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Transcriptional Regulation of the Homeobox Gene *NKX3.1* by all-*trans* Retinoic Acid in Prostate Cancer Cells

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Abstract *NKX3.1* is a homeobox gene, expression of which is largely restricted to the adult prostatic epithelium. Loss of NKX3.1 expression has been linked to prostate carcinogenesis and disease progression and occurs in the absence of mutations in the coding region of the *NKX3.1* gene. In this study, we have characterized regulation of NKX3.1 expression by all-*trans* retinoic acid (tRA), a naturally occurring vitamin A metabolite that is accumulated at high levels in the prostate. Using the prostate cancer cell line LNCaP, Western blot analysis revealed a ~twofold induction of NKX3.1 protein levels following tRA exposure, with sequential analysis of NKX3.1 protein levels in cycloheximide co-treated cells indicating that tRA does not alter NKX3.1 protein turnover. The ~1.6-fold increase in *NKX3.1* mRNA levels detected in tRA-treated LNCaP cells also occurred independently of new protein synthesis and was not mediated by changes in *NKX3.1* mRNA stability. In contrast, nuclear run-on assays indicated that tRA treatment increased *NKX3.1* transcription. To identify retinoid responsive regions of the *NKX3.1* gene, DNA sequences encompassing ~2 kb of the *NKX3.1* promoter or the entire 3′untranslated region (UTR) were cloned into luciferase reporter plasmids. Analysis of induced luciferase activity following transfection of these constructs into prostate cancer cells did not identify tRA responsiveness, however the 3′UTR was found to be strongly androgen responsive. These studies demonstrate that the *NKX3.1* gene is a direct target of retinoid receptors and suggest that androgen regulation of *NKX3.1* expression is mediated in part by the 3′UTR. J. Cell. Biochem. 99: 1409–1419, 2006. © 2006 Wiley-Liss, Inc.

Key words: retinoids; prostate cancer; NKX3.1; gene regulation

Prostate cancer is the most frequently diagnosed non-cutaneous malignancy of men living in western society. Despite being a leading cause of cancer-related morbidity and mortality, the etiology of the disease remains obscure [Gronberg, 2003; Hsing and Chokkalingam, 2006]. NKX3.1 is a homeodomain-containing transcription factor expressed predominantly in prostatic epithelial cells during prostate organogenesis and its expression is maintained in the adult prostate gland [Bieberich et al., 1996]. Several studies have reported reduced or complete loss of NKX3.1 protein expression in advanced prostate cancers, as well as in the pre-invasive lesion prostatic intraepithelial neoplasia (PIN), suggesting that NKX3.1 functions as a prostate-specific tumor suppressor [Bowen et al., 2000; Asatiani et al., 2005; Aslan et al., 2006]. Gene-targeting studies in mice support this role demonstrating that conditional knockout of *Nkx3.1* in the adult can predispose mice to prostate carcinoma by inducing the development of lesions resembling PIN [Abdulkadir et al., 2002]. Moreover, *Nkx3.1* knockout mice display severe developmental defects of the prostate, as well as prostatic epithelial hyperplasia and dysplasia [Bhatia-Gaur et al., 1999; Tanaka et al., 2000; Kim et al., 2002a], and can cooperate with other oncogenic mutations to augment carcinogenesis [Kim et al., 2002b; Gary et al., 2004]. These data

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indicate an important regulatory role for NKX3.1 in prostate epithelial cell proliferation and differentiation [Shen and Abate-Shen, 2003].

Mechanisms leading to the loss of NKX3.1 expression in prostate tumors are unknown. Although *NKX3.1* lies within 8p21, a region frequently disrupted by loss of heterozygosity (LOH) in advanced prostate cancers [Emmert-Buck et al., 1995; Vocke et al., 1996; He et al., 1997], gene alterations in the second allele that may account for NKX3.1 loss have not been identified. Transcriptional silencing due to chromatin structure modulation or histone modification seems the most plausible explanation as recent evidence suggests that hypermethylation of the *NKX3.1* promoter is not involved [Asatiani et al., 2005; Lind et al., 2005]. However, reports of several germ-line mutations detected in the *NKX3.1* promoter of patients with hereditary prostate cancer [Zheng et al., 2006], indicate that loss of expression may also arise due to deregulated transcription of the *NKX3.1* gene.

Regulation of *NKX3.1* expression and the mechanisms leading to its prostate-specific expression are poorly characterized. Preliminary data presented by Jiang et al. [2004], described the existence of both positive and negative regulatory elements in the initial 1 kb of the *NKX3.1* 5′ proximal promoter, which likely contribute to the control of *NKX3.1* expression. Recently, it was also proposed that DNA sequences downstream of the *Nkx3.1* coding region might direct prostate restricted expression [Chen et al., 2005]. However, the identities of the *trans*-acting factors mediating transcriptional control of *NKX3.1* in these studies were not identified. *NKX3.1* expression, like other prostate-specific genes, is known to be hormonally regulated. Androgens, which have an essential role in the development and maintenance of the male reproductive system [Dohle et al., 2003; Lombardo et al., 2005], strongly upregulate *NKX3.1* mRNA and protein levels in both the normal prostate and in prostate cancer cells [Bieberich et al., 1996; He et al., 1997; Prescott et al., 1998]. Likewise, estrogens have also been shown to markedly increase *NKX3.1* mRNA in LNCaP prostate cancer cells [Korkmaz et al., 2000]. In this study, we investigated the effects of the retinoid hormone responsive pathway in the regulation of *NKX3.1* gene expression.

