Androgen Regulation of Aldehyde Dehydrogenase 1A3 (ALDH1A3) in the Androgen-Responsive Human Prostate Cancer Cell Line LNCaP

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Previous gene array data from our laboratory identified the retinoic acid (RA) biosynthesis enzyme aldehyde dehydrogenase 1A3 (*ALDH1A3*) as a putative androgen-responsive gene in human prostate cancer epithelial (LNCaP) cells. In the present study, we attempted to identify if any of the three ALDH1A/RA synthesis enzymes are androgen responsive and how this may affect retinoid-mediated effects in LNCaP cells. We demonstrated that exposure of LNCaP cells to the androgen dihydrotestosterone (DHT) results in a 4-fold increase in *ALDH1A3* mRNA levels compared with the untreated control. The mRNA for two other *ALDH1A* family members, *ALDH1A1* and *ALDH1A2*, were not detected and not induced by DHT in LNCaP cells. Inhibition of androgen receptor (AR) with both the antiandrogen bicalutamide and small interfering RNA for AR support that *ALDH1A3* regulation by DHT is mediated by AR. Furthermore, specific inhibition of the extracellular signal–regulated kinase and Src family of kinases with PD98059 and PP1 supports that AR's regulation of *ALDH1A3* occurs by the typical AR nuclear-translocation cascade. Consistent with an increase in *ALDH1A3* mRNA, DHT-treated LNCaP cells showed an 8-fold increase in retinaldehyde-dependent NAD^+ reduction compared with control. Lastly, treatment of LNCaP with all-*trans* retinal (RAL) in the presence of DHT resulted in significant up-regulation of the RA-inducible, RA-metabolizing enzyme *CYP26A1* mRNA compared with RAL treatment alone. Taken together, these data suggest that (i) the RA biosynthesis enzyme *ALDH1A3* is androgen responsive and (ii) DHT up-regulation of *ALDH1A3* can increase the oxidation of retinal to RA and indirectly affect RA bioactivity and metabolism. Exp Biol Med 232:762–771, 2007

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Introduction

Prostate cancer (PCa) is the leading noncutaneous malignancy among men in the United States (1). Among the risk factors linked with the onset of PCa, circulating levels of male androgenic hormones remain one of the strongest biologic influences in the etiology of this disease (2). Androgens are required for the normal development and regulation of the adult prostate and have now been shown to play a critical role in the initiation and progression of prostate carcinogenesis (2, 3). In both normal prostate tissue and PCa, androgens exert their biologic effects through interaction with the androgen receptor (AR). Ligand-activated AR can regulate gene transcription either directly by nuclear translocation and interaction with androgen-responsive elements (ARE) in the promoter region of target genes or indirectly through activation of extracellular signal–regulated kinase (ERK) or Src cytosolic kinase pathways, which terminate at a number of downstream transcription factors (4–7). In the mature prostate, genes regulated by androgenic hormones are involved in many biologic processes, including proliferation, differentiation, apoptosis, and secretory functions such as expression of prostate-specific antigen (PSA) (8–10).

There is evidence that in addition to androgens, retinoids and more specifically retinoic acid (RA), acting through retinoic acid receptors (RARs) and retinoid X receptors, are required for normal prostate development and maintenance (11–13). Numerous experimental and population studies have also suggested that retinoids and RA can inhibit carcinogenesis of the prostate and that loss of normal RA metabolism is involved in PCa development (14–18). Two of these studies demonstrated that men with PCa possess significantly decreased prostatic levels of RA and

that PCa loses expression of lecithin:retinol acyltransferase, the enzyme responsible for synthesis of retinyl esters (the storage form of vitamin A). Nevertheless, despite evidence for "RA deficiency" in the etiology of PCa, little is known about the regulation of enzymes responsible for RA biosynthesis or metabolism in both normal and malignant prostatic tissues. There is some evidence of androgen-retinoid cross talk and ligand antagonism in the prostate and other hormone-responsive tissues (19, 20); however, interactions between androgens and retinoids in coordinating the expression and regulation of genes involved in RA metabolism and signaling remain unclear.

Epithelial cells that require RA synthesize it locally and control its concentration by balancing its synthesis and catabolism (21). RA biosynthesis begins with the reversible oxidation of retinol (vitamin A) to retinal by the enzyme retinol dehydrogenase, followed by the oxidation of retinal to RA by the aldehyde dehydrogenase 1A (ALDH1A) family of enzymes (22). Three vertebrate ALDH1A family members (ALDH1A1 [NCBI GeneID: 216], ALDH1A2 [NCBI GeneID: 8854], and ALDH1A3 [NCBI GeneID: 220]) have been identified, each possessing unique substrate specificity and tissue-specific expression patterns (23). ALDH1A-catalyzed synthesis of RA from retinal is non-reversible and is the rate-limiting reaction in RA biosynthesis (23). RA can modulate the expression of RA-responsive genes, which include genes responsible for its own catabolism (24). The cytochrome P450 hydroxylase family enzyme CYP26A1 is a retinoic-responsive gene that oxidizes RA to more polar metabolites and can be induced by RA through RAR-mediated events (24, 25).

The genetic and hormonal regulation of the ALDH1A family of enzymes, CYP26A1, and other enzymes responsible for regulating RA levels in the prostate is likely complex and likely involves both hormones and retinoids. However, given the potential for retinoids in the development of PCa and central role that androgens have in the pathogenesis of PCa, it is warranted to explore interactions between androgens and the RA metabolic pathway. In this study, we asked if the genes encoding the three ALDH1A isoforms are androgen responsive, and if so, whether such regulation could affect RA-mediated events. We demonstrate specific expression and differential regulation of the ALDH1A isoforms by androgen in LNCaP cells. Moreover, we also provide evidence supporting a role for androgen in the modulation of RA's biologic effects in this model.

