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FAST TRACK

RUNX1 (AML-1) and RUNX2 (AML-3) Cooperate With Prostate-Derived Ets Factor to Activate Transcription From the *PSA* Upstream Regulatory Region

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Abstract The RUNX transcription factors (RUNX1, RUNX2, and RUNX3) play essential roles in hematopoiesis and skeletal development. Consistent with these roles in differentiation and cell cycle, the activity of both RUNX1 and RUNX3 is perturbed in cancer. To determine a role for the RUNX factors in prostate biology, we investigated the expression of RUNX factors in prostate epithelial cell lines and normal prostate tissue. RUNX1, RUNX2, and RUNX3 were expressed in both normal prostate tissue and an immortalized, non-transformed cell line. We found that prostate cancer-derived cell lines expressed RUNX1 and RUNX2, but not RUNX3. Next, we sought to identify prostate-specific genes whose expression could be regulated by RUNX proteins. Four consensus RUNX sites are located within the prostate-specific antigen (PSA) regulatory region. Chromatin immunoprecipitation (ChIP) analysis showed that RUNX1 is specifically bound to the *PSA* regulatory region in LNCaP cells. RUNX1 and RUNX2 activated the *PSA* regulatory region alone or cooperatively with *p*rostate-*d*erived *E*TS *f*actor (PDEF) and RUNX1 physically associated with PDEF. Taken together, our results suggest that RUNX factors participate in prostate epithelial cell function and cooperate with an Ets transcription factor to regulate *PSA* gene expression. J. Cell. Biochem. 97: 1–17, 2006.

Key words: transcription; prostate cancer; RUNX; PDEF; prostate-specific antigen

The RUNX family of transcription factors is encoded by three genes, *RUNX1*, *RUNX2*, and *RUNX3* (AML-1, AML-3, and AML-2, respectively) [Levanon et al., 1994; Meyers et al., 1995]. The highly related proteins encoded by these genes bind DNA as heterodimers with the non-DNA binding protein CBFβ, creating a RUNX/CBFβ complex termed the core binding factor (CBF) [Wang and Speck, 1992; Ogawa et al., 1993]. The RUNX proteins bind to the consensus DNA site (TGT/CGGT) via the runt homology domain (rhd) and function as both transcriptional activators and repressors depending upon promoter and cellular context [Gergen and Butler, 1988; Meyers et al., 1993]. The rhd also mediates protein–protein interactions with a variety of partners including CBFβ and members of the Ets transcription factor family [Melnikova et al., 1993; Wotton et al., 1994].

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RUNX1 was first identified as the target of the t(8;21) chromosomal translocation found in acute myelogenous leukemia [Miyoshi et al., 1991]. Subsequent studies have suggested that RUNX1 is a master regulator of hematopoiesis, and *Runx1* null mice die in utero (E11.5) due to a complete lack of definitive myeloid and erythroid progenitors in the fetal liver [Okuda et al., 1996; Wang et al., 1996a]. Mice null for *CBFβ* show a very similar phenotype, providing genetic evidence that *Runx1* and *CBFβ* function together to regulate hematopoietic development [Sasaki et al., 1996; Wang et al., 1996b]. In addition to regulating hematopoiesis-specific genes, *RUNX1* is also implicated in the regulation of cell-cycle genes, including $p21^{WAF1/CIP1}$, which encodes a cyclin-dependent kinase inhibitor important for checkpoint controls and terminal differentiation [Lutterbach et al., 2000]. Over-expression of RUNX1 also accelerates cell-cycle progression and shortens the G_1 phase in the 32D.3 myeloid progenitor cell line [Strom et al., 2000]. Similar to RUNX1, mutations in RUNX3 and RUNX2 have been identified in human disease. RUNX3 haploinsufficiency is associated with gastric adenocarcinoma [Li et al., 2002]. *Runx*3 null mice exhibit gastrointestinal tract deficiencies including hyperplasia of the epithelial stomach lining and a loss of apoptosis secondary to an inability to respond TGFβ signaling [Ito and Miyazono, 2003]. RUNX2 plays an essential role in osteoblast differentiation, and haploinsufficiency of RUNX2 causes cleidocranial dysplasia (CCD), a syndrome characterized by generalized bone defects and growth retardation [Bergwitz et al., 2001; Otto et al., 2002]. Consistent with this, *Runx2* null mice show arrested osteoblast differentiation [Komori and Kishimoto, 1998]. These findings confirm a role for RUNX factors in the control of proliferation and differentiation outside of the hematopoietic system and suggest that the RUNX factors may be lost or deregulated in cancers of epithelial origin.

To date, most RUNX studies have focused on the roles of these proteins in the hematopoietic, bone and gastric systems, and it remains to be determined whether RUNX proteins regulate proliferation and differentiation in other tissues. Recent studies have suggested that RUNX2 is expressed in prostate cancer cells and may trigger the inappropriate expression of bone specific genes in these tissues [Yeung et al., 2002]. These reports led us to inquire about the roles of RUNX proteins in both the normal and malignant prostate. In addition to providing a broader understanding of basic transcriptional pathways underlying the development of the prostate, investigation into the roles of RUNX proteins might provide insight into the cancer-specific deregulation of these cell cycle and differentiation pathways.

We first investigated the expression of RUNX factors in prostate epithelial cell lines and normal prostate tissue. We found expression of RUNX1, RUNX2, and RUNX3 in normal prostate tissue and an immortalized, non-transformed cell line, and expression of RUNX1 and RUNX2, but not RUNX3, in prostate-cancer derived cell lines. We next sought to identify prostate-specific genes whose expression might be regulated by RUNX proteins. We identified four consensus RUNX sites in the prostate-specific antigen (PSA) regulatory region [Cleutjens et al., 1996, 1997b]. PSA, a serine protease component of prostatic secretion, is an important serum marker used for screening and staging prostate cancer, and its expression is highly prostate-specific [Duffy, 1996]. We found that RUNX1 is bound to the *PSA* regulatory region in vivo, and both RUNX1 and RUNX2 activated the *PSA* regulatory region alone or cooperatively with PDEF (*p*rostate-*d*erived *E*TS *f*actor), a novel Ets transcription factor [Oettgen et al., 2000]. In vitro GST-capture and co-immunoprecipitation assays demonstrated that RUNX1 physically associated with PDEF. Based on these results, we propose that RUNX factors participate in prostate epithelial cell function and may cooperate with PDEF to regulate *PSA* gene expression.

MATERIALS AND METHODS

Cloning and Expression Constructs

To create the PDEF expression construct, the cDNA for PDEF was PCR amplified from normal prostate cDNA (Becton-Dickinson Clontech, Palo Alto, California) with primers 5′-TATCTAGAATGGGCAGCGCCAGCCCGGGT-3 and 5′-TAGGATCCTCAGATGGGGTGCACGAACTG-3′. The amplified product was sequenced (Iowa State Sequencing Facility) and subcloned into the XbaI and BamHI sites of the J3M plasmid in frame with the Myc epitope. The resultant Myc-tagged PDEF cDNA was excised from J3M by HindIII and EcoRI digestion and subcloned into pCDNA3. For preparation of

RUNX1–GST recombinant proteins, RUNX1 was subcloned into pGex4T-1. The RUNX1 N-(amino acids 1–91) and C-(amino acids 229–480) termini were PCR amplified, confirmed by sequencing and then subcloned into the EcoR1 and XhoI sites of pGex 5X-3. PSAProLuc was constructed by first excising a portion of the *PSA* regulatory region containing the 632 bp *PSA* promoter [Cleutjens et al., 1996] from 7PSA-Luc by digestion with EcoRI and XhoI. The resulting fragment was subcloned into the EcoRI and XhoI sites of the pBluescript vector (Stratagene, La Jolla, California). This plasmid, pskPSAPro, was digested with SmaI and XhoI, and the fragment was inserted at the SmaI and XhoI sites in the PGL2Basic vector (Promega, Madison, Wisconsin) to yield PSA-ProLuc which contains the first 643 bases of the PSA regulatory region including the 632 bp promoter defined by Cleutjens et al. The identity of the insert was confirmed by sequencing. The following expression constructs were as previously described: pGex rhd (amino acids 85–214); the reporter construct 7PSA-Luc, which contains the firefly *luciferase* gene under the control of 5.8 kb of the *PSA* upstream regulatory region; pCMV5RUNX1; pCMV5RUNX2; and pCMV5RUNX1L148D [Lenny et al., 1995; Meyers et al., 1995, 1996]. Large-scale plasmid preparations were performed using alkaline lysis followed by PEG precipitation.

