells that were treated with hormones and their associated receptor antagonists. Further, these findings are consistent with previous studies that report similar up-regulation of *KLK4* mRNA in response to progesterone in breast cancer cells (8) and androgens in prostate cancer cells (10, 15). Conversely, the down-regulation of *KLK4* gene expression (4.2-fold for T47D cells and 2.4-fold for LNCaP cells) after cells were maintained in hormone-depleted medium further highlights the importance of hormones in mediating *KLK4* expression, despite the apparent modest level of these responses.

To date, the *KLK4* TIS, and consequently the promoter region of *KLK4*, have been ill defined. Early gene characterization studies predicted the *KLK4* gene to comprise of up to five exons based on sequencing of EST libraries and/or comparative exon prediction analyses (8, 10, 11). More recently, two groups identified two alternative *KLK4* TISs that would result in the expression of one transcript (TIS1 variant) that would be derived from six exons (9) and an alternative truncated transcript (TIS2a/b variant) that would be derived from four exons (15). The biological importance of each variant has been a source of recent controversy (35, 36). Nevertheless, our combined 5'-RACE and RT-qPCR results indicate that whereas the transcript arising from TIS2a/b is more abundant, both transcripts are expressed in T47D and LNCaP cells. Further, both TIS1 and TIS2a/b variants are hormonally regulated in LNCaP and T47D cells to a similar degree. Although there are no consensus TATA box within -25 to -30 bp upstream of TIS2a/b, the promoter region for TIS2a/b does have a cluster of GC box sites (Sp1 sites, CCCgCCC), which may play a role in mediating basal *KLK4* gene expression (data not shown). Indeed, a large-scale study of 1,031 genes found that only 32% of promoters harbored a consensus TATA box at -25 to -40 bp from the TIS (37). Further, 97% of these 1,031 genes contained a GC box (-74 to -45 bp from the TIS) and suggests that GC boxes play an important role in mediating transcription of TATA-less genes. Indeed, this is supported in the T47D luciferase reporter assays that showed higher basal activity of the minimal *KLK4* promoter construct (K4.898) when compared with the promoter-less pGL3-Basic vector.

Classically, the PR, AR, and glucocorticoid receptor recognizes HREs that comprise of a palindromic hexameric repeat sequence that is separated by three nucleotides to mediate target-gene transactivation (38). However, more recent studies suggest that the second zinc finger of the AR-DBD plays an important role in the ability of the AR to interact specifically with HREs that comprise of a direct hexameric repeat motif as opposed to the classic palindromic sequence (39-43). Significantly, reduced fertility and development of

