

Identification and characterization of a prostate-specific androgen-independent protein-binding site in the probasin promoter

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In this study we investigated the combination of transcription factors and proteins binding to the proximal part of the prostate-specific probasin (PB) promoter. Using DNaseI *in vitro* footprinting, several protected regions were identified on the proximal PB promoter (nucleotides –286 to +28 relative to the transcription start site) when nuclear extracts from LNCaP, a human prostate cancer cell line, were used. Four of the protected areas were observed only when LNCaP nuclear extracts treated with synthetic androgen (10 nM R1881) were used. Two other regions, referred to as FPI and FPII, showed protection regardless of the presence or absence of androgen. When DNaseI footprinting was done using other prostate and non-prostate nuclear extracts, protection of the FPII region was only seen in prostate cell lines. These androgen-independent regions were further tested for tissue and binding specificity using the electrophoretic mobility-shift assay. Eight complexes formed with the FPI probe while four complexes were observed with the FPII probe on incubation with the tested nuclear extracts. Methylation protection assays reveal that prostate cancer

cell lines yield slightly different protection patterns for some of the protein complexes formed with non-prostate-derived cell lines, suggesting the presence of prostate-enriched or -exclusive proteins. Site-directed mutagenesis of the protected nucleotides within FPII resulted in a significant reduction in expression from the PB promoter. Identification of proteins binding to the FPII region revealed the participation of nuclear factor I (NF-I) or a closely related protein, although other unknown proteins are also involved. Defining the DNA and protein components that dictate prostate-specific expression of the PB promoter in an androgen-independent manner would provide a strong basis for the design and development of a gene therapy for systemic treatment of androgen-independent prostate cancer.

Key words: footprint, methylation protection, nuclear factor I (NF-I).

INTRODUCTION

It is estimated that this year prostate cancer will account for almost 30 % of all new, potentially lethal cancer cases in men in North America [1]. Whereas most cases of advanced, metastatic prostate cancer can initially be controlled by androgen-withdrawal therapies, with progression to androgen independence the disease becomes incurable and deadly. One promising therapeutic strategy for treating advanced prostate cancer is to create a gene therapy vector in which a corrective or cytotoxic gene is placed under the control of a prostate-specific promoter and packaged into a suitable viral or chemical delivery system. In fact, there are several reports where the probasin (PB) promoter and other prostate-specific promoters such as prostate-specific antigen ('PSA') and prostatic steroid-binding protein have been tested in this manner in tumour model systems [2–8]. Unfortunately, an intrinsic limitation to the application of these promoters for expression in prostate tumour cells is that androgens and a functioning androgen receptor (AR) system are required for their activity. Hence they are not suitable for targeting androgen-independent prostate tumours.

Whereas the PB protein is a murine lipocalin whose *in vivo* expression is androgen-regulated and tightly confined to the dorsolateral prostate [9], a relatively small portion of its proximal gene promoter, running from –426 to +28 bp relative to the transcriptional start site, has been shown to be sufficient to confer androgen-regulated, prostate-specific expression in transgenic

mice [10]. *In vitro* transfection experiments using a variety of human cell lines have confirmed the prostate-specific expression of reporters attached to this region of the proximal PB promoter, with only low levels of expression detected in non-prostate-derived cell lines [2]. Further promoter analysis has revealed that a slightly smaller region of the PB promoter, running from –286 to +28 bp, had much higher levels of androgen-induced activity *in vitro* while retaining a high degree of preferential expression in human prostate cancer cell lines [2,8]. Similarly, in transgenic mouse models, the activity of a multimeric form of the –286 to +28 PB promoter, referred to as ARR₂PB (where ARR is androgen-response region; comprised of –244 to –96 in front of –286 to +28), was found to be confined to the prostate and to be much higher than in transgenics with the proximal PB promoter [11]. Currently, this is the optimal form of PB promoter for conferring prostate-specific gene expression while possessing substantially higher activity than the proximal promoter. Interestingly, a construct composed of three –244 to –96 ARR motifs attached to a thymidine kinase minimal promoter (ARR₃tk) was found in the presence of AR to have ≈10-fold higher activity than that seen with the proximal PB promoter [7], but could not be shown to direct prostate-specific gene expression (R. J. Matusik, personal communication).

While it is clear that the –286 to +28 PB promoter is highly androgen-regulated and that the regions of AR interactions

Abbreviations used: AR, androgen receptor; ARR, androgen-response region; NF-I, nuclear factor I; ARE, antigen-response element; DMS, dimethylsulphate; EMSA, electrophoretic mobility-shift assay; PB, probasin; TBE, Tris/borate/EDTA.

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are well mapped [8,12], the DNA motifs and protein factors that dictate its tight prostate-specific expression have not been identified. In this study we identify distinct DNA regions within the proximal PB promoter which on incubation with nuclear extracts derived from LNCaP human prostate cancer cells are protected from DNaseI digestion. At least two of these DNA regions demonstrated androgen-independent protection and were further characterized by electrophoretic mobility-shift assays (EMSA) and dimethylsulphate (DMS) footprinting (methylation-protection assay). DNA sequences in one of the protected regions contained elements closely resembling a c-Myb proto-oncogene binding site [13–15] and a nuclear factor I (NF-I) half site [16]. However, a detailed analysis revealed that only an NF-I-like protein was able to influence the unique binding pattern seen with nuclear extracts from prostate cancer cells and that this protein together with other unidentified DNA-binding proteins may contribute to the prostate-specific expression pattern associated with the PB promoter.

MATERIALS AND METHODS

Tissue culture

HeLa (human cervical cancer), HepG2 (human liver cancer), MCF-7 (human breast cancer) and PC-3 (human prostate cancer) cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) while LNCaP (human prostate cancer) and Jurkat (human B-cell cancer) cells were maintained in RPMI (Invitrogen). Both types of medium were supplemented with 5% (v/v) fetal bovine serum (Invitrogen) and 100 units/ml penicillin/streptomycin. For androgen-withdrawal experiments, medium was instead supplemented with 5% (v/v) charcoal-stripped fetal bovine serum and 100 units/ml penicillin/streptomycin for at least 24 h prior to harvesting of cells.

Oligonucleotides

Oligonucleotides were synthesized by Nucleic Acids Protein Services (University of British Columbia, Vancouver, BC, Canada). Oligonucleotide numbering for FPI and FPII is relative to the transcription start site of the murine PB gene. The sequences of the oligonucleotides were as follows: FPI –62 to –36 bp (27 bp), 5'-AGACACTGCCCATGCCAATCATCTGA-3' and 5'-TCAGGATGATTGGCATGGCAGTGTCT-3'; FPII –93 to –67 bp (27 bp), 5'-ATGTCTGTGTACAACGGCAACTGGGA-3' and 5'-TCCCAGTTGGCAGTTGTACACAGACAT-3'; c-Myb consensus binding site (18 bp), 5'-CCCAACGGCGGTTGGGG-3' and 5'-CCCCAACCGCCGTTGGG-3'; NF-I consensus binding site (34 bp), 5'-TGTGAGTTTTGGATTGAGCCAATATGATAGGA-3' and 5'-TCCTATCATATTGGCTTCAATCCAAAACCTCACA-3'.

EMSA

Individual oligonucleotides were 5'-end [γ - 32 P]dATP-labelled with T4 polynucleotide kinase. Labelled oligonucleotides (100 ng) were annealed to equimolar amounts of their complementary strands (unlabelled) by heating to 95 °C for 5 min in Tris/EDTA supplemented with 50 mM NaCl and slowly cooling to room temperature. Double-stranded oligonucleotide probes were purified on a 5% (w/v) polyacrylamide/0.5 × Tris/borate/EDTA (TBE) non-denaturing gel, eluted in 500 μ l of elution buffer [0.6 M ammonium acetate/0.1% (w/v) SDS/1 mM EDTA] and ethanol-precipitated prior to use in EMSA

experiments. Nuclear extracts used in EMSA were isolated from HeLa, Jurkat, LNCaP, MCF-7 and PC-3 cells, both androgen-depleted and supplemented with the synthetic androgen R1881 (10 nM), using a modified Dignam method [17]. Final nuclear protein preparations were collected in buffer C [400 mM NaCl/20 mM Hepes (pH 7.4)/25% (v/v) glycerol/1.5 mM MgCl₂/0.2 mM EDTA/0.5 mM dithiothreitol] and assayed for protein concentration by BCA assay (Pierce).

