

DD3: A New Prostate-specific Gene, Highly Overexpressed in Prostate Cancer¹

Marion J. G. Bussemakers,² Adrie van Bokhoven, Gerald W. Verhaegh, Frank P. Smit, Herbert F. M. Karthaus, Jack A. Schalken, Frans M. J. Debruyne, Ning Ru, and William B. Isaacs

Urology Research Laboratory, University Hospital Nijmegen, 6500 HB Nijmegen, the Netherlands [M. J. G. B., A. v. B., G. W. V., F. P. S., J. A. S., F. M. J. D.]; Department of Urology, Canisius Wilhelmina Hospital, 6500 GS Nijmegen, the Netherlands [H. F. M. K.]; and James Buchanan Brady Urological Institute, Johns Hopkins Hospital, Baltimore, Maryland 21287-2101 [N. R., W. B. I.]

ABSTRACT

Prostate cancer is the most commonly diagnosed malignancy and the second leading cause of cancer-related deaths in the Western male population. Despite the tremendous efforts that have been made to improve the early detection of this disease and to design new treatment modalities, there is still an urgent need for new markers and therapeutic targets for the management of prostate cancer patients. Using differential display analysis to compare the mRNA expression patterns of normal *versus* tumor tissue of the human prostate, we identified a cDNA, DD3, which is highly overexpressed in 53 of 56 prostatic tumors in comparison to nonneoplastic prostatic tissue of the same patients. Reverse transcription-PCR analysis using DD3-specific primers indicated that the expression of DD3 is very prostate specific because no product could be amplified in 18 different normal human tissues studied. Also, in a sampling of other tumor types and a large number of cell lines, no expression of DD3 could be detected. Molecular characterization of the DD3 transcription unit revealed that alternative splicing and alternative polyadenylation occur. The fact that no extensive open reading frame could be found suggests that DD3 may function as a noncoding RNA. The DD3 gene was mapped to chromosome 9q21-22, and no homology of DD3 to any gene present in the computer databases was found. Our data indicate that DD3 is one of the most prostate cancer-specific genes yet described, and this makes DD3 a promising marker for the early diagnosis of prostate cancer and provides a powerful tool for the development of new treatment strategies for prostate cancer patients.

INTRODUCTION

Prostate cancer is increasingly recognized as a major health problem: it is the most frequently diagnosed cancer in the Western male population and the second leading cause of male cancer deaths in this population (1). When this carcinoma has spread locally or distantly, no curative therapy can be offered. Therefore, studies to control the disease (*i.e.*, to decrease prostate cancer mortality) have focused on increasing detection of the cancer while it is still locally confined and potentially curable. Indeed, an appreciable increase in the early detection of prostate cancer has been achieved (2-4); however, it has not yet been demonstrated that the increased detection rate will decrease the prostate cancer-related mortality rates. Current methodologies for the staging of prostate cancer are not able to make a distinction between cancers that will remain indolent (and require no treatment) and those that will prove to be highly progressive and life threatening (5). Therefore, it is difficult to determine the optimal treatment strategy for the individual prostate cancer patient. Because there is no effective treatment available for patients with advanced, hormone-refractory prostate cancer, we can conclude that there is an urgent need not only to develop more sensitive and specific means to accurately determine the degree of malignancy of the prostatic disease but also to develop new approaches to treat patients with progressive prostate cancer.

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² To whom requests for reprints should be addressed, at Urology Research Laboratory (814 URL), University Hospital Nijmegen, P. O. Box 9101, 6500 HB Nijmegen, the Netherlands. Phone: 31-24-3615157; Fax: 31-24-3541222; E-mail: M.Bussemakers@hetnet.nl.

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 diagnostic and prognostic tools as well as new targets for therapy. Likewise, the recent identification of critical biochemical pathways, including angiogenesis, programmed cell death, cell adhesion, and signal transduction have offered promising targets for new therapeutic approaches (6). The identification and characterization of new prostate cancer-specific antigens or genes could provide new markers and could be instrumental for the development of new treatment modalities. Here we describe the identification and molecular characterization of a new prostate cancer-specific gene, DD3. DD3 is highly overexpressed in prostate cancer tissue in comparison to adjacent nonmalignant prostatic tissue. Moreover, because its expression appears to be restricted to the prostate, DD3 is one of the most prostate cancer-specific genes yet described and provides a promising tool for the early diagnosis of prostate cancer and the development of new treatment modalities.

