Nkx3.1 binds and negatively regulates the transcriptional activity of Sp-family members in prostate-derived cells

Steven O. SIMMONS and Jonathan M. HOROWITZ[1]

Graduate Program in Toxicology and Department of Molecular Biomedical Sciences, College of Veterinary Medicine, North Carolina State University, 4700 Hillsborough Street, Raleigh, NC 27606, U.S.A.

Nkx3.1 is a homeodomain-containing transcription factor that is expressed early in the development of the prostate gland and is believed to play an important role in the differentiation of prostatic epithelia. Loss of Nkx3.1 protein expression is often an early event in prostate tumorigenesis, and the abundance of Nkx3.1-negative epithelial cells increases with disease progression. In a number of systems, homeodomain proteins collaborate with zinc-finger-containing transcription factors to bind and regulate target genes. In the present paper, we report that Nkx3.1 collaborates with Sp-family members in the regulation of PSA (prostate-specific antigen) in prostate-derived cells. Nkx3.1 forms protein complexes with Sp proteins that are dependent on their respective DNA-binding domains and an N-terminal segment of Nkx3.1, and Nkx3.1 negatively regulates Sp-mediated transcription via Trichostatin A-sensitive and -insensitive mechanisms. A distal 1000 bp portion of the PSA promoter is required for transrepression by Nkx3.1, although Nkx3.1 DNA-binding activity is itself not required. We conclude that Nkx3.1 negatively regulates Sp-mediated transcription via the tethering of histone deacetylases and/or by inhibiting the association of Sp proteins with co-activators.

Key words: histone deacetylase, Nkx3.1, prostate, prostate-specific antigen, Sp protein, transrepression.

INTRODUCTION

Nkx3.1, a human orthologue of the *Drosophila Hox* gene *bagpipe* (*NK-3*), is expressed principally within the prostate gland [1,2]. *Nkx3.1* is expressed in the developing mouse rostral urogenital sinus as early as 15.5 days post-coitum and in all ductal derivatives in adult animals, suggesting that *Nkx3.1* is partly responsible for the development and differentiation of prostate tissue. *Nkx3.1* has also been implicated as a prostate-tumour-suppressor gene, as its loss is associated with prostate tumorigenesis in both humans and rodents. *Nkx3.1* maps to a region of the human genome, 8p21, which is subject to deletion in up to 75 % of human prostate cancers [3]. Expression of Nkx3.1 protein is lost in 20 % of patients with PIN (prostatic intraepithelial neoplasia), an early prostatic lesion, and the frequency of Nkx3.1-negative epithelial cells increases with disease progression [4]. For example, Nkx3.1 protein expression is absent from 34 % of hormone refractory prostate carcinomas and 78 % of metastatic prostate specimens. The maintenance of Nkx3.1 protein in at least some prostate tumours has led to speculation that haploinsufficiency may deregulate the proliferative potential of prostatic epithelia, and this proposition has been sustained by analyses of *Nkx3.1*-knockout animals. Mice hemi- or nulli-zygous for Nkx3.1 exhibit prostatic epithelial hyperplasia and dysplasia that increases in severity with age, defects in prostate ductal branching and a reduction in the overall size of the ventral prostate [1,5,6]. Animals that carry a single conditional allele of *Nkx3.1* have also been shown to develop pre-invasive lesions that are phenotypically similar to human PIN. Interestingly, PIN-like lesions that develop in these mice are devoid of Nkx3.1 protein, indicating that the wild-type allele has been lost or silenced [7]. Although *Nkx3.1*-deficient animals have been followed for years, frank cancer has not as yet been observed suggesting that loss of *Nkx3.1* is not sufficient to induce prostate tumorigenesis. Offspring from intercrosses between mice hemi- or nulli-zygous for *Nkx3.1* and the *Pten* (phosphatase and tensin homologue deleted on chromosome 10) tumour-suppressor gene exhibit greatly increased frequencies and severity of PIN, indicating that these genes co-operate in the suppression of prostate tumorigenesis [8].

Although it is widely believed that homeodomain proteins play an essential role in the specification of the body plan, it remains largely unclear how these transcriptional regulators promote development and differentiation. Most homeodomain proteins recognize similar consensus DNA sites containing the core motif 5′-TAAT/G-3′, and, as such, it is likely that they direct the expression of tissue-specific genes via combinatorial interactions with additional transcription factors. Using a DNA-binding site selection strategy, Steadman et al. [9] defined the Nkx3.1-consensus-binding site to be 5′-TAAGTA/G-3′ and showed that expression of Nkx3.1 reduced the transcriptional potential of a reporter gene carrying multimers of this sequence. Although such artificial reporter constructs are unlikely to mimic Nkx3.1 target genes precisely, these data suggested that Nkx3.1 may function as a transcriptional repressor in prostate cells. Based on the expression of *Nkx3.1* in vascular mesoderm, Carson et al. [10] identified the smooth muscle γ-actin gene as being a target of Nkx3.1 function. These workers showed that Nkx3.1 formed a physical complex with SRF (serum-response factor) and that each of these proteins bound adjacent sites within a proximal portion of the γ-actin promoter. Interactions between these proteins were shown to occur via their respective DNA-binding domains, and co-expression of Nkx3.1 and SRF led to synergistic transactivation of γ-actin transcription. In contrast with these results, Chen et al. [11] reported that Nkx3.1 forms a physical complex with a PDEF (prostate-derived Ets factor) and that Nkx3.1 blocked the capacity of PDEF to activate the PSA

Abbreviations used: btd, buttonhead; CAT, chloramphenicol acetyltransferase; ems, empty spiracles; GST, glutathione S-transferase; HDAC, histone deacetylase; PDEF, prostate-derived Ets factor; PIN, prostatic intraepithelial neoplasia; PSA, prostate-specific antigen; SRF, serum-response factor; TSA, Trichostatin A.

[1] To whom correspondence should be addressed (email jon_horowitz@ncsu.edu).

(prostate-specific antigen) promoter in human prostate cells. Physical interactions between PDEF and Nkx3.1 were shown later to require portions of their respective DNA-binding domains and sequences immediately downstream [12]. Additional studies appear to corroborate that Nkx3.1 may function as a transcriptional repressor as NK-3 has been shown to down-regulate its own transcription and to associate with Groucho and HDAC (histone deacetylase) 1, proteins shown previously to repress gene expression [13,14]. In summary, Nkx3.1 has been shown to function as both an activator and a repressor of transcription, although few target genes have been identified and its precise role in the specification of prostate development is far from clear.

A number of disparate observations suggest that homeodomain proteins may regulate gene expression at least in part via their physical and functional interaction with zinc-finger-containing transcription factors. It is well established that the yeast mating-type switching gene *HO* is regulated by SWI5, a transcription factor with a three zinc finger DNA-binding domain, and PHO2, a homeodomain protein [15]. A *Drosophila* homeodomain protein, termed ems (empty spiracles), specifies head segmentation and is required for proper differentiation of the anterior portion of the fly. These functions of ems have been shown to be dependent on the activities of a zinc-finger transcription factor termed btd (buttonhead), a probable orthologue of the mammalian Sp-family of transcription factors [16]. Indeed, many functions of btd can be supplanted by the expression of human Sp1. Analogous collaborations between homeodomain- and zinc-finger-containing transcription factors also appear to occur in mammals, as two recent reports have documented physical and functional interactions between Sp proteins and members of the Abd-B subfamily of *Hox* genes, as well as Crx, a homeodomain factor required for photoreceptor-specific gene expression [17,18]. Analyses of knockout mice appear to corroborate these findings as nullizygosity for Sp1, Sp3 or Sp4 can lead to global, as well as tissue-specific, developmental abnormalities [19–23]. Finally, placental-specific gene expression in humans has been shown to be dependent on physical and functional interactions between AP-2γ, a zinc-finger protein, and Dlx-3, a homeodomain protein and orthologue of the *Drosophila Distal-less* gene [24] To determine whether combinatorial interactions between Nkx3.1 and one or more Sp-family members play a role in the regulation of transcription in prostate-derived cells, we undertook a series of biochemical, molecular genetic and functional studies using the PSA promoter as a target gene. In the present paper, we report that: (i) a subset of Sp-family members stimulate PSA transcription in prostate-derived cells, (ii) co-expression of human or mouse Nkx3.1 negatively-regulates Sp-mediated transcription, (iii) Nkx3.1 antagonizes transactivation by Sp proteins via TSA (Trichostatin A)-sensitive and -insensitive mechanisms, (iv) Nkx3.1 and Sp proteins form specific protein complexes *in vitro* and *in vivo*, (v) the DNA-binding domains of Sp proteins are necessary and sufficient for the formation of protein complexes with Nkx3.1, (vi) the Nkx3.1 N-terminus and homeodomain carry binding sites for Sp-family members, (vii) portions of Nkx3.1 that bind Sp proteins are sufficient to block Sp-mediated transcription, (viii) Nkx3.1 DNA-binding activity is not required to antagonize Sp-mediated transcription, and (ix) a distal portion of the PSA promoter is required for Nkx3.1 to negatively regulate Sp-mediated transcription in prostate-derived cells. We conclude that Nkx3.1 negatively regulates Sp-mediated transcription, at least in part, via interactions with HDACs. Furthermore, we speculate that functional interactions between Nkx3.1 and Sp proteins may play an important role in prostate cell proliferation and differentiation.

MATERIALS AND METHODS

Cell culture

COS-1, LNCaP, PC-3 and DU145 cells were obtained from the Duke Comprehensive Cancer Center Cell Culture Facility (Duke University Medical Center, Durham, NC, U.S.A.). Cells were cultured in Dulbecco's modified Eagle's medium or RPMI 1640 (GIBCO) supplemented with 10 % (v/v) heat-inactivated foetal bovine serum and 50 μg/ml Pipracil in humidified incubators under 5 % CO_2. Sf9 insect cells were obtained from Invitrogen and were cultured in spinner flasks at 27 °C in Grace's insect medium supplemented with 10 % (v/v) heat-inactivated foetal bovine serum, 0.1 % (v/v) Pluronic F-68 and 10 μg/ml gentamicin.