For band-shift experiments, 10 000 c.p.m. of labelled oligoduplex probes were added to 18 μ g of nuclear extracts. To prevent non-specific binding of nuclear proteins, poly dI-dC was added to a concentration of 80 ng/ μ l and the binding reaction was incubated at room temperature for 10 min prior to electrophoresis. For supershift experiments, 2 μ g of rabbit anti-c-Myb polyclonal antibody (2 μ g/ μ l; Santa Cruz Biotechnology catalogue no. C-19X) or rabbit anti-NF-I polyclonal antibody (2 μ g/ μ l, Santa Cruz Biotechnology catalogue no. SC-5567) were added to the binding reaction and incubated for 30 min on ice prior to electrophoresis. To confirm that the observed protein–DNA complexes were the result of specific binding, a 100-fold molar excess of unlabelled oligoduplexes was added to reactions as a competitor to compete away the labelled oligoduplex. Protein–DNA complexes were separated from unbound DNA using a 5% (w/v) polyacrylamide/0.5 × TBE non-denaturing gel, run at a constant voltage of 300 V for 1 h.

Probe generation for DNaseI/DMS *in vitro* footprinting

Proximal PB promoter fragments PB_{–286} (–286 to +28 bp), FPI (–62 to –36 bp) and FPII (–93 to –67 bp) were generated with appropriate overhangs and cloned into the *Bam*HI/*Hind*III sites of the pBluescript SK[–] vector (Stratagene). Individual strands of the DNA region of interest were radiolabelled by first cutting with either *Xba*I or *Xho*I restriction enzymes to linearize the plasmid and generate a 5' overhang which was then Klenow-enzyme-labelled using [α - 32 P]dATP and [α - 32 P]dCTP. The second enzyme was then used to liberate the labelled promoter fragments from the plasmid backbones. Labelled PB fragments were separated from plasmids using a 5% (w/v) polyacrylamide/0.5 × TBE non-denaturing gel. Probes were then cut out of the gel, eluted in 500 μ l of elution buffer and ethanol-precipitated to generate a single-end-labelled probe for DNaseI or DMS *in vitro* footprinting.

DNaseI *in vitro* footprint analysis

Reactions were carried out by incubating the labelled PB_{–286} probe (20000 c.p.m.) with \approx 100 μ g of nuclear extract in binding buffer [5 μ g of poly dI-dC/10% (v/v) glycerol/20 mM Hepes (pH 7.4)/75 mM KCl/1 mM dithiothreitol] for 20 min at room temperature in a 60 μ l volume. BSA (\approx 4 μ g/reaction) was added to control reactions in the absence of nuclear extract. Mg²⁺/Ca²⁺ was added to each reaction to a final concentration of 10 mM together with 1–2 units of DNaseI and samples were incubated for 1 min at 37 °C. To stop reactions, 160 μ l of stop solution [192 mM sodium acetate/32 mM EDTA/0.14% (w/v) SDS] was added. Samples were then extracted with phenol/chloroform and ethanol-precipitated. A G/A ladder was also generated by sequencing the probe by the Maxam and Gilbert method. The G/A ladder and DNA fragments from DNaseI reactions were electrophoresed on a 6% (w/v) polyacrylamide/0.5 × TBE/8.3 M urea sequencing gel. Dried gels were exposed to MS or MR film (Kodak) for 24–72 h at –80 °C.

DMS *in vitro* footprinting: methylation-protection analysis

Reactions were carried out by incubating single-end-labelled FPI and FPII probes (1.0×10^6 c.p.m.) with $150 \mu\text{g}$ ($\approx 3 \mu\text{g}/\mu\text{l}$) of nuclear extract added to $200 \mu\text{l}$ of binding buffer II [$8 \mu\text{g}$ of poly dI-dC/10% (v/v) glycerol/20 mM Hepes (pH 7.4)] for 20 min at room temperature. The samples were incubated for 2 min with a one-tenth vol. of 2% (v/v) DMS freshly diluted with sterile filtered water. Unbound DNA and protein–DNA complexes were separated from the DMS by loading directly on to a 5% (w/v) polyacrylamide/0.5 \times TBE non-denaturing gel already running at 300 V [18]. Shifted DNA and unbound DNA bands were identified on a short (1 h) MS film exposure, cut out of the gel, eluted overnight in $500 \mu\text{l}$ of elution buffer and ethanol-precipitated. Samples were resuspended in $90 \mu\text{l}$ of sterile filtered water and piperidine-cleaved by the addition of a one-tenth vol. of piperidine followed by heating to 95°C for 20 min. Samples were then snap-frozen in liquid nitrogen and lyophilized to remove the piperidine. Sterile filtered water ($100 \mu\text{l}$) was added to each sample, and the samples were again snap-frozen and lyophilized until dry. Resulting DNA fragments were resuspended in $10 \mu\text{l}$ of formamide buffer [80% (w/v) formamide/10 mM NaOH/1 mM EDTA/0.1% (w/v) xylene cyanol/0.1% (w/v) Bromophenol Blue] and scintillation counted. Equal counts (≈ 2000 c.p.m.) of each sample were loaded and electrophoresed on a 6% (w/v) polyacrylamide/0.5 \times TBE/8.3 M urea sequencing gel. Dried gels were exposed to MS or MR film (Kodak) for 24–72 h at -80°C .

Western blotting

Nuclear lysates from different cell lines ($9 \mu\text{g}$) were boiled in Laemmli sample buffer and resolved by SDS/PAGE. Proteins were then transferred to a PVDF membrane. The membrane was blocked with 5% (w/v) non-fat milk in 0.05% (v/v) Tween 20 in TBS for 1 h and then incubated for 1–2 h at room temperature with primary NF-I, c-Myb or c-Myc antibodies (Santa Cruz Biotechnology). Detection was achieved with horseradish peroxidase-conjugated anti-rabbit antibody (Santa Cruz Biotechnology) and ECL Western blotting detection agents (Amersham Biosciences catalogue no. RPN 2106).

Plasmid construction

The reporter plasmids used for transfection were the pPBLuc plasmid, which contains the firefly luciferase gene under transcriptional control of the -286 to $+28$ fragment of the murine PB promoter [7], and the pPBLuc-NFneg plasmid, a pPBLuc plasmid in which a putative NF-I half site was removed by site-directed mutagenesis using the Stratagene QuikChange™ kit. The primers used to alter the pPBLuc plasmid, with the substituted bases underlined, were 5'-CAATGTCAATGTCTG-TCTACAACTCGACACTGGGATGCAAGAC-3' and the complement 5'-TCTTGCATCCCAGTGTCGAGTTGTAGAC-AGACATTGACATTG-3'. Mutant plasmids were sequenced to verify the base alterations. The pRL-TK (Promega) *Renilla* luciferase reporter plasmid was used as an internal transfection control.

Transfections and luciferase assay

LNCaP cells were plated in 12-well plates (2.0×10^4 cells/well) in RPMI with 5% (v/v) fetal bovine serum for 2 days or until they reached 50–60% confluency. Each well was transfected with $0.4 \mu\text{g}$ of pPBLuc or pPBLuc-NFneg reporter plasmid and

$0.01 \mu\text{g}$ of pRL-TK (Promega) for purposes of normalization. Plasmid DNA was mixed with Lipofectin reagent (Invitrogen) at a ratio of $1 \mu\text{g}$ of DNA/ $5 \mu\text{l}$ of Lipofectin in serum-free RPMI and incubated for 30 min at room temperature. Cells were incubated with the transfection mixture for 16 h, following which transfection medium was replaced by RPMI with 5% (v/v) charcoal-stripped serum with or without 10 nM R1881 and cells were collected after 24 h of incubation using passive cell-lysis buffer (Promega). Luciferase activities were measured using a commercial kit from Promega according to the manufacturer's protocol. Luciferase activities were measured as luminescence units/min and normalized to *Renilla* control values. All transfection experiments were carried out in replicates of four or six and repeated at least twice. Statistical analysis was done on the results using Student's *t* test.

Database analyses

DNA regions showing androgen-independent protection were searched against the ConSite (<http://forkhead.cgr.ki.se/cgi-bin/consite>) and MatInspector (<http://www.gsf.de/biodv/matinspector.html>) databases to detect potential transcription-factor-binding sites.

RESULTS

DNaseI footprinting of the proximal PB promoter reveals an androgen-independent protected region

To determine the regions within the PB promoter that are bound by proteins present in LNCaP human prostate cancer cells, DNaseI footprinting experiments were conducted using a pBluescript SK⁻-cloned, single-end-³²P-labelled proximal PB promoter probe spanning the region -286 to $+28$ bp relative to the transcriptional start site (Figure 1A). This probe was incubated with nuclear proteins extracted from the LNCaP cells cultured in the absence of androgen or in the presence of 10 nM of the synthetic androgen, R1881. Figure 1(B) shows the protection patterns observed under these conditions.

When using LNCaP nuclear extracts from cells treated with 10 nM R1881, protected regions were observed corresponding to the previously described antigen-response element (ARE)-1 and ARE-2 as well as newly characterized G-site 1 and G-site 2 AR-binding sites (Figure 1A, lane 4) [8,12]. In addition, two previously unidentified protected regions, referred to as FPI and FPII, were observed between the G-site 2 and the location of the transcriptional start site. When using androgen-depleted LNCaP nuclear extracts, AR-mediated protection patterns were absent and protection was only observed in the FPI and FPII regions (Figure 1A, lane 3). Thus the occupancy of FPI and FPII sites appears to be independent of the presence or absence of androgens.

