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Evidence that Sp1 positively and Sp3 negatively regulate and androgen does not directly regulate functional tumor suppressor 15-lipoxygenase 2 (15-LOX2) gene expression in normal human prostate epithelial cells

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In this project, we studied the gene regulation of 15-lipoxygenase 2 (15-LOX2), the most abundant arachidonate-metabolizing LOX in adult human prostate and a negative cell-cycle regulator in normal human prostate (NHP) epithelial cells. Through detailed *in silico* promoter examination and promoter deletion and activity analysis, we found that several Sp1 sites (i.e., three GC boxes and one CACCC box) in the proximal promoter region play a critical role in regulating 15-LOX2 expression in NHP cells. Several pieces of evidence further suggest that the Sp1 and Sp3 proteins play a physiologically important role in positively and negatively regulating the 15-LOX2 gene expression, respectively. First, mutations in the GC boxes affected the 15-LOX2 promoter activity. Second, both Sp1 and Sp3 proteins were detected in the protein complexes that bound the GC boxes revealed by electrophoretic mobility shift assay. Third, importantly, inhibition of Sp1 activity or overexpression of Sp3 both inhibited the endogenous 15-LOX2 mRNA expression. Since 15-LOX2 is normally expressed in the prostate luminal epithelial cells, we subsequently explored whether androgen/androgen receptor may directly regulate its gene expression. The results indicate that androgen does not directly regulate 15-LOX2 gene expression. Together, these observations provide insight on how 15-LOX2 gene expression may be regulated in NHP cells.
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Introduction

15-Lipoxygenase 2 (15-LOX2) shows the highest homology to murine 8-LOX, has at least three splice variants (termed 15-LOX2sv-a/b/c (15-LOX2 splice variant a, b or c)), mainly metabolizes arachidonic acid (AA) to 15(*S*)-hydroxyeicosatetraenoic acid [15(S)-HETE], and is primarily expressed in prostate, lung, skin, and cornea (Brash *et al*., 1997; Kilty *et al*., 1999; Tang *et al*., 2002). 15-LOX2 expression and activity are decreased in high-grade prostate intraepithelial neoplasia and prostate cancer (PCa) (Shappell *et al*., 1999). We recently reported that 15-LOX2 is a negative cell-cycle regulator in normal human prostate (NHP) epithelial cells (Tang *et al*., 2002), which may explain why it is advantageous for PCa cells to suppress its expression. Not surprisingly, re-expression of 15-LOX2 inhibits PCa cell proliferation *in vitro* and tumor development *in vivo* (Bhatia *et al*., 2003), suggesting that 15-LOX2 may represent a functional prostate tumor suppressor. Surprisingly, however, the tumor-suppressive function of 15-LOX2 does not appear to absolutely require its localization to the nucleus or its ability to metabolize AA, as 15-LOX2sv-b, a splice variant that does not localize to the nucleus and lacks obvious AA-metabolizing activity also demonstrates tumor-inhibitory effect (Bhatia *et al*., 2003).

To fully understand the role of 15-LOX2 in regulating prostate development and homeostasis and the contribution of its loss of expression to PCa development, we must first understand how the gene is regulated in NHP cells, which is the main goal of the current study. By utilizing a variety of cell biological, biochemical, and molecular approaches, we provide evidence that the Sp1 and Sp3 transcription factors positively and negatively regulate 15-LOX2 gene expression in NHP cells. In contrast, androgen/androgen receptor (AR) pathway does not directly regulate the 15-LOX2 gene expression.

Results

Determination of TSS and analysis of the putative 15-LOX2 promoter

We first cloned out a ~1. 2 kb 5′-flanking region (i.e. the P3–P8 fragment; Table 1) immediately upstream of

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ATG (Figure 1b). Using this fragment, we performed a primer extension analysis to pinpoint the TSS of the 15-LOX2 gene. As shown in Figure 1a, the TSS of 15-LOX2 was identified as an adenine in the sequence CAA TA***A***CCA, 87 bp upstream of ATG (Figure 1b and c).

Using the determined TSS, we performed *in silico* sequence analysis on 15-LOX2 promotor (Figure 1b), using TESS at www.cbil.upenn.edu/tess, TFSEARCH at www.cbrc.jp/research/db/TFSEARCH.html, Cister at http://zlab.bu.edu/~mfrith/cister.shtml, PROMO at www.lsi.upc.es/~alggen/recerca/promo_v2/ frame-promo.html, and Gene Regulation at www.gene-regulation.com, as well as searching several transcription factor compilations (Locker, 1993; Strachan and Read, 2000) and databases (e.g. TRANSFAC, TRANSCompel, and IMD) (Heinemeyer *et al*., 1998; Matys *et al*., 2000; Frith *et al*., 2001). This analysis revealed some interesting features for the 15-LOX2 promoter region (Figure 1a and c). First, the 15-LOX2 promoter does not have a TATA (consensus TATAAA) or TATA-like (TATTT) box, suggesting that 15-LOX2 is a tissue-specific 'housekeeping' gene. Second, most TATA-less promoters utilize the so-called initiator element (Inr; consensus PyA(A/T)PyPy, where Py is a pyrimdine) and/or downstream promoter element (DPE; consensus (AG)G(AT)CGTG) located at various distances downstream of the TSS to initiate transcription (Strachan and Read, 2000; Levine and Tijan, 2003). The 15-LOX2 TSS (CAATA***A***CC) conformed to the consensus Inr sequence and a DPE with sequence AGGCGTG that matches the consensus DPE sequence was found 16 bp downstream of the TSS (Figure 1b and c), suggesting that 15-LOX2 gene transcription might utilize these elements. Third, multiple potentially important transcription factor-binding sites were concentrated in the 15-LOX2 proximal promoter region (i.e. within ~120 bp region upstream of TSS). For example, a CAAT box (consensus (A/G)CCAATC) with the sequence GCCAATC was found at position −114 (Figure 1b and c). This *cis* element generally serves as

Table 1 Primers and probes used in the current study

Name	*Position*[a]	*Orientation*	*Sequence*
PCR primers			
P2	−3985/−3957	Forward	5′-GTCCTTGGAGGTGCAAGATCACAGGTT-3′
P3	−1116/−1093	Forward	5′-CCTTCCCACCTCTGCTTCTCACTC-3′
P4	−726/−706	Forward	5′-CAATAGCAGTGACGATAGCAC-3’
P5	−471/−452	Forward	5′-GGCTGAGGTAGGAGAATCAC-3′
P6	−163/−149	Forward	5′-CCCAAACCTCAGGGT-3′
P7	−102/−83	Forward	5′-CCCACTTTAGTTGCGTGTTC-3′
P8	+60/+80	Reverse	5′-GCCTAAGTCCAGCTCTCTACG-3′
P9	−151/−126	Reverse	5′-TGGGAACAGGAGGGACTAAGAATAC-3′
P10	+227/+250	Reverse	5′-CCCCATCCACTCCCACGCACTCA-3′
Site-specific mutagenesis primers[b]			
GC box 1/CACCC box			CACCC box
Wild type			5′-CAGCGCTGCCAATCCCCCGCCCACCCCCACTTTAGTTGC-3′
Mutant			5′-CAGCGCTGCCAATCCCCCG***TCTAGA***CCCACTTTAGTTGC-3′
			*Xba*I
GC box 2[c]			
Wild type			5′-CCAGCCTCTCCGCCCCGCCCCTCCCCGCCCTG-3′
Mutant			5′-CCAGCCTCTCCGCC***AAGCTT***CTCCCCGCCCTG-3′
			*Hind*III
GC box 3[d]			
Wild type			5′-CTCCGCCCCGCCCCTCCCCGCCCTGAAACGGACGTG-3′
Mutant			5′-CTCCGCCCCGCCCCTC***AACGCT***CTGAAACGGACGTG-3′
Double-strand oligonucleotide probes used in EMSA[b]			
Consensus Sp1 probe (Santa Cruz)			
Wild type			5′-ATTCGATCGGGGCGGGGCGAG-3′
Mutant			5′-ATTCGATCGG***TT***CGGGGCGAG-3′
GC1 probe			CAAT box CACCC box
Wild type			5′-CAGCGCTGCCAATCCCCCGCCCACCCCCACTTTAGTTGC-3′
Mutant			5′-CAGCGCTGCCAATCCCCCG***TCTAGA***CCCACTTTAGTTGC-3′
GC2/GC3 probe			
Wild type			5′-CTCCG*CCCCGCCCCTCCCCCGCCC*TGAAACG-3′
GC2 mutant[c]			5′-CTCCGCC***AAGCTT***CTCCCCGCCCTGAAACG-3′
GC3 mutant[d]			5′-CTCCGCCCCGCCCCTC***AACGCT***CTGAAACG-3′