Expression and reporter plasmids

The construction and characterization of pCMV4-Sp1/flu, pBSK-Sp1/flu, pCMV4-Sp3/flu, pBSK-Sp3/flu, pCMV4-Sp2/flu and pBSK-Sp2/flu has been described previously [25–27]. An Sp4 expression construct, pCMV4-Sp4/flu, carrying an N-terminal ten-amino-acid epitope from influenza virus haemagglutinin was prepared using PCR, a human Sp4 cDNA as template (a gift from Dr Richard Tsika, University of Missouri-Columbia, Columbia, MO, U.S.A.) and the primers, 5′-CAGATCTATGTATCCTTACGATGTGCCAGACTACGCTTCAGCAAAGATGAGCGATCAGAAGAAGGAGGAG-3′ and 5′-GGTCAGAATTCTTCCATGTTGGTTGAAACATTGGG-3′. These and all other oligonucleotides used in the present study were obtained from Invitrogen. The resulting PCR product was cloned in pCR-Blunt II-TOPO (Invitrogen) to generate TOPO-Sp4/flu and was then transferred to pCMV4 to generate pCMV4-Sp4/flu. An analogous Sp5 expression construct, pCMV4-Sp5/flu, carrying an N-terminal epitope tag was prepared as above, except for the substitution of the primers, 5′-CAGATCTATGTATCCTTACGATGTGCCAGACTACGCTTCAGCAAAGATGAGCGATCAGAAGAAGGAGGAG-3′ and 5′-CAGATCTTCATAGGTCCCGCGGATTCTCCCGCTTC-3′. The murine Sp5 cDNA employed as template was obtained from Dr Rosa S. P. Beddington (National Institute for Medical Research, London, U.K.). TOPO-Sp2ΔZn, carrying the transactivation domain of Sp2, was prepared by PCR using pBSK-Sp2/flu as template and the primers, 5′-GGGCCACCATGAGCGATCCACAGACCAGCATGGCTGCC-3′ and 5′-GGGTTAAACGTGCTTCTTCTTGCCCTGCTCTCC-3′. The resulting PCR product was cloned in pCR-Blunt II-TOPO, generating TOPO-Sp2ΔZn. A murine Nkx3.1 cDNA was a gift from Dr Michael M. Shen (Center for Advanced Biotechnology and Medicine, Piscataway, NJ, U.S.A.) and has been described in [2]. A mammalian expression vector (pcDNA3-hNkx3.1) carrying a human Nkx3.1 cDNA was a gift from Dr Charles J. Bieberich (University of Maryland-Baltimore County, Baltimore, MD, U.S.A.) and has been described in [11].

A GST (glutathione S-transferase)-fusion construct carrying the zinc-finger domain of Sp2, pGEX1N-Sp2Zn, was prepared by PCR using pBSK-Sp2/flu as template and the primers, 5′-GGGGGATCCCATGTGCCACATCCCCGACTGTGGCAAGACGTTCCG-3′ and 5′-GGGGAATTCTTACAAGTTCTTCGTGACCAGGTGGG-3′. The resulting PCR product was cleaved with BamHI and EcoRI and was cloned in pGEX1N (Amersham Biosciences). A GST-fusion construct, pGEX1N-mNkx3.1 was prepared by PCR using a murine Nkx3.1 cDNA (pcDNA3-mNkx3.1) as template and the primers, 5′-GGGGGATCCCATGCTTAGGGTAGCGGAGCCC-3′ and 5′-GGGGATTCCCTACCAGAAAGATGGATGCGAGCTG-3′. The resulting PCR product was cleaved with BamHI and EcoRI and was then cloned

in pGEX1N. Partial GST–mNkx3.1 fusion expression constructs were prepared using pcDNA3-mNkx3.1 as template and the following PCR primer pairs: pGEX(1–87), 5′-GGGGGATCCCATGCTTAGGGTAGCGGAG-3′ and 5′-GGGGAATTCTTAGGGGCTATGCCGGATACTTGG-3′; pGEX(1–128), 5′-GGGGGATCCCATGCTTAGGGTAGCGGAG-3′ and 5′-GGGGAATTCTTAGGAGCGCTTCTGTGGCTGCTTGG-3′; pGEX(1–216), 5′-GGGGGATCCCATGCTTAGGGTAGCGGAG-3′ and 5′-GGGGAATTCTTACACGGAGACCAAGGAGGTACTGGGCAGG-3′; and pGEX(129–216), 5′-GGGGGATCCCATGCGGGCCGCCTTCTCTCACAC-3′ and 5′-GGGGAATTCTTACACGGAGACCAAGGAGGTACTGGGCAGG-3′. The resulting PCR products were cleaved with BamHI and EcoRI and were cloned in pGEX1N. pGEX-FSH15 has been described in [28].

The *Renilla* luciferase fusion vectors pcDNA3.1-NhRL and pcDNA3.1-ChRL were a gift from Dr Sanjiv S. Gambhir (University of California-Los Angeles, Los Angeles, CA, U.S.A.) and are derivatives of vectors that have been described in [29]. pNhRL-Zn carries the zinc fingers of Sp2 fused downstream of the N-terminal 229 amino acids of *Renilla* luciferase and was prepared by PCR using pBSK-Sp2/flu as template and the primers, 5′-GGGGGATCCGGAGGAGGTGGTTCAGGAGGTGGAGGTAGCTGCCACATCCCCGACTGTGGCAAGACGTTCCG-3′ and 5′-GGGCTCGAGTTACAAGTTCTTCGTGACCAGGTGGG-3′. The resulting PCR product was cleaved with BamHI and XhoI and was cloned in pcDNA3.1-NhRL. pHD-ChRL carries the homeodomain of human Nkx3.1 fused upstream of the C-terminal 82 amino acids of *Renilla* luciferase and was prepared using pcDNA-hNkx3.1 as the template and the primers, 5′-GGGGCTAGCATGCTCAGGGTTCCGGAGCCG-3′ and 5′-GGGGGATCCCCAAAAAGCTGGGCTCCAGCTGC-3′. The resulting PCR product was cleaved with NheI and BamHI and was cloned in pcDNA3.1-ChRL. pChRL-HD carries the homeodomain of human Nkx3.1 fused downstream of the C-terminal 82 amino acids of *Renilla* luciferase and was prepared in two steps. An initial round of PCR was performed using pcDNA-hNkx3.1 as template and the primers, 5′-GGGGGATCCGGAGGAGGTGGTTCAGGAGGTGGAGGTAGCATGCTCAGGGTTCCGGAGCCG-3′ and 5′-GGGCTCGAGTTACCAAAAAGCTGGGCTCCAGCTGC-3′. The resulting PCR product was cleaved with BamHI and XhoI and was cloned in pcDNA3.1, generating pcDNA3.1-NkxHD. A second round of PCR was performed using pcDNA3.1-ChRL as the template and the primers, 5-GGGGCTACGATGAAGCCCGACGTCGTCCAGATTGTC-3′ and 5-GGGGGATCCCTGCTCGTTCTTCAGCACGCG-3′. The resulting PCR product was cleaved with NheI and BamHI and was cloned in pcDNA3.1-NkxHD such that the Nkx3.1 homeodomain was downstream of the C-terminal 82 amino acids of *Renilla* luciferase, generating pChRL-HD.

The mammalian expression vector pDXTAT was prepared by PCR using pTAT-HA (a gift from Dr Steven F. Dowdy, University of California-San Diego, San Diego, CA, U.S.A.; [30]) and the primers, 5′-GGGGAGCTCATGCGGGGTTCTCATCATCATCATC-3′ and 5′-GGGGGTCGACGGAGCCAGCATAGTCTGGGAC-3′. The resulting PCR product was cleaved with SacI and AccI and was cloned in pBK-CMV (Stratagene), generating pDXTAT. Derivatives of pDXTAT carrying full-length and partial human Nkx3.1 cDNAs were prepared by PCR using pcDNA3.1-hNkx3.1 as template and the following primers: pDXTAT(Nkx3.1), 5′-GGGGAATTCATGCTCAGGGTTCCGGAGC-3′ and 5′-GGGCTCGAGTTACCAAAAAGCTGGGCTCCAGCTG-3′; pDXTAT(1–90), 5′-GGGGAATTCATGCTCAGGGTTCCGGAGC-3′ and 5′-GGGCTCGAGTTACAGCGTCTCGGCCTCCTCCGG-3′; pDXTAT(1–123), 5′-GGGGAATTCATGCTCAGGGTTCCGGAGC-3′ and 5′-GGGCTCGAGTTACGGCTGCTTAGGGGTTTGGGG-3′; pDXTAT(1–183), 5′-GGGGAATTCATGCTCAGGGTTCCGGAGC-3′ and 5′-GGGCTCGAGTTACTGCTTTCGCTTAGTCTTATAGCG-3′; pDXTAT(124–183), 5′-GGGGAATTCATGCAGAAGCGCTCCCGAGCTGCC-3′ and 5′-GGGCTCGAGTTACTGCTTTCGCTTAGTCTTATAGCG-3′; and pDXTAT(124–234), 5′-GGGGAATTCATGCAGAAGCGCTCCCGAGCTGCC-3′ and 5′-GGGGAATTCATGCTCAGGGTTCCGGAGC-3′. The resulting PCR products were cleaved with EcoRI and XhoI and were cloned in pDXTAT.

Site-directed mutagenesis was employed to create a single amino acid change within the homeodomains of human ($Gln^{173} \rightarrow$ Glu) and murine ($Gln^{174} \rightarrow$ Glu) Nkx3.1. Briefly, pcDNA3.1-mNkx3.1 and pcDNA3.1-hNkx3.1 were used as templates for PCR using Platinum Pfx DNA polymerase (Invitrogen) and the following phosphorylated primers (mutations are indicated in bold): murine Nkx3.1, 5′-GAACCATATTTTGACTTGGGTTTCGG-3′ and 5′-**G**AGAACAGACGCTATAAGACCAAGCG-3′; and human Nkx3.1, 5′-GAACCATATCTTCACTTGGGTCTCCG-3′ and 5′-**G**AGAACAGACGCTATAAGACTAAGCGAAAAG-3′. The resulting PCR products were cleaved with DpnI to destroy template DNA, self-ligated, and used to transform *Escherichia coli*. Plasmids prepared from resulting transformants were sequenced to identify clones that harboured the desired mutations, creating pcDNA3.1-mNkx3.1mut and pcDNA3.1-hNkx3.1mut.