Cell Lines and Culture Conditions

C33A (human cervical carcinoma, ATCC number HTB-31), PC-3 (human prostate adenocarcinoma, ATCC number CRL-1435), and 293T (human kidney adenocarcimoma transformed with SV40 T antigen; a generous gift from Dr. C. Sherr, St. Jude Children's Research Hospital) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 1% penicillin–streptomycin, and 1% L-glutamine. PPC1 (human prostate carcimoma), DU145 (human prostate carcinoma, ATCC number HTB-81), and LNCaP (ATCC number CRL-1740) cells were maintained in RPMI supplemented as above. PZ-HPV7 (immortalized prostate epithelial, ATCC number: CRL-2221) and 276B1 (immortalized prostate epithelial) cells were grown in keratinocyte media supplemented with bovine pituitary extract and epidermal growth factor and BRFF-HPC1 media, respectively.

Electrophoretic Mobility Shift Assay (EMSA)

For preparation of whole cell extracts, cells were washed with phosphate-buffered saline (PBS) and sonicated on ice in microextraction buffer (20 mM *N*-2-hydroxyethylpeperazine-*N*-2-ethanesulfonic acid (HEPES; pH 7.4), 450 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 25% glycerol) containing protease inhibitors. The lysates were clarified by high-speed centrifugation, and equal amounts of protein were used in each EMSA reaction. DNA binding reactions were performed at room temperature in 20 mM HEPES (pH 7.8), 1 mM $MgCl_2$, 0.1 mM ethylene glycol-bis (β-aminoethylether-*N*,*N*,*N*′,*N*′-tetraacetic acid; EGTA), 0.4 mM dithiothreitol, 40 mM KCl, and salmon sperm DNA (60 μg/ml). EMSA was performed as described using previously reported oligonucleotides [Meyers et al., 1993]. Supershift experiments were performed with rabbit antisera, and antigenic peptides (2 μg) were used in competition assays as previously described [Meyers et al., 1996].

Transfection and Electroporation

C33A and 293T cells were transfected by a modified calcium phosphate procedure [Chen and Okayama, 1987]. PC-3 cells were electroporated as follows: 5×10^6 cells were resuspended in 1× PBS and placed in an electroporation cuvette (VWR). DNA constructs were added, and the mixtures were exposed to 240 V at room temperature using a Gene Pulser (Bio-Rad, Hercules, California), and cells were plated with fresh complete DMEM (10 ml) and allowed to recover for 36 h.

Transcription Assays

C33A and PC-3 cells were transiently transfected and electroporated, respectively, with 7PSA-Luc and pCMV5-RUNX1, pCMV5-RUNX2, or pCMV5 alone or together with pCDNA3MycPDEF. The Rous sarcoma virus (RSV)-secreted alkaline phosphatase (SEAP) and the Simian Virus 40-Renilla luciferase (phRL-SV40; Promega) plasmids were included in the C33A and PC-3 experiments, respectively, as internal controls for transfection efficiency. Thirty-six hours after transfection or electroporation, cells were harvested, cell lysates were prepared, and the lysates were assayed for protein concentration using the Bio-Rad Protein Assay Dye Reagent. C33A cell

lysates (40 μg total protein) were analyzed for luciferase activity (Promega Luciferase Assay System) and the medium was assayed for SEAP activity, which was used for comparative normalization of the luciferase data. PC-3 cell lysates (40 μg total protein) were analyzed for activity of both firefly and renilla luciferase using the Promega Dual Luciferase Assay System.

Primer Design, PCR, and RT-PCR

Primer pairs were designed to amplify each RUNX family member as follows: (RUNX1: 132 bp) 5′-CCATTGCCTCTCCTTCTGTGC-3′ and 5′-GGGTCGCTGAACGCTGTC-3′, (RUNX2: 106 bp) 5′-CCGTCCATCCACTCTACC-3′, and 5′-TGCCTGGCTCTTCTTACTG-3′, (RUNX3: 150 bp) 5′-CTCACTCAGCACCACAAG-3′ and 5′-GAAGGAGCGGTCAAACTG-3′. These primer pairs were designed so the 5′ and 3′ primers annealed to sequences within different exons to prevent amplification of any contaminating genomic DNA. The specificity of these primers was determined in control PCR reactions containing cDNAs for each family member. Amplifications were performed for 30 cycles at 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min. Products were separated on 2% agarose gels and then stained with ethidium bromide and visualized using UV light. For RT-PCR, RNA was isolated from 5×10^6 cells using the Trizol Reagent (Invitrogen Life Technologies, Carlsbad, California) according to the manufacturer's instructions, and the recovered RNA was diluted to 1 μg/μl. First strand cDNA synthesis was performed using the Super Script First Strand Synthesis System for RT-PCR (Invitrogen Life Technologies) according to the manufacturer's instructions. Reactions (50 μl) were amplified in a Gene Amp PCR System 9600 (Perkin Elmer, Wellesley, MA) as follows: 1 cycle at 94°C for 2 min followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, followed by a final soak of 72°C for 6 min. A fragment of the gene for glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 146 bp) was amplified as an internal control using primers 5′-GTCCACTGGCGTCTTCAC-3′ and 5′-CTTGAGGCTGTTGTCATACTTC-3′.

In Vitro GST-Capture Assays

GST and recombinant GST–RUNX1 and GST–RUNX1 N- and C-terminal segments and GST–RUNX1 Rhd were produced in *E. coli*, purified on glutathione-sepharose beads and incubated for 1 h at 4°C with 200 μl of whole cell lysates derived from 293T cells transiently expressing Myc-tagged PDEF. Immobilized protein complexes were then pelleted and washed five times in IP buffer (50 mM Tris-HCL (pH 7.5), 150 mM NaCl, 0.4% NP-40, 1 mM EDTA) + 0.1% BSA, and four times in IP buffer alone. Proteins were separated by 10% SDS–PAGE and transferred to nitrocellulose membranes. The membranes were subjected to immunoblot analysis using an anti-Myc monoclonal antibody (9E10, Developmental Studies Hybridoma Bank), and developed using the appropriate secondary antibody and the Immun-Star Detection Kit (Bio-Rad). Parallel 10% SDS–PAGE gels containing the same amount of purified GST proteins were run and stained with Coomassie blue for detection of GST proteins.

Immunoprecipitations

To detect in vivo associations between Myc-PDEF and RUNX1, 293T cells were transfected with pCDNA3Myc-PDEF and pCMV5RUNX1 as indicated in Figure 6. Twenty-four hours later, cell lysates were prepared and the proteins were immunoprecipitated with rabbit polyclonal antiserum directed against the N-terminus of RUNX1. Immunoprecipitated proteins were separated by 10% SDS–PAGE, transferred to nitrocellulose membranes and probed with 9E10 monoclonal antibody (specific for the Myc epitope; Santa Cruz), followed by an alkaline phosphatase-conjugated anti-mouse secondary antibody, and visualized using the Immun-Star kit (Bio-Rad).

Site Directed Mutagenesis

PCR-based site-directed mutagenesis was done to introduce a three base mutation in the RUNX1 binding site located within the *PSA* promoter in the pskPSApro plasmid. The primers were: 5′-CTGGGCTAGAGGATCTGTGGCTAACAAGATCTTTTTATG ATGACAGTAGC-3′ (forward) and 5′-GCTACTGTCATCATAAAAAGATCTTGTTA GCCACAGATCCTC TAGCCCAG-3′ (reverse). PCR was performed using the Pfu Turbo DNA polymerase (Stratagene) and the following PCR conditions: at 94°C for 30 s, at 55°C for 30 s, at 68°C for 13 min. Following 16 cycles, the PCR reactions were incubated at 37°C with DpnI (New

England Biolabs, Ipswich, MA) for 1 h to digest parental plasmid. After transformation into *E. coli* and plasmid preparation, site-specific mutagenesis was confirmed by sequencing. The pskPSAPro plasmid containing the targeted mutations in the RUNX binding site were then digested with SmaI and XhoI, and the resulting fragment was subcloned into the SmaI and XhoI sites of the PGL2Basic vector (Promega). This resulting reporter construct, PSAProLucs1m, was sequenced to verify the presence of the desired promoter mutation.