Retinoids, analogs of vitamin A, are well known to play highly diverse roles in both development and cell homeostasis through their regulatory effects on target genes involved in processes such as cell proliferation, differentiation, and apoptosis [Ross et al., 2000; Altucci and Gronemeyer, 2001]. Low dietary intake of retinoids and reduced plasma retinol (vitamin A) levels have been proposed as risk factors for development of a variety of cancer types including prostate cancer [Hayes et al., 1988; Boone et al., 1990]. In addition, serum retinol levels are lower in prostate cancer patients and levels of the vitamin A metabolite, all-*trans*- retinoic acid (tRA) are 5–8 times lower in prostate cancer tissues in comparison to normal or benign prostate [Reichman et al., 1990; Pasquali et al., 1996]. At pharmacological levels, natural and synthetic retinoids inhibit growth and induce differentiation of prostate cancer cells and act as chemopreventative agents in experimental prostate cancer models [Esquenet et al., 1996]. The biological roles of retinoids in embryonic and adult cells and their well-characterized regulation of homeobox genes including the *Hox* clusters have suggested that retinoid function in part, results from their tissue-specific effects on the expression of homeobox genes [Marshall et al., 1996].

The action of retinoids follows their binding to retinoic acid receptors (RARs) and retinoid X receptors (RXRs), which belong to the superfamily of steroid/thyroid hormone receptors [Mangelsdorf and Evans, 1995]. The two retinoid receptor subfamilies each comprise three isoforms designated α, β, or γ, and upon ligand binding, retinoid receptors heterodimerize in the nucleus of target cells. RAR/RXR heterodimers then mediate their effects by binding retinoic acid response elements (RAREs) located in the promoter/enhancer regions of target genes, thereby modulating transcriptional activity and gene expression [Bastien and Rochette-Egly, 2004]. Here, we show that the naturally occurring vitamin A metabolite, all-*trans* retinoic acid is a regulator of *NKX3.1* gene expression that acts to directly increase transcription of the *NKX3.1* gene in prostate cancer cells.

MATERIALS AND METHODS

Construction of Plasmids

To generate *NKX3.1*-luciferase reporter plasmids, *NKX3.1* sequences were PCR-amplified

from genomic DNA isolated from LNCaP prostate cancer cells. Using the primers, 5′-GCTAGCGGTATTCAACATCTCTAG-3′ and 5′-CTCGAGCACCGCTTTCAGTTTCC-3′, the *NKX3.1* proximal promoter encompassing nucleotides −2,062 to +6 (+1 represents transcription start site) was amplified and ligated into the *Nhe*I and *Xho*I sites of the pGL3-enhancer vector (Promega, Melbourne, Australia) creating the plasmid, pGL3-NKX-5′promoter. To develop the pGL3-NKX-3′UTRFL plasmid, the primers 5′-GTAATGCCAGCTCAGGTGAC-3′ and 5′-TTCAAAACACTCATATGTTGC-3′ were used to amplify the *NKX3.1* full-length 3′untranslated region (UTR) spanning nucleotides +1,714 to +4,215, which was initially ligated into pCR2.1 (Invitrogen, Melbourne, Australia) before being cloned into the *Kpn*I and *Xho*I sites of pGL3-promoter (Promega). The orientation and sequence of inserts in pGL3-NKX-5′propromoter and pGL3-NKX-3′UTRFL plasmids have been confirmed by DNA sequencing.

Cell Culture and Transfection

LNCaP and DU145 human prostate cancer cells, obtained from the American Type Culture Collection (Rockville, MD) were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin, and 100 μg/ml streptomycin in an atmosphere of humidified air/5% CO_2 at 37°C. For transient transfection experiments, 500 ng of plasmid DNA per well was transfected into cells in 24-well plates using Lipofectamine 2000™ reagent (Invitrogen), and the cells were allowed to recover for 6 h at 37°C prior to hormone treatment. Retinoid responsiveness was examined by exposing transfected cells to 5 μM tRA (Sigma, Sydney, Australia) or vehicle, 0.1% dimethylsulfoxide (DMSO). For studies investigating androgen-responsiveness, the medium was replaced with RPMI 1640/2% charcoal-stripped FCS containing 10^{-8} M 5α-dihydrotestosterone (DHT; Sigma) or 0.1% ethanol (vehicle). Luciferase assays were performed 24 h post-transfection by washing cells once in phosphate buffered saline (PBS), incubating in 1× passive lysis buffer (Promega) for 15 min with rocking before measuring luciferase activity in luciferase assay reagent (50 mM Tris pH 7.8, 15 mM $MgSO_4$, 33.3 mM DTT, 0.1 mM EDTA, 250 μM Lithium coenzyme A, 500 μM Na luciferin, 500 μM ATP, 0.5% Triton X-100) using a Microbeta 1516 luminometer (Wallac, Turku, Finland).