MATERIALS AND METHODS

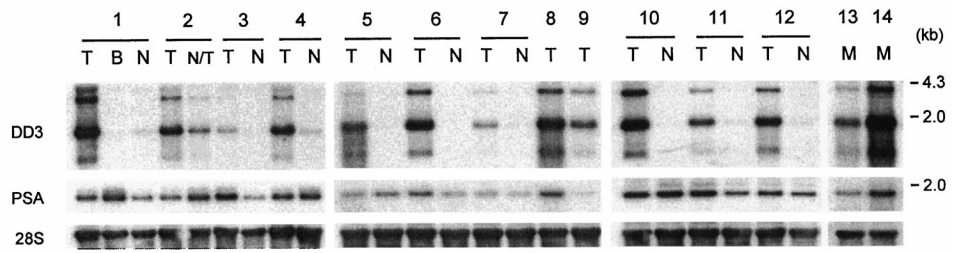
Differential Display Analysis. Upon radical prostatectomy, specimens were snap-frozen in liquid nitrogen. Normal, BPH³ and tumor prostatic tissues were selected for purity of benign cells or cancer cells, respectively, and processed by step sectioning. RNA was extracted using the LiCl-urea method (7). Differential display analysis was performed using RNAmapping Kit A (GenHunter): 20 different combinations of primers were tested (four anchored primers and five arbitrary primers). Upon autoradiography, mRNAs that appeared to be differentially expressed were selected, and cDNA fragments were recovered from gel, reamplified, and cloned using a TA-cloning system (pCRII; Invitrogen). Nucleotide sequence analysis was performed using Sequenase 2.0 (United States Biochemical), and sequences were analyzed by IntelliGenetics computer software.

Northern Blot Analysis. Total RNA (10 μ g) was glyoxalated, separated on a 1% agarose gel in 20 mM phosphate buffer, and transferred to Hybond-N⁺ (Amersham) by capillary blotting. Probes for DD3 (original differential display clone, nucleotides 513-987, GenBank accession number AF103907), PSA, and rRNA were radioactively labeled with [α -³²P]dATP by random prime labeling. Hybridizations were performed as described previously (8).

RT-PCR Analysis. Total RNA (1 μ g) was treated with DNase I, and cDNA was synthesized using Superscript Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.). Upon quantification of the reverse transcription reaction, 10 ng of cDNA were used for a PCR reaction using DD3-specific primers: (a) forward primer (located in exon 1), 5'-AGATTTGTGGTGCTGCAGCC-3'; and (b) reverse primer (located in exon 3), 5'-TCCTGCCATCCTTTAAGG-3'. The amplification was performed as follows: 30s, 94°C/30s, 60°C/30s, 72°C/35 cycles/10 min., 72°C. To verify the quality of the cDNA synthesized, control reactions were performed using primers derived from the β 2 microglobulin gene (9), a ubiquitous housekeeping gene: (a) forward primer (located in exon 2), 5'-AGCAGAGAATG-GAAAGTCAA-3'; and (b) reverse primer (located in exon 4), 5'-TGTT-GATGTTGGATAAGAGAA-3'. The amplification was performed as follows: 1 min., 94°C/1 min., 58°C/1 min., 72°C/35 cycles/10 min., 72°C. PCR products were analyzed by agarose gel electrophoresis. RNA that was not subjected to reverse transcription was used as a negative control for PCR amplification.

³ The abbreviations used are: BPH, benign prostatic hyperplasia; PSA, prostate-specific antigen; RT-PCR, reverse transcription-PCR; EST, expressed sequence tag; RISH, RNA *in situ* hybridization; GS, Gleason score.

Fig. 1. Northern blot analysis using DD3 and PSA as a probe. DD3 is highly overexpressed in prostatic tumors, whereas PSA cannot make this distinction at the mRNA level. Ten μg of total RNA were loaded per lane. T, tumor; B, benign prostatic hyperplasia; N, normal; NT, normal + 10% tumor cells; M, metastasis. Patients: 1, GS = 6; 2, GS = 7; 3, GS = 5; 4, GS = 8; 5, GS = 6; 6, GS = 7; 7, GS = 4; 8, GS = 9; 9, GS = 7; 10, GS = 7; 11, GS = 6; 12, GS = 8; 13 and 14, lymph node metastases. rRNA (28S) was used as a control for the quality and quantity of RNA loaded.