Chromatin Immunoprecipitations

ChIP assays were performed on the *PSA* regulatory region using LNCaP cells and the protocol and buffer recipes provided by the P.J. Farnham laboratory at: http://www.genomecenter.ucdavis.edu/farnham/protocols/chips.html. Cells (2×10^7) per sample were cross-linked with 1% formaldehyde for 10 min. Cross-linking was stopped with the addition of 0.125 M glycine, and cells were washed twice with cold PBS. Cells were collected by centrifugation and lysed in cell lysis buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% Triton-X-100) plus protease inhibitors. The nuclei were pelleted by centrifugation and then lysed in nuclei lysis buffer (50 mM Tris-Cl pH 8.1, 10 mM EDTA, 1% SDS) containing protease inhibitors. Chromatin was sheared to an average length of 1 kbp by sonication and precleared by the addition of protein A sepharose in 1× dialysis buffer (2 mM EDTA, 50 mM Tris-Cl pH 8.0, 0.2% Sarkosyl). Chromatin was immunoprecipitated overnight at 4°C with 10 μl of an IgG purified rabbit polyclonal RUNX1 N-terminus antibody or an equivalent concentration of non-immune rabbit IgG. A no antibody sample and a mock (no chromatin) sample were included as negative controls. To collect immunoprecipitated complexes, 75 μl protein A sepharose in 1× dialysis buffer was added to each sample and rotated at 4°C for 1 h. Immobilized antibody/protein/DNA complexes were washed four times with 1× dialysis buffer and six times with IP buffer (100 mM Tris-Cl pH 9.0, 500 mM LiCl, 1% Triton-X-100, 1% deoxycholic acid). Antibody/protein/DNA complexes were eluted twice by the addition of elution buffer (75 mM $NaHCO_3$, 1% of SDS) followed by vortexing for 15 min. Eluates were combined, 0.3 M NaCl was added to each sample, and the formaldehyde cross-links were reversed by incubation at 67°C for 5 hrs. DNA was precipitated and the samples were digested with proteinase K to remove protein. The DNA was then reprecipitated and resuspended in 30 μl of water. Immunoprecipitate (1–3 μl) and a 1:300 dilution of input chromatin were analyzed by PCR using the Eppendorf Mastercycler gradient thermal cycler and primers specific for three fragments of the PSA regulatory region as well as the β-actin promoter. The primers used for this analysis were: A (−675 to −398) 5′-TAGAGTGTACAGGTCTGGAG-3′ (forward) and 5′-ACCACTCTGAGACCAGAAAC-3′ (reverse); B (−2,905 to −2,676) 5′-TGTGCCAGCATCAGCCTTAT-3′ (forward) and 5′-CTCAATCCTATACCCAGCAC-3′ (reverse); C (−5,124 to −4,961) 5′-GAGATCACCTCTCAGGCTCT-3′ (forward) and 5′AGCATACACTTACACGGCAC-3′ (reverse); and β-actin 5′-TCCTCCTCTTCTCAATCTCG-3′ (forward) and 5′-AAGGCAACTTTCGGAACGG-3′ (reverse). Following 27–34 cycles of amplification, PCR products were run on a 1.8% agarose gel, stained with ethidium bromide, and visualized under UV light.

RESULTS

RUNX DNA Binding Activity and Messenger RNA Are Present in Prostate Tissue and Cell Lines

To test the hypothesis that RUNX factors play a role in prostate cell biology, we first had to establish that RUNX family members are expressed in prostate epithelial cells. We used a double-stranded DNA oligonucleotide containing the RUNX1 consensus binding site (TGTGGT) as a probe in electrophoretic mobility shift analysis (EMSA) [Meyers et al., 1993]. This assay identifies active DNA-binding proteins, including all three RUNX family members. EMSA with whole cell extracts of prostate epithelial cells identified a major DNA/protein complex that could be competed away by the addition of excess unlabelled RUNX-specific probe (Fig. 1). The major complex, CBF, was present in all four-cell lines. Complex A, which was first identified in hematopoietic cells, was specifically competed by unlabelled oligonucleotide and may contain a variant of RUNX1 lacking the C-terminal tail [Meyers et al., 1993]. Another complex (NS) was also identified, but appeared to be non-specific since unlabelled oligonucleotides did not strongly

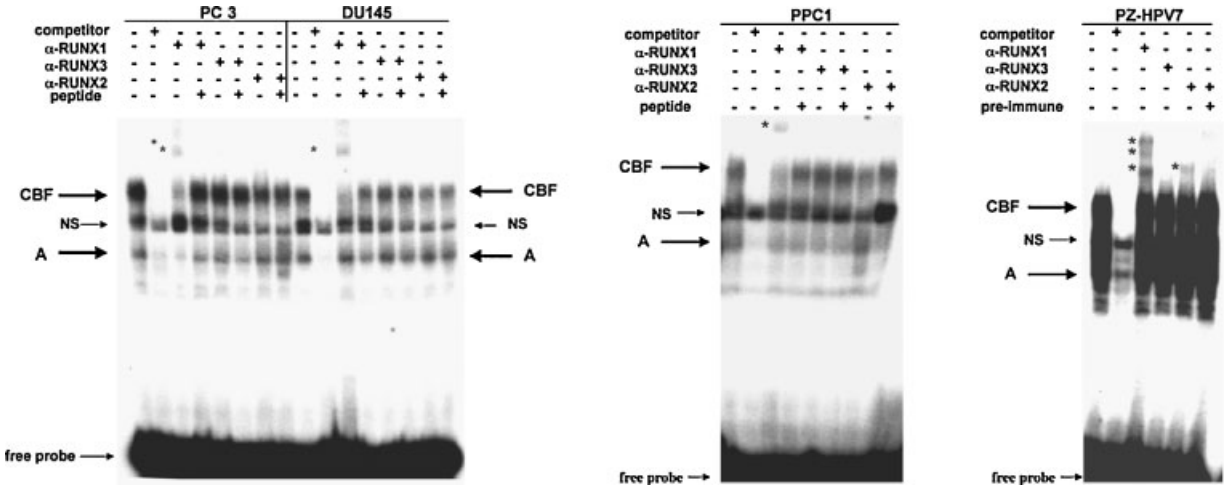


Fig. 1. Identification of RUNX1 and RUNX2 DNA binding activity in prostate cell lines. EMSA of CBF activity in the immortalized PZ-HPV7 cell line and prostate cancer-derived cell lines PC-3, DU145, and PPC1. Protein (10 μg) was incubated with a ^{32}P-labeled DNA probe containing the CBF consensus site (TGTGGT) [Meyers et al., 1993] in the absence or presence of the indicated antibody. Arrows indicate protein/DNA complexes and an asterisk (*) indicates the position of an antibody/protein/DNA complex (supershifted complex). NS, nonspecific complex; A, smaller specific complex previously observed when this oligomer was used with hematopoietic cell lysates; competitor, addition of 100 ng unlabeled oligomer; peptide, addition of the antigenic peptide (1 μg) for each antibody; pre-immune, addition of α-RUNX1 pre-immune sera.

compete for this interaction (Fig. 1). This complex is commonly observed in EMSA of hematopoietic cell line lysates using the RUNX-specific probe [Meyers et al., 1993]. These results demonstrate CBF activity in prostate epithelial cells.

Previously developed antibodies specific to native RUNX1, RUNX2, and RUNX3 were incorporated into the binding reactions [Meyers et al., 1993, 1996]. Addition of these antibodies to the EMSA incubations resulted in diminution of the original complex and the formation of slower migrating 'supershifted' complexes indicative of DNA/RUNX/antibody complexes (Fig. 1; asterisks denote supershifted complexes). The presence of these supershifted complexes together with the diminution of the original complex indicated that RUNX1 was the chief DNA binding component of CBF in the DU145 and PC-3 prostate cancer cell lines, whereas PPC1 and the immortalized PZ-HPV7 cells showed clear evidence of both RUNX1 and RUNX2, as indicated by formation of supershifted complexes and/or diminution of the RUNX1 and RUNX2 complex in extracts from these cells. In both the PC-3 and DU145 extracts, residual CBF DNA binding activity remains in the presence of the RUNX1 antibody; this residual activity is likely that of another RUNX protein. Further, the DU145 lysates exhibited a slight diminution of the CBF complex upon addition of the RUNX2 specific antisera, suggesting the presence of RUNX-2 DNA binding activity. The specificity of the supershift and/or diminution is evidenced by abolishment of this effect by the addition of antigenic peptide (Fig. 1; peptide, PC-3, DU145, and PPC1 panels). Moreover, addition of RUNX1 pre-immune sera did not alter DNA/RUNX migration indicating that the supershifts are specifically formed only with the addition of immune serum (Fig. 1; PZ-HPV7). Taken together, our data identified RUNX1 and RUNX2 DNA binding activity in several prostate cancer cell lines and in the immortalized prostate epithelial cell line PZ-HPV7.