[a]Relative to TSS, which is designated as +1. The overlapping GC box 1 and CACCC box are located at −113/−104 and the tandem GC box 2 and GC box 3 at −34/−53. See Figure 1 and text for details. [b]For the sake of simplicity, only the upper strand sequence is shown. GC boxes are underlined and CAAT and CACCC boxes are indicated on top. Mutated sequences are highlighted in italic. [c]In this mutant, GC box 3 remains intact (indicated by the second underlined sequence in wild type). [d]In this mutant, GC box 2 remains intact (indicated by the first underlined sequence in wild type)

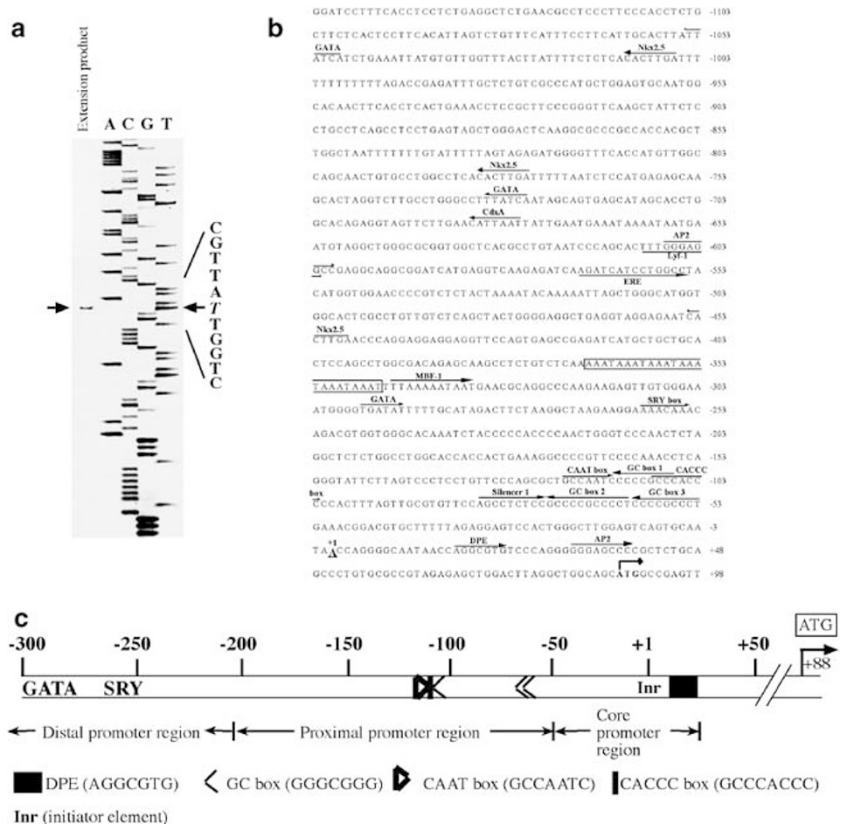

Figure 1 15-LOX2 TSS and *in silico* promoter analysis. (**a**) Determination of TSS. The location of the extension product is indicated by an arrow on the left. The sequence on the right is antisense and the arrow indicates the TSS. (**b**) *In silico* sequence analysis of the 15-LOX2 promoter region. The *cis* elements that can serve as putative binding sites for transcription factors are indicated (the arrows indicate the directions). The TSS nucleotide A is underlined and marked as +1 and ATG marked by an arrow. (**c**) Schematic illustrating the 15-LOX2 promoter region

the binding site for ubiquitous transcription factors NF-1 (also called CTF for *C*CAAT-binding *t*ranscription *f*actor), NF-Y (also called CBF for *C*CAAT box-*b*inding *f*actor), and C/EBP (Strachan and Read, 2000). Most prominently, a CACCC box with the sequence CCACCCC that matches the consensus and three GC boxes (consensus GGGGCGGGG) were found within ~120 bp upstream of the TSS (Figure 1b,c). CACCC box, GC box, and some other related GC-rich sequences are frequently called Sp1 sites, which generally are located at −160/+1 region and serve as binding sites for the Sp/KLF (Krüppel-like factors) transcription factors to modulate the basal and induced transcription of the core promoter as well as operate as essential enhancer sequences (Locker, 1993; Strachan and Read, 2000; Black *et al*., 2001). Fourth, multiple other perfectly matched transcription factor-binding sites including three GATA sites, three Nkx2.5 sites, one CdxA-binding site, one MBF-1 site, one silencer-1 site, two AP2 sites, and one SRY box were also found upstream of the TSS (Figure 1b). A stretch of six repeats containing sequence AAAT was present just upstream of the MBF-1 site (Figure 1b; boxed sequence). The significance of this interesting sequence feature remains unclear. Finally, examination of the 1.2 kb 15-LOX2 promoter region did not reveal androgen-responsive element (ARE), although a perfect estrogen-responsive element was found at ~500 bp upstream of the TSS (Figure 1b).

Sp1 sites as crucial cis *elements regulating the 15-LOX2 promoter activity*

Next, we carried out deletion analysis of the 1.2 kb 15-LOX2 promoter. The −1116/+80 (i.e. P3–P8; Table 1) fragment possessed 44-fold higher promoter activity than the vector (pGL3-basic) alone (not shown). The −726/+80 fragment demonstrated ~80% of the −1116/+80 promoter activity (Figure 2a). In contrast, the −471/+80 fragment showed a slightly higher promoter activity (Figure 2a), suggesting the presence of a potential negative regulatory element within the −726/−471 region. Notably, the −163/+80 fragment, which contained all four Sp1 sites, had nearly the full promoter activity as the −1116/+80 fragment, whereas the −102/+80 fragment, which contained only GC box 2 and GC box 3, demonstrated ~70% of the −1116/+80 promoter activity (Figure 2a). In contrast, the −726/−126 and −471/−126 fragments, both of which lack all four Sp1 sites (Figure 1b; Table 1), showed no promoter activity (Figure 2a).

In another set of experiments using a different batch of NHP6 cells (Figure 2b), we utilized the −726/+80 fragment as the baseline, which possessed 36-fold higher