Western Blot Analysis

LNCaP cells, treated with either 5 μM tRA or 0.1% DMSO, were washed twice in cold PBS, lysed in whole cell lysis buffer (50 mM Tris-Cl pH 6.8, 10% sucrose, 2% SDS, 5% β-mercaptoethanol) and harvested on ice by cell scraping. For NKX3.1 protein stability studies, LNCaP cells were pre-treated for 6 h with 5 μM tRA or 0.1% DMSO, and exposed to 10 μg/ml cycloheximide (Sigma) prior to cell lysis and harvesting as above at sequential timepoints. Cell lysates were heated at 95°C for 5 min, electrophoresed in 10% polyacrylamide gels, and transferred to nitrocellulose membranes (Hybond C-Extra, Amersham, Sydney, Australia). For immunoblot analysis, membranes were blocked for 1 h in Tris-buffered saline (TBS) containing 3% dried skim milk powder (Blotto) and probed for 1 h using antibodies directed against either NKX3.1 (Zymed, Melbourne, Australia) or β-actin (Santa Cruz, CA) diluted at 1:5,000 or 1:2,000, respectively, in TBS containing 0.2% Tween 20 (TBST) and 1% Blotto. Blots were washed in TBST and incubated for 1 h in peroxidase-conjugated anti-mouse (NKX3.1; Chemicon, Melbourne, Australia) or anti-goat (β-actin; Santa Cruz) secondary antibodies diluted at 1:2,000 in TBST/1% Blotto. Following 3 × 10 min washes in TBST, immunoreactivity was visualized using enhanced chemiluminescence (Amersham) and densitometric analysis performed using Quantity One™ quantitation software (Bio-Rad) with NKX3.1 protein levels standardized against β-actin.

Northern Blot Analysis

RNA extraction from LNCaP cells, treated with 0.1% DMSO or 5 μM tRA either alone or in combination with 10 μg/ml cycloheximide, was performed using UltraSpec reagent according to the manufacturer's instructions (Fisher Biotec, Perth, Australia). For studies of *NKX3.1* mRNA stability, cells were pre-treated for 6 h with 5 μM tRA (or vehicle), prior to the addition of 5 μg/ml actinomycin D (Sigma) and RNA extraction performed as above at sequential timepoints. Northern gel electrophoresis was performed loading 10 μg of total RNA per lane in 1% agarose MOPS/formaldehyde gels followed by overnight transfer to nylon membranes

(Hybond XL, Amersham) in 10 × SSC and UV-crosslinking. cDNA probes encoding *NKX3.1* (nucleotides 334–1,237 of mRNA, accession NM_006167) and β-actin (nucleotides 219–538 of mRNA, accession NM_001101) were ^{32}P-labeled using a Prime-a-Gene® random primer labeling kit (Promega) and membrane hybridization carried out at 65°C with rotation for 2 h in Rapid-hyb buffer (Amersham). Filters were washed in 2 × SSC/0.1%SDS for 20 min at 65°C, followed by sequential washing for 15 min in 1 × SSC/0.1%SDS and 0.1 × SSC/0.1%SDS at 65°C. Autoradiographs of Northern blots were quantified using Quantity One™ quantitation software (Bio-Rad) with *NKX3.1* mRNA levels standardized against either 28S ribosomal or β-actin RNA.

Nuclear Run-On Assays

Nuclei were prepared from untreated or 5 μM tRA-treated LNCaP cells by washing in ice-cold PBS followed by two rounds of lysis using NP-40 lysis buffer (10 mM Tris-Cl pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% Nonidet P-40) and centrifugation at 500*g* for 5 min at 4°C. The final pellet was resuspended in a storage buffer containing 50 mM Tris-Cl pH 8.3, 40% glycerol, 0.1 mM EDTA, and 0.1 mM DTT and stored at −80°C. Transcription and RNA labeling was performed for 30 min at 30°C in a reaction buffer containing 5 mM Tris-Cl pH 7.5, 2.5 mM $MgCl_2$, 150 mM KCl, 50 mM DTT, 0.5 mM each of ATP, CTP, GTP, and ^{32}P-UTP. RNA was isolated using UltraSpec reagent, according to the manufacturer's instructions, and precipitated with isopropanol before resuspension in 10 mM TES (*N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid) pH 7.4, 10 mM EDTA, 0.2% SDS, and 0.3 M NaCl. Nylon membranes (Hybond XL, Amersham) were slot blotted with 2.5 μg of cDNA sequences encoding *NKX3.1* (nucleotides 49–750 of cDNA), β-galactosidase (nucleotides 963–4,019 of pcDNA3.1/V5-His/LacZ vector, Invitrogen) or β-actin (nucleotides 427–885 of cDNA), slotted in separate wells. Hybridization was performed in 50% formamide, 5 × SSC, 5× Denhardt's solution, and 1% SDS at 42°C for 48 h followed by three washes in 2 × SSC at RT (5 min), 37°C (20 min), and RT (5 min), respectively. Autoradiographs were quantified using Quantity One™ quantitation software (Bio-Rad) and *NKX3.1* levels normalized against β-actin.

RESULTS

NKX3.1 Protein Expression Is Upregulated by all-*trans* Retinoic Acid (tRA)

To investigate retinoid effects on *NKX3.1* gene expression, LNCaP prostate cancer cells were treated with 5 μM tRA and NKX3.1 steady state protein levels determined by Western blot analysis. By 6 h of tRA exposure, NKX3.1 levels were increased ~twofold and remained elevated during 48 h of tRA treatment (Fig. 1). To determine if the tRA-induced increases in NKX3.1 levels were due to increased NKX3.1 protein stability, LNCaP cells were pre-treated for 6 h with tRA (or vehicle) prior to the addition of 10 μg/ml cycloheximide and sequential Western blot analysis of NKX3.1 levels performed. Quantitation of protein levels revealed NKX3.1 to be an extremely labile protein with a half-life estimated to be ~25 min (Fig. 2). tRA treatment of LNCaP cells did not alter NKX3.1 turnover indicating that the tRA-induced increases in