The following cell lines were analyzed for the expression of DD3: (a) human prostate cancer cell lines ALVA-31, DU-145, JCA-1, LNCaP, PC-3, PPC-1, and TSU-pr1; (b) human breast cancer cell lines 734B, BT-20, CAMA, MCF-7, MDA-MB-175, MDA-MB-231, MDA-MB-331, MDA-MB-361, and MDA-MB-469; (c) human bladder cancer cell lines 253J, 575A, 647V, 5637, J82, JON, RT-4, RT112, Scaber, SD, SW780, SW800, SW1710, T24, VMCUB-1, VMCUB-2, and VMCUB-3; (d) renal cell carcinoma cell lines SK-RC-1, SK-RC-7, SK-RC-10, SK-RC-12, SK-RC-14, SK-RC-17, SK-RC-28, SK-RC-35, SK-RC-42, SK-RC-52, and SK-RC-59; and (e) ovarian cancer cell lines COLO-316, OV2774, SK-OV-3, SK-OV-4, SK-OV-6, and SW626. Other human tumor specimens that were analyzed for DD3 expression included four breast, three ovarian, two testicular, two cervical, and two endometrial tumors.

Construction and Screening of a cDNA Library. A cDNA library was constructed from total cellular RNA extracted from human prostate cancer specimens using the ZAP-cDNA Gigapack II Gold Cloning kit (Stratagene). Approximately 1.5×10^6 phages were screened according to Sambrook *et al.* (10) using the original differential display clone DD3 as a probe. Positive clones were purified, and plasmids were rescued by *in vivo* excision, according to manufacturer's procedures.

Screening of the Genomic Library. A genomic library, constructed of human placenta genomic DNA, cloned in λ FIX2 (Stratagene) was screened according to Sambrook *et al.* (10) using the original differential display clone DD3 as a probe. Four different clones were obtained (λ FIX-ME1, λ FIX-ME2, λ FIX-ME3, and λ FIX-ME4). Screening of the genomic library with a more 5'-located probe, pME4.5.3, resulted in the identification of another two DD3-related genomic clones, λ FIX-IH1 and λ FIX-IH2.

Genomic Mapping. Screening of the National Institute of General Medical Sciences human X rodent somatic cell mapping panel (Coriell Cell Repositories, Camden, NJ) with DD3 as a probe indicated that the DD3 gene is located on chromosome 9. To more precisely determine the chromosomal location of the DD3 gene, we performed a hybridization of metaphase chromosomes of human lymphocytes using a probe specific for the centromere of chromosome 9 and a probe consisting of a mixture of four DD3-related genomic clones (λ FIX-ME1, λ FIX-ME2, λ FIX-ME3, and λ FIX-ME4). Chromosomes were counterstained using 4',6-diamidino-2-phenylindole.

RESULTS

Identification of DD3 as a Prostate Cancer-specific Gene. To identify unknown genes that are critically associated with the process of prostate tumorigenesis, we compared mRNA expression patterns of nonmalignant *versus* tumor tissue of the human prostate by differential display analysis (11). Using 20 different primer combinations, we identified 11 cDNAs that appeared to be differentially expressed. We selected one cDNA, DD3, which, by Northern blot analysis, is specifically and highly overexpressed in human prostatic tumors, whereas no DD3 expression or only very low levels of DD3 expression were found in normal prostate or BPH tissue from the same patients (see Fig. 1). In 53 of 56 human radical prostatectomy specimens examined, we found a 10–100-fold overexpression of DD3 in the tumor areas in comparison to the adjacent nonneoplastic prostate tissue, as assessed by densitometric scanning of autoradiographs obtained by Northern blot analysis. We also analyzed the expression of PSA in the same patients; however, as would be predicted (12), at the RNA level, PSA cannot make a clear distinction between benign and malignant pros-

tatic tissues (see Fig. 1). The up-regulation of DD3 expression appears to be an early event in prostate cancer development because expression is observed in almost all of the tumors studied. Moreover, DD3 expression is found in well-differentiated, moderately differentiated, and poorly differentiated tumors, with a trend toward more expression in the latter, although this correlation does not reach statistical significance in the samples studied. We also examined four prostate metastatic lesions and found DD3 expression in all samples.

