Isolation and Characterization of the Promoter of the Human 
Prostate Cancer-specific DD3 Gene*

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Recently, we have described a novel gene, DD3, which is one of the most prostate cancer-specific genes described to date (Bussemakers, M. J. G., van Bokhoven, A., Verhaegh, G. W., Smit, F. P., Karthaus, H. F. M., Schalken, J. A., Debruyne, F. M. J., Ru, N., and Isaacs, W. B. (1999) Cancer Res. 59, 5975–5979). The prostate cancer-specific expression of DD3 indicates that the DD3 gene promoter is a promising tool for the treatment of prostate cancer. To identify the promoter elements that are responsible for the prostate cancer-specific expression of DD3, we have isolated and characterized the DD3 promoter. Sequence analysis of the DD3 5′-flanking region was performed and several promoter-human growth hormone reporter constructs were prepared, which were transiently transfected in the DD3-positive cell line LNCaP and several DD3-negative cell lines. Using a 500-base pair DD3 promoter construct, we could detect promoter activity in LNCaP cells, which was not affected by increasing the size of the constructs. Truncated constructs, however, showed an increased transcriptional activity, suggesting the presence of a silencer that negatively regulates the expression of DD3. DNase-I footprint analysis, using nuclear extracts from LNCaP cells, revealed the presence of three DNase-I-protected areas within the DD3 proximal promoter. We show that the high mobility group I(Y) protein binds to one of the DNase-I-protected areas and recruits another, yet unidentified, protein to the DD3 promoter in LNCaP cells.

Prostate cancer is the most commonly diagnosed malignancy and the second leading cause of cancer-related deaths in the Western male population (1). When this carcinoma has locally or distantly spread, no curative therapy can be offered. Because there is no effective treatment available for patients with advanced and/or hormone-refractory prostate cancer, there is an urgent need to develop new approaches to treat patients with progressive prostate cancer. A better understanding of the molecular changes associated with the onset and progression of prostate cancer may provide a rational basis for the development of new treatment modalities. For example, gene therapy using prostate-specific gene promoters, i.e. linking up prostate-specific promoter sequences to genes that suppress tumor cell growth, induce apoptosis, and/or kill tumor cells, may provide a new way to attack this mordacious disease (2, 3).

A number of human genes have been identified that are specifically expressed in the human prostate, including prostate-specific antigen (PSA)1 (e.g. Ref. 4), prostatic acid phosphatase (e.g. Ref. 5), human kallikrein 2 (e.g. Ref. 6), prostate-specific membrane antigen (7, 8), prostate-specific transglutaminase (9), and prostate stem cell antigen (10). The promoter sequences responsible for the prostate-specific expression of these genes have been cloned, and the unraveling of their transcriptional regulation is ongoing and will provide prostate-specific promoter fragments that can activate therapeutic agents selectively in prostatic (cancer) cells.

The PSA gene promoter has been most extensively studied and revealed the existence of a proximal prostate-specific promoter with an upstream prostate-specific enhancer that are both required for high, androgen-regulated activation of PSA expression (11–15). The PSA enhancer-promoter was linked up to the HSV-tk gene, encoding a prodrug-converting enzyme, and was delivered by the human adenovirus into prostatic tumor cells growing subcutaneously in nude mice. As a result of the PSA promoter-driven HSV-tk expression, prostate tumor cell growth was significantly suppressed and life span of the animals was prolonged (16, 17). This proof of principle opens the way for the application of promoter-based gene therapy for prostate cancer patients.

Recently, we have cloned another human prostate-specific expressed gene, DD3 (18). In addition to its prostate-specific mRNA expression, DD3 was shown to be highly overexpressed in prostatic tumors. These data indicated that DD3 is one of the most prostate cancer-specific genes described. The prostate-specific DD3 expression and the sharp up-regulation in prostate cancer, suggest a unique transcriptional regulation. Consequently, the DD3 gene promoter becomes an interesting candidate to be used for the application of promoter-based gene therapy of prostate cancer patients.