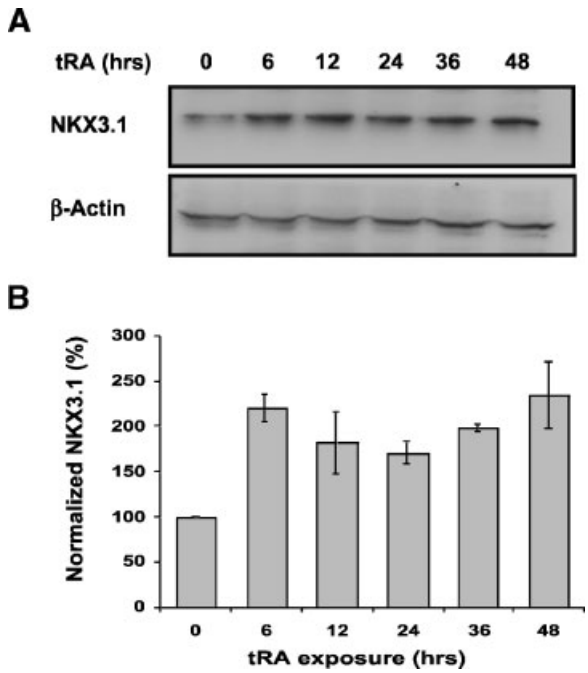

Fig. 1. NKX3.1 steady state protein levels are upregulated by tRA. **A**: LNCaP cells were treated for increasing time periods with 5 μM tRA and Western blot analysis performed on the cellular lysates using NKX3.1 (**upper panel**) and β-actin (**lower panel**) antibodies. **B**: Steady state NKX3.1 levels were quantified from experiments described in (A) and the densitometric values normalized using the matching β-actin protein levels in individual samples. The graphed results are expressed as mean ± SEM calculated from three separate time course experiments.

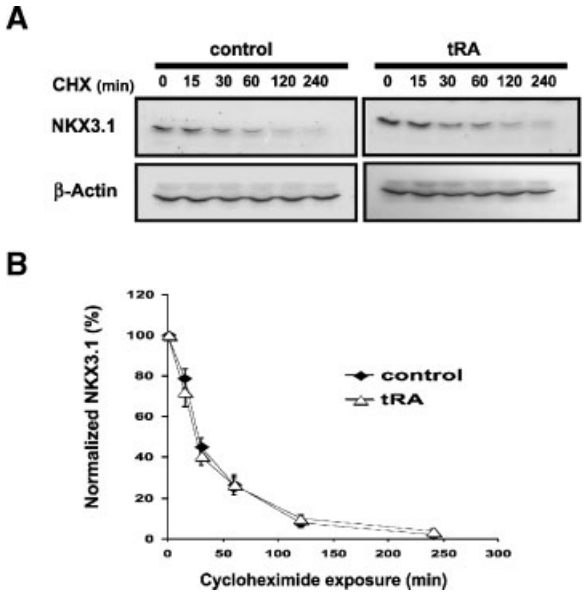

Fig. 2. NKX3.1 protein stability is not affected by tRA. **A**: LNCaP cells, pre-treated for 6 h with either DMSO (control) or 5 μM tRA, were exposed to the protein synthesis inhibitor, cycloheximide (CHX) for increasing time lengths prior to Western blot analysis using NKX3.1 (**upper panel**) and β-actin (**lower panel**) antibodies. **B**: NKX3.1 protein levels from experiments described in (A) were quantified and the densitometric values normalized using the matching β-actin levels in individual samples. The graphed results are expressed as mean ± SEM calculated from three separate experiments.

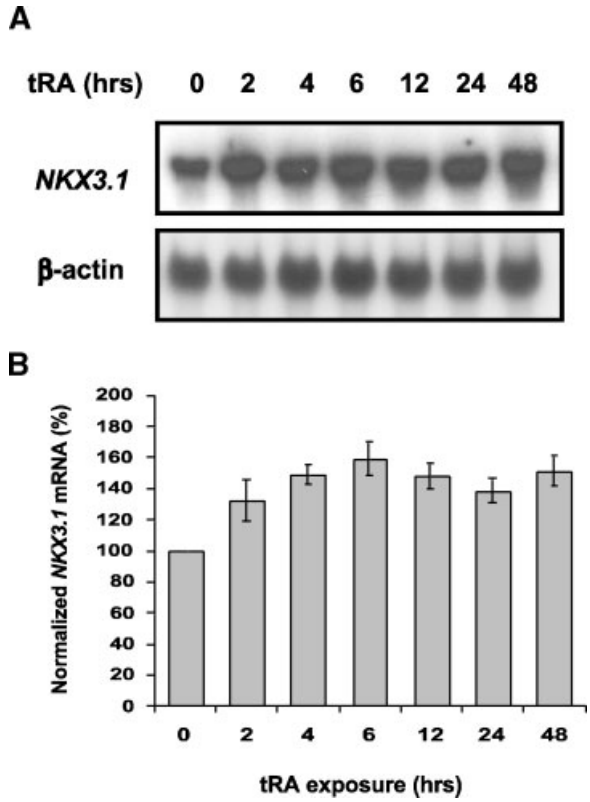

Fig. 3. tRA-induced upregulation of *NKX3.1* mRNA. **A**: LNCaP cells were treated for the indicated times with tRA (5 μM) and Northern blot analysis performed on total RNA using probes specific to *NKX3.1* (**upper panel**) and β-actin (**lower panel**). **B**: *NKX3.1* mRNA levels were quantified from experiments in (A) and normalized using the matching β-actin levels in individual samples. The graphed results are expressed as mean ± SEM from three separate experiments.

