THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 4, pp. 2278–2285, January 26, 2007

Differential Regulation of Clusterin and Its Isoforms by Androgens in Prostate Cells*

Received for publication, August 24, 2006, and in revised form, November 9, 2006 Published, JBC Papers in Press, December 4, 2006, DOI 10.1074/jbc.M608162200

Dawn R. Cochrane[‡§¶1], **Zhou Wang**[‖], **Motosugu Muramaki**[§¶], **Martin E. Gleave**[§¶], **and Colleen C. Nelson**[§¶2]

From the [‡]*Department of Genetics,* [§]*Department of Urologic Sciences, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada,* [¶]*The Prostate Centre, Vancouver General Hospital, Vancouver V6H 3Z6, Canada, and the* [‖]*Department of Urology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15232*

Clusterin mRNA levels were shown to increase dramatically in rat ventral prostate following castration, and clusterin was therefore originally thought to be repressed by androgens. It was later discovered that the increased clusterin levels are most likely due to castration-induced apoptosis of the prostatic epithelium rather than direct action of the androgen receptor (AR). In the studies presented here, LNCaP cells in culture and rat prostate organ culture were treated with androgens. Clusterin mRNA and protein are shown to increase with androgen treatment in a time- and dose-dependent manner. This induction of clusterin requires AR and can be inhibited by casodex, an AR antagonist. We have found that the first intron of the clusterin gene contains putative androgen response elements. The intronic region is shown to be bound by AR in chromatin immunoprecipitation assays and is transactivated by AR in reporter assays. Two isoforms of clusterin result from alternate transcriptional start sites. Both isoforms are cytoprotective; however, Isoform 1 has the capacity to produce a splice variant that is apoptotic. Real time PCR was used to determine the response of the two isoforms to androgens. Intriguingly, these results illustrated that Isoform 2 was up-regulated, whereas Isoform 1 was down-regulated by androgens. Isoform 2 was also increased as the LNCaP xenograft tumor progressed to androgen-independence, whereas Isoform 1 was unaltered. This androgen regulation of clusterin may underline the cytoprotective role of androgens in normal prostate physiology as well as play an antiapoptotic role in prostate cancer progression.

Clusterin, also known as TRPM-2 (testosterone-repressed prostate message), ApoJ, and SPG-2, is a heterodimeric sulfated glycoprotein, first isolated in ram rete testis fluid (1, 2). Clusterin is widely expressed in many tissues, including brain, ovary, testis, liver, heart, lung, breast, and prostate (3), and appears to be involved in a diverse number of biological processes. There is evidence that clusterin can act as a molecular chaperone (4), and it has been implicated in tissue remodeling (5), lipid transport (6, 7), cell-cell interactions (8), sperm maturation (9), and apoptosis (10). However, the exact role of clusterin in many of these processes remains unclear.

Upon castration, the prostatic epithelial cells undergo apoptosis in rat ventral prostate, and clusterin mRNA levels are greatly increased (11, 12); therefore, clusterin was originally thought to be repressed by testosterone. Clusterin also increases in the androgen-dependent Shionogi tumor model, in which castration causes a rapid apoptotic response following castration (13). However, in later studies it was shown that in mice bearing Shionogi tumors, when treated with calcium channel blockers that prevent apoptosis, clusterin is not up-regulated in the absence of testicular androgens (14). Similarly, if the rate of prostatic atrophy is decreased using glucocorticoid treatment, clusterin mRNA levels are reduced (15). Therefore, the current understanding is that clusterin induction is associated with apoptosis rather than an androgen-repressed gene.

Clusterin has been a somewhat enigmatic protein, being described as being both proapoptotic (16, 17) and antiapoptotic (18, 19). It was later discovered that the differential translation and post-translational processing result in either a secreted or nuclear clusterin. The secreted form of clusterin has been shown to be cytoprotective, whereas the nuclear form is proapoptotic (20, 21). An alternative splicing event generates nuclear clusterin (21–23). This splicing event removes the ER[3]-targeting signal and allows the protein to be transported into the nucleus using a nuclear localization sequence (21).

Recent updates to GenBank™ have highlighted that there are two transcriptional isoforms of human clusterin (Isoform 1, NM_001831; Isoform 2, NM_203339). These isoforms result from different transcriptional initiation sites and are only produced in humans and chimpanzees. These transcriptional isoforms result in proteins that have different N termini. Both clusterin isoforms produce proteins that are cytoprotective.[4] Intriguingly, it is only Isoform 1 that is capable of producing a splice variant that results in the nuclear, apoptotic form of clusterin (21). Many studies demonstrating that clusterin has anti-

* This work was supported by NCIC Program Grant 102003. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Senior Scholar of the Michael Smith Foundation for Health Research.

[2] Supported by Scholarship funds from the Michael Smith Foundation for Health Research, Canadian Institutes of Health Research, and the Canadian Prostate Cancer Research Initiative. To whom correspondence should be addressed: Prostate Centre at VGH, 2660 Oak St., Vancouver, British Columbia V6H 3Z6, Canada. Tel.: 604-875-5555 (ext. 64282); Fax: 604-875-5654; E-mail: colleen.nelson@ubc.ca.

[3] The abbreviations used are: ER, estrogen receptor; AR, androgen receptor; ARE, androgen response element; AI, androgen-independent; CSS, charcoal-stripped serum; DHT, dihydrotestosterone; PSA, prostate-specific antigen; ChIP, chromatin immunoprecipitation; MOPS, 4-morpholinepropanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; FAM, fluorescein; TAMRA, 6-carboxytetramethylrhodamine.

[4] M. Muramaki, E. Beraldi, D. Cochrane, M. Cox, P. Rennie, C. Nelson, and M. Gleave, submitted for publication.

apoptotic properties have been undertaken in mice or rats, and these species are only capable of transcribing the orthologue to Isoform 1 of clusterin; however, the first exon is predicted to not be translated due to the lack of an initiating methionine in nonprimate species.

Clusterin is expressed in many cancers, including breast, prostate, ovarian, pancreatic, and renal cancers (25–29). In prostate cancer, high levels of clusterin correlate with Gleason grade (30), which may suggest that clusterin plays a role in the aggressiveness of a tumor. The levels of clusterin increase significantly following androgen ablation therapy (31). Clusterin provides a route by which a subset of the prostate cancer cells can evade apoptosis due to androgen ablation and thereby allows the cells to grow into androgen-independent (AI) cancer. In xenografts of LNCaP cells overexpressing clusterin, the tumors reach androgen independence faster than the parental cell line, suggesting that clusterin plays a role in the acquisition of the AI phenotype (11).

Prostate cells overexpressing Isoform 2 of clusterin become more resistant to Fas-mediated apoptosis (32). Oligonucleotides that target Isoform 2 of clusterin endow the cells with sensitivity to radiation (33) and chemotherapeutic agents (34).

In the present study, we show that total clusterin expression is in fact up-regulated by androgens. In an androgen-dependent prostate cancer cell line, clusterin is up-regulated at both the RNA and protein levels when treated with androgens, and this effect is reversed with anti-androgen treatment. Clusterin mRNA levels are also seen increasing in rat ventral prostate organ culture when treated with androgens. This effect is specific to androgens, since exposure to other steroid hormones does not have an effect on clusterin levels. We show that the AR directly regulates clusterin expression through interaction with an intronic enhancer region on the clusterin gene. Furthermore, androgens have opposing effects on the two mRNA isoforms of clusterin that are generated from alternative start sites. Isoform 1 is repressed by androgens, whereas Isoform 2 is up-regulated by androgens through direct interaction with the first intron.

EXPERIMENTAL PROCEDURES

Cell Culture—LNCaP cells (passage 35–50) and PC3 cells (passage 10–20) were grown at 37 °C and 5% CO_2 in RPMI and Dulbecco's modified Eagle's medium, respectively, both containing 5% fetal bovine serum and penicillin/streptomycin. Charcoal-stripped serum (CSS) is prepared using fetal calf serum incubated with 1% charcoal and 0.1% dextran T-70 followed by filtration.

Hormone Treatments—R1881, dihydrotestosterone (DHT), and dexamethazone were purchased from Steraloids Inc. Progesterone, 17β-estradiol (estradiol), all-*trans*-retinoic acid, and 3,3′,5-triiodo-L-thyronine sodium salt are from Sigma. Casodex (bicalutamide) was from Astro Zenica. All hormone stock solutions were in absolute ethanol with the exception of the 3,3′,5-triiodo-L-thyronine sodium salt, which was dissolved in NaOH. Prior to any hormone treatments, cells were grown in media plus 2% CSS for 48 h. Cells were harvested for protein using radioimmune precipitation lysis buffer (150 mM NaCl, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris) and for RNA using Trizol (Invitrogen).