Next, we confirmed and extended the DNA binding data by RT-PCR examination of RUNX family member expression profiles at the mRNA level. Primer pairs specific for each RUNX cDNA were designed so that the 5′ and 3′ primers anneal to sequences within different exons to prevent amplification of genomic DNA. Figure 2A shows a schematic diagram of RUNX1, RUNX2, and RUNX3, illustrating the location of the primers within each gene. Control reactions showed that these primer pairs amplified the expected products from samples containing the appropriate RUNX cDNA but not from samples containing other cDNA templates (Fig. 2B). To analyze RUNX mRNA expression in prostate cell lines, total

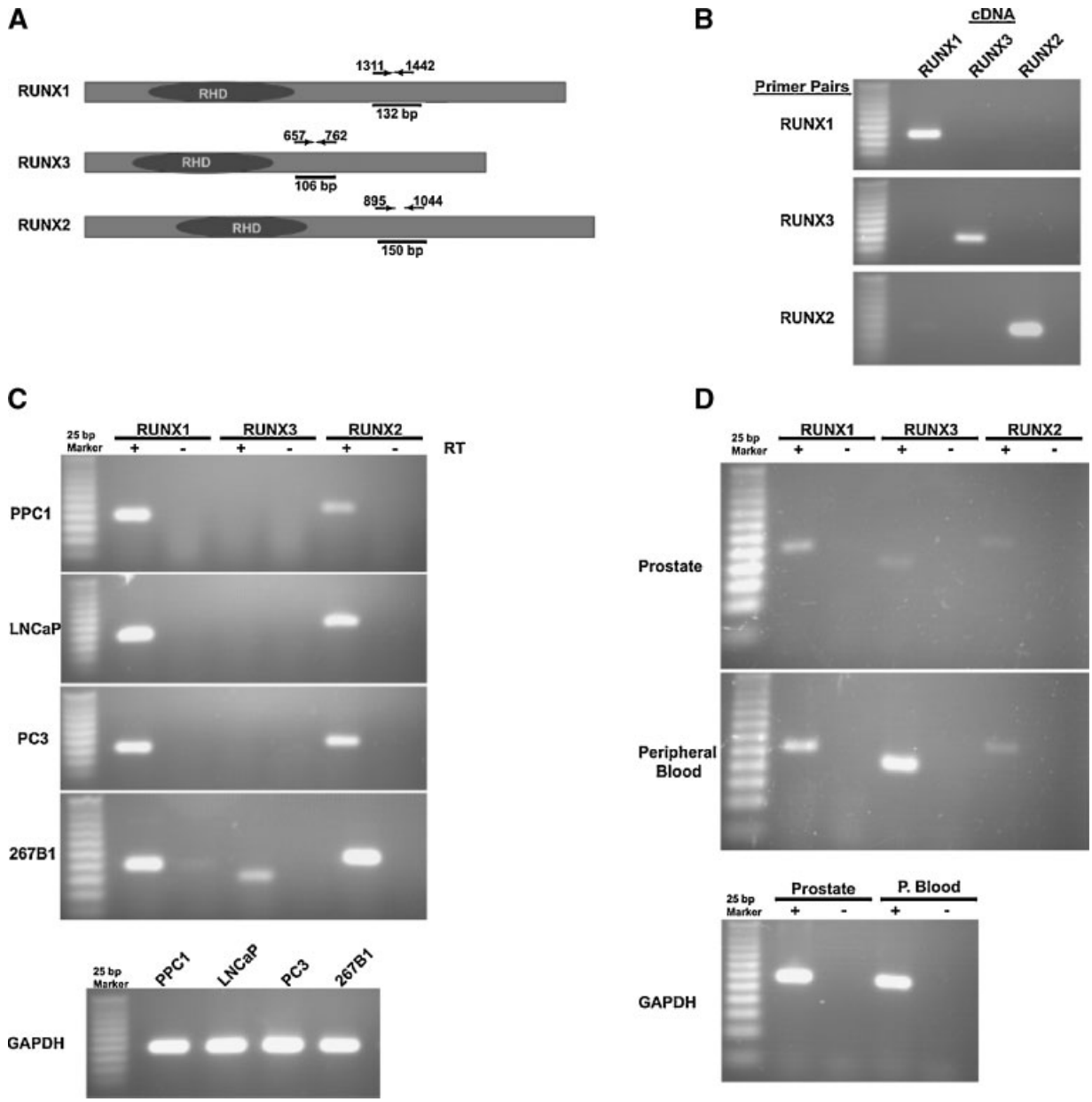


Fig. 2. RT-PCR amplification of RUNX mRNA in prostate and prostate-derived cell lines. Transcripts from RUNX1, 2, and 3 were specifically detected with RT-PCR amplification of mRNA isolated from various prostate-derived cell lines and prostate tissue. **A**: Schematic representation of the cDNAs for RUNX1, RUNX2, and RUNX3, showing the location of the various primers. Runt homology domain (RHD) refers to the runt homology domain. The base pair number for each primer is indicated and based upon the following GenBank sequences: RUNX1 (NM001754), RUNX3 (X79550), and RUNX (NM004348). **B**: Specific amplification of each RUNX family member. Primer specificity was demonstrated by testing the ability of each primer pair (**left**) to amplify the predicted product in PCR using cloned cDNAs for each RUNX family member (**top**). **C**: RT-PCR of RUNX family members (specific primers listed at the **top**) in prostate cancer-derived cell lines PPC1, PC-3, and LNCaP, and the immortalized, non-transformed prostate-derived cell line 267B1 (listed at **left**). Parallel reactions with (+) and without (−) reverse transcriptase served as controls. **D**: RUNX1 is expressed in normal prostate tissue. cDNAs prepared from normal human prostate and peripheral blood (Becton Dickson Clontech) were subjected to RT-PCR using the primer sets for each RUNX family (**top**) and 1 ng of cDNA. The addition or omission of cDNA is indicated (+ or −, respectively); amplification products were analyzed on 2% agarose gels, stained with ethidium bromide and visualized under ultraviolet light. Glyceraldehyde-3 phosphate dehydrogenase (GAPDH) was amplified to show the presence of equivalent amounts of cDNA in each reaction shown in panels C and D.

RNA was isolated from the prostate cancer cell lines LNCaP, PPC1, and PC-3, and the immortalized, non-transformed prostate epithelial cell line 267B1. RT-PCR was performed and the amplified products were separated on agarose gels, stained with ethidium bromide, and visualized under UV light. As seen in Figure 2C, specific amplified products demonstrated the presence of RUNX mRNA in all tested cell lines. The prostate cancer cell lines PPC1, LNCaP and PC-3 expressed both RUNX1 and RUNX2 mRNA. RUNX3 expression was not identified in these cell lines. In contrast, the immortalized, non-transformed 267B1 cell line yielded specific products for each RUNX family member (Fig. 2C). Amplification