NKX3.1 were not mediated at the level of protein stability.

NKX3.1 mRNA Levels Are Induced by tRA

To assess whether the effect of tRA on NKX3.1 protein levels was associated with an increase in *NKX3.1* mRNA, LNCaP cells were treated with 5 μM tRA and *NKX3.1* mRNA levels were examined by Northern blotting. *NKX3.1* mRNA was increased by 2 h of tRA exposure, peaking (~1.6-fold increase) at 6 h and remaining at elevated levels during 48 h of tRA treatment (Fig. 3), reflecting the persistence of elevated NKX3.1 protein levels in these cells. To determine whether *NKX3.1* was a direct or downstream target of tRA, *NKX3.1* mRNA levels were compared in tRA-treated LNCaP cells and in cultures co-treated with tRA and 10 μg/ml cycloheximide. Although cycloheximide treatment decreased *NKX3.1* mRNA levels, the tRA-induced increase in *NKX3.1* mRNA was maintained in the presence of cycloheximide with a similar increase (~1.78-fold) detected following tRA exposure (Fig. 4). These findings indicated that tRA effects on *NKX3.1* were independent of ongoing protein synthesis and suggested that tRA, acting through nuclear RARs, induced *NKX3.1* mRNA expression via a direct pathway.

tRA Increases the Rate of *NKX3.1* Gene Transcription

The tRA-induced increases in *NKX3.1* mRNA abundance could be due to elevated mRNA stability and/or increased *NKX3.1* gene transcription. To assess the effect of tRA on *NKX3.1* mRNA stability, LNCaP cells were treated for 6 h with tRA before the addition of the transcriptional inhibitor, actinomycin D (Act D). Total RNA was extracted after increasing lengths of Act D (5 μg/ml) exposure and *NKX3.1* and β-actin mRNA analyzed by Northern blotting. As observed in Figure 5, *NKX3.1* mRNA degrades rapidly with a half-life in LNCaP control cells of ~3.8 h, a result not

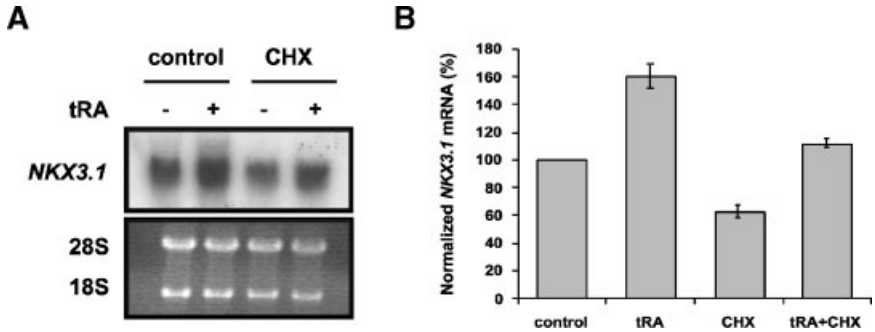

Fig. 4. tRA-induced upregulation of *NKX3.1* mRNA levels does not require new protein synthesis. **A**: LNCaP cells were treated for 6 h with either tRA (+) or DMSO (−) in the absence (control) or presence of the protein synthesis inhibitor, cycloheximide (CHX). Northern blot analysis was performed using an *NKX3.1* probe (**upper panel**) and RNA loading controlled using the 28S ribosomal marker (**lower panel**). **B**: *NKX3.1* mRNA levels were quantified from experiments described in (A) and normalized against the matching 28S marker in individual samples. The graphed results are expressed as mean ± SEM from three separate experiments.

markedly different from the mRNA half-life in tRA-treated cells (~4.2 h). Nuclear run on assays were therefore performed using nuclei extracted from LNCaP cells, pre-treated for 6 h with tRA, to determine if the elevated mRNA levels resulted from increases in transcription of *NKX3.1*. Transcription of *NKX3.1* was seen to be increased (~1.9–2.3-fold) following tRA exposure (lower panel; Fig. 6), in comparison

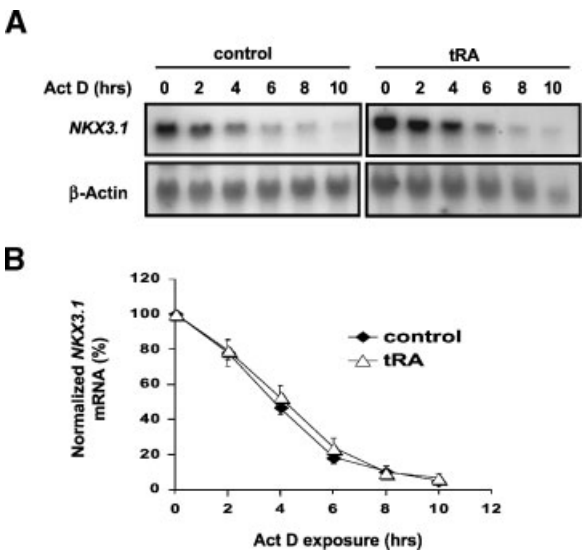

Fig. 5. *NKX3.1* mRNA stability is not affected by tRA. **A**: LNCaP cells treated for 6 h with either 5 μM tRA or DMSO (control) were exposed to the transcriptional inhibitor, actinomycin D (Act D) for the time periods indicated and Northern blot analysis was performed for *NKX3.1* (**upper panels**) or β-actin (**lower panels**). **B**: *NKX3.1* mRNA levels were quantified from experiments described in (A) and normalized using the matching β-actin mRNA levels in individual samples. The graphed results are expressed as mean ± SEM from three separate experiments.