Northern Blot Analysis—Total RNA (10 μg) was denatured in sample buffer and run through a denaturing 1% MOPS formaldehyde-agarose gel for 1 h at 80 V. The RNA was transferred to a nylon membrane (Biodyne B; Pall Gelman Laboratory) in 20× SSC (3 M sodium chloride, 0.3 M sodium citrate, pH 7.0) for 20 h. The RNA was cross-linked to the membrane using a UV Stratalinker (Stratagene) according to the manufacturer's instructions. Membranes were prehybridized in ExpressHyb (Clontech), containing denatured salmon testes DNA (Sigma) for 3 h at 65 °C. Clusterin probes were generated using reverse transcription-PCR from human kidney RNA using the following primers: 5′-AAGGAAATTCAAAAATGCTGTCAA-3′ and 5′-ACAGACAAGATCTCCCGGCACTT-3′. Radioactivity was incorporated into the probe using Ready-To-Go DNA labeling beads (Amersham Biosciences) to a specific activity of 1–2 × 10^8 disintegrations/min/μg. Probes were hybridized to the membrane overnight at 65 °C. High stringency washes were performed on the membranes at 65 °C. The membranes were exposed to Eastman Kodak Co. MR film.

Western Blot Analysis—Protein sample (15 μg) was boiled at 95 °C for 5 min in sample buffer containing β-mercaptoethanol. Samples were loaded on 10% polyacrylamide gels and subjected to electrophoresis for 1 h at 150 V. Proteins were transferred electrophoretically onto polyvinylidene difluoride membrane (Millipore). Blots were probed for clusterin using a goat polyclonal clusterin-β (C-18) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After incubation with a secondary antibody, the membranes were incubated with ECL reagents (Amersham Biosciences) and exposed to Eastman Kodak Co. Blue XB-1 film. For normalization purposes, membranes were reprobed with a rabbit polyclonal β-tubulin antibody (Santa Cruz Biotechnology).

Real Time PCR—To quantitatively evaluate the levels of clusterin in the samples, real time PCR was undertaken. Before generating the cDNA, 2 μg of the RNA was treated with DNAse 1 (Invitrogen) to remove any DNA contamination. The RNA was then reverse transcribed into cDNA in a reaction containing 1× reaction buffer, 0.01 M dithiothreitol, 1 mM dNTPs, 40 units RNAsin (Promega), random hexamers (250 ng), and 200 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen). The reaction proceeded at 25 °C for 10 min and then at 37 °C for 1 h. The levels of clusterin transcript were then assayed using real time PCR on the ABI 7900 HT sequence detection system. To determine the levels of total clusterin, a region of exon 3 was amplified. The primers used were as follows: forward, 5′-GAGCAGCTGAACGAGCAGTTT-3′; reverse, 5′-CTTCGCCTTGCGTGAGGT; probe, 5′-6-FAM-ACTGGGTG-TCCCGGCTGGCA-TAMRA-Q-3′. To differentiate Isoform 1 and 2 levels, primers and probes in the unique 5′-untranslated regions of the respective transcripts were used as follows: for Isoform 1, forward primer 5′-CGTGAGTCATGCAGGTTTGC-3′, reverse primer 5′-CTGGGAGGCGCCGAAT-3′, and probe 5′-6-FAM-TGTGTGCGCGAGCAGAGCGCTAT-TAMRA-Q-3′; for Isoform 2, forward primer 5′-CTCTACTCTCCGAAGGG-AATTGTC-3′, reverse primer 5′-CGGGCTGCCTGTGCAT-3′, and probe 5′-6-FAM-TTCCTGGCTTCCACTACTTCCAC-

CCC-TAMRA-Q-3′. For normalization purposes, rRNA control primers and probes (ABI) were used. The reported values are the averages and S.D. values of three biological replicates.

Organ Culture—Young adult male Harlan Sprague-Dawley rats (250–300 g) from Harlan, Inc. (Indianapolis, IN) were castrated by removing testes, fat pads, and epididymis in a room dedicated for animal manipulation according to a protocol approved by the Northwestern University Animal Care and Use Committee (Chicago, IL). The castrated animals were maintained in the Northwestern University animal facility. The ventral prostate was dissected out from the rats 7 days after castration. Organ culture was done as described previously (35, 36). Briefly, the prostates were minced with a scalpel to generate uniform fragments with ~1 mm in all dimensions. The prostate fragments were placed on lens paper supported at the media-air interface with a stainless steel screen in a 10-cm culture plate and incubated in 5% CO_2 incubator at 37 °C. The prostate fragments were in good contact with the medium but not submerged in the M-199 medium consisting of Earles salts, L-glutamine, and 2.2 g/liter sodium bicarbonate, without phenol red, from Invitrogen, with the addition of 10% charcoal-stripped, penicillin G sodium at 200 units/ml and streptomycin sulfate at 0.2 μg/ml. Treatment with DHT was at 1 μM final concentration for 24 h. After treatment, the prostate fragments were harvested and frozen immediately in liquid nitrogen N_2, and the RNA was isolated using the guanidinium/CsCl gradient method. Northern blot analysis of clusterin mRNA was carried out as described as previously using a clusterin cDNA probe.

Transfections—LNCaP cells were grown to 60% confluence before being transfected with 1.5 μg of the steroid receptor expression vectors. Prior to the transfections, cells were grown in RPMI + 2% CSS for 2 days. Lipofectin (Invitrogen) was incubated with serum-free RPMI for 30 min before the DNA was added and incubated for 10 min more. Cells were transfected with Lipofectin/DNA mix for 5 h before the medium was changed to RPMI + 2% CSS. Cells were treated with a 1 nM concentration of the indicated hormone ligand for 72 h prior to harvesting unless otherwise indicated.

Luciferase Assay—Consite (available on the World Wide Web at mordor.cgb.ki.se/cgi-bin/CONSITE/consite/) (37) was used to predict the location of androgen response elements (AREs) in the clusterin gene. Luciferase reporter plasmids were created by cloning the PCR-generated fragments into pTK-luc (ATCC). Both LNCaP and PC3 cells were plated into 6-well plates, and then using Lipofectin, the cells were transfected with 3 μg of reporter plasmid, 2 μg of rat AR in pRcCMV (Invitrogen), and 0.05 μg of the *Renilla* expression plasmid pRLTKS (Promega) per plate. After transfection, the cells were incubated with either 2% CSS medium alone or with R1881. Cells were washed and harvested with passive lysis buffer (Promega). PC3 cells were harvested after 48 h of hormone treatment, and LNCaP cells were harvested after 72 h. Luciferase activity of 20 μl aliquots of lysate was determined using the dual-luciferase reporter assay system (Promega) on a luminometer (Berthold, Germany). Luciferase activity was normalized for transfection efficiency using *Renilla* activity. Experiments were done in triplicate, averaged, and expressed as -fold induction.