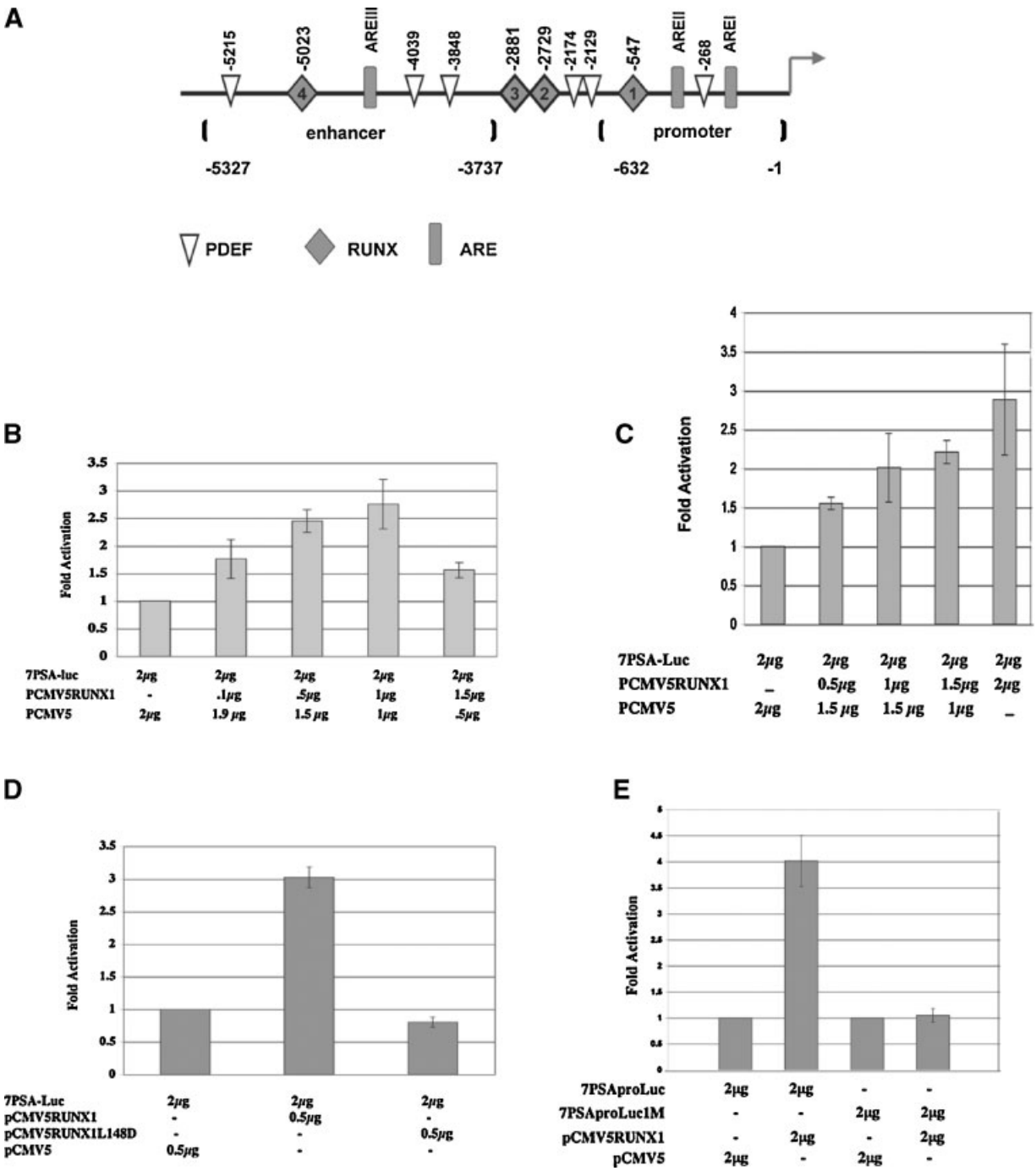


Fig. 3.

of a fragment of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a positive control (Fig. 2C, bottom). The negative control reaction, containing mRNA and reagents but not reverse transcriptase, showed no amplification products (Fig. 2C).

EMSA and RT-PCR experiments concurred in their identification of RUNX1 and RUNX2 DNA binding activity and mRNA expression, respectively, in the cell lines analyzed. For example, EMSA and RT-PCR identified both RUNX1 and RUNX2 in the PPC1 cell line. In contrast, EMSA identified only RUNX1 DNA binding activity in PC3 cell lysates, whereas RT-PCR amplification of PC3 mRNA provided evidence of RUNX1 and RUNX2 expression (Fig. 2C vs. Fig. 1). This most likely reflects the higher sensitivity of RT-PCR, and preliminary Real-Time RT-PCR analysis of PC3 mRNA suggests that RUNX1 is highly expressed, while RUNX2 is expressed at much lower levels [Boranzanci et al., in press], but could also be explained by the presence of untranslated RUNX2 mRNA or modified RUNX2 protein no longer recognized by our antibody. Taken together, the data derived from EMSA and RT-PCR analysis provide compelling evidence of RUNX expression in prostate epithelial cells.

We also analyzed RUNX expression in normal prostate tissue. cDNA from pooled human prostate mRNA was purchased and subjected to RT-PCR as above. Our results revealed expression of RUNX1, RUNX2, and RUNX3 messenger RNA in normal prostate tissue (Fig. 2D). As RUNX1 and RUNX3 expression occurs in peripheral blood in leukocytes and lymphocytes, we performed RT-PCR analysis of peripheral blood samples and compared the data to that from the normal prostate samples. The peripheral blood samples revealed expression of RUNX3 (most abundant), RUNX1 and RUNX2 (very low levels). This expression pattern is quite distinct from that observed in the prostate cDNA. Although we cannot rule out blood contamination of the pooled normal prostate sample, these results, combined with the observed expression of RUNX factors in the non-malignant cell line 267B1, lend support to the idea that the RUNX factors are expressed in normal prostate.

PSA 5′-Regulatory Region Contains Consensus Binding Sites for the RUNX Factors

Our demonstration of RUNX DNA binding activity in prostate cell lines suggests that these proteins may play a role controlling gene expression in normal and/or transformed prostate epithelial cells. Accordingly, we sought to identify putative RUNX target genes. A computer-assisted search of promoter sequences deposited in GenBank identified four previously unrecognized RUNX consensus sites in the regulatory region of the *PSA* gene. One site (−547) is located within the proximal promoter, one (−5,023) within the enhancer, and two (−2,729 and −2,881) are positioned between the minimal promoter and the enhancer (Fig. 3A) [Cleutjens et al., 1996, 1997b]. As

Fig. 3. RUNX1 activates transcription from the PSA regulatory region. **A**: Schematic representation of the upstream regulatory region of the PSA gene. The enhancer and promoter regions are indicated. Nucleotide position +1 corresponds to the 5′-most cap site, and the TATA box is located at −25 [Schuur et al., 1996]. The PSA upstream regulatory region contains four RUNX consensus-binding sites (TGTGGT) as marked. The androgen response elements (ARE) correspond to those defined by Cleutjens et al. [1996, 1997b]. The six PDEF binding sites shown correspond to those identified by Oettgen et al., 2000. These sites are bound by PDEF in EMSA. **B**: RUNX1 activates the PSA upstream regulatory region in C33A cells. C33A cells were transiently co-transfected with 7PSA-Luc and pCMV5RUNX1 or vector alone (pCMV5) at the concentrations indicated below the bar graph. Luciferase activity in the lysates was determined using the Promega Luciferase Assay System and 40 μg of total protein. Transfection efficiency was monitored using a RSV LTR-driven secreted alkaline phosphatase (RSV-SEAP) and light units were normalized to SEAP values in each panel. The results are represented as fold activation; the activity of 7PSA-LUC in the absence of RUNX1 was set at 1. **C**: RUNX1 activates the PSA upstream regulatory region in the prostate cancer cell line PC3. PC3 cells were electroporated with 7PSA-Luc (2 μg), and pCMV5RUNX1 or vector alone (pCMV5) at the concentrations indicated below the bar graph. Electroporation efficiency was monitored using a Simian Virus 40 (SV-40)-driven Renilla luciferase construct. Luciferase activity in the lysates was determined using the Promega Dual Luciferase Assay System. **D**: RUNXL148D fails to activate 7PSA-Luc. C33A cells were transiently transfected with 7PSA-Luc (2 μg), and pCMV5RUNX1 or pCMV5 RUN1L148D at the concentrations indicated below the bar graph. Luciferase activity in the lysates was determined as in (B). **E**: RUNX1 requires an intact binding site for activation of transcription driven by the PSA promoter. C33A cells were transfected with a vector containing the 632 base PSA promoter, 7 PSAProLuc, or a vector containing the PSA promoter with a mutation in the RUNX binding site, 7 PSAProLuc1m, as indicated. The response of these reporter vectors to RUNX1 was evaluated by cotransfection of PCMV5 or PCMV5RUNX1, and luciferase activity in the lysates was determined as in (B). The results in all panels are represented as fold activation; the activity of reporter vector alone was set at 1. The bar graphs represent the mean and standard deviation of three separate experiments.

PSA gene expression is highly prostate-specific, this regulatory region represents an important model that we may use to dissect the role of RUNX factors in prostate-specific gene transcription.

RUNX1 Transactivates a Reporter Construct Containing the *PSA* 5′-Regulatory Sequences Upstream of Firefly *Luciferase*

To determine whether the RUNX proteins activate or repress the *PSA* upstream regulatory region, we used a firefly *luciferase* reporter construct containing a 5.8 kbp fragment of the upstream control region, including the proximal promoter and upstream enhancer (7PSA-Luc). 7PSA-Luc was transiently transfected into the cervical carcinoma cell line C33A, which contains very little endogenous RUNX DNA binding activity, alone or with increasing amounts of a RUNX1 expression vector (pCMV5RUNX1). The amount of expression vector DNA in each transfection was kept constant by adjustment with empty vector, and transfection efficiency was assessed by co-transfection with an additional plasmid encoding secreted alkaline phosphatase expressed from the RSV LTR (RSV-SEAP). RUNX1 expression resulted in a modest (threefold) dose-dependent increase in reporter activity in the cervical carcinoma cell line C33A (Fig. 3B). RUNX1 expression had no effect on the parental luciferase vector lacking the *PSA* upstream regulatory region (data not shown). This result suggested that RUNX1 could regulate transcription via the PSA upstream regulatory region. To determine if RUNX1 could activate transcription of 7PSA-Luc in prostate epithelial cells we performed a similar experiment in the prostate cancer cell line PC-3. Co-electroporation of the RUNX1 expression constructs, but not empty vector, caused a modest dose-dependent rise in reporter gene activity (Fig. 3C).