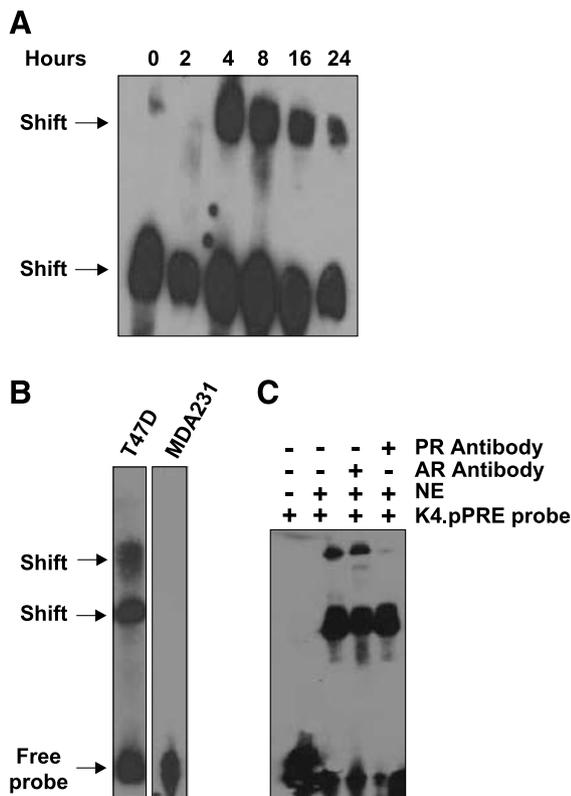


FIGURE 6. EMSA and blocked shift analyses of K4.pPRE. **A.** EMSA of 10 nmol/L progesterone-treated T47D cells over 24 h. Numbers at the top of the figure, the time in hours for each progesterone treatment; arrows, the two shifts. Note that the free probe is not shown. **B.** Control experiments for PR specificity. EMSA of the K4.pPRE was carried out using nuclear extracts from PR-positive (T47D) and PR-negative (MDA-MB-231) cells. Nuclear extracts from MDA-MB-231 cells failed to bind K4.pPRE. **C.** EMSA and blocked shift assay of K4.pPRE with PR and AR antibodies. Arrows denote the shifts and the free probe is shown at the bottom of the gel.

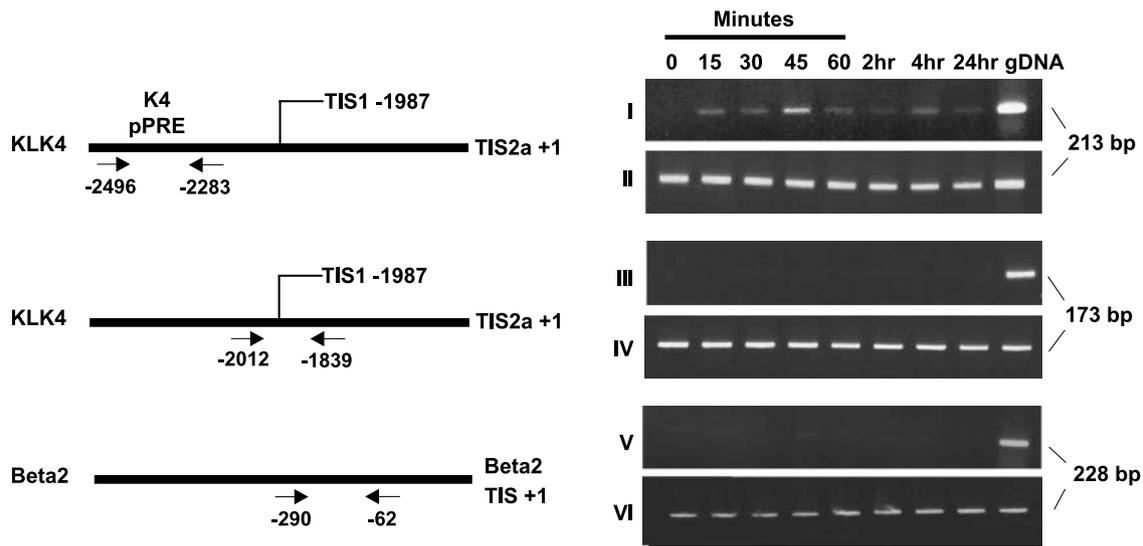


FIGURE 7. ChIP assay for the recruitment of the PR to the *KLK4* promoter. To the left of this figure are the schematic diagrams for the regions 5' of the TIS that were amplified for the *KLK4* (I, -2496 to -2238 bp; III, -2012 to -1839 bp) and β 2-microglobulin (V, -290 to -62 bp) genes. Top, the region of the *KLK4* promoter that contains a potential PRE (-2496 to -2283 bp). Middle, a region of the *KLK4* promoter that does not contain a PRE (-2012 to -1839 bp). Bottom, a promoter region of a non-progesterone-responsive gene, β 2-microglobulin (*Beta2*; -290 to -62 bp). II, IV, and VI are control samples that were assayed by PCR before the initial immunoprecipitation step and show that the input DNA contains the reference gene. I, III, and VI represent the PCR of each of the regions given schematically to the left of the gel following immunoprecipitation. Time points for analysis of 0, 15, 30, 45, 60 min and 2, 4, and 24 h are indicated at the top panel. To the far left of the panels are the positive controls of untreated genomic DNA (gDNA) from T47D cell lines.