A CAT (chloramphenicol acetyltransferase) reporter gene governed by a minimal portion of the adenovirus major late promoter, Δ53MLP-CAT (a gift from Dr Adrian R. Black, Roswell Park Cancer Institute, Buffalo, NY, U.S.A.) was employed in transcription experiments to normalize for plate-to-plate variations in transfection efficiency. The transcriptional activity of this construct has been shown not to be regulated by Sp family members [27,31]. A second adenovirus-derived reporter plasmid carrying this same promoter fragment linked to *Renilla* luciferase was prepared by annealing the following oligonucleotide and its complement, 5′-GGGCTCGAGGTTCACAATTTTCTGGTGGTGGGCTATAAAAAAAGCTTGGG-3′, digestion with XhoI and HindIII, and cloning into phRL-basic (Promega), generating phRL-Δ53MLP. A firefly luciferase reporter construct (PSA–Lux) carrying a 5.3-kb portion of the PSA promoter was a gift from Dr Charles J. Bieberich and has been described in [11]. PSA–Lux constructs lacking various portions of the PSA promoter were prepared by PCR using PSA–Lux as template, Platinum Pfx DNA polymerase, 5′-GAATGCCAAGCTTGGGGCTGGGGAG-3′ as a 3′-primer and the following 5′-primers: BstEII (-4243 to $+1$), 5′-GGGTCTAGACCAAATCTTGTAGGGTGACCAGAG-3′, EcoRV (-4075 to $+1$), 5′-GGGTCTAGACAAGCCTCGATCTGAGAGAGATATCATC-3′, Apa I (-2858 to $+1$), 5′-GGGTCTAGACCTGATGAACACCATGGTGTGTACAGG-3′, and SexAI (-1729 to $+1$), 5′-GGGTCTAGAGGCTGGCCTCGAACTCCTGACCTGG-3′. The resulting PCR products were cleaved with XbaI and HindIII and were cloned in pGL3-basic (Promega), generating pBstEII-Lux, pEcoRV-Lux, pApaI-Lux and pSexAI-Lux. Site-directed mutagenesis was employed to inactivate putative Nkx3.1-binding sites located at -4973 (site 1) and -4922 (site 2) of the PSA promoter. Briefly, PSA–Lux was used as template for two PCRs using Platinum Pfx DNA polymerase and the following phosphorylated primers (mutations are indicated in bold): site 1 mutation, 5′-**CCAC**GTATGCTTGCACTGCTGAATGCTTGGG-3′ and 5′-ACACGGCACTCCCCAGAGCCAGG-3; and site 2 mutation, 5′-**CCAC**GCTGAAGATTAACCCTGACACATCCC-3′ and 5′-CAGATGCTCATCTCATCCTCACAG-3′. The resulting PCR products were

cleaved with DpnI to destroy template DNA, DNAs were self-ligated and used to transform *E. coli*. Plasmids derived from resulting transformants were sequenced to identify clones, termed PSA–Lux mut1 and PSA–Lux mut2, carrying the desired mutations. A firefly luciferase reporter carrying mutations within both putative Nkx3.1-binding sites, PSA–Lux Dblmut, was prepared by PCR using PSA–Lux mut1 as template, Platinum Pfx DNA polymerase and the previously described site 2 primer pair. The resulting PCR product was cloned and sequenced as described above. Except where indicated, all PCRs employed Deep Vent polymerase (New England Biolabs). The integrity of all constructions was confirmed by dideoxy-sequencing using Sequenase version 2.0 DNA polymerase following a protocol supplied by the manufacturer (Amersham Biosciences).

Transient transfections and indirect immunofluorescence

Transient transfections were performed using SuperFect transfection reagent (Qiagen) according to the manufacturer's protocol. To analyse levels of transcription in the absence of HDAC activity, transfected cells were treated for 24 h with 100–300 nM TSA (Sigma–Aldrich). Cell extracts were prepared for luciferase and CAT assays 48 h after transfection. The Dual-Luciferase® Reporter Assay System (Promega) was employed to quantify luciferase activity precisely as recommended by the manufacturer. Luminescence was detected in a Lumat LB 9507 luminometer (EG&G Berthold), and results were normalized to the abundance of Δ53MLP-CAT or Δ53MLP-Renilla activity. CAT assays were performed using a liquid-scintillation assay as described previously [32]. Indirect immunofluorescence was performed precisely as described previously [27,31].

Protein–DNA-binding assays

Oligonucleotides were radiolabelled and used in protein–DNA-binding assays as described previously [33]. Recombinant Sp-family members were prepared from baculovirus-infected Sf9 cells as described previously [27,34]. Briefly, 100 ng of recombinant human Nkx3.1 (a gift from Dr Charles J. Bieberich) and/or 1 μl of baculovirus-infected Sf9 whole-cell lysate was incubated for 20 min at 4 °C with 50 000 c.p.m. of radiolabelled oligonucleotide probe, and protein–DNA complexes were resolved on 4.8 % polyacrylamide gels at 250 V for 1.5 h. In some experiments, protein–DNA complexes were challenged with 5 μl of anti-Nkx3.1 antibodies (T-19, N-15 or L-15) (Santa Cruz Biotechnology) before resolution on acrylamide gels. The following oligonucleotides and their complements were used in protein–DNA-binding assays (cognate binding sites are shown in bold): WT, 5′-AGACGGATCCTATGCGC-GATTTTT**TAAGTA**GTTTTTCAGTAGCTATCTGCAGGCGT-3′, Mut, 5′-AGACGGATCCTATGCGCGATTTTT**T**CCAC**A**G-TTTTTCAGTAGCTATCTGCAGGCGT-3′, NkxSp-5, 5′-GTAC-CTCGAGTA**TAAGTA**TATGT**GGGCGGGAC**TAAGGATCC-GCGG-3′, and NkxSp-10, 5′-GTACCTCGAGTA**TAAGTA**TA-TATATAGT**GGGCGGGAC**TAAGGATCCGCGG-3′.

In vitro transcription/translation and protein–protein-binding assays

In vitro transcribed/translated proteins were produced using pBSK-Sp1/flu, pBSK-Sp2/flu, pBSK-Sp3/flu, TOPO-Sp4, TOPO-Sp5, TOPO-Sp2ΔZn or pcDNA3.1-Nkx3.1 as templates and a coupled reticulocyte lysate system (TNT; Promega). *In vitro* translated proteins were radiolabelled with a proprietary cocktail of radiolabelled amino acids (^{35}S-Translabel; ICN Pharmaceuticals). Radiolabelled proteins were then employed in *in vitro* protein–protein-binding assays as described previously [35,36].

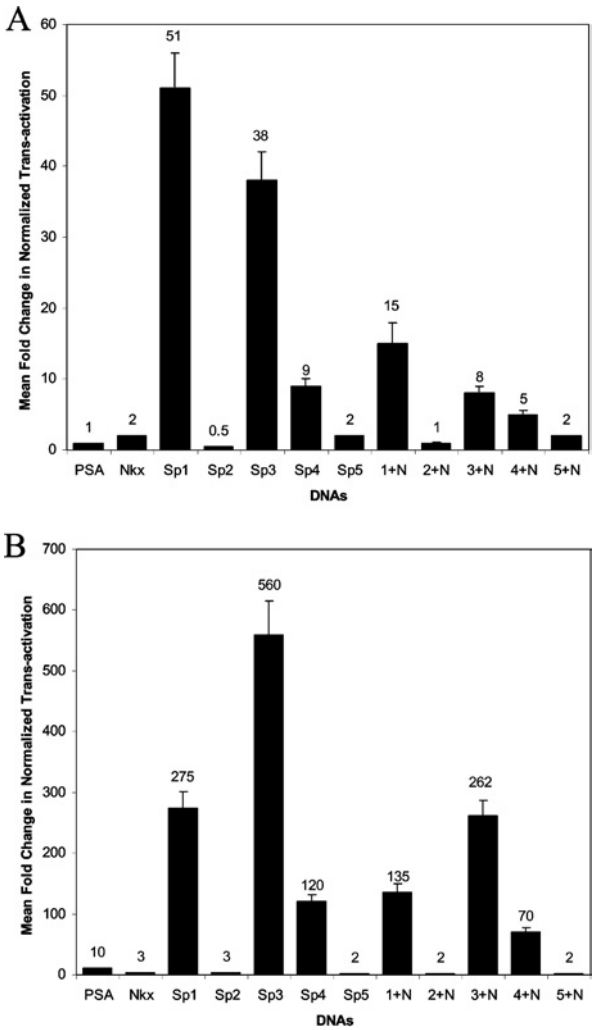

Figure 1 Transactivation of the human PSA promoter by Sp-family members and transrepression by mNkx3.1

(**A**) Effects of Sp-family members and Nkx3.1 on PSA transcription. Human DU145 prostate cells were transiently transfected with mNkx3.1 alone (500 ng; Nkx), with Sp1-5 alone (500 ng), or with mNkx3.1 (500 ng) and an Sp-family member (500 ng; denoted by Sp-family member + N, e.g. 1 + N). Basal levels of PSA transcription were set equal to 1, and the mean fold changes in PSA transactivation normalized to an internal firefly luciferase control following 48 h of cultivation post-transfection are plotted. Empty expression vector DNA was included in control reactions to maintain constant input DNA concentrations (2 μg per plate). Results are means ± S.D. for at least five independent plates of transfected cells. (**B**) Effects of Sp-family members and Nkx3.1 on PSA transcription following treatment with TSA. DU145 cells were transfected and processed as in (**A**), except that cells were treated with 100 nM TSA 24 h after transfection. Basal levels of PSA transcription were 10-fold greater than that shown in (**A**) and therefore PSA values were set equal to 10. Results are means ± S.D. for at least five independent plates of transfected cells.

RESULTS

Nkx3.1 antagonizes Sp-mediated transcription in prostate-derived cells

Human DU145 prostate epithelial cells were transiently transfected with a luciferase reporter construct carrying the PSA promoter (PSA–Lux; [37]) as well as mammalian expression constructs for mouse Nkx3.1 and human Sp-family members, and cell extracts were assayed for firefly luciferase activity. Resulting values were normalized to the abundance of *Renilla* luciferase activity elicited by a co-transfected reporter gene (phRL-MLP) driven by a minimal adenovirus major late promoter. As illustrated in Figure 1(A), co-transfection of PSA with Sp1 or Sp3 resulted in

a dramatic (40–50-fold) increase in levels of PSA transcription. In contrast with these results, transfection with equimolar amounts of Sp4 led to a more modest 9-fold increase in PSA-directed luciferase activity, and cells receiving Sp2 or Sp5 exhibited little or no change in PSA transcription. Having established levels of transcription induced by Sp-family members, DU145 cells were subsequently co-transfected with mouse Nkx3.1, and each Sp-family member and resulting levels of PSA transcription were quantified. Co-expression of mNkx3.1 with Sp1, Sp3, or Sp4 reduced Sp-mediated PSA transcription 2–5-fold, whereas co-expression of mNkx3.1 with Sp2 or Sp5 had little or no effect (Figure 1A). Western blots of transfected cell extracts showed that mNkx3.1 co-expression did not alter the abundance of Sp-family members (results not shown). The transient expression of mNkx3.1 alone did not appreciably alter basal levels of PSA transcription. Analogous transcriptional results were obtained in parallel studies with two additional human prostate epithelial cell lines, PC-3 and LNCaP (results not shown).

To determine whether the reduction in Sp-mediated transcription by Nkx3.1 is dependent on HDAC activity, we performed a similar series of transient transfections and quantified PSA transcription in cells treated for 24 h with 100 mM TSA, a potent inhibitor of HDAC activity. Consistent with the notion that the basal expression of PSA is dampened by HDACs, DU145 cells transfected with PSA–Lux alone and treated with TSA exhibited a 10-fold increase in transcription (Figure 1B). TSA-treated cells co-transfected with PSA–Lux and Sp1, Sp3 or Sp4 exhibited a 5–15-fold stimulation of Sp-mediated transcription above that noted without TSA treatment, whereas TSA did not facilitate the induction of PSA transcription by Sp2 or Sp5. Instead, basal PSA transcription was marginally reduced by Sp2 or Sp5 in TSA-treated cultures. Thus, in partial accord with results reported previously, treatment with TSA diminished the capacity of mNkx3.1 to negatively regulate the activity of a subset of Sp-family members [14]. However, Sp1-, Sp3- and Sp4-directed PSA transcription still remained limited in TSA-treated cells when co-expressed with mNkx3.1. Comparable results were also obtained with greater concentrations of TSA (up to 300 mM; results not shown). Given the results reported thus far we conclude that (i) PSA transcription is stimulated by a subset of Sp-family members in prostatic epithelia, (ii) Nkx3.1 antagonizes the transcriptional activities of this same subset of Sp-family members, and (iii) treatment with TSA reduces, but does not eliminate, the capacity of Nkx3.1 to negatively regulate Sp-mediated transcription. This latter conclusion raised the intriguing possibility that Nkx3.1 may antagonize Sp function via both TSA-sensitive and -insensitive mechanisms.