To analyse these androgen-independent regions in more detail, DNaseI footprinting was done with a smaller fragment of the promoter, encompassing the FPI and FPII regions, using HeLa and LNCaP nuclear extracts treated or untreated with 10 nM R1881 (Figure 1C, lanes 3–6). Protection of the FPI region was seen when using LNCaP and HeLa nuclear extracts while protection of the FPII region was only seen in the LNCaP (Figure 1C, lanes 3 and 4). This suggests that the protein binding to the FPII region may be enriched in or exclusive to prostate cell lines.

Specific proteins that bind to FPI sequences are enriched in prostate cell lines

To explore the tissue and binding specificity of proteins interacting with the FPI protected region, a radiolabelled double-stranded

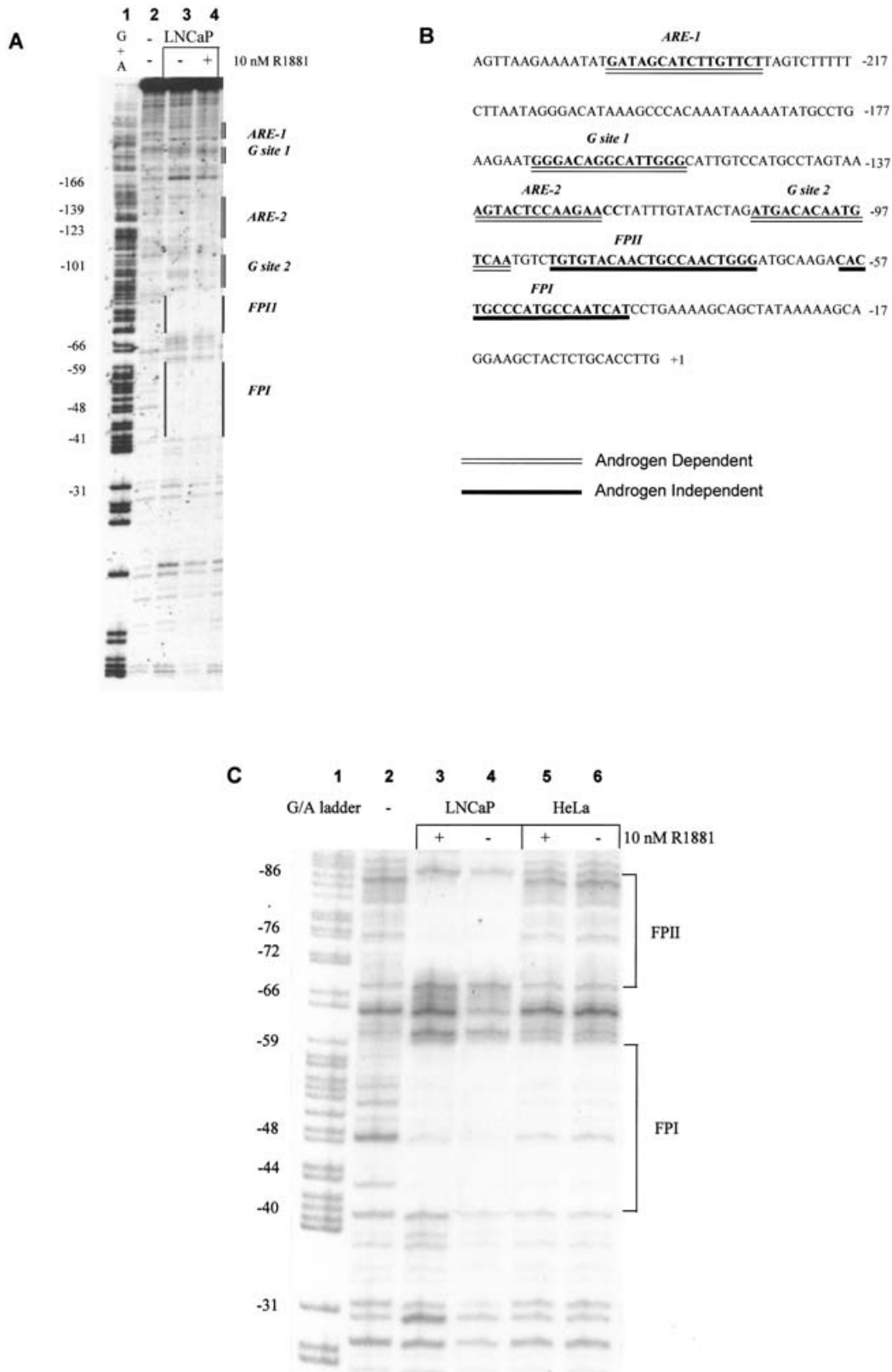


Figure 1 Identification of *in vitro* binding sites in the PB promoter by DNaseI footprinting

(A) DNaseI footprint of the PB promoter. Lane 1, G/A ladder; lane 2, DNaseI treatment of the PB promoter in the absence of nuclear extract; lane 3 and 4, LNCaP without and with 10 nM R1881. (B) Nucleic acid sequence of the proximal PB promoter from position -257 to +1 relative to the transcription start site. Previously and newly described protein-binding sites indicated are the ARE-1, G-site 1, ARE-2 and G-site 2 AR-binding sites and the FPI and FPII androgen-independent DNaseI-protected footprint regions. (C) DNaseI footprint of the FPI and FPII region. Lane 1, G/A ladder; lane 2, DNaseI treatment of the PB fragment in the absence of nuclear extract; lanes 3 and 4, LNCaP cells with and without 10 nM R1881; lanes 5 and 6, HeLa cells with and without 10 nM R1881.

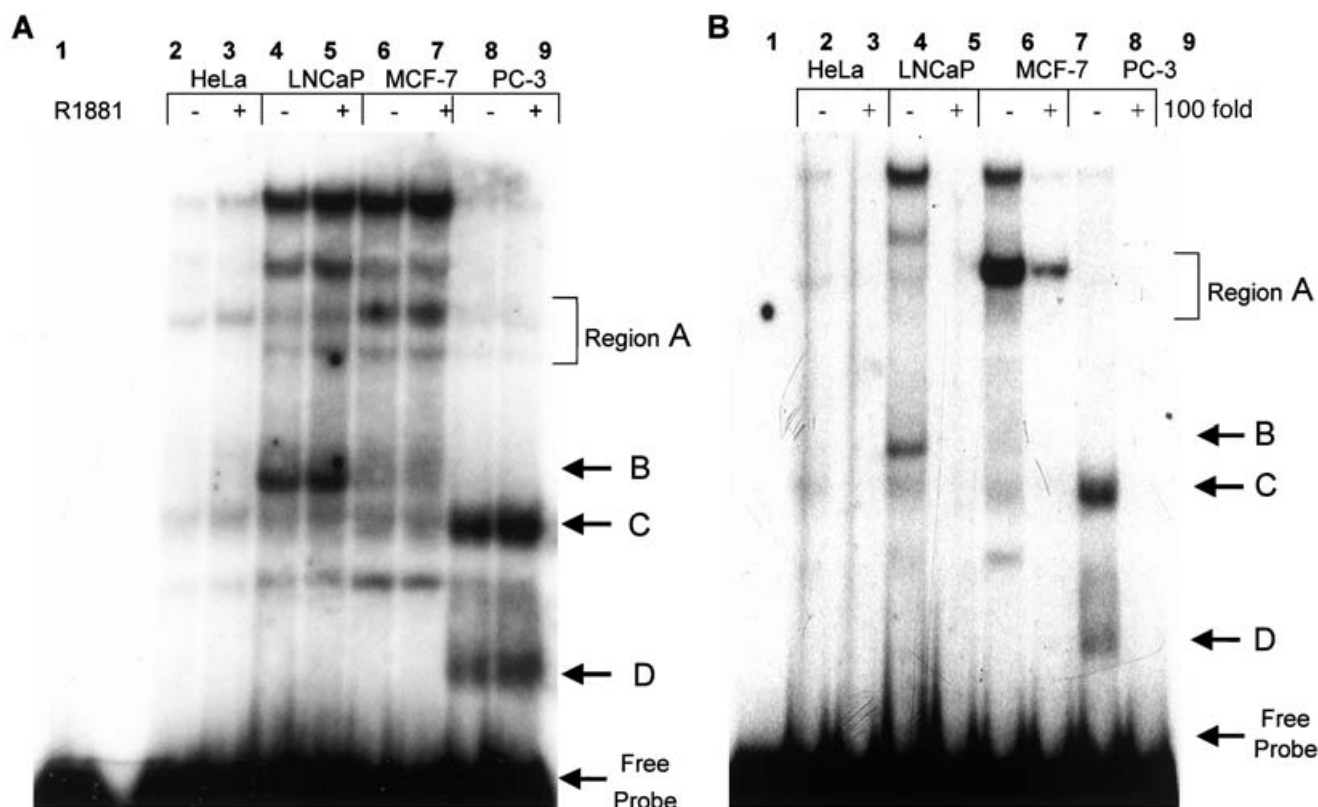


Figure 2 Determination of tissue and binding specificity of FPI protected region

EMSA were performed using labelled FPI probe incubated with nuclear extracts from HeLa, LNCaP, MCF-7 and PC-3 cells, treated with R1881 or hormone starved. **(A)** Eight major complexes formed with the various nuclear extracts. Lane 1, control; lanes 2 and 3, HeLa nuclear extracts; lanes 4 and 5, LNCaP nuclear extracts; lanes 6 and 7, MCF-7 nuclear extracts; lanes 8 and 9, PC-3 nuclear extracts. **(B)** 100-fold excess of unlabelled FPI probe was added to assess the binding specificity of FPI complexes A–D (lanes 2–9) in a competition EMSA.