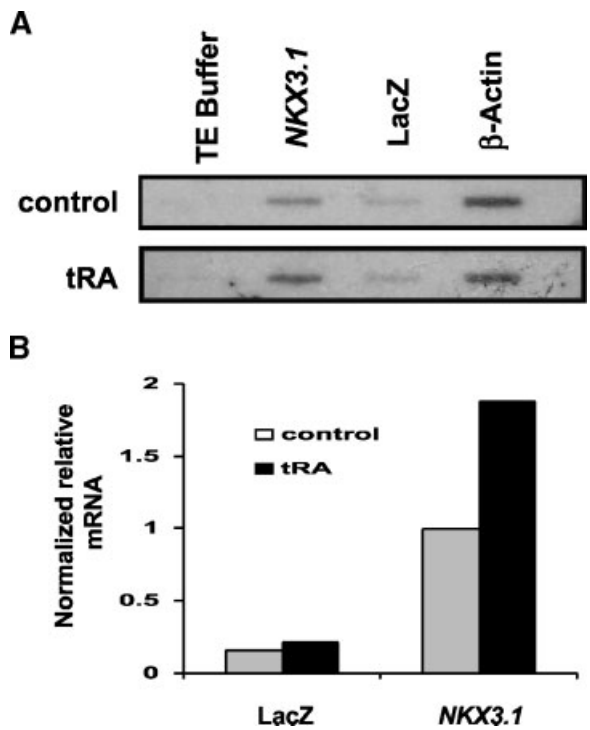

Fig. 6. tRA increases *NKX3.1* transcription. **A**: Nuclei were isolated from untreated LNCaP cells (control; **upper panel**) or LNCaP cells treated with 5×10^{-6}M tRA for 6 h (tRA; **lower panel**). In vitro transcription assays were performed and transcribed labeled mRNAs were hybridized to filters blotted with the cDNA detection probes, *NKX3.1*, β-galactosidase (LacZ), and β-actin. Displayed is a representative blot of two independent nuclear run on assays that were performed. **B**: Hybridized transcribed mRNA signals shown in (A) were quantified and densitometric values normalized against β-actin. tRA treatment of LNCaP cells specifically increased levels of *NKX3.1* transcripts produced from isolated nuclei.

to control levels of *NKX3.1* transcription from nuclei of untreated cells (upper panel; Fig. 6). In conjunction with the Northern blot data, these results suggest that tRA acts to increase *NKX3.1* mRNA levels in LNCaP cells by increasing the rate of *NKX3.1* gene transcription.

NKX3.1 Proximal Promoter and 3′UTR Are Not Targets of Retinoid Receptors

RARs bound by ligand mediate their transcriptional effects through interactions with specific response elements (RAREs) located in diverse regulatory regions of target genes [Bastien and Rochette-Egly, 2004]. Analysis of the proximal promoter of the *NKX3.1* gene identified two putative RAREs encompassing nts −116 to −100 and −753 to −737 relative to the transcription start site. Several additional putative response elements, spanning nucleotides +1,866 to +1,879, +3,029 to +3,041, and +4,011 to +4,023 were identified within the long 3′UTR of the *NKX3.1* mRNA (Fig. 7A).

To investigate their involvement in the tRA-mediated transcriptional activation of *NKX3.1,* a ∼2 kb region (−2,062 to +6) of the *NKX3.1* proximal promoter was cloned upstream of luciferase in the luciferase reporter vector, pGL3-enhancer. In addition, the 3′UTR of *NKX3.1* was cloned upstream of luciferase in the pGL3-promoter vector to test for retinoid responsive enhancer elements. The constructed plasmids, pGL3-NKX-5′promoter and