Materials and Methods

Chemicals and Reagents. Dihydrotestosterone (DHT) was purchased from NEN Life Science Products (Boston, MA). The antiandrogen bicalutamide was a gift from AstraZeneca, Inc. (Wilmington, DE). The selective ERK inhibitor PD98059 and the selective Src family of kinases inhibitor PP1 were purchased from BIOMOL International, LP (Plymouth Meeting, PA). Dimethyl sulfoxide (DMSO), Tris-HCl, β-mercaptoethanol, HEPES, EDTA, β-NAD^+, all-*trans* retinal (RAL), and RA were purchased from Sigma-Aldrich (St. Louis, MO). Phosphate-buffered saline (PBS) was purchased from Invitrogen (Carlsbad, CA).

Cell Culture and Treatments. The androgen-responsive LNCaP cell line was purchased from the American Type Culture Collection (Manassas, VA) and maintained in medium A (RPMI 1640 medium [Invitrogen] supplemented with 10% fetal bovine serum [Invitrogen], 1% glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin [Invitrogen]). Cells were incubated in the presence of 5% CO_2 at 37°C. For androgen-related experiments, cells were seeded as follows: 6-well plates at 250,000 cells/ml/well or 175-cm^2 flasks at 80,000 cells/ml/flask. After 24 hrs in medium A, cells were switched to medium B (RPMI 1640 [without phenol red] containing 10% charcoal-stripped fetal calf serum [HyClone, Logan, UT], 1% glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) for an additional 24 hrs to minimize background androgen levels. After androgen deprivation, cell treatments with test compounds in medium B were started. All treatments used DMSO as vehicle control.

RNA Isolation and Real-Time Quantitative Reverse Transcriptase Polymerase Chain Reaction (PCR). Total RNA was isolated using the TRIzol reagent (Invitrogen) and cDNA synthesized from 1 μg of total RNA using the StrataScript First-Strand cDNA Synthesis Kit (Stratagene, La Jolla, CA) as previously described (26). Real-time quantitative PCR was carried out using TaqMan Universal PCR Master Mix on a Prism 7000 Sequence Detection System (Applied Biosystems, Inc., Foster City, CA) as previously described (26). The TaqMan probes and primers were purchased from Applied Biosystems using inventoried TaqMan gene expression assays: *ALDH1A1* (assay ID: Hs00167445), *ALDH1A2* (assay ID: Hs00180254), *ALDH1A3* (assay ID: Hs00167476), *PSA* (assay ID: Hs02576345), and *CYP26A1* (assay ID: Hs00175627). Human glyceraldehyde-3-phosphate dehydrogenase (assay ID: Hs99999905) was used as an endogenous control. The following amplification parameters were used: 50°C for 2 mins, 95°C for 10 mins, followed by 46 cycles of amplification at 95°C for 15 secs and 60°C for 1 min. Quantitation of mRNA levels was performed using the $2^{-\Delta\Delta C_T}$ method as previously described (27).

Effects of Steroid Hormones. To examine the effects of steroids, LNCaP cells were seeded in 6-well plates as described above. Twenty-four hrs later the medium was removed and replaced with medium B. To examine the effects of DHT concentration, cells were treated with 0, 0.1, 1, or 10 n*M* DHT for 48 hrs. For time-course experiments, cells were treated for 0, 24, 48, or 72 hrs with 10 n*M* DHT. To examine the effects of the synthetic androgen R1881, cells were treated for 48 hrs with or without 1 n*M* R1881. To examine the effects of 17β-estradiol, cells were treated for 0, 24, 48, or 72 hrs with 1 n*M* 17β-estradiol. Culture

medium and treatments were replaced with fresh reagents daily. After treatments, cells were harvested at the indicated time points for RNA isolation and *ALDH1A* mRNA levels were analyzed as described above.

AR-Specific Inhibitor and Small Interfering RNA (siRNA) Inhibition Studies. Experiments using the AR-specific inhibitor bicalutamide and siRNA specific for AR were performed to confirm the role of AR in DHT regulation of *ALDH1A3* mRNA expression.

LNCaP cells were seeded in 6-well plates as described above. Twenty-four hrs after androgen deprivation, LNCaP cells were treated with or without 1 n*M* DHT and in the presence or absence of 25 μ*M* bicalutamide for 48 hrs. For siRNA experiments, siRNA was introduced into the cells using the RNAsi Human/Mouse Starter Kit (Invitrogen) according to the manufacturer's protocol. Preliminary experiments determined 5 n*M* siRNA to be optimal for AR inhibition. Forty-eight hours after transfection, cell culture media were replaced with fresh media in the presence or absence of 1 n*M* DHT for an additional 48 hrs. After treatment, cells were harvested for RNA isolation. SMARTPOOL siRNA against AR was purchased from Dharmacon (Lafayette, CO). Negative and positive controls were from the Invitrogen RNAsi Human/Mouse Starter Kit.

Effect of ERK and Src Family of Kinases Inhibitors on DHT Induction of *ALDH1A3* mRNA. To elucidate whether DHT induction of *ALDH1A3* mRNA occurs through nongenomic signal transduction pathways involving ERK- and/or Src kinase–mediated events, the effects of the ERK inhibitor PD98059 and selective Src tyrosine kinase inhibitor PP1 on DHT induction of *ALDH1A3* mRNA levels were determined. LNCaP cells were seeded in 6-well plates as described above. Twenty-four hours after androgen deprivation, LNCaP cells were treated with either vehicle (DMSO), 10 n*M* DHT, 25 μ*M* PD98059, 10 μ*M* PP1, 10 n*M* DHT + 25 μ*M* PD98059, or 10 n*M* DHT + 10 μ*M* PP1. After 48 hrs cells were harvested for RNA isolation. Cell culture media and treatments were replaced with fresh reagents daily.

