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Advances in Brief

Two Differentially Expressed Genes in Normal Human Prostate Tissue and in Carcinoma[1]

Fei-Lan Wang, Youbin Wang, Wai-Keung Wong, Yuan Liu, Frank John Addivinola, Peng Liang, Lan Bo Chen, Philip W. Kantoff, and Arthur B. Pardee[2]

Divisions of Cell Growth and Regulation [F-L. W., F. J. A., A. B. P.], Cellular and Molecular Biology [Y. W., W-K. W., Y. L., L. B. C.], and Medical Oncology [P. W. K.], Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115, and Vanderbilt Cancer Center [P. L.], Nashville, Tennessee 37232

Abstract

Alterations in transcriptional control may contribute directly to carcinogenesis. Using the differential display technique in prostate cancer cells compared to normal prostate epithelial cells, we identified a down-regulated gene and an up-regulated gene in cancer cells. The down-regulated gene encodes human epithelial tropomyosin (TMe1), a member of the family of actin filament-binding proteins. The up-regulated gene encodes cytochrome *c* oxidase subunit VIc (COSVIc), a protein of the respiration chain in the mitochondrial inner membrane. The differential display pattern was confirmed by Northern hybridization in both prostate tissue and cell lines. *In situ* hybridization of malignant prostate epithelial tissue using a digoxigenin-labeled antisense riboprobe detected strong staining for mRNA of COSVIc, as opposed to very weak staining in normal prostate epithelium. The expression pattern of COSVIc may be a useful marker for studying the alteration of energy metabolism in cancer cells and for the diagnosis of prostate cancer.

Introduction

The prostate in aging males is highly susceptible to benign and malignant proliferative changes. As a result, prostate cancer is the most common form of cancer and is the second leading cause of cancer-related deaths in American males (1). Mutations in oncogenes (2) and tumor suppressor genes (3), as well as alterations of androgen signaling have been studied in the multistep sequence of prostate carcinogenesis (4).

Energy metabolism of the prostate tumor should be part of a more comprehensive study in prostate carcinogenesis. Abnormality of energy metabolism in cancer cells has been a recurrent finding for many years. Warburg (5) described elevated glycolysis as characteristic of tumor cells, and suggested that the cancer cell originates as a heritable injury to mitochondria. Tumor cells have been associated with changes in mitochondria size, number, distribution, morphology (6), membrane lipid composition (7), membrane potential (8), loss of electron transport components, deficiencies in energy-related functions, increased state 4 respiration, and impaired protein synthesis (9).

Mitochondrial proteins are normally encoded either by the mtDNA or by the nuclear genome (10). Enhanced expressions of several mitochondrial genes in precancerous tissue and in transformed cell lines have been described (11, 12). Some nuclear genes for representative mitochondrial membrane proteins have high transcriptional levels in cancer cells (13). Researchers have found that the energy requirement in different cell types and different tissues is widely diversified; respiration and glycolysis were enhanced in some tumor tissues and either remained unchanged or decreased in other ones (14). No evidence for a relationship between the two genetic systems of mitochondria and carcinogenesis in human prostate tissue has been reported yet.

The fact that oncogenic elements are often transcription factors suggests that alteration of transcriptional regulation may directly contribute to cancer development. This alteration of regulation can be in a negative or positive direction, or both.

We screened normal *versus* tumor human prostate tissue by the DD[3] method (15–17) and identified a down-regulated gene in prostate cancer cells that encodes TMe1. Another nuclear gene that is up-regulated encodes COSVIc, a protein of the respiration chain in the mitochondrial inner membrane. In addition to its potential as tumor marker, the expression pattern of COSVIc may be useful to study the relationship between cancer cell respiration and oxidative phosphorylation.

Materials and Methods

Surgical Specimens and Cell Lines. Surgical samples of human prostate carcinoma and normal prostate tissue were obtained from the Department of Pathology at Brigham and Women's Hospital (Boston, MA). Immediately after surgery, normal epithelium was dissociated from tumor tissue under a dissection microscope. The human prostate carcinoma cell lines LNCaP, PC3, and DU145 were acquired from American Type Culture Collection (Rockville, MD). The human prostate immortalized cell line MLC 8891 was provided kindly by John S. Rhim (National Cancer Institute, Bethesda, MD). LNCaP, PC3, and DU145 were cultured in RPMI 1640 (Mediatech, Washington, DC) supplemented with 10% fetal bovine serum. MLC 8891 was cultured in BRFF-HPC1 (Biological Research Faculty and Facility, Ijamsville, MD).