DD3 Expression Is Very Prostate Specific. To establish whether DD3 is expressed in nonprostatic tissues or nonprostatic tumors, we performed a RT-PCR analysis. Presumably due to the increased sensitivity of this technique, we were now able to detect DD3 expression in normal prostate and BPH tissues. Importantly, we found that DD3 expression is very prostate specific, because even using this sensitive assay, no DD3 product could be detected under the same conditions in normal human artery, brain, breast, bladder, colon, duodenum, heart, liver, lung, ovary, pancreas, placenta, seminal vesicles, skeletal muscle, skin, spinal cord, spleen, or testis (see Fig. 2A). Furthermore, in a sampling of tumors originating in the breast, cervix, endometrium, ovary, and testis, no DD3-related product could be amplified (see Fig. 2B). We could detect DD3 expression in only one (LNCaP) of seven human prostate cancer cell lines studied (ALVA-31, DU-145, JCA-1, LNCaP, PC-3, PPC-1, and TSU-pr1; see Fig. 2B). In addition, we examined a large number of human bladder,

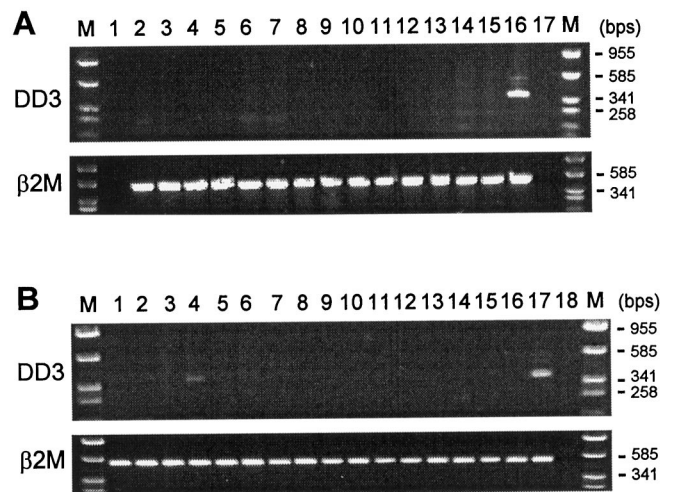


Fig. 2. RT-PCR analysis using DD3-specific primers. DD3 expression appears to be very prostate specific because no expression can be detected in any other normal human tissue or different tumor type studied. A: Lane M, marker; Lanes 1 and 17, negative control; Lane 2, testis; Lane 3, seminal vesicle; Lane 4, ovary; Lane 5, placenta; Lane 6, heart; Lane 7, duodenum; Lane 8, spinal cord; Lane 9, spleen; Lane 10, brain; Lane 11, artery; Lane 12, lung; Lane 13, liver; Lane 14, skeletal muscle; Lane 15, bladder; Lane 16, normal prostate. B: Lane M, marker; Lane 1, ALVA-31; Lane 2, DU-145; Lane 3, JCA-1; Lane 4, LNCaP; Lane 5, PC-3; Lane 6, PPC-1; Lane 7, TSU-pr1; Lane 8, cervix tumor; Lanes 9 and 10, endometrial tumors; Lanes 11 and 12, ovarian tumors; Lanes 13 and 14, testicular tumors; Lanes 15 and 16, breast tumors; Lane 17, normal prostate; and Lane 18, negative control. β 2M, β 2 microglobulin was used as a control for the quality of cDNA synthesis.