probe corresponding to the FPI region was synthesized for EMSA studies with prostate- and non-prostate-derived cell lines. Figure 2(A) shows the results of incubating the FPI probe with nuclear extracts from HeLa, LNCaP, MCF-7 and PC-3 cells left untreated or treated with 10 nM R1881. The 27 bp FPI probe yielded a very complex banding pattern consisting of eight protein–DNA complexes. None of the complexes were enriched in both prostate cell lines relative to non-prostate cell lines. In particular, the banding patterns of LNCaP and MCF-7 cells were found to be very similar except for the greater intensity of complex B in LNCaP cells. Complexes C and D formed with highest concentration when using PC-3 nuclear extracts and this binding was independent of hormone pre-treatment. Treatment with R1881 did not seem to affect the assembly of any of the eight complexes, verifying that the binding of these complexes is hormone-independent. To test for binding specificity, excess unlabelled FPI probe was added into the binding reactions to test its ability to displace protein binding to the labelled probe. All eight complexes seemed to represent specific protein–DNA interactions, as addition of a 100-fold molar excess of unlabelled FPI probe was able effectively to compete away protein–DNA complex formation (Figure 2B). The EMSA results suggest that there are large quantitative but minor qualitative differences with the binding patterns obtained with different sources of nuclear extract. To provide a more detailed assessment of the non-quantitative binding characteristics of each sample, methylation-protection analyses were performed.

Methylation-protection analyses show unique protection patterns in region A and complex C upon incubation with PC-3 nuclear extracts

Methylation-protection analyses were conducted using a pBluescript SK⁻-cloned, single-end-³²P-labelled FPI probe, to identify guanine–protein contacts for all eight protein–DNA complexes that formed during EMSA. The FPI probe was incubated with nuclear proteins extracted from LNCaP, HeLa, MCF-7 and PC-3 cells. Since no major differences in protein complex formation were observed between nuclear extracts derived from androgen-starved or -supplemented cells, methylation-protection assays were performed with extracts from cells treated with 10 nM R1881. In this assay, DMS is used to methylate unprotected guanines at position N-7 and adenines at position N-3 of the DNA double helix, while piperidine is used to cut these methylated bases. However, adenines at position N-3 of the DNA helix are not usually accessible to piperidine cleavage and are cleaved only when the DNA has been distorted by protein binding. Further, not all guanines are cleaved with equal efficiency, resulting in faint bands in areas where there is less-efficient cleavage. Because protein-bound DNA is electrophoretically separated from unbound radiolabelled probe, footprinting analyses can be performed with even minimal binding of protein to probe. This makes the assay very sensitive, enabling the resolution of DMS-protected regions using nuclear extracts displaying low binding levels for the described PB promoter elements, such as HeLa nuclear extract [19]. Following

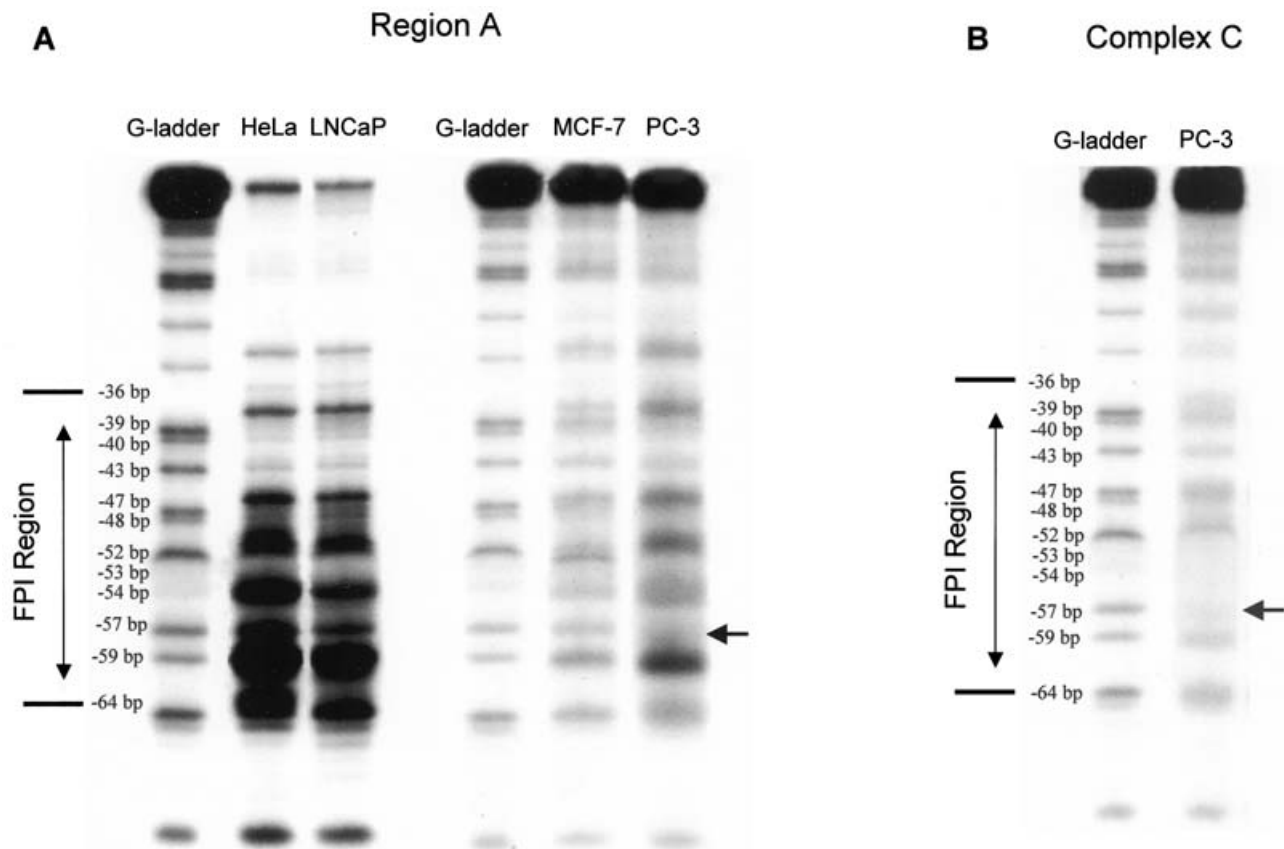


Figure 3 Determination of protein–DNA guanine contacts for (A) region A and (B) complex C binding to the FPI region

Methylation-protection analysis was performed on FPI region A and complex C. An arrow denotes the protected guanines at -57 bp. Each methylation-protection assay was repeated at least twice. The lower strand is shown in this Figure.

gel extraction and chemical cleavage, equal amounts of labelled DNA are then separated on a gel. Each protein–DNA complex was excised and footprinted individually and complexes that migrated closely on the gel were cut out collectively and footprinted together (e.g. region A on Figure 2). Footprints of region A and complex C both showed unique protection of a particular guanine located at -57 bp of the FPI region only upon incubation with PC-3 nuclear extracts (Figure 3). No other EMSA complex showed cell-line-specific guanine protection.

When bound to proteins, the conformation of DNA may change, causing certain bases to become more exposed and prone to methylation, resulting in hypersensitive sites. While the methylation-protection experiments revealed several guanine- and adenine-hypersensitive sites in the EMSA complexes, none of these appeared to be exclusively associated with a particular cell line (Figure 3). Similarly, complex D, which appeared in higher amounts in EMSA experiments with PC-3 cells, did not show unique cell-specific patterns of purine hypersensitivity or footprints of methylation protection (results not shown). Because only cell-line-specific hypersensitivities and protections were studied, universal differences shown on the footprints were not denoted. In the absence of major differences between the binding patterns of prostate- and non-prostate-derived nuclear extracts, no attempts were made to identify the proteins that bind to this region of the PB promoter.