***In Situ* Hybridization.** Digoxigenin-labeled probes were utilized for *in situ* hybridization. For digoxigenin-labeled probes, one μg of the recombinant plasmid containing a 420-bp fragment of human COSVIc insert was linearized by *Xba*I and *Hin*dIII to generate sense and antisense transcripts, respectively. Using the DIG RNA Labeling Kit (Boehringer Mannheim, Indianapolis, IN), riboprobes were generated with Sp6 and T7 RNA polymerase for 2 h at 37°C in 1× transcription buffer. For *in situ* hybridization, human cancer specimens from surgical operations were fixed in 3.7% formalin and embedded in paraffin. Routine sections were cut 5 nm thick, dried in an oven at 40°C for 4 h, deparaffinized in xylene, and rehydrated in alcohol, and were then ready for *in situ* hybridization. Five mM formalin-fixed paraffin-embedded sections were dewaxed, rehydrated, and washed in 2× SSC. Sections were digested with proteinase K (50 mg/ml) in 1 M Tris-EDTA buffer (pH 8.0) for 8 min at 37°C. Samples were then preincubated at 40°C for 5 min and denatured for 2 min. RNA probe was then added (100 μl/each slide, 1:20), hybridized, and washed with 2× SSC at 45°C for 30 min, 1× SSC at 45°C for 30 min, and 0.5× SSC at 50°C for 15 min. Sections were incubated for 28 min with alkaline phosphatase-conjugated antidigoxigenin polyclonal sera [normal sheep serum, diluted 1:500 in 2 mM Tris-HCl (pH 7.5) and 100 mM NaCl, at 20°C]. Bound

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[2] To whom requests for reprints should be addressed, at Division of Cell Growth and Regulation, Dana-Farber Cancer Institute, Harvard Medical School, 44 Binney Street, Boston, MA 02115.

[3] The abbreviations used are: DD, differential display; COSVIc, cytochrome *c* oxidase subunit VIc; TM, tropomyosin; TMe1, human epithelial TM; BPH, benign prostate hyperplasia.

antibody was detected by a standard immunoalkaline phosphatase reaction, using nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate as substrate. After development, sections were counterstained lightly with methyl green (0.5%) and mounted with Glycergel. Identical hybridization signals were obtained with digoxigenin-labeled riboprobes on an automated *in situ* hybridization instrument (Ventana Medical Systems, Tucson, AZ), in which duration and temperature of all the steps were standardized.

RNA Preparation and Northern Hybridization. Total RNA from tissues and cell lines were isolated with RNAzol B reagent (Biotecx Laboratories, Houston, TX; Ref. 18), and then treated with DNase I to remove genomic DNA. RNAs were separated on 1.1% agarose and 0.86 M formaldehyde gel and then transferred to a nylon filter (Micron Separations, Westboro, MA). The probes were labeled with ^{32}P using a random-prime DNA labeling kit (Boehringer Mannheim). The filters were hybridized to the probe overnight at 42°C .

DD. Two-base anchored oligodeoxythymidylate primers T12MC (5′-TTTTTTTTTTTTMC-3′) and T12MG (5′-TTTTTTTTTTTTMG-3′) were used to reverse transcribe total RNA from tissue and cell lines into first-strand cDNAs, which were amplified subsequently by PCR using the arbitrary upstream primers AP14 (5′-AATGGGCTGA-3′) and AP4 (5′-AGTATTCCAC-3′) of the RNAmap kit (GenHunter, Nashville, TN). PCR conditions used were the same as described previously (17). PCR products were analyzed on a 6% DNA sequencing gel using 0.5 mM [α-^{35}S]-labeled dATP (1200 Ci/mmol). The bands on the cDNA ladder that were unique to normal or to tumor prostate were cut out of the gel, eluted, and reamplified by PCR.

Cloning and Sequencing. The reamplified cDNA bands were cloned into plasmid PCRII using the TA cloning kit (Invitrogen, San Diego, CA). Individual clones were sequenced using an automated DNA sequencer (Applied Biosystems, Inc.).

RT-PCR of the Coding Region of COSVIc. RNA from PC3 cells was reverse transcribed using 30-mer oligodeoxythymidylate. The subunit cDNA was amplified by PCR using primers 5′-GAAGGACGTTGGTGTTGAGG-3′ and 5′-CTAGGGAATTCAACCTGAAG-3′. The 317-bp fragment was used as a probe for Northern hybridization.