Analysis of Human *ALDH1A3* Gene 5′ Flanking Region. We analyzed the 5′ untranslated region of the *ALDH1A3* gene, assembled from the Human Genome Browser database at the University of California Santa Cruz (28), for potential transcriptional start sites (TSS). Using a neural network promoter predictor tool (29), we analyzed approximately 1 kb from the ATG start codon for sites strongly predicted as TSS using a minimal promoter score cutoff of 0.8. A score of 0.85 yields a correlation coefficient of 0.60 and a false positive prediction of 0.1%–0.4%. Potential transcription start sites closest to the 5′ end with a score of 0.8 were further analyzed for proximity to the ATG codon and any TATA box elements. For the identification of ARE in an 8-kb region proximal to the *ALDH1A3* TSS, we used a computational transcription factor–binding site (TFBS) analysis tool, MAPPER (30). MAPPER derives its TFBS hidden Markov models using data from both the TRANSFAC and JASPAR databases. The search was conducted using MAPPER consensus binding site models corresponding to AR and other *cis* regulatory elements. For AR-binding sites, a total of six models were used for our search (factor numbers M00962, T0040, M00447, MA0007, M00481, and T0042). Predicted binding sites were ranked by score and E value. Score estimates the accuracy of the string query match computed against the matches against promoters in the MAPPER database. Scores are expressed as positive integers, and higher scores indicate stronger matches. The E value, which is also expressed as a positive integer, measures the probability that the match is a false positive; the smaller the E value, the stronger the probability of a positive match. Only results with a score of 2 or greater and E values of 0.5 or less were selected for further analysis and then assigned a percent homology to a common ARE consensus sequence (AGAACAnnnTGTTCT).

Determination of ALDH1A3 Enzyme Activity. LNCaP cells were plated in 175-cm^2 flasks as described above. Twenty-four hours after androgen deprivation, cells were treated in the presence or absence of 10 n*M* DHT for 48 hrs. After treatment, cells were harvested using a cell scraper and transferred to 50-ml Falcon tubes. Cells were then pelleted, washed with 1X PBS, pelleted again, and resuspended in 1 ml of lysis buffer (HEPES [pH 7.4], 150 m*M* KCl, 2 m*M* EDTA, 1 m*M* β-mercaptoethanol, complete mini protease inhibitor tablet [1 tablet/50 ml; Roche Applied Science, Indianapolis, IN], 10 m*M* pyrazole). To obtain the cytosolic fraction, cells were sonicated on ice with an Ultrasonics W220 cell disruptor (Misonix Inc., Farmingdale, NY) using four 10-sec pulses at high power and then centrifuging at 20,000 *g* for 45 mins. Cytosolic protein was measured using a bicinchoninic acid protein quantitation kit (Pierce Biotechnology, Rockford, IL) following the manufacturer's protocol. ALDH1A3 enzyme activity was determined using a standard dehydrogenase activity assay with slight modifications (31). Assay conditions were as follows: 200-μl final reaction volume; 180 μl of assay buffer (100 m*M* Tris-HCl [pH 8.5], 50 m*M* $MgCl_2$, 5 m*M* dithiothreitol), 50 μg of cytosolic protein (dissolved in 10 μl of lysis buffer), 500 μ*M* NAD^+ (dissolved in 5 μl of assay buffer), and 10 μ*M* RAL (dissolved in 5 μl of DMSO). Cytosolic protein was preincubated with NAD^+ for 10 mins, followed by initiation of the reaction by addition of the substrate retinal. The rate of NADH production was recorded on a Spectra 384 Plus spectrophotometer (Molecular Devices Corp., Sunnyvale, CA) at 340 nm at 37°C. The results were expressed as specific activity (μmoles NADH/min/μg protein). The extinction coefficient for NADH at 340 nm was 6.22. All experiments were done in triplicate and adjusted for the absorbance background of protein plus NAD^+ without retinal.

Effects of DHT on RA Bioactivity and Metabolism. Induction of RA-inducible RA-metabolizing *CYP26A1* mRNA was used as a surrogate end point to indicate putative