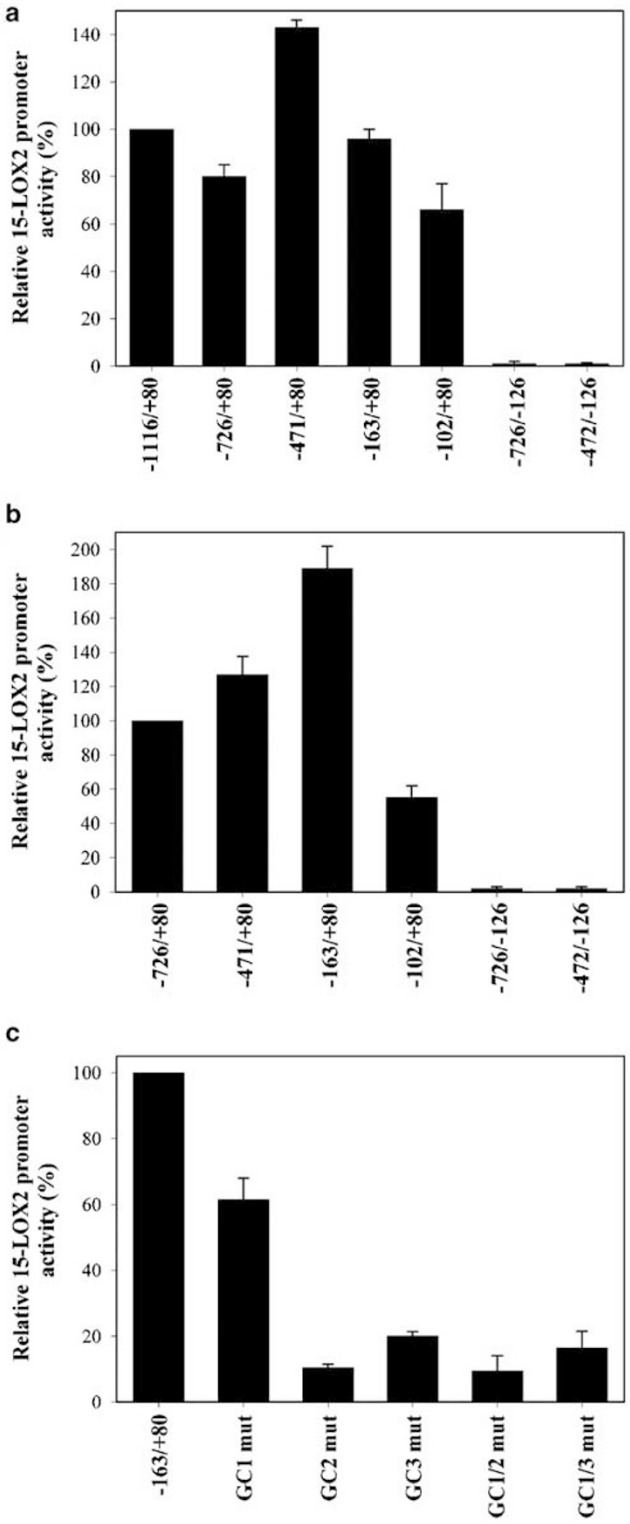

Figure 2 Critical roles of GC boxes. (**a**) Luciferase assays ($n=3$) performed using NHP6 cells (P5–7) and the relative promoter activities presented as the % of the $-1116/+80$ fragment. (**b**) Luciferase assays ($n=6$) performed using NHP6 cells (P6-7) and the relative promoter activities presented as the % of the $-726/+80$ fragment. Data in both (**a**) and (**b**) represent the mean$\pm$s.e.m. (**c**) Site-specific mutagenesis. Luciferase constructs were transfected into NHP6 cells (P6). The relative 15-LOX2 promoter activities were expressed as the % of the $(-163/+80)$-luc, which had a RLU of 44 over the pGL3-basic vector. The data represent the mean $\pm$s.e.m. ($n=3$)

promoter activity than the vector alone (not shown). Interestingly, the $-471/+80$ fragment had slightly higher promoter activity and the $-163/+80$ fragment consistently showed a $\sim$2-fold increase in the promoter activity compared to the $-726/+80$ fragment (Figure 2b), suggesting an inhibitory *cis* element(s) between $-471/-163$. Again, the $-102/+80$ fragment demonstrated $\sim$60% of the $-726/+80$ promoter activity, whereas both $-726/-151$ and $-471/-151$ fragments completely lacked promoter activity (Figure 2b). Similar results were obtained in experiments carried out in NHP2 cells (not shown).

Crucial role of GC box 2 and GC box 3 in regulating the 15-LOX2 promoter activity

Next, we carried out site-specific mutagenesis (Table 1). As shown in Figure 2c, mutation of GC box 1/CACCC box (GC1 mut) eliminated $\sim$40% of the promoter activity. In contrast, mutation of GC box 2 or GC box 3 eliminated 90 and 80% promoter activity, respectively (Figure 2c). Double mutations of GC box 1/CACCC box with GC box 2 or GC box 3 slightly reduced the promoter activity further (Figure 2c). These results together suggest that the GC box 2 and GC box 3 and, less significantly, GC1 box 1/CACCC box, play a crucial role in regulating the 15-LOX2 promoter activity.

Sp1 as a positive regulator of the 15-LOX2 promoter activity and gene expression

The above experiments suggest that the sequence in the proximal 15-LOX2 promoter region containing Sp1 sites is critical for the 15-LOX2 gene expression. Sp1 and related Sp family members such as Sp3 are the major transcription factors that bind to GC-rich Sp1 sites (Black *et al*., 2001). Sp1 sites and the Sp family proteins have been implicated in the constitutive expression of many 'housekeeping'genes as well as in tissue and cell specific and highly regulated expression of many other genes (Huang *et al*., 2000; Hong *et al*., 2002; Blais *et al*., 2002). Furthermore, Sp1 sites and the Sp1 protein have been implicated in regulating the basal expression of several other LOXs, including 12-LOX and 15-LOX 1 (Kritzik *et al*., 1997; Kelavkar *et al*., 1998; Chen and Chang, 2000).

To study the role of Sp1, we took advantage of *Drosophila melanogaster* Schneider SL2 cells, which lack endogenous Sp1 or other Sp family proteins (Black *et al*., 2001). As shown in Figure 3a, cotransfection of the $(-726/+80)$-luc or $(-102/+80)$-luc with an Sp1 expression vector driven by *Drosophila* actin promoter (pPacSp1) into SL2 cells enhanced, in a dose-dependent manner, their promoter activities. As expected, the $-102/+80$ fragment showed $\sim$50% luciferase activity compared to the $-726/+80$ fragment (Figure 3a), because the $-726/+80$ fragment contains four Sp1 sites, whereas the $-102/+80$ fragment only two Sp1 sites (Figure 1b). These results provide direct evidence that the Sp1 protein can activate the 15-LOX2 promoter.

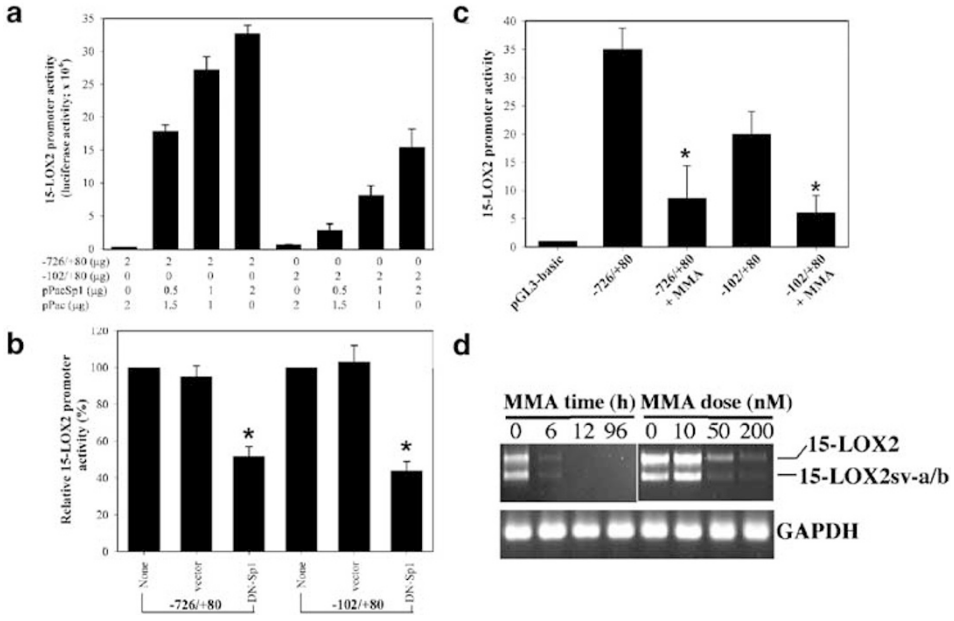

Figure 3 Sp1 as a positive regulator. (**a**) SL2 cells (2×10^5 cells/well) were transfected with the indicated plasmids at the respective doses (pPac was cotransfected to maintain equal plasmid amount). Cell lysates were made 48 h after transfection and equal amounts of proteins used for luciferase activity measurement. Results were expressed as RLU normalized to that obtained with the empty vector (pPac) alone. Data represent the mean ± s.e.m. ($n = 3$). $P < 0.01$ at all dose points (Student's *t*-test). (**b**) NHP6 cells (P6) were transfected with the promoter plasmids together with a DN-Sp1 vector (pEBG-Sp1) or its control vector (pEBG-N). At 48 h after transfection, cells were harvested for luciferase activity measurement. Data represent the mean ± s.e.m. derived ($n = 3$). *$P < 0.01$ (Student's *t*-test). (**c**) NHP6 cells (P5–6) were transfected with pGL3-basic control vector or promoter construct in the presence or absence of MMA (200 nM). After 48 h, cells were harvested for luciferase activity assays. The promoter activities were expressed as RLU (mean ± s.e.m.; $n = 3$). *$P < 0.01$ (Student's *t*-test). (**d**) NHP6 cells (P6) were treated with 200 nM MMA for various time intervals (time course) or with various doses of MMA for 48 h (dose study). RT–PCR was performed using primers C and D that could pick up both 15-LOX2 and 15-LOX2sv-a/b (Tang *et al*., 2002)