male reproductive organs are some of the outcomes for mice knock-in models that have a mutated AR-DBD, which results in loss of AR transactivation of AR-selective HREs (44). This highlights the importance of both the primary structure of the PR, AR, and glucocorticoid receptor, and the nature of the HREs within target genes in not only mediating gene transcription but also in disease pathology such as cancer. It was thus important to identify HREs within the *KLK4* promoter, which may mediate progesterone and androgen up-regulation of *KLK4*, using either receptor-selective or nonselective motifs. Using *in silico* modeling, we have identified two consensus-like motifs, one (K4.pARE) located in the promoter region of TIS2a/b (~1 kb) and another (K4.pPRE) located a further 1.5 kb upstream. EMSA analyses of K4.pARE resulted in the formation of two specific shifts that are likely to represent the formation of K4.pARE/AR complexes (as well as complexes incorporating other AR coregulators, which may account for the multiple shifts) as supported by the shifts being of the same molecular weight as shifts formed in reactions that were probed with *PSA* ARE I, and that abrogation of receptor/DNA complexes were observed in experiments that were incubated with AR antibodies. The specificity of K4.pARE and *PSA* ARE I shifts were further confirmed by the inability of the nonspecific IgG antibody to block these DNA receptor complexes. The differences in AR blocking patterns between experiments using LNCaP nuclear proteins from different R1881 treatment times also supports the hypothesis (45) that higher shifts represent higher-order AR/DNA complexes. However, binding assays of the putative K4.pARE with purified AR-DBD did not result in the formation of any shifts. Further, molar excess of K4.pARE was not able to outcompete binding of *PSA* ARE I/AR-DBD complexes. It is possible that

binding of the AR to K4.pARE may be ligand dependent. Indeed, the AR is ligand bound and dimerized when bound to the promoters of target genes (46). Moreover, formation of K4.pARE/AR complexes may require the association of other AR coregulators to permit the formation of energetically stable complexes. Finally, it is possible that K4.pARE is interacting indirectly with the AR through other AR coregulators.

To address these issues, luciferase reporter assays were carried out to assess if this element is able to mediate androgen-induced transactivation in prostate cancer cells. However, luciferase reporter assays using three tandem copies of K4.pARE (to enhance any potential signal) showed that this element was not responsive to androgens in either LNCaP or 22Rv1 prostate cancer cells. Luciferase reporter assays were then carried out on seven deletion promoter constructs spanning up to 2.8 kb of the *KLK4* promoter from TIS2b (853 bp from TIS1) to assess if androgen regulation of K4.pARE requires interaction with other *cis*-elements and to identify other potential HREs using a functional approach. However, androgen regulation experiments carried out on all *KLK4* promoter constructs in both LNCaP and 22Rv1 cells showed that the 2.8 kb of the *KLK4* promoter region is not responsive to androgens. Given our observations in the blocked shift experiments using AR antibodies for K4.pARE, these promoter assays support our hypothesis that this element is interacting indirectly with the AR. According to the androgen receptor mutation database,¹ the AR has been found to interact with up to 72 coregulators, some of which interact directly with DNA

¹ <http://www.androgendb.mcgill.ca/>

(47, 48). Alternatively, K4.pARE may mediate regulation through synergistic interaction with other distal HREs in the *KLK4* promoter, as has been reported for other androgen-responsive promoters (19). The androgen regulation of the *KLK4* PRE was not pursued given the lack of androgen response in the K4.2875 luciferase promoter construct (which contains the *KLK4* PRE motif).