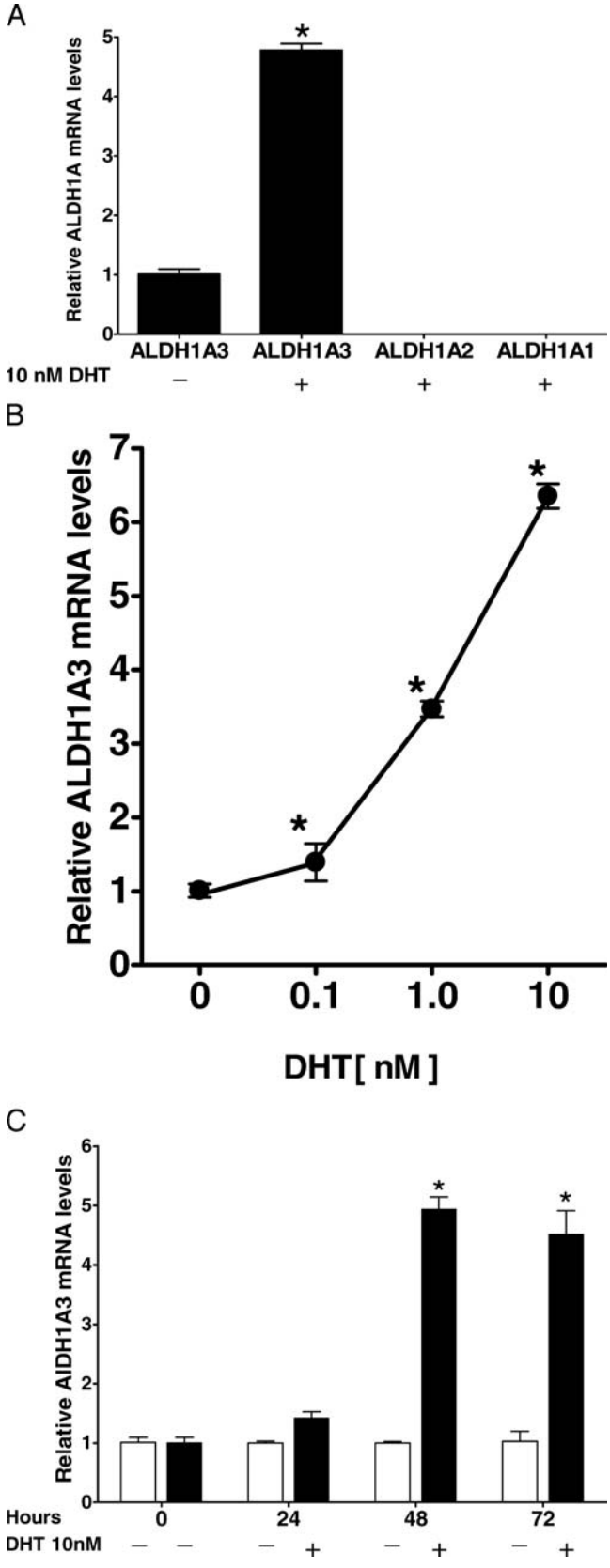


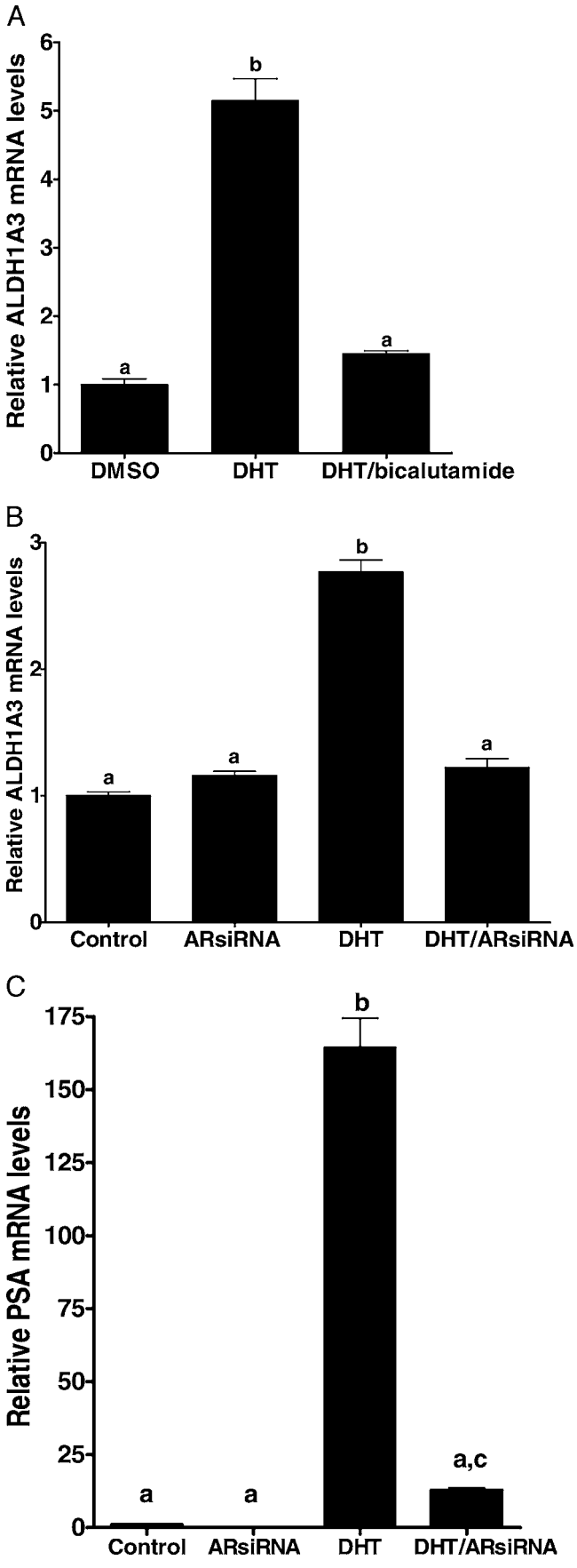
Figure 1. Effects of DHT on *LDH1A3* gene expression in LNCaP cells. (A) After androgen deprivation, LNCaP cells were treated daily with vehicle (DMSO) or 10 n*M* DHT for 72 hrs and then subjected to RNA isolation and real-time PCR analysis. Real-time PCR results are expressed as means ± SE of fold change relative to vehicle-treated control of three separate experiments with each experiment including triplicate PCR tubes. Error bars with an asterisk are significantly different than vehicle-treated control ($P < 0.05$). (B) Concentration-dependent effects of DHT on *ALDH1A3* mRNA levels. After androgen deprivation, LNCaP cells were treated with vehicle (DMSO), 0.1, 1.0, or 10 n*M* DHT for 48 hrs and then subjected to RNA isolation and real-time PCR analysis. Real-time PCR results are expressed as means ± SE of fold change relative to vehicle-treated control of three separate experiments with each experiment including triplicate PCR tubes. Points with an asterisk are significantly different than vehicle-treated control ($P < 0.05$). (C) Time course of DHT effects on *ALDH1A3* mRNA levels. After androgen deprivation, LNCaP cells were treated with either vehicle (DMSO) or 10 n*M* DHT for 0, 24, 48, or 72 hrs and then subjected to RNA isolation and real-time PCR analysis. Real-time PCR results are expressed as means ± SE of fold change relative to vehicle-treated control of three separate experiments with each experiment including triplicate PCR tubes. Error bars with an asterisk are significantly different than vehicle-treated control at the corresponding time point ($P < 0.05$).

RA bioactivity and metabolism. To establish RAL conversion to RA in this model, LNCaP cells were seeded in 6-well plates as described above. Twenty-four hrs after androgen deprivation, LNCaP cells were treated with or without 1 n*M* DHT in the presence or absence of 1 n*M* RAL for 48 hrs. Separate experiments were conducted to determine RA induction of *CYP26A1* in LNCaP cells. LNCaP cells were seeded in 6-well plates, and after androgen deprivation, cells were treated with 10, 50, 100, or 500 n*M* RA for 48 hrs. After treatments, cells were harvested, RNA isolated, and *CYP26A1* mRNA levels determined as described above.

Statistics. Statistical analysis of data was carried out with the GraphPad PRISM program (GraphPad Software Inc., San Diego, CA). Multiple group data were analyzed using analysis of variance (ANOVA) or two-way ANOVA followed by Bonferroni post hoc test. The unpaired Student's *t* test was used to compare experiments between two groups. Values were considered significant at $P < 0.05$.