Chromatin Immunoprecipitation—Plates of LNCaP cells were incubated with formaldehyde at a concentration of 1% for 10 min on a shaking platform. Glycine was then added to a final concentration of 0.125 M for 5 min. The cells were washed twice with ice-cold phosphate-buffered saline before being scraped off the plate with 5 mM EDTA in phosphate-buffered saline. The cells were then pelleted and washed with ice-cold phosphate-buffered saline. The pellet was resuspended in 500 μl of cell lysis buffer (5 mM PIPES, pH 8.0, 85 mM KCl, 0.5% Nonidet P-40) and incubated on ice for 10 min. The sample was centrifuged, and the supernatant was removed. The nuclei were then lysed by adding 1 ml of nuclei lysis buffer (50 mM Tris-Cl, pH 8.1, 10 mM EDTA, 1% SDS) and incubating on ice for 10 min. The samples are then sonicated on setting 3 (Sonic Dismembrator 550; Fisher), four pulses of 15 s, separated by 30 s. The sonicated chromatin was then precleared by rotating at 4 °C for 2 h with normal rabbit IgG (2 μg/ml), salmon sperm DNA (20 μg/ml), and 40 μl of protein A/G-agarose beads (Santa Cruz Biotechnology) that had been prewashed with dialysis buffer (2 mM EDTA, 50 mM Tris-Cl, pH 8.0, 0.2% Sarkosyl), followed by brief centrifugation. For the immunoprecipitation, 200 μl of the precleared sample was diluted with 400 μl of dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-Cl, pH 8.1, 167 mM NaCl). The sample was then rotated overnight at 4 °C with 2 μl of normal rabbit serum or 5 μl of rabbit polyclonal AR antibody (Upstate Biotechnology, Inc.). Protein A/G-agarose beads that had been prewashed with dialysis buffer were added, and the samples were rotated at 4 °C for 2 h. The samples were centrifuged briefly, and the resulting pellet was washed twice with dialysis buffer and four times with IP wash buffer (100 mM Tris-Cl, pH 9.0, 500 mM LiCl, 1% Nonidet P-40, 1% deoxycholic acid). Between washes, the samples were rotated at 4 °C for 3 min. To the pellet, 150 μl of elution buffer (50 mM $NaHCO_3$, 1% SDS) was added, and the samples were shaken vigorously at room temperature for 15 min. The samples were centrifuged briefly, the supernatant was collected, and the elution process was repeated. For input, 200 μl of the precleared DNA was diluted in 100 μl of elution buffer. To the pooled elutions and the input sample, NaCl (final concentration of 0.3 M) was added, and the samples were incubated at 67 °C overnight to decross-link. The DNA was ethanol-precipitated, and the protein was digested with proteinase K (Invitrogen). The DNA was purified using a PCR purification kit (Qiagen). Thirty-five cycles of PCR were then performed using 1 μl of the chromatin as template. Primers for the prostate-specific antigen enhancer were 5′- CATGTTCACATTAGTACACCTTGCC-3′ and 5′-TCTCAGATCCAGGCTTGCTTACTGTC-3′. Primers for Clu 1, which encompasses region +2094 to +2433 of the clusterin gene, were 5′-CAGCCCTGCAGTGATTCATA-3′ and 5′-CCCGACCCCTAGTCCAGTAT-3′. Primers for Clu 2, which encompasses region +2443 to +2761, were 5′-CTCTACTCCCAGGGTTACCA-3′ and 5′-CCCCTGAAAGCAACAACTTC-3′. Primes for Clu 3, encompassing +2779 and +3083 of the gene, were 5′-TTCTGGCTGGCTTTGTCTCT-3′ and 5′-GTTCCCCTTCCTGAAATGGT-3′.

LNCaP Xenografts—2×10^6 cultured LNCaP cells were combined with Matrigel (BD Biosciences) at a 50:50 volume ratio of

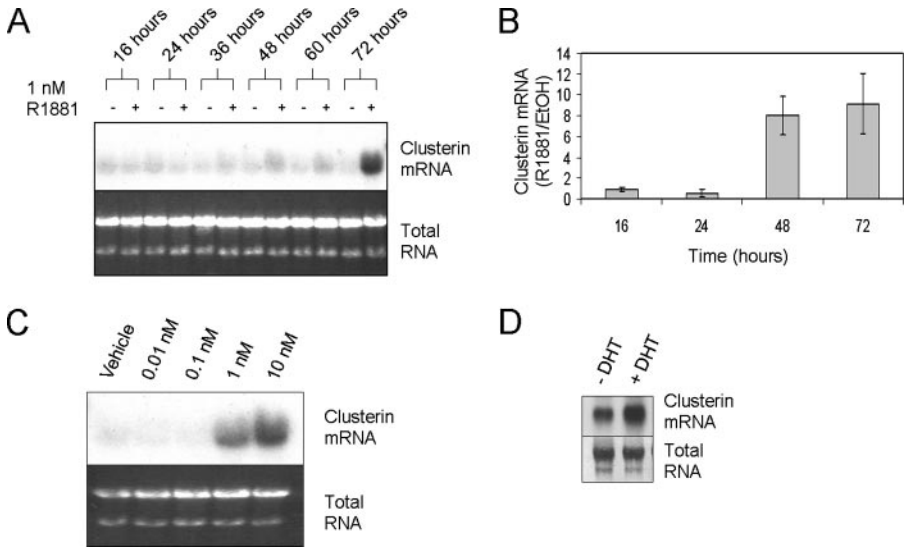

FIGURE 1. **Clusterin mRNA levels in response to androgen treatment.** *A*, *top*, Northern blot of an R1881 time course probed for clusterin; *bottom*, loading control. LNCaP cells were treated with 1 nM R1881 (+) or ethanol vehicle (−) for periods of time ranging from 16 to 72 h. *B*, real time PCR quantification of clusterin mRNA levels in response to androgen treatment. The graph shows the ratio of clusterin mRNA in R1881-treated cells to vehicle control-treated cells. *C*, Northern blot of R1881 titration. LNCaP cells were treated with doses of R1881 ranging from 0.01 to 10 nM R1881 for a time period of 72 h. *D*, the ventral prostates from 7-day castrated adult rats were minced into small pieces and cultured in dish, as described. After overnight culture, the prostates were treated with DHT at a final concentration of 1 μM or ethanol vehicle for 24 h. The tissues from organ culture were then harvested and isolated for Northern blot analysis. The *lower panel* shows the methylene blue staining pattern, indicating the amount and quality of total RNA loaded in each lane.

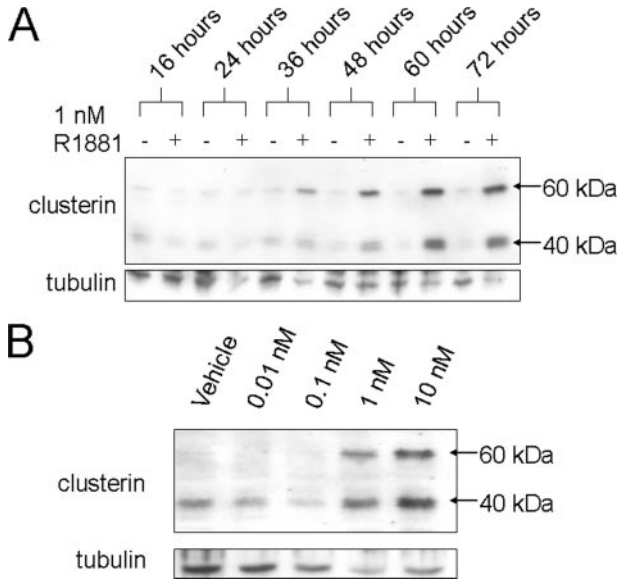

FIGURE 2. **Clusterin protein levels in response to androgen treatment.** *A*, *top*, Western blot of an R1881 time course probed for clusterin; *bottom*, β-tubulin levels as a loading control. LNCaP cells were treated with 1 nM R1881 (+) or ethanol vehicle (−) for periods of time ranging from 16 to 72 h. Shown are both the 60-kDa (full-length) and 40-kDa (processed) forms of clusterin. *B*, Western blot of R1881 titration. LNCaP cells were treated with doses of R1881 ranging from 0.01 to 10 nM R1881 for 72 h.

cells to Matrigel and injected subcutaneously into four sites of 6–8-week-old athymic nude mice (BALB/c strain; Charles River Laboratory). The mice were surgically castrated 8 weeks postinjection under methoxycluorane anesthesia. To harvest the tumors, the mice were sacrificed using carbon dioxide asphyxiation, and the tumors were removed and frozen immediately at −80 °C. Androgen-dependent tumors were harvested 8–14 days postcastration, whereas AI tumors were harvested 22–35 days postcastration.

Statistical Analysis—The *error bars* in the real time PCR graphs (see Figs. 1 and 4–6) represent S.E. The luciferase data were analyzed using a Kruskal-Wallis nonparametric test, followed by Dunn's multiple comparison test to determine statistically significant differences between EtOH- and R1881-treated samples using Prism (GraphPad). For the real time PCR, statistical significance was measured using a Student's *t* test (Microsoft Excel).

RESULTS

Clusterin is Up-regulated by Androgens in a Dose- and Time-dependent Manner—To determine if clusterin levels are affected by androgens *in vitro*, LNCaP cells were treated with 1 nM R1881 for a time period ranging from 16 to 72 h. Northern blot analysis of those samples reveals that when compared with the time-matched vehicle control, the levels of clusterin mRNA increase with androgen treatment starting at 36 h and continue to rise up to and including 72 h (Fig. 1*A*). Real time PCR was used to quantify the increase of clusterin levels in response to androgens (Fig. 1*B*). DNA laddering analysis of the androgen-treated cells revealed no apparent increase in apoptosis over the time period when clusterin levels increase (data not shown). To verify that the response being seen is dose-dependent, LNCaP cells were treated with R1881 for 72 h in doses ranging from 0.01 to 10 nM. There is no detectable response to low levels of androgens up to 0.1 nM R1881. Clusterin mRNA levels increase in cells treated with levels of R1881 greater than 1 nM (Fig. 1*C*). A similar increase in clusterin mRNA levels was seen with 10 nM DHT (data not shown).