Then, we sought to determine whether a RUNX protein deficient in DNA binding could transactivate 7PSA-Luc. To do this, a RUNX1 protein containing a single amino acid substitution (L148D) in the rhd, which abrogates both DNA binding and CBFβ interaction [Lenny et al., 1995], was tested for its ability to transactivate 7PSA-Luc. Wild-type RUNX1 but not RUNX1L148D activated transcription of 7PSA-Luc (Fig. 3D), indicating that activation of 7PSA-Luc by RUNX1 requires DNA binding and/or CBFβ association.

Next, we tested whether RUNX1 could activate the *PSA* promoter that contains a single RUNX DNA binding site (Fig. 3A). PSAProLuc, a reporter vector containing the 632 bp *PSA* promoter driving the expression of the luciferase gene, was transiently transfected into C33A cells along with empty vector or the PCMV5RUNX1 expression vector, as indicated (Fig. 3E). We found that, as with the entire *PSA* regulatory region, RUNX1 could transactivate PSAProLuc approximately fourfold (Fig. 3E). We then mutated the RUNX1 binding site, TGTGGT, to TGTTAG; this three nucleotide substitution completely abolishes the ability of RUNX1 to bind to this sequence. The resulting reporter vector, 7PSAProLuc1m, was tested for response to RUNX1 expression. As shown in Figure 3E, expression of RUNX1 has no effect on the transcriptional activity of the *PSA* promoter containing a mutation in the RUNX binding site. These results indicate that RUNX1 requires an intact consensus DNA binding site within the *PSA* promoter for regulation of reporter gene expression. These data support the idea that RUNX proteins specifically regulate expression driven by the *PSA* regulatory region, as this experiment demonstrates that the activation of luciferase expression is not due to a RUNX binding site located within the backbone of the reporter vector.

From these experiments, we concluded that RUNX1 could effect modest changes in transcription driven by the *PSA* upstream regulatory region in prostate- and non-prostate-derived epithelial cells. The modest transcriptional activation observed here is consistent with that observed in similar experiments using hematopoietic-specific promoters and RUNX1 [Meyers et al., 1995].

Endogenous RUNX1 Occupies the *PSA* Promoter in the LNCaP Cell Line

Chromatin immunoprecipitation (ChIP) assays were performed to identify PSA as an in vivo target for RUNX1 in the LNCaP prostate cancer cell line, which is androgen dependent and expresses PSA. To do this, the LNCaP cells were treated with formaldehyde to cross-link proteins to the DNA. Following cell lysis, the chromatin was sonicated to an average length of 1 kbp and precipitated with either an α-RUNX1 rabbit antibody or a non-immune rabbit antibody as indicated (Fig. 4B). No antibody and

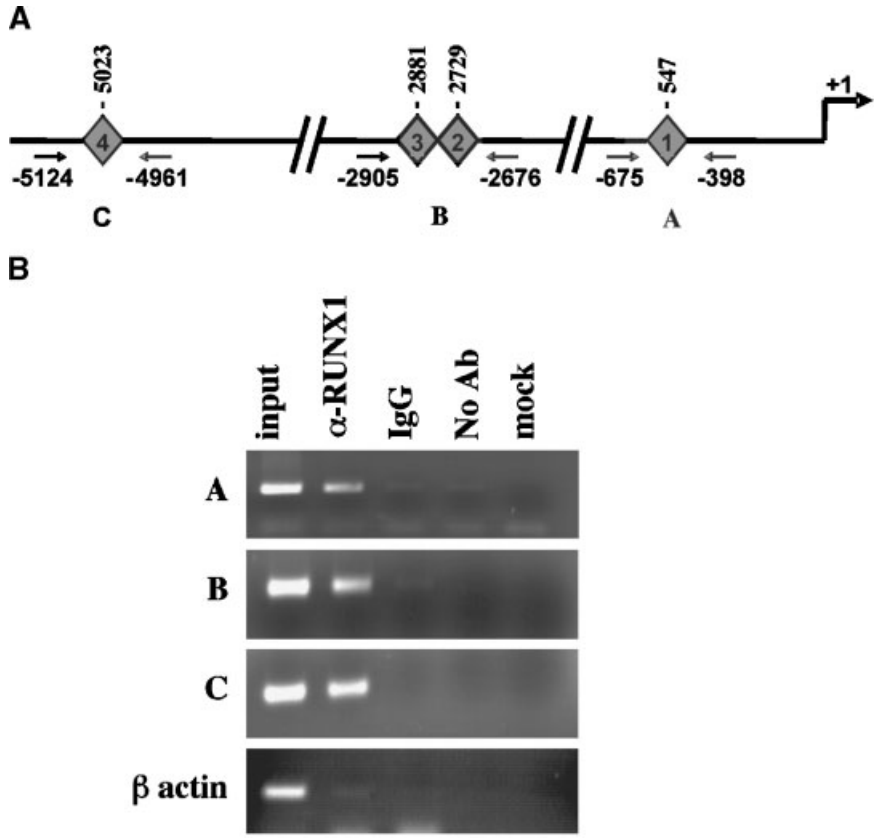


Fig. 4. Endogenous RUNX1 protein is specifically bound to the PSA regulatory region in LNCaP cells. **A**: Schematic diagram of the PSA regulatory region. The four consensus RUNX DNA binding sites are depicted as diamonds. The paired arrows, labeled A, B, and C, indicate the regions specifically amplified in PCR analysis of immunoprecipitates and total input chromatin. **B**: Occupancy of the PSA regulatory region by RUNX1. LNCaP cells were treated with formaldehyde to cross-link proteins to the DNA. The cells were then lysed and the chromatin was sheared to an average length of 1 kbp by sonication. The chromatin was precipitated with either IgG purified α-RUNX1 antibody or a non-specific antibody (IgG) as indicated. No antibody and mock (no chromatin) immunoprecipitations were included as negative controls. Following reversal of the cross-links, 1–3 μl of the immunoprecipitates and a 1:300 dilution of input chromatin were analyzed by PCR using primers specific for three fragments of the PSA regulatory region as shown in (A). PCR was also done using primers specific for sequences within the β-actin promoter.

mock (no chromatin) immunoprecipitations were included as negative controls. Following reversal of the cross-links, 1–3 μl of the immunoprecipitates and a 1:300 dilution of input chromatin were analyzed by PCR using primers specific for three fragments of the PSA regulatory region as shown (Fig. 4A). We found that endogenous RUNX1 was specifically bound to all three sections of the *PSA* regulatory region tested, as shown by the presence of PCR product only in those samples immunoprecipitated with the RUNX1 antibody but not in the IgG, no antibody, or mock immunoprecipitations (Fig. 4B). The RUNX1 antibody failed to immunoprecipitate sequences contained within the β-actin promoter, indicating that RUNX1 in vivo occupancy is specific for the *PSA* regulatory region. From these experiments, we conclude that the endogenous *PSA* regulatory region is bound by RUNX1 in the LNCaP cell line. These results suggest that *PSA* is a target for regulation by RUNX1 in prostate cells.

RUNX1 or RUNX2 and PDEF Synergistically Activate Transcription From the *PSA* 5′-Regulatory Region

Previously, the Ets-family transcription factor prostate-derived Ets factor (PDEF) had been shown to regulate the *PSA* promoter in both prostate and non-prostate-derived cell lines [Oettgen et al., 2000]. Oettgen et al. [2000] described eleven possible PDEF binding sites in the *PSA* regulatory region, six of which were bound by PDEF in EMSA (please refer to Fig. 3A). In agreement with published reports, we found that PDEF transactivated 7PSA-Luc approximately fourfold in the C33A cell line.