Results

Expression and Effect of Steroid Hormones on *ALDH1A* Gene Family Members in the Androgen-Responsive LNCaP Cell Line. To determine whether *ALDH1A* genes may be responsive to androgen, we treated the androgen-responsive LNCaP cell line for 48 hrs with or without 10 n*M* DHT. Treatment of LNCaP cells with 10 n*M* DHT for 48 hrs resulted in a 4-fold induction of *ALDH1A3* mRNA expression compared with controls. Under the same conditions, we were unable to detect mRNA expression of or induction by DHT of the two other members of the *ALDH1A* family: *ALDH1A1* and *ALDH1A2* (Fig. 1A). To further characterize the DHT induction of *ALDH1A3* expression, we examined the concentration and temporal effects of DHT on *ALDH1A3* mRNA levels. Treatment with DHT for 48 hrs increased expression of *ALDH1A3* in a dose-dependent manner (Fig. 1B). Temporally DHT treatments led to a time-dependent induction of *ALDH1A3* mRNA. *ALDH1A3* mRNA increased slightly at 24 hrs and



significantly at 48 hrs. Continuing treatment with DHT after 48 hrs did not lead to additional increases in *ALDH1A3* mRNA levels (Fig. 1C). Additionally, treatment of LNCaP cells with the synthetic androgen R1881 (1 n*M*) led to induction of *ALDH1A3* mRNA levels. We previously showed that the female sex steroid hormone 17β-estradiol can also induce certain androgen responsive genes through an estrogen receptor β-dependent pathway (26). To determine if *ALDH1A3* can also be regulated by 17β-estradiol, we treated LNCaP cells with 1 n*M* 17β-estradiol for 24, 48, and 72 hrs. We did not detect any changes in *ALDH1A3* mRNA levels compared with controls.

AR-Mediated Regulation of *ALDH1A3* mRNA Levels. Having established the specificity of *ALDH1A3* mRNA induction by androgen, we then determined the role of AR in regulation of *ALDH1A3* mRNA expression by testing the effects of the antiandrogen bicalutamide and AR siRNA on DHT induction of *ALDH1A3* mRNA. As shown in Figure 2A, LNCaP cell treatment with 25 μ*M* bicalutamide abolished the DHT-mediated increase in *ALDH1A3* mRNA levels. Furthermore, using an AR-specific siRNA, we observed that blocking mRNA expression of AR also led to complete inhibition of the DHT-induced increase in *ALDH1A3* mRNA levels (Fig. 2B) in a manner similar to inhibition of the androgen-responsive gene *PSA* (Fig. 2C). To further support the involvement of AR in these events, we analyzed the 5′ flanking region of the human *ALDH1A3* gene transcription start site. We identified a total of 21 putative AREs in the *ALDH1A3* promoter region within 6000 bp of the transcription start site.

Identification of Putative AR-Binding Site Motifs and Regulatory Elements. We identified a strong TSS located 75 bp upstream of the ATG start codon. A TATA box (CATAA) was found 29 bp upstream of the putative TSS. Among the 13 putative AR consensus sequences identified by MAPPER, two sequences with 73.0% homology (11 of 15 nucleotides) and one with 80%

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Figure 2. Effects of inhibition of AR and alternate AR signaling pathways on *ALDH1A3* expression. (A) Effects of the antiandrogen bicalutamide on *ALDH1A3* mRNA levels. After androgen deprivation, LNCaP cells were treated daily with vehicle (DMSO), 10 n*M* DHT, or 10 n*M* DHT + 25 μ*M* bicalutamide for 48 hrs and then subjected to RNA isolation and real-time PCR analysis. Real-time PCR results are expressed as means ± SE of fold change relative to vehicle-treated control of three separate experiments with each experiment including triplicate PCR tubes. Error bars with a different letter indicate significant differences between groups ($P < 0.05$). (B and C) Effects of siRNA on *ALDH1A3* mRNA levels. Forty-eight hrs after AR siRNA transfection, transfected LNCaP cells were treated with or without 1 n*M* DHT and nontransfected LNCaP cells with vehicle control (DMSO) for an additional 24 hrs. After treatment, cells were harvested for RNA isolation and the mRNA levels of *ALDH1A3* and *PSA* were determined using real-time PCR. Real-time PCR results are expressed as means ± SE of fold change relative to vehicle-treated control of three separate experiments with each experiment including triplicate PCR tubes. Error bars with a different letter indicate significant differences between groups ($P < 0.05$).