In this paper we describe the isolation of the DD3 5′-flanking sequences, and the identification of the proximal promoter that is required for the prostate-specific expression of DD3 mRNA. Furthermore, nuclear factor binding-sites in the DD3 promoter

1 The abbreviations used are: PSA, prostate-specific antigen; bHLH, basic helix-loop-helix; EMSA, electrophoretic mobility shift assay; FP, footprint; hGH, human growth hormone; HMG-I(Y), high mobility group proteins I and Y; HSV-tk, herpes simplex virus-thymidine kinase; NE, nuclear extract; PCR, polymerase chain reaction; bp, base pair(s); DTT, dithiothreitol; PIPES, 1,4-piperazinediethanesulfonic acid.
are identified, that may be important for the transcriptional regulation of the DD3 gene.

MATERIALS AND METHODS

Isolation and Sequence Analysis of DD3 Promoter Clones—Genomic clones containing the DD3 gene were described previously (18). λFIX-ME4 DNA, containing the DD3 exon 1-flanking region, was endonuclease digested and subcloned in plasmid vectors pGEM-3Zf(+) or pBluescript II SK(−). Double-stranded plasmid DNA was isolated by standard procedures (19), and sequenced using the Thermo Sequenase cycle sequencing kit (Amersham Pharmacia Biotech) and Texas Red-labeled universal primers. Sequencing products were separated and analyzed using the Vistra DNA sequencer 725.

Primer Extension Analysis—Total RNA of human tissue was isolated using guanidium isothiocyanate by standard procedures (19). DD3-specific antisense oligonucleotides BUS29 (5′-CTCTGTATCATCAGGTAAGGCCTTCC-3′, position +285 to +289), BUS39 (5′-CAGGATGGCAGAAA CAT-3′, position +420 to +401), and BUS28 (5′-GCTTCTCGGCACCTGT CATT-3′, position +28 to +29) were [γ-32P]dATP end-labeled (3000 cpm/mmol, Amersham Pharmacia Biotech) and annealed with 20 µg of RNA at 30 °C in 30 µl of hybridization buffer (40 mM PIPES, pH 6.7, 1 mM EDTA, 0.5 mM DTT, 40% formamide). Primers were extended M13 200 units of SuperScript II-Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) in 25 µl of reverse transcriptase buffer (Life Technologies, Inc.), supplemented with 0.5 mM dNTPs, 10 mM DTT, 0.5 µg of actinomycin D, and 1 unit/µl RNase inhibitor, for 1.5 h at 42 °C. RNase A-treated primer extension products were resolved by 6% polyacrylamide-urea gel electrophoresis. DD3 genomic sequence ladders, generated with the same oligonucleotide primers, were used as size markers. Gels were dried and exposed to Hyperfilm (Amersham Pharmacia Biotech) using two amplifying screens at −80 °C for approximately 3 days.

RNase Protection Assay—A DNA fragment encompassing the presumed transcription start site was used as a probe for the RNase protection assay. A 517-bp Sou5A1 DNA fragment from clone pME64.6 was inserted into the BamHI site of M13mp18. Single-stranded M13DNA was isolated, annealed with the DD3 exon 1-specific oligonucleotide BUS21 (5′-CTCTTCCACCATGAGATCTTCCGTGCAGGAGGACACCA-3′), and extended using [γ-32P]dATP and Klenow DNA polymerase. The radiolabeled probe was linearized with HindII and purified from a denaturing 5% polyacrylamide-urea gel. All reactions were performed according to standard protocols (19). The probe (10000 cpm) was annealed with 40 µg of total RNA at 30 °C in 30 µl of hybridization buffer (40 mM PIPES, pH 6.7, 1 mM EDTA, 0.5 mM NaCl, 80% formamide). DNA-RNA hybrids were digested with 300 units of S1 nuclease (Amersham Pharmacia Biotech) in 300 µl of S1 buffer (0.25 M NaCl, 0.05 M NaAc, pH 4.5, 4.5 mM MgSO4, supplemented with 20 µg/ml single-stranded herring sperm DNA) for 60 min at 37 °C. Digestions were terminated by addition of the addition of 25 mM Tris-HCl (pH 7.5), 4 mM NaAc, 50 mM EDTA, 50 µg/ml RNAse. Protected DNA fragments were resolved by 6% polyacrylamide-urea gel electrophoresis. DD3 genomic sequence ladder generated with the BUS21 primer was used as size marker. Autoradiography was performed as described above.