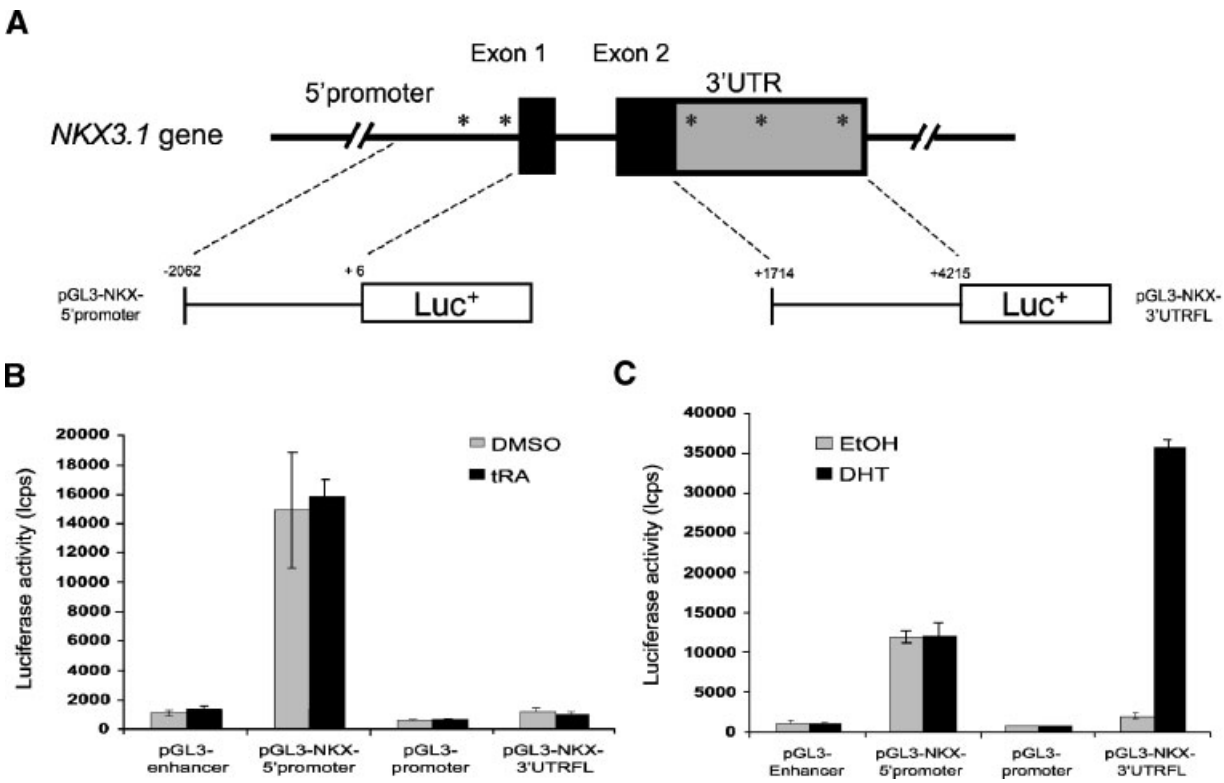

Fig. 7. Androgen but not retinoid responsiveness is detected in the *NKX3.1* 3′UTR. **A**: Schematic diagram of the *NKX3.1* gene and constructed luciferase reporter plasmids. The pGL3-NKX-5′promoter plasmid was generated by ligating nucleotides −2,062 to +6 (+1 indicates transcriptional start site) of the *NKX3.1* proximal promoter into pGL3-enhancer, while pGL3-NKX-3′UTRFL was developed by inserting the *NKX3.1* 3′UTR, encompassing nucleotides +1,714 to +4,215, into the pGL3-promoter vector. Putative RAREs located in the proximal promoter, encompassing nucleotides −753 to −737 and −116 to −100, or in the 3′UTR, spanning nucleotides +1,866 to +1,879, +3,029 to +3,041, and +4,011 to +4,023 are indicated by an asterisk. **B**: DU145 cells were transiently transfected with either the luciferase control vectors pGL3-enhancer or pGL3-promoter, or the *NKX3.1* reporter plasmids pGL3-NKX-5′promoter or pGL3-NKX-3′UTR. Following transfection, the cells were rested (6 h) and then treated overnight with either 5×10^{-6}M tRA or DMSO (vehicle) before assaying the cultures 24 h post-transfection for luciferase activity. The results (mean ± SEM) are expressed in actual light counts per second (lcps) and are representative of three transfection experiments. **C**: LNCaP cells were transiently transfected with luciferase plasmids indicated in (B), rested for 6 h and incubated overnight with either 10^{-8}M DHT or ethanol vehicle (ETOH). Cultures were assayed for luciferase activity 24 h post-transfection. The results (mean ± SEM) are expressed in actual lcps and are representative of three transfection experiments.

pGL3-NKX-3′UTRFL were then transiently transfected into the prostate cancer cell lines LNCaP and DU145 and luciferase assays performed 24 h later on cells cultured in either the absence or presence of tRA. As expected, strong basal promoter activity was observed from the *NKX3.1* promoter in DU145 cells with a ~14.5-fold increase in luciferase activity detected compared to the pGL3-enhancer control, while a modest twofold induction of luciferase activity was observed from the pGL3-NKX-3′UTRFL plasmid (Fig. 7B). However, tRA responsiveness was not detected from either of the *NKX3.1*-reporter plasmids with similar results also observed in LNCaP cells (data not shown).

Of interest in this study was the finding that the pGL3-NKX-3′UTRFL vector was highly androgen responsive with 10^{-8}M DHT inducing a ~19.5-fold increase in luciferase activity in LNCaP cells (Fig. 7C). No androgen responsiveness was detected in either control plasmids or the pGL3-NKX-5′promoter (Fig. 7C), and androgen responsiveness was not detected for any construct in DU145 cells, which do not express the androgen receptor (AR) ([Jarrard et al., 1998]; results not shown). These data indicate that the tRA-mediated activation of *NKX3.1* gene transcription does not occur via the *NKX3.1* proximal promoter or 3′UTR, while previously reported androgen regulation of *NKX3.1* gene expression is mediated, at least in part, by the *NKX3.1* 3′UTR.

DISCUSSION

NKX3.1 is a prostate-specific homeobox gene, expression of which is regulated by androgens and estrogens, with androgens directly increasing *NKX3.1* gene transcription in prostate cancer cells [He et al., 1997; Prescott et al., 1998; Korkmaz et al., 2000]. In this study, we have identified that the natural retinoid, tRA directly regulates *NKX3.1* expression in the human prostate cancer cell line, LNCaP by increasing transcription of the *NKX3.1* gene.