Results

A DD of mRNA present in normal and cancer prostate tissue is illustrated in Fig. 1*A*. A band is present in normal tissue lanes, and has a very weak signal or is absent in the four tumor lanes. To confirm that this pattern of identified fragments is displaying different expressions, this cDNA was reamplified, isolated, and used to probe a Northern blot containing RNAs from patients' prostate tissue (Fig. 1*B*). The down-regulated expression pattern was observed in Northern blot. The reamplified cDNA fragment was subcloned into the pCRII vector and sequenced. The result showed that the 290-bp fragment is identified as TMe1 (19).

We also performed DD using normal and cancer cell lines with different primer combination. In Fig. 2*A*, the arrow points to a band, p6, that came out only in the cancer cell line PC3 lane but not in the immortalized normal cell line MLC8891 lane. Northern blot containing RNAs from MLC 8891 and PC3 cell lines was probed with the p6 fragment (Fig. 2*B*). The expression pattern of the p6 fragment was consistent between DD and Northern blot. The reamplified p6 fragment was subcloned into the pCRII vector and sequenced (Fig. 3). The 261-bp cDNA fragment has 93% homology to human COSVIc (20). Homology in the coding region is 95%. COSVIc is a subunit of cytochrome *c* oxidase of the respiratory chain. COSVIc is encoded by a nuclear gene. The cytochrome *c* oxidase encoded by nuclear and mitochondria genes is a transmembrane complex of inner mitochondrial membrane, and its electron-carrier function is essential for oxidative phosphorylation (21). The full-length COSVIc coding region was cloned using designed primers and RT-PCR. The amplified cDNA fragment was used to probe a Northern membrane of cell lines and patients' prostate tissue. Results of the Northern blot showed that the mRNA signal of COSVIc was present in the androgen-dependent

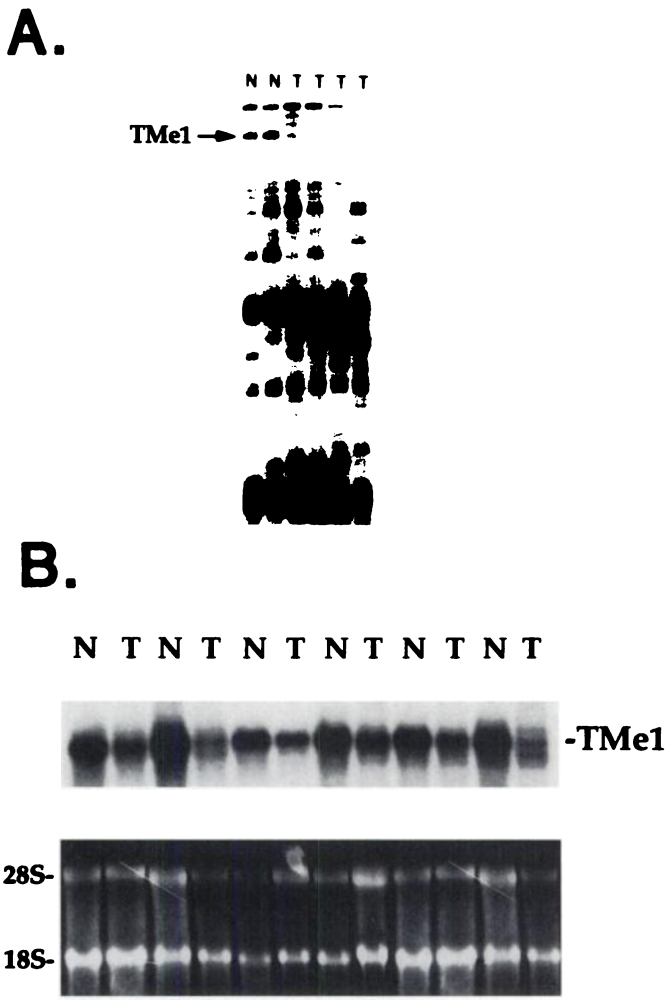

Fig. 1. Identification by DD of altered gene expression in normal and malignant prostate epithelial tissue. A DD was carried out using a 5′ arbitrary primer AP4 (5′-AGTATTCCAC-3′) and a 3′ T12MG (5′-TTTTTTTTTTTTMG-3′) anchored primer and resolved by electrophoresis. *A*, the candidate gene (*TMe1*) is indicated with an *arrow*. *B*, confirmation of DD pattern by Northern blotting (*top*). *Each two lanes* show a normal (*N*) and tumor (*T*) sample from one patient. Twenty μg of total RNA were loaded. Ethidium bromide staining demonstrates similar loading of undegraded RNA in each lane (*B*, *bottom*).

tumor cell line LNCaP and androgen-independent lines PC3 and DU145, and absent in normal MLC 8891. These results confirm further the expression pattern that had been identified initially by the p6 fragment of the 3′-terminal COSVIc cDNA (Fig. 4*A*). The mRNA of COSVIc in normal tissues was found to have a very low level, although the level was much higher in tumor tissues (Fig. 4*B*).