To determine whether the Sp1 protein is required for 15-LOX2 gene expression, we cotransfected a DN-Sp1 expression vector together with the promoter constructs into NHP6 cells. The DN-Sp1 has no transactivating function and selectively inhibits Sp1-dependent reporter gene expression (Ptersohn and Thiel, 1996; Grinstein *et al*., 2002). As shown in Figure 3b, DN-Sp1 at 0.5 μg/well inhibited the $-726/+80$ and $-102/+80$ promoter activities by 50–60%, respectively. At a higher concentration (i.e. 2 μg/ml), DN-Sp1 reduced both promoter activities to ~30% (not shown). As an alternative approach, NHP6 cells were treated with an Sp1-specific chemical inhibitor, MMA (Kaluz *et al*., 2003), simultaneously with the transfection of promoter fragments. As shown in Figure 3c, MMA significantly inhibited the promoter activities of both fragments. MMA inhibited not only the exogenous 15-LOX2 promoter activity but also the endogenous 15-LOX2 expression revealed by reverse transcriptase–polymerase chain reaction (RT—PCR) (Figure 3d). Time-course studies revealed that MMA (at 200 nM) nearly completely inhibited the expression of both 15-LOX2 and 15-LOX2sv-a/b as early as 6 h post-treatment (Figure 3d). Dose studies indicated that MMA at 50 nM inhibited 15-LOX2 mRNA expression in NHP6 cells by >90% at 48 h post-treatment (Figure 3d). Altogether, these results suggest that Sp1, through binding to the GC-rich sequences in the proximal 15-LOX2 promoter region, functions as a positive regulator of 15-LOX2 gene expression. The data also establish that 15-LOX2 gene expression in NHP cells are Sp1 dependent.

Sp3 as a negative regulator of the 15-LOX2 promoter activity and gene expression

Among the Sp family proteins, Sp3 has been shown to either positively (Garcia-Ruiz *et al*., 2002; Won *et al*., 2002; Schafer *et al*., 2003) or negatively (Hagen *et al*., 1994; Kumar and Butler, 1997) modulate the Sp1-dependent gene expression. To determine whether and how Sp3 may modulate the Sp1-dependent 15-LOX2 expression, we again made use of SL2 cells. As shown in Figure 4a, Sp3 dose dependently inhibited Sp1-dependent 15-LOX2 promoter activity in SL2 cells. When Sp3 was transfected into NHP6 cells, it completely inhibited the 15-LOX2 promoter, that is, $(-163/+80)$-luc, activity (Figure 4b). Importantly, overexpression of Sp3 also reduced the mRNA levels of both 15-LOX2 and 15-LOX2sv-a/15-LOX2sv-b (Figure 4c). Considering that the transfection efficiency in NHP cells was generally <10% (Tang *et al*., 2002), the inhibitory effect of Sp3 on endogenous 15-LOX2 mRNA expression was significant (Figure 4c). Interestingly, for unknown reasons, we consistently observed a shift of 15-LOX2sv-a/15-LOX2sv-b to 15-LOX2 upon transfection with the control vector (Figure 4c, the middle lane).

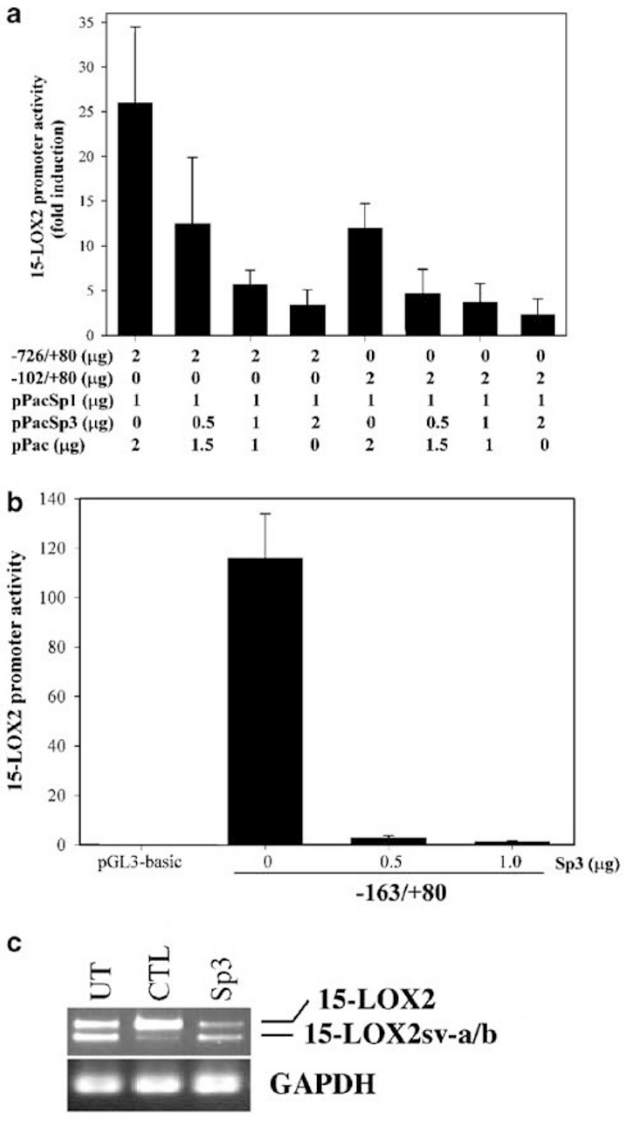

Figure 4 Sp3 as a negative regulator. (**a**) Experiments were performed as described in Figure 3a except for the addition of pPacSp3. The results are expressed as fold induction. Data represent the mean ± s.e.m. ($n=3$). $P<0.01$ at all dose points. (**b**) NHP6 cells (P7) were transfected with pGL3-basic or the (−163/+80)-luc in the presence of the control vector (i.e. pCMVneo; 0) or pCMV-Sp3. Luciferase assays were carried out 48 h after transfection. The 15-LOX2 promoter activity was expressed as RLU and data represent the mean ± s.e.m. ($n=2$). Sp3 at both doses significantly ($P<0.01$) inhibited the 15-LOX2 promoter activity. (**c**) Sp3 inhibits endogenous 15-LOX2 gene expression. NHP6 cells (P5) were either untransfected (UT) or transfected with pCMVneo (CTL) or pCMV-Sp3 (Sp3; 0.5 μg/well) for 48 h. RT–PCR was performed as in Figure 3d

These results, altogether, suggest that Sp3 negatively regulates the 15-LOX2 gene expression in NHP cells.

Multiple complexes form in the GC-rich regions of the 15-LOX2 promoter: Sp1 and Sp3, but not Sp2 or Sp4, exist in some of these complexes

To determine directly whether Sp1 and Sp3 interact with the GC boxes of the 15-LOX2 promoter, we carried out electrophoretic mobility shift assay (EMSA) experiments using labeled GC1 or GC2/GC3 probe (Table 1) and NE prepared from either NHP6 (Figure 5) or PCa (Figure 6) cells. There were at least four complexes, that is, complex I–IV (Figure 5a, lane 2) that bound to the GC box 1/CACCC box (Figure 1b; Table 1), as their binding could be completely competed out by cold GC1 probe (Figure 5a, lane 3). Note that a prominent band running faster than complex IV was not competed out by the cold GC1 probe (Figure 5a, lane 3) and perhaps represented a nonspecific band (Figure 5a; NS).