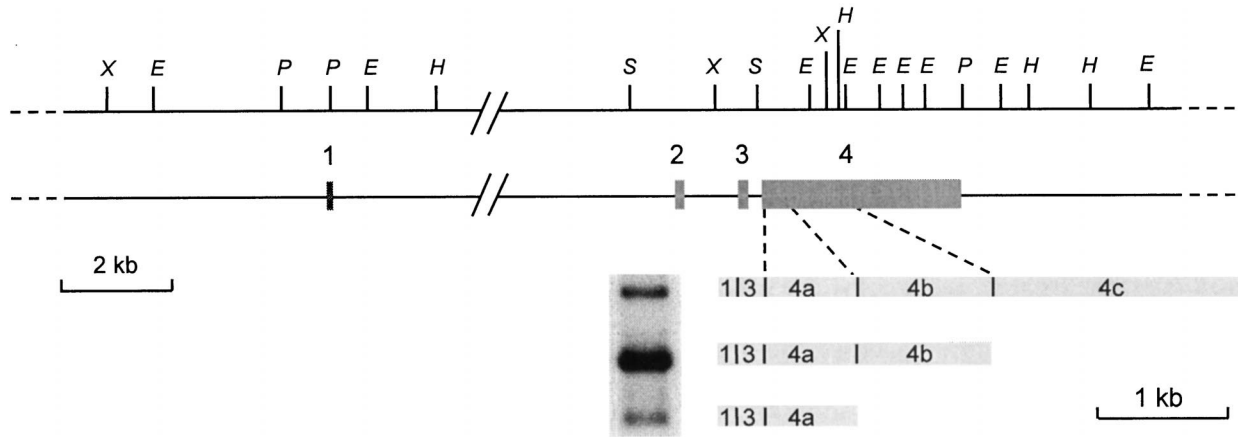


Fig. 3. Structure of the DD3 transcription unit. The DD3 gene consists of four exons; exon 2 is only present in 5% of the cDNA clones analyzed and is often skipped by alternative splicing. Alternative polyadenylation occurs at three different positions in exon 4 (4a, 4b, and 4c). Restriction sites: E, EcoRI; H, HindIII; P, PstI; S, SmaI; and X, XbaI.

Table 1 Exon-intron boundaries of the DD3 gene

Uppercase and lowercase letters indicate exon and intron sequences, respectively. Conserved splice donor and acceptor dinucleotide sequences are indicated in bold.

Exon	Exon 3' end	Intron length	Exon 5' end	Exon
1	TGATACAGAGgtctgtag. . .	~20 kb	. . .tgttctatgtcctaagGAATTACAAC	2
2	AATGGCAGGGgtgagta. . .	873 bp	. . .tcgtatgtattccagGTGAGAAATA	3
3	AAGCACAGAGgtaagtc. . .	227 bp	. . .tctccactttccacagATCCCTGGGA	4

breast, kidney, and ovarian cancer cell lines (see "Materials and Methods"), and a DD3-related product could not be amplified in any of these (data not shown).

Characterization of the DD3 Transcription Unit. Because screening of nonprostate-derived cDNA libraries would yield no DD3-related clones, a cDNA library of mRNA extracted from human prostate cancer specimens was constructed to isolate the full-length DD3 cDNA. Screening of this library resulted in the identification of more than 250 DD3-positive cDNA clones, of which we purified and characterized 80. Furthermore, we isolated and characterized six overlapping genomic phage clones that cover the DD3 gene from a human placenta genomic DNA library. Computer analysis of all obtained nucleotide sequence data allowed us to determine the structure of the DD3 transcription unit as it is shown in Fig. 3 (see also Table 1; GenBank accession numbers AF103907 and AF103908). The DD3 gene consists of four exons, and both alternative splicing (of exon 2) and alternative polyadenylation (at three different positions in exon 4) occur, giving rise to the differently sized transcripts as observed by Northern blot analysis. Exons 1, 3, and 4a are present in all three of the different transcripts seen on Northern blot (0.6, 2, and 4 kb), whereas exon 4b is present in the two larger transcripts, exon 4c is only present in the largest transcript (4 kb), and exon 2 can be part of all three transcripts but is only present in a limited number of the transcripts (5% of the cDNA clones analyzed). The most frequently found cDNA clone (60% of all cDNA clones analyzed) contains exons 1, 3, and 4a and 4b. This cDNA corresponds to the most prominent transcript detected on Northern blot (2 kb). The relative presence of the splice variants was confirmed by RT-PCR analysis using primers specific for exons 1 and 3: we found a pronounced PCR product of the size to be expected when only exons 1 and 3 are present in a transcript (confirmed by nucleotide sequence analysis of the PCR product) and a faint but clearly visible larger product that corresponds to exons 1, 2, and 3. Using exon-specific probes for Northern blot hybridization, we were able to confirm the data obtained by analysis of the cDNA clones. Computer-assisted database comparison revealed that the complete DD3 cDNA has no homology to any entry in the databases, with the exception of three

ESTs: (a) AA578773 was identified in human prostate epithelium and is almost identical to part of the DD3 cDNA; and (b) AI557225 and AI557495 were identified in human prostate tumor tissue. AI557225 is almost identical to part of the 3' end of the DD3 cDNA; AI557495 is a recombinant clone and contains part of exon 1 fused to sequences of intron 1 of the DD3 gene.