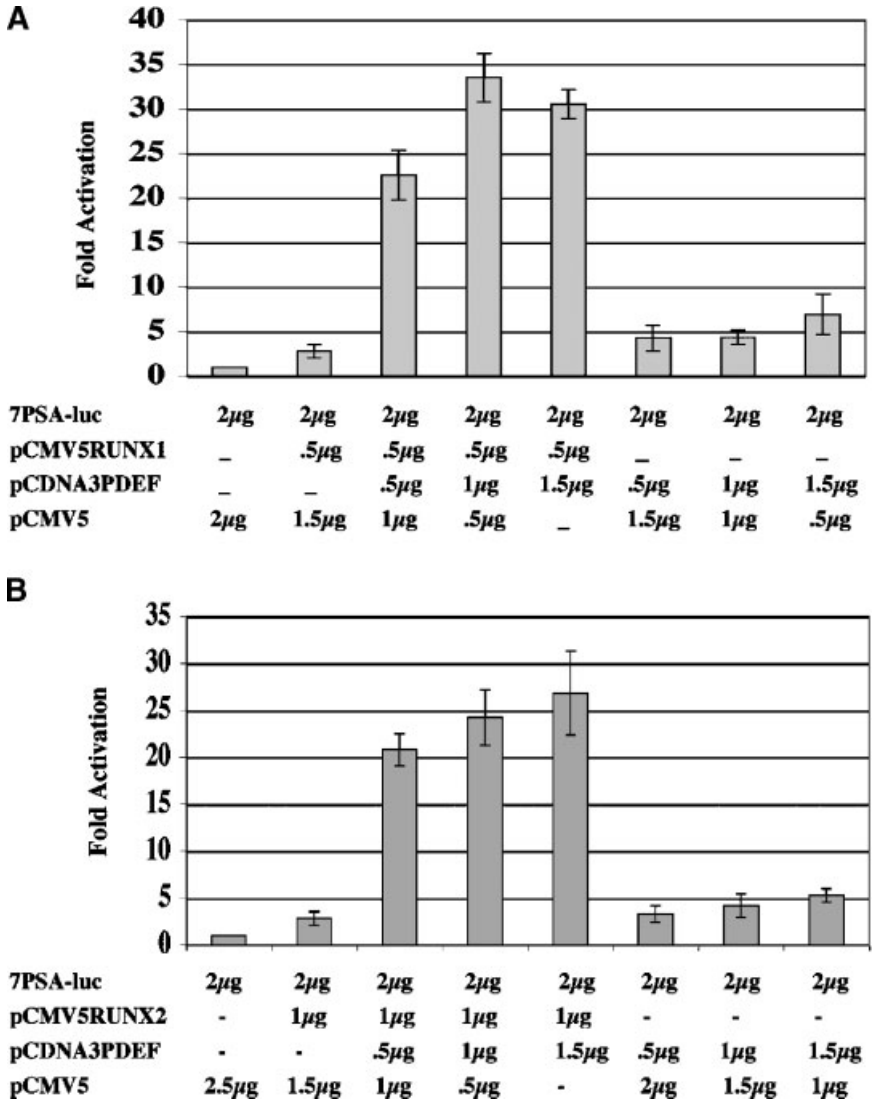


Fig. 5. RUNX1 and RUNX2 act synergistically with PDEF to activate transcription from the PSA regulatory region. **A**: C33A cells were transiently transfected with 7PSA-Luc (2 μg) together with expression vectors for RUNX1 or PDEF. The first bar shows basal activity of 7PSA-Luc, and the second bar shows the level of activation with 0.5 μg of pCMV5RUNX1. Bars 3–5 show the result of adding an increasing amount of pCDNA3PDEF to 0.5 μg pCMV5RUNX1. Bars 6–8 show the level of activation by PDEF. The results are represented as fold activation and the activity of 7PSA-Luc alone was set at 1. **B**: C33A cells were transiently transfected with 7PSA-Luc (2 μg) together with expression vectors for RUNX2 or PDEF. The bar graphs represent the mean and standard deviation of three separate experiments.

Since the RUNX family is known to cooperate with Ets proteins to achieve maximal activation of target promoters [Wotton et al., 1994; Kim et al., 1999], we investigated whether RUNX1 and PDEF could cooperatively regulate transcription of our reporter construct. As shown in Figure 5A, co-expression of RUNX1 and PDEF combined to generate almost 35-fold activation of the reporter. The observed activation is more than that predicted by an additive effect (~10-fold), indicating that RUNX1 and PDEF cooperatively transactivated 7PSA-Luc. Similar experiments demonstrated cooperation between RUNX2 and PDEF (Fig. 5B). These data suggest that RUNX and Ets factors can cooperatively regulate prostate gene transcription.

RUNX1 and PDEF Physically Associate

Next, we investigated whether PDEF and RUNX1 physically associate using glutathione-S-transferase (GST)-capture assays. These assays employed GST fusion proteins containing

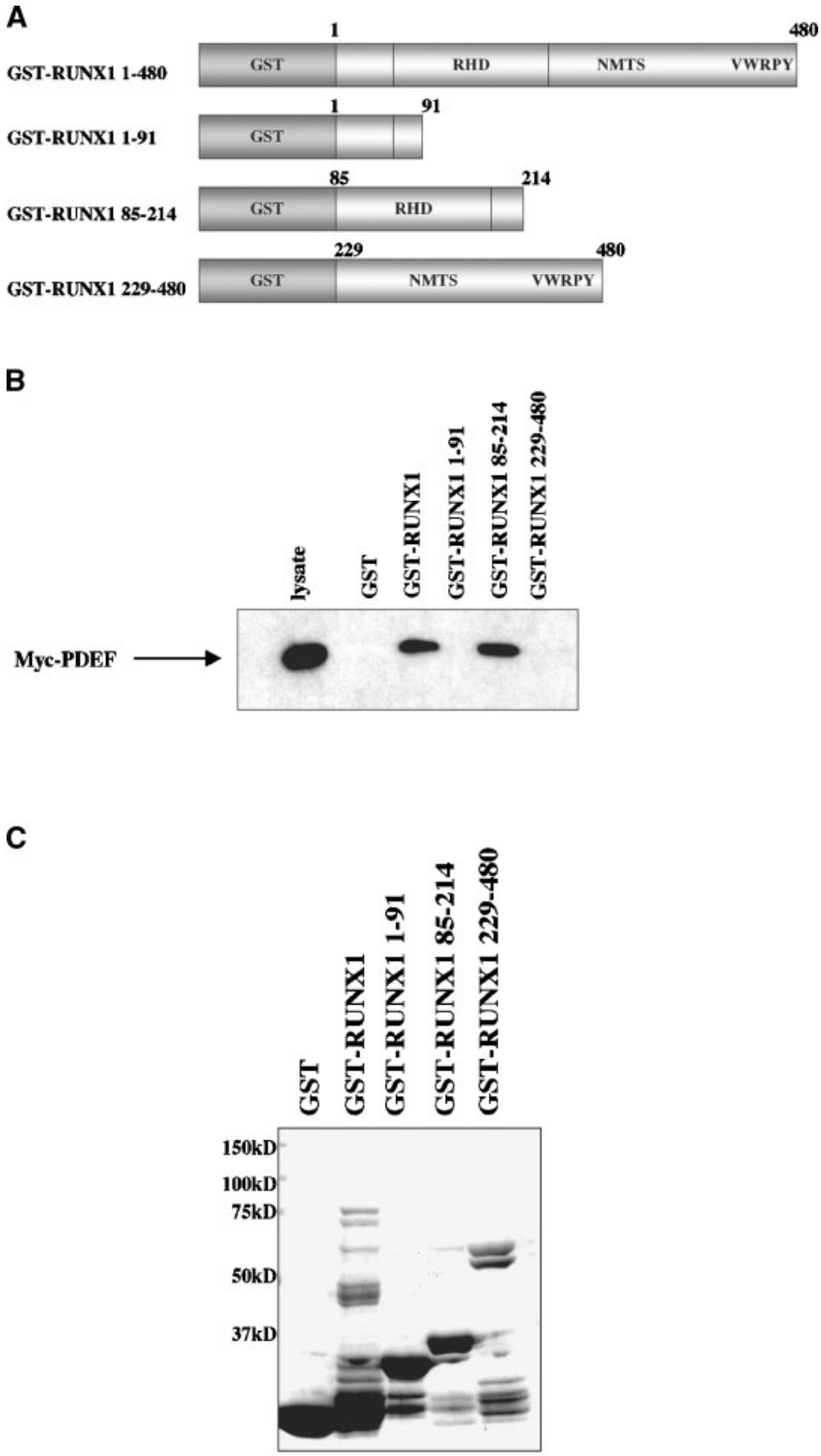
the full-length RUNX1 sequence or portions of RUNX1 containing the N-terminus (AA 1–91), the rhd and C-terminal sequences nearby (AA 85–214), and the C-terminus (AA 229–480) (Fig. 6A). Bacterially expressed GST recombinant proteins and GST alone were purified and mixed with whole cell lysates derived from 293T cells transiently expressing Myc-tagged PDEF (Myc-PDEF). Captured proteins were separated by 10% SDS–PAGE, transferred to a nitrocellulose membrane and immunoblotted with an anti-Myc antibody (9E10). Immunoblotting revealed that Myc-PDEF was specifically captured by GST–RUNX1 and GST–RUNX1 AA 85–214 (containing the rhd), but not by the other RUNX1 fragments or GST alone (Fig. 6B). Proper GST protein expression was confirmed by SDS–PAGE followed by Coomassie blue staining (Fig. 6C). Taken together, these results demonstrate an interaction between RUNX1 and PDEF and suggest that this interaction is mediated through the RUNX1 rhd.