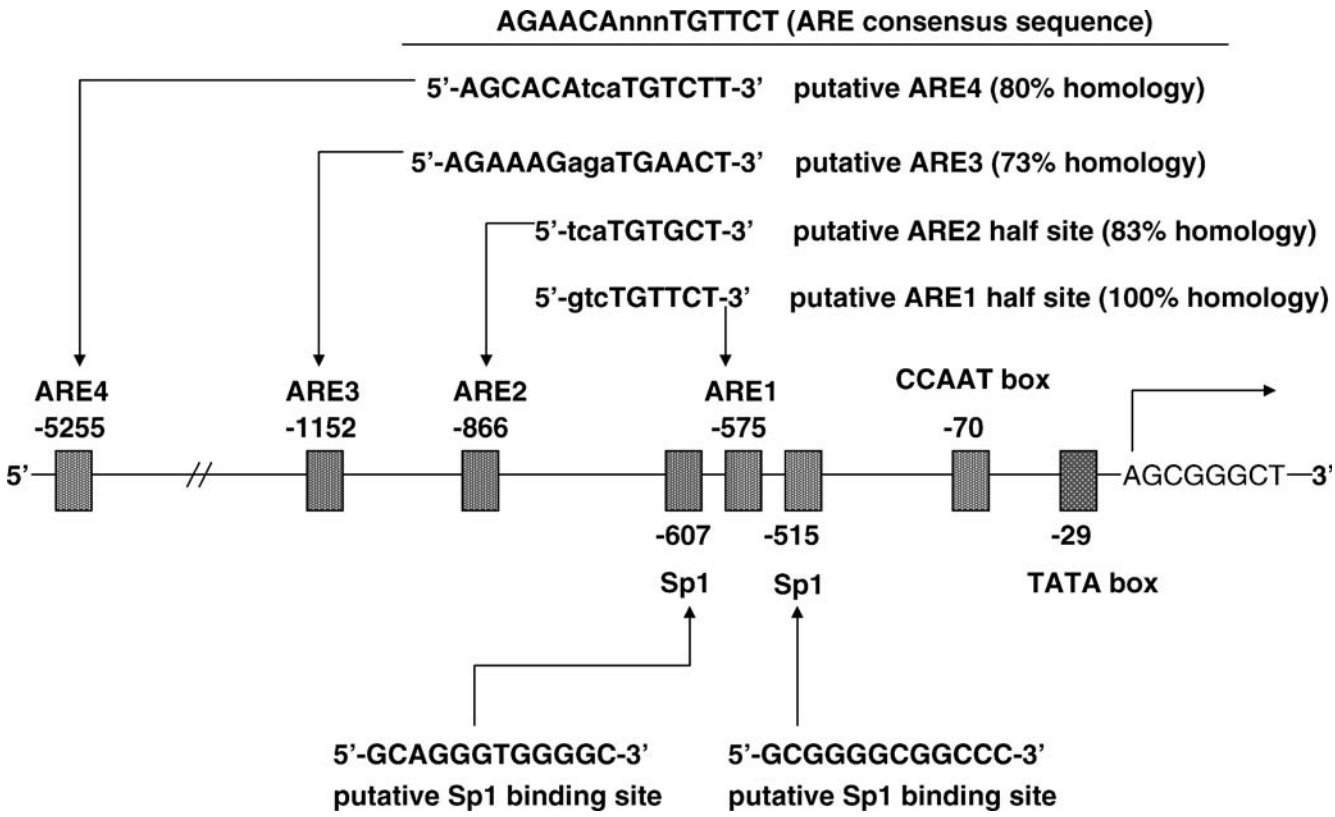

Figure 3. Identification of putative AR-binding site motifs and regulatory elements. We identified a strong TSS motif (5′-AGCGGGCT-3′) 75 bp 5′ of the ATG start codon. A TATA box (CATAA) was found 29 bp 5′ of the putative TSS. Two putative AR-binding site sequences with 80% homology and 73% homology to a consensus ARE (AGAACAnnnTGTTCT) were identified at 5.2 and 1.1 kb upstream of the TSS, respectively. Two ARE half sites were identified at 866 and 575 bp upstream to the putative TSS. Two AR *cis*-regulatory element binding sites (Sp1 and a CCAAT box) were also identified flanking ARE1 at 607, 515, and 70 bp upstream of the TSS.

homology (12 of 15 nucleotides) to the consensus ARE were identified at 5.2 and 1.17 kb upstream of the TSS, respectively. Two ARE half sites were identified with 100% and 83% homology to the highly conserved 5′-TGTTCT-3′ ARE motif at 866 and 575 bp upstream of the putative TSS. Potential binding sites for two known *cis* regulatory elements (Sp1 and NF1/CCAAT box), which were reported to be involved in AR transcriptional activity, were also identified proximal to ARE1 at 607, 515, and 70 bp upstream of the TSS (Fig. 3).

Effect of DHT on ALDH1A3 Enzyme Activity. To determine whether changes in *ALDH1A3* mRNA resulting from DHT treatment also led to changes in ALDH1A3 protein levels, we examined whether ALDH1A3 enzymatic activity is also increased in the presence of DHT. LNCaP cells treated with 10 n*M* DHT for 72 hrs showed an 8-fold increase in ALDH1A3 specific activity using RAL as substrate (Fig. 4).

Effect of DHT and RAL on *CYP26A1* mRNA Expression. To examine whether the effects of DHT on *ALDH1A3* influence RA bioactivity and metabolism, we studied the effects of DHT and RAL on the expression levels of the RA-metabolizing enzyme CYP26A1. *CYP26A1* is an RA-responsive gene (Fig. 5A) that serves as a surrogate marker for both RA biologic activity and catabolism. Treatment of LNCaP cells with RAL (1 n*M*) led to induction of *CYP26A1* levels (Fig. 5B), suggesting that RAL was converted to RA. The combined treatment of RAL and DHT resulted in a significant increase in *CYP26A1* expression compared with RAL-treated cells. DHT alone had no effect on *CYP26A1* expression levels (Fig. 5B).

Discussion

In this report, we provide evidence to support androgen induction of *ALDH1A3* mRNA as a possible mechanism by which androgen regulates RA biosynthesis in PCa cells. We demonstrates that treatment of LNCaP cells with DHT caused an increase in *ALDH1A3* mRNA and enzyme activity levels (Figs. 1 and 4). We also confirmed a previously reported microarray study that *ALDH1A3* is inducible by the synthetic androgen R1881 (32). The effect of DHT on *ALDH1A3* mRNA was blocked both by administration of the antiandrogen bicalutamide and by siRNA against AR (Fig. 2A and B), demonstrating that *ALDH1A3* induction is mediated through an AR-dependent process. While it has been demonstrated that AR's transcriptional activity can also occur by signal transduction pathways involving activation of cytosolic kinase ERK and Src kinase pathways, our experiments using the ERK

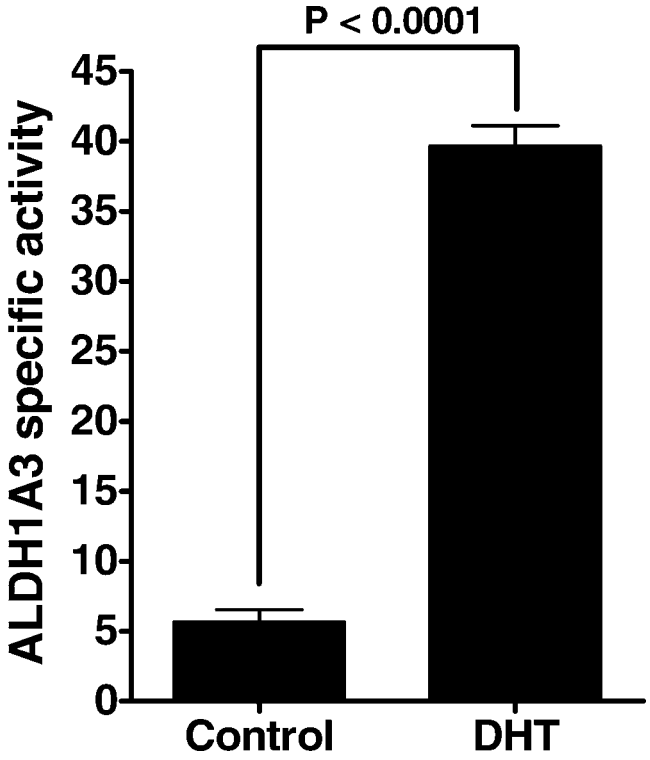

Figure 4. Effects of DHT on ALDH1A3 enzyme activity. LNCaP cells were treated with or without 10 n*M* DHT for 48 hrs followed by isolation of cytosolic protein fractions as described in Materials and Methods. ALDH1A3 enzyme activity was assessed using RAL and NAD^+ as substrates under conditions where the rate of NADH production was linear with time and with the amount of protein in the assay. Enzyme assay results are expressed as mean ± SE of enzyme activity expressed as μmol NADH/min/μg of protein lysate relative to vehicle treated control of three separate experiments with each experiment having triplicate wells.