Cell Culture—The following cell lines were used in transient transfection and/or electrophoretic mobility shift assays (EMSA): the human prostate adenocarcinoma cell lines LNCaP, PC-346C (kindly provided by Dr. W. van Weerden, Dept. of Urology, Erasmus University, Rotterdam, The Netherlands), and TSU-pr1; the human bladder cancer cell line SW800; the human colon carcinoma cell line HT-29; the renal cell carcinoma cell line SKRC-7; and the vulval epidermoid cancer cell line A431. All cells were grown in RPMI 1640 medium, supplemented with 10% fetal calf serum (Life Technologies, Inc.) in an atmosphere of 5% CO2 and 37 °C.

Production of Promoter Constructs—The promoterless plasmid pG5 (Nichols Institute) was used for cloning DD3 promoter fragments into the polylinker upstream of the human growth hormone gene. DD3 promoter fragments were produced by PCR using 5′-HindIII- and 3′-BamHI-tagged primers, using normal human genomic DNA as a template. Subsequently, the fragments were cleaved with HindIII and BamHI and cloned into pG5, using standard procedures (19). Mutant promoter constructs were generated using the “GeneEditor” in vitro site-directed mutagenesis system (Promega). Because the HGHI secretion capacity of different cell lines may vary, for each cell line the activity of the DD3-hGH constructs was compared with that of plasmid pTKGH containing the human growth hormone gene driven by the constitutive promoter of the HSV-tk gene (20). Transient Transfections—LNCaP cells were seeded at a density of 1 × 106 cells per 10-cm dish 2 days prior to transfection, and TSU-pr1, HT-29, A431, or SW800 cells were seeded at a density of 5 × 105 cells per 10-cm dish 1 day before transfection. For each transfection, 3 µg of the appropriate DD3-hGH construct and 2.5 µg of pCH110 (internal normalization marker) were complexed with Fugene-6 reagent (Roche Molecular Biochemicals) in serum-free medium for 15 min at room temperature. The Fugene-6 DNA complexes were added to the cell cultures, and cells were grown for an additional 72 h. Transfections were performed at least three times in duplicate.

Human Growth Hormone Assay—After transfection, medium was collected until use. Human growth hormone secretion in the medium was determined using the two-site fluorimunometric Delta IGH assay kit (Wallac Oy, Turku, Finland) according to the manufacturer’s instructions. IGH values were normalized to the β-galactosidase activities measured in the corresponding cell extracts. IGH values and relative induction values are expressed as mean ± standard error of the mean (S.E.).

DNase-I Footprinting Analysis—Nuclear protein extracts for footprinting were prepared as described (22, 23). Single strand-end-labeled DNA probes were generated by first labeling the 5′-end of DD3 promoter-specific primers with [γ-32P]dATP (~3000 cpm/mmol, Amersham Pharmacia Biotech), followed by PCR amplification of DNA fragments with one labeled and one unlabeled primer (as indicated below in Fig. 5A). For each footprint reaction, 20–40 µg of nuclear extract, or 100 ng of recombinant HMG-I protein (provided by Dr. R. Reeves, Dept. of Biochemistry and Biophysics, Washington State University, Pullman, WA), were preincubated for 5 min in 50 µl of foot print buffer (20 mM HEPES, pH 7.8, 50 mM KCl, 5% glycerol, 0.1 mM EDTA, 3 mM DTT, 10 mM ZnCl2, and 2 µg of herring sperm DNA as nonspecific inhibitor). Subsequently, 2 × 105 cpm radiolabeled probe was added and incubated for 20 min at room temperature. DNA (probe) was digested in DNase-I buffer (25 mM NaCl, 10 mM HEPES, 5 mM MgCl2, and 1 mM CaCl2) using 0.01–0.5 unit of DNase-I (Life Technologies, Inc.) for 1 min at room temperature. Reactions were terminated by adding 25 µl of stop buffer (200 mM NaCl, 30 mM EDTA, 1% SDS, and 100 µg/ml RNAse). After phenol/chloroform extraction and ethanol precipitation, samples were dissolved in sample buffer (95% formamide, 20 mM EDTA, 0.5 mM bромphenol blue, 0.5% xylene cyanol FF), denatured for 2 min at 100 °C, and resolved by 5% polyacrylamide-urea gel electrophoresis. DD3 genomic sequence ladders generated with the appropriate end-labeled oligonucleotide primers were used as size markers. Gels were dried and exposed to Hyperfilm (Amersham Pharmacia Biotech).