Increased COSVIc mRNA expression in a primary prostate carcinoma was observed, as judged by *in situ* hybridization and in comparison to adjacent normal tissue. In the samples of eight patients, six had positive staining in carcinoma samples. COSVIc mRNA expression was highest in scattered epithelial cells of the prostate carcinoma (Fig. 5*B*). The fibroblast of stroma also weakly expressed COSVIc mRNA, but BPH stained much more weakly than epithelial cells of prostate carcinoma (Fig. 5*A*).

Discussion

DD is a powerful and rapid tool for screening and identifying at the mRNA level altered gene expression between two or more cell populations. It provides an important addition to other techniques available to identify differentially expressed genes. We prepared RNA

from normal and tumor prostate tissues, and from normal (MLC8891) and tumor (PC3) prostate cell lines, performed DD, and identified a down-regulated gene, *TMe1*, and an up-regulated gene, *COSVIc*, in malignant prostate epithelial cells. This expression pattern was confirmed by Northern blot in four prostate cell lines, MLC 8891, LNCaP, PC3, and DU145, and in normal and prostate tumor tissues.

Cell lines are different from tissue in cellular homogeneity, growth status, microenvironment, and generation of passage. Also, in our experiments, the phenotype of immortalized normal cell line MLC 8891 is not completely the same as that of normal cells. In addition, different prostate cell lines grown in different media produce different COSVIc mRNA expression levels. To investigate further the mRNA expression of COSVIc *in vivo*, we proceeded to work with prostate tumor samples derived from radical prostectomy specimens. Finally, RNA *in situ* hybridization was performed. The expression of COSVIc mRNA can be visualized clearly under the microscope. Bluish-black staining of prostate cancer tissue *versus* green counterstaining of BPH gave a clear confirmation of elevated COSVIc mRNA expression in prostate tumor cells. Taking the DD, Northern blot, and *in situ* hybridization data together, we conclude that COSVIc is up-regulated at the mRNA level in prostate tumor tissue.