We were interested in determining if clusterin was also up-regulated by androgens in the rat prostate. The rat ventral prostate is highly androgen-responsive and has been used to study the effects of androgen withdrawal and as a model to study androgen-regulated genes. Young male Sprague-Dawley rats were castrated for 7 days, and prostates were removed, microdissected, and grown in organ culture with or without androgen treatment for 24 h. RNA was harvested and analyzed for a variety of genes (38, 39). In these *ex vivo* prostate samples, the levels of clusterin RNA increased in the prostate organ culture following treatment with DHT (Fig. 1*D*). This finding shows that clusterin is also up-regulated in the normal prostate secretory epithelium in response to androgens.

To verify that levels of clusterin protein corroborate the increase detected at the mRNA level, protein from androgen-treated LNCaP cells was harvested and probed by Western blot.

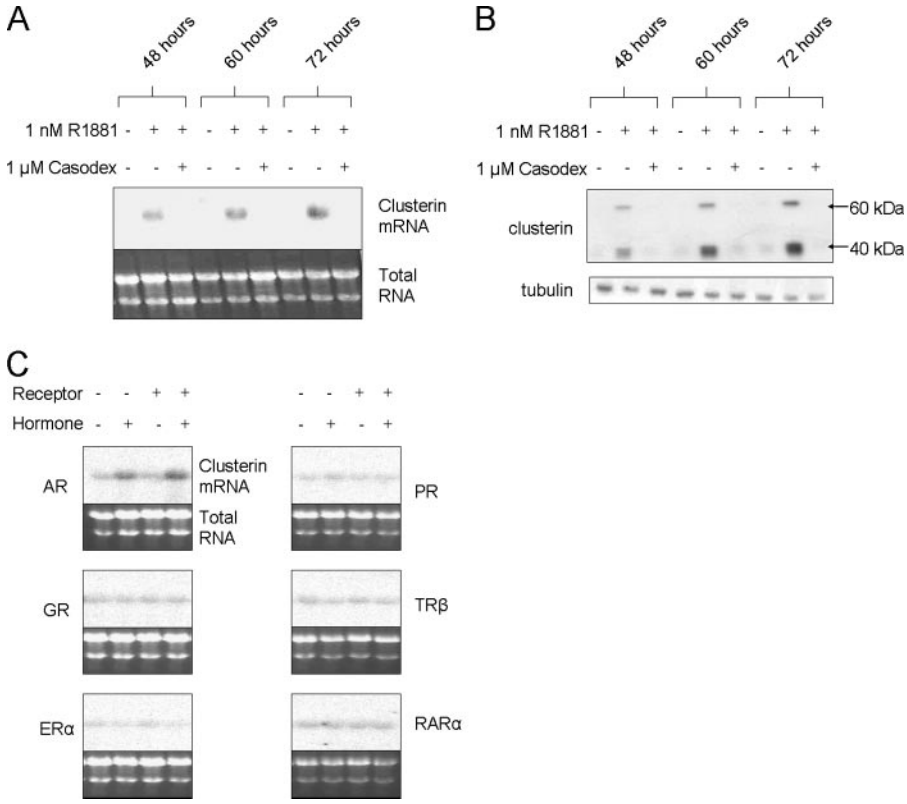

FIGURE 3. **Necessity of AR in clusterin up-regulation.** *A*, Northern blot showing that the up-regulation of clusterin is blocked by casodex, an AR antagonist. LNCaP cells were treated with 1 nM R1881 alone or in conjunction with 1 μM casodex for periods of time ranging from 48 to 72 h. *B*, Western blot showing that casodex also blocks androgen action on clusterin at the protein level. *C*, LNCaP cells were transfected with the steroid receptors AR, glucocorticoid receptor (*GR*), ER, progesterone receptor (*PR*), thyroid hormone receptor (*TR*), and retinoic acid receptor (*RAR*) and then treated with a 1 nM concentration of the cognate ligand (R1881, dexamethasone, estradiol, progesterone, 3,3′,5-triiodo-L-thyronine sodium salt or all-*trans*-retinoic acid, respectively). Shown are Northern blots of these samples, probed for clusterin.

A time course of androgen treatment is shown in Fig. 2*A*. In the absence of treatment, there are low levels of clusterin expression in LNCaP cells. With increasing duration of androgen treatment, the levels of clusterin increase starting at 36 h and continuing through to 72 h. The antibody used in the Western blots detects the β-chain of clusterin. The two bands seen in the Western blots are the 60-kDa form and the 40-kDa cleaved form of clusterin. Both the 40- and 60-kDa bands are seen increasing beginning at 36 h. The dose responsiveness of the clusterin is highly similar to that seen at the mRNA level, dramatically increasing with exposure of R1881 at 1 nM and above (Fig. 2*B*).

The AR Is Necessary for Clusterin to Be Up-regulated—To prove that AR mediates the up-regulation of clusterin, LNCaP cells were treated with R1881 alone or in conjunction with the AR-antagonist casodex for 48–72 h. At all time points tested, casodex was able to completely block the effect of R1881 up-regulation of clusterin (Fig. 3, *A* and *B*). The AR belongs to the steroid receptor subclass of nuclear receptors, which also includes ER, progesterone receptor, glucocorticoid receptor, and mineralocorticoid receptor (40). Nuclear receptors are structurally similar, containing a C-terminal ligand binding domain, a central DNA binding domain, and an N-terminal transactivation domain (41). It is possible that the response to androgens is not specific and that other steroid receptors and their cognate hormones could also regulate clusterin expression. To test this possibility, LNCaP cells were transfected with specific hormone receptors and treated with their respective ligands to examine whether the effect being seen is confined to androgens (Fig. 3*C*). The exogenous expression of receptors alone had no effect on clusterin levels; nor was there a noticeable effect on the mRNA levels (Fig. 3*C*) or protein levels (data not shown) when the receptor-transfected cells were ligand-activated. These data demonstrate that an increase in clusterin levels is mediated specifically by AR and not other steroid receptors in LNCaP cells.

The First Intron of the Clusterin Gene Contains Functional AREs—It has been reported previously that the first intron of human clusterin contained putative AREs to which the AR may bind (42, 43). To examine this further in our studies, the first intron was analyzed using Consite software, which predicts response elements within a DNA sequence and has shown the reliable prediction of validated AREs (44). Many AREs were predicted; however, a cluster of AREs was found between +2595 and +3211 of the clusterin gene (Fig. 4*A*). It has been shown previously that for maximal androgen responsiveness, AREs function cooperatively (45, 46); therefore, it is likely that the androgen-responsive region in the intron lies within the cluster of AREs. Two luciferase reporter constructs were made, both of which encompassed the region high in putative AREs. The reporter construct termed T21 contained the region high in AREs in addition to the region 5′, whereas the T23 construct contained the region high in putative AREs as well as the region 3′ to this. Both constructs were tested in luciferase assays done both in LNCaP and PC3 cells. As a positive control, the same amount of a luciferase reporter construct with the promoter region of the prostate-specific antigen (PSA) was used in parallel.

In LNCaP cells, the T21 construct was more responsive to androgens than the T23 construct and slightly more active than the PSA proximal promoter construct (Fig. 4*B*). This suggests that there may be AREs or other elements on the DNA 5′ to the region encompassed by both T21 and T23, which can further enhance the activity of the DNA. In PC3 cells transfected with AR, T21 has similar transcriptional activity as PSA in response to androgens; however, T23 shows no activity at all (Fig. 4*C*). The difference in the activity of the T23 construct between LNCaP and PC3 cells shows that the response is modulated by cell type and could require additional transcription factors.