Nkx3.1 forms specific protein complexes with Sp-family members *in vitro*

To determine whether Nkx3.1 can physically associate with Sp-family members, we performed a series of *in vitro* protein–protein-binding assays using bacterially expressed GST-fusion proteins prepared from a mouse Nkx3.1 cDNA and radiolabelled Sp proteins prepared in reticulocyte extracts. Full-length GST–Nkx3.1 fusion proteins were bound to glutathione–Sepharose beads, incubated with equivalent amounts of radiolabelled Sp proteins, loosely adherent proteins were removed by successive washes, and bead-bound proteins were resolved on acrylamide gels. As shown in Figure 2(A), each Sp-family member bound to GST–mNkx3.1, but not to a negative control fusion protein derived from a schistosome surface antigen (GST–FSH15). Interestingly, although each Sp-family member surveyed bound

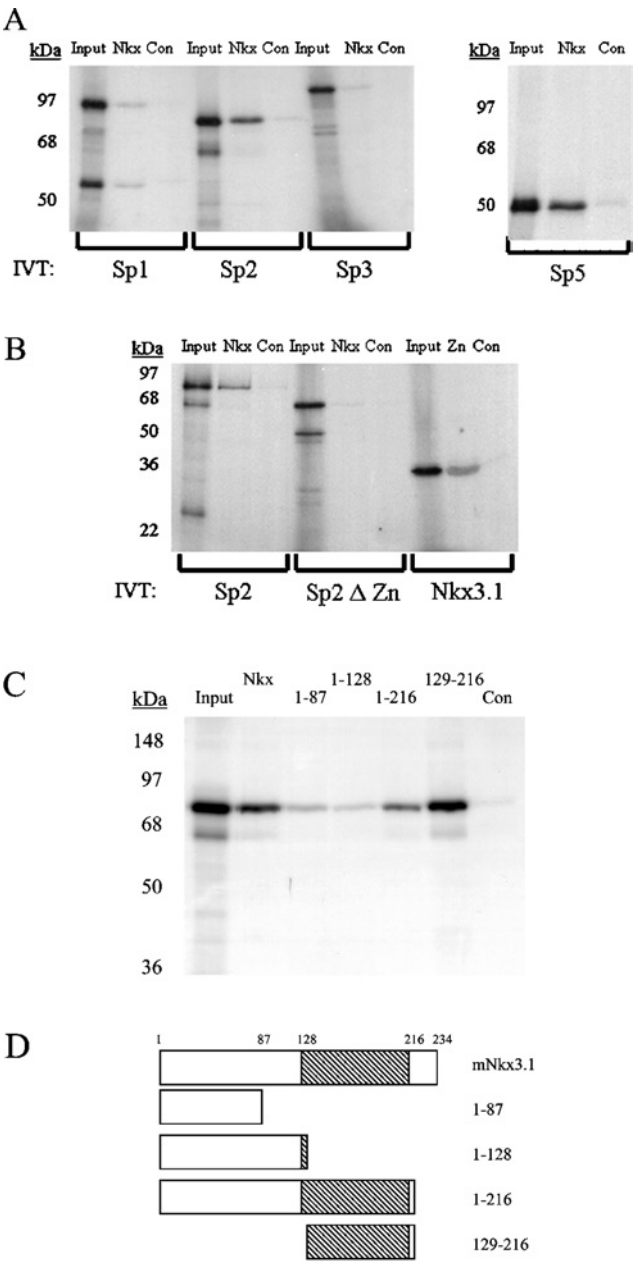

Figure 2 ***In vitro*** **protein–protein-binding assay**

(**A**) Specific binding of Nkx3.1 by Sp-family members. *In vitro* translated (IVT) radiolabelled Sp proteins were synthesized in reticulocyte lysates and incubated with a GST-fusion protein prepared using a full-length mNkx3.1 cDNA (Nkx) or a control fusion protein (GST–FSH; indicated by Con). *In vitro* translated proteins (10% of input) were resolved alone (indicated by Input) as controls. Molecular-mass markers (sizes in kDa) are indicated on the left. (**B**) The DNA-binding domain of Sp-family members is necessary and sufficient for complex formation with Nkx3.1. A full-length GST–mNkx3.1 fusion protein (Nkx), GST–FSH (indicated by Con) or a GST-fusion prepared from the Sp2 DNA-binding domain (pGEX1N-Sp2Zn; indicated by Zn) was challenged with *in vitro* translated radiolabelled Sp2 protein, an Sp2 derivative lacking the DNA-binding domain or mNkx3.1 respectively. *In vitro* translated (IVT) proteins (10% of input) were resolved alone as controls (indicated by Input), and radiolabelled proteins examined are indicated beneath the gel. Molecular-mass markers (sizes in kDa) are indicated on the left. (**C**) Two non-contiguous regions of Nkx3.1 carry binding sites for Sp-family members. *In vitro* translated radiolabelled Sp2 was synthesized in reticulocyte lysates and incubated with a full-length mNkx3.1 GST-fusion protein (Nkx) or derivatives carrying various portions of mNkx3.1 as depicted in (**D**) (indicated at the top of each lane). Sp2 (10% of input) was resolved as a control (indicated by Input), and GST–FSH was employed as a negative control (indicated by Con). Molecular-mass markers (sizes in kDa) are indicated on the left. (**D**) Schematic diagram of mNkx3.1 and truncated derivatives employed as GST-fusion proteins in (**C**). The homeodomain is indicated by a hatched box as are amino acid endpoints for Nkx3.1 deletions.

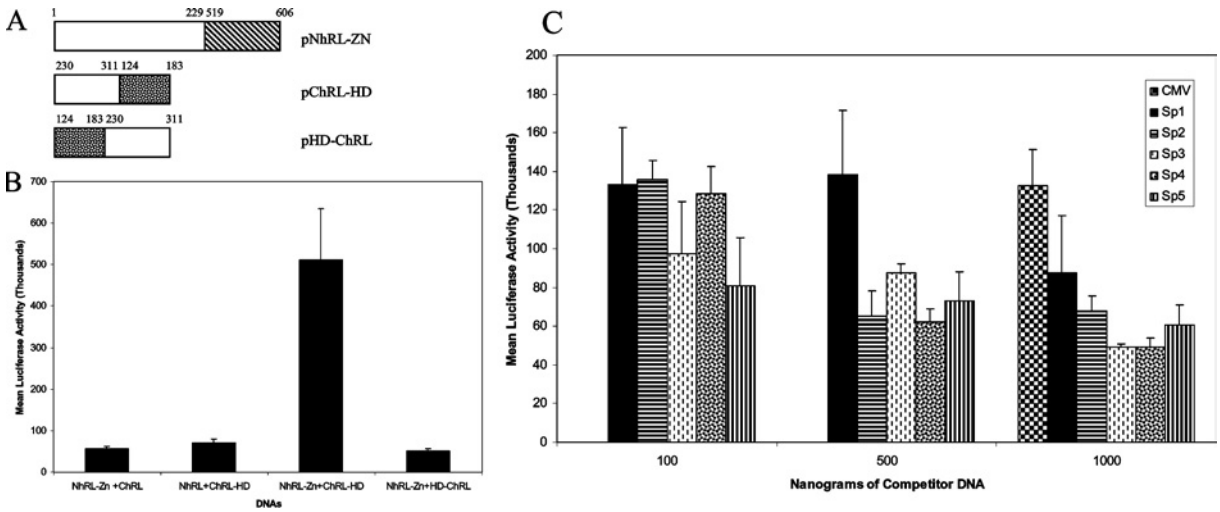

Figure 3 The DNA-binding domains of Nkx3.1 and Sp proteins form complexes in mammalian cells

(**A**) Schematic diagram of mammalian two-hybrid fusion proteins. The N-terminal 229 amino acids of *Renilla* luciferase were fused to the DNA-binding domain (amino acids 519–606) of Sp2, creating pNhRL-Zn. The C-terminal 82 amino acids of *Renilla* luciferase were fused to the human Nkx3.1 homeodomain (amino acids 124–183), creating pChRL-HD. An analogous construct carrying the human Nkx3.1 homeodomain upstream of the *Renilla* C-terminus created pHD-ChRL. The Sp2 DNA-binding domain is indicated by a hatched box, and the Nkx3.1 homeodomain is indicated by a stippled box. Amino acid endpoints are indicated above each schematic diagram. (**B**) Reconstitution of luciferase activity *in vivo*. COS-1 cells were transfected with 0.5 μg of pNhRL-ZN and 0.5 μg of pChRL, an empty control vector, or 0.5 μg of fusion constructs carrying the human Nkx3.1 homeodomain (pChRL-HD and pHD-ChRL). As an additional control, 0.5 μg of pChRL-HD was transfected with 0.5 μg of pNhRL, the corresponding empty control vector. An irrelevant plasmid was included in all transfections to maintain constant input DNA concentrations (2 μg per plate). Results are means $\pm$ S.D. of *Renilla* luciferase activity for six or more plates of transfected cells. (**C**) Competition for reconstitution of *Renilla* luciferase activity by wild-type Sp-family members. COS-1 cells were transfected with 0.5 μg of pNhRL-ZN and 0.5 μg of pChRL-HD as well as increasing amounts (100–1000 ng) of expression vectors carrying wild-type Sp-family members or 1 μg of empty expression vector (pCMV4). Where appropriate, an irrelevant plasmid was included in transfections to maintain constant input DNA concentrations (2 μg per plate). Results are means $\pm$ S.D. of *Renilla* luciferase activity for six or more plates of transfected cells.

mNkx3.1 specifically, Sp2 and Sp5 were reproducibly bound more efficiently than Sp1 or Sp3.