Given the lack of androgen responsiveness of the *KLK4* promoter, further *in vitro* analyses were focused on the progesterone regulation of K4.pPRE and the *KLK4* promoter in breast cancer cells. The EMSA analysis suggests that K4.pPRE is interacting with the PR as shown by the formation of a higher molecular weight shift in experiments that used nuclear proteins from T47D cells treated with progesterone. Further, binding of K4.pPRE/receptor complex was abrogated upon addition of PR antibodies in blocked shift experiments, supporting our hypothesis that these shifts represent PRE/PR complexes. To further characterize the K4.pPRE element and flanking sequences, progesterone-induced luciferase reporter assays were then performed in T47D cells using three promoter constructs, two (K4.2000 and K4.898) that did not harbor K4.pPRE and one (K4.2875) that did. Consistent with the location of the K4.pPRE, only the K4.2875 luciferase construct was significantly up-regulated (~ 1.4 -fold, $P = 0.002$) by progesterone. Although the K4.2875 construct showed moderate progesterone response, this was consistent with the marginal response observed at the mRNA level using quantitative RT-qPCR, and similar to other progesterone-responsive promoters in T47D cells, such as *c-myc* (~ 3 -fold), an important regulator of breast cell proliferation, differentiation, transformation, and apoptosis (49). Other HREs, such as those found in the *PSA* promoter (ARE I), have also been shown to have minimal sensitivity to androgens on its own (24, 25). Like the cooperative regulation of *PSA* AREs in androgen-mediated gene expression (19, 20), the *KLK4* PRE may act synergistically with other enhancer or intronic PREs. The ChIP data support our hypothesis that this element is a bona fide HRE as the PR was actively recruited to the *KLK4* promoter region, which harbors the PRE element. Further, recruitment of the PR to the *KLK4* PRE region seems to occur in a time-dependent manner that is consistent with other nuclear hormone receptor recruitment studies. For example, studies on the estrogen receptor α (ER α) and the cathepsin D promoter have suggested that the cyclic recruitment of ER to target promoters may represent a mechanism that facilitates continuous monitoring of the external environment (50).

In conclusion, we have quantified and confirmed previous reports that *KLK4* is exquisitely regulated by progesterone and androgens in breast and prostate cancer cells, respectively. We show that both *KLK4* variants are similarly hormone regulated despite differences in their abundance. We have also shown that the androgen-responsive region of *KLK4* is unlikely to lie within the ~ 2.8 kb promoter region from TIS2a/b investigated in this study, although the progesterone regulation of *KLK4* gene expression in breast cancer cells is likely mediated, at least in part, by the PRE identified at -2419 bp in the *KLK4* promoter. These studies lay the foundation for future analysis of the hormonal regulation of the *KLK4* promoter.

Materials and Methods

Steroids and Antibodies

Progesterone was obtained from Sigma Chemical Co., and the synthetic androgen R1881 was from Perkin-Elmer. The PR antagonist (RU486) was obtained from Sigma, and the AR antagonist (bicalutamide) was a gift from Prof. Wayne Tilley (Hanson Institute, Adelaide, Australia). The PR (PR-C19) and AR (C-19) antibodies used in EMSA and ChIP experiments were obtained from Santa Cruz Biotechnology. The rabbit IgG antibody was obtained from Zymed Laboratories.

Cell Culture

All cell lines used were obtained from American Type Culture Collection. Cells were maintained in either RPMI 1640 (LNCaP, 22Rv1, DU145, and PC-3) or phenol-red free DMEM/F12 (MCF7, MDA-MB-231, and T47D) medium (Invitrogen), supplemented with 10% FCS (Invitrogen) and 50 units/mL penicillin G and 50 μ g/mL streptomycin (CSL Biosciences). RWPE-1 and RWPE-2 cells were grown in keratinocyte serum-free medium with 50 μ g/mL bovine pituitary extract and 5 ng/mL recombinant human epidermal growth factor (Invitrogen). MDA-PCa-2B cells were maintained in BRFF-HPC1 medium from AthenaES (Sapphire Biosciences). MCF-10A cells were maintained in DMEM/F12 medium (Invitrogen) supplemented with 5% horse serum (Invitrogen), 10 μ g/mL insulin (Sigma), 20 ng/mL epidermal growth factor (Invitrogen), 100 ng/mL cholera enterotoxin (Sigma), 0.5 μ g/mL hydrocortisone (Sigma), and 100 units/mL penicillin/streptomycin. For steroid treatment experiments, cells were cultured until $\sim 70\%$ confluent and then maintained in 2% charcoal-stripped serum (HyClone) for 48 h followed by addition of steroid hormones (1 nmol/L R1881, 10 nmol/L progesterone) for an additional 24 h. In antagonist experiments, cells were pretreated with either 1 μ mol/L bicalutamide or 1 μ mol/L RU486 for 2 h before the addition of hormones and antagonists. For hormone starvation experiments, cells were maintained in phenol red-free medium containing 10% charcoal-stripped serum for 4 d before harvesting and RNA extraction.