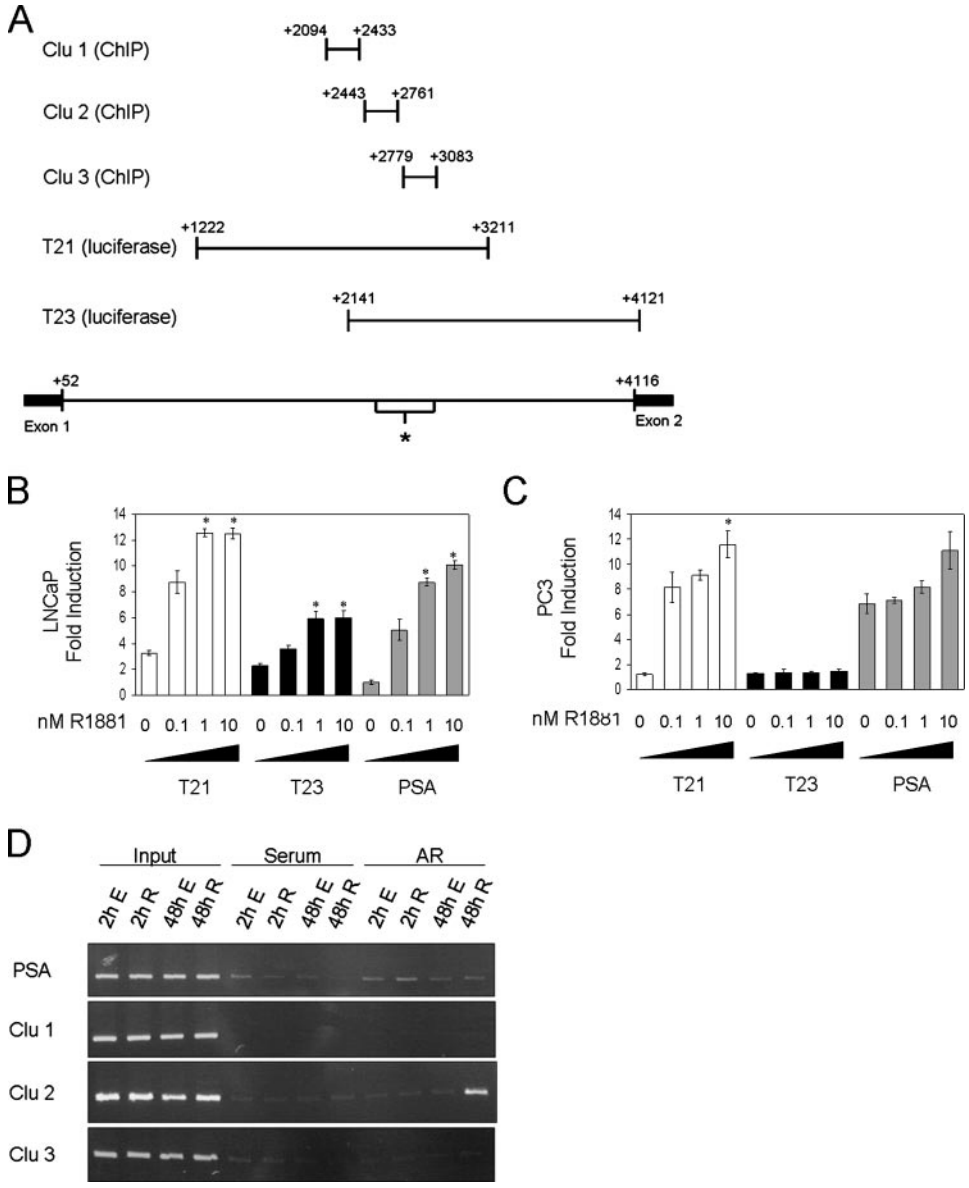

FIGURE 4. **Transcriptional activation due to AR interaction with an enhancer region within the first intron of the clusterin genomic DNA.** *A*, the clusterin gene and constructs made from it for the purposes of the luciferase assay and ChIP. The *asterisk* denotes the region containing clusters of putative AREs. *B*, luciferase assay in LNCaP cells. The LNCaPs were transfected with the reporter constructs, the *Renilla* construct, and an AR expression plasmid. The cells were treated with 0, 0.1, 1, and 10 nM R1881. PSA is included as a positive control. An *asterisk* indicates that the value is significantly different from the EtOH value (p value <0.05). *C*, luciferase assay in PC3 cells. The PC3 cells were transfected with the reporter constructs, the *Renilla* construct, and an AR expression plasmid. The cells were treated with 0, 0.01, 0.05, and 0.1 nM R1881. An *asterisk* indicates that the value is significantly different from the EtOH value (p value <0.05). *D*, PCR products from ChIP assay. The cells were treated for 2 or 48 h, with (*R*) or without (*E*) 1 nM R1881. Immunoprecipitation using rabbit serum was a negative control showing background levels of the PCR products. PSA was a positive control for AR binding. Clu 1, Clu 2, and Clu 3 are three regions within the clusterin first intron containing putative AREs.

showing that there is more AR bound. Three regions of the clusterin intron were assayed. There is no binding of AR observed in the regions of Clu 1 and Clu 3. In the Clu 2 region, however, there is more PCR product in the R1881-treated samples with a 48 h duration of treatment.

Differential Regulation of Clusterin Isoforms—In primates, there are two transcriptional isoforms of clusterin resulting from two independent transcriptional start sites (Fig. 5*A*). It is predicted that in humans, the two isoforms differ in their translational start sites, with Isoform 1 translating the first exon, whereas Isoform 2, whose transcriptional start site is in Intron 1, initiates translation in Exon 2. However, all available clusterin antibodies recognize the epitopes of the protein, which are common to both isoforms and are therefore not able to distinguish between isoforms. Since the ARE-containing region in the first intron is located between the two transcriptional start sites, we were interested in determining whether androgens can regulate the transcription of one or both isoforms. Primers and probes for real time PCR were designed to the unique 5′-untranslated regions of the two isoforms to measure their individual transcriptional levels. Interestingly, Isoform 2 is up-regulated by androgens by 24 h and is maintained throughout the 48-h time period, whereas Isoform 1 is down-regulated by androgens (Fig. 5, *B–E*). The up-regulation of total clusterin mRNA levels is not observed until 48 h, suggesting that at 24 h, the up-regulation of the Isoform 2 is masked by the down-regulation of Isoform 1, and there is no observable change in total clusterin levels.

To verify that AR is binding to the clusterin intron *in vivo*, chromatin immunoprecipitation (ChIP) was performed (Fig. 5*C*). The chromatin from LNCaP cells that had been cultured in the presence or absence of R1881 for 2 or 48 h was immunoprecipitated with either normal rabbit serum, as a negative control, or with an AR antibody. As a positive control, PCR was performed on a region of the PSA enhancer known to be bound by AR. At the 2-h time point, there is more PSA enhancer PCR product in the R1881-treated sample than the ethanol control,

The LNCaP xenograft tumor model is often used as a model for progression from androgen dependence to androgen independence. In this model, androgen-regulated genes, such as PSA, respond to castration by being down-regulated following castration up to about day 21. However, during progression to androgen independence, androgen-regulated genes, including PSA, are reactivated by about day 28 and remain elevated in AI tumors. To determine the kinetics of clusterin expression during progression to androgen independence, tumors from an