To identify regions of Sp proteins that are required for the formation of complexes with Nkx3.1, we employed PCR to generate a partial Sp2 cDNA that encodes the Sp2 transactivation domain (Sp2ΔZn) and subcloned this PCR fragment into an *in vitro* transcription vector. We chose Sp2 for these additional protein-binding assays, since it bound mNkx3.1 with high relative efficiency. Radiolabelled Sp2 and Sp2ΔZn proteins were generated by *in vitro* transcription/translation and employed in protein–protein-binding assays with GST–mNkx3.1 or GST–FSH15 (Figure 2B). Consistent with previous protein-binding assays, full-length Sp2 bound to GST–mNkx3.1, whereas an Sp2-derived protein carrying only its transactivation domain did not (Sp2ΔZn). These results suggested that the Sp2 DNA-binding domain carries at least one site that is required for physical interactions with Nkx3.1. To determine whether the Sp2 DNA-binding domain is both necessary and sufficient to bind Nkx3.1, we prepared another GST-fusion protein (GST–Sp2Zn) that carries the entirety of the Sp2 DNA-binding domain and performed protein–protein-binding assays with *in vitro* translated and radiolabelled mNkx3.1. In contrast with results obtained with Sp2ΔZn, Nkx3.1 bound efficiently and specifically to the Sp2 DNA-binding domain (Figure 2B, Zn). We conclude from these results that Sp-family members form specific protein complexes with Nkx3.1 *in vitro*, and that the DNA-binding domain of Sp-family members is necessary and sufficient for these physical interactions.

To delimit region(s) of mNkx3.1 required for the formation of physical complexes with Sp-family members, we used PCR to generate a series of partial cDNAs encoding the regions depicted in Figure 2(D). GST-fusion proteins were prepared from these partial mNkx3.1 cDNAs, and subsequently incubated with radiolabelled full-length Sp2 protein produced by *in vitro* transcription/translation. Interestingly, at least two independent portions of mNkx3.1 were noted to be sufficient to bind Sp2 *in vitro*. The Nkx3.1 homeodomain appears to carry a major site of protein–protein interaction as a partial fusion protein containing this portion of mNkx3.1 (amino acids 129–216) bound Sp2 as efficiently as full-length mNkx3.1 (Figure 2C). Yet another binding site for Sp proteins appears to reside within the N-terminal 87 amino acids of mNkx3.1, as a GST-fusion protein carrying this portion of mNkx3.1 (amino acids 1–87; Figure 2C) bound Sp2, albeit relatively weakly. Based on results from *in vitro* protein–protein-binding assays, we conclude that Nkx3.1 and Sp-family members form stable specific protein complexes largely through interactions between their respective DNA-binding domains. A second site of protein interaction is specified by amino acids within the N-terminal 87 residues of mNkx3.1.

The DNA-binding domains of Nkx3.1 and Sp proteins interact physically *in vivo*

To extend our protein–protein-binding results, we wished to determine whether physical complexes between Sp proteins and Nkx3.1 occurred *in vivo* and were specified by their respective DNA-binding domains. To address these issues, we employed a highly sensitive mammalian two-hybrid system that facilitates detection of protein–protein complexes via the reconstitution of *Renilla* luciferase activity when partial luciferase domains are brought together in close proximity [29]. We subcloned the DNA-binding domain of Sp2 (amino acids 519–606) into a mammalian expression vector, creating pNhRL-Zn, such that the Sp2 zinc fingers were linked in-frame with the N-terminal 229 residues of *Renilla* luciferase (Figure 3A). Similarly, the human Nkx3.1 homeodomain (amino acids 124–183) was subcloned into a mammalian expression vector, creating pChRL-HD, such that it was linked in-frame with the C-terminal 82 residues of *Renilla* luciferase (Figure 3A). COS-1 cells were transfected

with one or both of these constructs and then assayed for *Renilla* luciferase activity. As shown in Figure 3(B), extracts prepared from control cells that co-expressed partial *Renilla* fusion proteins and their complimentary empty vectors resulted in luciferase levels modestly above the background detected in extracts prepared from mock-transfected cells. In contrast, the co-expression of pNhRL-Zn and pChRL-HD resulted in 10-fold higher levels of luciferase activity. The specificity of these *in vivo* interactions between the Nkx3.1 and Sp2 DNA-binding domains is underscored by one further experiment shown in Figure 3(B). pNhRL-Zn was co-expressed with a third construct (pHD-ChRL) in which the Nkx3.1 homeodomain is sub-cloned upstream of the C-terminal 82 amino acids of *Renilla* luciferase (Figure 3A). This *Renilla* luciferase-fusion protein features a free N-terminal end of the Nkx3.1 homeodomain, whereas this end is tethered to the C-terminus of *Renilla* luciferase in pChRL-HD. Similar to the aforementioned control assays, extracts prepared from cells co-expressing pNhRL-Zn and pHD-ChRL resulted in minimal levels of luciferase activity. Western blots prepared from transfected cells indicated that each *Renilla* luciferase-fusion protein was expressed at equivalent levels *in vivo* (results not shown). Thus the reconstitution of *Renilla* luciferase activity appears to require orientation-specific interactions between *Renilla* luciferase-fusion proteins carrying the Nkx3.1 homeodomain and Sp2 zinc fingers.

To confirm the physiological relevance of these *in vivo* protein–protein interactions, we performed a competition experiment in which pNhRL-Zn and pChRL-HD were co-expressed in COS-1 cells in conjunction with increasing amounts of mammalian Sp-expression vectors or 1000 ng of empty expression vector (pCMV4). Should the interactions between *Renilla* luciferase-fusion proteins be physiologically relevant, we predicted that the co-expression of wild-type Sp proteins should prevent the reconstitution of *Renilla* luciferase activity by displacing pNhRL-Zn. Consistent with this prediction, co-expression of wild-type Sp proteins led to a dose-dependent decrease in *Renilla* luciferase activity (Figure 3C). In contrast, little or no reduction in luciferase activity was noted in extracts prepared from cells receiving empty expression vector. We conclude from these experiments that the DNA-binding domains of Sp-family members and Nkx3.1 interact specifically *in vivo*.

Physical interactions between the DNA-binding domains of Nkx3.1 and Sp proteins do not disrupt their capacity to bind DNA

To determine whether Nkx3.1 negatively regulates Sp-family members, at least in part, via competition for their capacity to bind DNA, we developed an Nkx3.1 protein–DNA-binding (gel-shift) assay and assessed the capacity of Sp-family members to bind DNA following incubation with recombinant Nkx3.1 protein. As a first step, we incubated 10–200 ng of bacterially expressed human Nkx3.1 protein with a radiolabelled oligonucleotide probe carrying a consensus DNA-binding site (WT; 5′-TAAGTA-3′) or an analogous probe carrying nucleotide substitutions (Mut; 5′-TCCACA-3′) that were expected to prevent the formation of hNkx3.1 protein–DNA complexes. As illustrated in Figure 4(A), reactions in which the wild-type probe was incubated with increasing amounts of hNkx3.1 led to the formation of two novel protein–DNA complexes (denoted ‘Nkx’). These novel protein–DNA complexes were barely detectable when hNkx3.1 was incubated with the mutated probe, consistent with the proposition that recombinant hNkx3.1 binds DNA specifically in these *in vitro* assays. To confirm the identity of the proteins comprising these complexes, gel-shift reactions were challenged with three anti-Nkx3.1 antibodies, and resulting antigen–antibody complexes

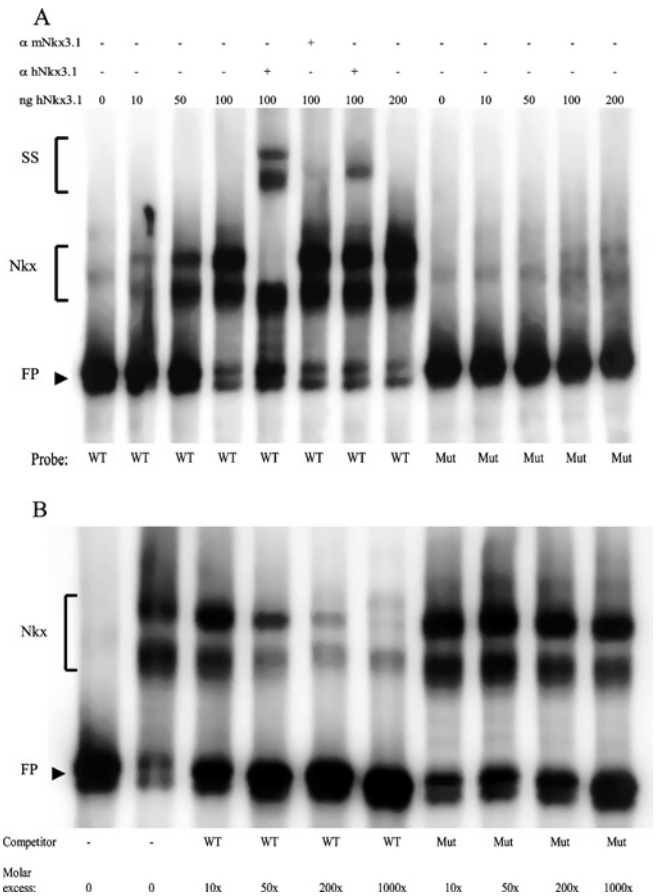

Figure 4 Protein–DNA-binding assays with recombinant human Nkx3.1 protein

(**A**) Recombinant hNkx3.1 binds DNA specifically *in vitro*. Radiolabelled oligonucleotide probes carrying consensus (WT) or mutated (Mut) Nkx3.1-binding sites were incubated with or without various amounts of bacterially expressed hNkx3.1 as indicated. Protein–DNA complexes were challenged with anti-human (T-19 and N-15) or anti-mouse (L-15) Nkx3.1 antibodies where indicated. Nkx3.1 protein–DNA complexes are indicated by Nkx, antibody-bound super-shifted complexes are indicated by SS, and free probe is indicated by FP. (**B**) Competition experiment using wild-type and mutated oligonucleotides. A radiolabelled oligonucleotide probe carrying a consensus Nkx3.1-binding site was incubated alone or with 100 ng of recombinant hNkx3.1 protein and challenged with increasing amounts of unlabelled wild-type (WT) or mutated (Mut) oligonucleotides. Molar excesses of unlabelled competitor DNAs are indicated. Nkx3.1 protein–DNA complexes are indicated by Nkx, and free probe is indicated by FP.

were resolved on acrylamide gels. Two anti-Nkx3.1 antibodies, T-19 and N-15, were prepared against human Nkx3.1 protein, whereas a third, L-15, was prepared against mouse Nkx3.1. Consistent with the antigenic specificities of the antibodies employed, addition of T-19 or N-15 to Nkx3.1 gel-shift reactions led to the generation of supershifted protein–DNA complexes (denoted ‘SS’; Figure 4A), whereas L-15 did not (Figure 4A). As one further test of DNA-binding specificity, we performed a competition experiment in which increasing amounts of unlabelled wild-type or mutated oligonucleotides were added to standard gel-shift assays pre-loaded with radiolabelled wild-type probe. Inclusion of a 50-fold molar excess of unlabelled wild-type oligonucleotides (Figure 4B) significantly diminished the abundance of Nkx3.1 protein–DNA complexes, whereas the abundance of such complexes was unaffected by the inclusion of up to 1000-fold molar excess of mutated oligonucleotides (Figure 4B). We conclude from these results that recombinant hNkx3.1 binds its cognate DNA-binding sequence specifically *in vitro*.