Finally, we tested whether RUNX1 and PDEF formed complexes in vivo by transfecting 293T cells with Myc-PDEF alone, or Myc-PDEF plus RUNX1-expressing vectors. Cell lysates were prepared and immunoprecipitated with an anti-RUNX1 N-terminus rabbit polyclonal antibody (N-term) or a non-immune (NI) rabbit polyclonal antibody. Immunoprecipitates were analyzed by immunoblotting with the 9E10 antiserum. Total cell lysate proteins were also subjected to immunoblot analysis using 9E10 as a marker for Myc-PDEF expression in the first lane (no antibody; "–," Fig. 7A). Myc-PDEF was detected in RUNX1 N-terminus antibody immunoprecipitates from lysates of Myc-PDEF/RUNX1 co-transfected cells. In contrast, Myc-PDEF was not detected in immunoblots of immunoprecipitated proteins from cells transfected only with Myc-PDEF (Fig. 7A). Lysates immunoprecipated with the non-immune antibody and immunoblotted with the 9E10 antiserum did not show evidence of Myc-PDEF. Immunoblots of the same lysates using either 9E10 or an antibody prepared against the RUNX1 RHD (RHD) demonstrated proper protein expression (Fig. 7B). Taken together, these experiments suggest that RUNX1 and PDEF can associate in vivo.

DISCUSSION

In the present study, we identified RUNX DNA binding activity and mRNA expression, respectively, in prostate cell lines and pooled normal prostate tissue. We observed RUNX1 and RUNX2 mRNA expression in every tested prostate cell line and expression of all RUNX family members in the immortalized, non-transformed cell line 267B1 and in the pooled normal prostate sample. DNA binding analysis identified RUNX1 and RUNX2 DNA binding activity in the prostate cell lines. Taken together, these data clearly show that RUNX family members are expressed in prostate cell lines and normal prostate tissue. This finding agrees with and extends several recently published reports demonstrating RUNX2 expression in the prostate cancer cell lines DU145, PC-3, and C4-2B (a subclone of LNCaP). These studies did not examine expression of the other RUNX genes [Yeung et al., 2002; Brubaker et al., 2003]. Strikingly, RUNX3 expression was observed in the pooled normal prostate and in the immortalized cell line 267B1, but not in the prostate-cancer derived cell lines. This finding is very intriguing in light of recent reports demonstrating loss of RUNX3 in gastric carcinomas [Li et al., 2002; Oshimo et al., 2004]. Further analysis of RUNX expression in matched tumor and normal prostate tissue samples will be required to address a possible role for the RUNX family members in prostate cancer.

To provide a model in which to dissect the role of RUNX factors in prostate-specific gene transcription, we looked for prostate-specific genes whose expression could be regulated by RUNX proteins. We identified RUNX binding sites in a number of promoters but decided to focus our efforts on PSA for several reasons. First, PSA is almost exclusively expressed in the luminal epithelial cells of the prostate, thus satisfying the requirement for prostate specificity [Lilja, 2003]. Second, the upstream regulatory elements required for tissue-specific expression of *PSA* are well characterized [Riegman et al., 1991; Cleutjens et al., 1996, 1997a,b]. Third, the *PSA* gene is regulated by the androgen receptor and by Ets factors [Oettgen et al., 2000], and members of the Ets family and the steroid hormone family cooperatively regulate transcription in combination with RUNX factors [Erman et al., 1998; Kim et al., 1999]. Thus, the *PSA* regulatory region presented an opportunity to analyze RUNX-regulated prostate gene expression in a context that allowed analysis of RUNX cooperativity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50kD
37kD

Fig. 6.

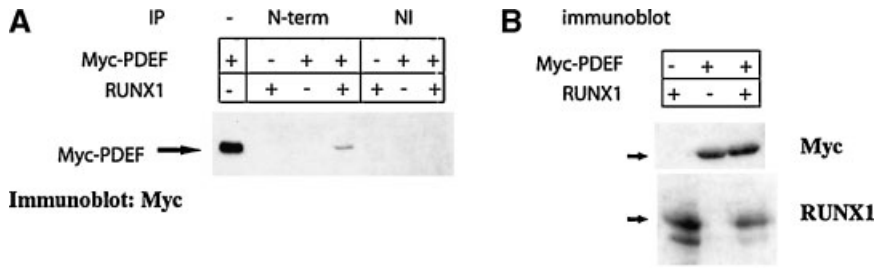

Fig. 7. RUNX1 associates with Myc-PDEF in vivo. **A**: 293T cells were transfected with the indicated expression constructs, lysates were prepared and proteins were immunoprecipitated with either anti-N-term (rabbit polyclonal antibody prepared against the N-terminal portion of RUNX1) or a non-immune (NI) rabbit polyclonal antiserum, and then immunoblotted with anti-Myc 9E10 antibody. Total lysates from Myc-PDEF transfected cells were also included as a size marker for Myc-PDEF (**first lane**: no antibody is indicated with a minus (−) sign). **B**: Expression of Myc-PDEF and RUNX1 in total lysates was determined by immunoblot using the 9E10 antibody or an antibody prepared against the RUNX1 RHD (RHD). The arrows denote full-length Myc-PDEF and RUNX1 proteins.

with Ets factors (this study) and with the androgen receptor. RUNX1 and RUNX2, like PDEF, regulate transcription from the *PSA* control region in prostate and non-prostate epithelial cells. Moreover, RUNX1 was bound to the upstream regulatory region of the endogenous *PSA* gene in the PSA-expressing LNCaP cell line. The PCR primers used in the ChIP experiment flank consensus RUNX1 sites, but we cannot determine whether RUNX1 is bound to the consensus sites, several near consensus sites proximally located, or a combination of the two. However, loss of RUNX1 dependent transactivation of the mutated *PSA* promoter together with the ChIP analysis provide strong evidence that the consensus site located in the *PSA* promoter is a relevant RUNX binding site. We have identified an additional PDEF binding site adjacent to the RUNX binding site in the *PSA* promoter (data not shown). However, further studies are necessary to determine if the observed cooperativity requires these adjacent sites.

Taken together, our data clearly show that RUNX1 and RUNX2 can activate transcription from the *PSA* upstream regulatory region. We also show that the *PSA* regulatory region is occupied by endogenous RUNX1, and thus the *PSA* regulatory region provides a good model on which to dissect RUNX-mediated prostate-specific gene transcription. Thus, RUNX1 and RUNX2 join the ranks of non-steroid hormone binding transcription factors that have been implicated in the regulation of *PSA*, including the homeodomain transcription factor NKX-3.1, NF-κB, and the GAGATA binding factor [Chen and Sawyers, 2002; Chen et al., 2002; Wang et al., 2003]. This growing list of transcription factors involved in *PSA* regulation indicates that control of PSA expression is complex and requires a variety of proteins in addition to the androgen receptor. Whether RUNX1 (or RUNX2) interact with these additional factors remains to be determined.

Our data suggests a role for RUNX proteins in prostate gene regulation in both normal and malignant cells. We have identified the first prostate-specific target for the RUNX proteins, *PSA*, and provided the first examination of the expression of all three RUNX proteins in prostate. Further definition of RUNX targets in prostate epithelial cells will help illuminate the biological role of this transcription factor family in prostate cell biology and disease, perhaps leading to the development of new treatment strategies for prostate cancer.

Fig. 6. RUNX1 interacts with PDEF in vitro. **A**: Schematic representation of the GST–RUNX1 proteins used in the GST-capture assays. The numbers refer to amino acids and correspond to RUNX1 accession number NM001754. **B**: A portion of RUNX1 containing the rhd is sufficient to associate with Myc-PDEF. GST–RUNX1, GST–RUNX1 portions, and GST were bacterially expressed, purified, incubated with lysates prepared from 293T cells transiently expressing Myc-PDEF, and separated by 10% SDS–PAGE. Two microliters of 293T lysate containing Myc-PDEF was electrophoresed for comparison (10% input). Proteins were transferred to nitrocellulose membranes and subjected to immunoblot using the anti-Myc 9E10 monoclonal antibody. **C**: Coomassie blue-stained gels showing GST protein expression. The uppermost band represents the full-length recombinant protein.

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