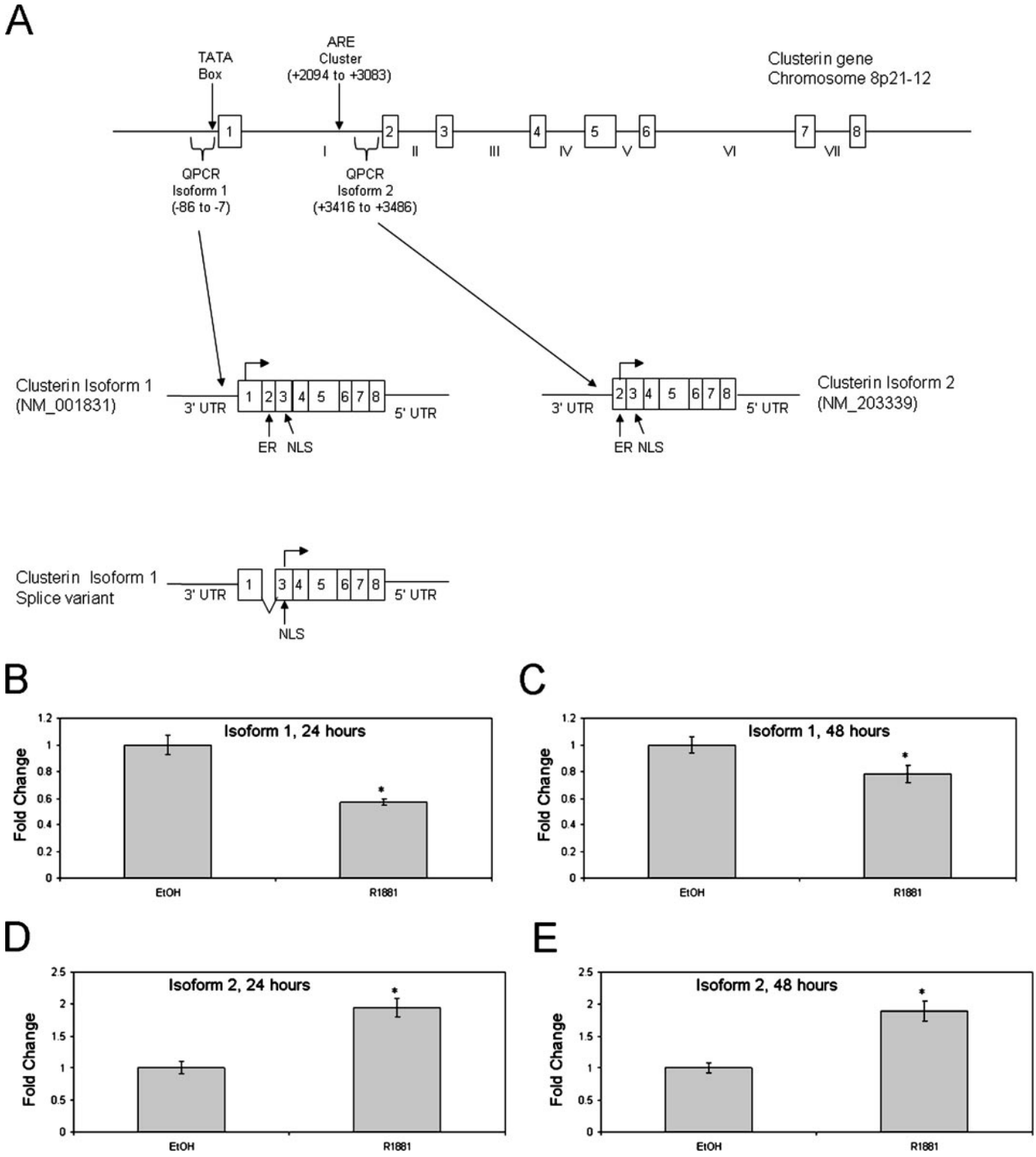

FIGURE 5. **Androgenic regulation of the two clusterin isoforms.** *A*, the clusterin gene and the two mRNA transcripts formed from alternate transcriptional start sites. The clusterin gene is pictured with the exons appearing as *numbered boxes* and the introns numbered in *roman numerals*. The locations of real time PCR primers used to distinguish the two isoforms are indicated on the clusterin gene as *QPCR*. The *bent arrows* show the translational start sites on the mRNAs. Splicing out of exon 2 results in the nuclear isoform of clusterin. The ER targeting signal is found in exon 2, and the nuclear localization signal (*NLS*) is found in exon 3. LNCaP cells were treated with 1 nM R1881 for 24 or 48 h. RNA from the cells was assayed for clustering Isoform 1 and Isoform 2 levels using real time PCR. Shown are clusterin Isoform 1 levels at 24 h (*B*) and 48 h (*C*) of treatment with R1881. Levels of clusterin Isoform 2 levels at 24 h (*D*) and 48 h (*E*) are shown. *, $p < 0.05$. *UTR*, untranslated region.

LNCaP tumor series were harvested for RNA. A Northern blot of PSA levels in the tumors prior to and following castration is shown (Fig. 6*A*), and the corresponding serum PSA levels are shown in Fig. 6*B*. The tumor volumes of the tumors are shown in Fig. 6*C*. Unlike normal prostate cells, LNCaP tumors do not undergo apoptosis in response to castration. The tumor vol-

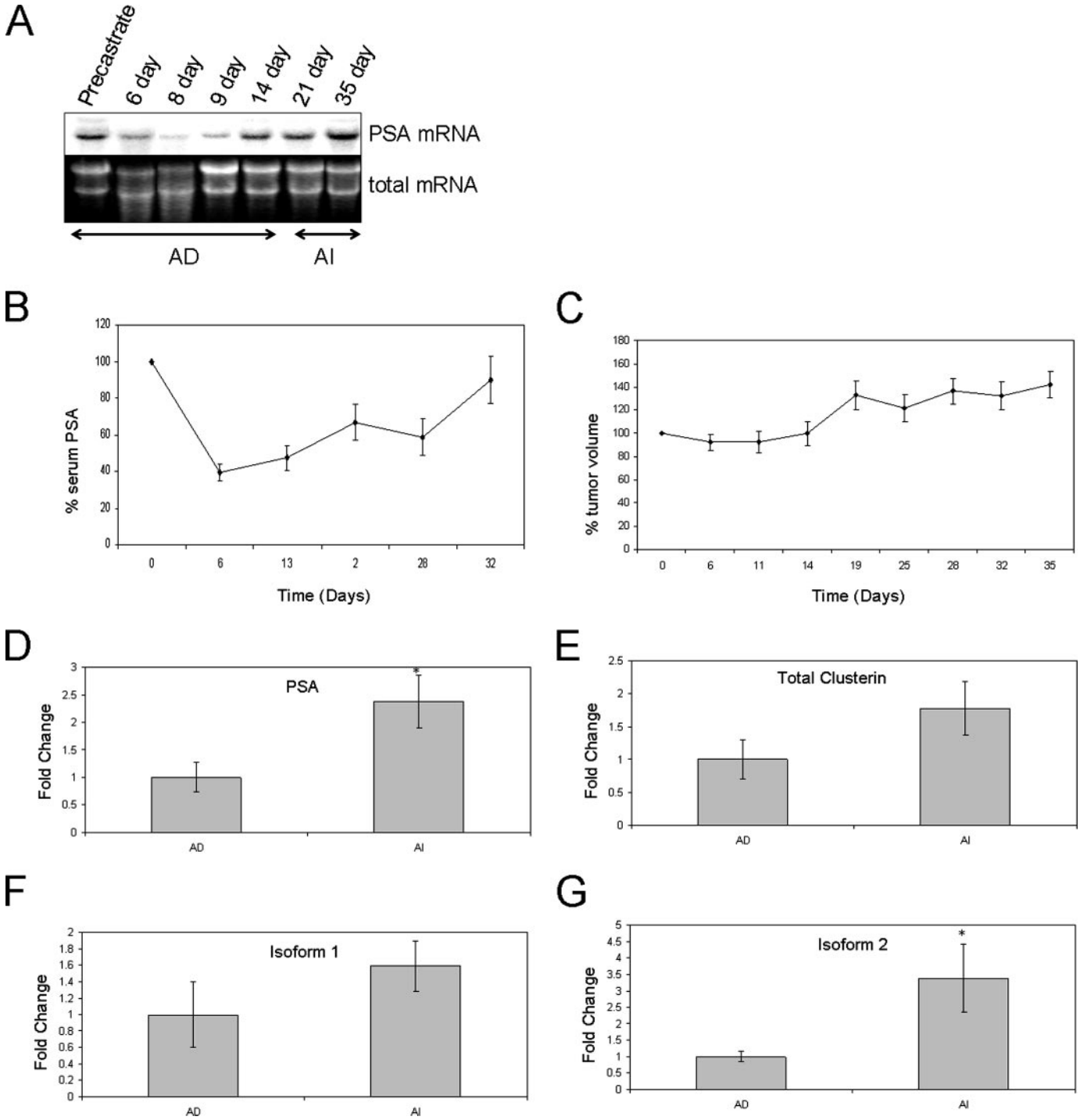

FIGURE 6. **Levels of clusterin isoforms in androgen-dependent and -independent LNCaP xenografts.** LNCaP cells were injected subcutaneously into athymic mice, and tumors were harvested prior to castration and at various time points after castration. *A*, Northern blots showing the PSA levels in the LNCaP tumors as it progressed to androgen independence. The serum PSA (*B*) and the tumor volume (*C*) of the tumor series is also shown. The mRNA levels of PSA (*D*), total clusterin (*E*), clusterin Isoform 1 (*F*), and Isoform 2 (*G*) were determined using real time PCR for androgen dependence (*AD*) (using day 8 samples) or androgen independence (*AI*) (using day 35 samples). *, $p < 0.05$.

umes instead cease growing until AI, where proliferation continues. Real time PCR was used to determine transcript levels of clusterin and its two isoforms. PSA is shown as a positive control, and its levels increase with progression (Fig. 6*B*). Both total clusterin and Isoform 1 levels increase moderately with progression to androgen independence (Fig. 6, *C* and *D*); however, the increase is not statistically significant. Isoform 2 levels do show a significant increase in levels at androgen independence as compared with androgen dependence (Fig. 6*E*), consistent with the androgen regulation of this isoform.

PCR has been used previously to detect the nuclear splice variant (21). Such PCR did not reveal the presence of the nuclear splice variant of clusterin (data not shown); however, neither androgen treatment *in vitro* nor progression to andro-

gen independence in the LNCaP tumor model is expected to have nuclear, apoptotic clusterin expressed.