Electrophoretic Mobility Shift Assay—Double-stranded oligonucleotides of DNase-I-protected DD3 promoter sequences were synthesized and end-labeled with [γ-32P]dATP (~3000 cpm/mmol, Amersham Pharmacia Biotech). Binding assays contained 200 µg of bovine serum albumin, 4 mM DTT, 10 mM ZnCl2, and 1.5 µg of herring sperm DNA, and were adjusted to a final volume of 30 µl with binding buffer (20 mM HEPES, pH 7.6, 50 mM NaCl, 10% glycerol, 0.1 mM EDTA). The antibody against the human HMG-I(Y) protein was provided by Dr. A. Fusco (Istituto Nazionale dei Tumori, Naples, Italy). Binding reactions were incubated for 30 min at room temperature. A 15-µl aliquot of each reaction was loaded onto a 4% non-denaturing polyacrylamide gel and run in TBE buffer at 120 V for approximately 3 h. Autoradiography was performed as described above.

Data Base Accession Number—The nucleotide sequence for the DD3 promoter region has been deposited into the GenBank® data base under accession number AF279290.

RESULTS

Structure of the 5′-Flanking Region of the Human DD3 Gene—Genomic clones, λFIX-ME3, -ME4, and -IH1, containing the 5′-end of the human DD3 cDNA were described previously (18). Lambda phage DNA was subcloned in plasmid vectors for DNA sequence analysis. In Fig. 1A, a restriction map is shown of the resulting clones pFS258, pGVS61, and pME4.6, containing DD3 exon 3 and its 5′-flanking sequences. The nucleotide sequence of the 5′-flanking region was determined (Fig. 1B) (GenBank® accession number AF279290).

Comparison of the DD3 5′-flanking nucleotide sequence with sequences in the non-redundant nucleotide data bases and the eukaryotic promoter data base, using BLAST (24), revealed no homology to any gene or promoter sequences described. Iden-
The location of exon 1 is indicated by a shaded box.

sites are identified by the MatInspector program using the TRANSFAC 3.5 matrices (ref. 25, and on the Web), with a core similarity of 1.00 and the transcription start site (position 1, *) of the DD3 gene. The GT splice donor site of intron 1 is underlined. Potential transcription factor binding sites are cloned upstream of the human growth hormone (hGH) reporter gene (construct pDDGH-1.9, position -433 to +62). When pDDGH-1.9 was transfected into LNCaP cells, a human prostate carcinoma cell line expressing DD3 mRNA (18), weak promoter activity was seen (Fig. 3), i.e. hGH production was about 20% of the HSV-tk-driven hGH production. Promoters are known to function unidirectionally. Therefore, the pDDGH-1.9 promoter sequences were cloned in the reverse orientation upstream of the hGH gene (pDDGH-2.1, position +62 to -433). This pDDGH-2.1 promoter construct was inactive in LNCaP cells, moreover, the hGH production was below that found in cells transfected with the promoterless p0GH construct. Sequences upstream of the 500-bp DD3 promoter (-433 to +62) had no effect on the DD3 promoter activity (Fig. 3, construct pDDGH-1.5, position -1900 to +62).