Specific EMSA complexes that formed with the FPII probe are prostate-enriched and androgen-independent

To test for tissue and binding specificity of proteins interacting with the FPII protected region, a FPII probe was generated for EMSA studies with prostate- and non-prostate-derived cell lines. Figure 4(A) shows the EMSA results after incubating the FPII probe with nuclear extracts from HeLa, LNCaP, MCF-7 and PC-3 cells untreated or treated with R1881. The FPII probe yielded four prominent protein–DNA complexes, labelled as complexes E, F, G and H. Whereas complex H was found in all nuclear extracts tested, only small amounts of complex E were formed upon incubation of FPII probe with HeLa nuclear extracts, although a prominent band was seen when using LNCaP, MCF-7 and PC-3 nuclear extracts (Figure 4A). By comparison, complex F was observed only with LNCaP and PC-3 nuclear extracts and complex G seemed to be present only when using nuclear extracts from PC-3 cells. However, the amount of complex G in PC-3 nuclear extracts was variable and occasionally absent. Complex H appears to contain a ubiquitous factor, since it formed with a similar affinity in FPII binding reactions with all nuclear extracts tested. Unlabelled FPII probe in 100-times molar excess was able to compete effectively away formation of complexes E–G with all tested nuclear extracts but was unable to compete away formation of complex H, suggesting that it represents a non-specific

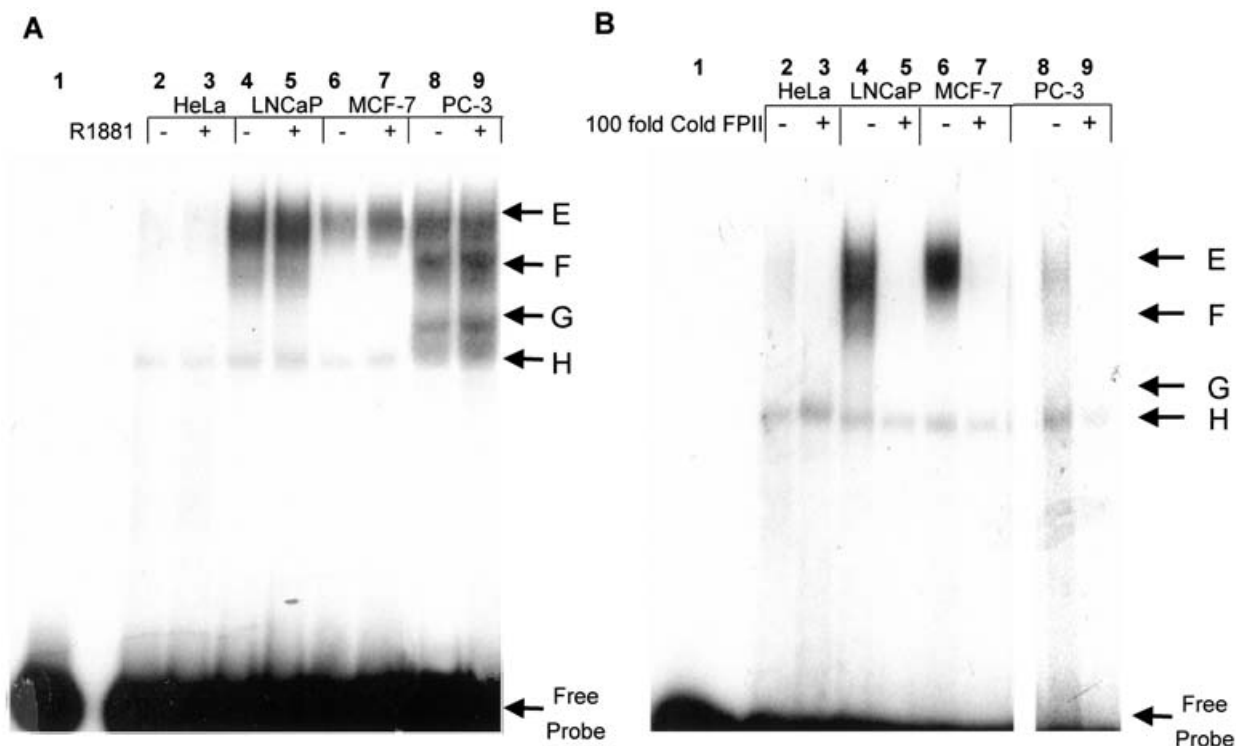


Figure 4 Determination of tissue and binding specificity of FPII protected region

EMSA were performed using labelled FPII probe incubated with nuclear extracts from HeLa, LNCaP, MCF-7 and PC-3 cells, treated or not treated with R1881. (A) Four complexes formed with the various nuclear extracts. Lane 1, control; lanes 2 and 3, HeLa nuclear extracts; lanes 4 and 5, LNCaP nuclear extracts; lanes 6 and 7, MCF-7 nuclear extracts; lanes 8 and 9, PC-3 nuclear extracts. (B) Binding specificity of complexes formed with FPII probe (E–H) were tested by addition of a 100-fold excess of unlabelled FPII probe to the binding reactions in a competition EMSA (lanes 2–9).

type of binding interaction (Figure 4B). Treatment with R1881 (Figure 4A) did not seem to affect the assembly of any of the complexes E–H, confirming that the binding is hormone-independent, as predicted from the DNaseI footprint analysis (Figure 1B).

Methylation-protection analysis show prostate-specific protection patterns in EMSA complexes

While the above EMSA experiments provide an indication of the relative quantitative differences with respect to the occurrence of proteins in different cell lines that bind to FPII sequences, to assess any qualitative differences in binding, highly sensitive methylation-protection analyses were conducted using a pBluescript SK⁻-cloned, single-end-³²P-labelled FPII probe for the identification of guanine–protein contacts for all four protein–DNA complexes (E–H) formed. The FPII probe was incubated with nuclear proteins extracted from LNCaP, HeLa, MCF-7 and PC-3 cells and each EMSA complex was isolated and analysed separately. Complexes E and F were cut out and methylation footprinted together because in EMSA preparations for DMS footprinting, where large amounts of radioactive probe were used, it was difficult to resolve the two complexes separately because they migrated so closely together. Whereas several guanines within the FPII region were DMS-protected by proteins in complexes from all sources of nuclear extracts tested (see the next section), only the guanine located at –79 bp of the PB promoter was found to be protected exclusively in methylation-protection experiments on complexes E and F with both LNCaP and PC-3 cell extracts (Figure 5). The universally protected guanines are not denoted on the footprint (Figure 5). This suggests

that there may be some proteins within complex E/F binding to the FPII region that are unique or greatly enriched in the prostate cancer cell lines. Again, universally protected guanines and hypersensitive sites were not denoted on the footprints.

As mentioned earlier, piperidine cleaves guanines and also adenines, but with less efficiency. It is possible that binding of proteins to these small DNA probes induces a conformational change that allows for more efficient cleavage of adenines. This accounts for the additional guanine- and adenine-hypersensitive sites seen in the footprinted regions when using the different nuclear extracts compared with the G/A ladder.

Database searches reveal two potential candidates binding to the FPII region

To determine which known transcription factors are able to bind to the FPII region of the PB promoter, database searches using MatInspector and ConSite were used to identify potential protein-binding sites within the FPII region. Results suggested that the FPII protected region contains motifs most closely resembling c-Myb or NF-I consensus binding sites (shown boxed in Figure 6), with 100% core similarity and 93.9% matrix similarity in both cases.

FPII oligonucleotides compete with c-Myb consensus oligonucleotides for binding of c-Myb

A radiolabelled probe of the c-Myb consensus binding site was used in EMSA studies to compare the binding characteristics of c-Myb to the FPII region. In these studies, nuclear extracts from