DISCUSSION

Levels of clusterin increase dramatically in the rat ventral prostate upon castration, leading to its designation as a testosterone-repressed message. Later studies demonstrated that the increase in clusterin was most likely a stress response brought about by castration-induced apoptosis of the prostatic epithelial cells and is perhaps regulated in concert with the heat shock proteins (16, 47). The androgenic regulation of clusterin has been primarily studied *in vivo* by removal of androgens by physical or hormonal castration of rats or on mice bearing xenograft tumors (12, 49, 50). However, in this context, it is difficult to dissociate the androgenic and the apoptotic stimuli. In the Shionogi tumor model, apoptosis is blocked by calcium channel blockers, and clusterin levels do not increase upon castration (14). Therefore, studying the effects of androgens on prostate cells in cell culture and in organ culture avoids the confounding influence of the multiple signals present in the *in vivo* system.

In the present study, we have demonstrated that in both human prostate cancer cells and in the rat ventral prostate, total clusterin is in fact up-regulated by androgens. Androgen treatment causes an increase in the levels of both the total clusterin mRNA and the resulting protein, clusterin. The effect seen is both time- and dose-dependent while independent of apoptosis.

Androgens are necessary for the growth and differentiation of normal prostatic epithelium. It has been shown that clusterin plays a role in many different types of cancer as a cell survival factor and helps cancerous cells to evade stress-induced apoptosis. We propose herein that clusterin may also play a cytoprotective role in normal prostate cells under the regulation of androgens. Androgens themselves have long been known to promote survival of prostatic epithelium, and they appear to increase the cell's ability to withstand apoptotic stimuli (47). Androgens have been shown to play a cytoprotective role by decreasing the levels of proapoptotic proteins, such as the caspases and members of the Bcl-2 family of proteins (51, 52), as well as preventing the release of cytochrome *c* from the mitochondria (53). It is therefore not surprising that androgens could increase the levels of antiapoptotic proteins, such as clusterin, which would act to protect prostate cells against apoptosis.

The transcriptional regulation of the clusterin gene is probably quite complex. When a cell undergoes a stress response to stimuli, such as heat shock, clusterin is induced. It was shown that in this scenario, the heat shock transcription factor, HSF-1, binds to a heat shock element in the 5′ promoter region of the murine clusterin gene and induces its expression (54). In addition to stress induction, there are various growth factors that can induce clusterin expression, including transforming growth factor β, nerve growth factor, and epidermal growth factor (55–57). These growth factors have been shown to require an AP-1 site found in the 5′-proximal promoter region of the clusterin gene to induce its expression.

Other steroid hormones have been shown to have effects on clusterin expression in other cell types. A number of studies have reported that estradiol treatment can cause an increase or decrease of clusterin levels in the uterus and endometrium (58–60). Also, vitamin D has been shown to increase clusterin levels in human breast cancer MCF-7 cells (61, 62). Although these steroids have been implicated in clusterin regulation, there has not been shown to be direct interaction of the cognate steroid receptor with the clusterin gene, and these are possibly downstream effects. In contrast, we have shown that the AR does interact with a regulatory region found in the first intron of the clusterin gene and increases the transcription of clusterin in prostate epithelial cells.

It has been postulated that there are AREs within the first intron of both the rat and human clusterin genes and that the AR could bind to these response elements and repress the expression of clusterin (42, 43, 63). We have shown that the AR does bind specifically to the clusterin intronic DNA, resulting in an increase in transactivation in a reporter assay. The ChIP assay demonstrated that although AR does bind a region of the clusterin first intron *in vivo*, binding does not occur at 2 h post-androgen treatment as it does for the PSA enhancer region. Rather, AR binding occurs later, within 48 h after androgen treatment. We therefore hypothesize that there may be repressor proteins at play that prevent AR from binding or other positive acting factors or chromatin remodeling needed for AR to bind and initiate assembly of a transcription complex.

AR is known to induce transcriptional activity through interaction with AREs. However, there is little known about its ability to directly cause transcriptional repression. Therefore, it is unclear as to whether the direct interaction of AR with the intronic region of clusterin is the cause of the down-regulation of Isoform 1 or if this is an effect of transcriptional interference.

The differential regulation of two clusterin isoforms may be critical to regulation of the opposing apoptotic and antiapoptotic functions of the two isoforms. Both isoforms are cytoprotective[4]; however, Isoform 1 has the capacity to produce the nuclear, apoptotic form of clusterin generated by alternative splicing (21). In the context of androgens, which are cytoprotective in prostate cells, we show that androgens specifically up-regulate Isoform 2 of clusterin while down-regulating Isoform 1. Studies performed in other species demonstrate the antiapoptotic nature of clusterin, and interestingly these other species only produce an orthologue equivalent to Isoform 2, since they lack an initiating methionine in the first exon.

Isoform 2 is shown to increase during progression to androgen independence in LNCaP xenograft tumors, and this correlates with the induction of PSA at androgen independence. This is consistent with Isoform 2 being an androgen-regulated gene, since there is a reactivation of AR signaling at androgen independence, demonstrated by the recurrence of PSA and other androgen-regulated genes (64, 65). AR remains important as tumors progress to androgen independence, and as such these tumors retain high expression levels of AR (66), and down-regulation of AR using small interfering RNA causes an increase in apoptosis (48). Therefore, as prostate tumors progress to androgen independence, the reactivation in AR signaling is functionally important for maintenance of cell viability. Clusterin is also important for evasion of apoptosis during progression to androgen independence (24, 31). In that context, AI prostate cells will seek to up-regulate the antiapoptotic form of

clusterin and, as such, will up-regulate Isoform 2, which cannot be spliced into the apoptotic form.

The data presented herein suggest that androgens may be cytoprotective in secretory prostatic epithelial cells in humans in part through up-regulation of clusterin Isoform 2 and down-regulation of clusterin Isoform 1. This is not only important in the maintenance of normal prostate but also during the progression to androgen-independent prostate cancer.