To investigate whether regions within the 500-bp DD3 promoter contributed to DD3 promoter activity, a series of 5'-deletion constructs were generated. Transfection of the deletion constructs pDDGH-1.10, -1.11 and -1.12 into LNCaP cells resulted in an increased promoter activity, compared with the activity observed in pDDGH-1.9 (Fig. 3). Construct pDDGH-1.12 (position -152 to +62) displayed the highest DD3 promoter activity of all constructs tested. Shortening the latter construct led to a decreased promoter activity (pDDGH-1.16) and a complete loss of activity in construct pDDGH-1.18 (position -41 to +62).

Cell Type Specificity of the DD3 Promoter —To define the specificity of the observed DD3 promoter activity, promoter
constructs pDDGH-1.9, displaying basal activity, and pDDGH-1.12, displaying maximum activity, were transfected into cell lines of different tissue origin. DD3 promoter activity of pDDGH-1.9 was found in LNCaP cells (Fig. 4), but not in A431 (vulval carcinoma), HT-29 (colon carcinoma), SKRC-7 (renal cell carcinoma), and SW800 (bladder carcinoma) cells. Importantly, this promoter construct is also silent in prostate carcinoma cell lines that do not express DD3 mRNA (TSU-pr1, Fig. 4, and PC-3, data not shown). The increased promoter activity of the truncated promoter construct pDDGH-1.12, however, was also observed in the DD3-negative cell lines, although the maximum promoter activity was significantly lower than in LNCaP cells.

**In Vitro DNase I Footprint Analysis of the Human DD3 Promoter**—DNase-I footprinting analysis was performed using nuclear extracts (NE) of LNCaP (DD3 mRNA-positive) and TSU-pr1 (DD3-negative) cells. Single-strand end-labeled probes, covering the DD3 promoter region from position -433 to +62 (Fig. 5A), were used to identify binding of nuclear factors to the DD3 promoter. Three DNase-I-protected regions

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**Fig. 2.** Determination of the DD3 transcription start site. A. Primer extension analysis of the DD3 5’-flanking region. 20 μg of total RNA from prostatic adenocarcinoma (lanes 4, 5, 7, 8, and 9), liver (lanes 1 and 10), or lung (lanes 2 and 11) tissue or tRNA (lanes 1 and 6) were used for primer extension using oligonucleotides BUS7 (lanes 1-5) and BUS2 (lanes 6-11) as primers as indicated under “Materials and Methods.” Primer extension products are indicated by arrows (+, BUS2, and ++, BUS7 products). A DNA sequence ladder of the DD3 genomic clone pME1.5S3 primed with BUS7 was used as a size marker. B. For RNase (S1 nuclease) protection analysis, a 252-bp radiolabeled single-stranded DNA probe was synthesized from plasmid pME4.6, containing exon 1 of DD3 and its 5’-flanking region, using BUS21 as a primer. The HinII-digested probe was hybridized to 40 μg of total RNA from prostatic adenocarcinoma (lanes 1-4), liver (lane 5), or lung (lanes 6) tissue or tRNA (lane 7) and treated with S1 nuclease at 37 °C (lanes 1-7) or at 30 °C (data not shown). The arrows mark protected fragments. A DNA sequence ladder of the DD3 genomic clone pME4.6 primed with BUS21 was used as a size marker. C. Nucleotide sequence surrounding the DD3 transcription start site. Primer extension products are indicated by triangles (closed triangle, major start site; open triangle, minor site), and S1 nuclease protected fragments by diamonds (closed diamond, major start site; open diamond, minor site).