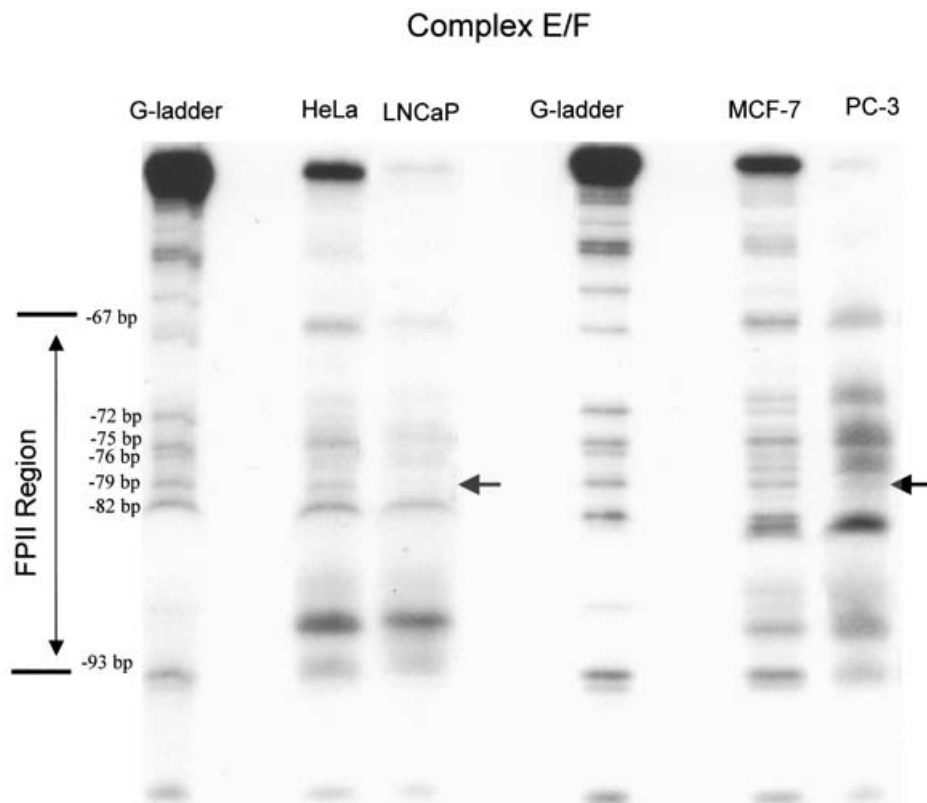


Figure 5 Determination of protein–DNA guanine contacts for complexes E and F, binding to the FPII region

Methylation-protection analysis was performed on FPII complexes E and F. The protected guanine at -79 bp is denoted by an arrow. Each methylation-protection assay was performed at least twice. The lower strand is shown in this Figure.

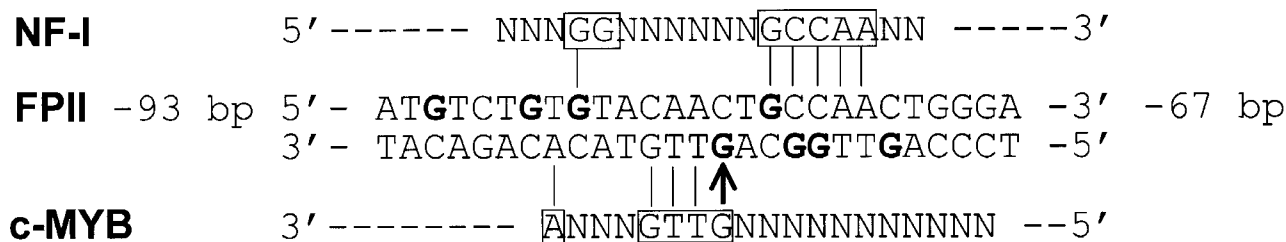


Figure 6 Comparison of FPII region-protected guanines with the c-Myb and NF-I consensus binding sites

Guanine protections from DMS modification of FPII region complexes E–H using LNCaP and PC-3 nuclear lysates are highlighted in bold. The core sequences of the binding sites of the c-Myb and NF-I proteins are denoted with boxes. The prostate cell line specific guanine protection at -79 bp is denoted by an arrow.

Jurkat cells (B-cell line) were used, as they have been shown to express large endogenous amounts of c-Myb [20,21]. EMSA studies using the 18 bp c-Myb probe and Jurkat nuclear extracts resulted in the formation of a high-molecular-mass complex (Figures 7A and 7B, lanes 2) that migrated to approximately the same position as FPII complexes E and F (Figure 4). Addition of c-Myb antibody to the binding reactions resulted in a supershift, verifying the presence of c-Myb protein within the complex (Figure 7A, lane 3). Further, when unlabelled c-Myb probe was added to the reaction in 100-fold excess, it effectively competed away formation of the protein–DNA complex, showing that binding was specific for c-Myb (Figure 7A, lane 4). To test how

closely the binding sites of the FPII region resemble that of the consensus c-Myb-binding site, unlabelled FPII probe was used as a competitor in c-Myb/Jurkat extract EMSA studies (Figure 7B). Unlabelled FPII probe used in 25-, 50-, 75- and 100-fold molar excesses in a competition EMSA against labelled c-Myb probe (Figure 7B, lanes 3–6 respectively) competed for c-Myb protein present in Jurkat nuclear extracts in a concentration-dependent manner. By comparison, addition of a 100-fold molar excess of unlabelled FPII was relatively ineffective (Figure 7B, lane 7).

The c-Myb probe was then used in binding reactions with LNCaP nuclear extracts to determine the level of c-Myb present

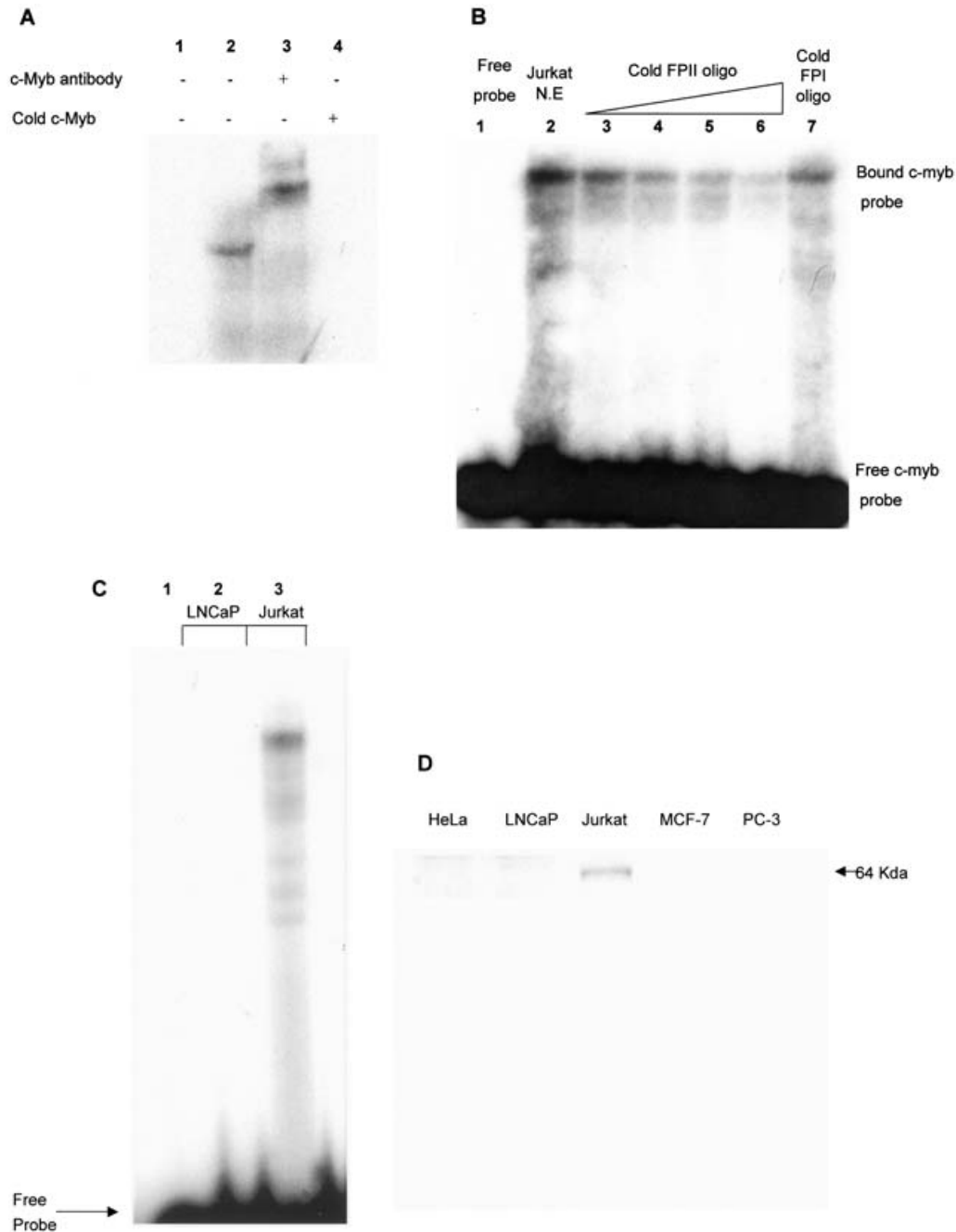


Figure 7 Competition between FPII probe and c-Myb consensus oligonucleotides for c-Myb protein binding