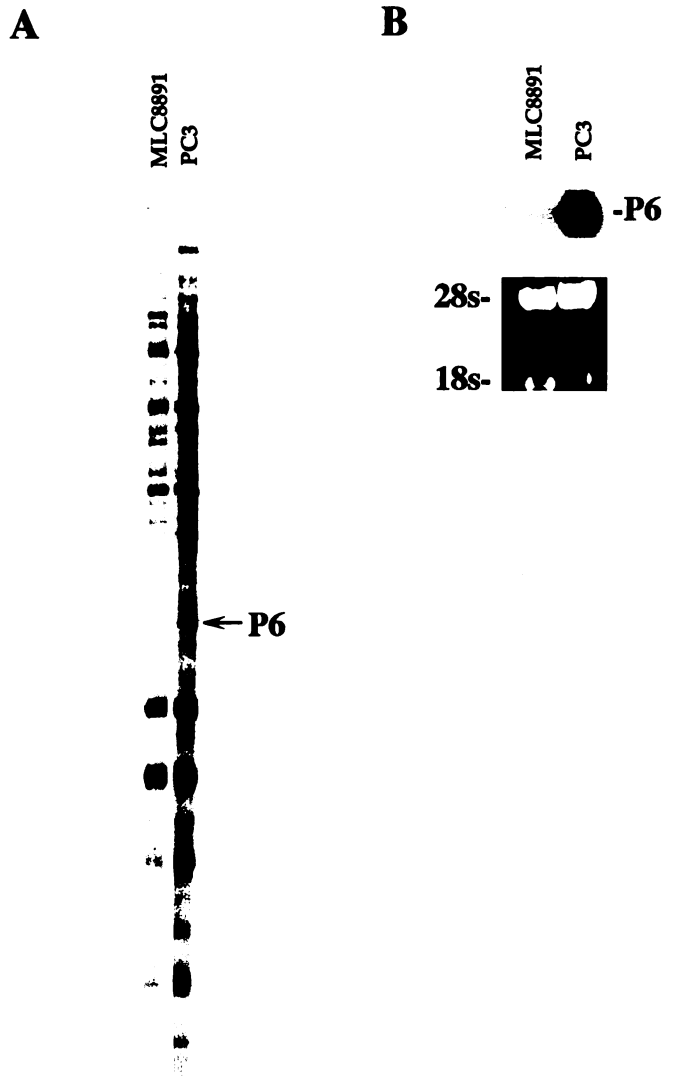

Fig. 2. Identification by DD of altered gene expression in prostate cancer cell line PC3. A DD was carried out using a 5′ arbitrary primer, AP14 (5′-AATGGGCTGA-3′), and 3′-T12MC (5′-TTTTTTTTTTTTMC-3′) anchored primer and resolved by electrophoresis. *Lane 1*, MLC 8891; *lane 2*, PC3. *A*, candidate gene (*p6*) is indicated with an *arrow*. *B*, confirmation of DD pattern by Northern blotting (*top*). Twenty μg of total RNA were loaded. Ethidium bromide staining demonstrates similar loading of undegraded RNA in each lane (*B*, *lower*).

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                10          20          30          40          50          60
P6  AATGGGCTGA TCAAAGAAAG AAGGCATACG CAGATTTCTA CAGAAACTAC GATGTCATGA
                70          80          90         100         110         120
    AAGATTTTGA GGAGATGAGG AAGGCTGGTA TCTTTCAGAG TGTAAAGTAA TCTTGGAATA
               130         140         150         160         170         180
    TAAAGAATTT CTTCAGGTTG AATTACCTAG AAGTTTGTCA CTGACTTGTG TTCCTGAACT
               190         200         210         220         230         240
    ATGACACATG AATATGTGGG CTAAGAAATA GTTCCTCTTG ATAAATAAAC AATTAAACAA
               250         260
    ATACTTTGGA AAAAAAAAAA A
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Fig. 3. Nucleotide sequence of p6 cDNA. The sequences of primers for the DD and polyadenylation signal AATAAA are *underlined*.

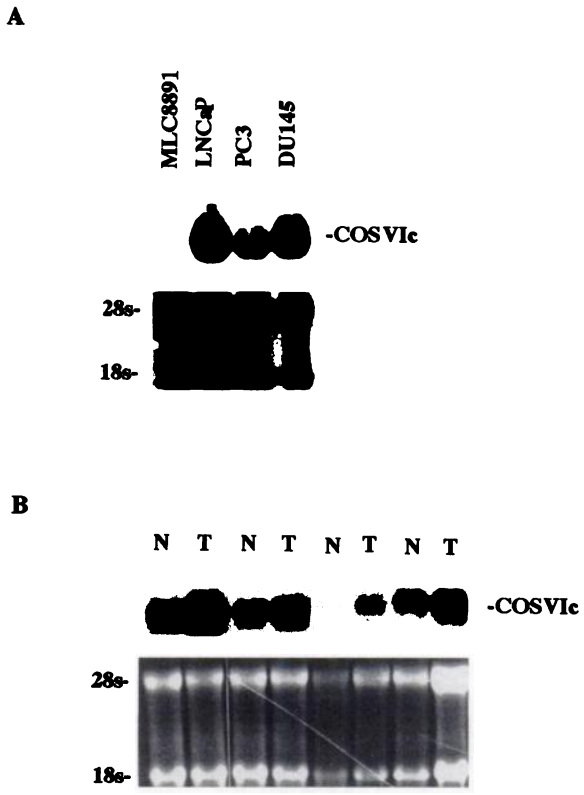

Fig. 4. Northern blot analysis of transcripts of COSVIc. Prostate cell lines (*A*); prostate tissues (*B*) from different patients, in which the two lanes in each pair show a normal (*N*) and tumor (*T*) sample from one patient. Twenty μg of total RNA were loaded in both *A* and *B*. *B*, *bottom*, ethidium bromide staining to demonstrate similar loading of undegraded RNA in each lane.

TM is an actin-binding protein the isoform expression of which involves multiple genes and alternative processing of RNA. The function of nonmuscle TM isoforms remains unclear (22). Consistent loss of expression of specific TM isoforms has been found in cells from human breast cancer cell lines (23). Some specific TM also showed tumor suppression in transformed cell lines (24, 25). Here, we show that the TMe1 encoded by the *b-tropomyosin* gene is down-regulated in prostate tumor, which provides additional evidence of reduced synthesis of various actin-binding proteins in cancer and transformed cells.