**Fig. 3.** DD3 promoter activity in the human prostatic cancer cell lines TSU-pr1 and LNCaP. DD3 promoter constructs used for transient transfections are shown at the left. Thin lines indicate vector sequences, boxes indicate the human growth hormone (hGH) gene, and the hatched boxes the DD3 promoter fragments. Positions relative to the transcription initiation site are indicated. Human growth hormone production in LNCaP cells (DD3-positive, black bars) and in TSU-pr1 cells (DD3-negative, light bars) were calculated using pTKGH (hGH gene expression driven by the HSV-tk promoter) as a reference. Error bars indicate the standard error of the mean of three independent experiments done in duplicates.
or footprints (FPs) could be identified within the DD3 5′-flanking region: FP1 −29 to −63, FP2 −173 to −201, and FP3 −281 to −307 (Fig. 5B). The protection pattern in FP1 was identical using NEs from LNCaP and TSU-pr1 cells. A qualitative different protection pattern was observed in FP2, i.e. NEs of TSU-pr1 cells gave protection from position −173 to −201, whereas a NE of LNCaP cells protected the same DNA probe from position −182 to −198. FP3 was only used observing NEs of LNCaP cells. All three footprints were confirmed using probes that were end-labeled at the complementary DNA strand (data not shown). Furthermore, all DNase-I footprints could be prevented by the addition of a 250-fold excess of unlabeled specific DNA, but not by a 250-fold excess of unlabeled unrelated DNA fragments (Fig. 5C (FP2) and data not shown (FP1 and FP3)). These data show that at least three nuclear protein(s) (complexes) sequence-specifically bind to the DD3 proximal promoter (Fig. 5D).

**Comparative EMSA of Footprinted Regions within the Human DD3 Promoter**—To further substantiate the sequence-specific binding of nuclear factors to the DD3 promoter, and to discriminate between DD3-specific and nonspecific interactions, DNA bandshift assays were performed. As shown in Fig. 6A, three protein-DNA complexes (indicated I, II, and III) were formed when NEs from LNCaP and PC-346C cells were incubated with an oligonucleotide enclosing FP1. PC-346C is another androgen-dependent, DD3-expressing, prostate cancer cell line described recently (26). The slowest migrating complex (I) was more pronounced with PC-346C nuclear extracts than with LNCaP extracts. Furthermore, this complex was not formed using NEs from TSU, DU-145, or A431 cells, which do not express DD3 mRNA. This suggests that, although FP1 was identical with NEs from both LNCaP and TSU-pr1 cells, a DD3-specific nuclear factor interacts with proteins in the FP1 region.

When an oligonucleotide enclosing FP2 (position −212 to −172) was used as a probe, a single specific bandshift was observed (Fig. 6B). The addition of NEs of LNCaP and PC-346C cells to the binding reactions resulted in a slower migrating complex compared with the bandshift using TSU and other NEs (Fig. 6B and data not shown). Protein-DNA complex formation could be prevented by the addition of an excess of unlabeled specific probe, but not by an excess of unlabeled unrelated oligonucleotides (data not shown), confirming the specificity of DNA binding. The DD3 promoter region −211 to −159 was found to be A+T-rich (Fig. 1B), and therefore, we investigated whether the high mobility group proteins I and Y (HMG-I(Y)), which preferentially bind to the minor groove of A+T-rich sequences (27), are involved in DD3 promoter binding. When recombinant HMG-I was incubated with probe 2, a similar bandshift was observed to that of TSU-pr1 extracts. The inclusion of HMG-I(Y)-specific antibodies to the binding assay, abolished the protein-DNA interactions observed using LNCaP and PC-346C extracts (Fig. 6B). The inclusion of the HMG-I(Y) antibody in the TSU-pr1 binding reaction diminished HMG-I(Y) binding to the FP2 oligonucleotide by 75%, as determined by densitometry (Fig. 6B). Other, non-HMG-I(Y) IgG molecules had no effect on the formation of protein-DNA interactions (data not shown). These data indicate that HMG-I(Y) interacts with the DD3 promoter at the FP2 region. In addition, another unidentified protein interacts with the HMG-I(Y)-DD3 promoter complex in the DD3-expressing cell lines LNCaP and PC-346C.

Despite the observed DD3-specific FP3 (Fig. 5B), no difference in binding of LNCaP and TSU-pr1 nuclear factors to the oligonucleotide enclosing FP3 (position −279 to −311) could be observed by EMSA so far (data not shown).