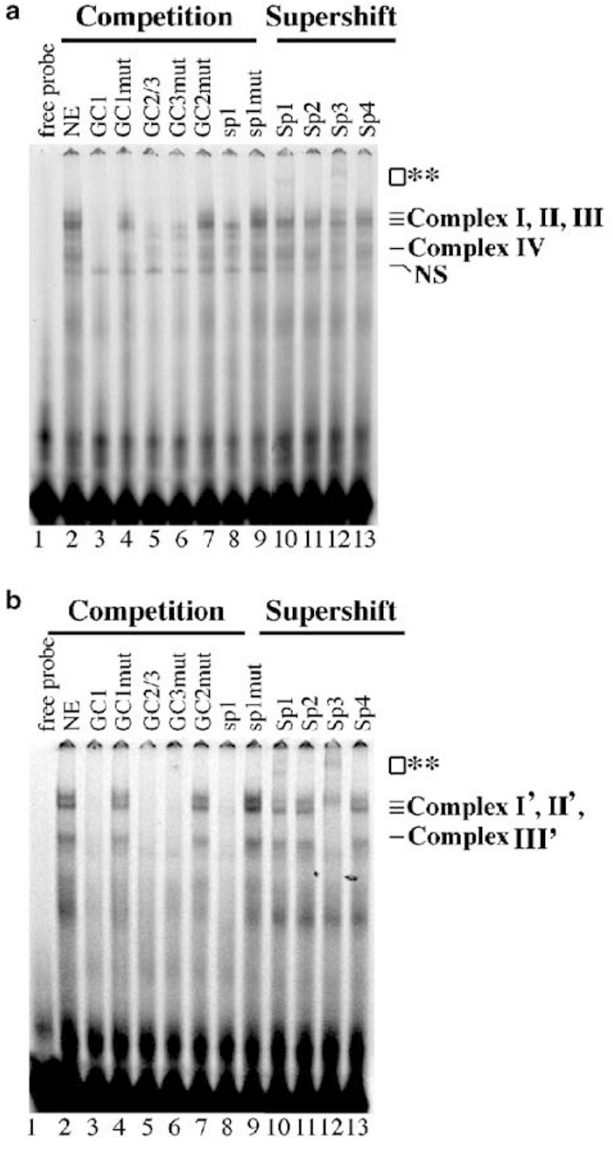

Figure 5 EMSA in NHP6 cells. (**a**) EMSA using GC1 probe and NE from NHP6 cells (P6). Two types of experiments, that is, competition and supershift, were performed. The unlabeled competition probes (either wild-type or mutant) and the supershifting antibodies used were indicated on the top. Distinct complexes were indicated on the right. Arrows, the supershifted bands. NE, nuclear extract; NS, nonspecific. (**b**) EMSA using GC2/GC3 probe. Legend is similar to that described in (**a**). In both (**a**) and (**b**), asterisks indicate the supershifted bands. Autoradiograms shown were representative of three to four repeat experiments with comparable results

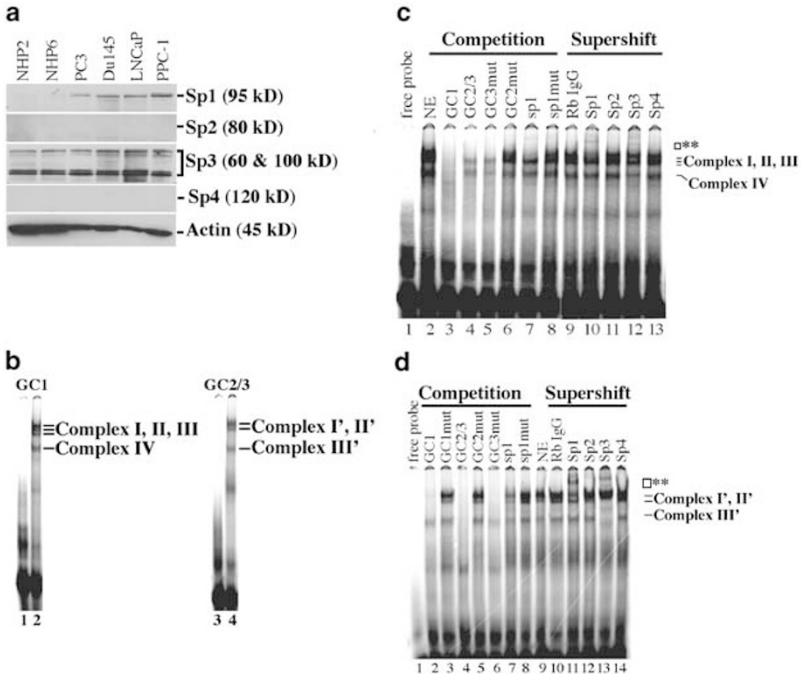

Figure 6 Overexpression of Sp1 and Sp3 proteins in PCa cells and EMSA in PPC1 cells. (**a**) PCa cells express higher levels of Sp1 and Sp3 proteins. Whole-cell lysates (80 μg/lane) from cells indicated were utilized for Western blotting of Sp1–Sp4 proteins. Both NHP2 and NHP6 cells were used at P6. (**b**) EMSA in PPC-1 cells using either GC1 probe (lane 2) or GC2/GC3 probe (lane 4). Lanes 1 and 3 were the NE alone. (**c** and **d**) EMSA competition and supershift assays in PPC-1 cells using either GC1 probe (**c**) or GC2/GC3 (**d**) probe. The legends are similar to those in Figure 5. Data shown in (**c**) and (**d**) were representative of three to four experiments with comparable results. RbIgG, non-immune rabbit IgG

Unlabeled, mutant GC1 probe, in which both GC box 1 and CACCC box were altered (Table 1), slightly reduced the binding of all four complexes, especially complex IV (Figure 5a, lane 4), suggesting that sequence(s) outside of the GC box 1/CACCC region may also help in the formation of these complexes. Unlabeled wild-type GC2/3 probe (Figure 5a, lane 5) as well as consensus Sp1 probe (Figure 5a, lane 8) but not mutant Sp1 probe, also showed strong competing effects, especially on complexes I and II. These results suggest that complexes I and II are formed mostly by transcription factors that bind to GC boxes. Surprisingly, unlabeled GC3 mutant (Figure 5a, lane 6), but not GC2 mutant (Figure 5a, lane 7) probe behaved just like the cold, wild-type GC2/3 probe and demonstrated strong competing effect on complexes I and II. As GC3 mutant retains intact GC box 2, whereas GC2 mutant retains intact GC box 3 (Table 1), these results suggest that the GC box 2 is more important. Supershift experiments revealed that the antibodies to Sp1 and Sp3 formed supershifted bands, whereas antibodies to Sp2 and Sp4 did not (Figure 5a, lanes 10–13). Sp1 appeared to exist in complexes I/II (Figure 5a, lane 10) whereas Sp3 in complex II (Figure 5a, lane 12).

When the GC2/GC3 probe (Table 1) was used, at least three complexes, that is, complex I′–III′, were observed (Figure 5b, lane 2). These complexes were all competed out with unlabeled, wild-type GC1, GC2/GC3, and Sp1 consensus oligonucleotides (Figure 5b, lanes 3, 5, and 8) but not with their corresponding mutants (Figure 5b, lanes 4, 7, and 9). Interestingly, just as observed in Figure 5a (lanes 6 and 7), only the GC3 mutant but not GC2 mutant competed out all three complexes (Figure 5b, lanes 6 and 7). Collectively, these data suggest that, likely, all three complexes are formed on GC box 2. Supershift experiments (Figure 5b, lanes 10–13) revealed that: (1) Sp1 existed in complex I′ whereas Sp3 in complexes II′ and III′ and (2) no supershifted bands were observed with antibodies to Sp2 and Sp4.

Taking advantage of the fact that cancer cells often overexpress Sp family proteins (Black *et al*., 2001), we also carried out EMSA experiments in PCa cells. As shown in Figure 6a, Sp1 was indeed expressed at much higher levels in PCa cells than in NHP cells. Sp1 was detected as a major $\sim$95 kDa protein with a minor slower-migrating band, which might represent the phosphorylated Sp1 (Jackson *et al*., 1990; Black *et al*., 2001). Sp3, which was detected as a doublet migrating at $\sim$60 and $\sim$100 kDa, was detected in all cell types but their levels, especially those of the $\sim$100 kDa doublet, were also slightly higher in PCa cells (Figure 6a). In contrast, Sp2 and Sp4 could not be detected in any cells (Figure 6a).

As in NHP6 cells (Figure 5), EMSA using GC1 probe detected four distinct complexes, whereas EMSA using GC2/GC3 probed at least three complexes in PPC-1 cells (Figure 6b) as well as in LNCaP, PC3, and Du145 cells (not shown). Competition and supershift experiments in PPC-1 cells using either GC1 probe (Figure 6c)

or GC2/GC3 probe (Figure 6d) revealed banding patterns essentially identical to those observed in NHP6 cells (compare with Figure 5a and b), except that the band intensities in PCa cells were stronger than in NHP6 cells. With GC1 probe, again four complexes were detected (Figure 6c, lane 2), which were all competed out by cold GC1 probe. As in NHP6 cells (Figure 5a), unlabeled wild-type GC2/GC3, GC3 mutant, and Sp1 oligonucleotides competed out primarily complexes I and II (Figure 6c, lanes 4, 5, and 7, respectively). Mutant Sp1 did not have any effect (Figure 6c, lane 8). Unlabeled GC2 mutant failed to show any competition effect (Figure 6c, lane 6). Supershift experiments also revealed banding patterns (Figure 6c) similar to those in NHP6 cells (Figure 5a). EMSA with GC2/GC3 probe revealed three complexes, I′, II′, and III′, which were competed out, to various degrees, by unlabeled wild-type GC1, GC2/3, and Sp1 oligonucleotides but not by their mutants (Figure 6d). As in NHP cells, GC3 mutant but not GC2 mutant showed strong competing effect (Figure 6d, lanes 5 and 6), and Sp1 primarily existed in complex I′ (Figure 6d, lane 10), whereas Sp3 in complexes II′ and III′ (Figure 6d, lane 13). A subtle difference consistently observed between PPC-1 and NHP6 cells was that the complex III′ was not effectively competed out in PPC-1 cells (compare Figure 6d and Figure 5b), perhaps due to higher levels of Sp3 (Figure 6a) and other transcription factors.