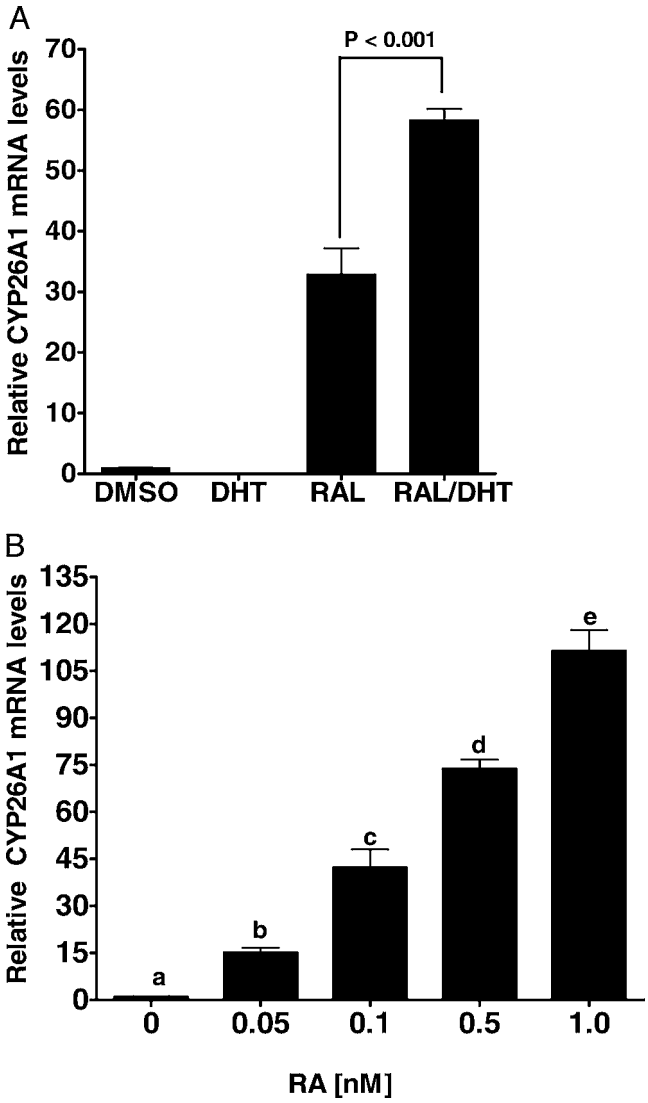

Figure 5. Effects of retinoids and DHT on mRNA expression of the RA-metabolizing enzyme *CYP26A1*. (A) Effects of DHT on RAL induction of *CYP26A1* mRNA levels. After androgen deprivation, LNCaP cells were treated with vehicle (DMSO), 1 n*M* RAL, or 1 n*M* RAL + 10 n*M* DHT for 48 hrs and then subjected to RNA isolation and real-time PCR analysis. Real-time PCR results are expressed as means ± SE of fold change relative to vehicle-treated control of three separate experiments with each experiment including triplicate PCR tubes. Bars with an asterisk are significantly different than vehicle-treated control ($P < 0.05$). (B) Effects of RA on *CYP26A1* mRNA levels. After androgen deprivation, LNCaP cells were treated with 0, 0.05, 0.1, 0.5, or 1.0 n*M* RA for 48 hrs and then subjected to RNA isolation and real-time PCR analysis. Real-time PCR results are expressed as means ± SE of fold change relative to vehicle-treated control of three separate experiments with each experiment including triplicate PCR tubes.

inhibitor PD98059 and Src family of kinases inhibitor PP1 did not affect DHT induction of *ALDH1A3* mRNA levels, suggesting that *ALDH1A3* regulation by DHT occurs by genomic events involving AR (data not shown). The effects of DHT on *ALDH1A3* also appeared to be steroid specific as treatment with the female sex steroid 17β-estradiol, which has been shown to induce selected androgen-response genes in LNCaP cells and the *ALDH1A2* isoform in rat tissue (33, 34), did not alter *ALDH1A3* mRNA expression.

Global tissue expression profiling suggests that all three ALDH1A isoforms are expressed in normal adult human prostate tissue (35, 36). However, relatively little is known about epithelial and stromal cell *ALDH1A* expression patterns in the adult prostate because most of the research on this enzyme family has focused on RA synthesis during embryonic and prenatal development (37, 38). Results from our gene expression analysis revealed that *ALDH1A1* and *ALDH1A2* isoforms are not detected in LNCaP cells, suggesting that *ALDH1A3* may be uniquely expressed in this model. Expression patterns of *ALDH1A* genes in PCa may be more complicated, as a recent study reported that *ALDH1A2* gene expression is epigenetically silenced in the androgen-independent DU-145 cell line and that immunohistochemical analysis of prostate tissue derived from matched pair donors (malignant and adjacent normal tissue) supports that ALDH1A2 protein expression is absent in PCa tissue (39). Moreover, we could not detect induction of *ALDH1A1* or *ALDH1A2* mRNA by DHT. Our data, coupled with previous reports, suggest that expression and regulation