(A) Jurkat nuclear extracts (18 μ g/binding reaction) were used to shift labelled c-Myb probe (lane 2). Binding specificity was confirmed by supershift with anti-c-Myb antibody (lane 3) and by competition with a 100-fold excess of unlabelled c-Myb probe: (lane 4). (B) Binding similarity of c-Myb and FPII protein is indicated by competition experiments with increasing amounts of unlabelled FPII oligonucleotide. Lane 1, control; lane 2, Jurkat nuclear lysate only; lanes 3–6, 25-, 50-, 75- and 100-fold excess unlabelled FPII. Unlabelled FPII probe, added in 100-fold molar excess, was used as a negative control (lane 7). (C) Nuclear lysates from LNCaP and Jurkat cells were used in an EMSA of the consensus c-Myb probe: lane 1, free probe; lane 2, LNCaP extract; lane 3, Jurkat extract. (D) Nuclear fractions were prepared from HeLa, LNCaP, Jurkat, MCF-7 and PC-3 cells. Protein (15 μ g) was loaded in each lane and separated by SDS/PAGE, blotted and analysed for the presence of c-Myb protein using the c-Myb antibody.

in these cells. Figure 7(C) shows the results of electrophoresing the c-Myb probe following incubation with nuclear extracts from androgen-supplemented LNCaP and Jurkat cells. No detectable shift was observed using LNCaP nuclear extracts, suggesting that there is little c-Myb protein present in LNCaP cells (Figure 7C, lane 2). This was further supported using Western blot analysis to check for the presence of c-Myb protein in HeLa, LNCaP,

Jurkat, MCF-7 and PC-3 cells. The blot revealed a 64 kDa c-Myb band in Jurkat nuclear lysate which was not detected in extracts from the other cell lines tested (Figure 7D). Together these results suggest that while c-Myb can bind to the FPII region, the low concentration of c-Myb in LNCaP and PC-3 nuclear extracts make it an unlikely candidate to account for the binding patterns seen with the prostate cell lines.

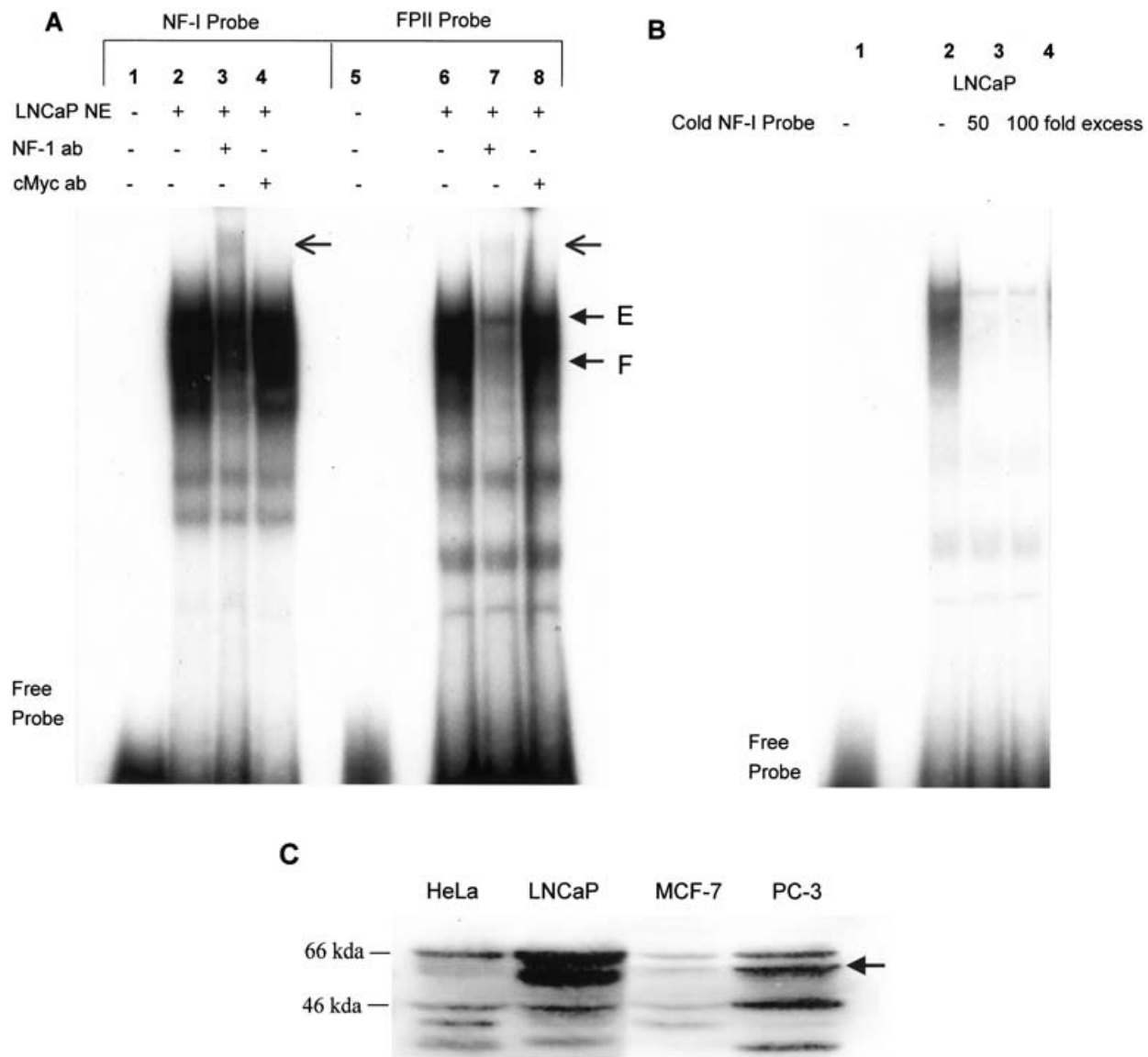


Figure 8 Protein binding to the FP1I region exhibit NF-I-like characteristics

(A) LNCaP nuclear lysates were incubated with labelled NF-I (lanes 1–4) or FP1I (lanes 5 and 6) probe. Supershift experiments were done to test for NF-I protein binding to the labelled probes (lanes 3 and 7, 2 μ g of NF-I antibody). c-Myc antibody was used as a negative control (lanes 4 and 8, 2 μ g of c-Myc antibody). Supershifted complexes are denoted by arrows. (B) Specificity of binding is indicated by competition experiments with unlabelled NF-I oligonucleotide. Lane 1, control; lane 2, LNCaP nuclear lysate only; lanes 3 and 4, 50- and 100-fold excess of unlabelled NF-I probe respectively. Each binding reaction contains 18 μ g of LNCaP nuclear lysate. (C) Nuclear fractions were prepared from HeLa, LNCaP, MCF-7 and PC-3. A total of 15 μ g of protein was loaded in each lane and separated by SDS/PAGE, blotted and analysed for NF-I protein using the NF-I antibody. The NF-I isoform that seems to be enriched in prostate cell lines is denoted by an arrow.

Protein binding to the FP1I oligonucleotide displays NF-I-like characteristics

To test for NF-I binding in LNCaP extracts, EMSA studies were carried out using LNCaP extracts with oligonucleotides corresponding to the consensus NF-I-binding site (Figure 8A). The 33 bp NF-I probe formed a protein–DNA complex (Figure 8A, lane 2) that migrated to approximately the same position as the 27 bp FP1I complex E/F (Figure 8A, lane 6). Addition of NF-I antibody resulted in a supershift of the NF-I probe, verifying that the protein–DNA complex forming with the probe contained the NF-I protein (Figure 8A, lane 3). No supershift or interference of binding was seen when c-Myc antibody (Figure 8A, lane 4) was used as a negative control. To test for NF-I protein binding to

the FP1I region, supershift studies were performed using the NF-I antibody on the 32 P-labelled FP1I probe shifted with LNCaP nuclear extracts. Addition of the anti-NF-I antibody resulted in a very weak supershift and partial blockage of complex E/F formation (Figure 8A, lane 7). Unlabelled NF-I consensus binding site added in 50- and 100-fold excess was also able to effectively compete away the binding of complex E/F to FP1I (Figure 8B, lanes 3 and 4), showing that complexes E and F have similar binding characteristics to that of NF-I. This suggests that complexes E and F contain the NF-I or an NF-I-like protein.