DD3 May Function as a Noncoding RNA. A striking feature of the entire DD3 cDNA sequence is the high density of stop codons in all three reading frames (see Fig. 4), which suggests that if DD3 is a coding RNA, the protein product is likely to be very small. Indeed, Grail computer software, an algorithm designed to detect coding regions in human DNA (13), revealed several small open reading frames scattered throughout the DD3 gene. The most likely open reading frame of DD3 (based on codon usage) was predicted to be located in exons 3 and 4a (nucleotides 401–553, accession number AF103907), a region that is present in all transcripts. The alternative splicing of exon 2 or the use of CTG or ACG as the initiation codon for translation (14) does not significantly lengthen any of the open reading frames. The fact that DD3 is expressed as a spliced and polyadenylated RNA molecule makes it highly unlikely that DD3 is a pseudogene.

The DD3 Gene Is Located on Chromosome 9q21–22. The DD3 gene spans a region of approximately 25 kb: the first intron is relatively large (approximately 20 kb); whereas intron 2 and 3 are small (873 and 227 bp, respectively; see Fig. 3 and Table 1). Screening of a panel of somatic hybrids indicated that the DD3 gene is located on chromosome 9. Using subsequent hybridization of metaphase chromosomes of human lymphocytes with a probe specific for the centromere of chromosome 9 and a probe consisting of a mixture of four DD3-related genomic clones (AFIX-ME1, AFIX-ME2, AFIX-ME3, and AFIX-ME4), we determined

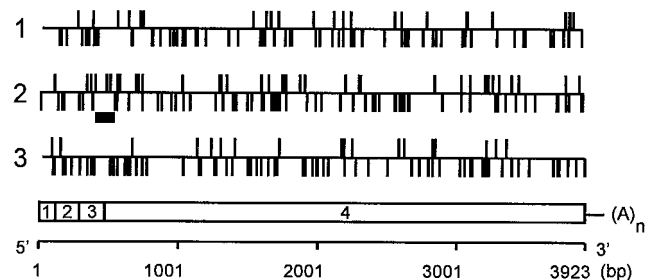


Fig. 4. Open reading frame analysis. The relative positions of ATG codons and termination codons (TGA, TAA, and TAG) are indicated by vertical lines, respectively, for each of the three reading frames. The small black rectangle indicates the most likely open reading frame of DD3 as predicted by Grail software. The four open boxes (□) represent exons 1–4 of the DD3 gene.

Fig. 5. Chromosomal mapping of the *DD3* gene. Hybridization of metaphase chromosomes of human lymphocytes indicates that the *DD3* gene is located on chromosome 9q21–22 (arrowhead).



that the *DD3* gene is located on human chromosome 9q21–22 (see Fig. 5). Hybridization of a zoo blot revealed that the *DD3*-encoding gene is conserved in the monkey, horse, cow, pig, goat, and sheep. In the dog and cat, a very faint signal was detected. In rodents, no signal was obtained under low-stringency hybridization conditions (40% formamide at 40°C). By comparison, it is interesting to note that the gene encoding PSA is only found in primates (15).

DISCUSSION

Using differential display analysis to compare the mRNA expression patterns of tumor tissue and adjacent nonneoplastic tissue of radical prostatectomy specimens, we identified a cDNA, *DD3*, which is highly overexpressed in almost all prostatic tumors and prostate metastatic lesions examined. Moreover, using a sensitive RT-PCR assay, we could not detect *DD3* expression in any of 18 normal tissues studied or in a small sampling of other human tumor types and a large number of tumor-derived cell lines. This indicates that the expression of *DD3* is restricted to the prostate. Only the human prostate cancer cell line LNCaP does express *DD3*. The strong overexpression of *DD3* observed in prostatic tumors in comparison to normal prostate or benign prostatic hyperplasia and the apparent prostate-restricted expression of *DD3* indicate that *DD3* is one of the most prostate cancer-specific genes yet described. This makes *DD3* an interesting candidate for use as a diagnostic and/or prognostic marker for prostate

cancer. Furthermore, *DD3* offers a powerful tool for the development of new treatment modalities for prostate cancer patients: the gene promoter that drives the prostate cancer-specific expression of *DD3* can be used for gene therapy approaches.