RNA Extraction, cDNA Synthesis, and RT-PCR

Total RNA was extracted using the TRI Reagent (Sigma) according to the manufacturer's protocol. RNA was then treated with DNaseI (Roche Diagnostics), purified through an RNeasy column (Qiagen) and electrophoresed to determine the integrity of the RNA before use in 5'-RACE experiments. Complementary DNA (cDNA) was synthesized from 2 μ g of total RNA using random hexamers (Proligo) and SuperScript III Reverse Transcriptase (Invitrogen). RT-PCR was carried out on a panel of four breast cell lines (T47D, MCF7, MCF-10A, MDA-MB-231) and seven prostate cell lines (LNCaP, 22Rv1, MDA-PCa-2b, RWPE-1, RWPE-2, DU145, PC-3) using primers targeting the *KLK4* (K4Ex2QS: 5'-ggcactgtgcatggaaacga-3' and K4Ex3QAS: 5'-tcaagactgtgcaggcccagcc-3'), *PR* (F: 5'-gattcagaagccagccagag-3', R: 5'-tgcctctcgctgattgatt-3'), *AR* (F: 5'-atcaggggcgaagtagagcatc-3', R: 5'-agccccactgaggggacaacc-3'; ref. 51), and $\beta 2$ -microglobulin ($\beta 2$ M-F: 5'-tgaattgctatgtctgggt-3', $\beta 2$ M-R: 5'-cctcatgatgctgcttaccat-3') genes. RT-PCR was performed using PCR annealing temperatures of 60°C for *KLK4*, *AR* and *PR*, and 55°C for $\beta 2$ M.

Quantitative RT-PCR

KLK4, 18S rRNA, prostate-specific antigen (*PSA*), and 11 β -hydroxysteroid dehydrogenase type 2 enzyme (*HSD11B2*) transcripts were analyzed by RT-qPCR in 20 μ L reactions with 1 \times Sybr Green (ABI, Applied Biosystems), 150 nmol/L of forward and reverse primers (see below), and the relevant cDNA (1 μ L of a 1:20 dilution for *KLK4*, *PSA*, and *HSD11B2* and 1:100 dilution for *18S*). PCR was done on an ABI Prism 7000 sequence detection system (Applied Biosystems) and cDNA was quantitated using known DNA concentration standards. Gene expression was calculated as the amount of *KLK4*, *PSA*, and *HSD11B2* cDNA relative to 18S cDNA copy number, and data are represented as the fold change in hormone-induced gene expression over vehicle control (0.1% ethanol). The primer sequences used in the RT-qPCR analyses are as follows: *KLK4* (K4Ex2QS and K4Ex3QAS from above), *18S* (18S-F: 5'-ttcgaactgaggccatgat-3'; 18S-R: 5'-cgaacctccgacttcg-3'), *PSA* (PSA-F: 5'-agtgcgagaagcattccaac-3'; PSA-R: 5'-ccagcaagatcacgcttttgg-3'), and *HSD11B2* (HSD11B2-F: 5'-tgccgctactcatggacacat-3'; HSD11B2-R: 5'-tttcccactgaccacgcttc-3').

Exon-specific RT-qPCR was carried out using primers K4Ex1QS and K4Ex2QAS and K4Ex2QS and K4Ex3QAS to detect *KLK4* variants that use either TIS1 or TIS2a/b (29). Standard curves of known copy number using amplicons cloned into pGEMT were used to quantify gene expression. Exon 1-2 values represent the TIS1 variant, whereas the expression of TIS2a/b is defined as (exon 2-3)-(exon1-2) because exons 2 and 3 are present in all *KLK4* variants. A two-tailed Student's *t* test was used to calculate statistical significance from three independent experiments that were each carried out in duplicate or triplicate.

KLK4 TIS Mapping

To identify the TIS, RNA ligase-mediated RACE (First-Choice RLM-RACE kit, Ambion) was carried out as per the manufacturer's instructions, except that Superscript II (instead of AMV) reverse transcriptase was used for cDNA synthesis. The first- and second-round *KLK4*-specific PCR primers used were 5'-agcccgatggtgtaggagtt-3' and 5'-cgatggtgtaggattctggaacagt-3', respectively. PCR cycling variables were 94°C for 5 min followed by 40 cycles of 94°C for 30 s, 55°C or 62°C for 30 s (first- and second-round PCR, respectively), 72°C for 30 s, and a final 72°C extension for 10 min. PCR amplicons were cloned into pGEMT easy vector (Promega) and clones were sequenced using the ABI PRISM Dye Terminator 2 protocol (Applied Biosystems) at the Australia Genome Research Facility, University of Queensland, Brisbane, Australia.