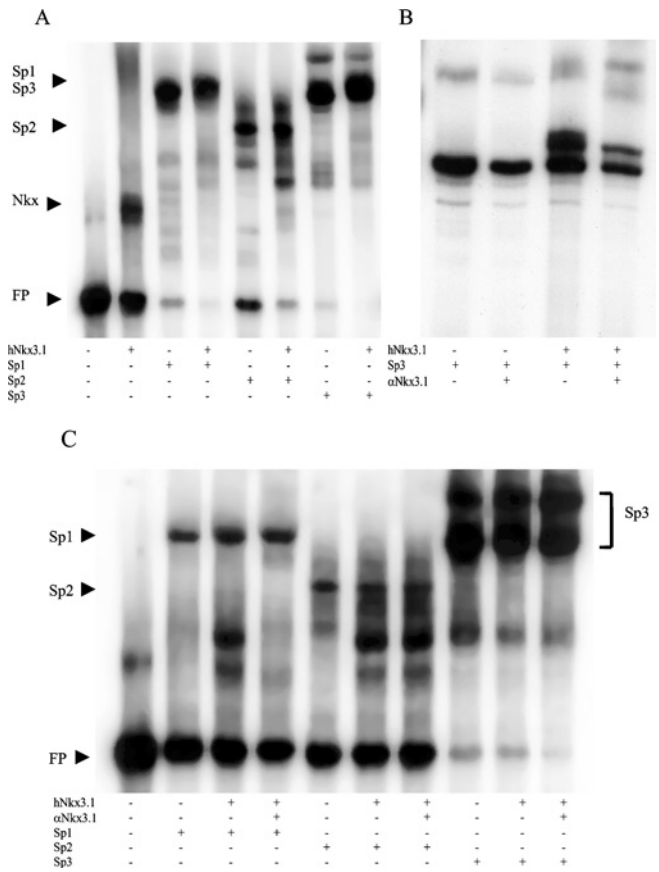

Figure 5 Protein–DNA-binding assays with recombinant human Nkx3.1 and Sp proteins

(**A**) Protein–DNA-binding assays with recombinant hNkx3.1 and human Sp-family members. A radiolabelled oligonucleotide probe (NkxSp-10) carrying consensus Nkx3.1- and Sp2-binding sites separated by 5 bp was incubated alone or with 100 ng of recombinant hNkx3.1 and baculovirus extracts carrying recombinant Sp1, Sp2 or Sp3 protein (proteins added are indicated by +). Nkx3.1 protein–DNA complexes are indicated by Nkx. Sp1, Sp2 and Sp3 indicate their respective protein–DNA complexes, and free probe is also indicated (FP). (**B**) Extended electrophoresis of protein–DNA-binding assays with recombinant hNkx3.1 and human Sp3 proteins. A radiolabelled NkxSp-5 probe was incubated with baculovirus extracts carrying recombinant Sp3 protein and 100 ng of recombinant hNkx3.1 (proteins added are indicated by +). Protein–DNA complexes were challenged with anti-hNkx3.1 antibody T-19 (where indicated by +). (**C**) Protein–DNA-binding assays with recombinant hNkx3.1 and human Sp-family members using a probe carrying a consensus Sp-binding site. A radiolabelled probe carrying a consensus Sp2-binding site was incubated alone or with baculovirus extracts carrying recombinant Sp1, Sp2 or Sp3 protein, and 100 ng of recombinant hNkx3.1 (proteins added are indicated by +). Protein–DNA complexes were challenged with anti-hNkx3.1 antibody T-19 (where indicated by +). Sp1, Sp2 and Sp3 indicate their respective protein–DNA complexes, and FP indicates free probe.

To determine whether physical interactions between the DNA-binding domains of Nkx3.1 and Sp proteins preclude DNA-binding activity, two double-stranded oligonucleotides were synthesized that carry consensus Nkx3.1 and Sp-binding sites separated by five (SpNkx-5) or ten (SpNkx-10) base pairs. These dual-binding-site oligonucleotides were subsequently challenged with bacterially expressed hNkx3.1 protein, recombinant Sp proteins expressed in insect cells via baculovirus infection, or mixtures of these proteins. The functional properties of these baculovirus-produced Sp proteins have been characterized extensively [27]. When each protein preparation was assayed alone, Nkx3.1, as well as Sp1, Sp2 and Sp3, bound the SpNkx-10 probe (Figure 5A). Incubation of Nkx3.1 and Sp1 or Sp3 proteins with SpNkx-10 resulted in the formation of a novel complex with a slightly reduced mobility compared with Sp1 and Sp3 protein–DNA complexes. Interestingly, mixtures of Nkx3.1, Sp2 and SpNkx-10 did not result in the formation of an analogous novel complex. Identical results were obtained for each of these protein mixtures in parallel gel-shift assays performed with the SpNkx-5 oligonucleotide (Figure 5B, and results not shown). To verify that the novel complexes we observed result from the formation of ternary protein–DNA complexes, we challenged gel-shift reactions with an anti-Nkx3.1 antibody and then employed an extended period of electrophoresis to resolve closely migrating protein–DNA complexes (Figure 5B). For this experiment, recombinant Sp3 protein was added to each gel-shift reaction with a radiolabelled SpNkx-5 probe (Figure 5B), whereas recombinant Nkx3.1 was added only to reaction mixtures resolved in lanes 3 and 4. As shown in Figure 5B, inclusion of an anti-Nkx3.1 antibody depleted the abundance of a novel complex formed by reactions prepared with both hNkx3.1 and Sp3. Addition of this same antibody to a control gel-shift reaction (Figure 5B) did not affect the abundance of a protein–DNA complex formed by recombinant Sp3 protein. We conclude from these results that interactions between the DNA-binding domains of Nkx3.1 and Sp-family members do not compete for their association with DNA, at least not under the experimental conditions that we employed.

Although binding sites for Nkx3.1 and Sp proteins were carried by oligonucleotides in the previous protein–DNA-binding assays, it remained unclear whether the formation of ternary complexes resulted from protein–protein or protein–DNA interactions. To determine whether ternary protein–DNA complexes require cognate DNA-binding sites for Nkx3.1 and Sp proteins, we performed a series of gel-shift assays using oligonucleotide probes that contained binding sites for Nkx3.1 or Sp-family members. As shown in Figure 5(C), Sp1, Sp2 and Sp3 bound a probe containing a single Sp-binding site, and inclusion of hNkx3.1 did not result in the formation of protein–DNA complexes with decreased mobility. Inclusion of anti-Nkx3.1 antibodies in parallel reactions confirmed the lack of ternary complex formation. Similar results were obtained using an oligonucleotide probe that carries a binding site for Nkx3.1, but not Sp-family members (results not shown). We conclude from these results that DNA-binding sites for both Nkx3.1 and Sp proteins are required for ternary complex formation. That is, neither Sp proteins nor Nkx3.1 can tether the other to DNA, at least under the experimental conditions that we employed. Taking together results illustrated in Figure 5, we also conclude that Nkx3.1 and Sp1 or Sp3 can occupy DNA-binding sites separated by as little as five base pairs. Further experiments will be required to determine why Sp2 and hNkx3.1 did not form ternary complexes under similar experimental conditions.

A distal portion of the human PSA promoter is required for Nkx3.1 to suppress Sp-mediated transcription in prostate-derived cells

Computer-assisted sequence analyses identified numerous predicted Sp-binding sites within the PSA promoter, as well as five predicted Nkx3.1 binding sites (Figure 6A). To map portions of the PSA promoter that are required for Nkx3.1 to suppress Sp-mediated transcription, we took advantage of a number of convenient restriction enzyme sites to create a series of nested deletions (Figure 6A). The basal activities of each of these deleted constructs were compared with that of the full-length PSA promoter in DU145 cells, and for their capacity to be regulated by the ectopic expression of Sp3 and/or hNkx3.1. Sp3-mediated transcription appears to be dependent on two regions of the PSA promoter: deletion of sequences upstream of the BstEII site reduced Sp3-directed transcription more than 3-fold, and a further

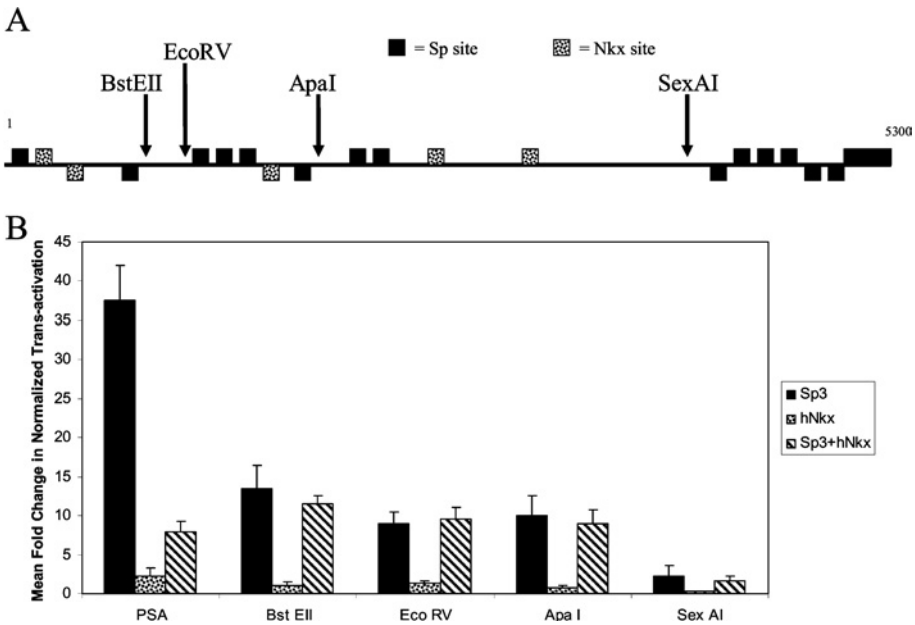

Figure 6 Sensitivity of PSA promoter deletion mutants to transactivation by Sp3 and transrepression by hNkx3.1

(**A**) Schematic diagram of human PSA promoter (5300 bp; drawn 5′ → 3′). Restriction sites used to generate deletion mutants are indicated as are predicted Nkx3.1- (stippled boxes) and Sp- (solid boxes) binding sites. Predicted protein-binding sites on the sense strand are shown above the line, and those on the antisense strand are shown below the line. (**B**) Transcriptional response of wild-type and deleted PSA promoter constructs following the ectopic expression of Sp3, hNkx3.1, or Sp3 and hNkx3.1 in DU145 cells. Results are changes ± S.D. of mean fold transactivation of each construction relative to its basal activity (set equal to 1) for five independent plates of cells transfected with each construction.