**Site-directed Mutagenesis of Factor Binding Sites**—To identify the potential significance of the three identified DNA-binding sites for the promoter activity, several base substitution mutants in the reporter constructs pDDGH-1.9, pDDGH-1.10, and pDDGH-1.12 were created. Base substitutions were introduced in those motifs that were predicted by the MatInspector program, using the TRANSFAC 3.5 matrices (25), with a core similarity of 1.0 and a matrix similarity of over 0.90 (see Fig. 1B). The positions and nature of the base substitutions are shown in Fig. 7A. The examined mutations in the C/EBPβ, CCAAT, and Th/IE47 sites (i.e. FP3 and upstream region) did not affect the promoter activity when compared with the wild-type pDDGH-1.9 reporter construct (Fig. 7B). Scrambling the A+T-rich sequence, present in FP2, resulted in a 50% reduced transcriptional activity (Fig. 7B, construct 1.10/51 versus pDDGH-1.10), indicating a relevant role of the HMG-I(Y) binding site in the transcriptional activation of DD3. Base substitutions in the FP1 region most clearly reduced promoter activity (Fig. 7B). Mutations in the NF-1 sequence motif reduced promoter activity of the 1.9/60a and 1.12/60a constructs to 27% and 18%, respectively, and mutations in the E-box motif reduced transcription from reporter constructs 1.9/60c and 1.12/60c to 43% and 67%, respectively. These data show that the cis-acting elements present in FP1 and FP2 are functionally involved in the initiation of DD3 transcription.

**DISCUSSION**

The DD3 gene was previously shown to be highly overexpressed in the majority of prostate adenocarcinomas (18). These data suggested that DD3 mRNA expression is regulated by a unique prostate cancer-specific transcriptional mechanism. In this paper we have described the cloning and the initial characterization of the prostate cancer-specific DD3 gene promoter.

Nucleotide sequence analysis did not reveal any obvious promoter elements. No known initiator motif, no TATA-box, no CAAT-box, and no GC-rich regions were found at consensus positions within the DD3 promoter. These canonical transcription factor binding sites are involved in the positioning of the transcription initiation complex. Interestingly, many TATA-less promoters initiate transcription at multiple start sites (reviewed in Ref. 28). Some TATA-less promoters, on the other hand, initiate transcription from one or a few clustered start sites. These transcription initiation sites are encompassed by an initiator element, which contains all the necessary information for correct initiation of transcription (reviewed in Ref. 28). The DD3 promoter initiates transcription mainly at a single location, which is consistent with the formation of a single transcription initiation complex.
site but so far lacks characterized initiator elements. A few promoters have been described that, like the DD3 promoter, are TATA-less and initiator-less and are not GC-rich (Ref. 29, and references therein). No further similarities to these gene promoters could be identified. Therefore, novel and tissue-specific cis-acting elements and trans-acting transcription factors might define the specific and characteristic expression of DD3.