Androgen does not directly regulate 15-LOX2 gene expression

15-LOX2 is expressed in differentiated (i.e. luminal) prostate epithelial cells (Shappell *et al*., 1999; Tang *et al*., 2002), which are normally regulated by androgen through the AR. We wonder whether 15-LOX2 itself might be directly regulated by androgen. To test this possibility, we first carried out *in silico* analysis, using various tools and databases described earlier, of ~16 kb 15-LOX2 DNA sequence upstream of ATG (NM_001141). We did not identify any ARE that matched the consensus sequence GG(T/A)ACANNNTGTTCT (the underlined sequences are the inverted repeat) derived from 29 AREs, except a partially matched ARE with the sequence GGCACAGCTTGTGCC located at −1437 upstream of the TSS (see Figure 8, below). To determine whether this partially matched ARE or some other sequences might regulate 15-LOX2 gene expression, we cloned a ~4.0 kb 15-LOX2 promoter fragment from NHP6 cells using primers P2 and P10 (Table 1) that encompasses the partially matched ARE and cloned it into the pGL3-basic vector, that is, (−3985/+250)-luc. When transfected into AR-positive LNCaP cells, the −3985/+250 fragment showed increased promoter activity over the empty vector, and this increased promoter activity was not affected by androgen, dihydrotestesterone (DHT) (Figure 7a). As expected, the promoter activity of (−726/+80)-luc transfected into the LNCaP was not affected by DHT, either (Figure 7a), as there was no ARE in this region (Figure 1b). Of note, the −3985/+250 fragment showed a similar promoter activity to that of −726/+80 (Figure 7a), consistent with our earlier findings that the basal promoter activity was mostly located in the GC-rich proximal promoter region. When both promoter constructs were cotransfected with the AR into the AR-negative PPC-1 cells, their promoter activities were similarly not affected by DHT (not shown). In contrast, as a positive control, DHT induced the expression of exogenous 15-LOX2 controlled by androgen-responsive promoter (ARR_2PB) (Zhang *et al*., 2000) in LNCaP cells (Figure 7b, lane 4), which expressed AR but little endogenous 15-LOX2 (Figure 7c, lane 7). Cotransfection of AR further induced the expression of the 15-LOX2 transgene by DHT (Figure 7b, lane 6), although it also induced 15-LOX2 transgene expression in the absence of DHT (Figure 7b, lane 5). Note that the antibody detected a faint nonspecific band that migrated slightly slower than 15-LOX2 (Figure 7b). These results overall are consistent with the lack of authentic ARE in the 15-LOX2 promoter and suggest that: (1) the partially matched ARE does not represent a functional ARE and (2) 15-LOX2 does not appear to be directly regulated by androgen.

To further explore this point, we carried out several sets of experiments in early-passage NHP6 cells (Figure 7c–f). We took advantage of our recent observations that the NHP cells are of the basal cell origin and do not express 15-LOX2 at early passages, that is, passages 2 and 3 (Figure 7c, lane 3). They gradually acquire 15-LOX2 expression by passages 4–6 (Figure 7c, lane 2), and by passage 7 essentially 100% cells express high levels of 15-LOX2 (Bhatia *et al*., manuscript in preparation). This is the main reason that we performed all luciferase experiments in late passage (i.e. P5–7) NHP6 cells. As shown in Figure 7c (lanes 2 and 3), NHP6 cells at passage 6 expressed mRNAs for both 15-LOX2 and 15-LOX2sva/sv-b, whereas NHP6 cells at passage 3 expressed neither. Late-passage NHP6 cells also showed increased AR mRNA expression (Figure 7c, lanes 2 *vs* 3), although no AR protein could be detected (not shown). When the ARR_2PB-15-LOX2 construct was cotransfected with the AR plasmid into the early-passage NHP6 cells, DHT induced strong expression of the mRNA (Figure 7c, lane 6) and protein expression of the 15-LOX2 transgene in AR-positive cells (Figure 7d). A counting of ~300 AR-transfected NHP6 (passage 3) cells revealed 100% positivity for 15-LOX2 staining. AR transfection in the absence of DHT did not result in increased 15-LOX2 mRNA or protein expression (not shown). In contrast, transfection of AR into the young NHP6 cells did not result in DHT-inducible expression of endogenous 15-LOX2 (Figure 7c, lane 5; and data not shown). Interestingly, DHT alone slightly upregulated endogenous AR mRNA levels, but the 15-LOX2 mRNA was not induced (Figure 7c, lane 3 *vs* lane 4). Similarly, the low, basal-level promoter activities of (−726/+80)-luc or the (−3985/+250)-luc transfected into passage 3 NHP6 cells were not affected by the cotransfected AR in the presence or absence of DHT

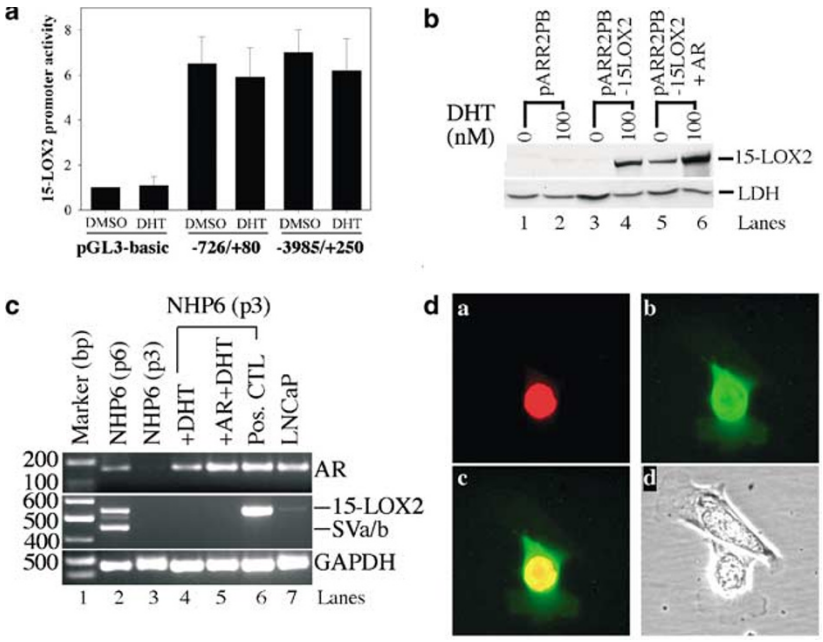

Figure 7 Androgen does not directly regulate 15-LOX2 gene expression. (**a**) Lack of 15-LOX2 promoter activity in LNCaP cells stimulated with DHT. LNCaP cells cultured in charcoal-stripped FBS (48 h) were transfected with the vectors indicated. After 24 h, cells were stimulated with DMSO or DHT (100 nM). Cells were harvested for luciferase activity assays 24 h later and the results were expressed as the relative 15-LOX2 promoter activity. Data represent the mean ± s.e.m. ($n = 5$). (**b**) DHT induces 15-LOX2 transgene expression. LNCaP cells were similarly treated with charcoal-stripped serum as in (**a**), except that cells were transfected with either the empty vector (lanes 1 and 2), pARR$_2$PB-15LOX2 (lanes 3 and 4) or cotransfected with pARR$_2$PB-15LOX2 and AR (lanes 5 and 6). Cells were harvested 24 h after DHT treatment and used for Western blotting of 15-LOX2 (upper panel) or lactate dehydrogenase (LDH; lower panel). (**c**) DHT/AR do not induce endogenous 15-LOX2 mRNA expression. Young (P3) NHP6 cells were either untreated (lane 3) or directly treated with DHT (100 nM) for 72 h (lane 4), or first transfected with AR for 48 h and then stimulated with DHT for 24 h (lane 5), or first transfected with pARR$_2$PB-15LOX2 for 48 h and then stimulated with DHT for 24 h (lane 6, positive control). At the end, cells were harvested and total RNA prepared for RT–PCR of AR (sense: 5′-GAAGCCATTGAGCCAGGTGT-3′; antisense, 5′-TCGTCCACGTGTAAGTT GCG-3′), 15-LOX2, or GAPDH. Late-passage NHP6 cells (lane 2) and LNCaP (lane 7) were used as controls for 15-LOX2 and AR, respectively. (**d**) DHT/AR induce exogenous 15-LOX2 protein expression. NHP6 (P3) cells were transfected with AR and pARR2PB-15-LOX2. After 48 h, cells were stimulated with DHT for 24 h and then dual labeled for AR (**a**) and 15-LOX2 (**b**). Micrograph in (**c**) represents overlays of images a and b and micrograph in (**d**) is the phase-contrast image. The images shown are representative of ~300 AR-transfected cells analysed, which were all positive for 15-LOX2. Original magnification: ×400