Mitochondria provide energy through oxidative phosphorylation metabolism (21). The proper function of mitochondria is essential for maintaining normal cellular metabolism; therefore, they are a target for many exogenous as well as endogenous insults. Two different genetic systems regulate mitochondrial function. Mitochondrial genes code for 13 mitochondrial peptides, and nuclear genes code for the rest of the mitochondrial proteins (9). Elevated expression of different

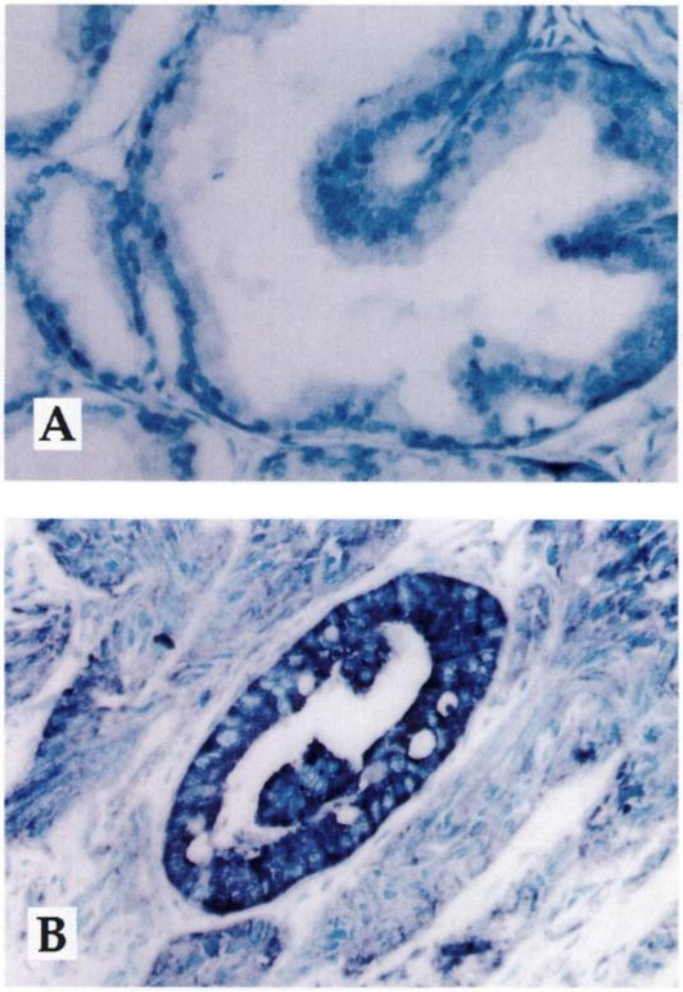

Fig. 5. *In situ* hybridization for COSVIc mRNA in prostate tissue using a digoxigenin-labeled riboprobe transcribed from a 317-bp of COSVIc coding fragment. It is detected with antidigoxigenin antibodies conjugated to an alkaline phosphatase enzyme after a 28-min exposure to nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate substrate. *A*, only a weak staining is detected with the antisense probe in BPH tissue (magnification, ×200). *B*, a strong tumor staining is seen on tumor tissue section (magnification, ×400).

mitochondrial and nuclear genes has been described in some precancerous tissues and transformed cell lines (12, 13). Previous studies show that some subunits of cytochrome *c* oxidase are highly expressed at the mRNA level in rat hepatoma cells (26). We found that in human tissue, COSVIc encoded by a nuclear gene was up-regulated in prostate cancer. COSVIc also had a similar expression pattern in many human breast tumor cell lines (data not shown). As one of the electron-driven proton pumps of oxidative phosphorylation, cytochrome *c* oxidase plays an important role in energy metabolism (21). There is no established universal principle of energy metabolism to malignancy. Previous evidence suggested that for some cell types, normal energy consumption might be sufficient to meet energy requirements of the cancerous state, whereas for others, this energy is insufficient for the process of malignancy (14). The human prostate has been found to have a uniquely high level of citrate in normal tissue and a significantly reduced level in cancerous tissue (27). This low level of citrate indicates that the citrate has been oxidized via the Krebs cycle, which is the pathway of energy production from carbohydrate and fat metabolism (21). Supplying the high energy requirement of cancer cells may depend on a high respiration rate, which could account for the elevated mRNA of some respiratory proteins in cancers. A contradiction between elevated transcript levels of respiratory proteins and a reduced level of content of some mitochondria proteins has been described in rat hepatoma cells (26). This may be the case in human prostate carcinoma.

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