We have shown that the 500-bp DD3 promoter region, encompassing the transcription start site, contains promoter activity upon transfection of reporter constructs into the DD3-expressing prostate cancer cell line LNCaP. The promoter activity increases 3-fold upon shortening of the 500-bp promoter fragment (Fig. 3, construct pDDGH-1.12 versus -1.9), indicating the presence of a silencer in the DD3 proximal promoter. The activity of the pDDGH-1.12 DD3 promoter construct was restricted to the DD3-positive cell line LNCaP, as DD3-negative prostatic and non-prostatic cell lines only marginally initiated transcription from the DD3 promoter. These
data suggest that the DD3 gene promoter is tissue and cell-type specific and, therefore, is a genuine prostate cancer-specific promoter. The absolute promoter activities of the DD3 promoter constructs tested, however, are rather low compared with the HSV-tk promoter activity. This correlates with the low level of endogenous DD3 mRNA expression observed in LNCaP cells, in contrast to the high DD3 expression in prostate cancer cells. Until now, we have found no other prostatic carcinoma cell lines that display high(er) DD3 expression levels; the expression of DD3 in the PC-346C cell line equals that of LNCaP cell lines that display high DD3 expression. This correlates with the low promoter activities of the DD3 promoter constructs examined (data not shown). The most pronounced effect on DD3 transcription was observed using constructs mutated in the NF-1 motif (position −58 to −62). Nevertheless, antibodies against the human NF-1 protein family, did not supershift or abolish any of the bandshifts shown in Fig. 6A (data not shown). These data indicate that the bHLH and NF-1 proteins are most probably not involved in the binding and regulation of DD3 transcription. Other factors might recognize and bind the NF-1 and E-box motif present in the DD3 promoter. Another footprint, FP2, was found further upstream at position −173 to −201 in the DD3 promoter. Mutation of this A+T-rich region, resulted in a decreased transcription rate, suggesting a positive role for this element in DD3 transcription. The high mobility group proteins I and Y (HMG-I(Y)) preferentially bind to the minor groove of A+T-rich sequences (27). In addition, the expression of HMG-I(Y) was shown to be up-regulated in prostate cancer cells and a significant correlation with tumor grade and stage was found (32). Therefore, we investigated whether HMG-I(Y) binds to the FP2 region in the DD3 promoter, and as such might be involved in the transcriptional regulation of DD3. Bandshift analysis using HMG-I(Y)-specific antibodies revealed that HMG-I(Y) interacts with DD3 promoter, and as such might be involved in the transcriptional regulation of DD3. Bandshift analysis using HMG-I(Y)-specific antibodies revealed that HMG-I(Y) interacts with DD3 promoter sequences in all prostate cancer cell lines studied. In addition, in the DD3-expressing cell lines LNCaP and PC-346C another unidentified factor is recruited to the HMG-I(Y)-DNA complex. Alternatively, a larger DNA-binding protein, antigenically similar to HMG-I(Y), may bind to the DD3 promoter instead of HMG-I(Y) itself. The DNA bending properties of HMG-I(Y) suggest that binding of HMG-I(Y) or HMG-I(Y)-like proteins could activate transcription initiation by altering the chromatin structure and/or by creating recognition sites for other trans-acting factors. DNA affinity purification methods will be approached to identify the protein(s) present in the slow migrating protein-DNA complex at FP2 of the DD3 promoter.

In addition to HMG-I(Y), the second footprint harbors a very
strong consensus motif for the FREAC-7/FKHL11 protein. FREAC-7 belongs to the forkhead/winged helix family of transcription factors (33). Several of the forkhead-related factors are tissue-specifically expressed and regulate cell-specific transcription, e.g. freac-6/HFH-3 is expressed in the distal tubules of the kidney (34) and HNF-3 in hepatocytes and respiratory and intestinal epithelia (35). No expression of freac-7 was reported in any adult human tissue (33). Therefore, despite the presence of a strong FREAC-7 consensus binding site in the DD3 promoter, the role of FREAC-7 in the regulation of DD3 expression seems speculative.

The putative silencer-containing region, position −132 to −366, was shown to lack promoter activity (Fig. 3, construct pDDGH-1.13). However, the cloning of fragments of the putative silencer (−132 to −254, −132 to −366, and −132 to −433) in front of the HSV-tk promoter did not reduce the HSV-tk promoter activity in transient transfection assays in LNCaP and TSU-pr1 cells (data not shown). This might indicate that, in contrast to data from the promoter assays (Fig. 3), the DD3 promoter region from position −132 to −433 does not harbor a functional silencer. On the other hand, the (DD3-specific) silencer may not repress transcription of a heterologous promoter. Alternatively, multiple repressor binding sites may be required to repress transcription from such promoters, as has been demonstrated for other silencer elements (36, 37).

Our data suggest that the binding of a prostate-specific repressor (complex) to the proximal DD3 promoter tightly regulates DD3 transcription. Such repressor complex may bind and inhibit the transcription initiation complex, the latter which most probably harbors one or more prostate-specific activators (like complex I in Fig. 6A). For re-activation of transcription in vitro (LNCaP cells) or up-regulation of transcription in vivo (prostate cancer), specific mechanisms are required to overcome repression, such as inactivation of the repressor by mutation, transcriptional inactivation, binding of an inhibitor, or otherwise. Further studies are required to identify the nature of the trans-acting factors that are involved in the transcriptional regulation of the DD3 gene in vitro.

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