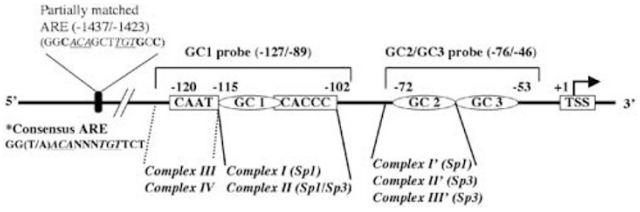

Figure 8 A model depicting the two GC-rich regions and potential transcription factor complexes formed on these regions in the 15-LOX2 gene promoter. Consensus ARE sequence and a partially matched ARE are also shown. TSS, transcription start site

(not shown). These observations in young NHP6 cells thus confirm the earlier conclusion that androgen does not directly or is not sufficient to regulate the 15-LOX2 gene expression.

Discussion

The present study addresses how 15-LOX2 expression may be regulated in NHP cells. The results suggest that: (1) several Sp1 sites in the proximal promoter region are important *cis* elements regulating the 15-LOX2 promoter activity; (2) Sp1 and Sp3 are two major GC-binding *trans* factors regulating 15-LOX2 gene expression with Sp1 being a positive and Sp3 a negative regulator; (3) multiple complexes form on the GC-rich regions and Sp1 and Sp3 exist in some of these complexes; and (4) androgen does not directly regulate 15-LOX2 gene expression.

Similar to 15-LOX1 and 12-LOX promoters (Kritzik *et al*., 1997; Kelavkar *et al*., 1998), the 15-LOX2 promoter is TATAA less, consistent with its constitutive expression in adult NHP cells *in vivo*. Sp1 sites including GC boxes and CACCC boxes (also called GT boxes) have been shown to be responsible for recruiting TATA-binding protein and fixing the TSS on TATAA-less promoters (Strachan and Read, 2000; Black *et al*., 2001; Levine and Tijan, 2003). *In silico* analysis identifies four potential Sp1 sites in the proximal promoter region of 15-LOX2 gene. Initial evidence that these Sp1 sites may play a role in regulating 15-LOX2 comes from deletion analysis. More definitive evidence comes from site-specific mutagenesis, which suggests that

GC box 2 and/or 3 may be more important than the GC box 1/CACCC box, as well as from EMSA competition experiments, which suggest that GC box 2 is the primary Sp1 site that multiple transcription factors bind.

Interestingly, promoter deletion experiments suggest that the sequence between −726 and −163 might contain a *cis* element(s) that negatively regulate the 15-LOX2 promoter activity. Furthermore, *in silico* analysis has also revealed multiple other perfectly matched transcription factor-binding sites. Many of these *cis* elements, together with their cognate transcription factors, have been implicated in physiological processes such as cell fate determination, proliferation, and differentiation as well as in pathological conditions including tumorigenesis. Conceivably, these elements may participate in the regulation of tissue-specific, differentiation-related, or stage-specific expression of 15-LOX2.

What *trans* factors bind to the Sp1 sites to regulate 15-LOX2 promoter activity? The Sp family proteins, that is, Sp1–Sp4, are the natural candidates as they primarily bind to GC-rich sequences. In the Sp subfamily, Sp1 and Sp3 are ubiquitously expressed and Sp4 is expressed mostly in central nervous and reproductive systems, whereas the expression pattern of Sp2 is largely unknown (Black *et al*., 2001). Sp1, Sp3, and Sp4 share a high affinity for GC boxes bearing consensus sequence GGGGCGGGG, while Sp2 only weakly binds to GT boxes (Kingsley and Winoto, 1992). NHP cells express low levels of Sp1, easily detectable Sp3, and undetectable Sp2 and Sp4 proteins, suggesting that Sp1 and Sp3 may be involved in regulating 15-LOX2 gene expression through binding to GC-rich Sp1 sites. Indeed, using SL2 cells that lack all endogenous Sp proteins, we demonstrate that Sp1 *can* activate 15-LOX2 promoter. Three additional lines of evidence establish that the Sp1 protein *is required* for 15-LOX2 gene expression. First, DN-Sp1 inhibits the 15-LOX2 promoter activity in transfected NHP cells. Second, the Sp1 inhibitor, MMA also inhibits 15-LOX2 promoter activity. Third, most importantly, MMA, in a time- and dose-dependent manner, suppresses endogenous 15-LOX2 mRNA expression.

In the Sp subfamily, Sp3 is the only protein that can either positively or negatively modulate the Sp1-dependent gene expression. Our subsequent experiments demonstrate that Sp3 dose dependently inhibits the Sp1-activated 15-LOX2 promoter activity in SL2 cells. Furthermore, Sp3 inhibits the 15-LOX2 promoter activity as well as endogenous 15-LOX2 expression in transfected NHP cells. Together, these results establish that Sp1 and Sp3 are biologically relevant and essential regulators of the 15-LOX2 gene expression with Sp1 being an activator and Sp3 an inhibitor via antagonizing Sp1 activity.

EMSA experiments demonstrate that multiple complexes form at the two GC-rich regions and both Sp1 and Sp3 exist in some of these complexes (Figure 8). Complex I and II are competed out by consensus Sp1 and GC2/GC3 oligonucleotides, suggesting that these two complexes are mainly formed of GC-binding proteins. Indeed, supershift experiments reveal the presence of Sp1 in complexes I and II and Sp3 mainly in complex II. What is the molecular nature of complexes III and IV that are competed out by unlabeled GC1 oligonucleotides but not significantly by Sp1 and GC2/GC3 oligonucleotides? Likely candidates may include those transcription factors (e.g. NF-1, NF-Y, and C/EBP) that normally bind to the CAAT box (Strachan and Read, 2000). In fact, NF-Y and Sp1 have recently been shown to cooperate in regulating the expression of several genes (Yamada *et al*., 2000; Liang *et al*., 2001).

When EMSA experiments are carried out using the GC2/GC3 probe, three major complexes are observed, which can be competed out, to different degrees, by GC1, GC2/3, or Sp1 oligonucleotides. Supershift experiments reveal that Sp1 exists in complex I′ and Sp3 in complexes II′ and III′ (Figure 8). Much to our surprise, the unlabeled GC3 mutant probe, but not mutant GC2 probe, behaves exactly like the wild-type GC2/3 oligonucleotide in competing out these complexes. The contrasting effects of GC2 and GC3 mutants are also observed in competition experiments using GC1 probe. These observations strongly suggest that the GC box 2 is *the* major binding site for Sp1, Sp3, and perhaps other related proteins (Figure 8), consistent with the site-specific mutagenesis data.

That 15-LOX2 is expressed exclusively in the androgen-sensitive luminal cells *in vivo* promoted us to address whether 15-LOX2 might be directly regulated by androgen. Several pieces of evidence argue against this possibility. First, there is no authentic ARE in the 16 kb upstream sequence of 15-LOX2 promoter. Second, although there is a partially matched ARE at ∼−1.4 kb kb location (Figure 8), a 4 kb fragment containing this partially matched ARE does not possess DHT-inducible promoter activity. This result is not surprising as this partially matched ARE has three conspicuous base differences (bold in Figure 8) from the ARE consensus. Third, androgen and AR do not induce endogenous 15-LOX2 gene expression in young NHP cells, although they can readily activate a 15-LOX2 transgene driven by ARR_2PB promoter. Fourth, we have observed that 15-LOX2 expression is cell autonomously upregulated in NHP cells cultured in the absence of androgen or serum, and that 15-LOX2 gene expression in human prostates comes after PSA expression, probably as a consequence of prostate differentiation, and appears to be involved in replicative cell senescence (Bhatia *et al*., manuscript in preparation). Finally, that androgen does not directly regulate 15-LOX2 gene expression is also consistent with clinical data that PCa tend to have heightened AR activity leading to increased PSA production, but they downregulate or lose 15-LOX2 expression. It should be noted that, although our data do not indicate a direct regulation of 15-LOX2 by androgen, it is still possible that androgen may indirectly regulate 15-LOX2 expression or its function.