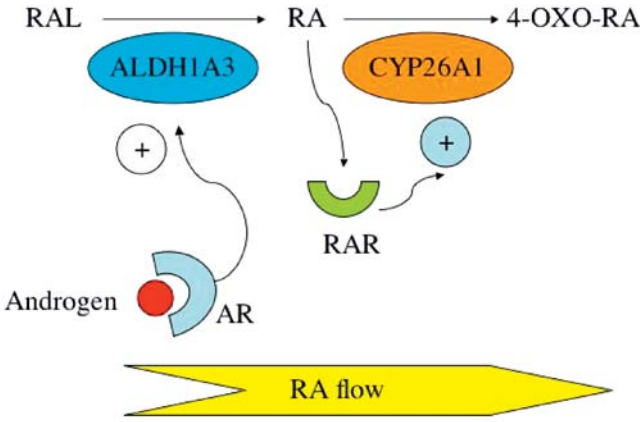

Figure 6. Hypothetical model of androgen regulation of RA metabolism in LNCaP cells. Exposure of LNCaP cells to DHT induces *ALDH1A3* gene expression. In the presence of substrate RAL, ALDH1A3 catalyzes the conversion of RAL to RA. The RA synthesized by ALDH1A3 will be available to exert biologic effects such as activation of *CYP26A1* through RAR-dependent mechanisms. The activation of *CYP26A1* allows for catabolism of RA to 4-oxo-RA. Increased exposure to androgens therefore will allow flow of RAL to RA catabolism.

of the ALDH1A enzyme family may vary among stages of PCa and degree of androgen dependence.

Informatics analysis of the *ALDH1A3* 5′ untranslated region revealed a number of potential AR-binding sites. We report sites that showed the strongest correlation to the MAPPER AR motif models and to a well-characterized ARE consensus sequence (Fig. 3; Ref. 40). One site was identified far upstream at 5.2 kb from the putative TSS and another at 1.1 kb. The latter ARE was within 600 bp of two nearly perfect ARE half sites (TGTCCT), which are highly conserved in vertebrates and have been shown to be sufficient for AR binding and gene regulation when proximal to a stronger palindromic ARE whole site (41, 42). It has been demonstrated that ligand-activated AR can regulate its own transcriptional activity and other target genes by interacting with a number of *cis* regulatory elements, including Sp1 and NF1/CCAAT box transcriptional factors (43–46). Interestingly, we discovered that the perfect ARE half site at 575 bp upstream of the TSS is flanked by GC-rich regions with strong homology to Sp1 transcription factor–binding sites. A CCAAT box/NF1 transcriptional factor–binding site was also identified 70 bp upstream of the TSS. In total, the presence of these potential AR-binding sites and regulatory elements supports our experimental data demonstrating androgen's regulation of *ALDH1A3*. Verification of these and possible other unknown AREs located in the coding region of this gene warrants further investigation.

In epithelial cells, the substrate for ALDH1A3, RAL, is synthesized from the oxidation of retinol derived from the storage form of vitamin A (retinyl esters) (47). To elucidate possible outcomes of androgen regulation of this RA synthesis enzyme, we measured the expression of the RA-metabolizing enzyme CYP26A1 in LNCaP cells exposed to RAL. The only known biologic role of CYP26A1 in vertebrates is to metabolize RA to inactive metabolites, and *CYP26A1* transcriptional activation by RA is known to be dynamic and well characterized in a number of tissue and cell types (48–51); therefore, *CYP26A1* mRNA levels are a reliable genetic marker for RA biologic activity (i.e., induction of *CYP26A1* by RA) and metabolism (i.e., RA catabolism). We observed that treatment of LNCaP cells with 1 n*M* RAL led to induction of *CYP26A1* mRNA levels (Fig. 5A), suggesting conversion of RAL to RA in this system. As a positive control, we conducted separate experiments to establish a range of *CYP26A1* transcriptional responses using various concentrations of RA (Fig. 5B). The marked induction of *CYP26A1* mRNA (Fig. 5B) in DHT + RAL–exposed cells compared with RAL exposure alone suggests to us that DHT signaling facilitates the flux of RA through its degradation pathway. This apparent increased catabolism of RA could be an indirect effect of DHT-mediated regulation of *ALDH1A3*. A hypothetical model is illustrated in Figure 6.

During embryonic development, *ALDH1A* and *CYP26A1* concerted gene expression patterns are responsible for maintaining the RA gradient during morphogenesis (52). The presence of *CYP26A1* gene expression and its induction by RA in this model supports other studies which have demonstrated that CYP26A1 hydroxylase activity serves as a barrier to increases in RA concentrations in adult tissue as it does during development (53). Still, relatively little is known about the enzymology and RA-mediated mRNA response of *CYP26A1* in mature human or animal prostate tissue, thereby making it difficult to compare with the *CYP26A1* response curve observed in our study to one expected in prostate *in vivo*. Nevertheless, concentrations of RAL and RA used in our experiments correlate to those found in adult prostate tissue (15), and the acute response of *CYP26A1* by RA that we observed also correlates to those typically observed in studies of RA's regulation of CYP26A1 in rat liver explants or during embryogenesis (53–55).

There is evidence that RA discharges its anticancer effect through induction of programmed cell death (apoptosis) in a number of models of PCa and other cancer types by down-regulating the proto-oncogene *bcl-2* (56, 57). *Bcl-2* is an antiapoptotic regulatory protein that has been shown to be involved in the molecular program of PCa progression to androgen-independent PCa (AIPCa; Ref. 58). AIPCa is defined by recurrent prostate tumor growth in the absence of androgens and resistance to apoptosis-induced chemotherapy. Although the etiology of PCa progression to androgen independence is not well understood, the regulation of *bcl-2* by RA and evidence of loss of RA metabolism in early stages of PCa raises the possibility that the degree of androgen responsiveness and degradation of RA in primary PCa tumors could correlate to loss of *bcl-2*

regulation by RA and propensity of prostate tumor to recur as AIPCa.

In summary, our results support that RA metabolism may be increased as a result of DHT-mediated induction of *ALDH1A3* mRNA activity. Regulation of *ALDH1A3* by DHT appears to be specific and mediated through an AR-dependent pathway. Thus, chronic exposure of prostate tissue to androgens over time could diminish intracellular RA concentrations and may lead to changes in RA homeostasis that could contribute to prostate carcinogenesis. Our results may also help explain why men with PCa have decreased prostatic RA concentrations, and this occurrence merits further investigation of RA metabolism in PCa.

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