Retinoids have been shown to play an important role in regulating the proliferation and differentiation of non-malignant and malignant prostate epithelial cells [Peehl et al., 1993; Goossens et al., 2002]. Alterations in retinoid signaling are associated with prostate carcinogenesis as evidenced by decreased accumulation of retinoids and reduced expression of retinoid receptors in prostate cancer cells compared to non-malignant prostate [Pasquali et al., 1996; Kikugawa et al., 2000; Lotan et al., 2000; Gyftopoulos et al., 2000a,b; Alfaro et al., 2003; Zhong et al., 2003; Mao et al., 2004]. As such, changes in the activity of retinoid signaling pathways may produce a wide range of biological effects via altered expression and therefore function of retinoid target genes. In this study, all-*trans* retinoic acid rapidly upregulated *NKX3.1* mRNA levels from as early as 2 h of tRA treatment of LNCaP prostate cancer cells, with peak levels detected at 6 h and persisting for up to 48 h of tRA treatment. Similar responses of NKX3.1 protein levels were evident, indicating that tRA produced a sustained effect on *NKX3.1* expression rather than an acute transient response.

tRA-transduced signals can regulate a diverse array of genes either directly by activating target gene transcription, or indirectly through downstream regulatory effects of newly synthesized intermediary proteins. Cycloheximide co-treatment did not attenuate tRA induction of *NKX3.1* levels, indicating that *NKX3.1* is a direct target of retinoid receptors. This result was supported by experiments that showed no tRA-mediated post-transcriptional effects (mRNA stability or protein stability) on *NKX3.1* expression. In combination with nuclear run on assays indicating that tRA induced *NKX3.1* mRNA synthesis, this study has generated strong evidence that retinoids directly regulate *NKX3.1* expression at the transcriptional level and led us to search for functional RAREs within the gene.

RAREs are highly pleiotropic but typically follow a loose consensus of two direct repeats of the core hexameric motif, PuG(G/T)TCA separated by 1 bp (DR1), 2 bp (DR2), or 5 bp (DR5) [Bastien and Rochette-Egly, 2004]. Several of these motif-like sequences were identified within the initial 2 kb of the *NKX3.1* proximal promoter and the unusually long 3′UTR (Fig. 7). However, retinoid responsiveness could not be demonstrated within either of these regions in the context of luciferase reporter plasmid analysis. Like many other enhancers, RAREs are often located within distant control elements and have been identified in intronic sequences (e.g., major histocompatibility complex (MHC) H2Kb and CD38 genes) [Jansa and Forejt, 1996; Kishimoto et al., 1998], as well as sites several kilobases downstream (erythropoietin, *Hox*a1, b1, a4, b4, and d4) or upstream

(*Hox*b1 and d4) of target genes [Langston et al., 1997; Morrison et al., 1997; Kambe et al., 2000]. It is therefore likely that tRA is mediating its transcriptional effects on *NKX3.1* at more distal sequences not examined in this study.

The crucial role of 3′UTRs in regulating gene expression is now widely acknowledged but is generally associated with mRNA localization, stability, and translation efficiency [Grzybowska et al., 2001]. There is however, increasing evidence that in specific circumstances, these sequences are also necessary for regulating transcription. An interesting finding of this study was the localization of strong androgen responsiveness in the 3′UTR of the *NKX3.1* gene. This effect was independent of the position and orientation of the 3′UTR within the pGL3-promoter plasmid (not shown), which suggests that the DHT-induced increase in luciferase activity is mediated by one or more androgen response elements (ARE) located within this region. In general, AREs are positioned in the 5′ flanking region of androgen target genes, however, several examples of functional hormone responsive elements located in the 3′UTR of genes have been reported [Bigler and Eisenman, 1995; Hyder et al., 2000; Butts et al., 2004]. Whilst putative AREs can be identified in the *NKX3.1* 3′UTR sequence, the exact location of the response elements mediating the androgen induction is unknown and is currently being investigated. These findings indicate that the *NKX3.1* 3′UTR, in combination with other regions of the gene [Yoon and Wong, 2005], plays a critical role in the androgen-mediated activation of NKX3.1 expression.

Retinoid regulation of *NKX3.1* has not been explored previously in either non-malignant or malignant prostate epithelial cells. Both *NKX3.1* and retinoids serve well-documented roles in proliferation control of prostatic cells and in inducing or maintaining differentiation of prostate cancer or non-malignant prostate cells [Peehl et al., 1993; Goossens et al., 2002; Shen and Abate-Shen, 2003]. As such, it is feasible that retinoids and retinoid signaling contribute to the regulation of expression of *NKX3.1* in the prostate and that the loss or reduction of NKX3.1 expression detected in early and advanced prostate tumors is due, in part, to aberrant retinoid signaling. It is also possible that retinoid induced growth inhibition and/or differentiation induction in prostate cancer cells is partly mediated by NKX3.1. Interest in retinoids as differentiation agents for the treatment and prevention of prostate cancer initially stemmed from work using chemically-induced mouse prostate cancer models, which demonstrated their effective inhibition of tumor growth and progression [Lasnitzki and Goodman, 1974; Chopra and Wilkoff, 1977; Pollard et al., 1991]. More recently, studies using human prostate cancer cell lines have reported that retinoids, either alone or in combination with other therapeutic agents, can inhibit cell proliferation and tumorigenicity [Dahiya et al., 1994; de Vos et al., 1997]. Although clinical trials using retinoids as sole or adjunct chemotherapeutic agents have shown limited efficacy to date [Trump et al., 1997; Culine et al., 1999], their therapeutic use remains clinically viable pending the development of synthetic retinoids with better pharmacokinetic profiles and identification of markers of retinoid sensitivity of individual tumors.

In summary, we have shown that NKX3.1 expression is upregulated by the retinoid tRA through the direct activation of *NKX3.1* gene transcription in prostate cancer cells. Identification of regulatory pathways controlling NKX3.1 expression will determine how this gene contributes to prostate development, as well as elucidate the mechanisms responsible for its deregulated expression during prostate carcinogenesis.

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