Materials and methods

Cells and reagents

NHP1–NHP6 cells were cultured in serum- and androgen-free, PrEBM medium (Clonetics) supplemented with insulin, EGF, hydrocortisone, bovine pituitary extract, and cholera toxin, and used at passages 3–7 (Chopra *et al*., 1996; Tang *et al*., 1998, 2002; Bhatia *et al*., 2003). PCa cell lines, PPC-1, PC3, LNCaP, and Du145, were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics.

Luciferase reporter plasmid, pGL3-basic, was purchased from Promega. Rabbit polyclonal anti-15-LOX2 antibody was described previously (Tang *et al*., 2002). Rabbit polyclonal antibodies to Sp1–Sp4 were obtained from Santa Cruz. Liposome FuGene 6 was bought from Roche. All chemicals were bought from Sigma unless specified otherwise.

Cloning of immediate upstream 15-LOX2 promoter region and mapping of transcription start site (TSS)

The $\sim$1.2 kb fragment upstream of ATG was cloned using primers P3 and P8 (Table 1) and genomic DNA prepared from several different NHP cell strains. The primer sequences were based on the genomic sequences deposited in the GenBank (Accession numbers AJ305028–AJ305031). A primer extension analysis was performed to determine the TSS of the 15-LOX2 gene using standard protocol (Sambrook and Russell, 2001). Briefly, the reverse primer P8 (Table 1) was labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The labeled primer was then annealed (65°C $\times$ 90 min) to 0.5 μg mRNA purified from NHP2 cells and extended (42°C $\times$ 60 min) with SuperScript II reverse transcriptase (Gibco). *In vitro* transcribed cDNA fragments were electrophoresed on denaturing polyacrylamide gels containing 8 M urea in parallel to a sequencing reaction (using the 1.2 kb fragment as the template) as a size marker.

Generation of the 15-LOX2 promoter deletion mutants and analysis of promoter activity

The 1.2 kb 15-LOX2 promoter (i.e. $-1116/+80$) fragment was used as the template to generate a series of truncated deletion mutants using PCR primers indicated in Table 1. An *Xho*I site (CTCGAG) was incorporated into the 5′-ends of the PCR primers and the resultant PCR fragments were first cloned into the pCRII-TOPO cloning vector and subsequently cloned into pGL3-basic. The orientation and sequence of each insert were confirmed by restriction digestion and sequencing. For luciferase reporter assays, NHP or PCa cells grown in six-well culture plates ($4–5 \times 10^4$ cells/well) were transiently transfected, in triplicate, with either empty vector (i.e. pGL3-basic) or various luc constructs (2 μg plasmid/well) together with the β-gal plasmid (0.125 μg plasmid/well) to normalize the transfection efficiency. One set of the triplicate wells was mock-transfected. Cells were harvested 48 h after transfection by scraping into the 1 $\times$ lysis buffer (Promega). Lysates containing equal amounts of protein were assayed for luciferase activity using luciferase assay kit (Promega). β-Galactosidase activity was measured using Tropix Galacto-Light Plus assay system. After subtracting the baseline values, the relative luciferase unit (RLU) activity was determined as the ratio of luciferase/β-gal activities. The relative promoter activities were expressed as either RLU or relative values (i.e. % or fold increase).

Site-specific mutagenesis of Sp1 sites

Site-specific mutagenesis was performed with the QuickChange Site-Specific Mutagenesis system (Stratagene) using the $(-163/+80)$-luc as template with the PCR primers and individual mutations indicated in Table 1. Double mutations of GC box 1 and GC box 2 or GC box 1 and GC box 3 were made using the $(-163/+80)$-luc containing mutated GC box 1 as the template to further mutate GC box 2 or 3. The successfully mutated sequences were confirmed by restriction digestion and sequencing.

Involvement of Sp1 family proteins in regulating 15-LOX2 gene expression D. melanogaster

Schneider SL2 cells were cotransfected with $(-726/+80)$-luc or $(-102/+80)$-luc and an Sp1 or Sp3 expression vector driven by *Drosophila* actin promoter (i.e. pPacSp1 or pPacSp3; Kumar and Butler, 1997). The Sp1 plasmid encodes amino acids 83–758 of human Sp1 under the control of the *Drosophila* actin 5C promoter. In another experiment, NHP6 cells were cotransfected with several luc constructs together with a dominant-negative (DN) Sp1 expression vector (pEBG-Sp1; Ptersohn and Thiel, 1996; Grinstein *et al*., 2002) or a mammalian Sp3 expression vector (Kumar and Butler, 1997). Alternatively, NHP6 cells were treated with an Sp1-specific chemical inhibitor mithramycin A (MMA; Kaluz *et al*., 2003) at 200 nM at the time of transfection of promoter constructs. All transfection experiments were carried out as described above. Finally, NHP6 cells were transfected with DN-Sp1 or Sp3 for various time periods or treated with various doses of MMA for different time periods followed by RNA isolation and RT–PCR analysis using 15-LOX2-specific primers C-D (Tang *et al*., 2002; Bhatia *et al*., 2003).

EMSA

Nuclear extract (NE) was prepared from NHP2, NHP6, or various PCa cells (Bhatia *et al*., 2003). For EMSA, three different double-stranded oligonucleotide probes (Table 1) were used. A probe containing consensus Sp1 site and the corresponding mutant probe were purchased from Santa Cruz. The second probe (i.e. GC1 probe) contained wild-type or mutant overlapping GC box 1 and CACCC box. The third probe (i.e. GC2/GC3 or GC2/3 probe) was derived from the proximal promoter of 15-LOX2 that contained GC box 2 and 3 (Table 1). Two mutant probes, that is, GC2 or GC3 mutant, were designed for the GC2/GC3 probe (Table 1). In all, 10 pmol of DNA fragment were end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. For EMSA, 6 μg NE was incubated in a 20 μl reaction containing 100 000 c.p.m. probe, 20 mM HEPES (pH 7.9), 5% glycerol, 2 mM $MgCl_2$, 0.2 mM EDTA, 50 mM KCl, and 0.5 mg/ml BSA. After 30 min incubation at room temperature, the DNA–protein complexes were separated on 5% polyacrylamide gel containing 5% glycerol and 0.5 $\times$ TBE. Competition assays used 100 $\times$ cold unlabeled probes. Supershift assays were performed using the antibodies (1 μg) to Sp1–Sp4.

Western blotting

Western blotting was performed using whole-cell lysates as described previously (Tang *et al*., 2002).

Potential effect of androgen on 15-LOX2 gene expression

Several sets of experiments including *in silico* promoter analysis, cloning, and characterization of a $\sim$4.0 kb. 15-LOX2 promoter sequence, luciferase transfection, RT–PCR, and immunostaining of 15-LOX2 upon transfection of exogenous AR were carried out to examine the potential role

of androgen in 15-LOX2 gene expression (detailed in Results). As a positive control, we cloned 15-LOX2 cDNA under the control of an ARR_2PB (Zhang *et al*., 2000). The vector, $pARR_2PB$-15LOX2, or various 15-LOX2 promoter constructs, was transfected into AR-positive LNCaP cells, which had been cultured in charcoal-stripped serum for 48 h. At 48 h after transfection, DHT was added to the culture medium at a final concentration of 100 nM. Cells were harvested, 24 h after DHT stimulation, for either luciferase activity measurement or Western blotting for 15-LOX2. In other experiments, these vectors were transfected into AR-negative cells by cotransfecting with a human AR expression plasmid and then RT–PCR (for 15-LOX2 expression), immmunofluorescence staining (for both 15-LOX2 and AR), or luciferase assays (for 15-LOX2 promoter activity) were carried out.

Abbreviations

AA, arachidonic acid; AR, androgen receptor; DN, dominant negative; EMSA, electrophoretic mobility shift assay; LOX, lipoxygenase; 15-LOX2, 15-lipoxygenase 2; 15-LOX2sv-a/b/c, 15-LOX2 splice variant a, b or c; MMA, mithramycin A; NE, nuclear extract; NHP, normal human prostate epithelial cells; PCa, prostate cancer; RLU, relative luciferase unit; TSS, transcription start site.

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