In silico Analysis

Four independent gene analysis programs, Cister, SigScan, MatInspector (52-54), and ConSite,² were used to identify putative HREs encompassing ~3 kb of the *KLK4* promoter.

² <http://asp.ii.uib.no:8090/cgi-bin/CONSITe/consite>

Extraction of Soluble Nuclear Protein Fractions

Soluble nuclear protein fractions were isolated from progesterone-treated T47D cells and R1881-treated LNCaP and 22Rv1 cells using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology) according to the manufacturer's instructions. Protein concentrations were quantified using the bicinchoninic acid protein assay (Pierce).

EMSA and Blocked Shift Assay

Nucleotide sequences encompassing a putative PRE (K4.pPRE) and a putative ARE (K4.pARE) in the *KLK4* promoter were synthesized (Prologo) with their complements. The forward sequences for K4.pPRE (5'-aaaaagagagaaAGAA-CATgagagAGAACAaggagagaatgag-3') and K4.pARE (5'-tcg-aaagccgagGGTGCaggaGATTGTGctcc-3') were end labeled with biotin (Pierce), then annealed to their labeled complementary oligomers. The putative HREs are underlined and the half sites are capitalized. *PSA* ARE I-A DNA probes (5'-tcgacttgcAGAACAgaAGTACTagctg-3) were also used in EMSA to serve as a positive control for interaction with the AR. Binding reactions were carried out using 5 μ g of nuclear proteins or 0.2 μ g of purified androgen receptor DNA binding domain (AR-DBD; ref. 45) with the LightShift Chemiluminescent EMSA kit (Pierce) as instructed. Blocked shift experiments were done using 1 or 2 μ g of either PR antibody (PR-C19) or AR antibody (C-19). PR and AR antibodies were incubated with nuclear proteins for 24 h at 4°C before addition to the labeled K4.pPRE or K4.pARE oligomer. Nuclear extracts from the PR-negative breast cancer cell line, MDA-MB-231, was used as a negative control to assess binding specificity in PR experiments. Controls using molar excess of unlabeled DNA probes were also included in EMSA analyses to assess for specificity of AR shifts.

KLK4 Reporter Constructs

A bacterial artificial chromosome clone (BC85745) and Cosmid clone (R28781; Lawrence Livermore Laboratory) were both used as template to generate seven deletion constructs encompassing 2,875 bp of the *KLK4* promoter. *Xho*I and *Hind*III restriction sites were added to the primers to facilitate orientation-specific cloning into the pGL3-Basic vector (Promega). Forward primer sequences were K4.170, 5'-tgt-gctcgagctgctctgaacctctgacc-3'; K4.445, 5'-cacctcgagctacctg-gaatccctgacca-3'; K4.688, 5'-cacctcgagaaaacggtgtttgtgtg-3'; K4.1298, 5'-cacctcgaggtgtgtgtctgacctgct-3'; K4.898, 5'-ctc-gagcaaacggtgtttgtgtg-3'; K4.2000, 5'-ctcagagcagtggaatccag-gagc-3' and K4.2875, 5'-cacaagctcagctctgcccgttatgat-3', and PCR was carried out using a common reverse primer (K4.Anchor, 5'-aagcttcagctctgcccgttatgat-3'). The *KLK4* promoter inserts were amplified using the "proofreading" enzyme *Pfx* or high-fidelity platinum Taq (Invitrogen) DNA polymerase and subcloned into the pGEM-T Easy (Promega) vector before cloning into pGL3-Basic (Promega). All constructs were sequenced to verify their sequence fidelity as described above. *KLK4* luciferase promoter constructs are designated in Results according to their corresponding forward primer name.

Oligonucleotides encoding three tandem copies of a putative ARE in the *KLK4* promoter (KLK4.pARE) and its native

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