REFERENCES

1. Fritz, I., Burdzy, K., Stechell, B., and Blaschuck, O. (1983) *Biol. Reprod.* **28,** 1173–1188
2. Blaschuck, O., Burdzy, K., and Fritz, I. (1983) *J. Biol. Chem.* **258,** 7714–7720
3. de Silva, H., Harmony, J., Stuart, W., Gil, C., and Robbins, J. (1990) *Biochemistry* **29,** 5380–5389
4. Poon, S., Easterbrook-Smith, S. B., Rybchyn, M. S., Carver, J. A., and Wilson, M. R. (2000) *Biochemistry* **39,** 15953–15960
5. Ahuja, H., Tenniswood, M., Lockshin, R., and Zakeri, Z. (1994) *Biochem. Cell Biol.* **72,** 523–530
6. Burkey, B., Stuart, W., and Harmony, J. (1992) *J. Lipid Res.* **33,** 1517–1526
7. Gelissen, I., Hochgrebe, T., Wilson, M., Easterbrook-Smith, S., Jessup, W., Dean, R., and Brown, A. (1998) *Biochem. J.* **331,** 231–237
8. Silkensen, J. R., Skubitz, K. M., Skubitz, A. P., Chmielewski, D. H., Manivel, J. C., Dvergsten, J. A., and Rosenberg, M. E. (1995) *J. Clin. Invest.* **96,** 2646–2653
9. Sylvester, S. R., Morales, C., Oko, R., and Griswold, M. D. (1991) *Biol. Reprod.* **45,** 195–207
10. Rosenberg, M., and Silkensen, J. (1995) *Int. J. Biochem. Cell Biol.* **27,** 633–645
11. Montpetit, M., Lawless, K., and Tenniswood, M. (1986) *Prostate* **8,** 25–36
12. Leger, J. G., Montpetit, M. L., and Tenniswood, M. P. (1987) *Biochem. Biophys. Res. Commun.* **147,** 196–203
13. Rennie, P. S., Bruchovsky, N., Akakura, K., Goldenberg, S. L., Otal, N., Akakura, S., Wong, P., and Tenniswood, M. (1994) *J. Steroid Biochem. Mol. Biol.* **50,** 31–40
14. Miyake, H., Nelson, C., Rennie, P., and Gleave, M. (2000) *Cancer Res.* **60,** 170–176
15. Rennie, P., Bowden, J., Freeman, S., Bruchovsky, N., Cheng, H., Lubahn, D., Wilson, E., French, F., and Main, L. (1989) *Mol. Endocrinol.* **3,** 703–708
16. Buttyan, R., Olsson, C. A., Pintar, J., Chang, C., Bandyk, M., Ng, P. Y., and Sawczuk, I. S. (1989) *Mol. Cell. Biol.* **9,** 3473–3481
17. Wong, P., Borst, D. E., Farber, D., Danciger, J. S., Tenniswood, M., Chader, G. J., and van Veen, T. (1994) *Biochem. Cell Biol.* **72,** 439–446
18. French, L., Sappino, A., Tschopp, J., and Schifferli, J. (1992) *J. Clin. Invest.* **90,** 1919–1925
19. French, L., Wohlwend, A., Sappino, A., Tschopp, J., and Schifferli, J. (1994) *J. Clin. Invest.* **93,** 877–884
20. Zhang, Q., Zhou, W., Kundu, S., Jang, T. L., Yang, X., Pins, M., Smith, N., Jovanovic, B., Xin, D., Liang, L., Guo, Y., and Lee, C. (2006) *BJU Int.* **98,** 452–460
21. Leskov, K. S., Klokov, D. Y., Li, J., Kinsella, T. J., and Boothman, D. A. (2003) *J. Biol. Chem.* **278,** 11590–11600
22. Yang, C. R., Leskov, K., Hosley-Eberlein, K., Criswell, T., Pink, J. J., Kinsella, T. J., and Boothman, D. A. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 5907–5912
23. Caccamo, A. E., Scaltriti, M., Caporali, A., D'Arca, D., Scorcioni, F., Candiano, G., Mangiola, M., and Bettuzzi, S. (2003) *Ann. N. Y. Acad. Sci.* **1010,** 514–519
24. Gleave, M. E., Zellweger, T., Chi, K., Miyake, H., Kiyama, S., July, L., and Leung, S. (2002) *Invest. New Drugs* **20,** 145–158
25. Redondo, M., Villar, E., Torres-Munoz, Tellez, T., Morell, M., and Petito, C. (2000) *Am. J. Pathol.* **157,** 393–399
26. Lee, C., Janulis, L., Ilio, K., Shah, A., Park, I., Kim, S., Cryns, V., Pins, M., and Bergan, R. (2000) *Prostate Suppl.* **9,** 21–24
27. Hough, C., Cho, K., Zonderman, A., Schwartz, D., and Morin, P. (2001) *Cancer Res.* **61,** 3869–3876
28. Xie, M., Motoo, Y., Mouri, H., Ohtsubo, K., Matsubara, F., and Sawabu, N. (2002) *Pancreas* **25,** 234–238
29. Dvergsten, J., Manivel, J. C., Correa-Rotter, R., and Rosenberg, M. E. (1994) *Kidney Int.* **45,** 828–835
30. Steinberg, J., Oyasu, R., Lang, S., Sintich, S., Rademaker, A., Lee, C., Kozlowski, J. M., and Sensibar, J. A. (1997) *Clin. Cancer Res.* **3,** 1707–1711
31. July, L. V., Akbari, M., Zellweger, T., Jones, E. C., Goldenberg, S. L., and Gleave, M. E. (2002) *Prostate* **50,** 179–188
32. Miyake, H., Hara, S., Zellweger, T., Kamidono, S., Gleave, M. E., and Hara, I. (2001) *Mol. Urol.* **5,** 105–111
33. Zellweger, T., Chi, K., Miyake, H., Adomat, H., Kiyama, S., Skov, K., and Gleave, M. E. (2002) *Clin. Cancer Res.* **8,** 3276–3284
34. Gleave, M. E., Miyake, H., Zellweger, T., Chi, K., July, L., Nelson, C., and Rennie, P. (2001) *Urology* **58,** 39–49
35. Freeman, A. E., and Hoffman, R. M. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 2694–2698
36. Martikainen, P., and Isaacs, J. T. (1990) *Endocrinology* **127,** 1268–1277
37. Sandelin, A., Wasserman, W. W., and Lenhard, B. (2004) *Nucleic Acids Res.* **32,** 249–252
38. Zhu, N., Pewitt, E. B., Cai, X., Cohn, E. B., Lang, S., Chen, R., and Wang, Z. (1998) *Endocrinology* **139,** 4337–4344
39. Pewitt, E. B., Haleem, R., and Wang, Z. (1999) *Endocrinology* **140,** 2382–2386
40. Beato, M., Herrlich, P., and Schultz, G. (1995) *Cell* **83,** 851–857
41. Truss, M., and Beato, M. (1993) *Endocr. Rev.* **14,** 459–479
42. Wong, P., Pineault, J., Lakins, J., Taillefer, D., Leger, J., Wang, C., and Tenniswood, M. (1993) *J. Biol. Chem.* **268,** 5021–5031
43. Rosemblit, N., and Chen, C. L. (1994) *J. Mol. Endocrinol.* **13,** 69–76
44. Yeung, L. H., Read, J. T., Sorenson, P., Nelson, C. C., Jia, W., and Rennie, P. S. (2003) *Biochem. J.* **371,** 843–855
45. Scheller, A., Hughes, E., Golden, K., and Robins, D. (1998) *J. Biol. Chem.* **273,** 24216–24222
46. Reid, K. J., Hendy, S. C., Saito, J., Sorensen, P., and Nelson, C. C. (2001) *J. Biol. Chem.* **276,** 2943–2952
47. Berchem, G. J., Bosseler, M., Sugars, L. Y., Voeller, H. J., Zeitlin, S., and Gelmann, E. P. (1995) *Cancer Res.* **55,** 735–738
48. Liao, X., Tang, S., Thrasher, J. B., Griebling, T. L., and Li, B. (2005) *Mol. Cancer Ther.* **4,** 505–515
49. Leger, J. G., Le Guellec, R., and Tenniswood, M. P. (1988) *Prostate* **13,** 131–142
50. Rennie, P. S., Bruchovsky, N., Buttyan, R., Benson, M., and Cheng, H. (1988) *Cancer Res.* **48,** 6309–6312
51. Kimura, K., Markowski, M., Bowen, C., and Gelmann, E. (2001) *Cancer Res.* **61,** 5611–5618
52. Coffey, R. N., Watson, R. W., O'Neill, A. J., McEleny, K., and Fitzpatrick, J. M. (2002) *Prostate* **53,** 300–309
53. Carson, J. P., Kulik, G., and Weber, M. J. (1999) *Cancer Res.* **59,** 1449–1453
54. Michel, D., Chatelain, G., North, S., and Brun, G. (1997) *Biochem. J.* **328,** 45–50
55. Gutacker, C., Klock, G., Diel, P., and Koch-Brandt, C. (1999) *Biochem. J.* **339,** 759–766
56. Jin, G., and Howe, P. H. (1997) *J. Biol. Chem.* **272,** 26620–26626
57. Jin, G., and Howe, P. H. (1999) *Eur. J. Biochem.* **263,** 534–542
58. Heikaus, S., Winterhager, E., Traub, O., and Grummer, R. (2002) *J. Mol. Endocrinol.* **29,** 239–249
59. Waters, K. M., Safe, S., and Gaido, K. W. (2001) *Toxicol. Sci.* **63,** 47–56
60. Wunsche, W., Tenniswood, M. P., Schneider, M. R., and Vollmer, G. (1998) *Int. J. Cancer* **76,** 684–688
61. James, S. Y., Mackay, A. G., and Colston, K. W. (1996) *J. Steroid Biochem. Mol. Biol.* **58,** 395–401
62. Simboli-Campbell, M., Narvaez, C. J., Tenniswood, M., and Welsh, J. (1996) *J. Steroid Biochem. Mol. Biol.* **58,** 367–376
63. Wong, P., Taillefer, D., Lakins, J., Pineault, J., Chader, G., and Tenniswood, M. (1994) *Eur. J. Biochem.* **221,** 917–925
64. Gleave, M. E., Hsieh, J. T., Wu, H. C., von Eschenbach, A. C., and Chung, L. W. (1992) *Cancer Res.* **52,** 1598–1605
65. Ettinger, S. L., Sobel, R., Whitmore, T. G., Akbari, M., Bradley, D. R., Gleave, M. E., and Nelson, C. C. (2004) *Cancer Res.* **64,** 2212–2221
66. Buchanan, G., Irvine, R. A., Coetzee, G. A., and Tilley, W. D. (2001) *Cancer Metastasis Rev.* **20,** 207–223

Differential Regulation of Clusterin and Its Isoforms by Androgens in Prostate Cells

Dawn R. Cochrane, Zhou Wang, Motosugu Muramaki, Martin E. Gleave and Colleen C. Nelson

J. Biol. Chem. 2007, 282:2278-2287.
doi: 10.1074/jbc.M608162200 originally published online December 4, 2006