5-fold reduction in transcription was noted following the deletion of sequences upstream of the SexAI site (Figure 6B). Little difference was noted between the full-length and deleted promoter constructs with respect to their sensitivity to hNkx3.1 when expressed alone. In contrast, the capacity of hNkx3.1 to down-regulate Sp3-directed transcription was completely dependent on sequences upstream of the BstEII site. To confirm this result, PSA promoter sequences between the BstEII and SexAI restriction sites were deleted, and this resulting construct was examined for its sensitivity to Sp3 and hNkx3.1. As shown in Figure 7(A), the transcriptional response of this internally deleted promoter construct was identical with that of the wild-type PSA promoter. Thus the distal end of the PSA promoter carries one or more elements required for the antagonism of Sp-mediated transcription by Nkx3.1.

Nkx3.1 DNA-binding activity is not required to down-regulate Sp-mediated transcription

Computer-assisted analyses identified two putative Nkx3.1-binding sites (5′-TAAGTG-3′, located on the promoter sense strand and termed site 1; and 5′-TAAGTG-3′, located on the antisense strand and termed site 2) within the distal segment of the PSA promoter required for the suppression of Sp-mediated transcription by hNkx3.1. To determine whether one or both of these putative DNA-binding sites is/are required for the negative regulation of Sp-mediated transcription, each was mutated via site-directed mutagenesis. Resulting mutated promoter constructs were employed in transient transfection experiments in comparison with the parental wild-type PSA promoter. The basal transcription activity of each mutated promoter construct was similar to that of wild-type PSA, and each was induced 18–36-fold following the transient expression of Sp3 (Figure 7A). Interestingly, the ablation of either or both putative Nkx3.1-binding sites had little or no effect on the capacity of hNkx3.1 to antagonize Sp-mediated transcription as the induced transcription of wild-type and mutated PSA promoters were each reduced at least 15-fold following co-expression of hNkx3.1 (Figure 7A).

Given that two predicted Nkx3.1-binding sites within the distal portion of the PSA promoter were not required for down-regulation of Sp-mediated transcription, we reasoned that Nkx3.1 might regulate Sp-mediated PSA transcription by binding to partially degenerate DNA sequences that would have been overlooked by our computer-assisted screen. To determine whether a functional Nkx3.1 DNA-binding domain is required to antagonize Sp-mediated transcription, a single amino acid substitution was introduced via site-directed mutagenesis at position 9 of the recognition helix (helix III) of the human ($Gln^{173} \rightarrow Gln$) and mouse ($Gln^{174} \rightarrow Glu$) Nkx3.1 homeodomains. As shown previously for several other homeodomain proteins, analogous amino acid substitutions inactivate DNA-binding activity due to the loss of a critical contact between residues at this position and DNA [38–42]. We then assessed the capacities of these homeodomain mutants to negatively regulate Sp-mediated transcription in parallel with their wild-type counterparts. Consistent with the notion that DNA-binding activity is not required for transcriptional repression by Nkx3.1, mutation of the human and mouse Nkx3.1 homeodomains resulted in only a modest reduction (approx. 2-fold) in their activity as inhibitors of Sp-mediated transcription (Figure 7B). To confirm that these mutated homeodomain constructs are karyophilic and localize to nuclei at least as efficiently as wild-type Nkx3.1, we utilized indirect immunofluorescence to monitor the subcellular localization of wild-type and mutated proteins. Transient expression of wild-type human or mouse Nkx3.1 in COS-1 cells led to protein expression throughout the nucleus, with much of the protein accumulating in perinuclear deposits (results not shown). The human and mouse homeodomain mutants were equally karyophilic, yet cells carrying these proteins exhibited a more uniform granular distribution of Nkx3.1 within the nucleoplasm (results not

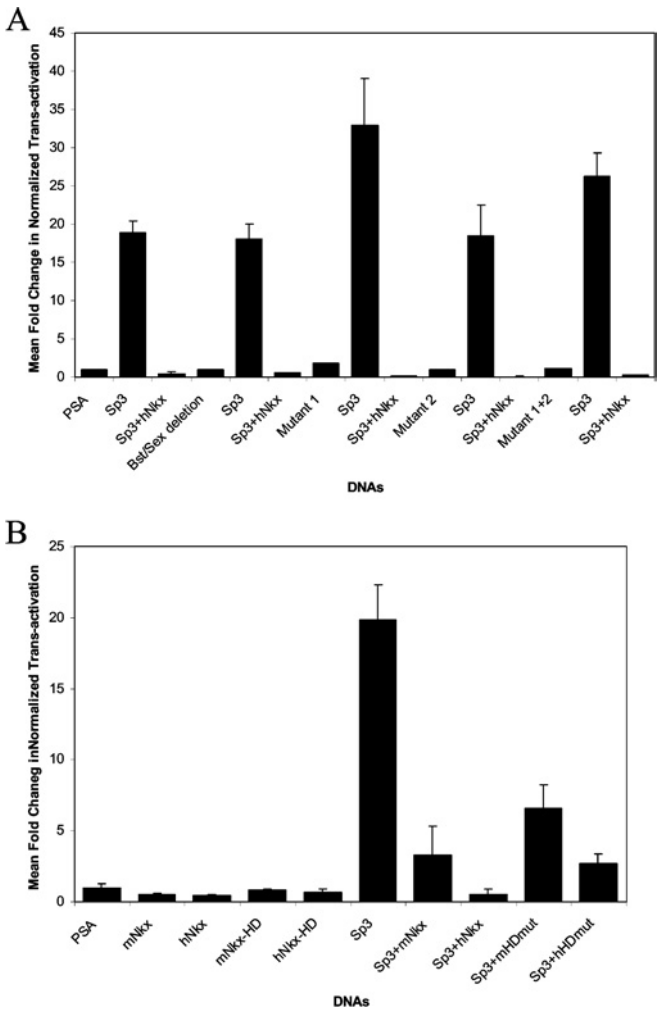

Figure 7 Transcriptional regulation of wild-type and mutated PSA promoter constructs by Sp3 and wild-type Nkx3.1 or DNA binding-deficient homeodomain mutants

(**A**) DU145 cells were transfected with promoter constructs alone, in conjunction with Sp3, or with Sp3 and hNkx3.1. Mutated promoter constructs are: deletion of sequence in between the unique BstEII and SexAI sites within the human PSA promoter (labelled Bst/Sex deletion), mutation of most distal putative Nkx3.1-binding site (site 1; labelled Mutant 1), mutation of other putative Nkx3.1-binding site (site 2; labelled Mutant 2), or both sites mutated (labelled Mutant 1 + 2). Results are are changes ± S.D. in mean fold transactivation relative to basal levels of PSA transcription (set equal to 1) for a minimum of six plates of transfected cells. (**B**) Transrepression by human and mouse Nkx3.1 proteins as well as derivatives carrying homeodomain mutations that ablate DNA-binding activity. DU145 cells were transfected with the human PSA promoter alone, in conjunction with wild-type mouse (mNkx) or human (hNkx) Nkx3.1 expression vectors or derivatives carrying homeodomain mutations (mNkx-HD and hNkx-HD), Sp3, or Sp3 and wild-type or mutated Nkx3.1 expression vectors. Results are changes ± S.D. in mean fold transactivation relative to basal levels of PSA transcription (set equal to 1) for a minimum of six plates of transfected cells.

shown). Whether this somewhat subtle difference in subnuclear distribution accounts for the relatively modest decline in Nkx3.1-directed transcriptional repression remains to be determined. We conclude that direct interactions between the Nkx3.1 homeodomain and DNA are not absolutely required for the suppression of Sp-mediated transcription.

Two non-contiguous portions of Nkx3.1 are each sufficient to antagonize Sp-mediated transcription

Nkx3.1 physically interacts with Sp-family members via at least two binding sites: a major site located within the Nkx3.1 homeodomain and a minor site located within the extreme N-terminus (Figure 2C). To determine whether these portions of Nkx3.1 are also required to inhibit Sp-mediated transcription, a series of expression vectors carrying partial hNkx3.1 cDNAs were constructed (Figure 8A). To ensure that the partial hNkx3.1 proteins elicited by these cDNAs would localize to the nucleus, each cDNA was linked in-frame to a nuclear localization sequence derived from the HIV Tat protein and ectopically expressed proteins were detected using an antibody prepared against a linked epitope tag. Each partial hNkx3.1 construct elicited stable karyophilic proteins (results not shown). Each was then ectopically expressed in DU145 cells, and their effects on Sp3-induced transcription were compared with that of wild-type hNkx3.1. As shown in Figure 8(B), transcriptional repression of PSA by hNkx3.1 deletions pDXTAT(1–90), pDXTAT(1–183), pDXTAT(124–183) and pDXTAT(124–234) ranged from 14- to 23-fold and was comparable with that of wild-type hNkx3.1 (23-fold). pDXTAT(1–123) was somewhat less potent as a negative regulator of Sp3, reducing PSA transcription 7-fold. Based on these results, at least two non-contiguous portions of Nkx3.1 appear to be sufficient for the negative regulation of Sp-mediated transcription. One region resides within the N-terminal 90 amino acids of hNkx3.1 [i.e. pDXTAT(1–90)], the other within the Nkx3.1 homeodomain [i.e. pDXTAT(124–183)]. Importantly, these two regions are coincident with portions of Nkx3.1 that we have shown previously are sufficient to direct physical interactions with Sp-family members *in vitro* (Figure 2C). Taking our *in vitro* and *in vivo* results together, we conclude that portions of Nkx3.1 required for physical interactions with Sp-family members are also required for the inhibition of Sp-mediated transcription in prostate-derived cells.