A number of prostate-specific genes are known, including PSA (16) and prostate-specific membrane antigen (17), which have already been studied extensively for their application in the management of prostate cancer patients as either a marker or a tool or target for therapy. More recently, human kallikrein 2 (18) and prostate stem cell antigen (19) were identified as prostate-specific genes. Their usefulness in prostate cancer diagnosis and treatment remains to be established.

Molecular characterization of the *DD3* transcription unit revealed that alternative polyadenylation at three different positions in exon 4 is the main mechanism giving rise to the differently sized transcripts detected by Northern blot analysis. In addition, alternative splicing occurs, by which exon 2 is deleted from most transcripts (exon 2 is present in only 5% of the transcripts). The most striking feature of the full-length *DD3* cDNA is the high density of stop codons in all three reading frames and the resulting lack of an extensive open reading frame. The alternative splicing of exon 2 or the use of CTG or ACG as the initiation codon for translation (14) does not result in the extension of potential protein-encoding regions. Recently, a number of genes (*e.g.*, H19, Xist, his-1, BORG) have been described, which are expressed as spliced and polyadenylated RNA molecules contain-

ing a high density of stop codons and lacking an extensive open reading frame (20–26). Analogous to DD3, these genes are unlikely to be pseudogenes, and, moreover, it has been suggested that these genes may function as noncoding RNAs. Available evidence indicates that H19 plays a role in mammalian development (20) and that Xist RNA acts in the nucleus and contributes to X chromosome inactivation (21, 22). BORG has been suggested to be a noncoding RNA that plays a role in the differentiation process induced by bone morphogenetic proteins (26). Recently, Lanz *et al.* (27) reported on SRA, a steroid receptor coactivator, which acts as a RNA transcript that functions as a component of a large multiprotein complex. DD3 may be another new member of the growing unique class of noncoding RNAs; however, we do not exclude the possibility that DD3 encodes a small peptide. Grail software predicted a potential protein-encoding region of DD3 to be located in exons 3 and 4a, which is present in all transcripts analyzed. *In vitro* transcription and translation of the full-length DD3 cDNA has not yet provided evidence that a small protein is produced. We are currently cloning the orthologues of DD3 from other mammalian species: if conservation of the nucleotide sequence is mainly limited to the potential open reading frame, this will indicate that a small protein may indeed be produced. However, if a strong conservation of the overall nucleotide sequence and genomic organization is found, as was shown for the his-1 gene (28), this will suggest that the RNA is the final and functional product of the DD3 gene.

Comparison of the nucleotide sequences of the full-length DD3 cDNA to the available computer databases revealed that no genes homologous to DD3 have been reported. However, three ESTs have been reported that are homologous to different parts of the DD3 cDNA. The fact that one of the ESTs was identified in human prostate epithelium supports our expectation that DD3 is expressed by the epithelial cells of the prostate rather than by the stromal cells, because DD3 is highly overexpressed in adenocarcinoma of the prostate, which arises from the epithelial cells. However, RISH will have to confirm this hypothesis. As mentioned before, several noncoding RNAs have been shown to localize to the nucleus and are involved in the regulation of expression of other genes. Therefore, determination of the subcellular localization of DD3 RNA by RISH may provide evidence for the functioning of DD3 as a noncoding RNA.

The gene encoding DD3 maps to chromosome 9q21–22, a region that has not been shown to be frequently affected in prostatic tumors. Preliminary data provide no evidence that the overexpression of DD3 is due to amplification or rearrangements on chromosome 9. Additional studies will be needed to disclose the mechanism responsible for the observed increase of DD3 expression in prostatic tumors. Moreover, studies are required to investigate the putative biological functional relation of DD3 in the process of prostate cancer development.

In our efforts to establish the usefulness of DD3 for the management of prostate cancer patients, the value of DD3 as a marker for prostate cancer needs to be assessed. Considering the fact that DD3 may be a noncoding RNA, it is difficult to envision its utility in a protein/antibody-based test. Therefore, RISH will be used to analyze human prostate cancer specimens to establish the potential usefulness of DD3 as a diagnostic and/or prognostic marker for prostate cancer. Furthermore, we are currently establishing a RT-PCR-based assay to determine the usefulness of DD3 as a marker for the detection of circulating neoplastic prostate cells. Likewise, the DD3 gene promoter needs to be characterized: identification and characterization of the elements that regulate the prostate cancer-specific expression of the DD3 gene may provide important tools for the development of new gene therapy approaches aimed at selectively driving the expression of therapeutic genes in prostate cancer cells.

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