To determine the extent of NF-I protein in the different nuclear extracts, Western blot analysis was performed using a polyclonal NF-I antibody which detects a number of NF-I isoforms. Bands ranging from \approx 40 to 66 kDa were detected in nuclear lysates

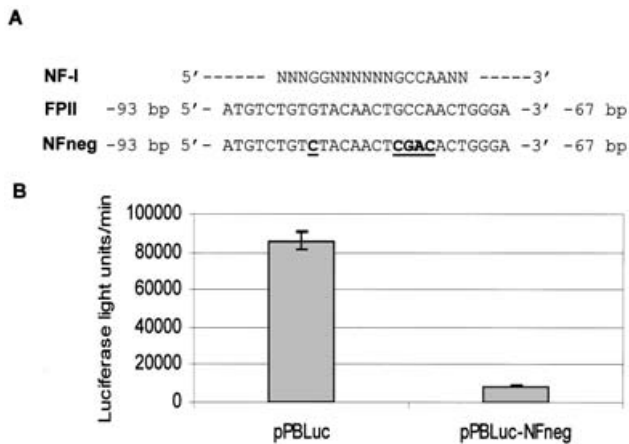


Figure 9 PB promoter activity is dependent on a putative NF-I half-site

(A) Site-directed mutagenesis was used to alter a putative NF-I half-site within the FPII region of the pPBLuc reporter plasmid. The sequences of the NF-I consensus binding site, the FPII region of the PB promoter and the mutation sequence (NFneg; altered bases bold and underlined) are shown. (B) LNCaP cells were transfected with pPBLuc or pPBLuc-NFneg reporter plasmid (0.4 μ g/well) and pRL-TK (0.01 μ g/well). Subsequent to transfection, cells were incubated for 24 h in RPMI medium supplemented with 5% charcoal-stripped serum in the presence of 10 nM R1881. Luciferase activities were corrected for transfection efficiency and are expressed as luciferase light units/min.

from HeLa, LNCaP, MCF-7 and PC-3 cells (Figure 8C). Four of the detected bands roughly correspond in size to the four human isoforms of NF-I (NF-IA, NF-IB, NF-IC and NF-IX), while the other band(s) present may represent splice variants of the protein [22]. Of all the bands observed in the Western blot, the second band from the top (denoted by an arrow in Figure 8C) seems to be the most prostate-enriched, suggesting that this isoform may bind to the FPII region of the PB promoter. The small range of sizes occupied by described NF-I isoforms makes positive identification of the prostate-enriched NF-I variant difficult.

PB promoter activity is highly dependent on a putative NF-I half site

Site-directed mutagenesis of the pPBLuc reporter plasmid was used to generate the pPBLuc-NFneg reporter plasmid. Five bases were altered to disable the core NF-I half site detected during database searches (Figure 9A). When the pPBLuc and pPBLuc-NFneg plasmids were transiently transfected into LNCaP cells the mutated vector showed expression levels that were $\approx 10\%$ of that displayed by the native pPBLuc plasmid (Figure 9B). This reduction in expression levels was statistically significant (t test, $P < 0.05$).

DISCUSSION

The PB promoter has previously been shown to confer high levels of androgen-regulated expression of reporters and other exogenous genes in the prostates of transgenic mice [10,23]. However, the critical proteins and transcription factors required for regulation of this high level of prostate specificity are currently unknown. In this study, several protected regions were identified in the proximal PB promoter (-286 to $+28$ bp) when using nuclear protein extracts from the LNCaP prostate cancer cell line in DNaseI footprint experiments (Figure 1). Many of these had been previously identified as AR E and G-sites to which AR bind

in the presence of androgens [12]. An additional pair of protected regions, referred to as FPI and FPII, were also observed, but shown to be insensitive to the presence or absence of androgens. Binding to the FPII region also seemed to be prostate-cell-line specific.

To examine the tissue and binding specificity of the FPI region in detail, EMSA studies were carried out using nuclear extracts from prostate- and non-prostate-derived cell lines (Figure 2). FPI formed at least eight different and specific bands or complexes with all nuclear extracts tested (HeLa, LNCaP, MCF-7 and PC-3). While there did not seem to be any complexes which were unique to both PC-3 and LNCaP prostate cancer cell lines, there was considerable variation in the relative intensity or concentration of individual complexes between different sources of nuclear extracts. However, in every case, the intensities were not dependent on the presence of androgens. With respect to prostate cell lines, complex B was most prominent when LNCaP nuclear extracts were used, while complexes C and D formed most readily when using nuclear extracts from PC-3 cells. Further detailed qualitative analysis of each of these complexes using highly sensitive DMS footprinting showed several protected and hypersensitive purine interactions, but failed to reveal any major differences between prostate and non-prostate sources. However, unique protection of one guanine (at -57 bp) in the FPI region was observed with PC-3 but not with LNCaP nuclear extracts. Overall, the complicated yet somewhat generic pattern of protein binding to the FPI region suggests that many proteins and nuclear factors common to all cells bind to this region. Since FPI is located ≈ 30 bp upstream of the transcription start site of the PB promoter and contains a potential CAAT box, much of the transcriptional machinery and basal transcription factors may bind to this region, accounting for the complicated pattern of binding observed in EMSA and methylation-protection experiments. Hence, it is unlikely that the FPI region plays a discriminating role or makes a major contribution to prostate specificity of the PB promoter.

In contrast, the binding profile associated with the FPII region of the PB promoter shows attributes that are uniquely associated with prostate-derived cell lines. EMSA revealed at least four androgen-independent, discrete bands or complexes (E-H), with considerably greater amounts of each measured with nuclear extracts from LNCaP and PC-3 prostate cancer lines (Figure 4). One of the complexes was non-specific, whereas the remaining three were displaceable with excess unlabelled FPII oligonucleotides. In addition to quantitative differences suggesting enrichment of these binding proteins in prostate cancer lines, methylation-protection analyses performed on each of the complexes indicated that one particular protein-guanine contact, located at -79 bp, was protected only when using LNCaP and PC-3 prostate cell lines (Figure 5); although several protected guanines were found to be common to all or most of the cell types tested (Figure 6). Together the results suggest that some of the proteins that bind to the FPII region may play an important role in specifically regulating gene transcription in prostate cell lines.

The FPII binding site was found to contain high sequence similarity to the consensus binding sites for both c-Myb and NF-I [13-15]. While unlabelled FPII probe was capable of competitively and specifically inhibiting the binding of c-Myb protein to its consensus binding site (Figure 7B), the reciprocal was not true in that LNCaP extracts did not show any detectable level of binding to c-Myb consensus sequences (Figure 7C). Furthermore, since Western blots indicated that little or no c-Myb was present in LNCaP cells (Figure 7D), it is unlikely that c-Myb or a closely related protein accounts for the relatively

unique FPII binding patterns seen with LNCaP and PC-3 cell extracts.

By comparison, the NF-I protein seemed to be a far more likely candidate to account for prostate-specific binding to the FPII region. While NF-I proteins are relatively ubiquitous transcription factors that participate in the expression of various genes [24], they have been implicated in tissue-specific gene expression, possibly by interacting with various other factors [25]. EMSA experiments implied that LNCaP nuclear extracts contain significant amounts of an NF-I-like protein that appear to bind equally well to an NF-I consensus sequence and to an FPII oligonucleotide (Figure 8A). Similarly, unlabelled NF-I consensus oligonucleotides could competitively displace binding to FPII sequences by LNCaP extracts (Figure 8B). Inclusion of antibodies to NF-I resulted in a very weak supershift and a partial blockage of the formation of E/F complexes (Figure 8A). This partial inhibition of binding is likely to be due to the antibody binding to an epitope which encompasses the highly conserved DNA-binding domain of the protein, located in the first 200 amino acids of the N-terminus [26–29].

All NF-I transcription factors analysed to date bind as protein dimers to composite DNA sites consisting of palindromic repeated motifs, with a spacing segment of either five or six nucleotides (underlined) between two dyad-symmetric pentameric half sites TTGGCNNNNN(N)GCCAA [29–32]. In addition, half sites alone have also been reported to be sufficient for promoter binding and for transcriptional activation [32,33], which illustrates the complex mode and variation of DNA sequence recognition by NF-I proteins [30]. Alteration of the putative NF-I half site within the FPII region of the pPBLuc plasmid resulted in a significant loss of expression activity (Figure 9). This further implicates an NF-I isoform or a related protein species in the regulation of the PB promoter. Western blots for NF-I proteins clearly indicate varying amounts of several NF-I isoforms in all the cell lines tested (Figure 8C), with perhaps one species particularly enriched in the prostate cancer lines. However, NF-I binding alone cannot account for the pattern of FPII guanine contact sites, including the prostate-specific one seen at –79 bp (Figure 6). Rather it is likely that NF-I or an NF-I-like protein contributes to a more complex mixture of FPII-binding proteins. The ability of NF-I proteins to form both homodimers and heterodimers, differences in the C-terminal region and alternative splicing have resulted in the large diversity of activities that can be attributed to different members of this family [26,34–36].

In order to develop a successful systemic gene therapy to target androgen-independent, metastatic prostate cancer, two criteria need to be met: first, the therapeutic transgene used to control the tumour should only be expressed in prostate tissue and, second, the expression should not be dependent on enhancement by androgens (because of the use of androgen-withdrawal therapy in advanced disease). Towards meeting these criteria, we have found that binding of nuclear proteins to at least one DNA region within the PB promoter, FPII, occurs independently of the presence of androgen or AR and that, relative to non-prostate cell lines, prostate cancer lines show both enhanced and specific binding to this region. The latter observation may be due in part to the contribution of an isoform of NF-I or related protein that is enriched or unique to prostate cells. Whether this region is sufficient to direct prostate-specific gene expression is uncertain and awaits tests in transgenic systems.

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