DISCUSSION

Homeodomain-containing transcription factors play a central role in the development of metazoans, orchestrating the development of the body plan, determining cell fate and stimulating organogenesis. Each is characterized by a conserved 60-amino-acid DNA-binding domain that interacts with a characteristic DNA sequence (5′-TAAT/G-3′) in the promoters of target genes. Given the variety of developmental outcomes that spring from their interaction with a common DNA-binding sequence, it is widely suspected that homeodomain proteins identify and regulate target genes in collaboration with a constellation of additional DNA-binding proteins. In the present paper, we report that Nkx3.1, a homeodomain protein linked to the development of the human and mouse prostate gland, collaborates with Sp-family members to regulate the transcription of PSA in prostate-derived cells. Nkx3.1 negatively regulates the transcriptional induction of PSA by Sp-family members, and does so via TSA-sensitive and -insensitive mechanisms. Consistent with the notion that one or more promoter elements is/are required for Nkx3.1 to suppress Sp-mediated transcription, a distal segment of the PSA promoter was identified as being necessary for transrepression. Additionally, we report that: (i) Nkx3.1 and Sp-family members form specific protein complexes *in vitro* and *in vivo*, and (ii) portions of Nkx3.1 that are required for complex formation with Sp-family members are also required to antagonize Sp-mediated transcription. We conclude that Nkx3.1 negatively regulates the transcriptional activity of Sp-family members in prostate-derived cells and speculate that these interactions, and their deregulation in tumour cells, may have important implications for prostate cell proliferation and differentiation. Moreover, when taken together with another recent report our results suggest that physical and functional

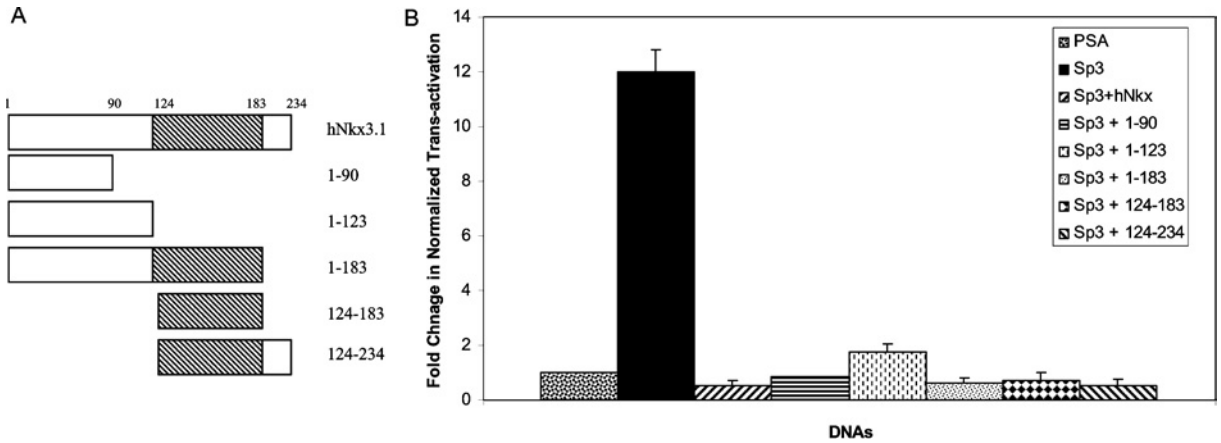

Figure 8 Transrepression of Sp-mediated transcription by two non-contiguous portions of Nkx3.1

(**A**) Schematic diagram of hNkx3.1 and truncated derivatives expressed in mammalian cells. The homeodomain is indicated by a hatched box as are amino acid endpoints for hNkx3.1 deletions. (**B**) Transrepression by wild-type and truncated hNkx3.1 derivatives. DU145 cells were transfected with the human PSA promoter alone, in conjunction with Sp3, or with Sp3 and expression vectors encoding wild-type or truncated hNkx3.1 mutants. Results are changes ± S.D. in mean fold transactivation relative to basal levels of PSA transcription (set equal to 1) for a minimum of six plates of transfected cells.

interactions between Sp-family members and homeodomain proteins may be quite widespread [18].

Despite the relative paucity of confirmed target genes, several previous studies have focused on Nkx3.1-mediated transcription, regulatory mechanisms and combinatorial interactions with additional sequence-specific DNA-binding proteins. When analysed, these studies appear to indicate that Nkx3.1 function is context-dependent. In non-prostate-derived cells, Nkx3.1 has been shown to repress its own transcription in association with Groucho and HDAC1, and to stimulate smooth muscle γ-actin transcription in conjunction with SRF [10,14]. Similar to results that we report for Nkx3.1 and Sp2, Nkx3.1 and SRF were shown to form physical complexes that require their respective DNA-binding domains, yet inclusion of SRF and Nkx3.1 in protein–DNA-binding assays did not lead to the formation of ternary complexes [10]. In prostate-derived cells, Nkx3.1 has thus far proved to function as a transcriptional repressor. In this setting, Nkx3.1 has been shown to repress transcription of both artificial and natural promoters, and to collaborate with factors such as PDEF in the regulation of PSA transcription [9,11]. Consistent with our results and studies with SRF, physical interactions between PDEF and Nkx3.1 were shown to be dependent on portions of their respective DNA-binding domains, as well as sequences immediately downstream [12]. Such collaborations between Nkx3.1 and additional sequence-specific DNA-binding proteins are in keeping with the widely held supposition that combinatorial interactions between transcription factors are likely to govern target gene identification and regulation by homeodomain proteins. Our results and those of others also underscore the importance of DNA-binding domains as substrates for protein–protein interactions.

In vitro protein-binding assays reported in the present paper identified Sp-binding sites within two non-contiguous portions of Nkx3.1 and at least one binding site for Nkx3.1 within the zinc fingers of Sp-family members. These interactions are likely to be physiologically relevant as: (i) we established that the Nkx3.1 homeodomain is necessary and sufficient for the formation of physical complexes with the zinc fingers of Sp proteins *in vivo* using a mammalian two-hybrid approach, and (ii) such complexes were disrupted in a dose-dependent fashion by the co-expression of full-length Sp-family members. It is worth noting that comparable physical interactions have been reported between the homeodomain of Nkx2.5 and the zinc fingers of GATA-4, two factors that are essential for cardiogenesis [43]. Unexpectedly, our mammalian two-hybrid results also suggest that the Nkx3.1 homeodomain and the zinc fingers of Sp proteins may interact in an orientation-specific fashion. That is, we detected protein–protein interactions when the Nkx3.1 homeodomain was linked downstream, but not upstream, of the C-terminal 82 amino acids of *Renilla* luciferase. These results are not due to problems inherent with *Renilla* reconstitution, since the C-terminal 82 amino acids reconstitute luciferase activity readily when fused downstream of control proteins, such as FKBP12 (FK506-binding protein 12) (S. O. Simmons and J. M. Horowitz, unpublished work). Given that (i) the Nkx3.1 homeodomain resulted in only background levels of luciferase activity when fused in this orientation, and (ii) homeodomains with free N-termini are capable of folding into functional units (e.g. capable of binding DNA; [44]), we speculate that Nkx3.1 and Sp proteins may interact in a specific orientation-dependent manner.

Our protein–protein-binding assays identified a second independent Sp protein-binding site within the N-terminus (residues 1–87) of mNkx3.1. At least *in vitro*, this binding site appears to be relatively weak compared with the Sp-binding site that is carried by the Nkx3.1 homeodomain, and the Sp amino acids with which this site interacts have not yet been determined. Nonetheless, our *in vivo* results indicate that Sp-mediated transcription is antagonized independently by the ectopic expression of the Nkx3.1 homeodomain or N-terminus. It is also worth pointing out that, although Nkx3.1 bound all Sp-family members *in vitro*, Sp2 and Sp5 appeared to be bound more efficiently than Sp1, Sp3 and Sp4. Thus it is possible that interactions between Nkx3.1 and Sp2/Sp5 may result in more potent effects on transcription than those documented in the present paper for Sp1, Sp3 and Sp4. Unfortunately, since Sp2 and Sp5 had little or no effect on PSA transcription, we were unable to compare their sensitivity to regulation by Nkx3.1 with other Sp-family members. Target genes of Sp2 or Sp5 have not as yet been identified, thus the functional consequence of the increased efficiency with which they interact with Nkx3.1 remains to be established.

The capacity of Nkx3.1 to down-regulate the induction of PSA by Sp-family members was found to be entirely dependent on a 1.0 kb promoter fragment at the distal end of the human PSA

promoter. This distal region has been shown to carry an ARE (androgen-response element), yet this site is not likely to be involved in the down-regulation of Sp-mediated transcription by Nkx3.1, since we have documented transrepression in prostate-derived cells that do not express the androgen receptor, such as DU145. Computer-assisted analyses identified two putative Sp-binding sites within this distal portion of the PSA promoter, and their potential relevance for the induction of PSA or regulation by Nkx3.1 has not been determined directly. Two putative Nkx3.1-binding sites within this distal region were also identified in this computerized screen, yet their mutational inactivation had little effect on the capacity of Nkx3.1 to antagonize Sp-directed transcription. Moreover, we have shown that DNA-binding-deficient homeodomain mutants remain potent inhibitors of Sp-mediated transcription. Given these mutational results and the capacity of homeodomain mutants to block Sp-mediated transcription, we speculate that Nkx3.1 associates indirectly with chromatin via one or more factors that bind the distal end of the PSA promoter. Additionally, since protein–DNA-binding assays indicate that neither Nkx3.1 nor Sp proteins can tether the other to DNA, it is unlikely that Nkx3.1 associates indirectly with chromatin via Sp-family members bound to the distal end of the PSA promoter. What then is the mechanism by which Nkx3.1 negatively regulates Sp-mediated transcription? Since partial Nkx3.1 proteins that are capable of binding Sp-family members are also quite potent inhibitors of Sp-mediated transcription, we speculate that Nkx3.1 regulates Sp-family members by attracting co-repressors to Sp protein–DNA complexes and/or by interfering with the interaction of co-activators necessary for Sp-mediated transcription. Nk proteins contain a conserved 23-amino-acid sequence, termed the TN domain, which, in some instances, can facilitate the recruitment of HDACs to DNA and the down-regulation of transcription [45–47]. Interestingly, a TN-like sequence in human and mouse Nkx3.1 is located within an N-terminal 90-amino-acid region that we have shown binds to Sp proteins *in vitro* and inhibits Sp-mediated transcription *in vivo*. We have also shown that the Nkx3.1 homeodomain harbours an Sp-protein-binding site and this portion of Nkx3.2, a closely related homeodomain-containing protein, has been shown to interact with HDAC1 and Smad proteins, and recruit co-repressors, such as Sin3A [46]. Yet another possibility is the influence of a family of HIPKs (homeodomain-interacting protein kinases) that function as co-repressors of transcription via association with the homeodomain of NK-3 proteins [13]. Consistent with the notion that HDAC association plays a role in the antagonism of Sp-mediated transcription, treatment of prostate-derived cells with a potent HDAC inhibitor, TSA, resulted in significant, albeit incomplete, relief from transrepression by Nkx3.1. Whether Nkx3.1 also inhibits the association of Sp proteins with one or more co-activators, perhaps providing a TSA-insensitive mechanism for transcriptional regulation of Sp-family members, remains to be determined.

Elevated levels of PSA in patient blood serum are correlated directly with prostatic hyperplasia and prostate tumorigenesis, and quantification of serum PSA levels is a clinically significant diagnostic tool. In the present study, we demonstrate that PSA is a transcriptional target for a subset of Sp-family members in prostate-derived cells and that co-expression of Nkx3.1 represses Sp-mediated transcription. Based on the results of the present study, one would predict that loss of Nkx3.1 protein expression, a common and early event in prostate tumorigenesis, would lead to unfettered Sp-mediated transcription and thus elevated levels of serum PSA. Whether such increased rates of PSA transcription contribute significantly to the elevation of serum PSA levels in patient serum remains to be determined. Given that Sp proteins regulate a wide variety of genes, loss of Nkx3.1 function and its consequent effects on transactivation by Sp proteins might be predicted to lead to global perturbations in prostate gene expression, and the deregulation of cell growth and differentiation. Such uncontrolled combinatorial interactions may account, at least in part, for the variety of genes that become transcriptionally deregulated within the prostates of hemizygous and nullizygous Nkx3.1 animals [7].

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